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NORE1A Tumor Suppressor Candidate Modulates p21^{CIP1} via p53

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Abstract

NORE1A (RASSF5) is a proapoptotic Ras effector that is frequently inactivated by promoter methylation in human tumors. It is structurally related to the RASSF1A tumor suppressor and is itself implicated as a tumor suppressor. In the presence of activated Ras, NORE1A is a potent inducer of apoptosis. However, when expressed at lower levels in the absence of activated Ras, NORE1A seems to promote cell cycle arrest rather than apoptosis. The mechanisms underlying NORE1A action are poorly understood. We have used microarray analysis of an inducible NORE1A system to screen for physiologic signaling targets of NORE1A action. Using this approach, we have identified several potential signaling pathways modulated by NORE1A. In particular, we identify the cyclin-dependent kinase inhibitor p21^{CIP1} as a target for NORE1A activation and show that it is a vital component of NORE1A-mediated growth inhibition. In primary human hepatocellular carcinomas (HCC), loss of NORE1A expression is frequent and correlates tightly with loss of p21^{CIP1} expression. NORE1A down-regulation in HCC also correlates with poor prognosis, enhanced proliferation, survival, and angiogenic tumor characteristics. Experimental inactivation of NORE1A results in the loss of p21^{CIP1} expression and promotes proliferation. The best characterized activator of p21^{CIP1} is the p53 master tumor suppressor. Further experiments showed that NORE1A activates p21^{CIP1} via promoting p53 nuclear localization. Thus, we define the molecular basis of NORE1A-mediated growth inhibition and implicate NORE1A as a potential component of the ill-defined connection between Ras and p53.

Introduction

NORE1A (novel Ras effector 1 or RASSF5) is a member of the RASSF family and is ~50% identical to the relatively well characterized RASSF1A tumor suppressor (1, 2). *NORE1A* is frequently down-regulated by promoter methylation in human tumors, and the *NORE1A*

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locus undergoes loss of heterozygosity (LOH) in some primary tumors (1, 3). Furthermore, a translocation involving *NORE1A* results in the manifestation of a hereditary human cancer syndrome (4). Exogenous expression of NORE1A can promote apoptosis (5, 6) or cell cycle arrest (7). Moreover, restoration of endogenous levels of NORE1A expression to a NORE1A-negative human tumor cell line blocks the tumorigenic phenotype (5). Thus, NORE1A seems to function as a human tumor suppressor, like the related RASSF1A (1, 2). However, the mechanism of action of NORE1A is poorly understood and the role of loss of NORE1A expression in tumor development has not been characterized.

NORE1A contains a Ras Association (RA) domain and was originally identified as a Ras binding protein in a two-hybrid screen (8). It directly binds the Ras oncoprotein in a GTP-dependent manner with an affinity comparable with that of other known Ras effectors (9). Moreover, NORE1A forms an endogenous complex with Ras in cells (6). Thus, NORE1A seems to be a *bona fide* Ras effector, which is also a tumor suppressor.

Activated forms of Ras, while being highly transforming, can also activate proapoptotic pathways (10). NORE1A has been identified as one of the proapoptotic effectors of Ras and may function in part by binding and activating the proapoptotic kinases MST1 and MST2 (6). Thus, loss of NORE1A function may enhance the transforming capacity of activated Ras by subverting its proapoptotic functions. This concept is supported by the observation that high levels of Ras activity correlate with low levels of NORE1A expression in hepatocellular carcinoma (HCC; ref. 11).

NORE1A promotes apoptosis when overexpressed or in the presence of activated Ras, but lower levels of NORE1A expression seem to promote G_1 cell cycle arrest (7). The signaling pathways involved in NORE1A function, with the exception of the MST kinases (6), are completely unknown. In an attempt to determine the mechanisms of action of NORE1A, we performed a microarray analysis of kidney cells induced to express NORE1A at levels comparable with those seen in cells that retain endogenous NORE1A expression. Several alterations in gene expression that would be compatible with the action of a tumor suppressor were detected and validated by quantitative reverse transcription–PCR (qRT-PCR). One of the most interesting was the induction of the cyclin-dependent kinase (cdk) inhibitor $p21^{CIP1}$. $p21^{CIP1}$ has a complicated role in the regulation of multiple signaling pathways and can promote, apparently, contradictory biological activities (12). However, overexpression of $p21^{CIP1}$ induces G_1 arrest (13), like NORE1A. Moreover, *p21*^{CIP1} knockout mice are tumor prone and more sensitive to tumor induction by additional genetic lesions (14). Thus, $p21^{CIP1}$ is a good candidate target for a novel NORE1A signaling pathway promoting G_1 arrest.

Here, we show that NORE1A overexpression up-regulates $p21^{CIP1}$ and that loss of NORE1A expression causes the reduction of $p21^{CIP1}$ levels. We confirm that $p21^{CIP1}$ is a key mediator of the growth inhibitory properties of NORE1A. Moreover, in primary liver tumors, loss of NORE1A expression correlated tightly with loss of $p21^{CIP1}$ expression and an enhanced proliferative index. Experimentally, we found that stable knockdown of NORE1A enhanced proliferation and reduced contact inhibition of a nontransformed cell line.

Perhaps, the best-characterized activator of $p21^{CIP1}$ is the master tumor suppressor p53 (15). Using dominant negatives and small interfering RNA (siRNA) against p53, we show that the activation of $p21^{CIP1}$ by NORE1A is p53 dependent. Although NORE1A expression did not seem to affect the protein levels of p53, it did seem to activate the nuclear translocation of p53 by an unknown mechanism. Thus, we have identified multiple proteins implicated in tumor suppression that are activated by near-physiologic levels of NORE1A and identify NORE1A as a component of $p53/p21^{CIP1}$ signaling pathways.

Materials and Methods

Plasmids.

pIND/SP1-NORE1A has been described previously (4), as have pCDNAFLAG and pZIP-NORE1A (5). HA-p53 wild-type and dominant-negative (16) expression plasmids were a generous gift of J. Isaacs (MUSC). p53 siRNA was from Applied Biosystems (ID 2714). The NORE1A short hairpin RNA (shRNA) sequences used were 367:GGCTGCTCAAGAAGTTCATGGTTGTGGAC and 971:GCGACGTGAGGAGCATCTTCGAGCAGCCG. Scram: CAGAAGATCGACAGCTACAACACGCGAGA cloned in the pRS vector (Origene) have been described previously (11).

Tissue culture, transfections, and treatment.

HEK-293ecr kidney (Invitrogen), HCT116 colonic (generous gift, C. Deng, NIH), A549 non–small cell lung cancer (NSCLC), WRL68 hepatoblast, and FOCUS, HuH2, HuH6, and HuH7 HCC (American Type Culture Collection) cell lines were grown in DMEM/10% fetal bovine serum. NIH 3T3 cells were grown as described previously (17). Transient transfections were performed using Lipofectamine 2000. NORE1A silencing experiments on FOCUS and WRL68 liver cell lines were performed, as previously reported (11), and results obtained at 24 and 36 h after transfection with siRNA were analyzed. For demethylating experiments, cell lines were plated and allowed 24-h growth before addition of 10 μmol/L 5-aza-cytidine (5-Aza-C; Sigma). All experiments were repeated at least thrice per each cell line.

Microarray analysis.

HEK-293ecr cells were stably transfected with vector or pIND/SP1 NORE1A, grown in triplicate, and induced with 5 μmol/L Ponasterone A for 48 h. Cells were lysed in Trizol, and extracted RNA was subjected to microarray analysis as described in Supplementary Materials and Methods. Differentially expressed genes between control versus NORE1A-expressing cells were identified with a $P < 0.05$ being considered statistically significant. qRT-PCR analysis was performed as described in the Supplementary Materials and Methods.

Tissue specimens, clinicopathologic data, methylation-specific PCR, microsatellite analysis, proliferation and apoptotic indices, and evaluation of microvessel density.

See Supplementary Materials and Methods section.

Western blots and immunoprecipitation.

The following antibodies were used: anti-$p21^{CIP1}$ (Novus Biologicals); mouse monoclonal anti–cyclin A, anti–cyclin E, rabbit polyclonal anti-CDK2, p53-D0–1 (Santa Cruz Biotechnology); rabbit polyclonal anti-NORE1A (5); HA and FLAG antibodies (Sigma). Secondary antibodies were from Amersham. CDK2–cyclin A and CDK2–cyclin E immunocomplexes were assessed by immunoprecipitation with the anti-CDK2 antibody and probing the membranes with either the anti–cyclin A or anti–cyclin E antibody. Blots were developed using an enhanced chemiluminescence kit from Amersham. Nuclear and cytoplasmic extracts from HuH6 and HuH7 cell lines were prepared by using the NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Inc.). Antirabbit polyclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase and histone H3 (Santa Cruz Biotechnology) were used to ascertain equal loading of cytoplasmic and nuclear pools, respectively.

Results

Microarray analysis of HEK-293ecr kidney cells stably induced for NORE1A implicates $p21^{CIP1}$ as a downstream effector of NORE1A.

NORE1A can induce apoptosis (5, 6) and cell cycle arrest (7). The apoptotic properties of NORE1A are activated by Ras and may be mediated via the MST1 and MST2 kinases (5, 6). However, the mechanisms underlying the effects of NORE1A on the cell cycle remain completely unknown. To address this question, we performed microarray analysis of HEK-293ecr kidney cells induced to express NORE1A. The Invitrogen pIND/SPI/293ecr system was deliberately used to avoid artifacts due to excessive protein expression generated by transient transfection studies. The pIND/SPI plasmid system is induced by an insect hormone mimic, Ponasterone A, which has minimal effects on mammalian gene transcription.

From these assays, we identified several genes that showed enhanced or reduced expression relative to the vector control (Table 1). Three of the targets that exhibited the greatest differential expression were subjected to qRT-PCR validation. EEF2 and SAT1 were confirmed as being down-regulated, whereas $p21^{CIP1}$ was confirmed as being up-regulated (Fig. 1A). Induced expression of NORE1A was confirmed by Western blot analysis (Fig. 1B). NORE1A has previously been shown to induce G_1 cell cycle arrest (7). As $p21^{CIP1}$ has also been shown to be capable of this type of cell cycle arrest (13), we selected it for further study as a potential NORE1A signaling pathway component.

NORE1A stimulates $p21^{CIP1}$ protein expression.

To confirm that the microarray data reflected the effects of NORE1A on $p21^{CIP1}$ protein expression, HEK-293ecr cells were transiently transfected with NORE1A and the levels of endogenous $p21^{CIP1}$ protein measured after 24 hours. Western blotting revealed a dramatic increase in the levels of $p21^{CIP1}$ protein in the NORE1A transfected cells (Fig. 1C). Further confirmation of the effects of NORE1A on $p21^{CIP1}$ expression was obtained by performing luciferase assays with a p21-Luc reporter (18) and increasing amounts of NORE1A expression plasmid (Fig. 1D).

NORE1A inhibition reduces p21^{CIP1} expression.

If NORE1A expression can enhance p21^{CIP1} levels, then inhibition of NORE1A should reduce the levels of p21^{CIP1} protein. The FOCUS (HCC) and WRL68 (hepatoblast) cell lines that retain NORE1A expression were transfected with siRNA against NORE1A, and the reduction of NORE1A protein expression was confirmed by Western blot analysis. The same protein samples were then analyzed for p21^{CIP1} expression (Fig. 2A). siRNA-mediated inhibition of NORE1A expression caused a corresponding decrease in the expression of p21^{CIP1}. Furthermore, suppression of NORE1A resulted in upregulation of CDK2, whose activity is inhibited by p21^{CIP1}. The CDK2 showed enhanced activation in the NORE1A knockdown cells, as measured by the presence of cyclin A and cyclin E in the complex, as well as by kinase activity toward histone H1 (Fig. 2A). These results support an enhanced G_1-S transition after inactivation of NORE1A.

NORE1A inhibition leads to enhanced proliferation/loss of contact inhibition of NIH3T3 cells.

Several studies have examined the effects of exogenous expression of NORE1A on the suppression of cell growth/survival and the tumorigenic phenotype (1). However, the biological consequences of NORE1A loss of function have not been described experimentally. We used a nontransformed murine cell line (NIH 3T3) as a model. Two different shRNAs against murine NORE1A were used to generate stable NIH3T3 cell lines knocked down for the expression of endogenous NORE1A. A scrambled shRNA served as the control. The cells were examined for the effects on proliferation by growth curve analysis (Fig. 2B). In each case, the NORE1A shRNA-transfected cells showed modest growth enhancement but pronounced loss of contact inhibition. Confirmation of NORE1A knockdown was obtained by qRT-PCR (Fig. 2C).

Activation of p21^{CIP1} plays a vital role in NORE1A-mediated growth/survival inhibition.

Having determined that NORE1A modulates p21^{CIP1}, we sought to determine the importance of the p21^{CIP1} activation in NORE1A-mediated suppression of growth and survival. We transfected isogenic HCT116 cells that are (+) or (−) for *p21*^{CIP1} (19) while retaining wild-type p53 with pZIP-NORE1A and selected the cells in G418. Cells retaining p21^{CIP1} expression did not survive the transfection with NORE1A. The *p21*$^{CIP1-/-}$cells showed multiple colony formation when transfected with NORE1A, although this was less than the cells transfected with vector (Fig. 3A). Confirmation that the *p21*$^{CIP1-/-}$cells were expressing NORE1A protein is shown in the adjacent panel. We then transfected HuH6 HCC cells with a NORE1A expression plasmid in the absence (Fig. 3B, i) or presence (Fig. 3B, ii) of a p21^{CIP1} siRNA and measured the effects on proliferation. NORE1A inhibited proliferation, but this was significantly reduced in the presence of the p21^{CIP1} siRNA.

Down-regulation of NORE1A expression correlates with poor prognosis and loss of p21^{CIP1} expression in HCC.

If NORE1A plays an important role in the regulation of basal p21^{CIP1} levels *in vivo*, then we might expect to see a correlation between the expression levels of NORE1A and p21^{CIP1} in primary tumors. We analyzed samples from a panel of HCC tumors that were classified as

poor prognosis (HCCP; survival, <3 years after partial liver resection) or better prognosis (HCCB; survival, >3 years). Promoter hypermethylation of *NORE1A* was detected in 21 of 60 (35%) tumor samples (Fig. 4A), with no hypermethylation being found in normal and surrounding nonneoplastic livers (data not shown). Together with *NORE1A* promoter hypermethylation, 9 of 60 (15%) HCCs displayed LOH of the *NORE1A* locus. Importantly, 18 of 21 (85.7%) HCCs displaying promoter hypermethylation of *NORE1A* were characterized by a poor prognosis (HCCP), indicating that its down-regulation is a feature of HCC aggressiveness. Analysis of the HCCP panel for the relative levels of NORE1A and $p21^{CIP1}$ protein expression showed promoter methylation of *NORE1A* correlated perfectly with reduced NORE1A expression (Fig. 4A). Furthermore, a significant, direct correlation was found between the levels of NORE1A and those of $p21^{CIP1}$ in HCC by the Pearson's correlation test ($r^2 = 0.6962$, $P < 0.001$). These results were further confirmed by qRT-PCR in pooled samples that were +/− for *NORE1A* promoter methylation (Supplementary Fig. S1). In these experiments, reduction of NORE1A expression also correlated with increased EEF2 and SAT1 expression (Supplementary Fig. S1). Thus, the microarray results are again predictive of the situation in primary tumors. A representative Western blot of a panel of tumors that were *NORE1A* nonmethylated and of good prognosis (HCCB) compared with a panel of *NORE1A*-methylated tumors that were of poor prognosis (HCCP) is presented in Fig. 4B to illustrate the levels of NORE1A observed in HCCB versus HCCP.

The tumor suppressor p53 is perhaps the best characterized regulator of $p21^{CIP1}$ (15). If the *NORE1A* methylated tumors were all *p53* mutant, then this might act as an alternative explanation of the reduction in $p21^{CIP1}$ expression. Intriguingly, our analysis of the *p53* mutation status of the tumors showed a total mutual exclusion of *NORE1A* promoter methylation/LOH and the mutation of *p53* (Fig. 4A, bottom).

Correlation of NORE1A levels with clinicopathologic parameters of human HCC.

To further characterize the role of NORE1A in human HCC, we evaluated the proliferation and apoptotic indices and microvessel density in liver tumors with levels of NORE1A comparable or lower than normal livers, respectively. HCC with low levels of NORE1A displayed higher proliferation (35.02 ± 6.77 versus 21.87 ± 6.33; $P = 1.92 \times 10^{-6}$) and lower apoptotic index (1.03 ± 0.49 versus 2.24 ± 0.74; $P = 8.19 \times 10^{-8}$), as well as higher microvessel density (303.79 ± 58.14 versus129.65 ± 29.79; $P = 3.03 \times 10^{-15}$), when compared with HCC displaying NORE1A levels comparable with normal livers. This resulted in a significant shorter patient survival length for HCC with lower than normal NORE1A levels (11.26 ± 3.63 versus 51.60 ± 19.77; $P = 3.93 \times 10^{-6}$). Taken together, these data indicate that suppression of NORE1A is associated with increased proliferation, reduced apoptosis, and angiogenic properties of human liver tumors, leading to a rapid, adverse outcome (Supplementary Table S2).

Epigenetic therapy restores NORE1A signaling pathways.

NORE1A is down-regulated by aberrant promoter methylation, and this can be reversed by treating cells with the DNA methyltransferase inhibiting drug 5-Aza-C (5). To determine if such treatment not only restores NORE1A expression but allows reconstitution of NORE1A signaling pathways, we treated the HuH2 HCC (Fig. 4C) and the A549 NSCLC (Fig. 4D)

cell lines that had suffered epigenetic inactivation of *NORE1A* (5)[5] with 5-Aza-C. We then examined the cells by Western blot for NORE1A and $p21^{CIP1}$ expression. 5-Aza-C not only increased NORE1A expression but also increased the expression of $p21^{CIP1}$ in both cell lines (Fig. 4C and D).

NORE1A activates *p21*^{CIP1} via the p53 tumor suppressor.

The best known activator of $p21^{CIP1}$ is the tumor suppressor *p53* (15). Activation of $p21^{CIP1}$ by p53 has been shown to play a key role in the ability of p53 to induce cell cycle arrest (19). Consequently, we performed experiments to determine if NORE1A was acting via p53. First, we transfected two HCC cell lines, one wild type for *p53* (HuH6) and one mutant for *p53* (HuH7) with pCDNAFLAG NORE1A and analyzed the two cell lines for the expression of both NORE1A and $p21^{CIP1}$ by Western blot. Figure 5A shows that $p21^{CIP1}$ was only activated in the *p53* wild-type cell line, despite the levels of transfected NORE1A expression being identical. Although the *p53* mutant cell line was defective for $p21^{CIP1}$ activation, we did not observe any apparent change in the levels of p53 protein (second to bottom panel). To confirm a role for p53 in the action of NORE1A, we transfected HEK-293ecr cells with NORE1A in the presence of a p53 dominant-negative (generous gift from J. Isaacs, MUSC) or p53 siRNA. Both inhibited the ability of NORE1A to stimulate $p21^{CIP1}$ protein expression (Fig. 5B).

Examination of the levels of p53 in NORE1A transfected cells failed to show any obvious change (Fig. 5A). However, when we fractionated NORE1A-transfected HuH6 and HuH7cells, we found that the levels of endogenous p53 in the nucleus were increased (Fig. 5C, i and ii) in the HuH6 cells (containing wild-type p53) but not in the HuH7 cells (containing mutant p53). Nuclear translocation of p53 is indicative of activation (20). Thus, NORE1A seems to act via promoting the nuclear localization of p53.

Discussion

NORE1A was originally identified as a Ras effector protein and was subsequently shown to mediate some of the proapoptotic functions of Ras (5, 6). NORE1A has no apparent enzymatic activity and is hypothesized to function as a scaffolding molecule, as it has been shown to bind the MST kinases and recruit them to the plasma membrane in a complex with activated Ras (6). In the absence of activated Ras, NORE1A expression can promote G_1 cell cycle arrest (7), but the mechanism of this action is not known. Thus, NORE1A can modulate apoptosis or cell cycle arrest, and the balance of these forces may be shifted toward apoptosis by the interaction with Ras.

Several lines of evidence suggest that in addition to serving as a Ras effector, NORE1A is also a tumor suppressor. Firstly, NORE1A expression is frequently impaired in a variety of tumors, particularly kidney, lung, and liver tumors (1). Secondly, restoration of NORE1A expression to normal levels in a NORE1A-negative tumor cell line blocks the ability of the cells to grow in soft agar (5). Thirdly, loss of NORE1A function is implicated in the development of a human hereditary cancer syndrome (4). Thus, NORE1A is strongly

[5]D.F. Calvisi, unpublished observation.

implicated as an important human tumor suppressor with a poorly understood mode of action.

To gain a better understanding of the function of NORE1A, we performed a microarray analysis to identify transcriptional alterations associated with the expression of NORE1A. In an attempt to maximize physiologic relevance, we used a kidney cell system stably transfected with an inducible form of NORE1A. Kidney tumors are the most frequently associated with NORE1A down-regulation (1), and the inducible system allowed us to control the expression levels of NORE1A, avoiding potential artifacts due to massive overexpression.

A series of transcriptional alterations due to NORE1A induction were observed. Three genes that showed some of the strongest changes in the microarray assay were subjected to quantification by qRT-PCR. These experiments confirmed that NORE1A expression induced decreases in *EEF2* and *SAT1* and an increase in $p21^{CIP1}$ expression. Further analysis showed that in primary HCC, NORE1A expression correlated with $p21^{CIP1}$ and inversely correlated with EEF2 and SAT1 expression (Supplementary Fig. S1).

EEF2 is a translation factor that mediates ribosomal translocation during peptide chain elongation and is activated by mitogenic stimuli (21). EEF2 is overexpressed in many tumors and seems to play an important role in rendering tumor cells resistant to the translation suppressing effects of hypoxia (22). Resistance to hypoxia is believed to play a critical role in the development of many tumors (23).

SAT1 is a spermidine kinase that plays a key role in the regulation of the intracellular levels of polyamines (24). Polyamines play an important role in neoplastic growth, and polyamine synthesis inhibitors are of interest as chemopreventive agents (25). Activated K-Ras, like NORE1A, inhibits SAT1 expression (25). Moreover, the related SAT2 has been implicated in the regulation of HIF-1α, a key component of the cellular response to hypoxia and an important component of the development of kidney cancer and HCC progression (26).

Previously, microarray analysis has been performed to determine the signaling profile of RASSF1A (27). RASSF1A is 50% identical to NORE1A, but the two proteins seem to promote quite different alterations in gene expression. SAT1 was the only target identified by both RASSF1A and NORE1A. This confirms that the functions of NORE1A and RASSF1A, while overlapping, are likely to be quite distinct.

Several other up-regulated targets identified in the array have also been associated with cell death and growth suppression. Among them, BTG3 is a putative tumor suppressor and target of p53 itself (28), and PDCD2 has been implicated in apoptosis (29) and proliferation control (30). Moreover, RAB32 down-regulation has been associated with colon cancer (31). Thus, NORE1A promotes a number of alterations in transcription that might be expected to repress transformation. Further work will be required to validate these targets and assess their role in NORE1A signaling.

The original purpose of initiating these experiments was to attempt to understand the basis of the nonapoptotic growth inhibition we had observed with relatively low levels of

NORE1A expression. Consequently, the most interesting effect of NORE1A that we detected was the activation of transcription of the $p21^{CIP1}$ cdk inhibitor.

Cell homeostasis is normally maintained by a complex, coordinated network of signaling pathways that balances proliferation, growth arrest, differentiation, and cell death. Errors in this balance can lead to the development of neoplasia. Correct regulation of the cell cycle is a key component of maintaining the homeostatic balance. Cell cycle regulation is mediated by cdks, and cdks are regulated, in part, by cdk inhibitors, such as $p21^{CIP1}$ (32). $p21^{CIP1}$ controls cell cycle progression through G_1-S at several levels (33).

$p21^{CIP1}$ has also been shown to induce resistance to apoptotic stimuli, such as DNA damage. The mechanism of this effect may be to delay the cell in G_1 while DNA repair is affected. However, the role of $p21^{CIP1}$ in the modulation of apoptosis is complex, and under some circumstances, $p21^{CIP1}$ may exhibit proapoptotic functions (34, 35). $p21^{CIP1}$ has also been identified as playing a key role in the induction of terminal differentiation and senescence (32, 36). Thus, $p21^{CIP1}$ plays a complex and, at times, apparently contradictory role in modulating cellular behavior and survival. However, on the whole, loss of $p21^{CIP1}$ expression seems to promote transformation. In primary human tumors, loss of $p21^{CIP1}$ protein expression has been correlated with poor prognosis (37). Moreover, the inactivation of $p21^{CIP1}$ in transgenic mice is, eventually, tumorigenic and enhances the transforming effects of other genetic lesions, including activated Ras (14, 38, 39). Therefore, $p21^{CIP1}$ seems to serve as a tumor suppressor.

The ability of NORE1A to activate $p21^{CIP1}$ explains the ability of NORE1A to induce G_1 cell cycle arrest (7), as $p21^{CIP1}$ blocks cell cycle at G_1 by inhibiting cdk2 (32). Conversely, loss of NORE1A expression reduces $p21^{CIP1}$ expression and enhances cdk2 activity. We also observed an increase in cdk2 protein levels, although this target was not identified in the microarray. Both effects could contribute to a large increase in kinase activity of the cdk2 kinase complex precipitated from the knockdown cells. The importance of $p21^{CIP1}$ activation to NORE1A-mediated growth inhibition was confirmed by our observation that loss of $p21^{CIP1}$ from the cell system impaired the growth/survival inhibitory effects of NORE1A.

NORE1A expression is often down-regulated in human tumors (1). Our siRNA experiments in cells lines suggested that cells require NORE1A for normal $p21^{CIP1}$ expression. Examination of a panel of primary HCC showed that loss of NORE1A expression correlated closely with down-regulation of $p21^{CIP1}$ expression. Preliminary data from our group show a similar pattern in a small collection of human renal, lung, and colorectal carcinomas (data not shown). These findings suggest a physiologic link between NORE1A and $p21^{CIP1}$ in tumors. Loss of NORE1A and $p21^{CIP1}$ expression also strongly correlated with poorer prognosis of HCC, and this may identify *NORE1A* as a good target for epigenetic therapy. Experimental restoration of NORE1A expression by demethylating drugs was successful in increasing $p21^{CIP1}$ expression. This confirms the ability of these drugs to restore pathway function, not just NORE1A expression.

As the p53 tumor suppressor is the best characterized regulator $p21^{CIP1}$ (40, 41), we examined the role of p53 in NORE1A mediated $p21^{CIP1}$ activation. Dominant-negative and siRNA reagents showed that p53 was necessary for NORE1A to activate $p21^{CIP1}$. Moreover, NORE1A could only activate $p21^{CIP1}$ in a wild type p53-containing tumor cell line. p53 seems to be the most important tumor suppressor yet identified in human cancer with over 50% of all primary tumors showing defective p53 function (42). Thus, NORE1A participates in the modulation of what may be considered the major human tumor suppressor pathway. This conclusion is supported by the observation that, in primary human HCC s, the mutation of *p53* and the inactivation of *NORE1A* are mutually exclusive.

The mechanism by which NORE1A can modulate p53 remains unknown. We have not detected NORE1A in complex with p53 nor have we observed changes in p53 protein levels due to NORE1A. However, we did observe an increase in the fraction of p53 present the nucleus in NORE1A-transfected cells. This suggests to us that NORE1A is promoting the nuclear localization of p53 via some posttranslational modification, such as phosphorylation or acetylation (43). We are currently examining which, if any, modifications p53 are affected by NORE1A.

Although NORE1A is frequently down-regulated in primary human tumors, the biological effects of inhibiting NORE1A function have not been determined. To identify the effects of NORE1A loss function on nontransformed p53 wild-type cells, we transfected NIH 3T3 cells with two different NORE1A shRNAs to make stable knockdown cell lines. The cells showed an enhanced rate of growth, but also a noticeable reduction in contact inhibition, and continued proliferate after the scrambled shRNA transfected cells had arrested. We have recently shown that defects in p53 function can lead to loss of contact inhibition in NIH 3T3 cells (44), so inactivation of NORE1A may have a similar effect via p53.

Thus, we identify a novel signaling pathway for NORE1A, connecting it to p53 and hence to the $p21^{CIP1}$ cdk inhibitor. This may help explain the frequency with which *NORE1A* is inactivated in primary human tumors and why *NORE1A* and *p53* inactivation is mutually exclusive in primary liver tumors. As NORE1A is a Ras effector, the data suggest that it could play a role in the well known but poorly understood connection between Ras and p53.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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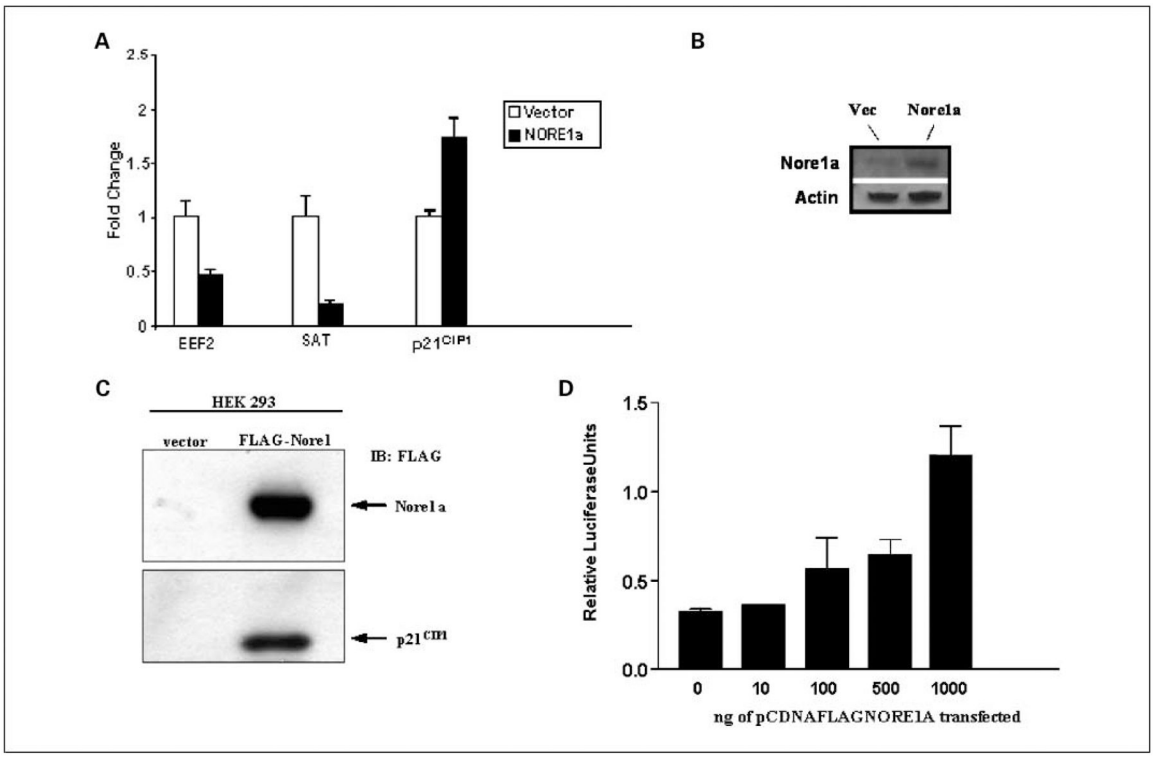

Figure 1.

A, qRT-PCR validation. qRT-PCR analysis was performed on the induced cells for EEF2, SAT1, and $p21^{CIP1}$. Values for the vector were normalized to 1, and the fold change due to NORE1A was plotted. The results confirmed the microarray results. *B*, protein expression of NORE1A in the induced cells. *C*, NORE1A induces $p21^{CIP1}$ protein expression. HEK-293 cells were transfected with pcDNAFLAG NORE1A or empty vector. After 24 h, the cells were lysed, and equal amounts of protein lysate were Western blotted for exogenous NORE1A (FLAG) and endogenous $p21^{CIP1}$. *D*, increasing amounts of pcDNAFLAG NORE1A expression plasmid were transfected into NIH 3T3 cells with a p21-luciferase reporter and a *Renilla*-luciferase internal control plasmid, essentially as described previously (45).

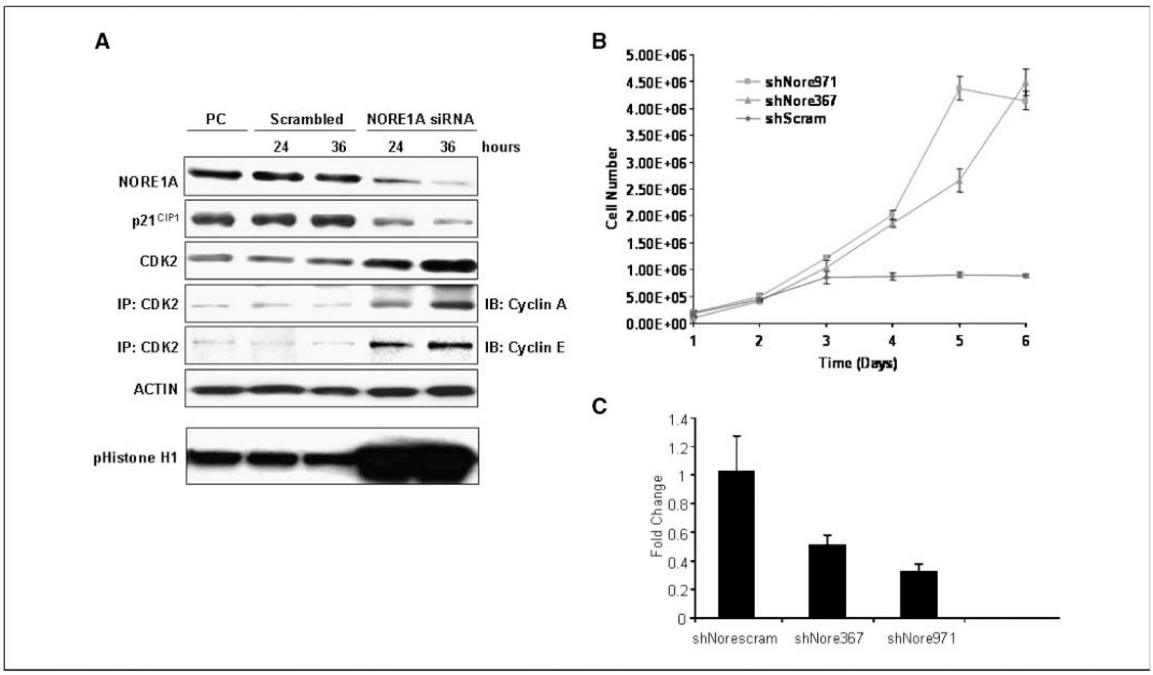

Figure 2.

A, inhibition of NORE1A expression leads to reduced $p21^{CIP1}$ expression and increased G_1-S transition markers in human liver cell lines. WRL68 and FOCUS cells were transfected with siRNA duplexes specific for human NORE1A. Results obtained from the FOCUS HCC cell line at 24 and 36 h after transfection with siRNA. Actin serves as the loading control. Equivalent results were obtained with WRL68 cells (data not shown). Experiments were repeated at least thrice per each cell line. *PC*, positive control (lysate from a human nontumor surrounding liver expressing NORE1A). The activity of CDK2 was measured by kinase assay of the immunoprecipitation (*IP*) on histone H1. *B*, down-regulation of NORE1A promotes modest enhanced growth and resistance to contact inhibition in NIH 3T3 cells *C*, down-regulation of NORE1A in the stable shRNA transfectants.

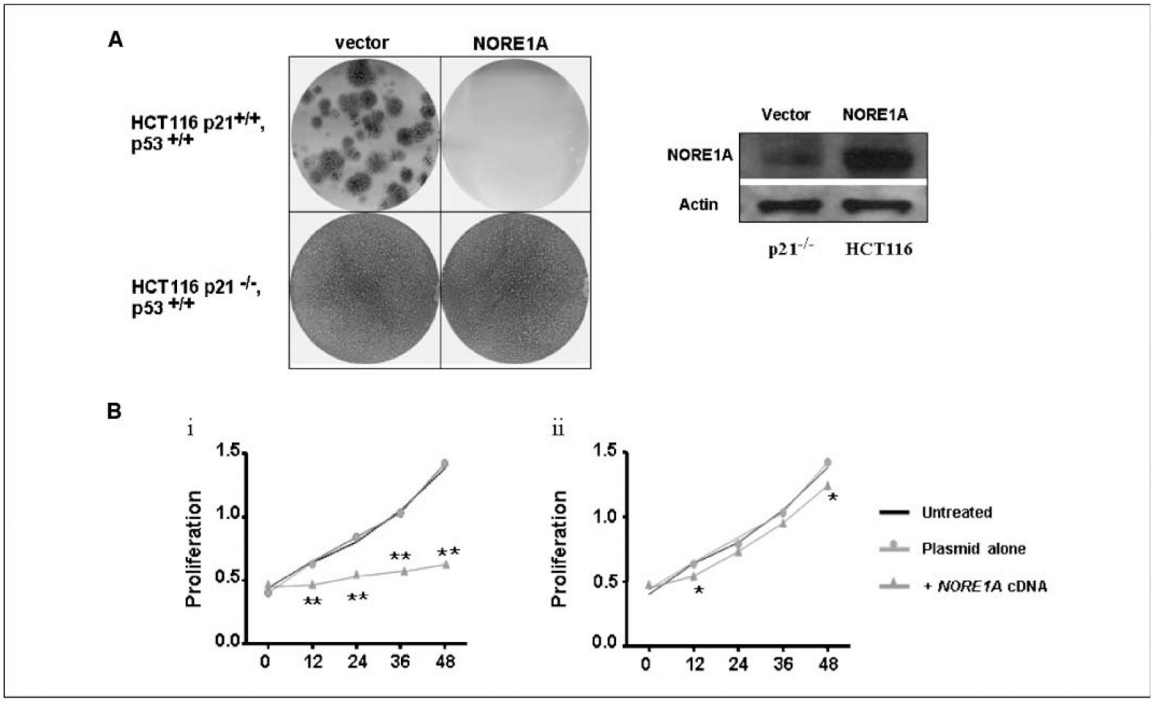

Figure 3.

NORE1A uses $p21^{CIP1}$ to inhibit cell growth. *A*, a matched pair of HCT116 cells that were (+/−) for $p21^{CIP1}$ were transfected with pZIP-NORE1A and selected in G418. Surviving colonies were stained with crystal violet (*top*). *B*, transfection of pCDNAF NORE1A led to marked growth restraint in the HuH6 cell line (*i*), which was lost when $p21^{CIP1}$ was suppressed by siRNA (*ii*). Points, means of three experiments in triplicate. Tuckey-Kramer test, NORE1A-transfected versus control and plasmid alone; *, $P < 0.05$; **, $P < 0.0001$. Cell viability was determined by the WST-1 cell proliferation reagent (Roche Diagnostics).

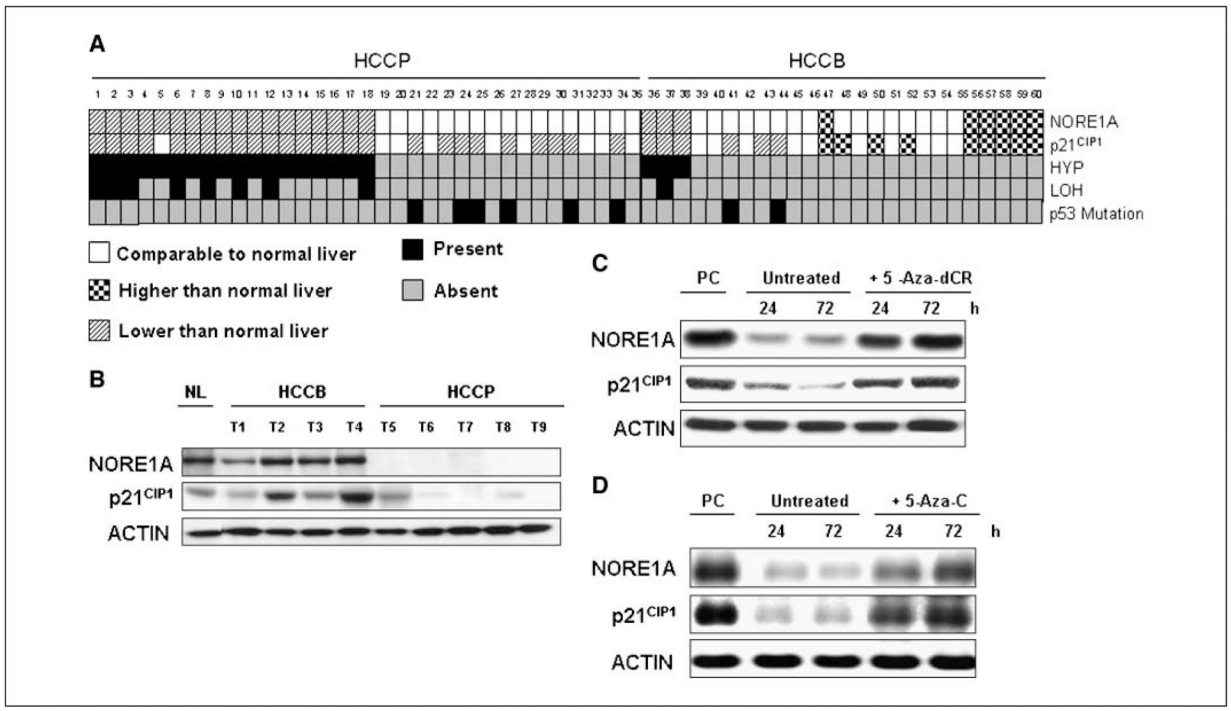

Figure 4.

A, loss of NORE1A expression correlates with loss of $p21^{CIP1}$ expression and inversely with p53 mutation in human HCC. HCC samples were examined for *NORE1A* promoter hypermethylation (*HYP*) and LOH, as well as for NORE1A and $p21^{CIP1}$ expression and *p53* mutation. Tumors 1 to 35 are HCCP (survival, <3 y). Tumors 36 to 60 are HCCB (survival, >3 y). Protein lysates were immunoblotted with NORE1A and $p21^{CIP1}$ antibodies. In each case, Western blot analysis revealed that *NORE1A* methylation corresponded with reduced NORE1A expression compared with normal liver and, in every case but one, reduced $p21^{CIP1}$ expression. *NORE1A* inactivation and *p53* mutation were mutually exclusive. *B*, representative Western blot analysis is shown. Actin serves as a loading control. *HCCB*, HCC with better prognosis; *HCCP*, HCC with poorer prognosis; NL, normal livers. HuH2 human HCC (*C*) and A549 NSCLC (*D*) cell lines were treated with 5-Aza-C and analyzed by Western blot for the expression of NORE1A and $p21^{CIP1}$. Actin serves as a loading control.

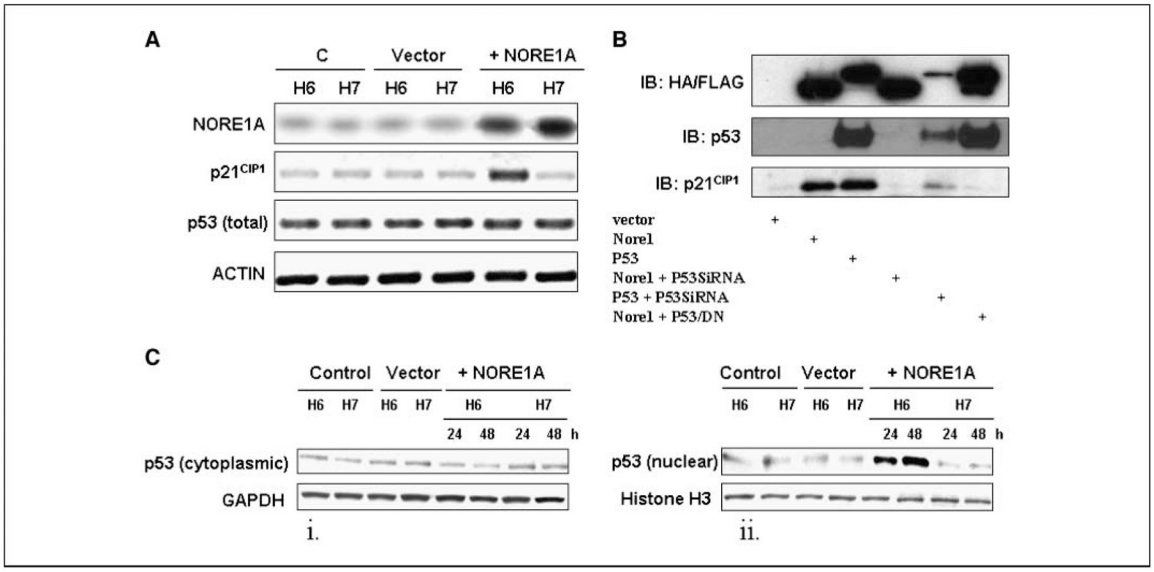

Figure 5.

NORE1A activates p21^{CIP1} protein expression via p53. A, HuH6 (p53 wild type; *H6*) and HuH7 (p53 mutant; *H7*) human HCC cell lines were transfected with pCDNAFLAG NORE1A, and the expression of NORE1A and p21^{CIP1} protein was determined by Western blot. NORE1A induced p21^{CIP1} only in the p53 wild-type cell line, whereas the total levels of p53 did not change in both cell lines. *B*, Hek-293 cells were transfected with NORE1A in the presence or absence of p53siRNA or a p53 dominant-negative construct. An HA-tagged p53 expression vector serves as a positive control. siRNA to p53 or the p53 dominant-negative inhibited NORE1A induced p21^{CIP1} expression. *C*, NORE1A induces nuclear translocation of wild-type p53. HuH6 (p53 wild type; *H6*) and HuH7 (p53 mutant; *H7*) HCC cell lines were transiently transfected with pCDNAFLAG NORE1A and fractionated into cytoplasmic (*i*) and nuclear (*ii*) fractions before Western blotting for p53. NORE1A had no effect on p53 levels in HuH7 cells, but it promoted progressive nuclear accumulation of p53 in HuH6 cells.

Table 1.

Summary of the most overt alterations in gene expression in 293ecr cells induced to express NORE1A compared with induced cells transfected with the empty vector

Entrez gene ID	Gene	Description	Fold difference	Chromosomal location	Function
1026	CDKN1A	CDK inhibitor 1A (p21, Cip1)	2.257365	6p21.2	CDK inhibitor
10981	RAB32	RAB32, member RAS oncogene family	2.135154	6q24.3	Participates in mitochondrial anchoring of PKA
5906	RAP1A	RAP1A, member of RAS oncogene family	1.980178	1p13.3	Interacts with RAS GAPs and RAF to counteract the mitogenic function of RAS
5134	PDCD2	Programmed cell death 2	1.948819	6q27	May play a role in cell death
9689	BZW1	Basic leucine zipper and W2 domains 1	1.896422	2q33	Enhances histone H4 gene transcription
5116	PCNT	Pericentrin	1.883309	21q22.3	Integral component of the pericentriolar material
10950	BTG3	BTG family, member 3	1.881548	21q21.1-q21.2	Involved in the negative regulation of cell proliferation
5877	RABIF	RAB interacting factor	1.813152	1q32-q41	Guanine nucleotide-releasing protein that acts on members of the SCE4/YPT1/RAB subfamily
3301	DNAJA1	DnaJ (Hsp40) homologue, subfamily A, member 1	1.783573	9pl3-pl2	May play a role in protein import into mitochondria
9208	LRRFIP1	Leucine rich repeat (in FLII) interacting protein 1	1.714645	2q37.3	Transcriptional repressor, may regulate expression of TNF, EGFR, and PDGFA
1841	DTYMK	Deoxythymidylate kinase (thymidylate kinase)	1.698028	2q37.3	Catalyzes the conversion of dTMP to dTDP
4675	NAP1L3	Nucleosome assembly protein 1-like 3	1.676265	Xq21.3-q22	Involved in nucleosome assembly
5707	PSMD1	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 1	1.667157	2q37.1	Regulatory subunit of the 26 proteasome, which is involved in the ATP-dependent degradation of ubiquitinated proteins
5110	PCMT1	Protein L-isoaspartate (L-aspartate) *O*-methyltransferase	1.627503	6q24-q25	Catalyzes the methyl esterification of L-isoaspartyl and D-aspartyl residues
7307	U2AF1	U2 small nRNA auxiliary factor 1	1.618806	21q22.3	Plays a critical role in both constitutive and enhancer-dependent splicing
2971	GTF3A	General transcription factor IIIA	1.615342	13q12.3-q13.1	RNA polymerase III transcription factor inducing transcription of the 5S rRNA genes
4192	MDK	Midkine (neurite growth-promoting factor 2)	1.605163	11p11.2	Has heparin binding activity and growth-promoting activity
23369	PUM2	Pumilio homologue 2 (*Drosophila*)	0.756976	2p22-p21	Sequence-specific RNA-binding protein that regulates translation and mRNA stability
2195	FAT1	FAT tumor suppressor homologue 1 (*Drosophila*)	0.749822	4q35	May function as a cell-adhesion protein
51690	LSM7	LSM7 homologue, U6 small nRNA associated (*S. cerevisiae*)	0.74828	19p13.3	Binds specifically to the 3'-terminal U-tract of U6 snRNA
6209	RPS15	Ribosomal protein S15	0.733836	19p13.3	component of the 40S ribosomal subunit
4267	CD99	CD99 molecule	0.708318	Xp22.32; Yp11.3	Involved in cell adhesion processes

Entrez gene ID	Gene	Description	Fold difference	Chromosomal location	Function
1153	CIRBP	Cold inducible RNA binding protein	0.700366	19p13.3	May play an essential role in cold-induced suppression of cell proliferation
10975	UQCR	Ubiquinol-cytochrome *c* reductase, 6.4-kDa subunit	0.663303	19p13.3	May function as an iron-sulfur protein-binding factor
1938	EEF2	Eukaryotic translation elongation factor 2	0.588235	19pter-q12	Essential factor for protein synthesis
6303	SAT1	Spermidine/spermine N1-acetyltransferase 1	0.427843	Xp22.1	Rate-limiting enzyme in the catabolic pathway of polyamine metabolism

NOTE: Results are derived from three separate assays.