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Supplemental Data

The SIRT2 Deacetylase Regulates

Autoacetylation of p300

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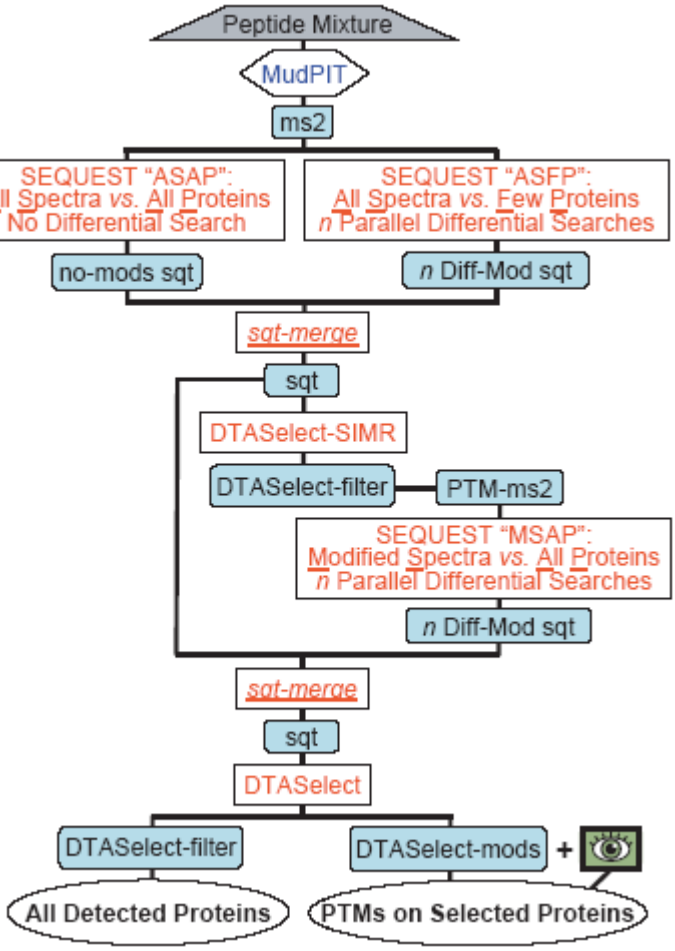
IDENTIFICATION OF ACETYL-LYSINE MODIFICATIONS IN THE P300 DATASET

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Preparation of peptide mixtures — TCA-precipitated protein samples were solubilized in 30µl of 8M urea, 0.1 M Tris-HCl, pH 8.5, and 5mM TCEP. After 30 minutes at room temperature, 0.5M IAM was added to a final concentration of 10mM, and the samples were left at room temperature for another 30 minutes in the dark. The samples were then digested with LysC/Trypsin (Roche/Promega), Chymotrypsin (Roche), or GluC (Roche) according to the manufacturer's recommendations.

Data Acquisition — Each sample was analyzed independently by Multidimensional Protein Identification Technology (MudPIT) as described previously (Washburn, Wolters et al. 2001; Wolters, Washburn et al. 2001). Peptide mixtures were pressure-loaded onto a 250 µM ID fused silica capillary column with a filtered union (UpChurch Scientific) that had been packed with 2 cm of 5 µm Partisphere strong cation exchange resin (Whatman) followed by 2 cm of 5 µm Aqua C18 resin (Phenomenex). The filter union was attached to a pulled tip packed with 8 cm of 5 µm Aqua C18 material. Loaded split columns were placed in line to a LTQ ion trap mass spectrometer (ThermoFisher) connected to an Agilent 1100 quaternary pump (Agilent Technologies). During the course of the fully automated chromatography, twelve to fourteen 120-minutes cycles of increasing salt concentrations followed by organic gradients slowly released peptides directly into the mass spectrometer (Florens and Washburn, 2006). Following each full MS scan, five tandem mass spectra were acquired on the 1^{st} to 5^{th} most intense ions.

Data Analysis — RAW files were extracted into ms2 file format (McDonald et al., 2004) using RAW_Xtract (J.R. Yates, Scripps Research Institute). MS/MS spectra were queried for peptide sequence information on a 157-node dual processor Beowulf Linux cluster dedicated to SEQUEST analyses (Eng et al., 1994). Because differential modification searches are a computational bottleneck, MS/MS spectra were searched in a recursive manner following the flowchart described in Supplemental Chart 1.



Supplemental Chart 1 - Acetylation Search Flowchart

First, ms2 files were searched without specifying differential modifications against a database combining non-redundant protein sequences downloaded from NCBI for the Autographa californica nucleopolyhedrovirus, and known proteins sequences for Spodoptera frugiperda (downloaded on 05/22/2007). The database also included the sequences for human

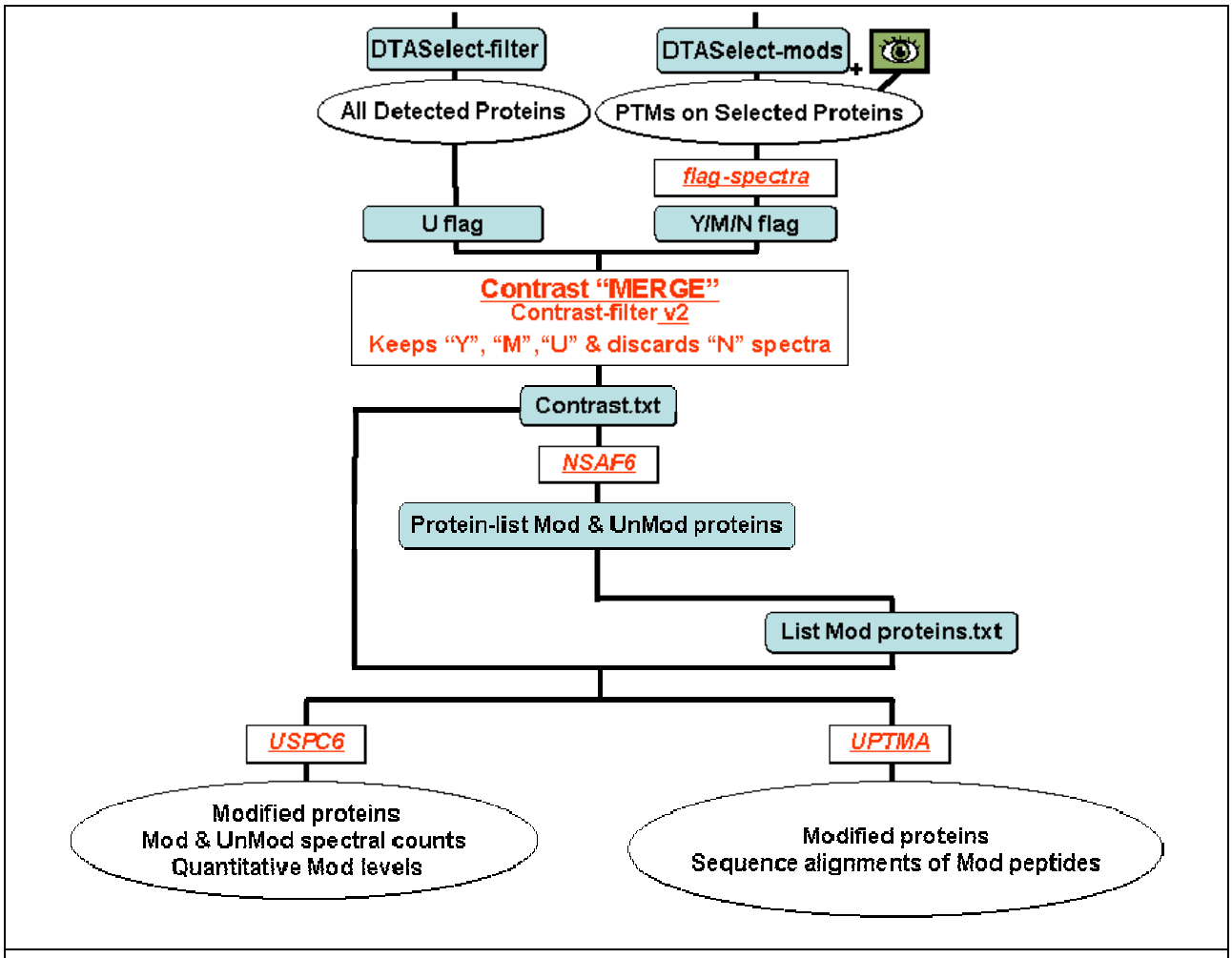
sirtuin 2 and p300 (gi|13775602|ref|NP_085096.1| and gi|50345997|ref|NP_001420.2|, respectively). Since the Spodoptera frugiperda genome has not been fully sequenced, sequences for Drosophila melanogaster (downloaded 11/28/2006) were also included as well as sequences for usual contaminants (keratins, proteases, IgGs…) for a total of 21005 protein sequences. In addition, to estimate false discovery rates, each non-redundant protein entry was randomized and the resulting sequences were added to the database for a final search space of 42010 amino acid sequences. This SEQUEST "ASAP" (All Spectra against All Proteins) step allowed establishing the list of proteins detected in the samples. To account for carboxamidomethylation, +57 Da were added statically to cysteine residues for all the searches.

Second, the same ms2 files were also searched for post-translational modifications (PTMs) against a database containing only the sequence for the protein of interest, i.e. p300. In SEQUEST "ASFP" (All Spectra against Few Proteins), a differential modification search was performed to query the p300 sequence for peptides containing acetylated lysines (+42) AND/OR oxidized methionines (+16). The maximum number of modified amino acids per differential modification in a peptide was limited to 6.

After this round of searches, an in-house developed script, sqt-merge (Zybailov et al., 2005), was used to combine the SEQUEST output files (sqt files) generated from the normal search "ASAP" (i.e. without modifications) and PTMs "ASFP" searches described above into one set (Supplemental Chart 1). The sqt-merge script read in sqt files from normal and differentially modified searches generated from the same ms2 files, then merged and ranked the spectrum/peptide matches based on cross-correlation scores (XCorrs). Normalized differences in XCorrs (DeltCn) values were re-calculated. This merging step allowed only the best matches out of n differential SEQUEST queries to be ranked first. The peptide matches contained in the merged sqt files was compiled and sorted into protein-level information using DTASelect (Tabb et al., 2002).

For the third round of searches, only spectra matching modified peptides were selected if they passed conservative filtering criteria: a minimum XCorr of 1.8 for +1, 2.0 for +2, and 3.0 for +3 spectra, maximum Sp rank of 10, and minimum length of 7 amino acids. Because Xcorr scores for isopeptides in which any of several adjacent residues could be modified tend to close — which leads to low DeltaCn values — the DeltaCn cut-off was set at 0.01 to allow such peptides to be further examined. Peptide end requirements were also employed based on the protease used for digestion: LysC/Trypsin digests required ends of R or K, GluC digests

required ends of E or D, and Chymotrypsin digests required ends of F, W, Y, N, L, A, D, or E. The coordinates for spectra passing these criteria were written out into smaller ms2 files using the "-- copy" utility of DTASelect ("PTM_ms2" in Supplemental Chart 1). Because these subsetted ms2 files contained at best a few hundreds of MS/MS spectra, they can be subjected to the same two differential searches against the complete database, without having to pay too high of a computer-hour price (SEQUEST "MSAP": Modified Spectra against All Proteins). This step allowed us to check that spectra matching modified peptides from p300 did not find a better match against the larger protein database.



Supplemental Chart 2 – PTMs Analysis Flowchart

DTASelect was used to create reports listing all detected proteins ("--DB" utility) and the modified residues ("--mods" utility) on p300. All spectra matching modified peptides were

visually assessed. All assessed peptide/spectrum matches (except those for oxidized methionines) were assigned an evaluation flag (Y/M/N for yes/maybe/no) using flag-spectra (Mike Coleman), which reads in the evaluation flags from an input file and switches spectrum flags in DTASelect.txt and sqt files in a directory (Supplemental Chart 2). The "no" matches were removed from the final data (-v 2 parameter in DTASelect). Since trypsin will not digest modified lysine residues, peptides containing C-terminal lysine acetylations were not kept.

Replicate experiments were merged using CONTRAST "Merge" option (Supplemental Chart 2). Results from different replicates were compared using CONTRAST. Spectrum/peptide matches were only retained if peptides had to be at least 7 amino acids long and match the end requirements mentioned above for the different proteases. The DeltCn had to be at least 0.08, with minimum XCorrs of 1.8 for singly-, 2.5 for doubly-, and 3.5 for triply-charged spectra, and a maximum Sp rank of 10.

U_SPC6 (Tim Wen) was used to extract total and modified spectral counts for each amino acid within the proteins of interest and calculate modification levels based on local spectral counts:

$$AA_x\_PTM_y\_Level = \frac{AA_x\_PTM_y\_SpectralCounts}{AA_x\_Total\_SpectralCounts} x100$$

Data from each biological replicate was combined from the different protease digests in order to obtain the total observed peptides from each replicate only after filtering each search with the appropriate peptide end requirements. This output was used for calculation of the total percent modification for each biological replicate for the acetylated (Ac) and SIRT2-treated (DeAc) samples. Since p300 contains multiple stretches of basic amino acids, some peptides/amino acids were observed in the acetylated sample that may not have been observed in the deacetylated sample when digested with LysC/Trypsin since peptides were required to be at least 7 amino acids in length. U_PTMA (an in-house software by Tim Wen) was used to display sequence overlaps between modified peptides detected for p300.

Supplemental Experimental Procedures

Tissue Culture

HEK293 and U2OS Tet-ON cells were grown in DMEM (GIBCO) supplemented with 10% tetracycline free FBS (Clontech). Cells were treated with 100ng/ml TSA (Sigma), 2mM Nicotinamide (Sigma), or 25nM Leptomycin B (Sigma) for 16 hours as indicated.

Immunofluorescence

Cells were grown on 12mm BD BioCoat glass coverslips (Fisher) in 6-well plates. Cells were transfected with wildtype FLAG-SIRT2 using Lipofectamine 2000 according to the manufacturer's instructions. 24 hours following transfection, cells were treated with Leptomycin B for 16 hours as indicated in the figures. The cells were washed with PBS and fixed with 4% paraformaldehyde. Following fixation, cells were washed with PBS and permeabilized in PBS with 1% Triton X-100, and blocked with 10% FBS in PBS. Cells were stained with rabbit anti-p300 (Santa Cruz, SC-585) and anti-FLAG (Sigma). Anti-rabbit Alexa 488 and anti-mouse Alexa 555 secondary antibodies were purchased from Invitrogen. The stained cover slips were mounted on glass slides using Prolong Anti-Fade Gold (Molecular Probes) and dried overnight. Images were acquired on a LSM 5 PASCAL laser-scanning confocal microscope equipped with a Plan-Neofluar 100x/1.3 NA objective (both from Carl Zeiss MicroImaging, Inc.). Images were analyzed for colocalization using the PASCAL software histogram analysis suite. Equal intensities were set as cut-offs for each image set.

Immunoprecipitation

$5x10^6$ cells were resuspended and lysed in RIPA buffer (20mM Tris pH 7.5, 150mM NaCl, x NP-40) containing 0.5mM PMSF, 2mM Nicotinamide (Sigma), 10μM Sodium Butyrate (Sigma). Lysate from $2x10^6$ cells was diluted 1:2 with IP buffer (20mM Tris pH 7.5, 150mM NaCl, 0.1% NP-40) and incubated overnight with 4μg of the indicated antibody. Antibody complexes were

recovered on magnetic protein A beads (Invitrogen) and washed five times with IP buffer supplemented to 0.25% NP-40. Immunoprecipitated material was analyzed by SDS-PAGE and immunoblot.

Deacetylation Assays

The Sirtuins were incubated with 2µg HeLa core histones and 100ng ac-p300 in Deacetylation Buffer for two hours at 30°C in the presence of NAD^+. Following deacetylation, proteins were analyzed by immunoblot.

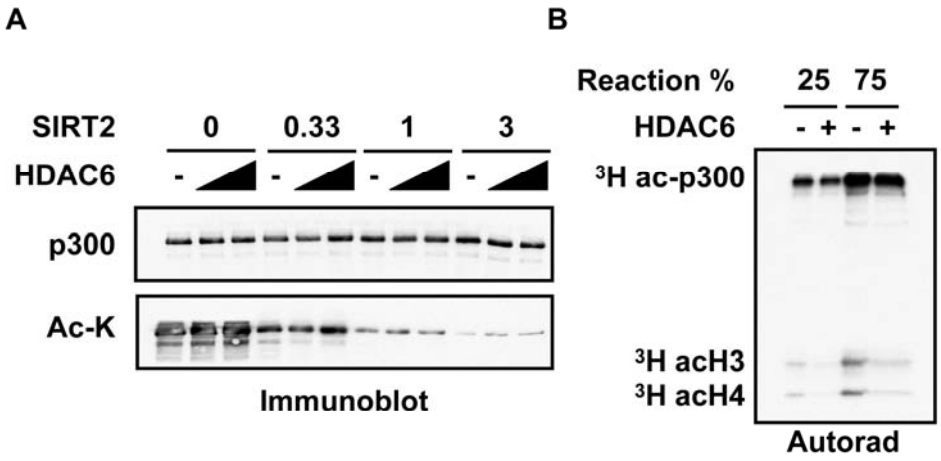


Figure S1: HDAC6 does not deacetylate p300. a.) Purified recombinant HDAC6 and SIRT2 were titrated against each other in an in vitro deacetylation assay in the presence of NAD^+. Reaction products were assayed by immunoblot. Titrations represent a 4 fold step of HDAC6. b.) HDAC6 is active. HDAC6 activity on ^{3}H-ac-p300 and ^{3}H-ac-HeLa core histones was assayed. HDAC6 deacetylates core histones but not p300. The two sets of lanes represent a 3-fold difference in the amount of each deacetylation reaction loaded on the gel.

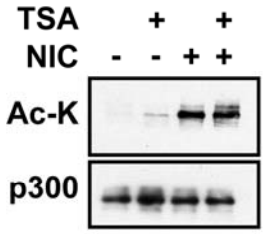


Figure S2: Nicotinamide is more effective than TSA at inhibition of p300 deacetylation in cells. U2OS cells were treated with Trichostatin A (TSA) alone, Nicotinamide (NIC) alone, or TSA and NIC for 16 hrs. p300 was immunoprecipitated from whole cell extracts and its acetylation status was assayed by immunoblotting with anti acetyl-Lysine.

Black Supplemental Figure 3

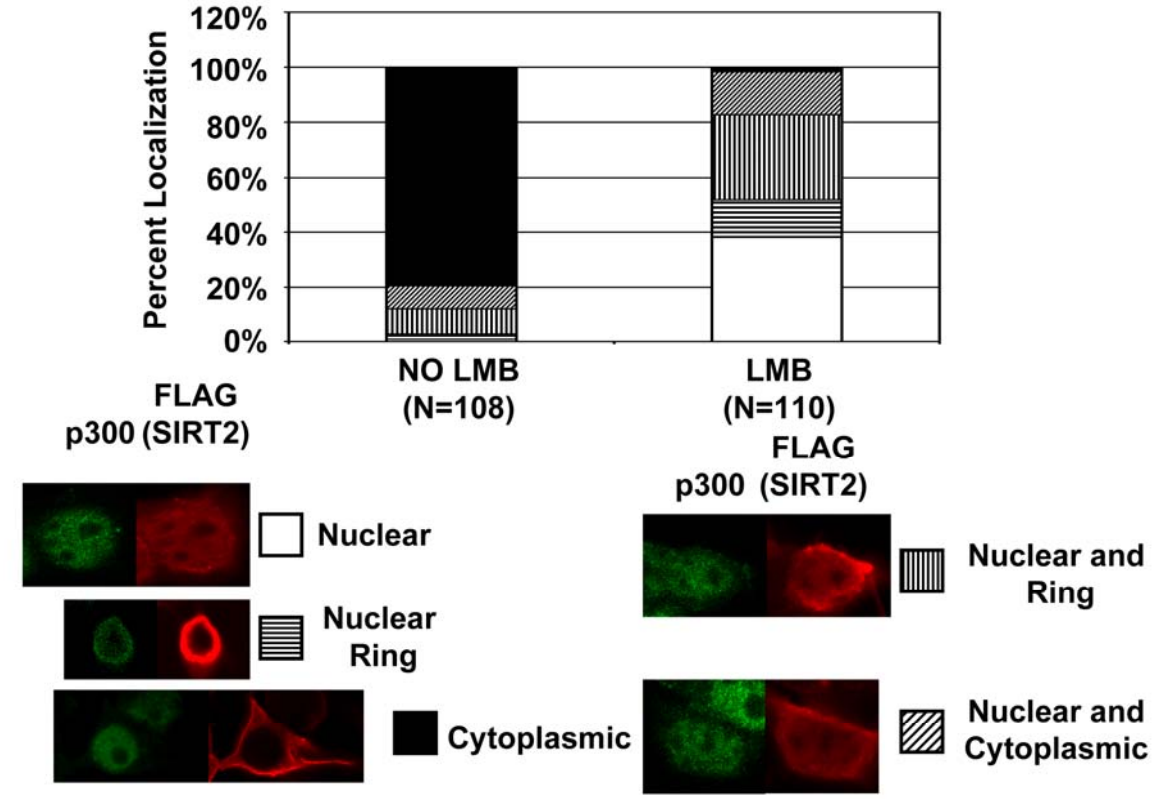


Figure S3: Leptomycin B induces nuclear localization of SIRT2. HEK293 cells were transfected with FLAG-SIRT2. Following 24 hours of expression, transfected cells were treated with or without Leptomycin B (LMB) for 16 hours. Cells were fixed for localization analysis of p300 (green) and FLAG (SIRT2, red). Stained cells were scored into one of 5 categories, represented in the bottom panels.

Black Supplemental Figure 4

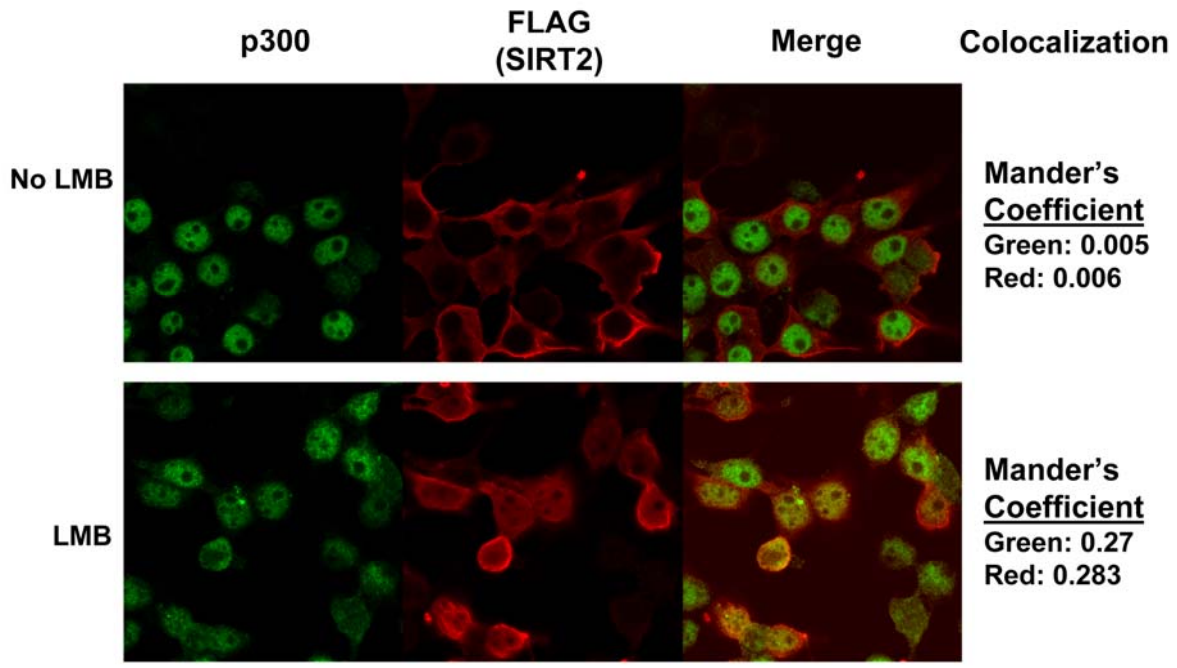


Figure S4: Leptomycin B induces nuclear localization of SIRT2. HEK293 cells were transfected with FLAG-SIRT2. Following 24 hours of expression, transfected cells were treated with or without Leptomycin B (LMB) for 16 hours. Cells were fixed for localization analysis of p300 (green), and FLAG (SIRT2, red). A representative field of cells for no LMB and LMB treated cells is shown with the Merge image on the right. To the right of each set of images is the Mander's colocalization coefficients calculated using the PASCAL software suite.

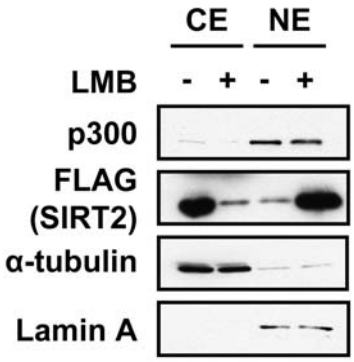


Figure S5: Leptomycin B causes retention of SIRT2 in the nucleus. Cytoplasmic (CE) and Nuclear (NE) extracts were prepared from U2OS cells transfected with FLAG-SIRT2. Following treatment with or without Leptomycin B (LMB) for 16 hours, cells were harvested and extracts prepared. The sub-cellular localization of SIRT2 was assayed by immunoblotting with anti-FLAG. Anti α-tubulin antibody was used as a cytoplasmic marker, while Lamin A served as a nuclear marker.

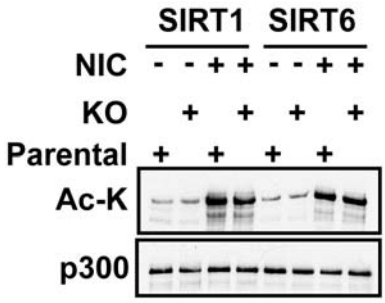


Figure S6: Nicotinamide inhibits p300 deacetylation in SIRT1 and SIRT6 Knockout MEFs. MEFs containing (parental) or knocked out (KO) for SIRT1 or SIRT6 were treated with nicotinamide (NIC) as indicated. Following 16 hours of treatment, p300 was immunoprecipitated from whole cell extracts and its acetylation levels were probed by immunoblot with anti acetyl-Lysine.

References

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