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Non-visual arrestins regulate the focal adhesion formation via small GTPases RhoA and Rac1 independently of GPCRs

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Abstract

Arrestins recruit a variety of signaling proteins to active phosphorylated G protein-coupled receptors in the plasma membrane and to the cytoskeleton. Loss of arrestins leads to decreased cell migration, altered cell shape, and an increase in focal adhesions. Small GTPases of the Rho family are molecular switches that regulate actin cytoskeleton and affect a variety of dynamic cellular functions including cell migration and cell morphology. Here we show that non-visual arrestins differentially regulate RhoA and Rac1 activity to promote cell spreading via actin reorganization, and focal adhesion formation via two distinct mechanisms. Arrestins regulate these small GTPases mutants effectively reduced the size of DKO cells to WT independently of G-protein-coupled receptor activation.

Keywords

arrestin; small GTPases; RhoA; Rac1; cell spreading; cell motility; actin; focal adhesions

1. Introduction

Cell migration and chemotaxis are essential processes in embryonic development, the inflammatory response, and play a key role in metastatic cancers [1-3]. The signaling mechanisms cells use to sense chemical gradients in their environment are complex and include multiple functional steps involving activation of chemokine G protein-coupled receptors (GPCRs), as well as other GPCRs [4, 5] and a network of actin regulatory signaling pathways. To ensure correct navigation of different cells to distinct destinations, the availability of the guiding cues and the cell's responsiveness to them must be tightly controlled. Thus, as the cell migrates, signaling must be quenched at the trailing edge.

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Arrestins, together with their partners in the GPCR desensitization process, G protein-coupled receptor kinases, are known to play the key role in regulating the sensitivity to chemokines and the signaling of other GPCRs involved in migration [6, 7]. Migration requires the coordinated activation of hundreds of proteins in distinct compartments of the cell [8]. Because arrestins are multi-functional regulators capable of orchestrating signaling and localizing proteins to distinct subcellular compartments [9, 10], they are also likely to affect the activity of various signaling proteins involved in generating the forces that promote movement. Indeed, over the last few years, arrestins have emerged as important regulators of the actin cytoskeleton [11-13].

Rho family GTPases are small G proteins that act as molecular switches that regulate the signal transduction pathways connecting plasma membrane receptors to the cytoskeleton [14, 15]. GTPases of the Rho family, which includes 20 proteins from three distinct types, Rho, Rac and Cdc42, control separate signal transduction pathways regulating the remodeling of actin cytoskeleton [15]. Rac activation induces the formation of protrusions known as lamellipodia that drive the cell migration. Cdc42 activity produces filopodia, a different type of cell protrusions involving actin polymerization [16]. Cdc42 activity may be involved in the control of the movement direction in response to external cues [17]. Rho proteins also regulate the actin-myosin contractility required to propel the cell forward [15, 18]. The functional information about other members of the Rho family is limited.

There is growing evidence for a role of the non-visual arrestins in facilitating small GTPase-mediated events. First, it was shown that arrestin-2¹ activates the small GTPase RhoA coordinately with Gα_q following the activation of the angiotensin II 1A receptor (ATII_{1A}R) [11]. Arrestin-2 also regulates RhoA activity by binding and inhibiting ARHGAP21, a RhoA GTPase activating protein, in response to ATII_{1A}R stimulation [19]. Arrestin-3 interacts with the actin treadmilling protein cofilin upon activation of another GPCR, PAR2 [13], and both arrestins inhibit PAR-2-stimulated Cdk2 activity [20]. In contrast, the transforming growth factor beta (TGF-beta) superfamily co-receptor, the type III TGF receptor, activates Cdk2 via direct interaction with arrestin-3, which leads to inhibition of directed cell migration [21]. Both arrestin-2 and -3 regulate small GTPase guanyl nucleotide dissociation stimulator ralGDS upon activation of the fMLP receptor [22], and activates the ELMO-ARF cascade upon stimulation of the calcium-sensing receptor [12]. Furthermore, arrestins interact with tumor suppressor PTEN, and this interaction is enhanced by stimulation of the G₁₂-coupled lysophosphatidic acid receptor and subsequent activation of RhoA [23]. In the context of 3-D culture, PTEN regulates the arrestin-2 interaction with ARHGAP21/Cdk2 and the activity of Cdk2, which is essential for the multicellular morphogenesis [24]. Thus, collectively the data suggests that arrestins could act both upstream as RhoA regulators as well as downstream as RhoA effectors.

We were interested in determining whether ubiquitous non-visual arrestins [10] regulate the activity of these GTPases. Arrestins have been shown to regulate a variety of proteins

¹Different names of arrestin proteins are used in the field. We use the systematic names of arrestins, where the number after the dash indicates the order of cloning: arrestin-1 (historic names S-antigen, 48-kDa protein, visual or rod arrestin), arrestin-2 (β-arrestin or β-arrestin1), arrestin-3 (β-arrestin2 or hTHY-ARRX), and arrestin-4 (cone or X-arrestin).

independently of G-protein coupled receptor (GPCR) activation [25-30], but the effect of arrestins on the small GTPases under basal conditions has not been explored. Recently we found that arrestins promote focal adhesion disassembly, likely by recruiting clathrin to microtubules targeting focal adhesions to facilitate integrin internalization [31]. Here we show that arrestins regulate the actin cytoskeleton to limit cell spreading by affecting the activity of the small GTPases RhoA and Rac1 in a receptor-independent manner. We also show that, in addition to microtubule dependent FA disassembly, arrestin-mediated regulation of the small GTPase RhoA likely contributes to the FA phenotype in arrestin null cells.

2. Material and methods

2.1. Materials

Restriction endonucleases and other DNA modifying enzymes were from New England Biolabs (Ipswich, MA). Cell culture reagents and media were from Mediatech-Corning (Manassas, VA) or Life-technologies (Carlsbad, CA). DNA purification kits were from Zymo Research (Irvine, CA). All other reagents were from Amresco (Solon, OH) or Sigma-Aldrich (St Louis, MO).

Antibodies—Rhodamine-phalloidin (for actin staining) was from Invitrogen (Carlsbad, CA); anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-HA, monoclonal anti-Cdc42 antibodies, anti-GFP monoclonal antibody, and monoclonal paxillin antibodies were from BD Biosciences (Palo Alto, CA); monoclonal rat from Roche Molecular Biochemicals (Indianapolis, IN) or monoclonal rabbit (from Cell Signaling Technology) anti-HA antibodies were used for cell staining; antibodies against mouse FAK were from Abcam (Cambridge, MA). Pan-arrestin mouse monoclonal F4C1 antibody [32] was a gift of Dr. L. A. Donoso. Monoclonal RhoA antibody was a part of the Rho activation pulldown kit (Cytoskeleton, Denver, CO). Anti-Rac1 mouse monoclonal antibody was from Cytoskeleton (Denver, CO).

2.2. Cell culture, transfection, and retroviral infection of cells

Arrestin DKO and WT MEF cell lines [33, 34] (a generous gift from Dr. R.J. Lefkowitz, Duke University) were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin (P/S) at 37 °C and 5% CO₂. HA-tagged RhoN19, RhoV14, RacN17, RacV12 genes were cloned into PFB murine retrovirus vector (Stratagene). Cells were retrovirally infected after transfecting the retroviral vector using Lipofectamine 2000 (Sigma, St. Louis, MO) into Phoenix cell line. Eugene HD (Promega, Fitchburg, WI) (1:3 DNA:lipid) or Lipofectamine2000 (1:2.5 DNA:lipid) was used to transfect cells in some cases. Cells were kept in serum-free DMEM with 1% (P/S) for approximately 24 hours prior to every experiment.

2.3. Protein Preparation and Western-blotting

Cells were lysed in Lysis solution (Ambion) or 1% SDS lysis buffer and heated for 5 min at 95°C. Protein concentration was measured with Bradford reagent (BioRad). The protein was precipitated with nine volumes of methanol, pelleted by centrifugation (10,000xg, 10 min at

RT), washed with 90% methanol, dried and dissolved in SDS sample buffer at 0.5 mg/ml. Equal amounts of protein were analyzed by reducing SDS-PAGE and Western blotting onto Immobilon-P (Millipore, Bedford, MA). The membrane was blocked with 5% non-fat dry milk in TBS with 0.1% Triton X-100 (TBST) and incubated with appropriate primary and then secondary antibodies coupled with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) in TBST with 1% BSA. Bands were visualized with SuperSignal enhanced chemiluminescence reagent (Pierce, Rockford, IL) and detected by exposure to X-ray film. The bands were quantified using VersaDoc and QuantityOne software (BioRad).

2.4. GTPase Pulldown Assays

The levels of GTP-liganded Rho were analyzed using the Rho activation pulldown kit according to manufacturer's instructions (Millipore, Temecula, CA). Equal volumes of cell lysates were used to measure total Rho. PAK1-PBD-conjugated glutathione-Sepharose beads were prepared as described [35]. Cells were serum-starved for 24 h and lysed. Equal volumes of lysates were added to 30 μ l of PAK1-PBD conjugated glutathione-Sepharose beads and incubated at 4°C for 1 h with gentle rocking. After four washes with 125 mM HEPES, pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 50 mM MgCl₂, 5 mM EDTA and 10% glycerol, the beads were resuspended in 15 μ l of 2 $\times$ Laemmli SDS sample buffer, boiled for 5 min, and resolved on 15% SDS-PAGE. Equal volumes of total lysates were run for comparison. The proteins were transferred to a nitrocellulose membrane and incubated with anti-Rac1 or anti-Cdc42 antibodies in 2% BSA at 4°C overnight.

2.5. RhoA Inhibition

WT cells were infected with retrovirus to express either dominant-negative RhoN19-HA or GFP and plated on fibronectin or poly-D for 2 hours. Cells were stained with Rhodamine-phalloidin and anti-HA or anti-paxillin and anti-HA. Alternatively, cells were allowed to spread for 2 h, and then incubated with 0.5 μ g/mL C3 Transferase (Cytoskeleton, Denver, CO), a permeable Rho inhibitor, or with 1 μ M Y-27632, a selective inhibitor for Rho-associated kinases (ROCK) (EMD Chemicals, Inc., Darmstadt, Germany) for 4 h. Cells were fixed and stained with rhodamine-phalloidin and anti-paxillin antibody.

2.6. Image and Statistical Analysis

Cell size, FA size, and FA number analysis was from 10-15 randomly selected fields per experiment, cell area was measured using Image J or Nikon NIS software. Cells with moderate expression of HA-GTPases were selected for cell size and focal adhesion number rescue measurements. Cells were imaged either on Nikon TE2000-E automated inverted microscope with 40x oil objective with additional 1.5 optic magnification or on LSM 510 Meta Confocal with 40X oil objective. Focal adhesion size and number were measured from confocal images using ImageJ with qualifications for focal adhesion area: 0.5-100 μ m². Using threshold tool, global background staining was removed from the images, leaving only punctate focal adhesions. Using Analyze Particles feature of ImageJ, Focal adhesion size and number were binned with qualifications for focal adhesion area: 0.5–100 μ m². When three or more groups were compared, ANOVA analysis was used. To minimize the probability of Type I error, Bonferroni correction for multiple comparison was applied.

When the data deviated significantly from normal distribution, non-parametric statistics was used: Mann-Whitney U-test for two-group comparison and Kruskal-Wallis non-parametric ANOVA for more than two groups. In all experiments a p value of <0.05 was considered significant.

3. Results

3.1. Activity of the small GTPases is altered in cells lacking arrestin-2 or arrestin-3

To test whether non-visual arrestins play a direct role in regulating cell shape under basal conditions, arrestin-2/3 double knock-out (DKO) mouse embryonic fibroblasts (MEFs) [33, 34] were plated on fibronectin (FN), and compared to wild type (WT) MEFs (Fig. 1A) in serum-free media. DKO MEFs are twice as large as WT cells, and show a dramatically different arrangement of the actin cytoskeleton [31]. DKO cells spread similarly on poly-D-lysine (PDL), which binds integrins but does not promote their clustering and activation, and FN, which binds and activates integrins (Fig. 1A). In contrast, WT cells do not spread well on PDL: the average cell area was reduced nearly by half, as compared to FN (Fig. 1A). This phenotype can be rescued by expressing either arrestin-2 or arrestin-3 in the DKO cells [31].

Rho family GTPases RhoA, Rac1, and Cdc42 regulate cytoskeleton via multiple effectors, controlling actin dynamics and cell shape [36]. Altered cytoskeleton and abnormal spreading of DKO cells suggest that the activity of small GTPases is dysregulated. Therefore, we measured the activity of RhoA, Rac1, and Cdc42 [35]. The activity of RhoA and Rac1, but not Cdc42, was significantly decreased in DKO cells relative to WT (Fig. 1B,C,D,E). MEFs with only arrestin-2 or arrestin-3 missing had similarly decreased Rac1 activity (Fig. 1D), suggesting that both arrestin-2 and arrestin-3 participate in the regulation of Rac1 and play non-redundant roles. In contrast, cells lacking individual arrestins had near-normal RhoA activity (Fig. 1B), suggesting that either non-visual arrestin can enhance RhoA activity and compensate for the loss of the other subtype. Interestingly, MEFs lacking either arrestin-2 or arrestin-3 had significantly elevated activity of Cdc42, whereas DKO cells did not differ from WT cells (Fig. 1B,E). No change in the expression levels of RhoA or Rac1 was seen in any of the cell lines (Fig. 1B). Indeed, overexpression of arrestin-2 or arrestin-3 in HEK293A cells dramatically increased RhoA activity (Fig. 1F,G,H). Thus, both non-visual arrestins independently facilitate RhoA activation in different cell types. Therefore, the absence of both non-visual arrestins accounts for the decreased activity of RhoA and Rac1 in DKO cells.

3.2. Activity of small GTPase RhoA plays a role in DKO phenotype

To test the contribution of reduced RhoA and/or Rac1 activity to DKO phenotype, in DKO cells we expressed HA-tagged constitutively active and dominant-negative RhoA and Rac1 mutants [37]. Cells were incubated overnight in serum-free media to ensure the absence of receptor activity before being allowed to spread for two hours. DKO and WT cells expressing GFP served as controls (Fig. 2A). The cells were stained for HA (to visualize HA-tagged RhoA and Rac1 proteins) and actin (Fig. 2B), and the expression of the mutant GTPases was determined by Western blot (Fig. 2C). The expression of constitutively active RacV12 did not reduce DKO cell size on FN, and even significantly increased it on PDL

(Fig. 2B,D,E). Since Rac1 activation promotes cell spreading and membrane ruffling [38–40], this was not surprising. Further suppression of Rho or Rac activity by dominant-negative RhoN19 or RacN17 did not change the morphology of DKO cells (Fig. 2B,D,E). However, the expression of constitutively active RhoV14 significantly reduced DKO cell size to WT levels on both FN and PDL (Fig. 2B,D,E). Thus, arrestin-dependent activation of RhoA, but not Rac1, contributes to the spreading phenotype of DKO cells.

To test whether this effect required arrestin binding to GPCRs, we used receptor binding-deficient arrestin mutants with a 7-residue deletion in the inter-domain hinge (Δ7) [30, 41], which prevents domain twist that accompanies receptor binding [42, 43]. Both Δ7 mutants effectively reduced the size of DKO cells to WT level on FN and PDL (Fig 2F,G). Thus, GPCR binding is not essential for arrestin-dependent regulation of cell spreading via RhoA.

To further explore the effect of decreased RhoA on spreading, we expressed dominant-negative RhoN19 in WT cells and plated them on FN or PDL (Fig. 3A). As expected, WT cells expressing RhoN19 had fewer stress fibers than surrounding cells, but their size remained similar to GFP-expressing controls when plated on FN (Fig. 3B). Importantly, on PDL WT cells expressing RhoN19 spread significantly better than WT-GFP cells (Fig. 3B). Next, we tested the effect of suppressing RhoA signaling in WT cells by a direct Rho inhibitor C3-transferase (C3T), or indirectly through ROCK inhibitor Y-27632. In contrast with RhoN19 that did not affect spreading on FN, WT cells incubated with either inhibitor did not spread on FN as well as untreated cells (Fig. 3C,D). We also observed small but significant increase in the size of WT cells treated with either inhibitor when plated on PDL (Fig. 3C,D). Thus, constitutively active RhoA reduces DKO cell size to WT level, whereas suppression of RhoA activity in WT cells increases their size on PDL, suggesting that decreased RhoA activity significantly contributes to abnormal spreading of DKO MEFs.

3.3. Altered RhoA activity plays a role in focal adhesion formation

The two main functions of RhoA are to promote stress fiber formation and to regulate focal adhesion accumulation [44, 45]. In particular, actin-myosin contractility mediated by ROCK activation via active RhoA provides tension within the cell that promotes the aggregation of integrins and an increase in associated focal adhesions (FAs) [46]. To determine whether arrestin-dependent regulation of RhoA leads to defects in FA formation, we stained DKO and WT MEFs with an antibody against FA marker paxillin. There was a dramatic increase in FA number in DKO MEFs compared to WT on both FN and PDL (Fig. 4A,B) [31]. FA size on FN did not differ between the cell types, but DKO cells plated on PDL have FAs similar in size to cells plated on FN, while FAs in WT cells were significantly smaller on PDL (Fig. 4C).

If decreased RhoA activity in DKO cells is the main cause of increased FA number, the reduction of RhoA activity in WT cells should yield DKO-like phenotype. To test this idea, we expressed dominant-negative RhoN19 in WT cells and plated them on FN or PDL (Fig. 5A). The FA number in WT cells expressing RhoN19 was lower than in WT controls when plated on FN (Fig. 5B), and FAs were smaller (Fig. 5C). However, FAs were increased in number and size when WT-RhoN19 cells were plated on PDL as compared to WT-GFP cells (Fig. 5B,C). Since WT-RhoN19 plated on PDL cells have large sizes (Fig. 3A,B), we asked

how these changes were related to the increased cell size, and measured FA number per $100\mu\text{m}^2$ of the cell area and the percentage of the cell area taken up by FAs. We found that FA number/ $100\mu\text{m}^2$ cell area was decreased in WT-RhoN19 cells, whereas FA area percentage remained unchanged (Fig. 5D,E). Importantly, all FA parameters, the number and FA area in cells, were compromised in RhoN19-expressing WT cells when plated on FN (Fig. 5B-E), which is contrary to the DKO phenotype.

Next, we examined the effect of suppressing RhoA signaling in WT cells by Rho inhibitor C3-transferase or ROCK inhibitor Y-27632 on the FA formation (Fig. 6A). Under both conditions, the numbers and size of FAs were significantly decreased on FN (Fig. 6B,C), which is similar to the effect of RhoN19. Neither Rho inhibitor C3-transferase nor ROCK inhibitor Y-27632 promoted the formation of new FA on PDL, as evidenced by the lack of increase in the FA number per cell (Fig. 6B). However, both drugs significantly increased in WT cells plated on PD the size of FAs that did form, with the ROCK inhibition being more effective (Fig. 6C), the effect similar to that of RhoN19. Similarly to RhoN19, the inhibitors had no significant effect on the FA number/ $100\mu\text{m}^2$ cell area or the fraction of cell area occupied by FAs (Fig. 6D,E).

The data suggest that the regulation RhoA by arrestins appears to be predominately responsible for their effects on cell spreading. Since inhibition of Rho or ROCK shifts the FA towards the DKO phenotype (Fig. 6C), the Rho-ROCK pathway may mediate arrestin-dependent focal adhesion regulation when integrins are not active.

4. Discussion

Small GTPases control cell shape by interacting with a variety of effectors that regulate the cytoskeleton [15, 36]. The dramatically altered morphology of DKO cells suggested that small GTPases are dysregulated. Indeed, we found that basal activity of RhoA and Rac1 was significantly reduced by the deletion of both non-visual arrestins. In contrast to the previous report, which detected enhanced Rac activity in MEFs lacking arrestin-2 [47], we found no changes in the Rac activity in MEFs with one arrestin isoform deleted (Fig. 1A,B). Interestingly, in that study morphological differences between WT and arrestin-2 knockout MEFs were observed that were independent of the presence of serum or the surface coating. In contrast, in our study, although the morphological differences between WT and DKO MEFs were evident regardless of the surface coating, the difference was much more pronounced in the absence of integrin activation, i.e. on PDL coating. RhoA was previously identified as an arrestin-2 target. RhoA-induced stress fiber formation was shown to be dependent on arrestin-2, but not arrestin-3, following activation of the ATIII1 angiotensin receptor [48]. Arrestin-2 was shown to bind Rho GTPase activating protein, ARHGAP21, a known inhibitor of RhoA activity [19]. In both studies arrestin knockdown reduced RhoA activity, similar to our observations in DKO cells (Fig. 2B). However, arrestin-3 was shown to mediate RhoA activation by the β -adrenergic receptor, the mechanism that seems to be involved in the regulation of focal adhesions by this particular receptor [49].

Although arrestins were previously shown to regulate RhoA activation, our study yielded several novel insights. First, knockout of either arrestin individually does not change RhoA

activity in MEFs, whereas over-expression of individual non-visual arrestins in HEK293A increased RhoA activity. Thus, both arrestin-2 and arrestin-3 are capable of facilitating RhoA activation independently of each other. The fact that cell shape is not dramatically altered when arrestin-2 or arrestin-3 was individually knocked out provides further support for this notion (Fig. 1A). However, our data are in direct contrast with the previous report that stable knockdown of arrestin-3 in HEK293 cells enhanced basal RhoA activity, and the basal RhoA activity was also significantly elevated in MEFs lacking arrestin-3 [49]. The reason for this discrepancy is unclear. A possible explanation could be that in our experiments we performed extensive serum starvation in order to exclude any possible receptor activity, which does not seem to be the case in the study by Ma et al. [49]. We found that only when both arrestins isoforms were absent the RhoA activity in MEFs was significantly reduced, which is in agreement with the data that either arrestin can maintain the RhoA activity.

Conversely, normal Rac1 activity required both arrestin-2 and arrestin-3, since Rac1 activity was similarly compromised in MEFs lacking individual arrestins and in DKO MEFs. This suggests that the two non-visual arrestins differentially regulate RhoA and Rac1. Our data are in contrast with the previous finding of enhanced Rac activity in MEFs lacking arrestin-2 [49]. The study did not examine Rac activity in DKO MEFs. Again, the key experimental difference affecting the results could be prolonged serum starvation that we employed in order to isolate receptor-independent effects of arrestins. Additionally, we found no change in the activity of Cdc42 in cells lacking both arrestins and upregulation of the Cdc42 activity in cells lacking either arrestin isoform (Fig. 1E), suggesting a complex mode the Cdc42 regulation by arrestins under basal conditions. Previous reports found that arrestins regulate the Cdk2 activation in the receptor-dependent manner [20, 21], but the basal Cdk2 activity did not seem to be affected by the loss of both arrestins [20] or that of arrestin-2 [47], which partially agrees with our data.

Arrestins are regulatory proteins known to play diverse roles in many facets of cell signaling. Because arrestins move freely in the cytoplasm, they can be recruited to various compartments of the cell to regulate signaling in response to a variety of stimuli. Our studies showed that arrestins regulate cell migration, shape, and adhesion [31]. These processes are complex, requiring hundreds of proteins concordantly acting at the leading edge, cytoskeleton, and at the tail end of the cell. Arrestins bind a wide array of the regulatory proteins involved [50-52]. Therefore, it is not unreasonable to speculate that arrestins may regulate various steps in cell migration. Indeed we have seen this to be true. First, arrestin acts locally at focal adhesions to regulate disassembly via recruitment of clathrin to microtubules targeting FAs [31]. Without arrestins present, FA numbers and FA lifetime dramatically increase. In this study, we describe a different function of arrestins in the regulation of the cell size and adhesion.

Arrestins could affect the cytoskeleton through regulating the basal activity of Rac1 and RhoA. When both arrestins are knocked out, RhoA activity decreases nearly 3-fold, causing a proportional increase in the cell size. We found that the expression of constitutively active RhoA mutant returned DKO cell size back to WT, suggesting that arrestin-dependent regulation of RhoA activity directly affects cell spreading via actin cytoskeleton

rearrangement and FA formation. Importantly, we showed that both arrestin-2 and arrestin-3 regulate RhoA activity independently of receptor activation. To this end, experiments were performed in cells incubated overnight in serum-free medium to ensure the absence of receptor stimulation. Additionally, we demonstrated the rescue of DKO MEF cell size phenotype by expression of arrestin 7 mutants incapable of receptor binding [30]. The reversed rescue experiments in WT cells aimed at reducing the Rho activity also supported a notion that arrestin-dependent regulation of Rho GTPase plays an important role in controlling cell size. Therefore, arrestins keep RhoA activity in balance to maintain proper cell spreading and size.

It is important to note that although the lack of arrestins in MEF cells results in reduced Rac1 activity, constitutively active Rac1 expressed in DKO cells does not rescue cell size. This underscores the complexity of arrestin regulation of the small GTPases. Rho and Rac GTPases act downstream of the integrin signaling, and the formation and maturation of FA depend on the extensive cross-talk between the two classes [53, 54]. Integrin activation results in initial adhesion mediated by nascent FA, the process associated with increased activity of Rac and reduced activity of Rho, whereas maturation of FA anchored to actin stress fibers is associated with reduced Rac and increased Rho activity [53-56]. As we have shown previously, DKO cells have an abundance of mature and very stable FAs [31] and, thus, should be expected have reduced activity of Rac, which they do. At the same time, these cells also should have the elevated Rho activity, but we observed the exact opposite. Considering that DKO cells have equally abundant FAs regardless whether integrins are activated or not, collectively the data suggest that the loss of both arrestins causes the entire signaling network to be rearranged giving the leading role in the control of FA formation to Rho rather than Rac. Elucidation of the precise mechanisms governing arrestin regulation of Rac1 and well as cross-talk among small GTPases yielding distinct functional outcomes requires further investigation.

The most striking result obtained in this study is the difference in the arrestin effect on the cell behavior depending on the nature of the extracellular matrix. A conspicuous property of DKO cells we described before [31] is their ability to spread and form a large number FA equally well when plated on FN, the substrate that supports integrin activation, or PDL, which does not. WT cells, on the other hand, always form significantly fewer FAs, than DKO cells, and very few FAs on PDL, which are also very small (Fig. 4). Here we found that the manipulation of the Rho activity, similarly to arrestins, affects the cells differently depending on the plating substrate. When Rho activity is inhibited in WT cells by the expression of the dominant negative RhoA, inhibition of Rho by C3T, or ROCK by Y27632, the cell size on FN was unchanged or reduced. However in WT cells plated on PDL, where they normally have difficulty spreading, a secondary effect of RhoA inhibition is unmasked that partially recapitulates the arrestin DKO phenotype, i.e. enhanced spreading resulting in a significantly larger cell size. The inhibition of RhoA activity also causes measurable changes in FAs. RhoA is typically known to cause increased FA formation due to its tensile regulation of the actin cytoskeleton and subsequent aggregation of integrins and FA proteins [45, 46, 57]. In particular, arrestins might play a role in mediating GPCR-dependent RhoA activation and subsequent FA formation [49]. In agreement with this notion, we found that in WT cells plated on FN, over-expression of RhoN19 reduced RhoA activity and caused

reduced number and size of FAs. RhoA inhibitor C3-transferase and ROCK inhibitor Y-27632 both have similar effects. What is of particular interest, however, is that when FAs are compromised by plating on PDL, a secondary effect of RhoA inhibition is revealed that partially recapitulates the arrestin DKO phenotype, i.e. increased number and size of FA shifting the FA phenotype of RhoN19-expressing WT cells closer to that of DKO cells.

RhoA inhibitor C3-transferase and ROCK inhibitor Y-27632 only partially replicated the effect of dominant negative RhoA, RhoN19, on FAs in WT cells. As opposed to RhoN19, the inhibitors failed to induce a measurable increase in the FA number, but both drugs significantly increased the size of FAs that do form was, similarly to the effect of RhoN19. The discrepancy between the effect of RhoN19 and the inhibitor drugs on FA could be due to their side effects unrelated to their purported targets. For example, C3-transferase is known to inhibit, in addition to RhoA, its homologues RhoB and RhoC, which have distinct roles in controlling the cell attachment [58]. Y-27632, in addition to ROCK, also inhibits two other Rho-dependent kinases, PRK1 and PRK2, with similar potency [59]. However, the increase in the FA size on PLD caused by Y-27632 is similar in magnitude to the increase caused by RhoN19, which suggests that RhoA-ROCK pathway could be largely mediating this effect. Furthermore, the FA size and not the FA number seems to be the most important determinant of the cell spreading and size [60]. Taken together the data indicate that reduced Rho activity promotes FA formation, improves spreading, and increases the cell size on PDL. Since WT MEFs have difficulties forming FA on PDL and do not spread, it is likely that the increased cell size following Rho inhibition is a direct result of a better spreading on PDL due to an improvement in the FA formation (increase in the FA size or both FA size and number).

This study implicates arrestin-mediated RhoA activation in the FA dynamics and cell spreading and attachment. This role appears to be particularly prominent when the cell attachment occurs without integrin activation and clustering. This suggests that arrestin-dependent (and GPCR-independent) RhoA activation, unlike many other signaling events at FAs [61, 62] can occur in an integrin signaling-independent manner and can in its turn regulate the attachment of cells to such extracellular matrices that do not support integrin signaling. The mechanisms of such attachment are poorly understood and have been studied mostly from the technical standpoint in search of the optimal surface coating for various cell types. Importantly, some cells attach quite well to the surfaces coated with polymers, such as PDL, poly-L-lysine, or polyethylenimine. For example, neuronal cells attach to PDL much stronger than to the surfaces coated with extracellular matrix proteins, such as FN, and develop more neuritis [63]. However, the morphology of neurons attached to PDL differ from that attached to proteins like FN [63] suggesting the involvement of an entirely different signaling network. The ability of DKO MEFs to form FAs equally well with or without integrin activation, which MEFs do not normally possess, suggests a significant rearrangement of the signaling network controlling cell adhesion and spreading. Such rearrangement could be due to the absence regulatory action of arrestins on other components of the normal integrin signaling. It is tempting to speculate that these effects could be via the loss of arrestin-dependent regulation of the activity of c-Src [64], an important component of the integrin signaling complex (reviewed in [65]).

RhoA has two well-known effectors, ROCK and mDia. RhoA activation of mDia causes outgrowth of microtubules and adhesion formation at the leading edge of cells [66] and, therefore, is a likely candidate for future investigation. However, the overall decrease in FAs when RhoA is inhibited suggests that this is not the main mechanism of arrestin-dependent regulation of FAs. Therefore we conclude that arrestins play a role in cell motility, shape and adhesion through regulating actin dynamics, as well as microtubule-directed focal adhesion disassembly.

5. Conclusions

Both non-visual arrestin subtypes regulate the activity of small GTPases RhoA and Rac1, thereby affecting cell spreading and motility. Non-visual arrestins differentially regulate RhoA and Rac1 activity to promote cell spreading via actin reorganization, and focal adhesion formation via two distinct mechanisms. Arrestins act independently of GPCRs. Our data reveal a completely novel function of arrestins. Arrestin-2 and arrestin-3 individually regulate RhoA independently of GPCR stimulation to promote FA formation and proper cell spreading.

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Highlights

- Arrestin-2 and arrestin-3 differentially regulate small GTPases RhoA, Rac1, and Cdc42
- The absence of arrestin-2 and arrestin-3 enhances cell spreading
- Arrestin-2 and arrestin-3 regulate small GTPases and cytoskeleton independently of GPCRs
- Inhibition of RhoA and loss of arrestins improves spreading independently of integrin signaling

Fig. 1. Arrestins regulate the activity of small GTPases RhoA and Rac1

A. Rhodamine Phalloidin staining of cells lacking arrestin-2 (A2KO), arrestin3 (A3KO) or both (DKO) were compared to WT cells after spreading for 2 h on FN or PDL. **B.** Representative Western blots of active and total RhoA, Rac1, and Cdc42 performed as described in Methods. Please, note that the total measurements were made from aliquots the same lysates used to pulldown active GTPases and were not normalized by the protein level. The ratios of active (Active) to total (Total) were computed for each sample, and these data were analyzed by one-way ANOVA with cell type as the main factor. The total levels of

GTPases were measured separately in lysate samples normalized by the protein concentration (Normalized Total) to evaluate the expression levels. **C,D,E.** Active RhoA (**C**), Rac1 (**D**), and Cdc42 (**E**). Means $\pm$ SD (from three experiments) of the levels of active RhoA and Rac1 in arrestin-2 and arrestin-3 single knockout, DKO, and WT cells. The graphs show the data from 3-4 experiments expressed as a percent of the WT levels. * $p < 0.05$ compared to A3KO, *** $p < 0.001$ compared to WT; # - $p < 0.01$ to DKO according to Bonferroni/Dunn post hoc test with correction for multiple comparisons. **F.** RhoA activity in HEK293a cells overexpressing arrestin-2 (Arr2) or arrestin-3 (Arr3). Means $\pm$ SD are shown. Data from two experiments were analyzed by one-way ANOVA, followed by post hoc Scheffe test with correction for multiple comparisons: ** $p = 0.045$ arrestin-2 compared to control, ** $p = 0.0046$ arrestin-3 compared to control. **G.** Representative blot showing increase in RhoA activity in HEK293a cells overexpressing indicated arrestins. **H.** Expression levels of arrestin were measured by Western blot with pan-arrestin (F4C1) antibody [32].

Fig. 2. Small GTPase RhoA affects cell spreading and contributes to the DKO phenotype in a GPCR-independent manner

DKO and WT cells were retrovirally infected with GFP as control (**A**) and DKO cells were infected with HA-RhoV14, HA-RhoN19, HA-RacV12, HA-RacN17 (**B**). Cells were plated on FN or PDL. Scale bar = 10 μm . **C**, Expression of HA-tagged small GTPase mutants or GFP was determined by Western blot. GAPDH was used as a loading control. **D**, Cell size was measured on FN (27-50 cells from 3 experiments) and analyzed by one-way ANOVA, ***p<0.001 to WT-GFP. **E**, Cell size was also measured on PDL (26-40 cells from 3 experiments), ***p<0.001 to WT-GFP and DKO-RhoV14 cells. DKO cells expressing HA-RacV12 were significantly larger than control (GFP expressing) DKO cells, ##p<0.01. **F**, DKO cells expressing receptor binding deficient arrestin mutants, HA-Arr2 Δ 7 and HA-

Arr3 7 were plated on FN and PDL. Scale bar = 10 μ M. **G.** Cell size was measured on FN and PDL. *** $p < 0.001$ from other conditions. FN data are from 37-82 cells/condition from 3-4 experiments. PDL data are from 29-54 cells from three experiments.

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Fig. 3. Decreasing RhoA in WT cells promotes DKO-like phenotype

A. WT cells were infected with retroviruses encoding dominant-negative HA-RhoN19 or GFP; the expression levels are shown on the right. Cells were plated on FN or PDL and stained for actin plus HA or GFP. **B.** Cell size measurements are shown as scatterplots. Data are taken from 30-41 cells from 3-4 experiments. Statistical significance at $p < 0.001$ shown is for WT-RhoN19 on PDL compared to WT-GFP control on PDL according to Mann-Whitney non-parametric test. No significant difference was found between control and WT-RhoN19 cells plated on FN. **C.** WT cells were plated and treated with C3-transferase (RhoA inhibitor; C3T), Y-27632 (ROCK inhibitor), or DMSO (control) for 4 hours. Cells were fixed and stained with rhodamine-phalloidin (for actin). Note the overall reduced size of

cells treated with C3T or Y-27632 as compared to control plated on FN. Also note the appearance of larger cells following treatment of WT cells plated on PLD with C3T or Y-27632, while small cells still remain. **D.** Scatterplots of the cell size data measured in 3-4 experiments (37-41 cells each). The significance levels shown are to respective controls according to Mann-Whitney non-parametric test.

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Fig. 4. Arrestins are critical for focal adhesion formation

A. DKO and WT cells were plated for two hours on FN or PDL and were stained with rhodamine-phalloidin (for actin; red) and with anti-paxillin antibody (green). **B.** FA number was measured in 3-4 independent experiments (37-41 cells total). The significance level by Mann-Whitney non-parametric test shown to corresponding groups of WT on FN or on PDL. The dashed line separates the dataset plotted on the left Y-axis (left) or on the right Y-axis (right). **C.** FA size was measured from same data set as in **(B)**, *** $p < 0.001$ to WT on PDL.

Fig. 5. Arrestin-regulated RhoA activity controls focal adhesion formation

A. WT cells were infected with dominant-negative HA-RhoN19 or GFP and were co-stained with paxillin antibody (red) or HA (RhoN19) or GFP (control) (green). Both red and green channels are shown as black and white. Yellow asterisks indicate co-labeled cells. Focal adhesions were seen in cells expressing HA-RhoN19 when plated on PDL indicated by white arrows (shown in magnified inset). **B.** FA number was measured in WT cells expressing either GFP or HA-RhoN19 and were compared based on substrate. **C.** FA size measured in WT cells expressing either GFP or HA-RhoN19 in the same dataset as in **B.** **D.** FA number per 100 μm of cell area measured in WT cells expressing either GFP or HA-RhoN19 plated on FN or PDL. **E.** Total FA area expressed as a percentage of the cell area.

The data are shown as scatterplots. The significance levels shown are to corresponding WT-GFP groups analyzed separately for the FN and PDL plating by Mann-Whitney non-parametric U-test. Data (**B-E**) were taken from 31-37 cells from 3-4 independent experiments. Note that many WT cells plated on PDL do not have FA, so the number of measurements for FA size and FA area (**C** and **E**) was smaller as compared to WT plated on FN.

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Fig. 6. Arrestin regulated RhoA activity controls focal adhesion formation through two pathways

A. WT cells were plated on either FN or PLD and treated with DMSO (control), C3-transferase (RhoA inhibitor), or Y-27632 (ROCK inhibitor) for 4 hours. Cells were stained with rhodamine-phalloidin (for actin) and anti-paxillin antibody. Paxillin-stained focal adhesions in cells treated with either C3-transferase or Y-27632 and plated on PDL were magnified in the insets and indicated by white arrows. **B.** FA number per cell shown as a scatterplot from 3-4 independent experiments. **C.** FA size was measured from same data set as in **B.** **D.** FA number per 100 μm^2 of cell area. **E.** Total FA area expressed as a percentage of the cell area. In **B-E:** The data were analyzed by Kruskal-Wallis non-parametric analysis of variance separately for the cells plated on FN or PDL; the significance shown is from the Kruskal-Wallis analysis for the respective group; the data were taken from 37-41 cells from

3-4 independent experiments; note that cells plated on PDL have few FA; therefore FA size was measured in 14-16 cells (in **C** and **E**).

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