

Nore1 inhibits tumor cell growth independent of Ras or the MST1/2 kinases

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Nore1, a noncatalytic protein identified by its ability to bind selectively to active Ras, is most closely related in amino-acid sequence to the tumor suppressor RASSF1. Both are expressed predominantly as a longer (Nore1A/RASSF1A) and/or shorter (Nore1B/RASSF1C) polypeptide; all four polypeptides contain a Ras-association domain and bind, through their conserved carboxytermini, the proapoptotic protein kinases MST1 and MST2. Moreover, the expression of the longer polypeptide is downregulated in human tumor cell lines through promoter methylation (frequently for RASSF1A, less regularly for Nore1A). Forced expression of RASSF1A in several such lines (including the NSCLC line A549) has been shown to suppress tumorigenicity; herein we inquire whether Nore1 has growth inhibitory activity. Four tumor cell lines were tested, selected for their low expression of both Nore1A and Nore1B; the two NSCLC lines, A549 and NCI-H460, each have a mutant active Ras oncogene, whereas the two melanoma lines G361 and M14 each contain the constitutively active BRaf(V599E) oncogene and wild-type Ras. The expression of Nore1A or Nore1B suppresses colony formation by the A549 and G361 lines, as effectively in A549 as does RASSF1A; colony formation in the NCI-H460 and M14 lines is unaffected. Nore1A inhibits anchorage-independent growth by A549 cells and delays A549 progression through G1 without evidence of increased apoptosis. The growth suppressive action of Nore1A is largely unaffected by deletion of both the MST- and Ras-binding domains, as well as by mutation of the Nore1A zinc finger. Thus, Nore1 suppresses the growth of some tumor cell lines through as yet unidentified effectors, independent of Ras-like proteins or MST1/2.

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Introduction

Nore1 (also named RASSF5) is the founding member (Vavvas *et al.*, 1998) of a small gene family that includes RASSF1-6. Each of these genes is expressed as a variety of mRNA splice variants; the dominant human Nore1 polypeptides are Nore1A and Nore1B (Tommasi *et al.*, 2002; Hesson *et al.*, 2003). Human Nore1A is 418 amino acids, encompassing an aminoterminal proline-rich segment, a central zinc finger of the C1 type, a Ras/Rap association (RA) domain of the RalGDS/AF6 variety (Ponting and Benjamin, 1996) followed by a carboxyterminal tail (Figure 1). Nore1B, 265 amino acids, contains a unique 40 residue aminoterminal followed by 225 amino acids identical to Nore1A that contains the RA domain and carboxyterminal tail. The amino-acid sequence of the carboxyterminal tail is as strongly conserved within the family as that of the RA domain; the tail serves as the binding site for the Ste-20 related protein kinases MST1/2, which also bind to the carboxytermini of RASSF1-4 (Khokhlatchev *et al.*, 2002). Nore1A is widely expressed (Tommasi *et al.*, 2002; Hesson *et al.*, 2003), whereas Nore1B (also called RAPL) is expressed predominantly in lymphoid tissues (Katagiri *et al.*, 2003). Nore1 was originally isolated through its ability to bind selectively and with high affinity to the GTP-liganded form of Ras, and endogenous Nore1A in KB cells has been shown to transiently associate with endogenous Ras after serum or EGF stimulation (Vavvas *et al.*, 1998). Nore1A also binds to other members of the Ras subfamily, especially Rap1, R-Ras and MRas/R-Ras3 (Ortiz-Vega *et al.*, 2002), and Nore1B/RAPL has been proposed as a mediator of the Rap1 induction of integrin clustering and activation after TCR stimulation (Katagiri *et al.*, 2003). The role of Nore1A in the biologic programs controlled by these GTPases is, however, unclear. Although overexpression of wild-type Nore1A does not promote apoptosis, targeting Nore1A to the membrane by fusion to the carboxyterminal polybasic and prenylation motif from Ki-Ras4B (to give Nore1CAAX) confers some proapoptotic activity, and augments substantially the proapoptotic efficacy of coexpressed MST1, to an extent comparable to that achieved by targeting MST1 itself to the membrane. Moreover, interfering with the endogenous, constitutive

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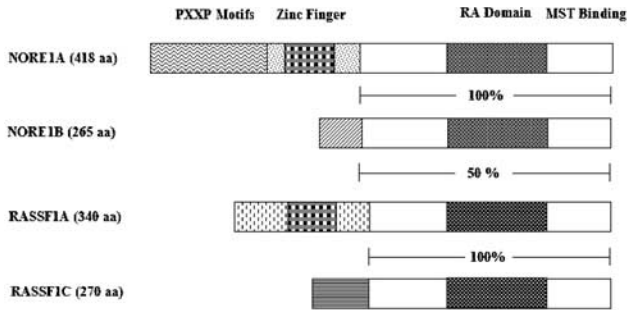


Figure 1 Domain structure of the Nore and RASSF1 polypeptides. See text for details

complex of MST1 with a Nore/RASSF polypeptide completely suppresses the ability of overexpressed Ki-Ras(G12V) to cause apoptosis (Khokhlatchev *et al.*, 2002). The physiologic significance of the antiproliferative or proapoptotic programs initiated by overexpression of these small GTPases is unclear, hence these findings do not reveal the physiologic functions of Nore. Nevertheless, they do suggest that Nore may serve as a growth inhibitor. Moreover, Nore1A and Nore1B are each approximately 50% identical to RASSF1A (340 amino acids) and RASSF1C (270 amino acids), respectively, and considerable evidence indicates that RASSF1A is a bona fide tumor suppressor (Zabarovsky *et al.*, 2002; Dammann *et al.*, 2003). The RASSF1 gene is located within a segment on chromosome 3p21.3 that is frequently deleted in many tumors of epithelial origin, and the expression of the RASSF1A isoform is often lost selectively through hypermethylation of its CpG-rich promoter, whereas expression of the shorter RASSF1C isoform is well maintained. Re-expression of RASSF1A in the NSCLC cell lines A549 and H1299 (Dammann *et al.*, 2000; Burbee *et al.*, 2001) or in the renal clear cell cancer-derived line KRC/Y (Dreijerink *et al.*, 2001) inhibits tumorigenicity. Deletions/mutations of chromosome 1q32.1–2 in the vicinity of the Nore1 gene are uncommon in human tumors, although reportedly frequent in renal collecting duct carcinoma, a rare malignant neoplasm of distal nephron origin (Steiner *et al.*, 1996). Conversely, hypermethylation of the Nore1A (but not the Nore1B) promoter has been observed to be moderately prevalent in human tumor cell lines (Tommasi *et al.*, 2002; Hesson *et al.*, 2003; Morris *et al.*, 2003), and Nore1 polypeptide expression is frequently very low in human tumor lines and in some primary tumors (Vos *et al.*, 2003). Moreover, Vos *et al.* recently reported that ectopic expression of Nore1A in the NSCLC line A549 and in other tumor cells suppressed growth in a Ras-dependent manner through the induction of apoptosis. These findings impelled us to inquire further as to whether Nore1 has antiproliferative activity in human tumor cell lines, and if so, whether this activity is dependent on its ability to bind to Ras-related GTPases or the proapoptotic kinases, MST1/2. The present results demonstrate that both Nore1A and Nore1B have antiproliferative activity comparable in potency to that of RASSF1A in some, but not all tumor

cell lines. This antiproliferative activity is, however, independent of the presence of an activated Ras oncogene and of the ability of Nore1A to bind to the endogenous Ras-like GTPases or to MST1/2. Growth inhibition is attributable, at least in part, to interference with progression through G1 to S, whereas no evidence for increased apoptosis is detected.

Results

Nore1A or Nore1B suppress colony formation in A549 NSCLC and G361 melanoma cells

Previous studies have shown that expression of RASSF1A, as estimated by Northern blot, is very low or absent in the A549 non-small-cell-lung-cancer (NSCLC) and G361 melanoma cell lines, and stable expression of RASSF1A in A549 cells potently inhibits colony formation (Dammann *et al.*, 2000; Tommasi *et al.*, 2002). Nore1A and Nore1B expression were also reported to be lacking or extremely low in these two cell lines, estimated by Northern blot using exon-specific probes (Tommasi *et al.*, 2002). We surveyed a variety of human tumor cell lines by PCR and observed that a majority of those examined exhibited very low levels of Nore1A mRNA, with Nore1B expression being more variable. To evaluate the significance of this low Nore1 expression to the transformed phenotype, we inquired whether reintroduction of Nore1A or Nore1B could suppress aspects of the transformed phenotype. For this purpose, we selected two NSCLC cell lines, A549 and NCI-H460, and two melanoma cell lines, G361 and M14. All four lines exhibited very low levels of Nore1A and Nore1B mRNA, estimated by quantitative PCR, in comparison to the nontransformed human lung fibroblast cell line WI-38 (Figure 2a). Nore1A mRNA abundance in the melanoma lines is also much lower than in primary human melanocytes (Nore1B abundance in primary melanocytes could not be determined because of nonspecific PCR products). Immunoblot of these lines with a polyclonal antibody described previously (Vavvas *et al.*, 1998), reactive with Nore1A and B (Figure 2b), revealed detectable Nore1A expression only in KB cells and keratinocytes, and no expression of Nore1B. The A549 and NCI-H460 lines each contain an activating mutation in Ki-Ras (G12S and Q61H, respectively), whereas both melanoma cell lines contain only wild-type Ras genes, but have a BRaf oncoprotein (V599E) whose mutant activation loop renders the kinase constitutively active and independent of further activation by Ras-GTP (Davies *et al.*, 2002).

The mammalian expression plasmid pcDNA 3.1, unmodified or encoding Nore1A, Nore1B or RASSF1A, was introduced in triplicate into each of these four cell lines by transfection. G418 was added to the medium 48 h later; after 2 weeks' growth in selective medium, the cultures were fixed, stained with crystal violet and the number of colonies was counted. As shown in Table 1, both Nore1A and Nore1B suppressed colony formation by A549 cells by more than 60% in comparison to the

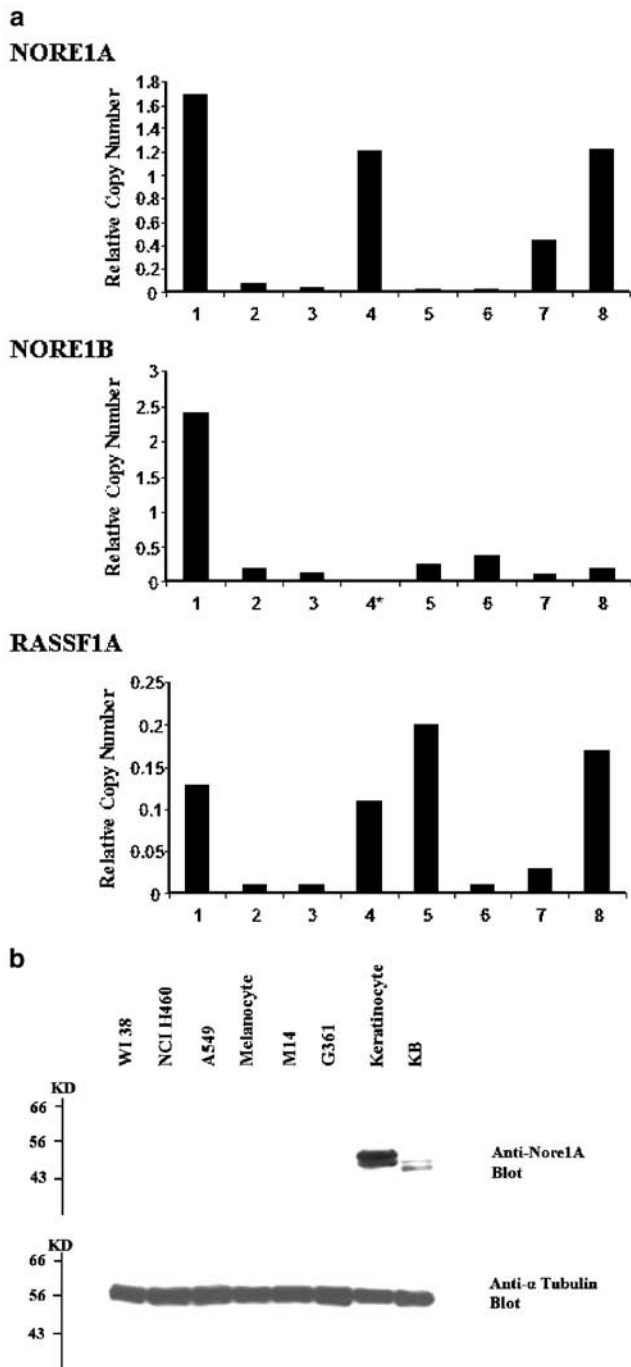


Figure 2 Relative expression levels of Nore1A, Nore1B and RASSF1A in human tumor cell lines and primary cells as determined by QRT-PCR and Immunoblot (**a**) Relative mRNA levels as determined by QRT-PCR. Quantitative RT-PCR (QRT-PCR) was performed in duplicate on total RNA using the Brilliant One-Step QRT-PCR kit. The amount of specific Nore1A, Nore1B and RASSF1A mRNA in each sample was normalized using the amount of TATA Binding Protein mRNA determined in parallel. 1: WI-38, 2: NCI-H460, 3: A549, 4: primary human melanocytes, 5: M14, 6: G361, 7: primary human keratinocyte, 8: HeLa-KB; *not available. (**b**) Immunoblot blot of cell extracts using a polyclonal antibody previously described (Vavvas *et al.*, 1998), raised against a prokaryotic recombinant Nore1A (188-413) fragment (upper panel). Anti- α tubulin blot was used as control for equal protein loading (lower panel)

Table 1 Suppression of colony formation by Nore1A and Nore1B in A549 and G361 cells

	NSCLC		Melanoma	
	A549	NCI-H460	G361	M14
NORE1A	73 \pm 3 (n=9)	23 \pm 11 (n=8)	75, 80	2, 0
NORE1B	64 \pm 12 (n=6)	12 \pm 13 (n=3)	79, 67	11 \pm 11 (n=3)
RASSF1A	76 \pm 7 (n=3)	ND	ND	ND

% reduction in number of colonies relative to vector, shown as mean \pm s.d. if $n \geq 3$; ND — not determined

empty vector control, an inhibition of focus formation comparable in extent to that caused by reintroduction of RASSF1A. Nore1A and Nore1B also strongly suppressed colony formation in G361 melanoma cells. In contrast, the development of foci in NCI-H460 and M14 cells was not significantly affected by Nore1A or Nore1B. The disparate efficacy of Nore1A and Nore1B is unrelated to the initial level of Nore expression (Figure 2); moreover, the A549, NCI-H460 and M14 cells were each comparably transfectable by a variety of mammalian expression vectors (data not shown), whereas the G361 melanoma cells, which were relatively poorly transfectable, nevertheless, showed a robust suppression of colony formation in response to Nore1A or Nore1B. Thus, both Nore1A and Nore1B are capable of suppressing tumor cell growth, and this inhibition is independent of the presence of a mutant activated Ras. We chose to focus further characterization of Nore-induced growth suppression on the action of Nore1A in A549 cells.

Nore1A expression in A549 cells inhibits anchorage-independent growth

Tumor cell growth in soft agar measures the ability of cells to grow in an anchorage-independent manner, and is an *in vitro* property that correlates with *in vivo* tumorigenicity much better than does growth rate in monolayer (Cifone, 1982). To examine the effect of Nore1A on growth in soft agar, we screened the Nore1A-transfected A549 lines emerging from the G418 selection for expression of Nore1A by immunoblot, using the polyclonal anti-Nore1 antibody. Most clones showed only a slight increase in Nore1A abundance over the pcDNA3.1-transfected control lines; however, we did identify an A549 line that expressed Nore1A at levels comparable to that endogenous to BC3H1 cells (Figure 3a). This Nore1A-expressing A549 line and a pcDNA3.1 transfected control A549 line were seeded at identical abundance onto soft agar and the size of the resultant colonies 10 days thereafter is shown in Figure 3b. It is apparent that stable expression of Nore1A markedly reduces the growth of A549 cells in soft agar.

Nore1A expression in A549 cells does not induce apoptosis but retards cell cycle progression

To address the mechanisms by which Nore1A suppresses the growth of A549 cells, we examined the

consequences of transient expression of Nore1A on cell viability and cell cycle progression. Examination of A549 cells transfected with plasmids expressing either a Nore1A-GFP fusion protein or GFP alone did not reveal evidence of toxicity in either population of GFP-positive cells, for example, cell rounding or detachment, as compared with the surrounding GFP-negative cells. TUNEL staining (Table 2) revealed comparably low levels of TUNEL-positive cells among the GFP positive or negative A549 cell populations, and no difference in the frequency of TUNEL staining in cells expressing GFP or GFP-Nore1A fusion protein; by contrast, brief treatment with H₂O₂ resulted in widespread, strongly positive staining by TUNEL. We conclude that expression of Nore1A in A549 cells does not induce apoptosis.

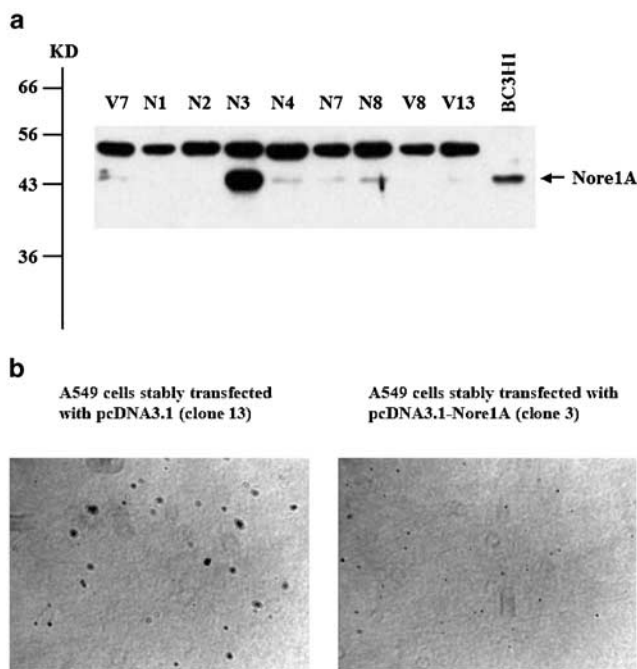


Figure 3 Ectopic expression of Nore1A inhibits anchorage-independent growth in A549 cells (a) Immunoblot using a polyclonal anti-Nore antibody (Vavvas *et al.*, 1998) of cell extracts prepared from clones of A549 cells selected with G418 after transfection with pcDNA3.1 empty vector (V) or pcDNA3.1 encoding Nore1A (N); an extract of untransfected B3CH1 cells is present in the far right lane. (b) Images of G418-resistant A549 cells, stably expressing recombinant Nore1A (clone N3) or control vector (clone V13), grown in soft agar for 10 days

Table 2 Overexpression of GFP-Nore1A in A549 cells does not induce apoptosis

	GFP-positive cells counted	GFP + TUNEL-positive cells	% TUNEL-positive cells positive for GFP
phr-GFP	53 ± 3.5	3 ± 1.2	5.1 ± 2.4
phr-GFP-Nore1A	55 ± 6.4	2 ± 0.6	3.0 ± 0.9
H ₂ O ₂	53 ± 5.3 ^a	30 ± 4.6 ^b	56.5 ± 6.5 ^c

Composite results from three independent experiments are shown, mean ± s.d. ^aNumber of cells counted. ^bTUNEL-positive cells. ^c% TUNEL-positive cells

To assess the effect of Nore1A expression on cell cycle progression, we examined BrdU incorporation in A549 cells expressing either GFP or a Nore1A-GFP fusion protein (Table 3). In A549 cells transfected with GFP alone, BrdU staining is positive in 15.6% of total GFP-positive cells; in comparison, in cells comparably labeled but expressing the GFP-Nore1A fusion, only 5.3% are positive for BrdU. This result indicates the existence of a significant delay in A549 cell cycle progression into the S phase as a result of Nore1A expression.

To further characterize the nature of altered cell cycle progression induced by Nore1A, and to ensure that any delay is directly attributable to the Nore1A polypeptide, we constructed a bicistronic retroviral expression vector (pB-IRES-GFP) encoding Nore1A. A549 cells were infected with recombinant retroviruses expressing GFP alone or GFP together with Nore1A. At 48 h after infection, nearly 100% of cells exhibited the expression of GFP. Nore1A expression was verified by Western blotting (data not shown). Cycling cells were fixed, stained with propidium iodide and analysed for DNA content by FACS. As shown in Table 4, expression of Nore1A causes a significant decrease in the number of cells in the S phase and a reciprocal increase of the fraction in the G₁ phase.

Suppression of colony formation by Nore1A does not require MST1 binding or an intact RA domain or Zinc finger

We next sought to determine which domains in Nore1A are important for its growth-suppressing activity. As depicted in Figure 1, Nore1A contains at least four conserved structural/functional domains, that is, an aminoterminal proline-rich domain, a central C1-type, putative DAG-PE-binding zinc-finger domain, an RA domain and a carboxyterminal MST1 binding domain. We progressively deleted and/or inactivated each of these domains (Table 5). Nore1A (1–358) is incapable of

Table 3 Effect of Nore1A and RASSF1A on BrdU incorporation in A549 cells

Plasmid	GFP-positive cells counted	GFP + BrdU-positive cells	% of GFP-positive cells positive for BrdU
phrGFP	102.6 ± 4.5	16.0 ± 1.0	15.6 ± 0.8
phrGFP-NORE1A	94.0 ± 7.5	5.0 ± 1.0	5.3 ± 0.9
phrGFP-RASSF1A	88.0 ± 7.2	3.3 ± 1.5	3.9 ± 2.1

Composite results from three independent experiments are shown

Table 4 Effect of Nore1A on cell cycle distribution in A549 cells (% of cells in each phase)

Retrovirus	G0/G1	S	G2/M
pB-IRES-GFP	53.2 ± 5.2	26.9 ± 1.3	19.9 ± 5.0
pB-IRES-GFP-NORE1A	62.4 ± 0.3	18.4 ± 0.1	19.2 ± 0.3

Composite results from three independent infections are shown

Table 5 Suppression of colony formation in A549 Cells by Nore1A mutants

<i>pCDNA 3.1-</i>	Inhibition %
Vector	0
Nore1A	61 ± 10
Nore1A (A ³⁰¹ AAA)	59 ± 12
Nore1A (S ¹³² S and S ¹³⁵ S)	58 ± 7
Nore1A (1–358)	67 ± 3
Nore1A (1–250)	43 ± 3
Nore1A (1–188)	6 ± 6

% of reduction in numbers of colonies relative to vector, mean ± s.d., *n* = 3

binding MST1, whereas GST-Nore1A (359-413) binds MST1 as well as full-length Nore. Nore1A (1–250) lacks both the MST binding segment and the RA domain; additionally, four conserved amino acids (L³⁰¹KKF) in the RA domain of full-length Nore1A were converted to Ala to generate Nore1A (A³⁰¹AAA), a mutant previously shown to have essentially no ability to bind Ras-GTP (Ortiz-Vega *et al.*, 2002). All of the amino acids in Nore1A that are shared with Nore1B were deleted, to give Nore1A (1–188). These three deletions do not disturb the Nore1A zinc finger; therefore two cysteines in the zinc finger of full-length Nore1A were converted to serines to make Nore1A (S¹³² and S¹³⁵). The corresponding mutations in the cRaf-1 zinc finger prevent Ras-dependent activation completely (Luo *et al.*, 1997). All these mutants were found to be expressed comparably to full-length, wild-type Nore1A during transient expression in HEK293 cells.

We examined the ability of these Nore1A mutants to suppress colony formation in A549 cells as compared to wild-type Nore1A (Table 5). It is evident that deletion of the MST binding domain (67 ± 3% suppression) and mutational inactivation (Nore1A (A³⁰¹AAA)) of the Ras binding domain (59 ± 12% suppression) has essentially no effect on the ability of Nore1A to suppress colony formation in A549 cells in comparison to wild-type Nore1A (61 ± 10% suppression). Deletion of both domains (Nore1A (1–250) causes only a minor drop in growth suppression (to 43 ± 3%). Mutation of the Nore1A zinc finger (Nore1A (S¹³²S and S¹³⁵S)) has no significant effect (58 ± 7% suppression); however, the deletion mutant Nore1A (1–188) failed completely to inhibit colony formation (6 ± 6% suppression). These results demonstrate that Nore1A binding to MST or to Ras-like GTPases is almost completely dispensable for growth suppression, whereas the segment between Nore1A amino acids 188 and 250 is necessary for this action.

Discussion

The present studies sought to determine whether Nore1, like its closest homolog the tumor suppressor RASSF1A, has growth inhibitory activity in human tumor cells. This seemed likely, based on the nearly 50%

identity in amino acid sequence between RASSF1 and Nore1, their similarities in gene structure and pattern of mRNA splice variants (Dammann *et al.*, 2000; Tommasi *et al.*, 2002), the very low levels of Nore1A and Nore1B expression in many tumor cell lines (Tommasi *et al.*, 2002; Hesson *et al.*, 2003; Vos *et al.*, 2003 and Figure 2) and our previous finding that Nore1 binds the proapoptotic kinase MST1 and is a component of a Ki-Ras(G12V)-activated apoptotic pathway (Khokhlatchev *et al.*, 2002). The present results confirm this bias in part, but in contrast to the report of Vos *et al.*, indicate that Nore1A suppresses growth through a mechanism independent of its ability to bind to activated Ras-like GTPases and to the MST1/2 kinases, and primarily by inducing cell cycle delay rather than apoptosis.

In preliminary studies, we observed that neither Nore1A nor NoreCAAX caused NIH3T3 cells to form foci or augmented the transforming activity of a weak Raf allele. Moreover, Nore did not interfere with the transforming activity of Ha-Ras(G12V) in this cell background. Nevertheless, because Nore binds with high affinity to Ras-GTP, it seemed possible that Nore overexpression might interfere with the ability of endogenous Ras-GTP to engage endogenous effectors. We therefore sought to examine the effect of Nore on the growth of tumor cell lines that express a mutant active Ras as well as lines that lacked a mutant Ras and were transformed by other oncogenes. The NSCLC lines A549 and NCI-H460 each contain a mutant active Ki-Ras (G12S and Q61H, respectively), whereas the melanoma lines G361 and M14 do not have Ras mutations, but bear a BRaf(V599E) mutation that renders the kinase constitutively active and independent of further activation by Ras-GTP (Davies *et al.*, 2002). Nore strongly inhibited the ability of A549 cells to establish foci and form colonies in soft agar, but had little or no effect on the growth of NCI-H460. This discordant response is surprising, inasmuch as mRNA expression profiles of these two NSCLC cell lines (Ross *et al.*, 2000) and their responses to a variety of chemotherapeutic agents (Scherf *et al.*, 2000) exhibit a very high degree of similarity. Similarly, Nore1A strongly inhibited the growth of the G361 melanoma line with little effect on the M14 cells. This discordance is not explained by the endogenous expression of Nore1A and Nore1B, which is very low in all four lines, and nor by differences in transfectability. Thus Nore1A can act as a potent inhibitor of tumor cell growth, and the presence of a mutant active Ras oncogene is neither necessary nor sufficient for growth inhibition. The inhibition of growth in the A549 line does not appear to involve an increase in cell death, but rather a delay in cell cycle progression through G1 into S, the basis for which is under continued investigation; the inhibition of cell cycle progression through G1 has also been reported as the dominant mechanism for the antiproliferative action of RASSF1A (Shivakumar *et al.*, 2002).

The structure-function analysis (Table 5) establishes that the Nore1A RA domain and the carboxyterminal MST1/2 binding domain are both largely dispensable

for the ability of Nore to inhibit the growth of A549 cells; the aminoterminal Nore1A(1–250) fragment, which lacks both domains, exhibits ~70% of the inhibitory potency of full-length Nore1A. Thus the ability of Nore1A to inhibit growth is not mediated through the binding of a Ras-like GTPase or MST1/2. This finding was surprising, in view of our previous demonstration that a Nore/MST complex serves a necessary role in the apoptosis induced by transient overexpression of Ki-Ras(Gly12Val). The marked overexpression of mutant, active Ki-Ras in that model does not, however, reflect the situation found in spontaneously transformed cells, and the present work, examining the effect of reintroducing Nore1A into human tumor cell lines that exhibit low expression, was undertaken to examine a more plausible paradigm within which to address the function of Nore1A in tumorigenesis. Clearly, the independence of the growth suppressive action of Nore1A in the A549 human tumor cell line from its ability to bind Ras or MST indicates that Nore1A participates in a second growth inhibitory pathway whose components are yet to be defined. The present findings do not contradict the previous implication of Nore in the regulation of a cell death pathway involving MST, which can be engaged by overexpressed Ki-Ras(Gly12Val). The ability of a regulator to participate in proapoptotic or cytostatic pathways is not mutually exclusive, as is well known for p53 (Oren, 2003; Bartek and Lukas, 2001). Spontaneous endogenous complexes of MST with Nore and RASSF1 are readily detectable in cells that express Nore, such as KB cells; MST kinase activity in these complexes is strongly suppressed, perhaps awaiting an activating upstream input. The unanswered questions as regards the Nore/MST complex are how the complex is engaged under physiologic circumstances, and whether apoptosis is among its physiologic outputs. An indication that MST can signal both growth arrest and apoptosis is provided by the phenotype of the *Drosophila* MST1/2 homolog (*hippo*) (Harvey *et al.*, 2003; Wu *et al.*, 2003), which can suppress cell proliferation in the developing eye through either cell cycle arrest or apoptosis, depending on circumstances as yet incompletely understood.

The independence of Nore1's growth inhibitory action from the ability to bind Ras-GTP is a feature shared with RASSF1A, in that the latter polypeptide, although possessing a canonical RA domain, exhibits very little or no ability to bind directly to Ras-like GTPases (Ortiz-Vega *et al.*, 2002). It is unlikely that the Nore1A C1-type zinc finger (118–165) is critical inasmuch as mutation of this structure does not alter the inhibitory potency of full-length Nore1A. Conversely, the aminoterminal proline-rich segment, amino acids 17–108, is not sufficient to inhibit A549 growth. Thus the 62 amino-acid segment from Nore1A(189–250) is indispensable for Nore1A-induced growth inhibition. This segment of Nore1A(1–250) (starting at NVC through PAG), which lacks known motifs, is the only portion of Nore1A(1–250) that is shared with Nore1B. Nevertheless, the pattern of growth inhibition caused by Nore1B in these four cell lines is identical to that seen

with Nore1A. The amino-acid sequence of this segment of Nore1A/B is also highly conserved in RASSF1A, with about 50% identity overall, mostly concentrated in the carboxyterminal half. Moreover, point mutations in this region of RASSF1A identified in human tumors (S131F, A133S) (Burbee *et al.*, 2001) severely compromise the tumor suppressing activity of RASSF1A (Shivakumar *et al.*, 2002); RASSF1A serine 131 is a potential ATM/DNA_PK site (SQ) and the defective growth suppression by these two RASSF1A mutants seems to be correlated with their significantly reduced phosphorylation *in vivo* (Shivakumar *et al.*, 2002). Nevertheless, this entire segment of RASSF1A is also present in RASSF1C, which exhibits no growth inhibitory activity. Thus, although this central region of Nore1A/B and RASSF1A is important and perhaps necessary to their growth suppressing activity, its presence (in RASSF1C) is not sufficient to confer growth inhibitory activity; an additional input is necessary, probably contributed by the divergent aminotermini of these polypeptides. The aminotermini of Nore1A and RASSF1A mediate the ability of these polypeptides to homodimerize and to heterodimerize with each other (Ortiz-Vega *et al.*, 2002); nevertheless, inasmuch as the endogenous expression of both polypeptides is essentially undetectable in A549 cells (Figure 2), it is unlikely that this ability contributes to the antiproliferative action of recombinant Nore1A. The aminoterminal of Nore1A contains a variety of proline-rich motifs capable of binding SH3 and WW domains, and this property may be contributory. Inasmuch as the segments of Nore1A, Nore1B and RASSF1A aminoterminal to their homologous central region bear no similarity in amino-acid sequence to each other, the mechanism of growth inhibition by these three polypeptides may involve the recruitment of distinct sets of effectors, each perhaps sharing a domain that interacts with the central 62 amino-acid segment of Nore1A, Nore1B and RASSF1A whose integrity is necessary for growth suppression.

Materials and methods

Cell culture

A549, G361 cell lines were purchased from ATCC. NCI-H460 and M14 were kind gifts from Dr Daniel A Haber and Dr Daphne Bell (Cancer Research Center Massachusetts General Hospital Charlestown MA, USA). Human primary melanocyte was kindly provided by Dr David E Fisher. A549 cells were grown in F12 Ham's, G361 cells in McCoy's 5a, NCI-H460 and M14 cells in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum plus glutamine and penicillin/streptomycin at 37°C with 5% CO₂.

cDNA constructions

Full-length cDNA encoding Nore1A, Nore1B or RASSF1A was subcloned into pCDNA3.1 (In vitro)gen to make pCDNA3.1-Nore1A, pCDNA3.1-Nore1B and pCDNA3.1-RASSF1A. The Nore1A(A³⁰¹AAA) and Nore1A(S¹³² and S¹³⁵) mutants were made in the vector pCMV5-FLAG using QuickChange™ Site-

Directed Mutagenesis kit (Stratagene) and then subcloned into pCDNA3.1. Nore1A truncation mutants Nore1A (1–358), Nore1A (1–250) and Nore1A (1–188) were made by PCR. Full-length cDNA encoding Nore1A or Rassf1A was subcloned into pHRGFP-N1 (Stratagene) to make pHRGFP-Nore1A and pHRGFP-Rassf1A. All constructs were verified by DNA sequencing.

Colony formation assay

Healthy growing cells in tissue culture dishes were trypsinized and plated into six-well plates the previous day in such a density that cells would be about 40% confluent the next day for transfection. Cells were transfected using the Genejuice™ cationic lipid transfection reagent according to the manufacturer's instructions (EMD Biosciences, Inc. Novagen Brand 441 Charmany Drive Madison, WI 53719, USA). 0.2 µg DNA/well was used. At 48 h after adding DNA:lipid mixtures to the cells, culture medium was changed to fresh medium containing G418 (2 mg/ml for A549 and NIH-H460, 1 mg/ml for G361 and M14 cells). The medium containing G418 was refreshed every 3–4 days. At 14 days after selection in G418 containing medium, cells were washed three times with PBS, fixed and stained with 0.5% Crystal Violet in 85% ethanol (Sigma, catalog # C-6158). The plates were rinsed with distilled water, air-dried, photo-scanned and cell colonies were counted using NIH ImageJ software.

Anchorage-independent growth in soft agar

A549 cells were transfected with pCDNA3.1 or pCDNA3.1-Nore1A. Stable clones were obtained through cloning and selection in culture medium containing 2 mg/ml of G418. For soft agar assay, 5000 cells/35 mm dish in culture medium containing 0.35% agar were overlaid onto 0.5% bottom agar in culture medium. Cells were fed every 5 days with 0.5 ml/dish culture medium. After 10 days, pictures of colonies formed were taken under a microscope.

Retroviral expression

Full-length cDNA encoding murine Nore1A was subcloned into the bicistronic retroviral expression vector pB-IRES-GFP (Liu *et al.*, 2000). Recombinant retroviruses were generated in 293T cells by cotransfection with two other plasmids encoding VSVG and Gag-Pol, respectively, using calcium phosphate precipitation method. A549 cells were infected with the retroviruses in the presence of 8 µg/ml of polybrene followed by centrifugation at 2560 r.p.m. (J6M rotor, Beckman) for 80 min at 32°.

Flow cytometry analysis

A549 cells were infected with pB-IRES-GFP or pB-IRES-GFP-NORE1A. At 7 days thereafter, cells were trypsinized, washed twice in PBS and fixed in 70% ethanol for 30 min at 4°C. The cells were again washed twice in PBS, and incubated in 100 µg/ml RNase and 50 µg/ml propidium iodide for 20 min at room temperature. The relative DNA content of 100 000 cells was analysed with a FACScan machine (Epics Elite, Coulter).

Quantitative PCR

QPCR was performed as described previously (Belham *et al.*, 2003). Total RNA was extracted using TRIzol (Invitrogen), further purified using the Qiagen (Chatsworth, CA, USA) RNeasy total RNA isolation kit according to the instruction

manual, and quantified using Ribogreen (Molecular Probes, Inc., Eugene, OR, USA). After DNase I treatment, quantitative RT-PCR was performed in duplicate using the Brilliant One-Step quantitative RT-PCR kit (Stratagene, La Jolla, CA, USA) containing SYBR Green I (1:30 000, Sigma), forward and reverse primers (25 nM each), and sample RNA (1 µg). The nucleotide sequence of the primers used were: Nore1A-F (5'-TGTGCGGACGAGAGGTGCT-3') and Nore1A-R (5'-GGTGCTTTCTGGAGAGGGT-3') with Nore1A-specific product size of 143 bp; Nore1B-F (5'-GTAGATGACCGTG-GACAGCAG-3') and Nore1B-R (5'-GCAGTCTTC-CAGTTCCTCGTC-3') with Nore1B-specific product size of 67 bp; Rassf1A-F (5'-TCTGTGGCGACTTCATCTG-3') Rassf1A-R (5'-AACAGTCCAGGCAGACGAG-3') with Rassf1A-specific product size of 105bp; and TATA box binding protein (TBP)-F (5'-CACATCACAGCTCCCCAC-CA-3') TBP-R (5'-GCACGGTATGAGCAACTCACA 3') with a TBP-specific product size of 132 bp. The thermal cycling conditions comprised an initial RT reaction step at 48°C for 30 min and 40 cycles at 95°C for 30 s and 65°C for 1 min. Accumulation of PCR product was monitored in real time and the crossing threshold (Ct) value was measured by using Mx4000 (Stratagene), with appropriate controls. A standard curve was generated for each molecule as Ct value = $-a \log(\text{starting quantity, copy number}) + b$; a is the slope and b is the intercept. The starting copy number of mRNA was calculated based on Ct value and standard curve for each molecule. The starting copy number of mRNA of each molecule was normalized with the starting copy number of TBP mRNA in each sample.

BrdU-labeling experiments

The GFP tagged Nore1A or Rassf1A expression plasmid (pHRGFP-N1, Stratagene) was transfected by Lipofectamin (Invitrogen). At 48 h after transfection, cells were labeled with BrdU (1:500 dilution, cell proliferation labeling reagent (Amersham-Pharmacia Biotech, Piscataway, NJ, USA)) for 4 h. Labeled cells were fixed with 3% paraformaldehyde for 20 min. After fixation, cells were incubated in 2 N HCl 0.5% triton X-100 PBS for 20 min. Incorporated BrdU was detected by immunofluorescence using anti-BrdU monoclonal antibodies followed by TRITC-conjugated anti-mouse IgG antibody. Tagged GFP was enhanced by anti-GFP antibody (Living Colors A.v. peptide antibody: BD Biosciences Clonthech, CA, USA) followed by FITC-conjugated goat anti-rabbit IgG antibody. The images of the BrdU positive cell and transfected cells were collected using the Texas red filter and FITC filter of the Zeiss Axiovert S100M microscope (Carl Zeiss) connected to a CCD camera. The Texas red filter image and FITC filter image were overlapped by using MetaMorph Imaging software (Universal Imaging).

TUNEL assay

The TUNEL assay was performed to detect apoptotic cells. The DeadEnd™ Colorimetric TUNEL System (Promega Madison, WI, USA) was used according to the manufacturer's instructions. Cultured A549 cells on coverslip were transfected with pHRGFP Nore1A and pHRGFP control vector using Lipofectamin (Invitrogen). A549 cells were treated with 2 mM hydrogen peroxide for 8 h to induce apoptosis as a positive control for TUNEL assay. After 48 h transfection, cells were fixed with 3% paraformaldehyde for 20 min. Fixed cells were permeabilized in 0.2% Triton X-100 PBS for 5 min. Cells were incubated with biotinylated nucleotide mix and terminal deoxynucleotidyl transferase for 60 min at 37°C. The reaction

was terminated by adding the $2 \times$ SSC for 15 min at RT. The endogenous peroxidase was blocked in 0.3% hydrogen peroxide for 5 min at RT. Cells were incubated with the streptavidin HRP solution (diluted 1 : 500 in PBS) for 30 min at RT. Biotinylated nucleotides incorporated at the 3'-OH DNA ends were bound to streptavidin HRP, then detected using the peroxidase substrate, hydrogen peroxides and the stable chromogen, diaminobenzidine. The image of apoptotic nuclei stained dark brown and GFP positive transfected cells were

collected using the phase-contrast filter and FITC filter of the Zeiss Axiovert S100M microscope connected to a CCD camera. The phase-contrast image and FITC filter image were overlapped by using MetaMorph Imaging software.

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