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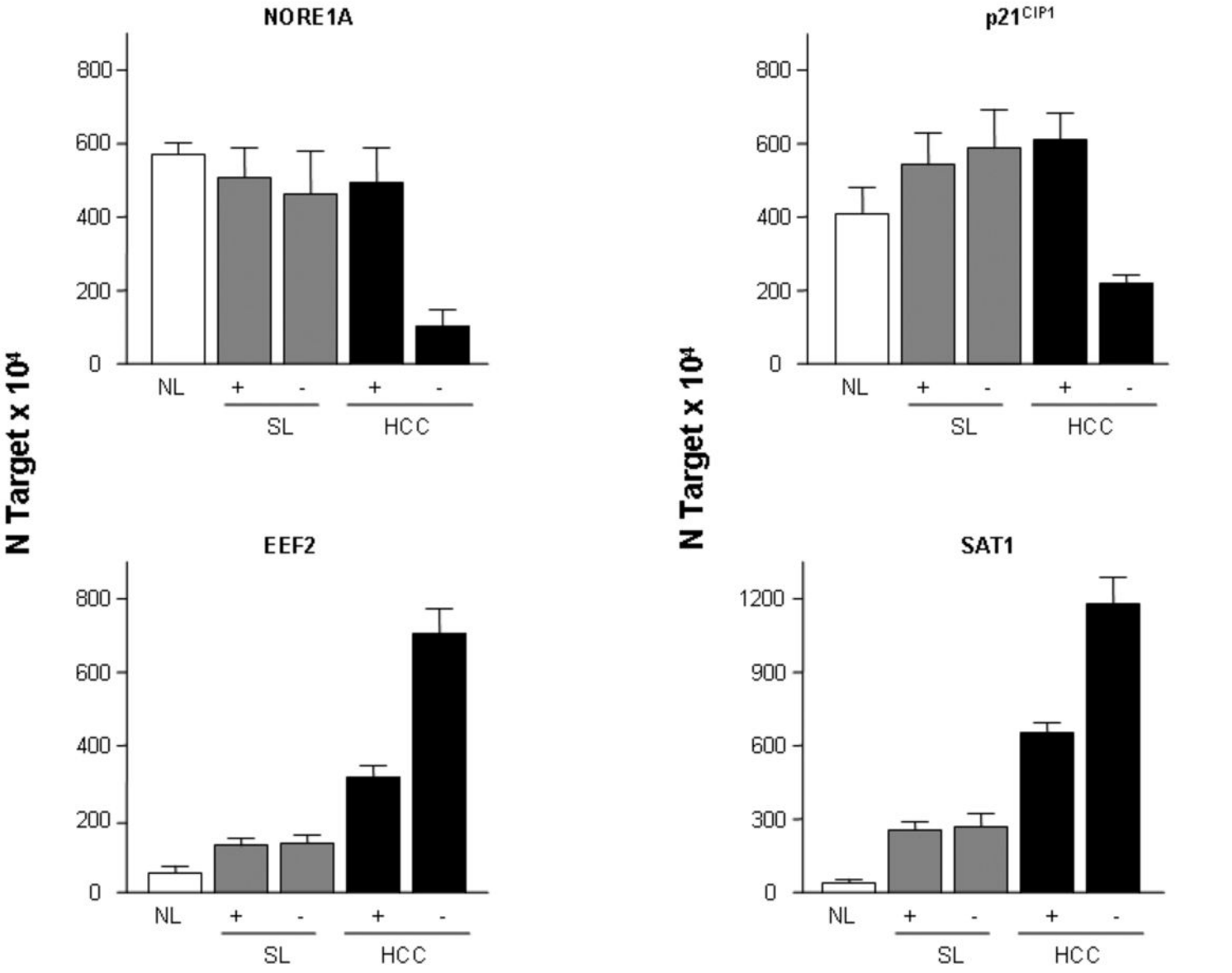
Supplementary Figure 1



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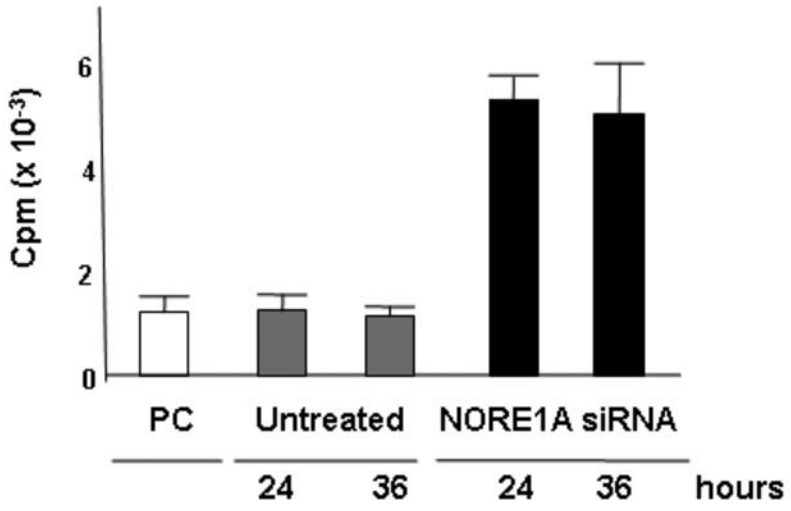
Supplementary Figure 2



SUPPLEMENTARY METHODS

Microarray analysis. HEK-293ecr cells stably transfected with vector or pIND/SP1 Nore1a were grown in triplicate and induced with 5 μM Ponasterone A for 48 hours. Cells were lysed in Trizol and extracted RNA was amplified using the Eberwine method. Total RNA was reverse transcribed using an oligo dT primer containing the T7 RNA polymerase binding site (5'-GGCCAGTGAATTGAATACGACTCCTATAGGGAGGCGG-(dT)24-3'). Second strand cDNA synthesis was performed using RNAse H, DNA Polymerase 1 and DNA Ligase (Gibco). The cDNA was blunt ended and amplified RNA prepared using the T7 Megascript Kit 1334 (Ambion). Five μg of amplified human reference RNA (Stratagene) was labeled with cy3-dUTP and 5μg amplified RNA from vector control and Nore1a-expressing cells were labeled with cy5-dUTP by reverse transcription using 6μg of random primers and 400 units of superscript II-reverse transcriptase enzyme (Gibco). The probes were purified on centricon-30 microconcentrator columns (Amicon). Labelled human reference cDNA was combined with cell line cDNA, 2μg human COT1 DNA (Gibco), 16 μg polyA (Sigma), 8 μg yeast tRNA and mixed with an equal volume of 2X hybridization solution (10X SSC, 50% formamide and 0.05% SDS). The probes were denatured at 95°C for 1 min; snap cooled and hybridized to 11 000-element cDNA microarrays produced by the National Cancer Institute's Microarray Facility using Incyte Genomics UniGEM clones. The slides were prehybridized in 5X SSC, 0.1% SDS and 10mg/ml BSA for 1hr, washed in ddH_2O and isopropanol, and incubated with the labeled probes for 16hr in humidified slide chambers in a 42°C water bath. Cover slips were removed by dipping in 2X SSC, 0.1% SDS and the arrays washed in 1X SSC, 0.1% SDS for 4 min, 0.2X SSC for 4 min and in 0.05X SSC for 1 min. The arrays were dried and scanned using a 10mm resolution GenePix 4000 scanner (Axon Instruments Inc). The resulting TIFF images were analyzed with GenePix software version 3 and result files exported to the NCI microarray database (MaDB)[1] developed by Dr. John Powel.

Differentially expressed genes between control vs Nore expressing cells were identified in MaDB and a $p < 0.05$ was considered statistical significant.

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). qRT-PCR was performed on total RNA isolated from the cells with Trizol using an iCycler Real-Time Detection System (Bio-Rad Laboratories, Inc., Hercules, CA) with the Quantitect SYBR Green RT-PCR Kit (Qiagen, Inc., Valencia, CA) as per the manufacturer's instructions. The fold change for each gene was calculated using the $2^{-\Delta\Delta C}{}_T$ method with ß-actin as the reference gene. The primers for each gene validated by qRT-PCR were: $p21^{CIP1}$: 5'-CCGTGAGCGATGGAACTTC, 5'-CCTGCCTCCTCCCAACTCS; EEF2: 5'-GTGCTGATGATGAACAAGAT, 5'-TCGCCGTAGGTGGAGATG; SAT1: 5'-TTACCTATGACCCGTGGATT, 5'-GCAACCTGGCTTAGATTCTT. For human liver samples, primers for NORE1A, $p21^{CIP1}$, EEF2, SAT1, and RNR-18 genes were chosen with the assistance of the Assay-on-Demand™ Products" (Applied Biosystems, Foster City, CA). Quantitative values were calculated by using the PE Biosystems Analysis software and expressed as N target (NT). $NT = 2^{-\Delta Ct}$, where ΔCt value of each sample was calculated by subtracting the average Ct value of the target gene from the average Ct value of the *RNR-18* gene.

[1] http://nciarray.nci.nih.gov/

Human tissue samples. Five normal livers, 60 surgically-resected HCCs and corresponding surrounding non-tumor liver tissues were used. Clinicopathological features of the patients enrolled in this study are shown in Supplementary Table 1. HCCs were divided in two groups based on patient's survival length: HCCs with poor prognosis (HCCP) were characterized by a shorter (< 3 years) survival and HCCs with better prognosis (HCCB) by a longer (> 3 years) survival following liver partial resection.

Proliferation and apoptotic indices. Proliferation and apoptotic indices were scored on human hepatocellular carcinomas (HCC) and at least 2000 hepatocyte nuclei per sample were counted. Proliferation index was determined by counting Ki-67-positive cells. Apoptotic index was calculated by counting the apoptotic figures on tumor sections stained with the ApoTag peroxidase in situ apoptosis detection kit (Millipore, Millerica, MA) and expressed as percentage of the total number of cells counted.

Evaluation of Microvessel Density (MVD. HCCs were subjected to immunostaining with mouse monoclonal anti-CD34 antibody (Vector Laboratories, Burlingame, CA). HCCs were screened at low power (X40) to identify the areas of highest MVD. The four highest MVD areas for each tumor were photographed at high power (X200) and the size of each area standardized using the ImageJ software. MVD was expressed as the percentage (mean + SE) of the total CD34 stained spots per section area (0.94 mm^2).

Mutation analysis of p53 gene. To detect p53 gene mutations, exons 4–8 were amplified and processed for direct DNA sequencing as published (Supplementary Reference 1).

Methylation-specific PCR and microsatellite analysis. High molecular weight DNA from human samples was isolated and modified with the EZ DNA methylation kit (Zymo Research, Orange, CA) as reported (11). The CpGenome Universal Methylated DNA and CpG Universal Unmethylated DNA (Millipore, Billerica, MA) were used as positive and negative control for each reaction, respectively. Two distinct sets of primers specific for methylated and unmethylated NORE1A promoter were used (19;20). Loss of heterozygosity (LOH) of the NORE1A locus was investigated with D1S3553 and RH98662 primer pairs, as published (11).

LOH was recorded when a 50% or greater reduction in electrophoretic band intensity was detected with silver nitrate staining (Silver Stain Plus, Bio-Rad, Hercules CA).

Cdk2 kinase assay. Cdk2 assay was performed as described by Mazumder et al (Supplementary Reference 2). In brief, liver tissues were homogenized on ice in cdk lysis buffer (50mM HEPES pH7.0, 250mM NaCl, 5mM EDTA pH8.0, 0.5% NP-40, 1mM PMSF, 1mM NaF, 20 mM sodium pyrophosphate, 1 mM sodim vanadate, 10 ug/ml each of leupeptin, aprotinin and pepstatin). Protein concentration was determined with the Bio-Rad assay (BioRad). A total 50 ug of protein lysate was incubated with 2 ug of rabbit anti-Cdk2 antibody (Santa Cruz) at 4ºC for 1 hour followed by immunoprecipitation with protein A agarose conjugate (Santa Cruz) at 4ºC for 1 hour. The beads were washed three times with kinase buffer, containing 50mM HEPES pH7.0, 5mM MgCl2, and 10mM DTT. Beads were suspended in 30 µl of kinase buffer containing 1 µg of histone H1 as the substrate (Calbiochem), 2.5 mM EGTA, 10 mM ß-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM NaF, 20 µM ATP, and 10 µCi of [γ-^{32}P]ATP. After incubation for 30 min at 30°C with occasional mixing, the reactions were stopped with 10 µl of 4x Laemmli buffer, boiled for 10 min, and resolved by SDS-PAGE. Phosphorylated histone H1 was visualized by autoradiography. The gel was dried on a whatman filter and exposed to phosphoimager for quantification analysis.

Supplementary references

1. Anzola M, Saiz A, Cuevas N, Lopez-Martinez M, Martinez de Pancorbo MA, Burgos JJ. High levels of p53 protein expression do not correlate with p53 mutations in hepatocellular carcinoma. J Viral Hepatitis 2004; 11:502-510.

2. Mazumder S, Gong B, Chen Q, Drazba JA, Buchsbaum JC, Almasan A. Proteolytic cleavage of cyclin E leads to inactivation of associated kinase activity and amplification of apoptosis in hematopoietic cells. Mol Cell Biol 2002; 22:2398-409.

Supplementary Figure and Table legends

Supplementary Table 1. Clinical characteristics of primary tumor samples examined. Liver tissues were kindly provided by Dr. S.S. Thorgeirsson (Laboratory of Experimental Carcinogenesis, NCI, Bethesda, MD). Institutional Review Board approval was obtained at participating hospitals in the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis, MN, and University of Pittsburgh, Pittsburgh, PA, USA) and the NIH.

Supplementary Table 2. Analysis of the differences in apoptosis, proliferation and MVD (microvessel density) in primary liver tumors scored as + or – for NORE1A expression.

Supplementary Figure 1. NORE1A levels directly correlate with those of $p21^{CIP1}$ and inversely with EEF2 and SAT1 in human HCC. Liver tumors and corresponding surrounding livers were divided in two groups, depending on the levels of NORE1A when compared to normal livers (+ means comparable to normal liver, and – indicates lower than normal liver). The groups were assayed by qRT-PCR for the expression of each gene and the results averaged. Abbreviations: NL, normal liver; SL, surrounding liver; HCC, Hepatocellular carcinoma. Thus, the microarray data was predictive of the situation in primary tumors.

Supplementary Figure 2. Quantification of CDK2 activity in NORE1A knockdown FOCUS hepatoma cells shown in Figure 2A.

Supplementary Table 1. Clinicopathological features of HCC patients

Variable	No. of cases
No. of patients	38
Male	35
Female	3
Age	
Mean	56.4
SD	9.4
Etiology	
HBV	20
HCV	11
Alcohol	5
Hemochromatosis	2
AFP (> 300 ng/ml)	
+	26
-	12
Cirrhosis	
+	29
-	9
Tumor size	
< 5 cm	25
> 5 cm	13
Survival	
< 3 years (HCCP)	22
> 3 years (HCCB)	16

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Supplementary Table 2

	Proliferation	Apoptosis	MVD
Nore1a+	21.87	2.05	129.65
Std. Dev	6.33	0.86	29.79
Nore1a-	35.02	1.08	303.79
Std. dev	6.77	0.49	58.14