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Supplemental Material to:

Lu-Zhe Pan, Dae-Gyun Ahn, Tanveer Sharif,
Derek Clements, Shashi A Gujar, and Patrick WK Lee

The NAD+ synthesizing enzyme nicotinamide mononucleotide adenylyltransferase 2 (NMNAT-2) is a p53 downstream target

Cell Cycle 2014; 13(7)
http://dx.doi.org/10.4161/cc.28128

http://www.landesbioscience.com/journals/cc/article/28128

SUPPLEMENTARY INFORMATION

MATERIALS AND METHODS

Cell lines and reagents. All human cell lines utilized in this study were purchased from American Type Culture Collection (ATCC). U2OS (human osteosarcoma cell) and HCT116 (human colon cancer cell) cells were cultured in Dulbecco's modified eagle medium (Gibco). H1299 human lung cancer cell line was grown in RPMI 1640 medium. All culture media were supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and antimycotics and antibiotics (Invitrogen). Cells were grown in a humidified incubator with 5% CO_2 at 37 °C. Actinomycin D (Act. D), doxorubicin (Dox) and etoposide (Etp) were purchased from Sigma-Aldrich and Nutlin-3 from Roche. Antibodies against p53 (DO-1), normal mouse IgG and β-actin were purchased from Santa Cruz Biotechnology. NMNAT-2 antibody was purchased from Abcam.

Plasmid construction. The p53 inducible gene expression system was constructed by modifying the TRIPZ-Tet On vector from Openbiosystems. pTRIPZ vector was originally designed for inducible expression of shRNAmir. To convert it into a gene expressing vector, the shRNA cassette was replaced with p53 open reading frame. Briefly, TRIPZ vector was digested with *Age*I and *Mlu*I restriction enzymes and the vector backbone was recovered. p53 coding sequence was amplified by PCR from IMR-90 cell (human lung fibroblast with wild-type p53 gene) cDNA with forward primer containing *Age*I linker sequence and reverse primer containing *Mlu*I linker sequence. PCR product was digested with *Age*I and *Mlu*I then ligated with pre-cut TRIPZ vector. The clone was confirmed by DNA sequencing. The new p53 inducible vector was named TPZ-p53-Tet On and used for generating lentiviruses. H1299 human lung cancer cell (p53 null) was transduced with TPZ-p53-Tet On construct. Primers used for amplification ofp53 are listed in supplementary table S1. shRNAs for knock-down of NMNAT-2 and p53 were cloned into pSMP vector (Openbiosystems). For each target gene, at least three shRNA sequences were designed. Oligomers containing each shRNA sequence were amplified by PCR using SMP-forward and SMP-reverse primers, which contain *Xho*I and *Eco*RI restriction site, respectively. PCR products and SMP vector were digested with *Xho*I and *Eco*RI and ligated together. Primer sequences and shRNA sequences are listed in supplementary table S1. For luciferase reportor plasmids containing NMNAT-2 p53-binding sites, NF-κB luciferase reporter vector, pNF-κB-luc (Clontech), was digested with *Mlu*I and *Bgl*II to remove NF-κB responsive sequence. Forward and reverse primers (about 60 bases) containing NMNAT-2 p53-binding sites (20 bases) with extra linker sequences of *Mlu*I (forward oligomers) and *Bgl*II (reverse oligomers) were annealed. NMNAT-2 p53 binding site #1 (p53BS#1) and p53BS#2, either wild-type or mutant, were cloned into luciferase reporter vector (NF-κB RE removed). All constructs were confirmed by DNA sequencing.

Real-time quantitative PCR. Total RNA was extracted with Trizol reagent (Invitrogen) and RNA-containing phase was further purified using PureLink RNA extraction kit (Invitrogen). cDNAs were reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and subjected to real-time quantitative PCR reaction. Real-time quantitative PCR was carried out using Quantifast Sybr-green qPCR kit (Qiagen) and Stratagenee MX 300 thermocycler. Data were analysed with MxPro software (Stratagene). mRNA levels were normalized to actin or GAPDH mRNAs as internal controls. PCR primers used for detecting NAMPT, NMNAT-1, NMNAT-2, NMNAT-3 and p53 are listed in supplementary table S1.

Flow cytometry analysis. The fixation and permeabilization of cells for intra-cellular staining for NMNAT-2 was conducted using Foxp3 staining buffer set (eBioscience) following the manufacturer's instructions. With the use of an anti-NMNAT-2 primary antibody and Alexa Fluor 488 secondary antibody (Invitrogen), the expression of NMNAT-2 was analysed with FACS Calibur flow cytometer (BD Biosciences), CellQuest Pro (BD Biosciences), FCS Express V3 (DeNovo Software), and ModFit LT software (Verity Software House).

Table S1. Primer and shRNA sequences used in this study

Name	Sequences
Primers for cloning	
TPZ-p53 forward	GTCAGAACCGGTTTAATTAAGCCACCATGGAGGAGCCGCAGTCAGATCC
TPZ-p53 reverse	TGAGACACGCGTATCGATTCAGTCTGAGTCAGGCCCTTCTG
NMNAT-2 p53 BS#1 WT forward	GCATACGCGTAGGTTTGGGCTGGAGAGTCAAGAACAAGTCGTGGCTTGCCAAGTGTGGACCACATGGCGAT
NMNAT-2 p53 BS#1 WT forward	GCTAAGATCTATCGCCATGTGGTCCACACTTGGCAAGCCACGACTTGTTCTTGACTCTCCAGCCCAAACCT
NMNAT-2 p53 BS#1 mutant forward	GCATACGCGTAGGTTTGGGCTGGAGAGTCAAGAATAATTCGTGGTTTTCCAAGTGTGGACCACATGGCGAT
NMNAT-2 p53 BS#1 mutant forward	GCTAAGATCTATCGCCATGTGGTCCACACTTGGAAAACCACGAATTATTCTTGACTCTCCAGCCCAAACCT
NMNAT-2 p53 BS#2 WT forward	GCATACGCGTTGGGATCAAACTTATTACAGTGGCATGTATGGACTTGTGGAAGGACAGATCATTCCAGCT
NMNAT-2 p53 BS#2 WT forward	GCTAAGATCTAGCTGGAATGATCTGTCCTTCCACAAGTCCATACATGCCACTGTAATAAGTTTGATCCCA
NMNAT-2 p53 BS#2 mutant forward	GCATACGCGTTGGGATCAAACTTATTACAGTGGAATATATGGAATTATGGAAGGACAGATCATTCCAGCT
NMNAT-2 p53 BS#2 mutant forward	GCTAAGATCTAGCTGGAATGATCTGTCCTTCCATAATTCCATATATTCCACTGTAATAAGTTTGATCCCA
Primers for Chip analysis	
NMNAT-2 p53 BS#1 Chip forward	GCTAAGGGCCAGAGAGGTTT
NMNAT-2 p53 BS#1 Chip reverse	GTGGCAGAGGCAAGATTCA
NMNAT-2 p53 BS#2 Chip forward	GTGCCAGACACTGGACAAGA
NMNAT-2 p53 BS#2 Chip reverse	CCCTTCAAAATCCAACTTGC
p21 Chip forward	AGGTCAGCTGCGTTAGAGGA
p21 Chip reverse	TCTGTGCCTGAAACATTTGC
MDM2 forward	TCGGGTCACTAGTGTGAACG
MDM2 reverse	CACTGAACACAGCTGGGAAA
Primers for real-time quantitative PCR	
p53 forward	CACATGACGGAGGTTGTGAG
p53 reverse	TGGTACAGTCAGAGCCAACCT
GAPDH forward	GAAGGTGAAGGTCGGAGTC
GAPDH reverse	GAAGATGGTGATGGGATTTC
NAMPT forward	ATCCTGTTCCAGGCTATTCTGT
NAMPT reverse	CCCCATATTTTCTCACACGCAT
NMNAT-1 forward	TCATCATGGCAGAACTTGCT
NMNAT-1 reverse	TGGCACAGCTTTTGTTTTTG
[a]NMNAT-2 common forward	GGCACCGTCTCATCATGTGTCAG
[a]NMNAT-2 common reverse	CCCAAGATCTTGGCTGCAGTG
NMNAT-3 forward	CCCTGCAAATAGCAGCTACC
NMNAT-3 reverse	TGAGAAGCTGCGAGGTCTTT
[b]NMNAT-2-TV1 specific forward	ATGACCGAGACCACCAAGAC
[b]NMNAT-2-TV2 specific forward	TGGAAATCCAGGAACTAGAGGA
[b]NMNAT-2 specific reverse	CCATAGGAGTCGTGGACAGG
shRNA sequences for gene knock-down	
SMP-Control shRNA	CGCTACACAAATCAGCGATTTATAGTGAAGCCACAGATGTATAAATCGCTGATTTGTGTAGCG
SMP-p53 shRNA	CGGAGGATTTCATCTCTTGTATTAGTGAAGCCACAGATGTAATACAAGAGATGAAATCCTCCA
SMP-NMNAT-2 shRNA	AGCCACTGTTAGGTTTCCTTTATAGTGAAGCCACAGATGTATAAAGGAAACCTAACAGTGGCC

[a]primers amplifying common region of NMNAT-2 variants, thereby detecting all transcriptional variants

[b]primers amplifying specific NMNAT-2 transcriptional variants, tv1 and tv2