

## Analysis of KRAP expression and localization, and genes regulated by KRAP in a human colon cancer cell line

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**Abstract** We previously identified the human *KRAP* (Ki-ras-induced actin-interacting protein) gene from the cDNA library of human colon cancer HCT116 cells as one of the genes whose expression levels were up-regulated by activated Ki-ras. Although the *KRAP* gene is structurally conserved from fish to mammalian species, the expression pattern and function of KRAP still remain to be elucidated. Here, we have generated a specific polyclonal antibody for KRAP and characterized the histological expression of KRAP in mouse tissues. KRAP was ubiquitously expressed in mouse tissues, with high levels in pancreas, liver, and brown adipose tissues, and KRAP was co-localized with filamentous actin along the apical membranes in both pancreas and liver tissues. A subfractionation study

revealed that KRAP is a cytoplasmic protein and that the majority is associated with the cytoskeleton. Furthermore, microarray gene expression profile by inhibiting KRAP expression in HCT116 cells showed that several receptors and signal molecules frequently deregulated in cancers were differentially expressed in the KRAP-knockdown cells. All of these results suggested that KRAP might be a cytoskeleton-associated protein involving the structural integrity and/or signal transductions in human cancers.

**Keywords** KRAP · F-actin · Cytoskeletal protein · Pancreas · Liver · Acinar cells · Bile canaliculi · Microarray

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### Introduction

The human *KRAP* (Ki-ras-induced actin-interacting protein) gene was originally identified as one of the genes whose expression levels were up-regulated by activated Ki-ras in human colon cancer HCT116 cells (Inokuchi et al. 2004). While KRAP was rarely expressed in normal colon epithelium, deregulated constitutive KRAP expression was observed in some cancer cells (Inokuchi et al. 2004). In humans, KRAP mRNA was most strongly expressed in the pancreas and testis, with ubiquitous distribution among the other organs at the lower levels (Inokuchi et al. 2004). KRAP is structurally predicted to possess a coiled-coil motif within its C terminus and exogenous KRAP expression was localized along the actin stress fibers in NIH3T3 cells (Inokuchi et al. 2004), suggesting that the KRAP might be involved in the regulation of F-actin. KRAP is structurally well conserved from fish to mammalian species (UCSC Genome Browser) and mouse KRAP has high amino acid sequence identity with

human KRAP, together suggesting that KRAP may play physiologically critical roles. Here, as the first step toward understanding the functional role of KRAP, we generated a polyclonal antibody specific for this protein and investigated the tissue distribution and cellular expression of KRAP by northern blotting, in situ hybridization, western blotting, and immunohistochemical staining in mouse tissues and cells. Furthermore, we analyzed the microarray gene expression profile by inhibiting KRAP expression in HCT116 cells.

## Materials and methods

### Animals

All of the animals used in this study were treated in accordance with the rules of Fukuoka University, Japan.

### Northern blot analysis

Total RNA was extracted from C57BL/6J mouse adult tissues with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The total RNA (15 µg) was electrophoresed in 0.9% agarose/formaldehyde gel and transferred onto a nylon membrane. After fixation by UV crosslinking, the membrane was hybridized with mouse KRAP cDNA (GenBank accession no. AB120565), as described previously (Okumura et al. 1999).

### Generation of polyclonal antiserum

Recombinant human KRAP (amino acids 1039–1246) was expressed as a bacterial fusion protein using the pGEX6P-1 vector (Amersham Pharmacia). The fusion protein was soluble in nondenaturing buffer and was purified with Glutathione Sepharose 4B (Amersham Pharmacia). Antiserum was obtained by injecting the recombinant KRAP protein into Japanese White Rabbit, followed by booster injection. Antiserum was purified with an affinity column prepared by cross-linking the recombinant protein to CNBr-activated Sepharose 4B (Amersham Pharmacia).

### Cell culture

HCT116 cells and HKe3 cells were cultured at 37°C with 5% CO<sub>2</sub> in DMEM containing 10% fetal calf serum (FCS), as described previously (Okumura et al. 1999). NIH3T3 cells were maintained at 37°C with 5% CO<sub>2</sub> in DMEM containing 10% FCS and penicillin–streptomycin–

glutamine. NIH3T3 cells were transfected with a small inhibitory RNA (siRNA) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Two distinct siRNAs were designed to target the coding region of the mouse *KRAP* gene (nucleotides 729–753 or 1970–1994, GenBank accession no. AB120565). Scrambled RNAs containing the same number of each nucleotide as the siRNAs targeting the *KRAP* gene were used as controls. The following siRNA duplexes were used in this study: KRAP #1, 5'-C CUG ACU ACU GUG GCC AAU GCA UUU-3' and 3'-G GAC UGA UGA CAC CGG UUA CGU AAA-5'; scramble RNA #1, 5'-C CUC AUC GUG UAC CGC GUA AAG UUU-3' and 3'-G GAG UAG CAC AUG GCG CAU UUC AAA-5'; KRAP #2, 5'-C CAC ACA CCA UAU UCU CAG AUC CUU-3' and 3'-G GUG UGU GGU AUA AGA GUC UAG GAA-5'; scramble RNA #2, 5'-C CAA CCC UAU ACU CUG AUA CAC CUU-3' and 3'-G GUU GGG AUA UGA GAC UAU GUG GAA-5'.

### RT-PCR

Total RNA was extracted from NIH3T3 cells 48 h after the transfection of *KRAP*-specific siRNAs as described above. RT-PCR was done with Superscript III (Invitrogen) and LA Taq™ Polymerase (Takara), using primers for *KRAP*, 5'-C ATATGACAGAGGAGGACA-3' and 5'-GTGGCTGTCC TGCTTAGG-3'; and *β-actin*, 5'-ATGGATGACGATATC GCTGCG-3' and 5'-GAAGCTGTAGCCACGCTCGG-3'.

### Western blot analysis

C57BL/6J mouse tissues or cultured cells were lysed in RIPA buffer [50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, protease inhibitor cocktail (Roche)] and subjected to western blotting, as described previously (Okumura et al. 1999). Antibodies used for western blotting were as follows: affinity purified anti-KRAP antibodies (dilution 1:2,000), anti-ERK1 antibody (K-23; Santa Cruz Biotechnology; 1:1,000), anti-actin antibody (H-300; Santa Cruz Biotechnology; 1:1,000), anti-CREB antibody (Cell Signaling; 1:500), anti-E-cadherin antibody (BD Biosciences; 1:1,000), and anti-Radixin antibody (C-15; Santa Cruz Biotechnology; 1:500).

### In situ hybridization

An adult ICR mouse was perfused with 10% neutral formalin and the pancreas was dissected. Then, the pancreas was embedded in paraffin. Tissue sections (4 µm) were

dewaxed and hybridized as described previously (Hoshino et al. 1999). A 510-bp DNA fragment corresponding to the nucleotide positions 3250–3759 of mouse *KRAP* cDNA (GenBank accession no. AB120565) in pBluescript II SK(+) was used for the generation of digoxigenin-labeled sense or antisense RNA probes. The expressions were detected using NBT (nitro-blue tetrazolium chloride)–BCIP (5-bromo-4-chloro-3'-indolylphosphatase *p*-toluidine salt) and tissue slides were counterstained with Kernechtrot stain solution (Hoshino et al. 1999).

### Immunohistochemistry

C57BL/6J mouse tissues were frozen in an ethanol/dry ice bath, followed by the preparation of sections (6  $\mu$ m) using a cryomicrotome. Sections were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min at room temperature (RT), blocked with 5% bovine serum, 0.1% Tx-100, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5 for 30 min at RT, and then subjected to double-immunostaining with rabbit polyclonal anti-*KRAP* antibodies (dilution 1:400) and mouse monoclonal anti-insulin antibody: clone K36aC10 (Sigma-Aldrich; 1:2,000). Primary antibodies were visualized with goat anti-rabbit IgG conjugated to FITC or goat anti-mouse IgG conjugated to Alexa Fluor 594. F-actin was visualized with rhodamine-phalloidin (molecular probe) according to the manufacturer's protocol. Fluorescence images were acquired by confocal fluorescence microscopy, as described previously (Inokuchi et al. 2004).

### Subcellular fractionation

The preparation of cytoplasmic and nuclear fractions was performed as described previously (Joo et al. 2004).

Subcellular fractionation of C57BL/6J mouse liver was performed using a compartmental protein extraction kit (Chemicon) according to the manufacturer's protocol. In this assay, cytoplasmic, membrane, and cytoskeletal fractions were sequentially obtained from liver homogenate. Proportional amounts of each fraction were analyzed by western blotting.

### Gene expression profile by inhibiting *KRAP* expression in HCT116 cells

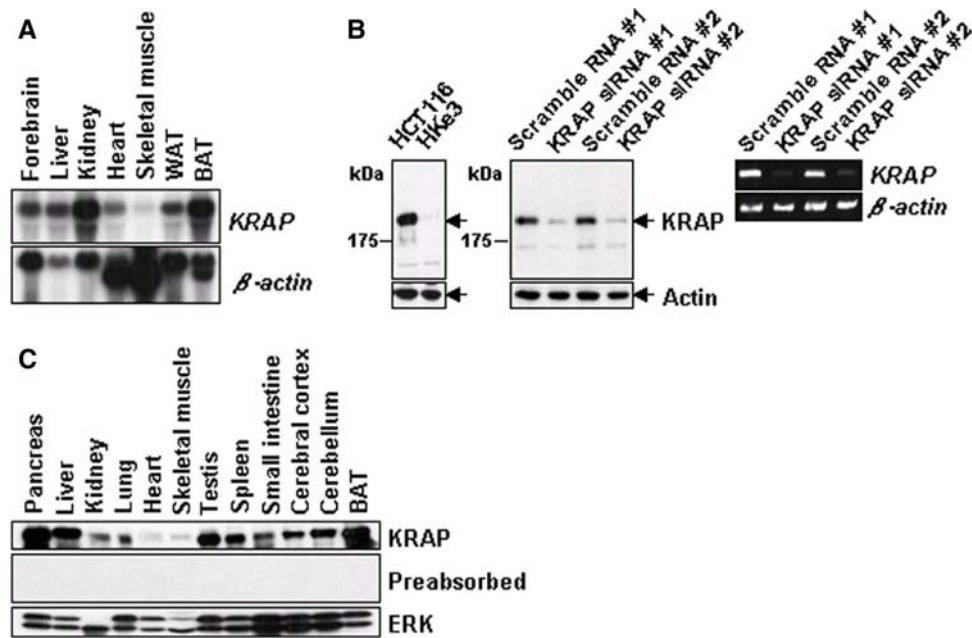
HCT116 cells were transfected with a small inhibitory RNA (siRNA) using MicroPorator MP-100 (Digital Bio) according to the manufacturer's protocol. Three distinct siRNAs were designed to target the coding region of the

human *KRAP* gene (nucleotides 1179–1203, 1524–1548, or 1732–1756, GenBank accession no. AB116937). Scrambled RNAs containing the same number of each nucleotide as the siRNAs targeting the *KRAP* gene were used as controls. The following siRNA duplexes were used in this study: *KRAP* #1, 5'-G GAG AAU GCU GAU AGU GAU AGA AUU-3' and 3'-C CUC UUA CGA CUA UCA CUA UCU UAA-5'; scramble RNA #1, 5'-G GAC GUA UAG UGU GAG AUA AAG AUU-3' and 3'-C CUG CAU AUC ACA CUC UAU UUC UAA-5'; *KRAP* #2, 5'-C CAG CUA GGU CUU ACG AAG UCG AAA-3' and 3'-G GUC GAU CCA GAA UGC UUC AGC UUU-5'; scramble RNA #2, 5'-C CAU AGG UCU UAC GAA GUC GGC AAA-3' and 3'-G GUA UCC AGA AUG CUU CAG CCG UUU-5'; *KRAP* #3, 5'-C CAA CAG CAC AAG ACC AGC CUU AUU-3' and 3'-G GUU GUC GUG UUC UGG UCG GAA UAA-5'; scramble RNA #3, 5'-C CAG AAC ACG ACA ACC GUC UCA AUU-3' and 3'-G GUC UUG UGC UGU UGG CAG AGU UAA-5'. Twenty-four hours after the transfection, the total RNA was isolated from  $1 \times 10^6$  cells with the RNeasy Mini Kit (Qiagen). Biotinylated antisense cRNA was prepared using GeneChip Expression 3'-Amplification One-Cycle cDNA Synthesis kit and GeneChip Expression 3'-Amplification Reagent (Affymetrix) according to the manufacturer's protocol. Biotinylated cRNAs were fragmented and hybridized to Affymetrix GeneChip Human Genome U133Plus2.0 arrays. The arrays were washed and scanned on a GeneChip Fluidics Station 450 and GeneChip Scanner Model 3000 controlled by a GeneChip Operating System v1.4 (GCOS) (Affymetrix) according to standard Affymetrix protocols. The data were annotated according the NetAffx database (<http://www.affymetrix.com/analysis/index.affx>) as of July 2007. Three pairs of comparisons of the gene expression profile between *KRAP*-knockdown cells and the control cells (*KRAP* #1 vs. scramble #1, *KRAP* #2 vs. scramble #2, and *KRAP* #3 vs. scramble #3) was performed. A cut-off value of 1.5-fold or more change between the *KRAP*-knockdown (KD) and the control (Ctl) cells was used.

## Results and discussion

### Tissue distribution of the mouse *KRAP* mRNA and protein

To reveal the tissue distribution of the mouse *KRAP* transcript, a northern blot analysis was performed on the adult mouse tissues (Fig. 1a). *KRAP* mRNA were strongly expressed in the forebrain, liver, kidney, white adipose tissue (WAT), and brown adipose tissue (BAT), whereas it was weakly expressed in the heart and skeletal muscle tissues. Next, to examine the exact tissue distribution of the



**Fig. 1a–c** Tissue distribution of the mouse *KRAP* mRNA and protein. **a** Northern blot analysis of the *KRAP* transcript. Total RNA was extracted from seven kinds of adult mouse tissues and northern blotting was carried out using the probes of *KRAP* (upper panel) and  $\beta$ -actin (lower panel). **b** Detection of *KRAP* protein by western blotting using the anti-*KRAP* polyclonal antibody. *KRAP* expression in HCT116 and HKe3 cells (left panel). *KRAP* protein (middle panel) and mRNA (right panel) expressions in NIH3T3 cells

48 h after the transfection of *KRAP*-specific siRNAs. Scramble RNAs were used as the transfection control. **c** Western blot analysis for *KRAP* expression in the 12 kinds of adult mouse tissues. The specificity of the band was confirmed using anti-*KRAP* preabsorbed with immunizing recombinant protein (middle panel). Western blotting using anti-ERK antibody was done as a loading control (lower panel)

*KRAP* protein, a polyclonal antibody was generated by immunizing a recombinant *KRAP* protein in a rabbit. To validate the reactivity and specificity of the *KRAP* polyclonal antibody, HCT116 cells and HKe3 cells disrupted at activated Ki-ras by homologous recombination (Shirasawa et al. 1993) were examined using this *KRAP* antibody. A 180-kDa band was evidently detected in HCT116 cell extracts (Fig. 1b), whereas the corresponding band in HKe3 cell extracts was rarely detected (Fig. 1b). Furthermore, in NIH3T3 mouse cells, *KRAP* was evidently detected as a 180-kDa protein (Fig. 1b), while the immunoreactive band was dramatically reduced in NIH3T3 cells treated with siRNAs for *KRAP* (Fig. 1b). The RT-PCR analysis of *KRAP* mRNA expression in NIH3T3 cells treated with the siRNAs for *KRAP* validated the specificities of the siRNAs used in this study (Fig. 1b). These results together indicated that the antibody generated here specifically recognizes both human and mouse *KRAP* proteins. Then, western blot analysis using this anti-*KRAP* polyclonal antibody was performed against the homogenates from various tissues of adult mice. Strong expressions of *KRAP* were detected in the pancreas, liver, brown adipose, and testis, and moderate expressions were detected in the kidney, lung, spleen, small intestine, and

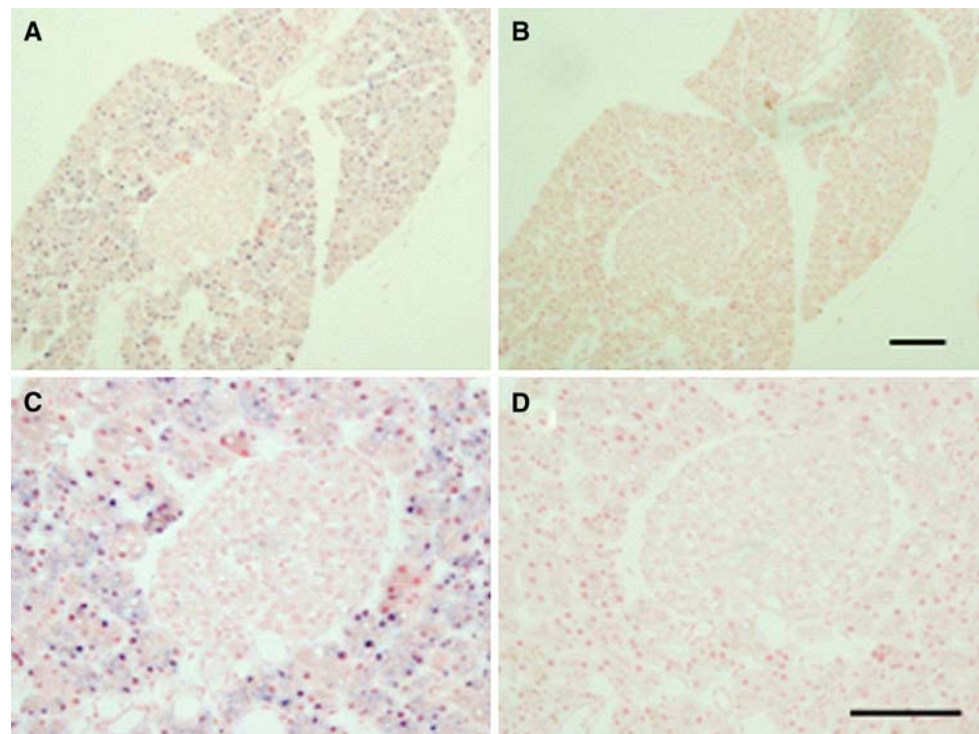
brain (Fig. 1c). On the other hand, *KRAP* was rarely detected in the heart and skeletal muscle tissues (Fig. 1c). The relative amount of *KRAP* protein among the mouse tissues was well correlated with the results from the northern blot analysis (Fig. 1a). Taken together, *KRAP* is an ubiquitous protein in adult mouse tissues.

#### Regional and cellular localization of *KRAP*

In situ hybridization was performed to reveal the distribution of the *KRAP* transcript in the adult mouse pancreas. *KRAP* was restricted to the exocrine acinar region of the pancreas and no significant signals were observed in the islet region (Fig. 2a, c). To examine the precise regional and cellular localization of the *KRAP* protein, we carried out immunohistochemical analysis on the mouse pancreas and liver sections. In the pancreas, *KRAP* staining was observed in the exocrine acinar cells, while the signals were not detected in the insulin-positive islet region (Fig. 3a–c), confirming the previous result obtained by in situ hybridization that the *KRAP* transcript was restricted to the exocrine acinar region in the pancreas (Fig. 2a, c). Double immunohistochemical staining using the *KRAP*



**Fig. 2a–d** In situ hybridization analysis of *KRAP* mRNA expression. A section of an adult mouse pancreas was hybridized with the *KRAP* antisense RNA probe (**a** and **c**). Control hybridization was performed with the sense probe on consecutive sections (**b** and **d**). The scale bar represents 100  $\mu$ m



antibody and phalloidin showed that KRAP was co-localized with F-actin along the apical membrane (Fig. 3d–f). In the liver, KRAP was detected as a belt-like localization along the bile canalicular membrane of hepatocytes and was co-localized with F-actin (Fig. 3g–i). These cellular localizations were correlated with the former observation that ectopically expressed KRAP protein was localized along the F-actin in NIH3T3 cells (Inokuchi et al. 2004). Furthermore, the common feature of the KRAP localization along the apical membrane in the two distinct tissues suggested the possibility that KRAP might be localized in a similar fashion to other tissues.

#### Subcellular distribution of KRAP

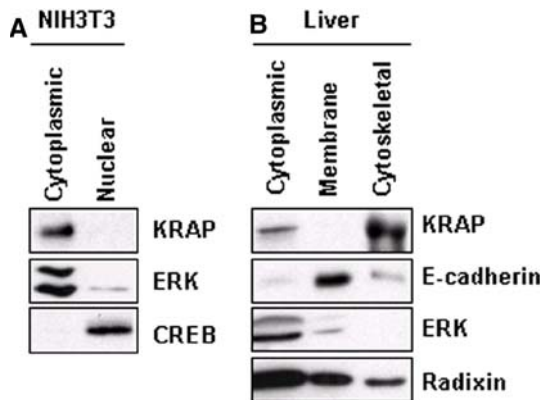
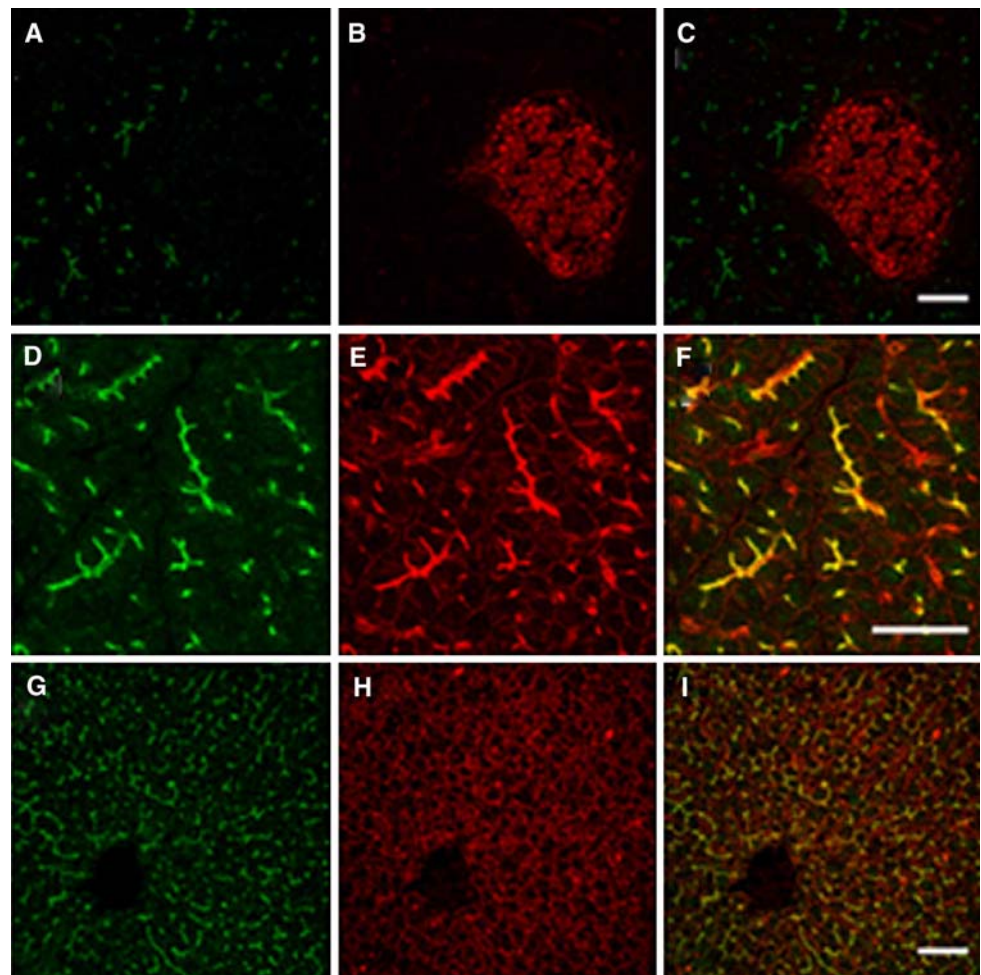
The subcellular fractionation of NIH3T3 cells was carried out to understand the localization of KRAP. A cytoplasmic protein-ERK (p44/42 MAP kinase) and a nuclear protein-CREB (cyclic-AMP response element-binding) were expectedly fractionated into the cytoplasmic and nuclear fractions, respectively (Fig. 4a). In this condition, KRAP was exclusively detected in the cytoplasmic fraction. Next, the subcellular localization of KRAP was examined by the biochemical fractionation of mouse liver tissue. ERK and an integral protein-E-cadherin were expectedly fractionated into the cytoplasmic and membrane fractions, respectively (Fig. 4b). In this condition, KRAP was detected in the cytoplasmic fraction and the majority of KRAP protein was

concentrated in the cytoskeletal fraction (Fig. 4b), suggesting that KRAP is a cytoplasmic protein associated with the cytoskeleton. Radixin is an adaptor protein concentrated in the apical membranes in hepatocytes (Kikuchi et al. 2002; Fouassier et al. 2001). In our assay, Radixin was expectedly detected in the cytoskeletal fraction, but the significant amount of this protein was readily solubilized in the cytoplasmic fraction compared with KRAP (Fig. 4). All of these results suggested the possibility that KRAP may be a constituent of cytoskeleton-associated proteins anchoring integral and/or signal proteins.

#### Gene expression profile by inhibiting KRAP expression in HCT116 cells

To reveal the functions of KRAP, KRAP expression was inhibited using siRNAs and microarray gene expression analysis was performed in a colon cancer cell line, HCT116. The treatment of HCT116 cells with three distinct siRNAs specific for *KRAP* sequence led to a significant decrease in KRAP protein expression ( $81.4 \pm 2.2\%$ ) compared to scramble RNA-treated controls (Fig. 5a). For the microarray gene expression analysis, a cut-off value of 1.5-fold or more change between the KRAP-knockdown (KD) and the control (Ctl) cells was used. A total of 113 probesets on the microarray were commonly changed in the three pairs of comparisons. Out of 113 probesets, 70 and 43 were up- and down-regulated

**Fig. 3a–i**  
 Immunohistochemical localization of KRAP in the mouse pancreas and liver. Immunofluorescence photomicrographs for KRAP (**a** and **d**), insulin (**b**), F-actin (**e**), and merged images (**c** and **f**) in the pancreas. Immunofluorescence photomicrographs for KRAP (**g**), F-actin (**h**), and merged images (**i**) in the liver. The scale bar represents 50  $\mu$ m

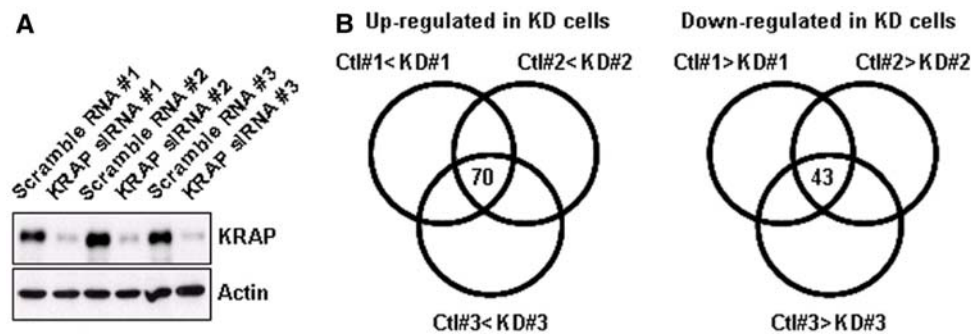


**Fig. 4a, b** Subcellular distribution of KRAP. **a** NIH3T3 cells were fractionated into cytoplasmic and nuclear fractions. **b** Mouse liver homogenate was fractionated into cytoplasmic, membrane, and cytoskeletal fractions. Proportional amounts of each fraction were analyzed by western blotting using anti-KRAP, anti-CREB, anti-E-cadherin, anti-ERK, and anti-Radixin antibodies

in KD cells, respectively (Fig. 5b). The expression data for these probesets are provided in the supplementary material (Supplementary Table S1). Two distinct probesets for *KRAP* (236207\_at, 202506\_at) were down-regulated by

siRNA treatments, showing the reduction of *KRAP* expression (Supplementary Table S1).

Gene ontology analysis by GeneSpring GX 7.3.1 showed that genes with differential expression levels between KD and Ctl cells were associated with protein kinase C activation, cellular morphogenesis, lipoprotein catabolism, inositol phosphate-mediated signaling, G-protein signaling, the regulation of MAPK activity, and cell-surface-receptor-linked signal transduction (Supplementary Table S2), suggesting that *KRAP* expression might affect the cellular malignancy by altering the diverse signaling pathways. Among the differentially expressed genes (Supplementary Table S1),  $\alpha_V$  integrin,  $\beta_1$  ntegrin, *GNAS complex locus (GNAS)*, *mitogen-activated protein kinase kinase kinase (MAP4K3)*, *interleukin 6 receptor (IL6R)*, *interleukin 6 signal transducer (IL6ST)*, *interleukin 1 $\beta$  convertase (CASP1)*, and *EPH receptor B2 (EPHB2)* were remarkably interesting from the viewpoint of the cancer biology. Among the integrin receptors,  $\alpha_V\beta_1$ ,  $\alpha_3\beta_1$ ,  $\alpha_5\beta_1$ , and  $\alpha_6\beta_4$  integrins are associated with enhanced cell motility and cancer metastasis (Felding-Habermann 2003). One of the important functions of the  $\beta_1$  integrin is to



**Fig. 5a, b** Gene expression profile by inhibiting KRAP expression in HCT116 cells. **a** KRAP protein expression in HCT116 cells 24 h after the transfection of three distinct *KRAP*-specific siRNAs. Scramble RNAs were used as the transfection controls. **b** Venn diagram showing the up-regulated 70 probesets and down-regulated 43 probesets in *KRAP*-knockdown (*KD*) cells. A cut-off value of

1.5-fold or more change between the *KD* and the control (*Ctl*) cells was used. The up-regulated genes included  $\alpha_v$  integrin,  $\beta_1$  integrin, *GNAS*, *MAP4K3*, *IL6R*, and *IL6ST*. The down-regulated genes included *CASP1* and *EPHB2*. The data of the differentially expressed genes is provided in Supplementary Table S1

suppress apoptosis in mammary epithelial cells through the negative regulation of interleukin  $1\beta$  convertase (*CASP1*) (Boudreau et al. 1995). Both  $\beta_1$  integrin and *CASP1* were changed in *KD* cells in our analysis (Supplementary Table S1), suggesting that there might be a functional interaction between  $\beta_1$  integrin and *CASP1* in HCT116 cells. Loss- and gain-of-function mutations of *GNAS* involved in the cyclic AMP-dependent pathway cause human diseases (Weinstein et al. 2006). *MAP4K3* is a component in the nutrient-responsive pathway affecting cell growth (Findlay et al. 2007). Deregulated expression of various EPH receptors, including *EPHB2*, has been reported in diverse tumor types (Guo et al. 2006; Jubb et al. 2005). All of these results suggested that *KRAP* might play critical roles in cancer-associated signaling pathways, including Ras, MAPKs (MAP kinases), integrins, EPH receptors, and protein kinase C.

In this study, we demonstrated that *KRAP* was ubiquitously expressed in mouse tissues and was associated with cytoskeletal fraction, and the microarray-based gene expression analysis revealed that *KRAP* affected particular receptors and signal molecules involved in cellular malignancy. These findings together suggested that *KRAP* may influence the cellular events related to attachment, migration, proliferation, and apoptosis. Further analyses of *KRAP* should be awaited for the exact understanding of *KRAP* functions in cancer cells.

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