NORE1B Is a Putative Tumor Suppressor in Hepatocarcinogenesis and May Act via RASSF1A

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Abstract

Recently, we found epigenetic silencing of the Ras effector genes NORE1B and/or RASSF1A in 97% of the hepatocellular carcinoma (HCC) investigated. This is strong evidence that the two genes are of major significance in hepatocarcinogenesis. Although RASSF1A serves as a tumor suppressor gene, the functions of NORE1B are largely unknown. Here, we studied the role of NORE1B for growth and transformation of cells. To understand the molecular mechanisms of action of the gene, we used the wild-type form and deletion mutants without the NH2 terminus and CENTRAL domain, the gene, we used the wild-type form and deletion mutants (36x418) to interact closely with RASSF1A, as determined with fluorescence resonance energy transfer. In further studies, cell cycle delay by NORE1B was equally effective in hepatocyte cell lines with wild-type or mutant Ras suggesting that NORE1B does not interact with either Ras. In conclusion, NORE1B suppresses replication and transformation of cells as effectively as RASSF1A and thus is a putative tumor suppressor gene. NORE1B interacts physically with RASSF1A and functional loss of one of the interacting partners may lead to uncontrolled growth and transformation of hepatocytes. This may explain the frequent epigenetic silencing of NORE1B and/or RASSF1A in HCC. [Cancer Res 2009;69(1):235–42]

Introduction

Hepatocellular carcinoma (HCC) is among the most frequent types of cancer worldwide accounting for approximately half a million deaths per year (1). The molecular mechanisms underlying the development of this disease are not well-understood (2, 3). In sharp contrast to other types of human malignancies, activating mutations in one of the Ras genes occur rarely in HCC (3–5). We recently found epigenetic inactivation of the Ras effector genes NORE1B and RASSF1A in the majority of HCC due to CpG-site hypermethylation in the promoter regions. As a result, 97% of the 28 tumors studied revealed epigenetic inactivation of NORE1B, RASSF1A, or both (6). However, the Ras effector NORE1A was not affected by hypermethylation. This raised our interest in the significance of NORE1B and RASSF1A for the development of liver cancer.

Ras effectors are defined as proteins with strong affinity to GTP-charged forms of Ras, Rap-1, and several other Ras subfamily GTPases (5). Currently, there are >10 different Ras effectors, such as RAF, RalGDS, phosphatidylinositol 3-kinase, and the RASSF gene family comprising RASSF1 and NORE1 (RASSF5; refs. 5, 7–11). The interaction of these effectors with GTP-charged Ras may trigger various signaling cascades, which induce not only growth, survival, and migration of cells but also cell cycle arrest, differentiation, senescence, and apoptosis (5, 7–10). These diverse biological effects may serve for the fine tuning of the cellular homeostasis. However, details in the interplay between the various Ras effector-mediated pathways and the biological consequences are still unknown.

Members of the RASSF family are deleted or repressed selectively by gene loss and/or epigenetic mechanisms in a considerable fraction of epithelial cancers and cell lines derived thereof. Re-expression usually suppresses the proliferation and tumorigenicity of these cells (7, 8–14). Accordingly, RASSF1A knockout mice show enhanced tumor susceptibility, and loss of function of the NORE1 gene is linked to a familial form of kidney cancer (15, 16). Thus, RASSF proteins act as tumor suppressors, which contrasts to the oncogenic effects of most other Ras effectors known, such as Raf or PI3-K.

The predominant RASSF family members are all characterized by the presence of a Ras association domain (RA) of the RalGDS/Raf-like GTPases but heterodimerizes with NORE1A, serving as link of these effectors with GTP-charged Ras suggesting that NORE1B does not interact with either Ras. In conclusion, NORE1B suppresses replication and transformation of cells as effectively as RASSF1A and thus is a putative tumor suppressor gene. NORE1B interacts physically with RASSF1A and functional loss of one of the interacting partners may lead to uncontrolled growth and transformation of hepatocytes. This may explain the frequent epigenetic silencing of NORE1B and/or RASSF1A in HCC. [Cancer Res 2009;69(1):235–42]
RASSF1A. These signals require the RA and SARAH-domain, which serve to interact with RASSF1A and not with GTP-charged Ras. In conclusion, our data indicate a close cooperation of NORE1B and RASSF1A in the maintenance of cell homeostasis. Thus, the functional loss of NORE1B and/or RASSF1A, as observed in almost all HCCs, may contribute significantly to uncontrolled growth and transformation of hepatocytes.

Materials and Methods

Plasmids and constructs. Human NORE1B, NORE1A, RASSF1A, pECFP-tagged Ha-Ras-wt, and pECPF-tagged Ha-Ras-G12V were kind gifts from J. Avruch (Massachusetts General Hospital, Boston, MA), A. Khokhlatchev (University of Virginia, Charlottesville, VA), R. Dammann (University of Halle, Germany), and A. Wittinghofer (Max-Planck Institute, Düsseldorf, Germany), respectively. NORE1B-deletion mutants lacking the NH2 terminus or the SARAH-domain were generated by PCR, deletants without RA-domain by site-directed mutagenesis, as described (22). For a list of primers and PCR conditions, see Supplementary Data. The coding regions of NORE1Bwt, deletion mutants, RASSF1Awat, and NORE1Awat, were sequenced and subcloned in-frame into pEYFP or pECFP.

Cell lines. The human hepatocarcinoma cell-line Hep3B (ATCC-No HB-8064) was maintained as described (6). The absence of mutations in exons 2 and 3 of N-, Ha-, and Ki-Ras was verified by sequencing. Primer sequences are given in the Supplementary Data and methodical details in a study by Macheiner and colleagues (6). MIM-1-4 immortalized murine hepatocytes, deriving from p19ARF−/− mice, and MIM-M hepatocytes, established from p19ARF−/− and transformed with oncogenic v-Ha-Ras, were generated and cultured as described elsewhere (23). Cells were retrovirally transduced with pMSCV-GFP (ClonTech) and in a second round with pBABEpuro harboring NORE1Bwt, NORE1Awat, or RASSF1Awat. Cells were kept in 5 μg of puromycin/mL medium for ~2 wk and resistant clones were isolated and propagated.

Reverse transcription-PCR. Total RNA was extracted, reversely transcribed applying random primers and Moloney murine leukemia virus reverse transcriptase, and PCR products were analyzed by electrophoretic separation, as described (6).

siRNA. Silencer Select siRNAs targeting human NORE1B with the sequences 5′-CACUGCCUAAGACUACCUUTT-3′ and 5′-AAAGGUAGCUAGGCGUAA-3′ were custom-made by Ambion (‘‘siNORE1B’’). A non-silencing Silencer Select scrambled siRNA (‘‘siSCRAMB,’’ No AM4390843; Ambion) and Cy3-labeled Silencer gyceraldehyde-3-phosphate dehydrogenase (GAPDH; ‘‘siGAPDH,’’ No AM46949; Ambion) were used as controls. Cells were transfected with either 30 nmol/L (siSCRAMB and siNORE1B) or 10 nmol/L (siGAPDH) siRNAs using siLentFect (BioRad) according to the manufacturer’s instructions and were incubated for 24 h until analyses.

Confocal laser scanning microscopy. Hep3B-cells were transfected with 1 μg of pEYFP- or pECFP-tagged constructs or with 0.5 μg of each plasmid DNA in case of double-transfections applying Fugene 6 (Roche Applied Bioscience); 24 h later, cells were fixed with formalin for 30 min, and the DNA was stained with Hoechst 33258 (0.8 μg/mL in PBS). Images were obtained by a LSM510 Meta-confocal microscope (Carl Zeiss).

Fluorescence resonance energy transfer. Cells were transfected as described above. After 24 h, images were obtained with a Nikon Eclipse TE300 microscope (Nikon), equipped with a SenSys camera, and applying a CFP (donor) - YFP (acceptor), and a RAW-fluorescence resonance energy transfer (FRET; acceptor emission at donor excitation)-filter. The FRET signals were calculated with MetaMorph 5.0 software as an intensity-modulated display image (24). After determination of the correction factor for CFP and YFP, FRET was calculated using the formula: FF = (a × dF) - (a × AF). FF, FRET signals; d, donor correction factor; DF, donor filtersignal; a, acceptor correction factor; AF, acceptor filtersignal. For further details see ref. 25. The fusion construct pEYFP/CFP served as positive control (a kind gift from JA Schmid, Medical University of Vienna, Vienna, Austria).
The wild-type form and the deletion mutants on growth, transformation, and on subcellular localization were studied.

**NORE1B delays proliferation of hepatoma and hepatocyte lines.** By fluorescence-activated cell sorting (FACS) analyses, we first checked the effect of NORE1B, NORE1A, and RASSF1A on the cell cycle of the human hepatoma cells Hep3B, a line with inherently low endogenous expression of these genes (6). The transfection stressed the cells as indicated by absence of the G2-M phase. Expression of NORE1Bwt induced an increase of cells in G2-M phase. NORE1Awt and RASSF1Awt also retrovirally into murine hepatocyte lines without (MIM-1-4) and with mutant Ha-Ras (MIM-R). However, replication was significantly reduced in MIM-1-4 and MIM-R cells expressing NORE1B, NORE1A, or RASSF1A as manifested by significantly decreased cell numbers after a cultivation period of 3 days. When

**Results**

Human NORE1B encodes a 265 amino acid (aa) protein, which consists of a unique 40 residue NH2 terminus, followed by the CENTRAL-domain (aa 41–115), RA-domain (aa 116–207), and COOH-terminal SARAH-domain (aa 208–265; refs. 7, 9). To understand the functions of these domains we generated the following deletants: NORE1B_Nterm lacking aa 1-107, NORE1B_RA lacking aa 113-226, and NORE1B_SARAH without aa 222-265. For further information, see Supplementary Data. The effects of the
calculating the mean duration of each cycle phase, NORE1Bwt prolonged the G_{0}-G_{1} phase fraction in both cells types and the G_{2}-M phase in MIM-1-4 cells (Fig. 2D). A similar effect was observed with NORE1A and RASSF1A.

NORE1B antagonizes cell transformation. RECs were transfected with plasmids encoding for c-Myc and Ha-Ras expression. Subgroups were additionally cotransfected with plasmids encoding NORE1Bwt, NORE1B deletion mutants, RASSF1Awt, or NORE1Awt. A, the number of colonies generated by Ha-Ron-c-Myc alone was arbitrarily set 100%, corresponding to a mean of 28.4 clones per plate. In each treatment group, two to six dishes were analyzed per experiment. Experiments were done in triplicate; columns, mean; bars, SE. Statistics by Kruskal-Wallis test. *, P < 0.05. B, phase contrast microscopy of clones. Magnifications, ×150.

NORE1B antagonizes cell transformation. RECs were transfected with plasmids encoding for c-Myc/Ha-Ras, which produced numerous transformed cell clones (Fig. 3). Cotransfection with NORE1Bwt or NORE1B_Nterm significantly lowered focus formation by 24%. NORE1B_RA and NORE1B_SARAH exerted no decrease indicating that the RA- and SARAH-domain may be important for interference with c-Myc/Ha-Ras induced cell transformation. The number of foci was not significantly affected with mutant Ha-Ras only and not with the wild-type form (Fig. 6C). Thus, the lack of cytoplasmic occurrence of the NORE1B_RA or NORE1B_SARAH was probably due to lack or steric hindrance of the NES, respectively. The unaltered cytoplasmic location of NORE1B_Nterm under leptomycin B-treatment may be due to the deletion of a hitherto unidentified nuclear localization signal close to the NH_{2} terminus. These data indicate that NORE1B may be active in the cytoplasmic and nuclear compartment of the cell.

NORE1B interacts with RASSF1A but not with wild-type or mutant Ha-Ras. To elucidate the molecular target of NORE1B, we double-transfected Hep3B cells. The presence of RASSF1Awt altered the subcellular localization of NORE1Bwt and NORE1B_Nterm to a RASSF1A-like pattern but had no effect on the other NORE1B-deletants (Fig. 6A). As a result, NORE1Bwt or NORE1B_Nterm distributed differently when compared with single transfections and exhibited colocalization with RASSF1A. To check for interactions between the two molecules, we performed FRET analyses (Fig. 6B). The cotransfection of RASSF1A and NORE1A, a known RASSF1A-binding partner, produced a significant FRET signal. Transfecting RASSF1A with either NORE1Bwt or NORE1B_Nterm also resulted in considerable signals, whereas the combinations of NORE1Bwt/NORE1Awt, NORE1B_RA/RASSF1A, and NORE1B_SARAH/RASSF1A were ineffective (Fig. 6C). This strongly suggests that NORE1B interacts physically with RASSF1A, which involves the RA- and the SARAH-domain.

NORE1A has been described to bind GTP-charged Ras proteins by its RA-domain (20). Accordingly, in double-transfected Hep3B-cells NORE1A altered the subcellular distribution and colocalized with mutant Ha-Ras only and not with the wild-type form (Fig. 6C). The absence of the FRET signal does not exclude interaction of NORE1A and mutated Ras because the energy transfer requires parallel orientation of donor and acceptor dipoles of the two fluorophores (35). These preconditions are often not met by the labels of the transfectants. When cotransfecting NORE1Bwt with either wild-type or mutant Ha-Ras, NORE1B neither changed its subcellular localization nor did it produce a significant FRET signal (Fig. 6D). Thus, a physical interaction of NORE1B with Ras in its wild-type or mutated form seems unlikely.

Discussion

In the majority of HCCs investigated, NORE1B was found to be epigenetically silenced (6). The present report provides several lines of evidence supporting that NORE1B is a putative tumor suppressor gene and cooperates closely with RASSF1A, as outlined in the following.
NORE1B, RASSF1A, and cell cycle. In hepatocyte and hepatoma cell lines, NORE1B, NORE1A, and RASSF1A increased the percentage of cells in G0-G1 at the expense of the S-phase fraction. Retroviral insertion of NORE1B and RASSF1A in hepatocyte lines tended to increase additionally the G2-M fraction. As a result, cell cycle progression was significantly delayed and growth of the lines suppressed. Identical effects were reported for RASSF1A and NORE1A in cells derived from melanoma, kidney, cervix, breast, lung, and prostate cancer (27–30, 32, 36). Data on hepatocytes or hepatoma cells have not been available. The molecular mechanisms of the G1-S and G2-M arrest by RASSF1A are largely identified. The protein complexes with the transcription factor p120E4F, thereby delaying the G1-S transition via interference with p14ARF, retinoblastoma, p53, and cyclin A (9, 10, 27, 31). During the interphase RASSF1A localizes to microtubules and is found in centrosomes and spindles during mitosis by binding to RABP1 (also called C19ORF5; ref. 37). Overexpression of RASSF1A causes a prometaphase arrest by preventing activation of the anaphase-promoting complex/cyclosome, whereas depletion of RASSF1A accelerates mitotic progression and causes mitotic defects (9, 10, 29, 33). Details of the molecular mechanisms underlying the reduced S-phase fraction by NORE1B, as observed in the present study, have yet to be defined. According to our data, the SARAH-domain and to some extent also the RA-domain of NORE1B are essential for growth suppression.

NORE1B and cell transformation. Under our experimental conditions, NORE1B antagonized c-Myc/Ha-Ras–induced transformation of embryonal cells. Only NORE1B constructs harboring the RA-/SARAH-domains and being capable of binding to RASSF1A, were effective. Interestingly, RASSF1A alone did not significantly antagonize cell transformation but enhanced greatly the NORE1B effect, which indicates cooperation of these genes. In the clones generated, the effects of NORE1B and RASSF1A seemed to persist as being evident by a reduced population doubling time over an observation period of several weeks.

Previous studies described a colony-suppressing activity of NORE1B, NORE1A, and RASSF1A in cancer cell lines deriving from kidney, lung, or melanoma (8, 12, 13, 21, 38). Colony formation in already transformed cell lines is due to altered cell-cell adhesion and anchorage independence, partly reflects the process of tumor progression, and differs greatly from the transformation of unaltered and mortal primary cells. Our data add to the profile of NORE1B and RASSF1A, indicating that these genes interfere with processes already at the very beginning of carcinogenesis.

Putative interaction partners of NORE1B. Harboring a RA-domain like NORE1A, NORE1B has been anticipated to bind GTP-charged Ras (20). However, after cotransfection of NORE1Bwt and mutant Ha-Ras, there was no evidence for any interaction. Furthermore, NORE1B affected the cell cycle in Hep3B cells harboring wt-forms of Ha-, Ki-, and N-Ras and in MIM-hepatocytes without or with activated Ras. Thus, NORE1B seems to act independently of Ras and loss of NORE1B function may render a growth advantage to hepatocytes with or without Ras mutation.

Figure 4. Effect of NORE1B and RASSF1A on cloning efficiency. RECs were transfected with plasmids encoding c-Myc/Ha-Ras only or additionally with NORE1Bwt- or RASSF1Awt-constructs. Representatives of stable clones generated were isolated. Plasmid expression in clones was verified by RT-PCR in A and by immunoblotting in B. β-actin was used as control for sample integrity and equal loading. Co, untransfected RECs. C, cell number in clones was determined at each replating and served to calculate the population doubling time. Statistics gives significance at the 95% confidence interval (CI). Slopes of curves: c-Myc/Ha-Ras only, 0.43 ± 0.03 cell doublings per day (CI, 0.36–0.45); NORE1Bwt, 0.37 ± 0.04 cell doublings per day (CI, 0.29–0.46); RASSF1Awt, 0.2 ± 0.04 cell doublings per day (CI, 0.12–0.28).
NORE1B lacks the NH₂-terminal DAG-domain, the putative RASSF1A binding site, and nevertheless interacts with this gene via the RA- and SARAH-domain. Cotransfections of RASSF1A with NORE1B-constructs containing the critical domains produced significant FRET-signals. Generally, efficient energy transfer of >50% through the resonant coupling of the dipole moments of the donor (CFP-tagged NORE1B) and acceptor (YFP-tagged RASSF1A) requires parallel orientation and proximity of <7 nm of the two fluorophores, and an interaction time beyond random molecular collisions (35). Thus, FRET proves the molecular proximity between the two macromolecules, NORE1B and RASSF1A, and strongly suggests close physical interaction of the two peptides. Furthermore, FRET analyses are superior to conventional biochemical binding studies in that they allow to study living cells (35). 

Via heterodimerization NORE1B seems to be recruited to the microtubular binding sites of RASSF1A, as the colocalizing gene products seem to arrange as fibers. The molecular mechanisms of growth arrest by RASSF1A largely involve interactions with microtubules, as outlined above. Similar mechanisms may underlie the growth suppressing effects of NORE1B. Furthermore, as essential components of cell adhesion, intercellular contacts, and/or the cytoskeleton microtubuli affect cell morphology (39). Accordingly, by binding to the microtubuli, NORE1B may induce the observed shift from fibroblastoid to an epitheloid, cobblestone-like morphology in the embryonal cells.

**Lack of NORE1A silencing and hepatocarcinogenesis.** As part of a multiprotein complex RASSF1A controls the cell cycle and recruits protein kinases to induce apoptosis (9, 10, 17–19, 27–33). Our data show that RASSF1A heterodimerizes with both, NORE1A and NORE1B, but that only NORE1A seems to serve as link to GTP-charged Ras. Thus, it may be a critical point for a cell whether the RASSF1A-containing protein complex attaches to NORE1A or NORE1B and thus may be linked to active Ras or not. In human HCC NORE1B and RASSF1A are epigenetically silenced but not NORE1A (6). Impaired function of NORE1A by mutations of the gene has already been excluded (6). Because most of the HCCs lack Ras mutations, the adaptor function of NORE1A between RASSF1A and GTP-charged Ras-proteins may be of minor importance for hepatocytes to abrogate harmful Ras effects. This may explain that NORE1A is not frequently epigenetically silenced in the HCCs.

In conclusion, our data suggest that NORE1B and RASSF1A interact closely to antagonize cell transformation and to control cell morphology.

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**Figure 5.** Confocal laser scanning microscopy of subcellular localization of NORE1Bwt, NORE1B deletion mutants, NORE1Awt, and RASSF1Awt. Hep3B cells were transiently transfected with pEYFP-tagged plasmids (green) and cultured for 24 h with leptomycin B (solved in ethanol; applied at 10 ng/mL medium; Sigma) or solvent. Cells were fixed and counterstained with Hoechst 33258 (blue). Images were obtained by LSM510Meta confocal microscope (Carl Zeiss) using a ×63 oil objective.
growth of hepatocytes. Thus, functional integrity of both genes seems to be fundamental in preventing the development of liver cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Figure 6. Close spatial interactions of NORE1B with RASSF1A and not with wild-type or mutant Ha-Ras. Hep3B cells were transiently cotransfected with RASSF1A and NORE1B wt, NORE1B-deletants, NORE1A, and/or Ha-Ras in its wild-type or mutant form. Constructs were tagged with either CFP (light green) or YFP (turquoise). A, nuclei were counterstained with Hoechst (blue). FRET-analyses are shown in B: images of each of the samples were acquired with filters for CFP (pseudo light green), YFP (pseudo turquoise), and for FRET (pseudo green) using identical microscope and camera settings. After determination of the correction factor for CFP (donor) and YFP (acceptor), the intensity of the FRET signal was calculated. Note that in C, the left column shows single transfections with wild-type and mutant Ha-Ras and the middle and right columns cotransfections of NORE1A with wild-type or mutant Ha-Ras. For further details, see Materials and Methods. Magnifications, ×50. A summary on colocalization (co. local) and FRET-signals of the double-transfectants are given in (D).


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