



Down-regulation of Rab5 decreases characteristics associated with maintenance of cell transformation



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ARTICLE INFO

Article history:

Received 12 June 2015

Accepted 23 June 2015

Available online 10 July 2015

Keywords:

Rab5

Viability

Cell death

Transformation

Cancer

ABSTRACT

The early endosomal protein Rab5 is highly expressed in tumor samples, although a causal relationship between Rab5 expression and cell transformation has not been established. Here, we report the functional effects of targeting endogenous Rab5 with specific shRNA sequences in different tumor cell lines. Rab5 down-regulation in B16-F10 cells decreased tumor formation by subcutaneous injection into C57/BL6 mice. Accordingly, Rab5 targeting in B16-F10 and A549, but not MDA-MB-231 cells was followed by decreased cell proliferation, increased apoptosis and decreased anchorage-independent growth. These findings suggest that Rab5 expression is required to maintain characteristics associated with cell transformation.

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1. Introduction

Cancer progression is characterized by the acquisition of several traits, including sustained proliferation, resistance to cell death, evading anti-growth factors, replicative immortality, angiogenesis and metastasis [1]. Several of these traits have been shown to be influenced by deregulated organelle and endosome trafficking [2], although the molecular players implicated in these phenomena remain to be fully characterized. Rab GTPases are critical regulators of endosome trafficking with functions ranging from endosome and vesicle formation through tethering, targeting, fusion and transport [3]. In recent years, several Rab proteins have been shown to be altered in cancer, supporting the view that deregulation of components required for intracellular trafficking may be important in cancer progression (reviewed in Refs. [4,5]). In this respect, the early endocytic protein Rab5 is particularly relevant, because it binds a wide range of molecules and effectors, and additional functions have been documented for this GTPase. For instance, Rab5 is required for mitosis progression, by controlling

chromosome alignment, kinetochore assembly and nuclear envelop disassembly [6,7]; moreover, Rab5 is known to promote cell migration and invasion in cancer cells [8–10]. Intriguingly, Rab5 levels are upregulated in lung adenocarcinoma [11], breast cancer [12], hepatocellular carcinoma [13], ovarian cancer [14], thyroid adenoma [15] and cervical carcinoma [9]. Importantly, increased expression of Rab5 is associated with elevated incidence of metastasis [12,16]. Despite this evidence, the precise role of Rab5 in determining characteristics associated with malignant transformation and cancer progression remain poorly understood. In this report, we show that Rab5 is important for the maintenance of characteristics associated with malignant transformation in different cancer cell lines, as shown using both *in vitro* and *in vivo* assays.

2. Materials and methods

2.1. Materials

Monoclonal anti-Rab5 (sc46692) was from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit and goat anti-mouse antibodies coupled to horseradish peroxidase (HRP) and anti-actin antibody (number A5316) were from Bio-Rad Laboratories (Hercules, CA). Tissue culture medium, antibiotics and fetal bovine serum (FBS) were from GIBCO Life Technologies (Grand Island, NY)

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and HyClone Laboratories (Logan, UT). The EZ-ECL chemiluminescent substrate was from Pierce Chemical (Rockford, IL). Rab5 lentiviral short hairpin RNAs (shRNA) were from Open Biosystems (Huntsville, AL).

2.2. Cell culture

MDA-MB-231 human breast cancer cells, A549 lung carcinoma cells and B16-F10 murine melanoma cells were cultured in DMEM-F12, DMEM-high glucose and RPMI medium, respectively, supplemented with 10% FBS and antibiotics. Rab5 targeting was performed as previously described, by using shRNA constructs targeting Rab5A [8,10]. To this end, shRNA sequence #B5, #F8 and #F10 (Open Biosystems) were used to target endogenous Rab5A in A549, B16-F10 and MDA-MB-231 cells, respectively. Control cells were infected with a lentivirus encoding a non-specific shRNA sequence (plasmid 1864; Adgene, Cambridge, MA). Stable cell lines were selected and maintained in puromycin-containing culture medium, as previously reported [10].

2.3. Western blotting

Cells were washed twice with ice-cold PBS and lysed in 0.2 mM HEPES (pH 7.4) buffer containing 0.1% SDS, phosphatase inhibitors (1 mM Na_3VO_4), as well as protease inhibitors. Total protein extracts (50 $\mu\text{g}/\text{lane}$, unless indicated) were separated by SDS-PAGE and transferred onto nitrocellulose membrane. Blots were blocked with 5% milk in 0.1% Tween-TBS and then probed with primary antibodies. Bound primary antibodies were detected with HRP-conjugated secondary antibodies and the EZ-ECL system.

2.4. Apoptosis measurement

Cell death by apoptosis was measured by flow cytometry, following propidium iodide (PI) staining, as previously described [26]. Cells were cultured for 24 h in medium supplemented with 0%, 2% and 10% serum, then harvested and resuspended in PBS. Samples were acquired by FACS (BD FACSCanto) and the extent of apoptosis was determined by plotting PI fluorescence versus the forward scatter parameter, using the *WinMDI 2.8* software. Methanol and TNF α -treated cells were used as controls for normodiploid and hypodiploid (apoptotic) cells, as previously described [26].

2.5. Proliferation assay

Cells were seeded on 96-well plates at a density of 1×10^4 cells per well. Cells were incubated for 24, 48 and 72 h, and optical density (O.D.) was assessed with the MTS[®] kit, by measuring the absorbance at 490 nm, according to the manufacturer's instructions (Promega, Madison, WI, US).

2.6. Anchorage independent growth

Cells (1.5×10^3) were re-suspended in 500 μl culture medium (DMEM-F12, DMEM-HG, RPMI, depending on the cell type) containing 10% FBS and 0.3% low-melting point agarose (Invitrogen, Carlsbad, CA, US). This layer was poured on top of a 500 μl solidified-bottom layer containing 0.8% low-melting point agar in a 24-well plate, allowed to solidify at room temperature and then returned to 37 °C. Samples were photographed at different time points and the number of colonies was determined in 5 random fields per well. Three wells per experiment were analyzed. Data were averaged from three independent experiments. Colony formation was defined as the number of colonies per total of cells in the visual field analyzed.

2.7. Tumor growth assay

Tumorigenicity assays were performed as previously reported by us [27]. Briefly, B16-F10 cells (3×10^5) in 100 μl physiological saline (0.9% NaCl) were injected sub-cutaneously into the flanks of C57/BL6 mice. Appearance of tumors was monitored by palpitation. The largest perpendicular diameters of the resulting tumors were periodically measured, and tumor volumes were calculated according to the following formula: $\text{width}^2 \times \text{length} \times \pi/6$ (Current Protocols in Immunology, 2000). Animals were sacrificed when tumors reached the bioethically permitted limit of 2500 mm^3 . Animal survival is defined as the period post-injection of tumor cells until animals were sacrificed. The experimental protocols employed were approved by the institutional bioethics committee (CBA 0271 CMUCH).

2.8. Statistical analysis

Data were analyzed with unpaired Student's t-tests, by using the *GraphPad Prism 5* software (San Diego, CA, US). Three independent experiments were analyzed, and $p < 0.05$ was considered significant.

3. Results

3.1. Down-regulation of endogenous Rab5 in tumor cell lines decreases cell proliferation and promotes cell death

We previously showed that Rab5 is required to sustain migration and invasion of A549 lung cancer and MDA-MB-231 breast cancer cells [10], as well as B16-F10 mouse melanoma cells [8]. Intriguingly, Rab5 has been shown to be increased in several tumor samples, when compared to non-tumor tissue [9,11–15], although the functional relevance of this increase is unknown. Therefore, we sought to target the endogenous protein in different tumor cell lines using a shRNA-based approach. Rab5 was efficiently down-regulated in A549 and MDA-MB-231 cells, as well as B16-F10 cells using species specific shRNA sequences (Fig. 1A). Intriguingly, down-regulation of Rab5 substantially decreased cell proliferation of A549 and to a lesser extent B16-F10 cells, whereas a negligible decrease in proliferation was observed for MDA-MB-231 cells (Fig. 1B). Additionally, the extent of cell death was evaluated in tumor cells treated with shRNA-targeting endogenous Rab5. As anticipated, Rab5 down-regulation was followed by increased apoptosis levels in A549 and B16-F10 cells, but not in MDA-MB-231 cells (Fig. 1C). No differences were observed for other forms of cell death, such as necrosis (data not shown). Since both cell proliferation and apoptosis are key events de-regulated in cancer cells, these observations prompted us to investigate the requirement of Rab5 in maintenance of further characteristics of cell transformation, including anchorage-independent growth and tumor formation.

3.2. Expression of Rab5 is associated with anchorage independent growth and tumorigenicity

The ability of tumor cells with low endogenous Rab5 levels to grow in an anchorage independent manner was evaluated in soft agar assays. Indeed, A549, B16-F10 and MDA-MB-231 cells readily formed colonies in soft agar and treatment with a control shRNA did not affect this pattern (data not shown). However, shRNA-mediated targeting of Rab5 decreased the ability of A549 and B16-F10, but not MDA-MB-231 cells, to form colonies in soft agar (Fig. 2). Importantly, decreased anchorage-independent growth in

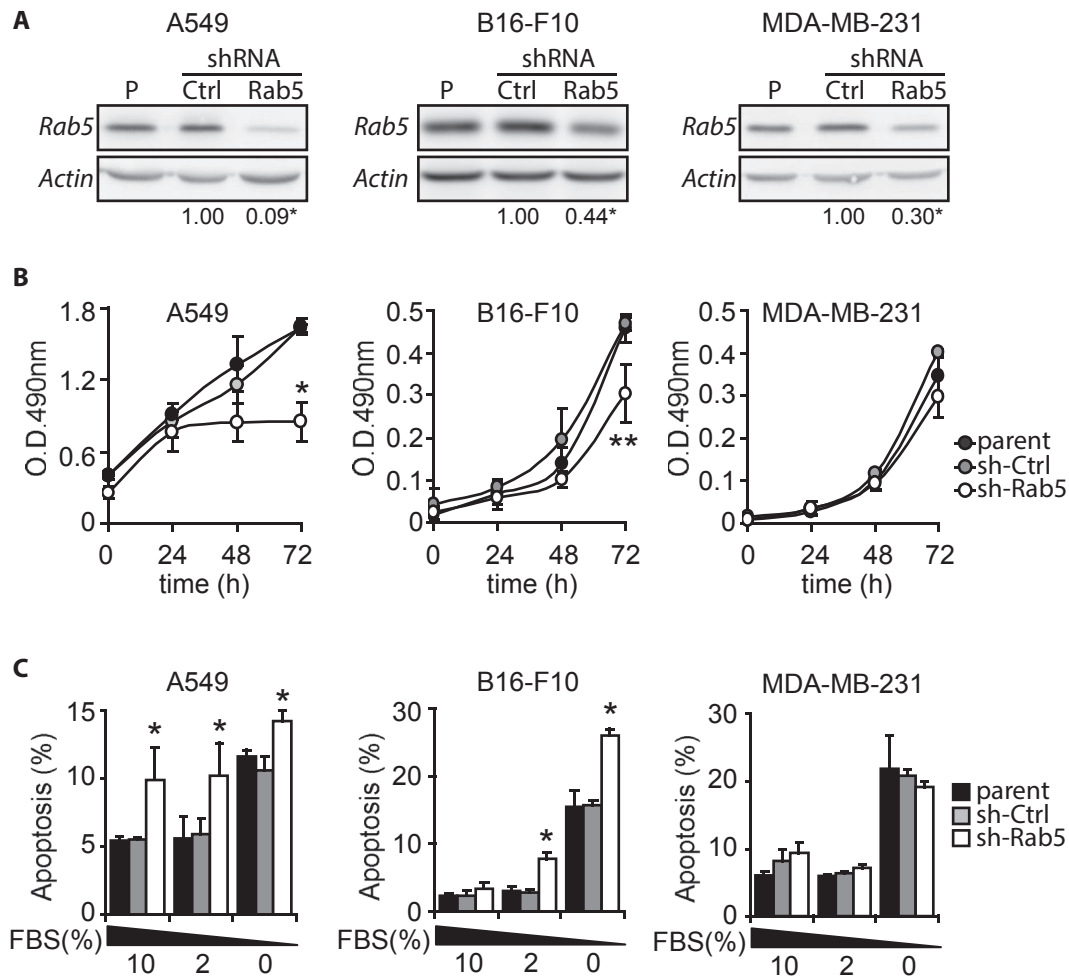


Fig. 1. Rab5 down-regulation by shRNA decreased cell proliferation and increased apoptosis. A549, B16-F10 and MDA-MB-231 cells were transduced with either control or Rab5-specific shRNA constructs (sequences #B5, #F8, #F10; Open Biosystems), as described in the [Materials and methods](#). Stable puromycin-resistant cells were used for subsequent analysis. (A) Whole cell lysates were prepared and proteins were analyzed by Western blotting for Rab5 and actin. Representative images are shown for non-treated cells (parental, P) and cells treated with shRNA-control (Ctrl) or shRNA-Rab5 (Rab5). Residual Rab5 levels were quantified by scanning densitometry and normalized to actin (numerical data below panel). Residual Rab5 levels in A549 (0.09 ± 0.05), B16-F10 (0.44 ± 0.06) and MDA-MB-231 (0.30 ± 0.05) were calculated from three independent measurements (mean \pm s.e.m.). (B) Cell proliferation was measured by the MTS[®] assay, as indicated in the [Materials and methods](#). Cells were allowed to grow for 24, 48 and 72 h, and optical density (O.D.) at 490 nm was recorded. Data represent the average from three independent experiments (mean \pm s.e.m.). * $p < 0.05$; ** $p = 0.06$. (C) Cells were grown for 24 h in culture medium supplemented with 0, 2 and 10% fetal bovine serum (FBS), harvested and incubated with propidium iodide. Apoptosis was measured by flow cytometry, as previously reported [26] (for details, see [Materials and methods](#)). Plotted data were calculated from three independent measurements (mean \pm s.e.m.). * $p < 0.05$.

shRNA-Rab5 cells was also noted at different periods of evaluation in soft agar (data not shown).

In order to validate these findings *in vivo*, we used an isogenic mouse model for tumor formation using B16-F10 cells. Here, B16-F10 cells were injected subcutaneously into C57BL/6 mice and the tumor-forming ability of cells treated with shRNA-Control and shRNA-Rab5 was evaluated. In agreement with data shown in [Figs. 1 and 2](#), down-regulation of endogenous Rab5 in B16-F10 cells led to decreased tumor formation, as shown by comparing shRNA-Rab5 versus shRNA-Control cells ([Fig. 3A](#)). Moreover, treatment with shRNA-Rab5, but not shRNA-Control substantially increased mouse survival ([Fig. 3B](#)). Taken together, these results suggest that the expression of Rab5 is required for different characteristics associated with cell transformation in lung carcinoma and melanoma, but not breast cancer cells.

4. Discussion

Evidence obtained during the last decade has shown that Rab5 is up-regulated in different tumor samples [9,11–15] and its

expression has been associated with poor patient prognosis [12]. In addition to its classical role in intracellular trafficking, Rab5 is necessary for sustaining tumor cell migration and invasion, as shown by both *in vitro* and *in vivo* approaches [8–10,12,16–18] (reviewed in Ref. [19]). However, the precise role of Rab5 in features otherwise commonly associated with early stages of malignant transformation has not been addressed. In this study, we show that Rab5 expression is required for sustaining tumor cell viability and that Rab5 promotes anchorage-independent growth in A549 lung carcinoma and B16-F10 melanoma cells. In addition, we found that Rab5 expression is required for efficient tumor formation *in vivo*, further indicating that Rab5 is necessary to sustain characteristics of malignant transformation. These data are in agreement with earlier studies showing that expression of a GAP-deficient form of p85 α – the regulatory subunit of PI3K – leads to transformation of fibroblasts, as shown by anchorage independence and tumorigenicity *in vivo* [20]. Given that p85 α is a GAP for Rab4 and Rab5 [21], expression of a GAP-deficient p85 α mutant leads to increased cell proliferation and signaling by growth factors [20,22]. However, these studies did not address the precise role of

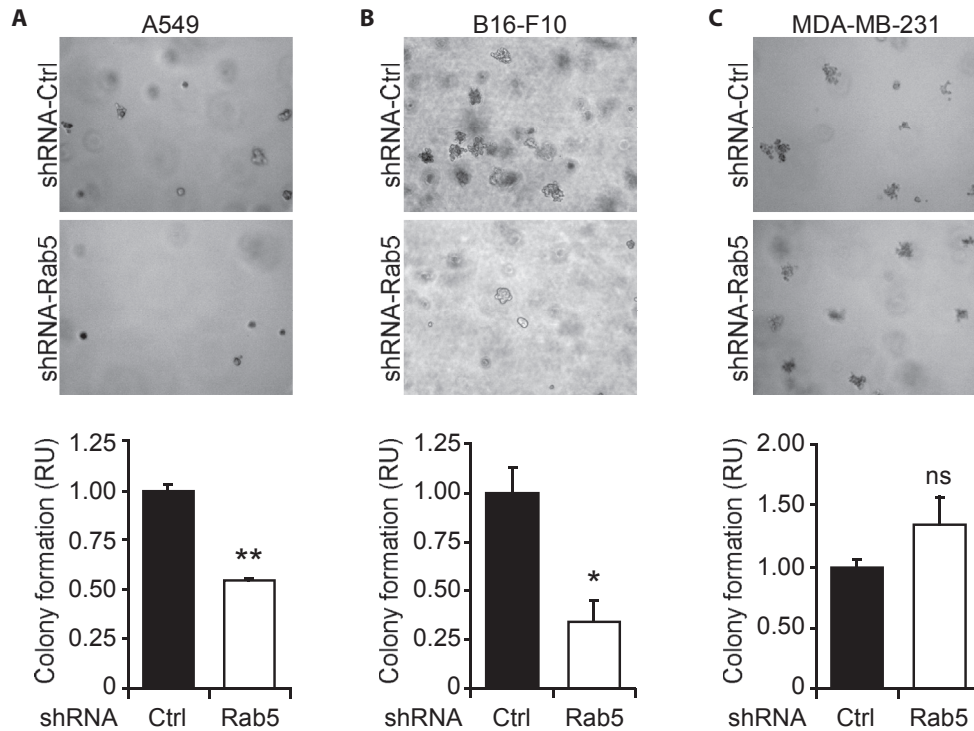


Fig. 2. Targeting of Rab5 decreased anchorage-independent growth. A549, B16-F10 and MDA-MB-231 cells were transduced with either control or Rab5-specific shRNA constructs (sequences #B5, #F8, #F10; Open Biosystems), as described in the [Materials and methods](#). Stable puromycin-resistant cells were used for subsequent analysis. (A) A549, (B) B16-F10 and (C) MDA-MB-231 cells (1.5×10^3 cells/well) were allowed to grow in soft agar for 10, 5 and 6 days, respectively, as described in the [Materials and methods](#). Plates were photographed and the number of colonies recorded in five random fields was determined. Colony forming efficiency was defined as the number of colonies per total number of cells per field. *Upper panels*, representative images from three independent experiments. *Lower graphs* represent the average from three independent experiments (mean \pm s.e.m). * $p < 0.05$; ** $p < 0.01$; ns = no significant.

either Rab4 or Rab5 in maintenance of cell transformation associated characteristics, and this is particularly relevant, because both GTPases are targets of p85 α . Here, we provide evidence indicating that Rab5 is required to maintain tumor cell viability, anchorage-independent growth and tumorigenicity *in vivo*. Whether this phenomenon applies to Rab4 remains to be determined, although recent evidence suggests that both Rab proteins may play similar roles during cancer progression [12].

Rab5 absence reduced tumor cell viability and anchorage independent growth in A549 and B16-F10, but not in MDA-MB-231 cells. One may suspect that these discrepancies are due to the cellular context. In this respect, early endocytic Rabs are known to be functionally redundant to some extent in endosome trafficking [23]. For instance, the early endosome protein Rab21 is required

during cytokinesis and cell proliferation [24,25] and loss of Rab21 function was reported to promote malignant transformation [24]. Thus, Rab5 and Rab21 may converge functionally in MDA-MB-231 cells. This is particularly intriguing given the demonstrated participation of Rab5 in other characteristics associated with tumor progression, including cell migration and invasion not only in the cell models described here (A549, MDA-MB-231, B16-F10), but also other cancer cell lines, including HT-29 colon cancer and neuroblastoma cells [8,10,16]. Understanding precisely the mechanisms underlying these differences requires further investigation.

In summary, our data identify Rab5, a central trafficking protein, as an important factor in maintenance of specific cell traits associated with the transformed state of some, but not all cancer cells.

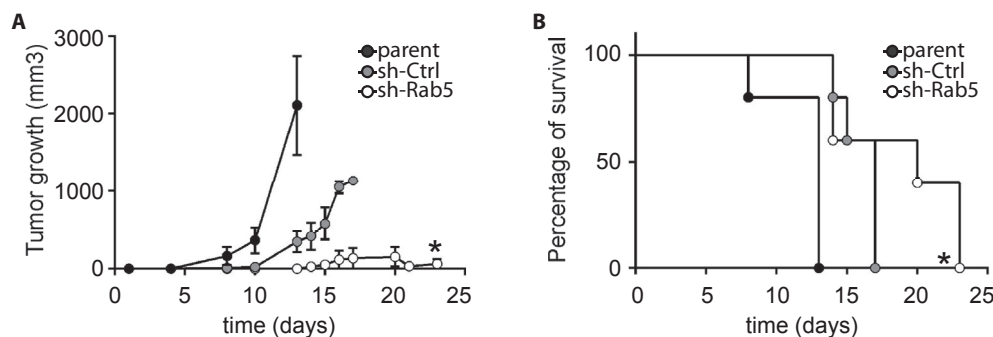


Fig. 3. Down-regulation of Rab5 in B16-F10 cells decreases tumorigenicity. (A) B16-F10 cells were transduced with either control or a Rab5-specific shRNA construct (sequence #F8; Open Biosystems). Stable puromycin-resistant cells (3×10^5) were injected subcutaneously into the one flank of C57BL/6 mice, as described in the materials and methods. Tumor volume was monitored between days 1 and 23. Results shown were averaged from data obtained with 5 mice (mean \pm s.e.m). * $p < 0.05$, compared to sh-Ctrl. (B) Animal survival is plotted as a function of time (mean \pm s.e.m). * $p < 0.05$.

Conflict of interests

The authors have declared no conflict of interests.

Acknowledgments

This work was supported by National Fund for Scientific and Technological Development (FONDECYT) 1140907 and 11100287 (VT); FONDECYT 1130250 (AFGQ); CONICYT-FONDAP 15130011 (VT, AFGQ); Conicyt PhD Student Fellowships (PS, JD, ND).

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.bbrc.2015.06.176>.

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