SUPPLEMENTAL INFORMATION

Identification and molecular basis for SIRT6 loss-of-function point mutations in cancer

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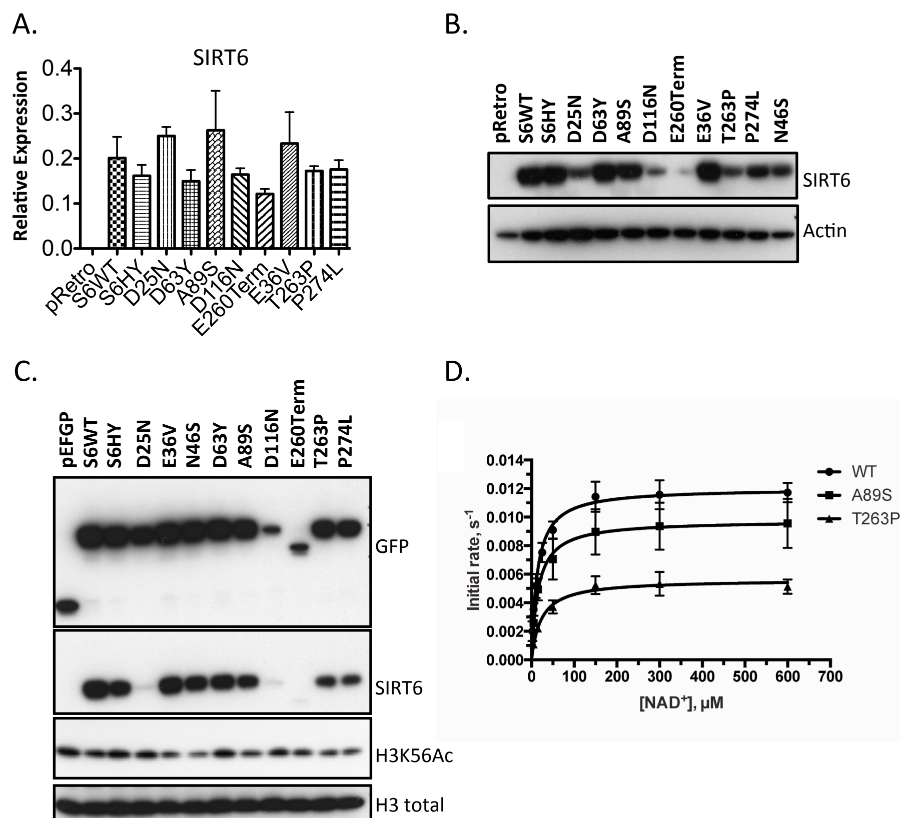
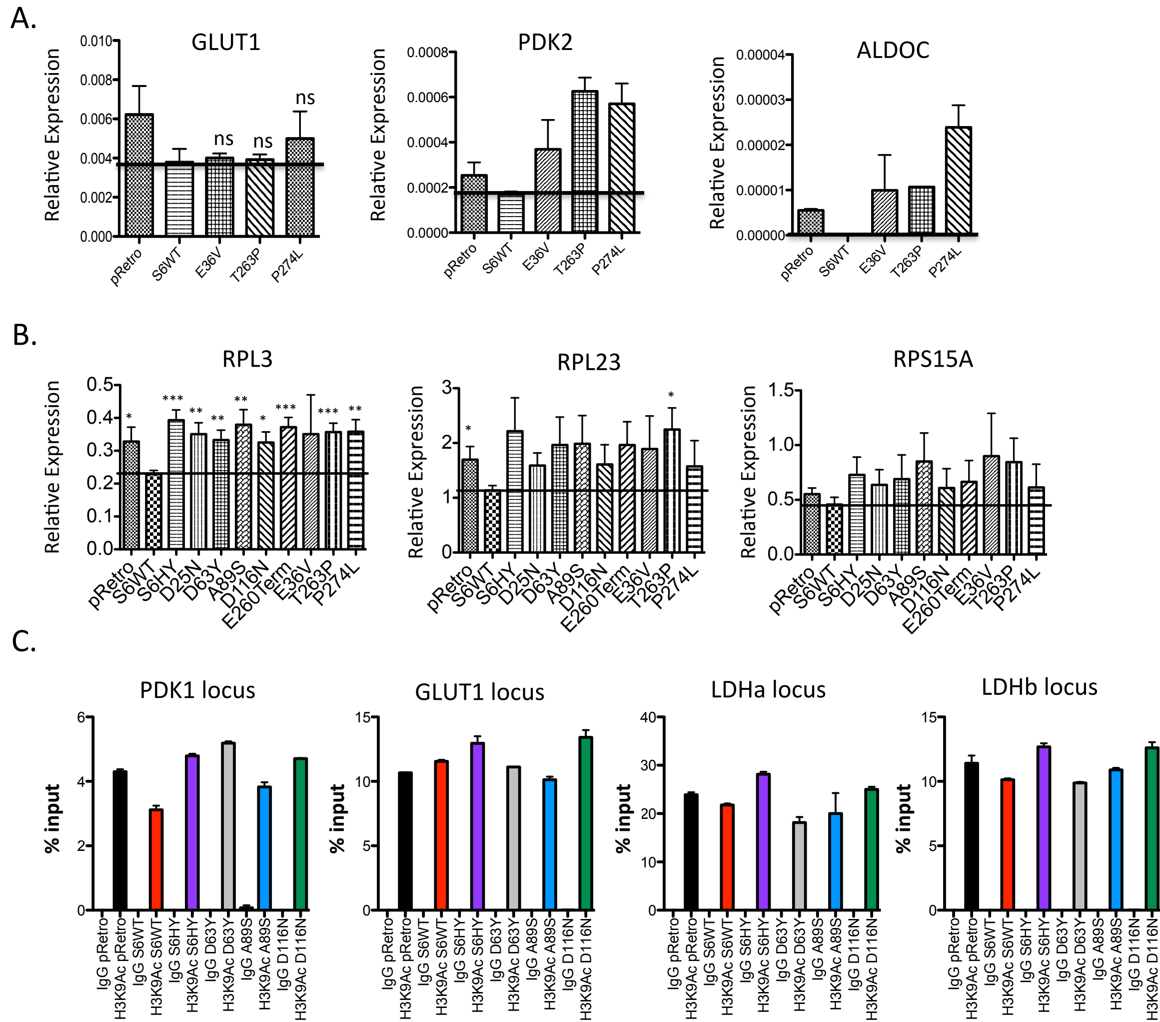


Supplemental Figure 1, related to Figures 1 and 2. (A) Quantitative real-time PCR showing the expression of SIRT6. Data are shown as mean ± s.e.m. between duplicates and are representative of six independent experiments. **(B)** Western blot of whole cells lysate for SIRT6 in SIRT6 knock-out (KO) MEFs expressing the indicated proteins. **(C)** Western blot of chromatin fraction for SIRT6 and indicated histone marks in 293T cells with transient overexpression of wild-type EGFP-SIRT6 and SIRT6 mutants. **(D)** Steady-state rates were measured by varying NAD^+ (2–600 µM) in the presence of 0.5 µM WT (*circle*), A89S (*square*) and T263P (*triangle*) SIRT6 and 50 µM H3K9Myr peptide. (n≥3, ± standard deviation). WT SIRT6 values previously published (Feldman et al., 2015).



Supplemental Figure 2, related to Figure 3. (A&B) Quantitative real-time PCR (qRTPCR) showing the expression of indicated genes. Data are shown as mean ± s.e.m. between duplicates and are representative of six independent experiments (GLUT1) and two independent experiments (PDK2 and ALDOC). **(C)** Chromatin immunoprecipitation assay for H3K9Ac in SIRT6 KO MEFs expressing the indicated SIRT6 constructs followed by qRTPCR amplification of the indicated glycolytic genes. Data are shown as mean ± s.e.m. between duplicates.

Supplemental Table 1, related to Figures 1,2,3 and 4.

Assay	WT	D25N	D63Y	A89S	D116N	E260	E36V	T263P	P274L	N46S
Nuclear localization	+++	+++	+++	+++	+	-	+++	+++	+++	+++
Stability	+++	+++	+++	+++	-	ND	+++	+++	+++	+++
In vitro deacetylase	+++	+	-	+	-	ND	+	+	+	+++
In vitro deacetylase +FA	+++	+++	-	-	-	ND	+++	++	++	+++
In vitro demyristoylase	+++	+++	-	+++	-	ND	+++	+++	+++	+++
Bulk chromatin deacetylation	+++	-	-	+++	-	-	+++	+++	+++	+++
Myc luciferase	+++	-	-	-	-	-	-	-	-	ND
HIF1α luciferase	+++	-	-	-	-	-	-	-	-	ND
Glucose uptake	+++	-	-	-	-	-	+++	+++	+++	ND
SIRT6 ChIP	+++	ND	+++	+++	-		ND	ND	ND	ND
H3K56Ac ChIP	+++	ND	-	+	+		ND	ND	ND	ND
Soft agar	+++	+	-	+	-	-	++	+++	+++	+++
In vivo	+++	+	ND	-	-	-	ND	ND	ND	+++

Supplemental Table 2, related to Figure 3.

Gene name	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
hSIRT6	AGGATGTCGGTGAATTACGC	CCAGTTCCCACACCTTCC
mPDK1	ATCTTCTCAGGACCCCATCC	GCATCTTCAGCACTTTTGTCTTT
mPFKm	GCCGTGTTGACCTCTGGT	GCCCGTGAAGATACCAACTC
mLDHB	TGGTGGACAGTGCCTATGAA	GAATCCGGGAGAGGTTTTTC
mPDK4	GATTGACATCCTGCCTGACC	CTTCTGGGCTCTTCTCATGG
mALDOC	GGAGACCATGACCTCAAACG	ACCATATTGGGCTTGAGCAG
mRPL3	CTTCCAAGCCCGTTCACCTC	GTTCACCTTAGATCCTGGCCT
mRPL23	ATCAAGGGACGGCTGAACAG	ACTGCTGGATGTACCTTTTTCC
mRPS15A	ATGGTGCGAATGAATGTTCTGG	TCTTCTCAGCGTTGTTGATGC
mLDHA +475 ChIP	TGCCCTAAACGCATTTTGTT	CGTCTAGGGTGCAGAGGAAG
mLDHB +255 ChIP	GCCCCAAAGAGTGGATGTGT	TTGGCAGAACAGGAGGAAGG
mGLUT1 +32 ChIP	CCTACACCCCAGAACCAATG	GACGCACTTAAGACCCCGTA
mPDK1 ChIP	CACACCCCACAAAGCTCAG	TTAAAGACACGCCCATGTAGG

SUPPLEMENTAL EXPERIMENTAL PROCEDURES.

Tissue Culture Conditions. Cells were cultured at 37 °C under 5% CO2 in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin (100 U/ml)/streptomycin (100 Ug/ml) (Invitrogen), 2mM L-glutamine, 0.1mM NEAA, 1mM sodium pyruvate and 20mM HEPES. 293T were cultured in high glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin (100 U/ml)/streptomycin (100 Ug/ml) (Invitrogen).

Preparation of SIRT6 pRetro and Generation of Stable SIRT6 Transfectants. Full-length wild-type SIRT6 cDNA was amplified from the normal pancreatic ductal epithelial cell line (HPDE) using the following primers (CA**GGATCC** TTGTTCCCGTGGGGCAGTCGAGG; bold sequence indicates BamHI site) and (CA**GAATTC**CTACAAAAAGCCCCACCCTCCC; bold sequence indicates EcoRI site). Following PCR amplification and subcloning into pGEMT (Promega), SIRT6 constructs were digested with BamHI and EcoRI, and purified with the QIAquick Gel extraction kit (Qiagen). Digested SIRT6 was subcloned into pRetroX-TIGHT-Pur

plasmid (Clontech) and site-directed mutagenesis of wild-type SIRT6 used the QuikChange Lightning site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). pLVX-Tet-On was obtained from Clontech. Viral particles containing the above mentioned plasmids was synthesized using retroviral packaging plasmids pCL-ECO (Addgene) and pCMV-VSV-G (Addgene). SIRT6 KO MEFs were infected by incubating with virus and 10 µg/ml polybrene. Forty-eight hours later, cells were selected in 2.5 µg/ml puromycin and the pooled populations were used for various experiments. For all experiments involving the dox-inducible SIRT6 KO MEFs, cells were treated with 1µg/mL dox for 48-96 hrs.

Chromatin isolation and western blot. Cell pellet was resuspended in lysis buffer containing 10mM HEPES pH 7.4, 10 mM KCl, 0.05% NP-40 supplemented with a protease inhibitor cocktail (Complete EDTA-free, Roche Applied Science), 5µM TSA, 5mM sodium butyrate, 1mM DTT, 1mM PMSF, 50mM NaF, 0.2mM sodium orthovanadate and phosphatase inhibitors (Phosphatase Inhibitor Cocktail Sets I and II, Calbiochem) and incubated on ice for 20 mins. The lysate was then centrifuged at 14,000 rpm for 10 mins at 4°C. The supernatant was removed (cytosolic fraction) and the pellet (nuclei) was acid-extracted using 0.2N HCl and incubated on ice for 20 mins. The lysate was then centrifuged at 14,000 rpm for 10 mins at 4°C. The supernatant (contains acid soluble proteins) was neutralized using 1M Tris-HCl pH 8. Protein concentration was quantified by Biorad Protein Assay. Ten-micrograms protein was electrophoresed on a 10-20% gradient polyacrylamide gel with SDS (Biorad) and electroblotted onto polyvinylidene difluoride membranes (PVDF) (Millipore) (Matsudaira, 1987). Membranes were blocked in TBS with 5% non-fat milk and 0.1% Tween and probed with antibodies. Bound proteins were detected with horseradish-peroxidase-conjugated secondary antibodies (Vector Biolaboratories) and SuperSignal West Pico Luminol/Enhancer Solution (Thermo Scientific).

Luciferase reporter assay. Briefly, 293T cells were cotransfected using Trans-IT 293 (Mirus) with 950 ng of pMYC-luc (Signosis, Inc., Sunnyvale, CA), 50ng of pGL3-Renilla and either 1 μg of empty vector, pEGFP-SIRT6 or the SIRT6 mutant constructs. HIF1α transcriptional activity was determined by cotransfecting 950 ng mpGL3:HRE4, 50ng of pGL3-Renilla and either 1 μg of empty vector, pEGFP-SIRT6 or the SIRT6 mutant constructs. Twenty-four hours after transfection, cells were harvested and luciferase activity was determined using the Dual-Luciferase Reporter Assay system (Promega). In order to measure HIF1α luciferase activity cells were placed in a 1% O_2 incubator (hypoxia) for 8 hrs before harvesting cells for analysis.

HPLC deacylation assay. For deacetylation assays, 2 μM WT or mutant SIRT6 was incubated with 500 μM NAD^+ and 50 μM acetyl-lysine peptide in the presence and absence of 300 μM myristic acid in 20 mM sodium phosphate, pH 7.5, at 37 °C. For demyristoylation assays, 0.5 μM WT or mutant SIRT6 was incubated with 500 μM NAD^+ and 50 μM myristoyl-lysine peptide in 20 mM sodium phosphate, pH 7.5, at 37 °C. Reactions were quenched with TFA at various time points and centrifuged at 21,000xg for 5 min. Time points were chosen such that all reactions remained within steady-state initial velocity during the course of the reaction. The product and substrate peaks were quantified, and rates of deacylation were determined.

Real-Time RT-PCR Analysis. Was performed using the SYBR green master mix (Roche), following the manufacturer's instructions, with the exception that the final volume was 12.5 μl of SYBR green reaction mix. Real-time monitoring of PCR amplification was performed using the LightCycler 480 detection system (Roche).

Chromatin Immunoprecipitation Assays. Cells were cross-linked with 1% paraformaldehyde for 15 min at room temperature. The reaction was quenched for 5 min at room temperature by adding 0.125 M glycine. After three washes with 1X PBS, cells were lysed with lysis buffer (1%

SDS, 10 mM EDTA pH 8, 50 mM Tris-HCl pH 8) supplemented with protease and deacetylase (TSA) inhibitors. Lysates were sonicated on ice using a Branson Sonifier 250 sonicator (10 second pulses of output 3 at constant duty repeated 5 times at 4°C, keep on ice between cycles of sonication). Size of fragments obtained (between 200 and 1,200 bp) was confirmed by electrophoresis. Soluble chromatin was collected after centrifugation at 14,000 rpm at 4°C for 10 min and 1 million cells was diluted to 1/5 in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1) supplemented with protease and deacetylase inhibitors. Soluble chromatin (1%) was kept as input control. Soluble chromatin was precleared with 100 µg/ml of salmon sperm (Amersham Biosciences), 2.5 µg/ml of unspecific IgGs, and protein-A-Sepharose at 50% overnight at 4°C in rotation. After centrifugation, supernatants were collected and specific antibodies were added. Mixtures were incubated at 4°C for 8 hrs in rotation and then incubated overnight at 4°C in rotation with protein-A-Sepharose at 50% (Roche). Beads were collected and washed sequentially at 4°C for 10 min with TSE I (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 20 mM Tris-HCl (pH 8.1)), TSE II (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 20 mM Tris-HCl (pH 8.1)), and buffer III (0.25 LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.1)). Beads were washed once with 1X PBS by pipetting and immunoprecipitates were eluted two times (20 min incubation) with elution buffer (0.1 M $NaHCO_3$ and 1% SDS). Reversion of cross-linking was performed overnight by adding 0.2M NaCl and heating samples and input controls at 65°C. Samples were then treated with 0.2 mg/mL RNAse A (Qiagen) and incubated for 1hr at 37°C followed by addition of 0.01M EDTA, 0.04M Tris-HCl pH 6.5 and 4 U/mL of Proteinase K (Promega) and samples were incubated at 45°C for 1 hr. DNA was then purified using the QIAquick spin kit (Qiagen). Real time RT-PCR was performed with primers listed in Supplemental Table 1.