RbAp48 Regulates Cytoskeletal Organization and Morphology by Increasing K-Ras Activity and Signaling through Mitogen-Activated Protein Kinase

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Abstract

RbAp48 is a WD-40 protein that plays an important role in chromatin metabolism and regulates Ras signaling. Here, we report that RbAp48 is involved in the regulation of cytoskeletal organization, a novel function. First, we show that transfection of RbAp48 into Hs-578T breast cancer cells (Hs-RbAp48-hi) leads to cell size reduction, a rounded cell shape, decreased cellular protrusions, and a higher nuclear/ cytoplasmic ratio. Furthermore, we observed cytoskeletal F-actin organization disruption with loss of actin stress fibers and formation of membranous F-actin rings in Hs-RbAp48-hi cells. These morphologic changes were partially reversed by RbAp48 knockdown. Interestingly, mitogen-activated protein kinase (MAPK) was activated in Hs-RbAp48-hi cells, and this activity was also partly reversed by RbAp48 down-regulation. Furthermore, pharmacologic inhibition of MAPK led to the reappearance of organized actin fibers and focal contacts, suggesting MAPK as the effector pathway. Moreover, we show an increase in total Ras activity in Hs-RbAp48-hi cells with K-Ras-GTP becoming the dominant isoform. This reverted to baseline activity levels on RbAp48 small interfering RNA transfection, thus suggesting a direct role for RbAp48 in Ras regulation. Finally, we tested the model in transformed 3T3-K-Ras-G12V fibroblasts. As expected, RbAp48 knockdown in 3T3-K-Ras-hi fibroblasts resulted in reappearance of an organized cytoskeleton and shutdown of K-Ras activity. In conclusion, our data support a model whereby RbAp48 regulates cellular morphogenesis and cytoskeletal organization by increasing K-Ras activity and signaling through MAPK. [Cancer Res 2007;67(21):10317–24]

Introduction

RbAp48 is a member of the WD-40 protein family (1), originally identified as a binding partner for the retinoblastoma protein (2, 3) and subsequently found to cofractionate with histone deacetylase 1 (4, 5). RbAp48 has been shown to play an important role in several areas of chromatin metabolism, including nucleosome assembly as well as histone modification (6–10). Additionally, it has been found to be a component of several important protein complexes that target chromatin including chromatin assembly factor 1 (11), the Sin3 complex (12), and the Mi-2/NuRD complex (13–17). Furthermore, Hat2p, a yeast protein highly related to RbAp48, is a component of a cytoplasmic histone acetyltransferase with Hat1p (18). Thus, depending on subunit composition, RbAp48 is capable of affecting all stages of chromatin metabolism. Importantly, RbAp48 binds histone H4 (19), thus targeting the enzymatic activity in the complex and allowing histone modification. Consistent with its role in chromatin metabolism, developmental studies in plants have suggested that RbAp48 may play a key role in maintaining epigenetic changes throughout cell division (20).

Although its role in chromatin metabolism is perhaps the best studied of its functions, RbAp48 is involved in other important cellular processes. For example, it has been proposed to play a role in the regulation of Ras-regulated pathways in Caenorhabditis elegans and Saccharomyces cerevisiae (2, 3, 21, 22). Lin-53 (C. elegans homologue) was proposed to antagonize Ras signaling during vulval development in the nematode (21). Additionally, overexpression of MSI1 (S. cerevisiae homologue) led to decreased cyclic AMP (cAMP) levels in response to glucose, which is also consistent with a negative regulatory role of Ras signaling (22).

The Ras signaling pathway is involved in several important cellular processes including proliferation, apoptosis, cytoskeletal organization, and differentiation (23–27). Furthermore, it has been proposed that Ras mediates a central pathway in radiation resistance (28–30). In previous studies, we identified RbAp48 as a key predictive gene in a gene expression radiation sensitivity classifier. Furthermore, we showed that overexpression of RbAp48 in three cell lines led to enhanced radiation sensitivity and dephosphorylation of Akt, suggesting that RbAp48 enhanced radiation response by antagonizing the PI3K Ras effector pathway (31).

One of the phenotypic hallmarks of transformed cells is the disruption of the F-actin cytoskeleton (32–37). This leads to a decrease in the adhesiveness of transformed cells, which is important in cellular migration. The Ras signaling pathway has been shown to mediate these phenotypic changes via activation of either the mitogen-activated protein kinase (MAPK; refs. 36, 37) or PI3K effector pathways (34). In this study, we show that transfection of RbAp48 into Hs-578T breast cancer cells leads to a profound disruption of the F-actin cytoskeleton and cellular morphology. We show that these changes are partially reversible by transfection with RbAp48 small interfering RNA (siRNA) and are dependent on signaling via the MAPK effector pathway. Furthermore, we show that high baseline levels of H-Ras-GTP and N-Ras-GTP activity characterized both Hs-578T parental cells and Hs-578-EV cells. In contrast, K-Ras-GTP becomes the dominant active isoform in Hs-RbAp48-hi cells. Finally, we tested our hypothesis in K-Ras-G12V–transformed 3T3 fibroblasts. Indeed, RbAp48 knockdown in these cells led to reappearance of an organized cytoskeleton and a decrease in K-Ras-GTP activity.
We propose a model whereby RbAp48 regulates cytoskeletal organization by increasing K-Ras-GTP activity and signaling through MAPK.

Materials and Methods

Cells and cell culture conditions. Cells were cultured in DMEM supplemented with 5% BGS and 50 units/mL penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO2. Stably RbAp48-transfected Hs-57ST (Hs-RbAp48-hi) cells and empty vector–transfected Hs-57ST control cells (Hs-57ST-EV) were previously generated by our group (31). G418 (500 μg/mL; Mediatech, Inc.) was used as the selection agent for transfected cells. 3T3-K-Ras-G12V fibroblasts were kindly provided by S. Sebti (Department of Interdisciplinary Oncology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL).

Scanning electron microscopy. Cells at 90% confluence were rinsed twice in serum-free medium at 37°C and fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.2). Cells were initially fixed at 37°C and then refrigerated for 1 h. Cells were rinsed 3× 5 min each in the above buffer at 4°C and dehydrated through a graded series of ethanol, 5 min per change (35%, 50%, 70%, 95% ethanol in distilled water), and in 100% ethanol, 3× 5 min each. Cells were infiltrated with 100% hexamethyldisilazane, 2× 5 min each. After pouring off the hexamethyldisilazane, cells were dried in a vacuum desiccator. Regions of the plastic Petri dishes where the cells were grown were cut out and mounted on aluminum sample holders for the scanning electron microscopy with double-sided carbon tape. Samples were coated with a 20-nm-thick coating of gold/palladium in a sputter coater, observed and photographed with a Philips 515 Scanning Electron Microscope at 15-kV accelerating voltage. Polaroid type 55 film was used to photograph the cells. Random regions of each sample were photographed at a magnification of ×775.

siRNA transfection. Hs-RbAp48-hi cells (3×10⁵) in 2-mL antibiotic-free complete medium were plated in each well of a six-well plate and, after 24 h of incubation, were transfected following the basic dharmaFECT transfection protocol (Dharmacon, Inc.) with either a pool of four negative control siRNAs (siRNA pool) or RbAp48 siRNA designed by Dharmacon with FITC conjugation. The siRNA transfection was performed with Fugene 6 according to the manufacturer's protocol (Roche Molecular Biochemicals). The siRNA transfection efficiency was evaluated by the incorporation of siRNA into the cell nucleus after 24 h of incubation.

Drug treatments. Hs-RbAp48-hi cells were treated every day for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 30 s, then blocked for 30 min with 1% bovine serum albumin (BSA) in PBS at room temperature. Incubations with primary antibodies in 1% BSA against ILK (1:200; mouse monoclonal IgG2b, clone 65.1; Upstate) and paxillin (1:20; mouse monoclonal IgG1, clone 165; BD Bioscience, Pharmingen) were conducted at room temperature for 1 h. After washing, cells were incubated with Alexa Fluor 546 goat anti-mouse IgG2b (1:200; AlexFluor 546 goat anti-mouse IgG1 (γ) secondary antibodies (1:200; Molecular Probes) and with FITC-conjugated phalloidin (10 ng/μL Sigma-Aldrich) for 1 h at room temperature. After washing, coverslips were finally mounted using Vectashield medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Inc.). Samples were examined and pictures acquired on a Leica DMLB upright fluorescent microscope equipped with a QImaging Retina 1300 cooled charge-coupled device camera using the ScionImage software (Scion corporation, Frederick, MD) for image analysis. All photographs were taken at ×40 or ×60 magnification.

Results

Transfection of RbAp48 induces changes in morphology and cytoskeletal disruption in Hs-57ST breast cancer cells. Cellular morphology of stably RbAp48-transfected Hs-57ST (Hs-RbAp48-hi) was assessed by electron microscopy. As shown in Fig. 1A, Hs-RbAp48-hi cells adopted profound RbAp48-dependent changes in morphology, characterized by a rounded cell shape, cell size reduction, decreased cellular protrusions, and a higher nuclear/cytoplasmic ratio, when compared with Hs-57ST and Hs-57ST-EV control cells.

Given the significant morphologic changes observed by electron microscopy, we explored whether the actin cytoskeleton had been disrupted in Hs-RbAp48-hi cells. Indeed, using immunofluorescence, we observed cytoskeletal F-actin organization disruption with loss of stress fibers and strong formation of membranous F-actin rings in Hs-RbAp48-hi cells when compared with Hs-57ST-EV and wild-type controls (Fig. 1B). Because ILK and its interacting protein paxillin are implicated in the assembly of the actin cytoskeleton process (38, 39), we determined whether ILK and paxillin protein levels and their cellular localization were affected. As shown in Fig. 1B and C, RbAp48 overexpression induced a decrease in both ILK and paxillin protein levels. Furthermore, ILK and paxillin, which colocalized to the membrane and the cytoplasm in the parental and empty vector–transfected cell lines, were mainly localized in the cytoplasm in Hs-RbAp48-hi cells. Finally, there was a complete disappearance of focal adhesions in Hs-RbAp48-hi cells when compared with the parental and empty vector–transfected cell lines.

RbAp48 siRNA transfection causes partial reversion of morphology, reorganization of cytoskeleton, and assembly of focal adhesions in Hs-RbAp48-hi cells. To determine whether the cytoskeletal changes were a direct or indirect effect of RbAp48, we transfected RbAp48 siRNA into Hs-RbAp48-hi cells (Fig. 2A), siRNA...
transfection was successful at significantly down-regulating RbAp48, although we could not achieve a complete silencing of the gene. Interestingly, RbAp48 siRNA transfection partially reversed the rounded phenotype of Hs-RbAp48-hi cells to a more flattened phenotype. Furthermore, within 3 days of siRNA transfection, there was a reappearance of organized F-actin fibers. A time course experiment confirmed that these changes were still present up to 10 days after siRNA transfection (Fig. 2B). We then determined whether similar changes would be seen when ILK and paxillin were assessed. Indeed, down-regulation of RbAp48 partially restored cellular colocalization of ILK, paxillin, and F-actin with an objective increase in focal adhesions within 3 days (data not shown) up to 10 days (Fig. 2C), suggesting that the observed morphologic, cytoskeletal, and focal adhesion changes are at least partially a direct effect of RbAp48.

Cytoskeletal disruption, morphologic changes, and loss of focal contacts in Hs-RbAp48-hi cells occur via a MAPK-dependent pathway. Because the MAPK signaling pathway has been reported to inhibit the formation of stress fibers (37, 40), we assessed the activation of MAPK in Hs-RbAp48-hi cells by Western blot (Fig. 3A). Indeed, we found higher levels of phosphorylated MAPK in Hs-RbAp48-hi cells when compared with Hs-578T-EV control cells. Additionally, RbAp48 down-regulation resulted in partial dephosphorylation of MAPK, suggesting that the disruption of the cytoskeleton may be occurring through this pathway. We then assessed the effect of PD098059, a MAPK inhibitor, on the cellular and cytoskeletal morphology of Hs-RbAp48-hi cells. As shown in Fig. 3B, PD098059 was successful in inducing dephosphorylation of MAPK. Seventy-two hours after pharmacologic treatment, we observed, by immunofluorescence, reversion of the Hs-RbAp48-hi cells rounded phenotype to a polygonal shape with restoration of stress fibers (Fig. 3C) and assembly of focal contacts (Fig. 3D), suggesting that the observed RbAp48-induced morphologic, cytoskeletal, and focal adhesion changes were mediated through MAPK activation. In contrast, treatment of cells with LY294002, a PI3K inhibitor, did not affect the morphology or the cytoskeleton of the cells (Fig. 3C).

RbAp48 functions as a positive regulator of K-Ras but a negative regulator of H-Ras and N-Ras. RbAp48 has previously been implicated as a negative regulator of Ras. Furthermore, in previous studies, we had shown that Akt was dephosphorylated in Hs-RbAp48-hi cells (31), which appears to be in conflict with the observation of MAPK activation in the same cells. To reconcile these data, we assessed Ras protein levels and carried out Ras-GTP activation assays (Fig. 4). Interestingly, RbAp48 overexpression induced an overall decrease in Ras protein levels, consistent with its prior attributed role as a negative regulator of Ras (Fig. 4A). In contrast, there was an increase in Ras-GTP activity in Hs-RbAp48-hi cells when compared with the parental and empty vector-transfected cell lines. We then assessed each individual Ras isoform (Fig. 4A). Interestingly, RbAp48 overexpression induced down-regulation of all three Ras isoforms at the protein level. Activation assays showed that, at baseline, Hs-578T cells (parental and empty vector) are predominantly characterized by high H-Ras and N-Ras activities. In contrast, in Hs-RbAp48-hi cells, K-Ras becomes the dominant isoform as the activities of both H-Ras and N-Ras are significantly down-regulated, whereas K-Ras-GTP is up-regulated. Therefore, K-Ras is primarily responsible for the overall increase in total Ras activity, whereas both H-Ras and N-Ras activities are almost completely suppressed in Hs-RbAp48-hi cells. To determine...
whether the increased K-Ras-GTP activity was a direct effect of RbAp48, we transfected Hs-RbAp48-hi cells with RbAp48 siRNA and carried out K-Ras-GTP activation assays. As shown in Fig. 4B, down-regulation of RbAp48 resulted in diminished K-Ras activity, which is similar to the baseline level in parental and empty vector-transfected Hs-578T cells, suggesting a direct role for RbAp48 in the regulation of K-Ras activity.

**RbAp48 knockdown results in reappearance of the F-actin cytoskeleton in 3T3-K-Ras-hi fibroblasts.** The experiments detailed above suggested a direct role for RbAp48 in the regulation of cellular morphology via a K-Ras-MAPK–dependent mechanism. To further test this hypothesis, we did similar experiments in a 3T3 fibroblast model. First, as previously shown by others, transformation with K-Ras-V12 led to disappearance of stress fibers in 3T3 fibroblasts, consistent with K-Ras role in regulation of morphology (Fig. 5). Importantly, down-regulation of RbAp48 in 3T3-K-Ras-hi fibroblasts resulted in reappearance of the stress fibers. Furthermore, as shown in Fig. 5B, RbAp48 knockdown resulted in inhibition of K-Ras-GTP activity, thus confirming our earlier observations in the Hs-578T model.

**RbAp48 is localized in the nucleus and not the cytoplasm in Hs-RbAp48-hi cells.** RbAp48 is a major component of several protein complexes involved in chromatin metabolism. Additionally, RbAp48 has been predicted to form a β-propeller structure similar to β-transducin (41) that allows interaction with multiple proteins (8). To determine whether RbAp48 regulatory influence over K-Ras was due to direct binding between the proteins, we assessed RbAp48 intracellular localization by immunofluorescence. As

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**Figure 2.** RbAp48 siRNA transfection causes partial reversion of morphology, reorganization of cytoskeleton, and assembly of focal adhesion in Hs-578T-RbAp48-hi cells. Hs-RbAp48-hi cells were transfected with either a pool of four negative control siRNAs or RbAp48 siRNA. Forty-eight hours, 6 d, and 9 d after transfection, cells were either plated on fibronectin-coated glass coverslips for immunofluorescence staining 24 h later or harvested for Western blot. A, whole-cell extracts from the three conditions were immunoblotted with anti-RbAp48 and anti–β-actin antibodies. B, Hs-578T-RbAp48-hi transfected for 3, 7, and 10 d with RbAp48 siRNA or pool negative control siRNA were grown on fibronectin-coated coverslips and fixed. Actin stress fibers were visualized by indirect immunofluorescence with FITC-phalloidin. Representative photograph (×40) of three independent experiments. C, focal contacts of Hs-RbAp48-hi cells treated for 10 d with pool negative control are compared with focal contacts of Hs-578T-RbAp48 cells treated for 10 d with RbAp48 siRNA. Fixed cells were stained with phalloidin, anti-ILK, and anti-paxillin monoclonal antibody. Representative photograph (×40) of three independent experiments.
shown in Fig. 6, RbAp48 was only found in the nuclei of both Hs-578-EV and Hs-578-RbAp48-hi cells, suggesting that RbAp48 regulates K-Ras via transcriptional regulation and not by direct interaction. Nuclear localization was also confirmed in 3T3 fibroblasts (data not shown).

Discussion

In this article, we present evidence to support a novel function for RbAp48 in the regulation of cellular morphology. Hs-578T-RbAp48-hi cells were characterized by a round cell shape, cell size reduction, a higher nuclear/cytoplasmic ratio, and a disruption of the actin cytoskeleton. Additionally, RbAp48 overexpression led to down-regulation of both paxillin and ILK and an objective disappearance of focal adhesions. Importantly, transfection of RbAp48 siRNA into Hs-RbAp48-hi cells led to a partial reversion of the morphologic changes, thus supporting a direct role for RbAp48 in mediating them. Furthermore, our data suggest that the RbAp48-induced morphologic and cytoskeletal changes were dependent on activation of MAPK because siRNA knockdown of RbAp48 dephosphorylated MAPK and pharmacologic inhibition of MAPK using PD098059 led to reappearance of stress actin fibers and colocalization of paxillin and ILK. In contrast, inhibition of PI3K did not reverse the morphologic and cytoskeletal changes.

Figure 3. Morphologic, cytoskeletal, and focal adhesion disruption in Hs-578T-RbAp48-hi cells are rescued by reversion of MAPK activation in Hs-578T cells. A, whole-cell extracts from Hs-578T-EV, Hs-RbAp48-hi cells, and Hs-RbAp48-hi cells treated with either pool control siRNA or RbAp48 siRNA were immunoblotted with anti-MAPK and anti–phospho-MAPK antibodies. Representative of three independent experiments. B to D, Hs-RbAp48-hi cells were plated on fibronectin-coated glass coverslips and treated with 0.1% DMSO, 50 μmol/L PD098059, 100 μmol/L PD098059, or 100 μmol/L LY294002 in a six-well plate. After 72 h of incubation, cells grown on the coverslips were fixed for immunofluorescence staining and the remaining cells grown on the six-well plate were lysed for Western blotting. B, whole-cell extracts from Hs-RbAp48-hi cells treated for 72 h with 0.1% DMSO, 50 μmol/L PD098059, or 100 μmol/L PD098059 were immunoblotted with anti-MAPK and anti–phospho-MAPK antibodies. C, Hs-RbAp48-hi cells treated for 72 h with 0.1% DMSO, 50 μmol/L PD098059, 100 μmol/L PD098059, or 100 μmol/L LY294002 were immunoblotted with anti-MAPK and anti–phospho-MAPK antibodies. C, Hs-RbAp48-hi cells treated for 72 h with 0.1% DMSO, 50 μmol/L PD098059, 100 μmol/L PD098059, or 100 μmol/L LY294002 were grown on fibronectin-coated coverslips and fixed. Actin stress fibers were visualized by indirect immunofluorescence with FITC-phalloidin. Representative photograph (×40) of three independent experiments. D, Hs-RbAp48-hi cells either treated or not treated with 100 μmol/L PD098059 for 72 h were evaluated by immunofluorescence. Fixed cells were stained with anti–ILK and anti–paxillin monoclonal antibodies followed by Alexa Fluor 546- and Alexa Fluor 546-conjugated antibodies, respectively. Photographs were taken at ×40 magnification with an inverted confocal microscope. Representative of three independent experiments.
Furthermore, we show that in Hs-RbAp48-hi cells there is an increase in total Ras activity, with K-Ras-GTP becoming the dominant isoform while both H-Ras-GTP and N-Ras-GTP activities were decreased. Interestingly, this occurs in the presence of a general down-regulation at the protein level of all Ras isoforms. Finally, we confirm RbAp48 involvement in K-Ras and cytoskeletal regulation in a 3T3 fibroblast model, suggesting that these observations are part of a general biological process and not limited to the particular context of Hs-RbAp48-hi cells. These experiments support a model in which RbAp48 regulates changes in cellular morphology and F-actin cytoskeleton by up-regulating K-Ras activity and signaling through the MAPK effector pathway.

RbAp48 has previously been reported as a negative regulator of the Ras pathway (2, 3, 21). Ruggieri et al. (22) showed that in S. cerevisiae, overexpression of MSI1 (RbAp48 yeast homologue) resulted in a decreased cAMP level in response to an exogenous growth stimulatory signal (glucose). Furthermore, lin-53, an RbAp48 homologue, has been shown to antagonize vulval induction in C. elegans during development, a process regulated by Ras (21). Our study provides the first evidence that RbAp48 may

![Figure 4](image)

Figure 4. RbAp48 functions as a positive regulator of K-Ras but as a negative regulator of H-Ras and N-Ras. A, Ras activation assays were done in Hs-578T parental (WT), Hs-578T-EV (EV), and Hs-RbAp48 hi cells as detailed in Materials and Methods. Right: expression level of total Ras as well as each of the Ras isoforms. Left, activity level of total Ras and each of the isoforms. β-Tubulin was used as a loading control. Representative of at least three independent experiments. B, Ras activation assays were done in Hs-RbAp48-hi cells untreated or transfected with either RbAp48 siRNA or a pool of four negative controls for 72 h as detailed in Materials and Methods. Left, expression level of total Ras and K-Ras. Right, total Ras and K-Ras activities. β-Tubulin was used as a loading control. Representative of at least three independent experiments.

![Figure 5](image)

Figure 5. RbAp48 knockdown leads to reappearance of F-actin fibers and decreased K-Ras activity in 3T3-K-Ras-G12V fibroblasts. A, 3T3-EV and 3T3-K-Ras-G12V (untreated or treated with either negative control siRNA pool or RbAp48 siRNA for 48 h) were attached to fibronectin-coated coverslips for an additional 24 h. Actin stress fibers were visualized by indirect immunofluorescence with FITC-phalloidin. Representative photograph (×40) of two independent experiments. B, Ras activation assays were done in 3T3-K-Ras-G-12V fibroblasts treated with either negative control siRNA pool or RbAp48 siRNA for a total of 72 h. Representative of two independent experiments.
exert regulatory influence on Ras signaling in mammals. However, our data suggest that RbAp48 may function as either a positive or negative regulator of Ras activity. The mechanistic details of how RbAp48 regulates Ras activity are unclear. However, because RbAp48 is part of the Mi-2/NuRD complex (13–17), one possibility is that it may transcriptionally repress/activate direct regulators of Ras activity. Although Ras-GEFs and GTPase-activating proteins would be the first candidates to consider, the observation that RbAp48 knockdown in 3T3 fibroblasts results in down-regulation of K-Ras-G12V activity argues that a different mechanism might be in play. An alternative is that the regulation occurs via a specific K-Ras binding partner, such as galectin-3, which has been shown to promote strong activation of both wild-type and mutated forms (42).

Previous studies have shown that transformation by Ras leads to profound morphologic and cytoskeletal changes. Interestingly, the effector pathway responsible for the reorganization of the actin cytoskeleton appears to be cell context specific. The PI3Ks have been implicated in membrane ruffling in REF-52 fibroblasts transformed with active Ras (34). In contrast, the MAPK pathway has been implicated as the key effector in Swiss 3T3 cells, Rat1 fibroblasts, rat kidney cells (NRK), and Madin-Darby canine kidney cells transformed with Ras (36, 37, 43, 44). In our experiments, MAPK appears to be the key effector pathway. Several lines of evidence support this conclusion. First, there is increased activation of MAPK in Hs-RbAp48-hi cells when compared with both parental and Hs-578T-EV controls. Further, RbAp48 knockdown results in MAPK dephosphorylation. Additionally, pharmacologic inhibition of MAPK resulted in at least partial reversion of the morphologic and cytoskeletal changes. In contrast, inhibition of the PI3K pathway did not reverse the morphologic and cytoskeletal changes. Additionally, in previous studies, we had shown that Akt is dephosphorylated (31) in Hs-RbAp48-hi cells, which further argues for the involvement of this Ras effector pathway (PI3K) in explaining our observations.

Several investigators have shown that Ras isoforms may preferentially signal through one specific effector pathway (45). For example, Yan et al. (46) have shown that H-Ras is a more potent activator of phosphatidylinositol 3-kinase (PI3K) than K-Ras. In contrast, K-Ras activates both Raf-1 and Rac more efficiently than H-Ras. In addition, Choi and colleagues proposed this differential signal ability by H-Ras and K-Ras as a potential explanation for the

Figure 6. RbAp48 is localized to the nucleus in Hs-RbAp48-hi cells. Hs-RbAp48-hi cells were harvested and stained with anti-RbAp48 and DAPI as described in Materials and Methods and evaluated by immunofluorescence. Representative photographs for each condition.
opposite effects on radiation sensitivity induced by transfection of mutated forms of each isoform into Rast2 cells. These investigators show that whereas H-Ras induced radiation resistance, constitutive activation of K-Ras induced radiation sensitivity (47). Interestingly, we have previously shown that Hs-RbAp48 hi-cells are more radiosensitive than both parental and Hs-578T-EV cells (31). Additionally, K-Ras has been shown to be critical in maintaining the transformed state in colon cancer cells by uncoupling Rho from stress fiber formation (48). It is possible that this may be the downstream mechanism in play in our system because uncoupling of Rho from stress fiber formation required continued signaling through the MAPK effector pathway.

In summary, we provide evidence that RbAp48 leads to morphologic and cytoskeletal changes by increasing K-Ras activity and signaling through the MAPK effector pathway. We propose that RbAp48 may exert its regulatory influence on Ras activity by transcripational regulation of either binding partners or genes upstream of K-Ras activation.

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