Supplementary information

METHODS

Yeast two-hybrid assay. For bait construction, a DNA fragment encoding amino acids 520-1209 of HIPK1 was subcloned into the *EcoRI*/*SalI* sites of pGBKT7 (Clontech) and transformed into the AH109 yeast strain, followed by mating with yeast Y187 cells pretransformed with a mouse embryonic day 11 cDNA library (Clontech). Approximately 10^7 transformants were screened, and positive colonies were confirmed with β-galactosidase colony lift assays. The specificity of the interaction and binding affinity between the two fusion proteins were verified by streaking several colonies onto synthetic dropout agar plates lacking TL (tryptophan and leucine) or TLH (tryptophan, leucine, and histidine).

Co-immunoprecipitation and Western blotting. Co-immunoprecipitation of endogenous proteins was performed after the lysis of 2 x 10^7 cells in high salt lysis buffer (50 mM Hepes, 350 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1 mM EDTA). After incubation on ice for 10 min and centrifugation for 20 min at 4°C, equal volumes of protein were diluted with dilution buffer (lysis buffer lacking NaCl), then incubated overnight with antibody and protein A/G-Sepharose beads at 4°C on a rotating wheel. After incubation, the beads were washed three times with washing buffer. Whole cell lysates and immunoprecipitates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were immunoblotted with anti-Myc and anti-HA antibodies (Roche). Rabbit polyclonal antibodies to HIPK1 were

raised against a GST fusion protein containing an N-terminal fragment of mouse HIPK1(residues 1-285). Other antibodies were purchased as follows: anti-Myc and anti-HA (Roche); anti-Flag and anti-GFP (Sigma); anti-MDM2 (SMP14) and anti-p53 (DO-1) (Santa Cruz Biotechnology).

Immunocytochemistry. U2OS cells were grown on coverslips and transfected with EGFP-HIPK1, Flag-Nore1A or HA-Mdm2 expression plasmid. Thirty-six hours after transfection, cells were fixed with 100% methanol for 5 min at -20°C and incubated in a solution of 1× phosphate-buffered saline and 0.5% Triton X-100. Cells were rinsed with 1× phosphate-buffered saline containing 1% bovine serum albumin and incubated with anti-Flag or anti-HA antibody for 1 hr. After washing three times with 1× phosphate-buffered saline, cells were incubated with anti-mouse secondary antibody conjugated with rhodamine red (Molecular Probes, Inc., Eugene, OR). Fluorescence microscopy was performed with a Zeiss Axiophot 2 microscope, using excitation wavelengths of 543 nm (rhodamine red), 488 nm (GFP), and 433 nm (CFP).

Reverse transcription PCR. First strand cDNA synthesis was performed with 500 ng of total RNA using oligo(dT) primers and avian myeloblastosis virus reverse transcriptase (Roche Applied Science). One-twentieth of the reaction product was used for PCR amplification (DNA denaturation at 94°C for 30 s, primer annealing at 61°C for 30 s, primer extension at 72°C for 30 s). The following human NORE1A-specific primers were used for PCR amplification: NORE1A nt 304, 5′-GACGTGCGGAGCATCTTC-3′; NORE1A 527 nt, 5′-TAAACCCTCCTGCTGACT-3′.

Methylation-specific PCR. The bisulfite method was used to detect DNA methylation of the *NORE1A* promoter. Genomic DNA (500ng) was treated with bisulfite, which converts unmethylated cytosine to uracil, using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA). Two primer sets for *NORE1A* were prepared corresponding to methylated and unmethylated DNA as described previously (Irimia *et al*, 2004).

Establishment of cell lines. A549 shHIPK1 cell lines were established by transfection of shHIPK1 vector, followed by selection with puromycin (1.2 μg/ml) for 2 weeks. H1299 cells and NIH3T3 cells expressing either GFP or GFP-HIPK1 were established by transfection of either EGFP expression plasmid or GFP-HIPK1 expression plasmid, followed by selection with G418 (1500 μg/ml) for three weeks.

Human lung cancer tissue specimens. For analysis of HIPK1 expression in human tissue samples, 25 pairs of freshly frozen cancer tissue and matching normal lung tissue were obtained. Consent was obtained from the patients to use the tissue specimens for research purposes, and the use of the specimens was authorized by the Institutional Review Board of the Samsung Medical Center.

REFERENCE

Irimia M, Fraga MF, Sanchez-Cespedes M, Esteller M (2004) CpG island promoter hypermethylation of the Ras-effector gene NORE1A occurs in the context of a wild-type K-ras in lung cancer. *Oncogene* **23**(53)**:** 8695-8699

Legends to Supplementary figures

Fig S1| HIPK1 interacts with Nore1 *in vivo* and *in vitro* (**A**) U2OS cells were treated with MG132 for 12 hrs and cell lysates were immunoprecipitated with either rabbit IgG or anti-HIPK1 antibody, followed by Western blotting using anti-HIPK1 and anti-Nore1 antibodies. (**B**) A GST pull-down assay showing physical interaction of GST-Nore1 with Myc-HIPK1. Coomassie Brilliant Blue (CBB) staining of the gel is shown below. (**C**) Co-localization of GFP-HIPK1 with Flag-Nore1 in U2OS cells. GFP-HIPK1 localizes to dots in the nucleus, and GFP-Nore1 localizes to dots in the nucleus as well as the cytoplasm (top panels). Forced co-expression of GFP-HIPK1 and Flag-Nore1 in the presence of MG132 showed co-localization of HIPK1 and Nore1 in nuclear dots (bottom panels).

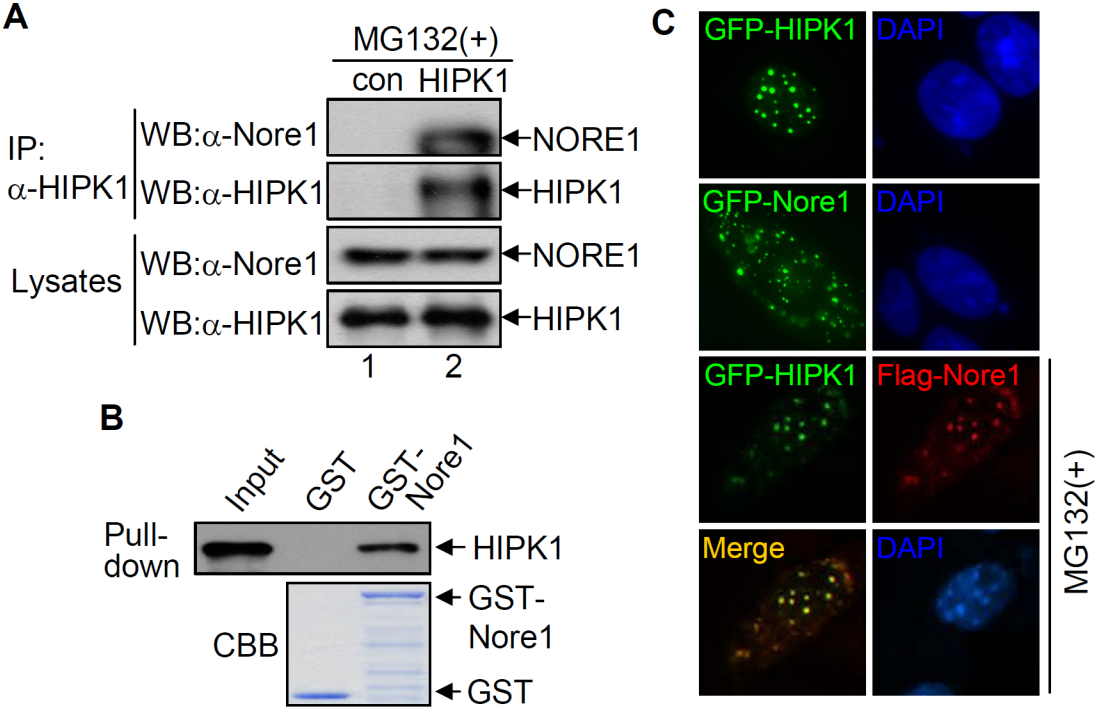
Fig S2| HIPK1 is a positive regulator for Mdm2 and p53 expression. U2OS cells were knocked down with each siRNA against HIPK1, Mdm2 and p53. Transfected cells were subjected to Western blotting using anti-HIPK1, anti-Mdm2 and anti-p53 antibodies.

Fig S3| Knockdown of HIPK1 blocks tumorigenesis of A549 cells *in vivo*. (**A**) A549 cell lines stably expressing control shRNA (shcontrol) or HIPK1 shRNA (A549-shHIPK1 #1 and A549-shHIPK1 #2) were established, and subcutaneous xenografts were established in the flanks of athymic nude mice (n = 2 per group) using 5×10^6 cells of each cell line. After injection, tumor volumes were measured twice a week with a caliper, and calculated using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2. (**B**) The tumors were excised six weeks after injection and images were taken. Scale bars: 1 mm.

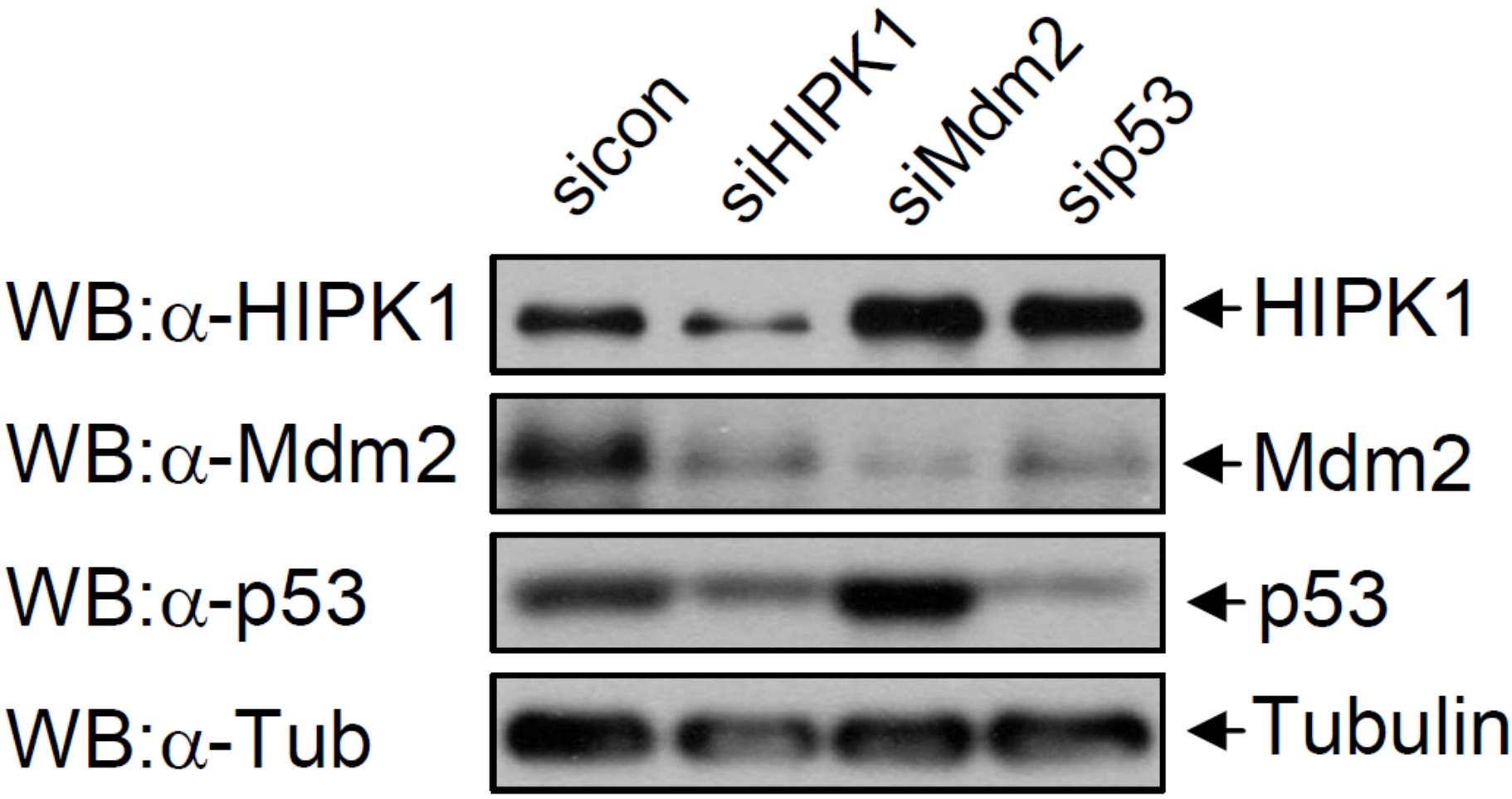
Fig S4| Establishment of H1299 and NIH3T3 cells stably expressing GFP-HIPK1. (**A**) G418-resistant H1299 cell lines stably expressing GFP-HIPK1 were subjected to Western blotting using either anti-GFP antibody or anti-HIPK1 antibody. Two H1299-GFP-HIPK1 cell lines (#2 and #9) were further utilized in a soft agar assay. (**B**) Images of GFP fluorescence were taken from either H1299-GFP control cell line or H1299-GFP-HIPK1 cell lines. (**C**) G418-resistant NIH3T3 cell lines stably expressing GFP-HIPK1 were subjected to Western blotting using either anti-GFP antibody or anti-HIPK1 antibody. Two NIH3T3-GFP-HIPK1 cell lines (#1 and #2) were utilized in a soft agar assay. (**D**) G418-resistant NIH3T3 cells stably expressing H-Ras(Q61L) activated mutant were subjected to Western blotting using anti-H-Ras antibody. Endogenous H-Ras was detected in parental NIH3T3 cells (lane 1), and H-Ras expression was increased presumably due to stable expression of H-Ras(Q61L) mutant (lanes 2 and 3). (**E**) Images of GFP fluorescence were taken from each of NIH3T3-GFP-HIPK1 cell line (#1 and #2).

Fig S5| HIPK1 is highly expressed in primary lung tumors. (**A**) Some primary lung tumors have methylated *NORE1A* promoters. Genomic DNA was isolated from 25 pairs of primary lung tumors and their normal tissue counterparts, and utilized as a template for *NORE1* promoter methylation-specific PCR using primers specific for the methylated CpG island (M) and unmethylated nucleotide (UM) as a control. The PCR products were separated on a 10% acrylamide gel and stained with ethidium bromide. (**B**) HIPK1 protein levels are increased in primary lung tumors with methylated *NORE1* promoter. Immunoblot analysis of

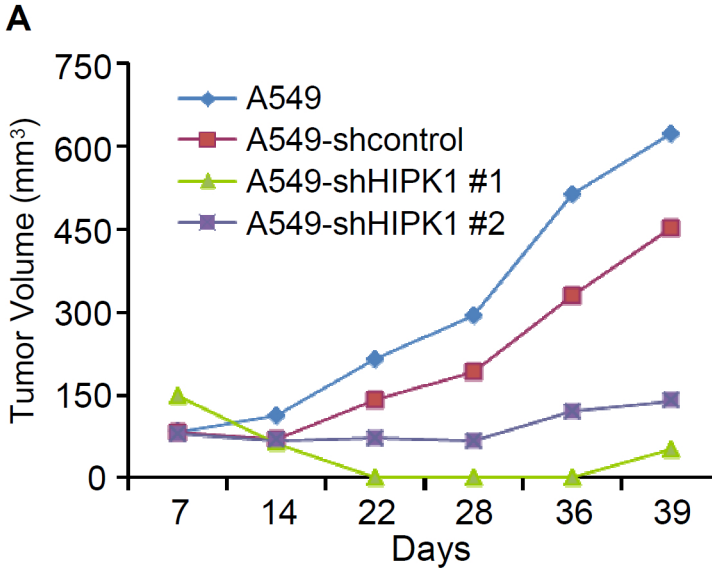
human primary lung tumors (T) and their normal lung tissue counterparts (N) using anti-HIPK1 and anti-Actin antibodies. (**C**) Immunohistochemistry of primary lung tumors and corresponding normal lung tissues with anti-HIPK1 antibody.

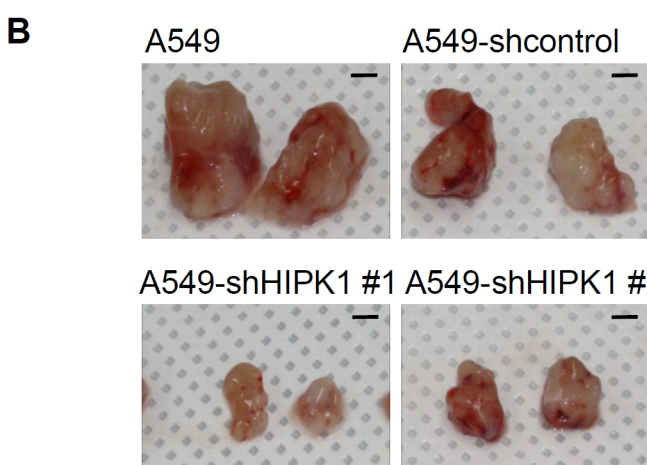


Deresa *et al.*, Fig S1

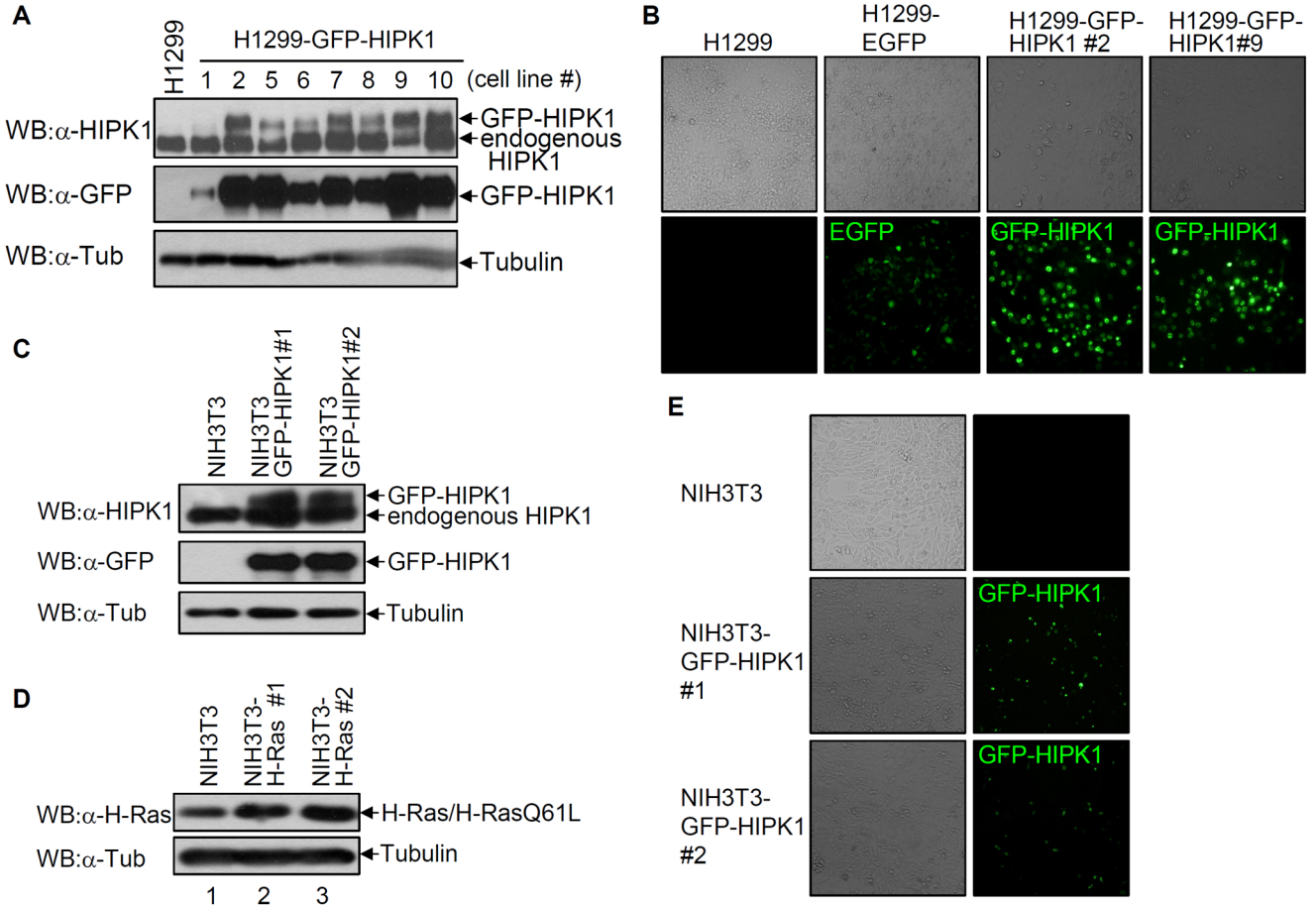


Deresa *et al.*, Fig S2

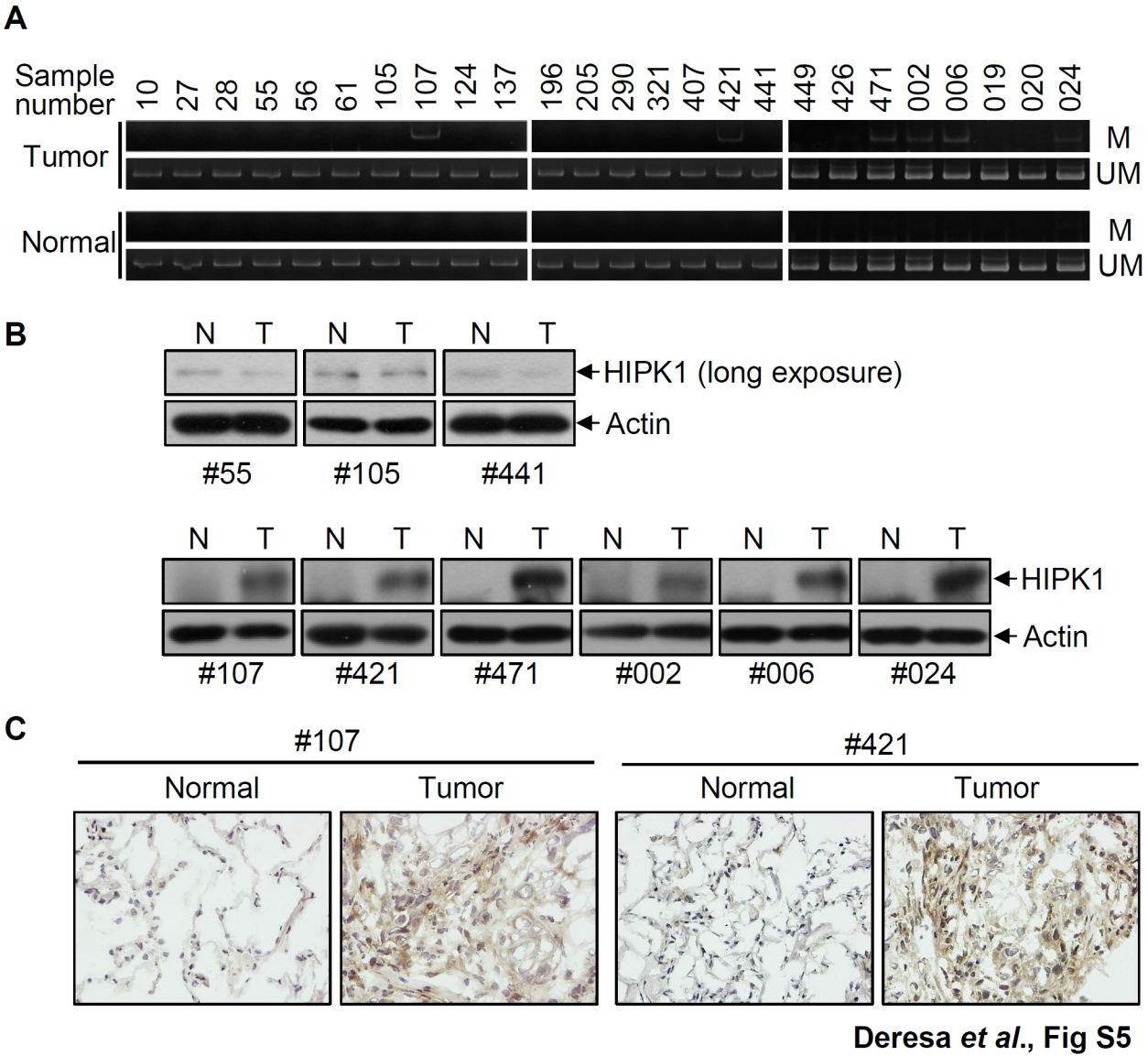




Deresa *et al.*, Fig S3



Deresa *et al.*, Fig S4



Deresa *et al.*, Fig S5