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Identification of HDAC4 as a target of γ-catenin that regulates the oncogenic K-Ras-mediated malignant phenotype of Rat2 cells

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ABSTRACT

The mechanisms by which activated Ras accelerates malignant transformation of normal cells are not fully understood. Here, we characterized the role and molecular mechanism of γ-catenin in regulating the malignant phenotype of Rat2 cells induced by codon 12-mutant K-Ras (K-Ras12V). Suppression of γ-catenin signaling by K-Ras12V was an early event and played a crucial role in promoting the acquisition of a highly metastatic phenotype of Rat2 cells. Notably, the gene encoding histone deacetylase 4 (HDAC4) was identified as a target of γ-catenin during this process. The transcription factor, lymphoid enhancer-binding factor-1 (Lef1), was involved in the modulation of *HDAC4* transcription, and disruption of this pathway was a key event in promoting the invasion and migration of K-Ras12V-transduced Rat2 cells. Thus, our findings extend the range of targets for the development of new drugs for the therapy of oncogenic K-Ras-driven cancer.

1. Introduction

Activated Ras family proteins can preferentially induce the malignant transformation of cells, a process that is involved in tumor initiation and progression [1]. Ectopic overexpression of these proteins in noncancerous cells can lead to an invasive and metastatic phenotype [2], whereas elimination of Ras function in a nude mouse xenograft model of K-Ras-mutated colorectal carcinoma DLD1 cells inhibits tumor growth [3]. During the malignant transformation process, Ras interacts with and regulates multiple downstream effectors that mediate the pro-oncogenic activity of Ras, such as Raf and Ral guanine nucleotide exchange factor (RalGEF) [4,5], or negatively modulate the oncogenic properties of Ras, such as Ras association (RalGDS/AF-6) domain family (RASSF) members (e.g., Nore1) [6]. Despite the accumulated information on Ras signaling mechanisms, much about Ras-mediated molecular events associated with the malignant phenotypic conversion of cells remains unknown.

γ-Catenin is a major plaque constituent of both desmosomes and adherens junctions. Similar to β-catenin, this protein is anchored to the cytoplasmic tail of cadherin and to the actin cytoskeleton at submembrane sites via α-catenin [7]. The interactions of cell adhesion molecules are necessary for tissue integrity. Thus, changes in the expression of γ-catenin can have both positive and negative cell type-dependent effects on cell fates. Elevated expression of γ-catenin promotes neoplastic transformation of rat kidney epithelial cells [8] and has oncogenic properties in hepatocellular carcinoma [9]. In addition to cell–cell adhesive roles, γ-catenin interacts with signaling proteins and transcription factors to induce cell growth [10]. Notably, when regulated by the tumor suppressor, adenomatous polyposis coli (APC), γ-catenin exerts oncogenic activity via upregulation of c-Myc expression and activation of T-cell factor/lymphoid enhancing factor-1 (Lef/Tcf1) target genes [8]. In contrast, γ-catenin acts as a tumor/metastasis suppressor in mouse and human cells, an action that is dependent on its ability to activate downstream signaling cascades [11,12]. γ-Catenin also readily induces apoptosis in etoposide-treated keratinocytes via regulation of the apoptotic signaling cascade mediated by Bcl-2 family members [13]. However, γ-catenin expression is unchanged during the transformation process induced in PC C13 rat thyroid cells *in vitro* by introduction of the activated Ras [14]. Although γ-catenin is known to have dual functions in neoplastic progression, its involvement in Ras-induced malignant transformation and the potential underlying mechanism is not clear.

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In this paper, we examine the role and signaling functions of γ-catenin in regulating induction of the malignant phenotype of Rat2 cells by activated K-Ras. We found that mutated K-Ras suppressed the expression of γ-catenin and transcription of histone deacetylase 4 (*HDAC4*), which we identified as a genetic target of the γ-catenin/Lef1 complex. These pathways play a critical role in acquisition of the transformed phenotype and thus may provide targets for the development of new therapeutic agents for cancer therapy.

2. Materials and methods

2.1. Cell lines

Rat2 cells were cultured in RPMI-1640 medium supplemented with 5% FBS, 50 μg/mL streptomycin, and 50 units/mL penicillin. Activated K-Ras12V-expressing, transformed Rat2 stable cells (Rat-K-Ras) were established by infecting Rat2 cells with retrovirus encoding K-Ras12V using the Metafectene method (Biontex, Munich, Germany). The infected cells were selected by culturing with 1 μg/mL of puromycin (InvivoGen, San Diego, CA) for 10–15 d.

2.2. Ras activation

Ras-GTP, the activated form of Ras, was assessed using a Ras activation assay kit (Upstate Biotechnology Inc., Lake Placid, NY) according to the manufacturer's instructions. Cells were lysed by incubating with magnesium-containing lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, and protease inhibitors) for 15 min at 4 °C. Cell lysates containing 500 μg protein were incubated with 30 μL of the glutathione *S*-transferase-Ras-binding domain of Raf-1 (amino acids 1–149) for 2 h at 4 °C. The captured active Ras complexes were detected by Western blotting with anti-Ras antibody.

2.3. Retroviral production

The K-Ras12V cDNA (GGT to GTT mutation at codon 12), a gift from Dr. P. Kirshmeier (Schering-Plough Research Institute), was ligated as a *Hpa*I/*Stu*I fragment into the corresponding sites of the MFG retroviral vector. H29D virus-packaging cells were transfected with retroviral vector containing the K-Ras12V gene using the Metafectene method (Biontex). After 48–72 h, virus-containing supernatant was collected, 0.45 μm-filter sterilized, and transferred to Rat2 cells using 8 μg/mL Polybrene (Sigma, St. Louis, MO) or stored in aliquots at −70 °C for later use.

2.4. Construction of expression vectors

The HDAC4 expression construct, pEGFP-HDAC4, was provided by Dr. Claudio Brancolini (Università degli Studi di Udine, Italy). Wild-type α-catenin [15] and non-ubiquitinatable S37A β-catenin [16] were constructed as described previously. The wild-type γ-catenin was generated with the primer pair, 5′-GAATTCATGGAGGTGATGAAC-3′ (sense) and 5′-CTC GAGGGCCAGCATGTGGTC-3′ (antisense). These primers contained either *Eco*RI or *Xho*I restriction enzyme sites (underlined) to facilitate subcloning. The PCR product was subcloned into the pcDNA3.1/myc-His vector (Invitrogen, Carlsbad, CA).

2.5. Knockdown of proteins by small interference RNA (siRNA)

The siRNA oligonucleotides for γ-catenin (sense, 5′-UGAUG AACCUUAUUGAACAUU-3′; antisense, 5′-UGUUCAAUAAGGUUCAUCAUU-3′), Lef1 (sense, 5′-GAAUUAGCGCGG AAAGAAAUU-3′; antisense, 5′-UUUCUUUCCGCGCUAAUUCUU-3′), and HDAC4 (sense, 5′-CAUGGGUUUCUGCUACUUUAAUU-3′; antisense, 5′-UUAAAGUA GCAGAAACCC AUGUU-3′) were synthesized by Genolution Pharmaceuticals, Inc (Seoul, Korea). A scrambled siRNA that showed

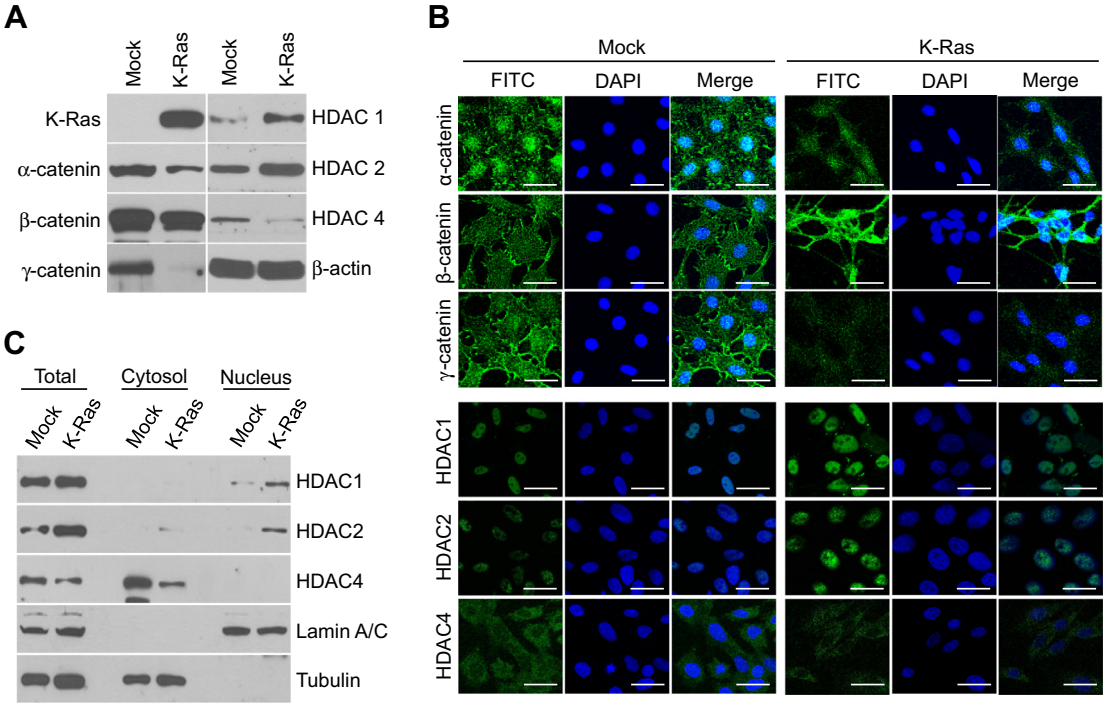

Fig. 1. Changes in the protein expression of catenins and HDACs by oncogenic K-Ras in Rat2 cells. Rat2 cells were infected with control retrovirus or retrovirus encoding activated K-Ras12V for 48 h. (A and B) Protein levels and distributions of catenins and HDACs were determined by Western blotting (A), and by immunofluorescence confocal microscopy (B, scale bar: 50 μm). (C) Cytosolic and nuclear fractions were prepared from each cell line, and HDACs protein levels were determined by Western blotting. Purity of cytosolic and nuclear fractions was determined by detecting tubulin and lamin, respectively.

no significant homology to known gene sequences was used as a negative control. Transfection of siRNAs into cells was performed as described previously [17].

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed as previously described [17]. The following primer pairs were employed for conventional RT-PCR: HDAC4 (330 bp product), 5′-ATGAGCTTCCAAAGCCATCC-3′ (sense) and 5′-ATGTTCATGCAGCTGTGCCT-3′ (antisense); Lef1 (219 bp product), 5′-GCAGCTATCAACCAGATCC-3′ (sense) and 5′-GATGTAGGCAGC TGTCATTC-3′ (antisense); γ-catenin (491 bp product), 5′-GTCTCA GGCGAGGACAGTTC-3′ (sense) and 5′-TCCTGGTAGAGCAGCAGG TT-3′ (antisense); K-Ras (377 bp product), 5′-TGTGGTAGTTG-GAGCTGGTG-3′ (sense) and 5′-CCTGAGCCTGTTTCGTGTCT-3′ (antisense); glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 440 bp product), 5′-ATGGGAAGCTGGTCATCAAC-3′ (sense) and 5′-GGATGCAGGGATGATGTTCT-3′ (antisense). The amplification signals of target genes were normalized against that of GAPDH in the same reaction.

2.7. Western blot analysis

Western blot analysis was done as described previously [17] using primary antibodies against HDAC1, HDAC2, HDAC9, Lef-1, Lamin A/C and tubulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA); α-catenin, β-catenin and γ-catenin (BD Transduction Laboratories, Franklin Lakes, NJ); K-Ras (Calbiochem, La Jolla, CA); and HDAC4 (Cell Signaling Technology Inc., Beverly, MA). β-Actin (Sigma) was used as a loading control.

2.8. Cell fractionation

Cells were lysed with hypotonic buffer (20 mM HEPES pH 7.5, 250 mM sucrose, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail) for 20 min on ice and homogenized by 26 strokes of a Dounce glass homogenizer with a loose pestle (Wheaton, Millville, NJ). The homogenate was centrifuged at 13,000 rpm for 30 s at 4 °C, and the supernatant was collected (cytosol fraction). For isolation of the nuclear fraction, the remaining pellet was suspended in 20 mM HEPES buffer (pH 7.9) containing 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and protease inhibitor cocktail, and centrifuged at 13,000 rpm for 5 min at 4 °C.

2.9. Immunohistochemistry

Cells were fixed with 3.5% paraformaldehyde in PBS for 10 min at room temperature, as described previously [15]. For staining, cells were incubated for 1 h with the following primary antibodies: anti-α-catenin, anti-β-catenin, and anti-γ-catenin (BD Transduction Laboratories); anti-HDAC1 and anti-HDAC2 (Santa Cruz Biotechnology Inc.); anti-HDAC4 (Cell Signaling Technology Inc.). The cells were washed with PBS and incubated with rhodamine- or fluorescein isothiocyanate-conjugated secondary

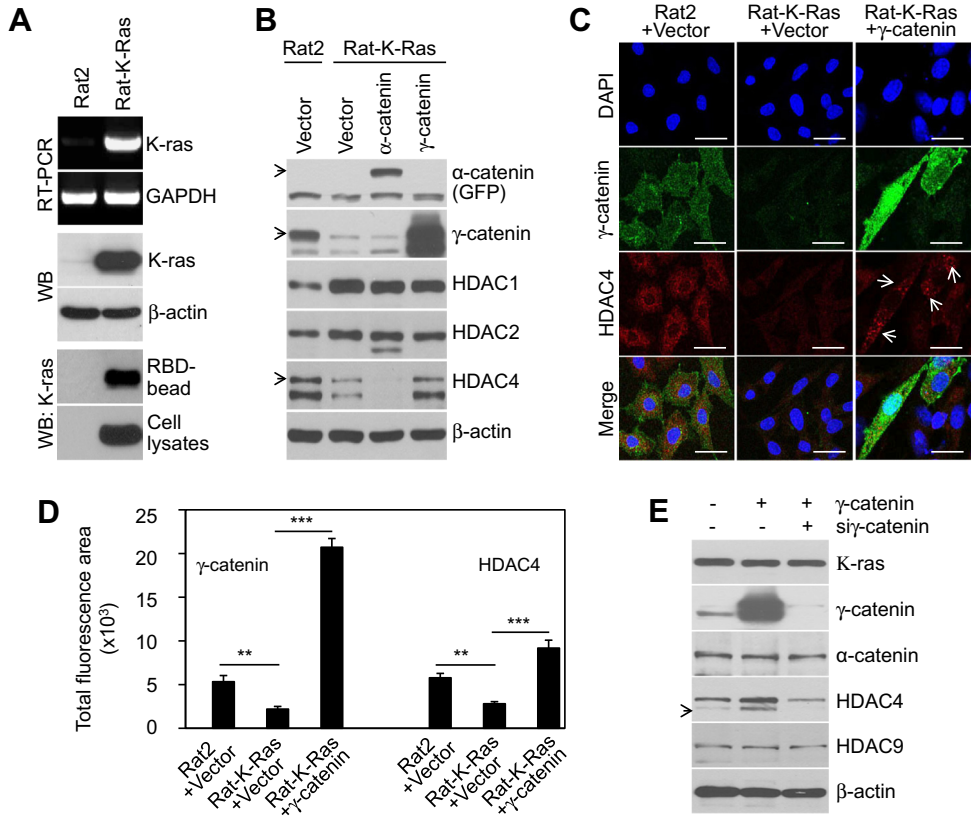

Fig. 2. γ-Catenin-dependent regulation of HDAC4 expression in Rat-K-Ras cells. (A) K-Ras expression and activity were compared between Rat2 and Rat-K-Ras cells. Transcript (*top*) and protein levels (*middle*) of K-Ras were determined by RT-PCR and Western blotting, respectively. K-Ras activity was determined by affinity-precipitation of Ras-GTP using the GST-RBD of Raf-1 conjugated with agarose microspheres (*bottom*). (B–D) Rat2 and Rat-K-Ras cells were transfected with 2 μg control vector, α-catenin expression vector, or γ-catenin expression vector for 48 h, as indicated. (B) Catenins and HDACs protein levels were determined by Western blotting. (C) γ-Catenin and HDAC4 expression were determined by double immunostaining (scale bar: 50 μm). (D) Quantification of the fluorescent area of cells, representing the expressional changes of proteins among cells ($n = 6$, $^{**}p < 0.01$ vs Rat2 cells, $^{***}p < 0.001$ vs Rat-K-Ras cells). (E) Rat-K-Ras cells were transfected with 2 μg control vector or γ-catenin expression vector in the absence (−) or presence (+) of 100 nM γ-catenin- targeting siRNA for 48 h, as indicated. Catenins and HDACs protein levels were determined by Western blotting.

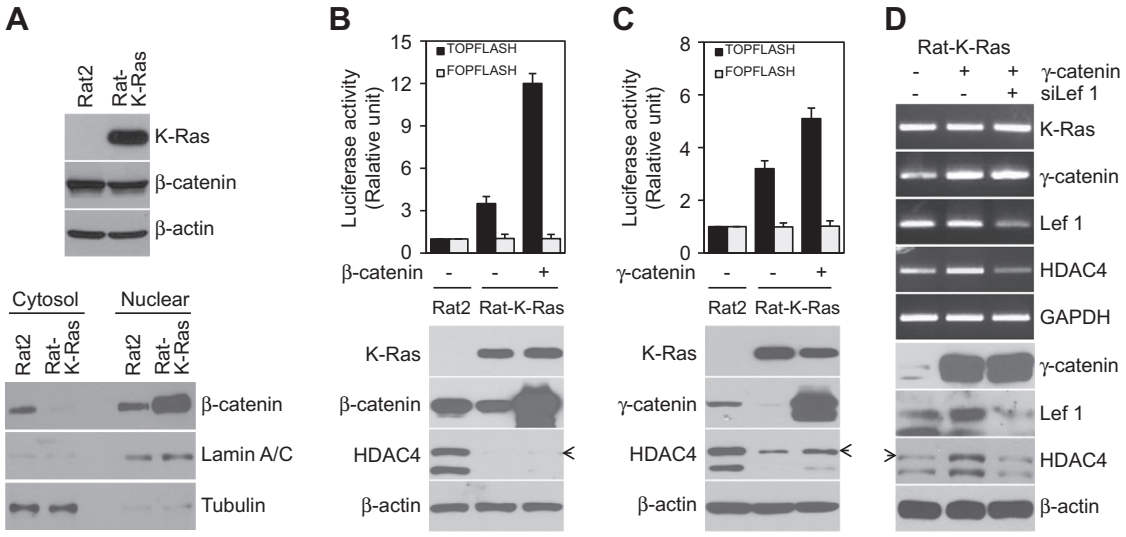

Fig. 3. Involvement of Lef1 with γ-catenin, but not β-catenin, in the regulation of HDAC4. (A) β-catenin protein level was determined by Western blotting using total cell lysates (*top*) and cell fractions (*bottom*). Purity of cytosolic and nuclear fractions was determined by detecting tubulin and lamin, respectively. (B and C) Rat2 and Rat-K-Ras cells were transfected with 2 μg control vector, β-catenin expression vector, or γ-catenin expression vector for 48 h, as indicated. *Top*: Transcriptional activity of Tcf/Lef was determined using active (TOPFLASH) or inactive (FOPFLASH) Tcf/Lef reporter genes. *Bottom*: K-Ras, catenins, and HDAC4 protein levels were determined by Western blotting. (D) Rat-K-Ras cells were transfected with 2 μg control vector or γ-catenin expression vector in the absence (−) or presence (+) of 100 nM Lef1-targeting siRNA for 48 h, as indicated. Transcript (*top*) and protein levels (*bottom*) of K-Ras, γ-catenin, Lef1, and HDAC4 were determined by RT-PCR and Western blotting, respectively.

antibodies (Invitrogen). Cell nuclei were identified by staining with 4,6-diamidino-2-phenylindole (DAPI).

2.10. Assay for metastasis

In vitro invasion and migration assays were performed as described previously [18]. For mobility assays, the monolayer was scraped with a 200-μl micropipette tip to create a wound of 1–2 mm width. The area was subsequently photographed using an inverted phase-contrast microscope at 100× magnification at given time points after scratching (TE300, Nikon, Japan), and the wounded area was defined in each image by positioning lines corresponding to the original scratch.

2.11. Reporter gene assay

The transcriptional activity of the catenin-Tcf/Lef complex was determined using a previously described reporter gene assay [15]. Luciferase activity was measured and expressed relative to β-galactosidase activity to normalize for transfection efficiency.

2.12. Statistical analysis

All data are expressed as mean ± standard error. Statistical evaluation was conducted using a one-way analysis of variance. A p-value <0.05 was considered significant.

3. Results

3.1. Oncogenic K-Ras modulates expression and localization of catenin and HDAC proteins in Rat2 cells

Transient infection of Rat2 cells with retrovirus expressing a K-Ras12V oncogene led to a decrease in the adhesion junction proteins α-catenin and γ-catenin, but not β-catenin, compared to control Rat2 cells (Fig. 1A, left). Exogenous overexpression of K-Ras12V significantly downregulated the class II HDAC protein, HDAC4, and conversely, upregulated the class I HDAC proteins, HDAC1 and HDAC2 (Fig. 1A, right). Quantification analysis using fluorescence intensity confirmed that γ-Catenin and HDAC4 were reduced to 81 ± 6.8% ($n = 7$) and 56 ± 3.9% ($n = 7$) in K-Ras12V-treated Rat2 cells, respectively (Supplementary Fig. 1). Notably, K-Ras12V overexpression in Rat2 cells induced β-catenin translocation from the cytosol to the nucleus without changing total β-catenin protein level (Fig. 1B, top). An analysis of subcellular localization by immunofluorescence staining revealed that HDAC1 and HDAC2 were mainly distributed in the nucleus, whereas HDAC4 was localized to the cytoplasm (Fig. 1B, bottom). We confirmed the modulation and localization of these HDACs by Western blot analysis of subcellular fractionations obtaining results that were completely consistent with immunofluorescence staining experiments (Fig. 1C). These results suggest that alterations of catenins and HDACs may play an important role in the malignant transformation of Rat2 cells by activated K-Ras.

3.2. γ-Catenin regulates expression of HDAC4, but not HDAC1 and HDAC2

Because γ-catenin acts as a transcriptional co-activator through its interaction with members of the Tcf/Lef transcription factor family [19], we examined the effect of γ-catenin on K-Ras12V-induced modulation of HDACs. For this experiment, we established Rat2 cells stably expressing K-Ras12V (Rat-K-Ras) and characterized this cell line by determining K-Ras transcript and protein levels and analyzing mutant K-Ras activation (Fig. 2A). Ectopic overexpression of α-catenin in Rat-K-Ras cells did not change the expression levels of HDACs. Notably, however, we found that overexpression of γ-catenin markedly rescued the downregulation of HDAC4 expression in Rat-K-Ras cells without affecting HDAC1 or HDAC2 level (Fig. 2B). Consistent with the results of Western blotting, HDAC4 protein restored by γ-catenin overexpression was localized in the cytosolic compartment (Fig. 2C). Quantification analysis using fluorescence intensity confirmed that HDAC4 expression by γ-catenin overexpression was increased by 310 ± 9.9% ($n = 6$) in Rat-K-Ras cells (Fig. 2D). To better understand the γ-catenin-dependent induction of HDAC4, we examined the

effect of loss of endogenous γ-catenin function. As shown in Fig. 2E, a γ-catenin-specific siRNA effectively knocked down this protein in γ-catenin-overexpressing Rat-K-Ras cells without changing α-catenin expression, and completely eliminated the strong upregulation of HDAC4 protein induced by γ-catenin accumulation. Notably, depletion of γ-catenin did not alter the levels of HDAC9, a class II HDAC used as a negative control (Fig. 2E). Thus, our results demonstrate that HDAC4 is selectively and positively regulated by γ-catenin, but not by α-catenin.

3.3. Lef1 interacts with γ-catenin, but not β-catenin, to play a role in regulating HDAC4 transcription

We confirmed the nuclear translocation of β-catenin in Rat2 cells transiently infected with K-Ras12V (see Fig. 1B) by examining subcellular fractionations prepared from Rat-K-Ras cell line. As expected, β-catenin was reduced in the cytosolic fraction of Rat-K-Ras cells and showed a reciprocal increase in the nuclear fraction compared to parental Rat2 cells (Fig. 3A, bottom) without changing total protein level (Fig. 3A, top). To examine whether β-catenin, plays a role in the regulation of HDAC4 expression, we ectopically expressed β-catenin in Rat-K-Ras cells. Overexpression of β-catenin in Rat-K-Ras cells caused a significant increase in β-catenin/Lef1 complex transcriptional activity compared to untransfected control and parental Rat2 cells (Fig. 3B, top), but it had no effect on HDAC4 expression (Fig. 3B, bottom). To better understand the relationship between γ-catenin and HDAC4 expression, we transfected Rat-K-Ras cells with a wild-type γ-catenin expression vector. Overexpression of γ-catenin effectively induced an increase in γ-catenin/Lef1 complex transcriptional activity in Rat-K-Ras cells compared to untransfected control cells (Fig. 3C, top). This γ-catenin-induced increase in γ-catenin/Lef1 activity was subsequently accompanied by a restoration of HDAC4 protein levels that had been decreased by activated K-Ras12V (Fig. 3C, bottom). Next, using siRNA to knockdown endogenous Lef1, we examined whether the Lef1 pathway is involved in γ-catenin-mediated HDAC4 regulation. Depletion of Lef1 in Rat-K-Ras cells caused a decrease in both transcript (Fig. 3D, top) and protein (Fig. 3D, bottom) levels of HDAC4, essentially reversing the restoration of HDAC4 expression induced by γ-catenin overexpression. These results suggest that the Lef1 signaling pathway acts as a positive mediator of γ-catenin-induced HDAC4 expression.

3.4. HDAC4 is essential for regulating the oncogenic K-Ras-induced malignant phenotype of Rat2 cells

To elucidate whether HDAC4 is involved in regulating the oncogenic K-Ras-mediated malignant phenotype of Rat2 cells, we tested HDAC4 effects on invasion and migration status. HDAC4-specific siRNA effectively knocked down endogenous HDAC4 expression in Rat2 cells (Fig. 4A, top). Interestingly, depletion of HDAC4 in Rat2 cells induced a slight increase in invasion (29 ± 3.1%, $n = 3$), migration (34 ± 4.5%, $n = 3$), and motility (30 ± 3.6%, $n = 3$), compared to control Rat2 cells (Fig. 4,B and C).

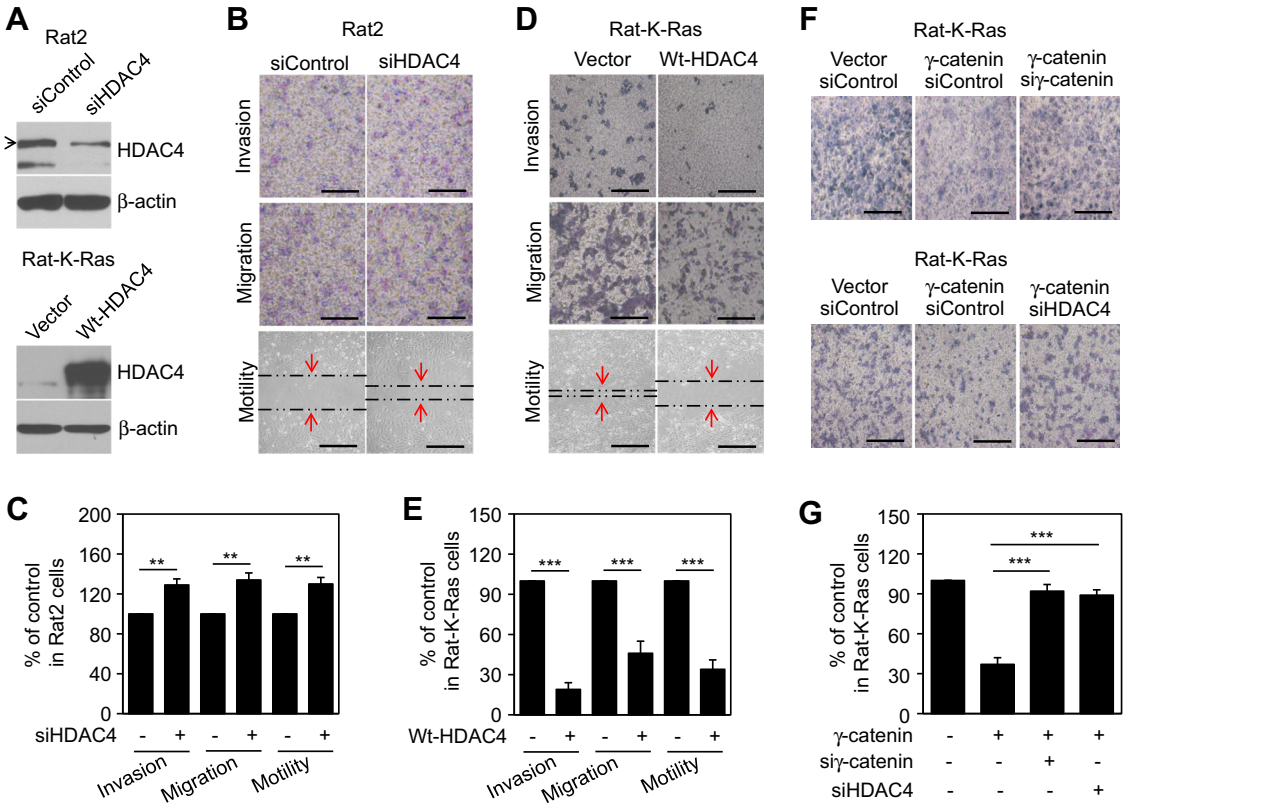

Fig. 4. Inhibitory effect of HDAC4 and γ-catenin on the K-Ras12V-driven malignant phenotype of Rat2 cells. (A–C) Rat2 cells were transfected with 100 nM nonspecific siRNA or HDAC4-targeting siRNA for 48 h; (A, D, and E) Rat-K-Ras cells were transfected with 2 μg control vector or HDAC4 expression vector for 48 h. HDAC4 protein level was determined by Western blotting (A). Transfected cells were seeded in Matrigel-coated transwells and allowed to invade for 24 h. Invading cells were visualized by light microscopy (B and D, *Top* and *Middle*, scale bar: 1 mm). Confluent cells were scratched with a micropipette tip 48 h after transfection, and images were obtained 24 h after creating the scratch (B and D, *Bottom*, scale bar: 1 mm). Data are expressed as means ± SD ($^{**}p < 0.01$, $^{***}p < 0.001$ vs untransfected controls) (C and E). (F and G) Rat-K-Ras cells were transfected with 2 μg control vector or γ-catenin expression vector in the presence of 100 nM nonspecific siRNA, γ-catenin-targeting siRNA, or HDAC4-targeting siRNA for 48 h, as indicated. Invasion assays were performed as in B (F, scale bar: 1 mm). Data are expressed as means ± SD ($^{***}p < 0.001$ vs γ-catenin alone-transfected control) (G).

We next ectopically overexpressed wild-type HDAC4 in Rat-K-Ras cells (Fig. 4A, bottom). As expected, Rat-K-Ras cells displayed much higher levels of invasion, migration, and motility than Rat2 cells (data not shown). However, overexpression of HDAC4 in Rat-K-Ras cells had a dramatic effect on the transformed phenotype, reducing invasion, migration, and mobility by approximately 82 ± 6.3%, 54 ± 4.1%, and 66 ± 5.5% ($n = 3$), respectively compared to control malignant R-K-Ras cells (Fig. 4,D and E). To examine whether γ-catenin regulates the transformed phenotype of Rat-K-Ras cells, we first ectopically overexpressed wild-type γ-catenin in Rat-K-Ras cells. Overexpression of γ-catenin in Rat-K-Ras cells reduced cell penetration through Matrigel-coated membranes to low levels compared to control R-K-Ras cells, whereas siRNA-mediated depletion of γ-catenin in the γ-catenin-overexpressing Rat-K-Ras cells restored invasion activity to its former high levels (Fig. 4F, top). Consistent with the pattern of γ-catenin modulation, siRNA-mediated knockdown of HDAC4 also restored invasive activity to γ-catenin-overexpressing Rat-K-Ras cells (Fig. 4F, bottom). Invading cells were increased by 250 ± 9.2% ($n = 3$) by γ-catenin depletion and 230 ± 8.7% ($n = 3$) by HDAC4 depletion in γ-catenin overexpressing Rat-K-Ras cells (Fig. 4G). Collectively, these results suggest that γ-catenin and its target HDAC4 act as key regulators of activated K-Ras-driven malignant transformation in Rat2 cells.

4. Discussion

Investigating prognostic markers and their signaling pathways is critical in defining the risks of individual cancer patients for radiotherapy or chemotherapy. Identifying molecular mechanisms that promote increased mitogenic signaling through alteration of Ras signal transduction is especially important because Ras mutations are found in approximately 30% of all human malignancies [20]. K-Ras mutations, which are also useful biomarkers for detecting colorectal cancer, have been reported to occur early in tumorigenesis [21]. The Ras/Raf/MEK/ERK cascade is well defined as an essential component of intracellular signaling from activated cell surface receptors to transcription factors in the nucleus, and is associated with tumor initiation and development [22]. However, certain aspects of its function and the role of other signaling pathways in cancer development remain ill defined.

Consistent with the previous demonstration that H-Ras induces invasion and migration of breast epithelial cells [23], we also observed that K-Ras activation led to an increase in invasive and migratory properties of Rat2 fibroblast cells, indicating that this property of Ras extends to different Ras isoforms and cell lines. During this process, activated K-Ras downregulated γ-catenin expression, a phenomenon that played a role in promoting the malignant phenotype of Rat2 cells. How oncogenic K-Ras suppresses γ-catenin expression is unclear and is a subject for future study. To date, the potential role of γ-catenin as oncogene or tumor suppressor has been a matter of controversy [8,9,11,12]. Studies examining γ-catenin signaling in SCC9 cells and non-small cell lung cancer cell lines have concluded that γ-catenin acts through inhibition of the oncogenic activity of β-catenin to function as a tumor suppressor [24]. Our data also suggest that γ-catenin is able to act as a tumor suppressor in normal Rat2 cells, but the mechanism is independent of β-catenin and instead depends on gene regulation by γ-catenin. Similar to β-catenin, γ-catenin regulates gene expression by binding to DNA via interaction with Tcf/Lef family transcription factors. In fact, our results showed that exogenous expression of γ-catenin resulted in increased Tcf/Lef transcriptional activity in oncogenic K-Ras-transduced Rat2 cells, although this activity was less than that of the β-catenin-Tcf/Lef complex (Fig. 3). Importantly, we identified the gene for the class II HDAC family member HDAC4 as a target of γ-catenin, which promoted *HDAC4* transcription in a Lef1-dependent manner, suggesting that the β-catenin/Lef1 complex is unable to promote *HDAC4* gene expression.

Transcription is controlled, in part, by the dynamic acetylation and deacetylation of histone proteins, and HDACs are important mediators of this latter process. The primary role of HDAC4 is to repress gene expression following loss of its interaction with 14-3-3 in the cytoplasm and subsequent interaction with myocyte enhancer factor 2 (MEF2) in the nucleus [25]. Here, we presented evidence that activated K-Ras downregulates HDAC4 expression. This suggests the possibility that the oncogenic-K-Ras signal cascade promotes malignant phenotype-related gene expression by diminishing the function of the HDAC4 repressor, thereby inducing cancer initiation and development. Although additional studies are needed to identify new molecular targets of HDAC4, our gain-of-function and loss-of-function strategies, which showed that overexpression inhibited the malignant phenotype of Rat-K-Ras cells and depletion induced the malignant transformation of Rat2 cells, clearly establish the role of HDAC4 in this process.

In conclusion, γ-catenin has both oncogenic and tumor suppressor functions, reflecting its ability to activate different downstream signaling pathways. Therefore, changes in the expression of γ-catenin can be an important factor governing cell fates, including transformation.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.122.

References

[1] D. Hanahan, R.A. Weinberg, The hallmarks of cancer, Cell 100 (2000) 57–70.
[2] M. Barbacid, Ras genes, Annu. Rev. Biochem. 56 (1987) 779–827.
[3] S. Shirasawa, M. Furuse, N. Yokoyama, T. Sasazuki, Altered growth of human colon cancer cell lines disrupted at activated Ki-ras, Science 260 (1993) 85–88.
[4] P.M. Campbell, A. Singh, F.J. Williams, K. Frantz, A.S. Ulku, G.G. Kelley, C.J. Der, Genetic and pharmacologic dissection of Ras effector utilization in oncogenesis, Methods Enzymol. 407 (2006) 195–217.
[5] A. De Luca, M.R. Maiello, A. D'Alessio, M. Pergameno, N. Normanno, The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: role in cancer pathogenesis and implications for therapeutic approaches, Expert Opin. Ther. Targets 16 (Suppl. 2) (2012) S17–27.
[6] P.M. Campbell, C.J. Der, Oncogenic Ras and its role in tumor cell invasion and metastasis, Semin. Cancer Biol. 14 (2004) 105–114.
[7] C.L. Adams, W.J. Nelson, Cytomechanics of cadherin-mediated cell-cell adhesion, Curr. Opin. Cell Biol. 10 (1998) 572–577.
[8] F.T. Kolligs, B. Kolligs, K.M. Hajra, G. Hu, M. Tani, K.R. Cho, E.R. Fearon, Gamma-catenin is regulated by the APC tumor suppressor and its oncogenic activity is distinct from that of beta-catenin, Genes Dev. 14 (2000) 1319–1331.
[9] K. Endo, T. Ueda, J. Ueyama, T. Ohta, T. Terada, Immunoreactive E-cadherin, alpha-catenin, beta-catenin, and gamma-catenin proteins in hepatocellular carcinoma: relationships with tumor grade, clinicopathologic parameters, and patients' survival, Hum. Pathol. 31 (2000) 558–565.
[10] J. Zhurinsky, M. Shtutman, A. Ben-Ze'ev, Plakoglobin and beta-catenin: protein interactions, regulation and biological roles, J. Cell Sci. 113 (Pt 18) (2000) 3127–3139.
[11] I. Simcha, B. Geiger, S. Yehuda-Levenberg, D. Salomon, A. Ben-Ze'ev, Suppression of tumorigenicity by plakoglobin: an augmenting effect of N-cadherin, J. Cell Biol. 133 (1996) 199–209.
[12] K.M. Rieger-Christ, L. Ng, R.S. Hanley, O. Durrani, H. Ma, A.S. Yee, J.A. Libertino, I.C. Summerhayes, Restoration of plakoglobin expression in bladder carcinoma cell lines suppresses cell migration and tumorigenic potential, Br. J. Cancer 92 (2005) 2153–2159.

[13] R.L. Dusek, L.M. Godsel, F. Chen, A.M. Strohecker, S. Getsios, R. Harmon, E.J. Muller, R. Caldelari, V.L. Cryns, K.J. Green, Plakoglobin deficiency protects keratinocytes from apoptosis, J. Invest. Dermatol. 127 (2007) 792–801.
[14] A. Celetti, C. Garbi, C. Consales, A. Cerrato, D. Greco, E. Mele, L. Nitsch, M. Grieco, Analysis of cadherin/catenin complexes in transformed thyroid epithelial cells: modulation by beta 1 integrin subunit, Eur. J. Cell Biol. 79 (2000) 583–593.
[15] S.G. Hwang, S.S. Yu, J.H. Ryu, H.B. Jeon, Y.J. Yoo, S.H. Eom, J.S. Chun, Regulation of beta-catenin signaling and maintenance of chondrocyte differentiation by ubiquitin-independent proteasomal degradation of alpha-catenin, J. Biol. Chem. 280 (2005) 12758–12765.
[16] J.H. Ryu, S.J. Kim, S.H. Kim, C.D. Oh, S.G. Hwang, C.H. Chun, S.H. Oh, J.K. Seong, T.L. Huh, J.S. Chun, Regulation of the chondrocyte phenotype by beta-catenin, Development 129 (2002) 5541–5550.
[17] J.S. Kim, J.W. Chang, H.S. Yun, K.M. Yang, E.H. Hong, D.H. Kim, H.D. Um, K.H. Lee, S.J. Lee, S.G. Hwang, Chloride intracellular channel 1 identified using proteomic analysis plays an important role in the radiosensitivity of HEp-2 cells via reactive oxygen species production, Proteomics 10 (2010) 2589–2604.
[18] H. Kausar, J. Jeyabalan, F. Aqil, D. Chabba, J. Sidana, I.P. Singh, R.C. Gupta, Berry anthocyanidins synergistically suppress growth and invasive potential of human non-small-cell lung cancer cells, Cancer Lett. 325 (2012) 54–62.
[19] O. Maeda, N. Usami, M. Kondo, M. Takahashi, H. Goto, K. Shimokata, K. Kusugami, Y. Sekido, Plakoglobin (gamma-catenin) has TCF/LEF family-dependent transcriptional activity in beta-catenin-deficient cell line, Oncogene 23 (2004) 964–972.
[20] M. Malumbres, M. Barbacid, RAS oncogenes: the first 30 years, Nat. Rev. Cancer 3 (2003) 459–465.
[21] Z. Lev, D. Kislitsin, G. Rennert, A. Lerner, Utilization of K-ras mutations identified in stool DNA for the early detection of colorectal cancer, J. Cell. Biochem. Suppl. 34 (2000) 35–39.
[22] P.J. Roberts, C.J. Der, Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer, Oncogene 26 (2007) 3291–3310.
[23] I. Shin, S. Kim, H. Song, H.R. Kim, A. Moon, H-Ras-specific activation of Rac-MKK3/6-p38 pathway: its critical role in invasion and migration of breast epithelial cells, J. Biol. Chem. 280 (2005) 14675–14683.
[24] R.A. Winn, R.M. Bremnes, L. Bemis, W.A. Franklin, Y.E. Miller, C. Cool, L.E. Heasley, Gamma-Catenin expression is reduced or absent in a subset of human lung cancers and re-expression inhibits transformed cell growth, Oncogene 21 (2002) 7497–7506.
[25] C.M. Grozinger, S.L. Schreiber, Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization, Proc. Natl. Acad. Sci. USA 97 (2000) 7835–7840.