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Supplemental Data

SIRT6 Links Histone H3 Lysine 9 Deacetylation to NF-κB-Dependent Gene Expression and Organismal Life Span

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. SIRT6 and RELA interaction.

(A) SIRT6 interacts specifically with RELA in endogenous co-IPs. Shown are Western blots of endogenous SIRT6 IPs or negative control anti-Flag IPs probed with antibodies specific for the indicated NF-κB family members. *, antibody heavy chain; arrows indicate the expected sizes of p50 and p52.

(B) Co-IP of endogenous SIRT6 with endogenous RELA.

(C) RELA-SIRT6 interaction is independent of DNA bridging. Shown is Western blot analysis of RELA following FLAG IP from 293T cells expressing FLAG-SIRT6 or empty vector; IPs carried out +/- ethidium bromide (EtBr,100 ug/ml).

(D) SIRT6 expression is not induced upon NF-κB activation. Western analysis with the indicated antibodies shows that SIRT6 levels do not change following TNF-α treatment. The expected acute decrease in IκB levels (upper band) is observed under these conditions, confirming the efficacy of the TNF-α treatment. The lower band is a non-specific cross-reacting protein.

Figure S2. Interplay of SIRT6 and RELA on chromatin.

(A) Immunoblot analysis of RELA in cells transfected with siRELA 1 and 2. siRELA 1 targets the same sequence as the shRELA used in Figure 2. siRELA 2 target sequence: CCCACGAGCTTGTAGGAAATT.

(B) RELA knock-down inhibits SIRT6 recruitment to promoters of NF-κB target genes. SIRT6 ChIPs were performed in HeLa cells transfected with siRELA 2 or control siRNAs, following treatment with TNF-α (20 ng/ml). SIRT6 occupancy (mean ± s.e) is shown relative to untreated control cells.

(C) SIRT6 recruitment to NF-κB target genes is selective. *ATP2C1*, *PDZD2*, *TRIM25*, and *IAP2* are all induced by TNF-α and known to be direct RELA targets (Lim et al., 2007); however, SIRT6 is only detected at the *IAP2* promoter (upon TNF-α stimulation). Gene expression analysis in these same cells (Figure S4) confirmed that *IAP2* becomes hyperactivated in SIRT6 depleted cells while the other three genes do not. SIRT6 occupancy (mean ± s.e) is shown relative to untreated control cells.
(D) SIRT6 limits RELA occupancy at the promoter of RELA target gene promoters. ChIP with α-RELA antibodies was performed following continuous TNF-α treatment (20 ng/mL); RELA occupancy (mean ± s.e) at the *IAP2* promoter relative to untreated control samples is shown.
(E) Hyper-acetylation of other histone acetylation sites is not observed in SIRT6-depleted cells. H4K8 acetylation (H4K8Ac) levels are not increased in SIRT6-knockdown (S6 sh2) cells compared to control (pSR) cells. H4K8Ac levels (mean ± s.e) are normalized to control untreated cells. Similar results were observed at other promoters, and for H4K16 acetylation (data not shown).

Figure S3. SIRT6, H3K9Ac and RELA ChIP at NF-κB promoters in MEFs.
(A) Wild-type and *RelA*-/- MEFs were treated with TNF-α (20 ng/mL) for 1 hour, and ChIP with an α-Sirt6 antibody was performed. Shown is Sirt6 occupancy (mean ± s.e) at the *Birc3* (murine homologue to *IAP2*) and *Il1rl1* promoters relative to untreated control samples. *, $p<0.05$.
(B) Wild-type and *Sirt6-/-* MEFs were treated with TNF-α (20 ng/mL) for 1.5 hours, and ChIP with α-H3K9Ac and α-H3 antibodies was performed. Shown are H3K9Ac levels (mean ± s.e) at the *Birc3* and *Il1rl1* promoters relative to untreated control samples and normalized to total H3 levels. *, $p<0.05$.
(C) Wild-type and *Sirt6-/-* MEFs were treated with TNF-α (20 ng/mL) for 1.5 hours, and ChIP with α-RELA antibodies was performed. Shown is RELA occupancy (mean ± s.e) at the *Birc3* and *Il1rl1* promoters relative to untreated control samples. *, $p<0.05$.

Figure S4. SIRT6 knockdown leads to NF-κB target gene induction
(A) Gene expression analysis of genes enriched for NF-κB motifs in promoters and known NF-κB target genes (Hinata et al., 2003; Hinz et al., 2001) following TNF-α treatment (10 ng/ml) for the indicated times. *, Genes verified by qRT-PCR.

(B) Increased expression of NF-κB target genes in SIRT6 knock-down (S6 sh2) cells. Shown is quantitative TaqMan real-time PCR analysis of the indicated mRNAs (mean ± s.e), normalized to *GAPDH*.
(C) Increased expression of NF-κB target genes with SIRT6 knockdown depends on RELA. (Top) Western blots confirming siRNA-mediated RELA knockdown. (Bottom) Quantitative TaqMan real-time PCR analysis (mean ± s.e) of *MnSOD*, normalized to *GAPDH*. A similar trend is observed for several other genes (data not shown).
(D) Levels of expression of RELA-dependent NF-κB family members in SIRT6 knockdown cells. Slight increases are seen for several family members, consistent with the observed increase in mRNA levels.

Figure S5. No effects of SIRT6 on NF-κB release from IκB or direct deacetylation of RELA.

(A) SIRT6 depletion has no effect on release and re-sequestration of RELA. Shown is the RELA DNA binding activity in HeLa cells following a 30 minute pulse of TNF-α treatment (2.5 ng/ml) by the NoShift ELISA assay (Novagen) (mean ± s.d.).
(B) SIRT6 depletion does not affect IκB degradation but induces increased re-synthesis of IκB-α. Immunoblot of IκB-α in pSR and S6 sh2 HeLa cells following a 30 minute pulse of TNF-α treatment (2.5 ng/ml). Arrows point to time-points of IκB-α re-synthesis.
(C) SIRT6 does not deacetylate RELA in vitro. In vitro deacetylation reactions were carried out with the indicated purified recombinant proteins. Acetylated RELA was purified as follows: 293T cells were co-transfected with T7-RELA and p300 acetyltransferase and treated with TNF-α (20 ng/mL for .5 hours), and RELA protein was affinity purified with anti-T7 antibodies. Unacetylated RELA was purified from cells in the absence of p300 expression or TNF-α. These preparations of RELA were incubated with recombinant purified SIRT6 or SIRT1, or buffer control in NAD-dependent deacetylation reactions. SIRT1 efficiency deacetylates RELA, whereas SIRT6 does not.

Figure S6. Representative genotyping and IGF-1 levels of cohort mice.

(A) Representative genotyping of cohort mice. PCR analysis with primers designed for the wild-type and null alleles of *Sirt6* and *RelA* was performed to identify mice of the indicated genotypes. Genotyping primer sequences are:
GTGCATCTCAATGGTGCAGTGCATGTT (wild-type, 5’),

GCAATAGCATCACAAATTTCACAAATA (knockout, 5'), and
GTGTGATTGGTAGAGAGGCACGTGGAT (common, 3').
(B) Serum IGF-1 levels (mean ± s.e.) in wild-type (n=5) and *Sirt6-/-RelA+/-* (n=6) mice at 25 and >90 days. *p*-value is indicated.

Figure S7. Murine gene expression data.
A) Shown is the expression of all direct NF-κB targets in wild-type and *Sirt6*-/- MEFs, based on Lim et al. (Lim et al., 2007). Sirt6-dependent targets are indicated on the right (56%); a gene is considered Sirt6 dependent if its average fold induction upon TNF-α treatment is higher in *Sirt6*-/- as compared to wild-type.
(B) Shown is the average expression of genes within significantly induced or repressed Gene Ontology terms following treatment with TNF-α in wild-type and *Sirt6*-/- MEFs.
(C) Shown is the expression of NF-κB motif module genes in wild-type, *Sirt6*-/- and *Sirt6*-/-*RelA*+/- spleen tissues, all normalized to wild-type expression levels.

Figure S8. Sirt6 Expression in young and old murine tissues.
Shown is the western blot analysis of Sirt6 protein in young (~3 month) and old (24–27 month) spleen and liver tissue.

Table S1. Sirt6-dependent motif modules induced or repressed in seven *Sirt6*-/- tissues. Listed are the motif modules that are induced (+) or repressed (–) upon *Sirt6* knockout in mouse. Notations following some transcription factor names (ex. Q6_01) are identifiers for variants of the motifs according to TRANSFAC. Standalone sequences or sequences before a transcription factor name represent consensus binding motifs (key for combination of nucleotides: Y = C or T; R = A or G; W = A or T; S = C or G; K = T or G; M = C or A; N = unknown).

Table S2. Primer sequences for the promoter regions of NF-κB target genes.

SUPPLEMENTAL REFERENCES

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