Supplemental Information

SIRT2 regulates cellular iron through NRF2 deacetylation and nuclear localization

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Supplemental Experimental Procedures

Isolation of primary hepatocytes

Isolation of primary hepatocytes was performed as previously described (1). After anesthetization with freshly prepared Tribromoethanol (250 mg/kg, Sigma), the portal vein of each mouse (males, 8-12 weeks old) was cannulated with a 24G x 3/4" intravenous catheter (Terumo) and the liver was perfused with Ca^{2+}- and Mg^{2+}-free Hanks' buffered saline solution (HBSS, GIBCO) for 5 minutes, followed by perfusion with HBSS containing 0.05% type IV collagenase (Sigma) for 5 minutes. The perfusate was drained by an incision of the femoral artery. After perfusion, the liver was removed and placed in ice-cold DMEM (with 4.5 g/L glucose and L-glutamine lacking sodium pyruvate, Corning). The liver sac was cut and cells were released by gently shaking the liver in the ice-cold DMEM. The cell suspension was filtered through a 100 μM nylon strainer (BD Falcon) and centrifuged at 50 g for 1 minute. The cell pellets were washed three times with ice-cold DMEM, and then resuspended in DMEM containing 5% fetal bovine serum (FBS). Cell viability (~80%) was determined by Trypan blue exclusion. Cells were plated on type I collagen-coated dishes at a density of 5 x 10^4 cells/cm^2 and incubated at 37 °C under 5% CO_2. After 1.5 h of attachment, the media was aspirated, cells were washed with PBS, and cells were incubated in DMEM containing 10% FBS and 1% P/S/amphotericin B. For cell death and cell viability studies, cells were treated with 100 μM DFO or PBS for 16 hours. All experiments involving cultured hepatocytes were performed within 48 h of culturing.

Quantitative RT-PCR

RNA was isolated with RNA STAT-60 (TEL-TEST, Inc, TX), reverse-transcribed with a Random Hexamer (Applied Biosystems, CA), and amplified on a 7500 Fast Real-Time PCR system with SYBR Green PCR Master Mix (Applied Biosystems, CA). Primers were designed using Primer3 (v. 0.4.0) software to target sequences spanning an exon-intron-exon boundary and their specificity was confirmed by running a dissociation curve. mRNA levels were calculated by the comparative threshold cycle method and normalized to *18S* or *ACTB*.

Western blot and immunoprecipitation

Protein (20-40 µg) were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes (Invitrogen, CA). The membranes were probed with antibodies against SIRT2, FLAG, V5, Acetyl lysine, HPRT1, KEAP1, NRF2, TFRC, FTL, Lamin A/C, GAPDH, Fpn1, and Tubulin. HRP-conjugated donkey anti-rabbit or donkey anti-mouse were used as secondary antibodies (Santa Cruz, CA) and visualized by Pierce SuperSignal Chemiluminescent Substrates.

For IP, cells or tissue were lysed using IP buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40 and 5% glycerol), and cell extracts were incubated overnight with appropriate antibodies followed by incubation with protein A or G agarose beads for 4 h at 4 ^{o}C. After washing five times with IP buffer, immunocomplexes were resolved using SDS-PAGE and analyzed by western blot.

Immunohistochemistry

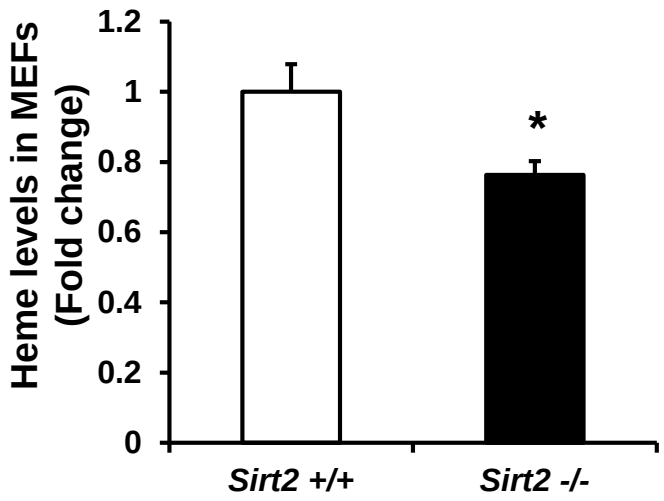
Paraffin-embedded liver tissues were sectioned to 5-µm thickness. Sections were deparaffinized and stained with Perl's Prussian blue for detection of ferric iron(2). One section of each was also stained with SIRT2 primary antibody followed by fluorophore-conjugated

secondary antibody. Nuclei were stained with DAPI. Images were taken using Zeiss AxioObserver.Z1 fluorescent microscope. Images were analyzed with Image J (NIH).

Supplemental References

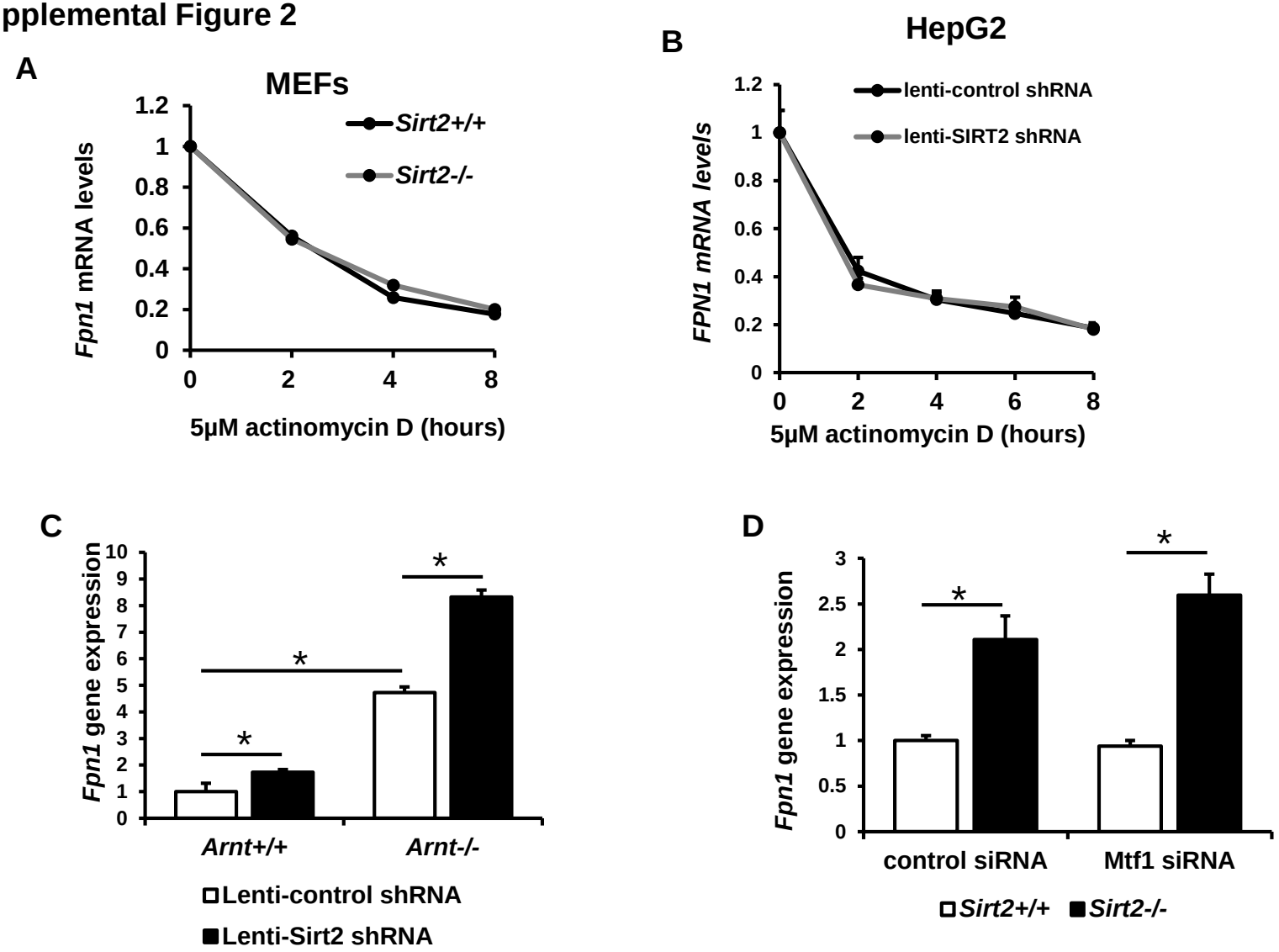
1. McCommis KS, Chen Z, Fu X, McDonald WG, Colca JR, Kletzien RF, Burgess SC, and Finck BN. Loss of Mitochondrial Pyruvate Carrier 2 in the Liver Leads to Defects in Gluconeogenesis and Compensation via Pyruvate-Alanine Cycling. *Cell Metab.* 2015;22(4):682-94.
2. Orchard GE. In: Suvarna KS, Layton C, and Bancroft JD eds. *Bancroft's Theory and Practice of Histological Techniques*. Elsevier; 2013:239-70.

Supplemental Figure 1



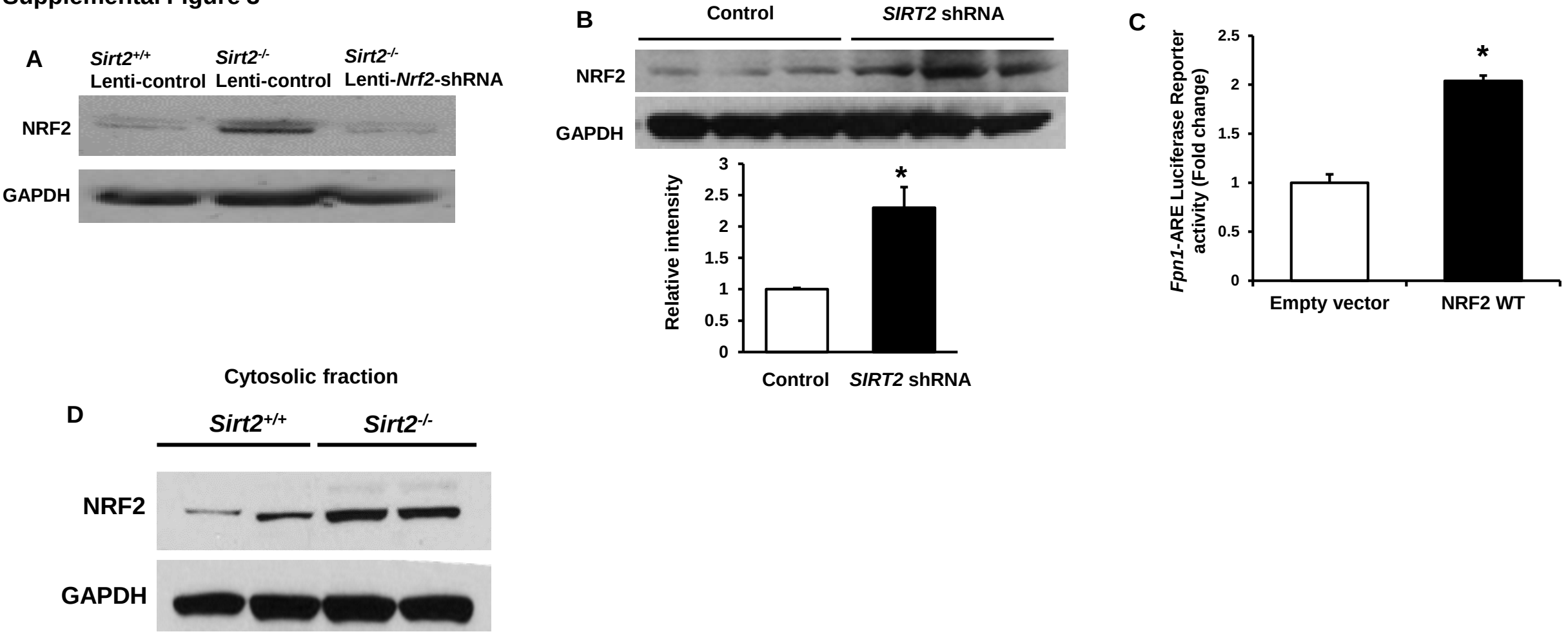
Supplemental Figure 1. SIRT2 regulates heme iron in MEFs.
Heme iron levels in *Sirt2*$^{+/+}$ and *Sirt2*$^{-/-}$ MEFs (n=11-12). Data are presented as mean ± SEM, **P* < 0.05 by Student's t-test .

Supplemental Figure 2



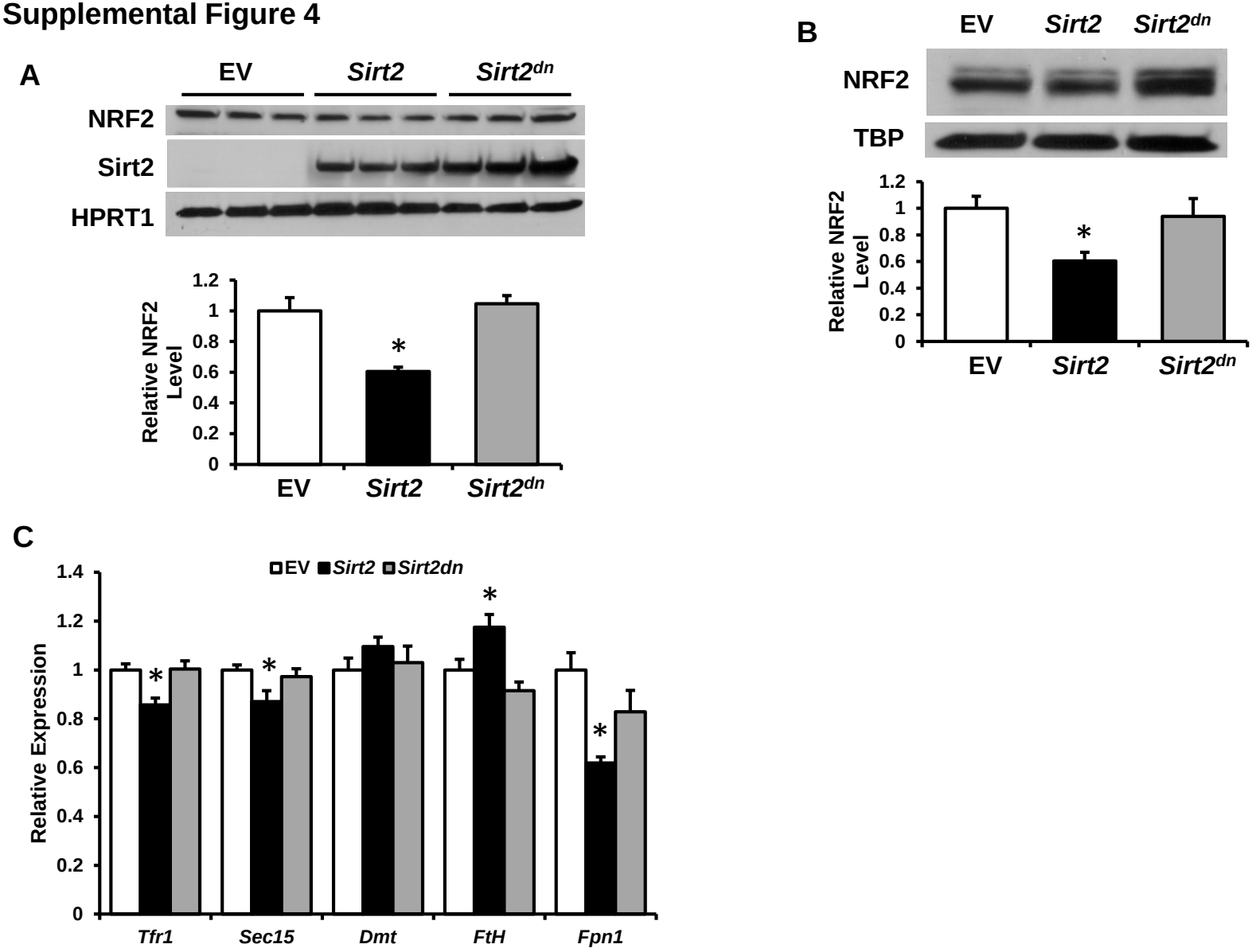
Supplemental Figure 2. FPN1 is transcriptionally upregulated with SIRT2 knockout or knockdown.
(**A**) *Fpn1* mRNA decay assay in *Sirt2*$^{+/+}$ and *Sirt2*$^{-/-}$ MEFs (n=5-6). (**B**) *FPN1* mRNA decay assay in HepG2 cells infected with lenti-control shRNA and lenti-*SIRT2* shRNA (n=5-6). (**C**) *Fpn1* gene expression on *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ MEFs infected with lenti-control shRNA and lenti-*Sirt2* shRNA (n=5-6). (**D**) *Fpn1* gene expression on *Sirt2*$^{+/+}$ and *Sirt2*$^{-/-}$ MEFs with or without silencing of *Mtf1* (n=5-6). Data are presented as mean ± SEM, *$P < 0.05$ by ANOVA with Bonferroni correction for multiple comparisons.

Supplemental Figure 3



Supplemental Figure 3. NRF2 expression is regulated by SIRT2.
(**A**) NRF2 expression in MEFs infected with lentivirus (lenti-control shRNA or lenti-*Nrf2* shRNA). (**B**) NRF2 expression in HepG2 cells infected with lentivirus (lenti-control shRNA or lenti-*SIRT2* shRNA). (**C**) Luciferase activity in HepG2 cells transfected with murine *Fpn1* ARE-luciferase reporter construct along with *NRF2* plasmid or empty vector (n=6). (**D**) NRF2 expression in the cytoplasm of MEFs. Data are presented as mean ± SEM, **P* < 0.05 by Student's t-test.

Supplemental Figure 4



Supplemental Figure 4. Enzymatic activity of SIRT2 is required for its regulation of NRF2.
Total cellular (**A**) and nuclear (**B**) levels of NRF2 in *Sirt2*$^{-/-}$ MEFs with overexpression of control (empty vector or EV), WT or deacetylation-null SIRT2 (*Sirt2*^{dn}) (n=3). (**C**) Expression of key iron homeostasis genes in *Sirt2*$^{-/-}$ MEFs with overexpression of WT *Sirt2* or *Sirt2*^{dn} (n=6). Data are presented as mean ± SEM, *P < 0.05 by ANOVA with Bonferroni correction for multiple comparisons.

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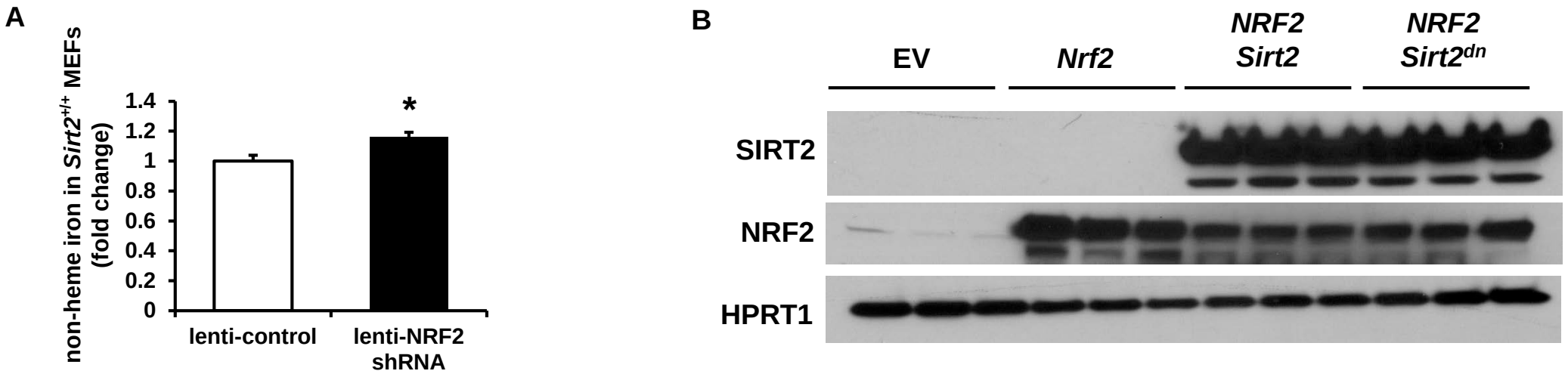
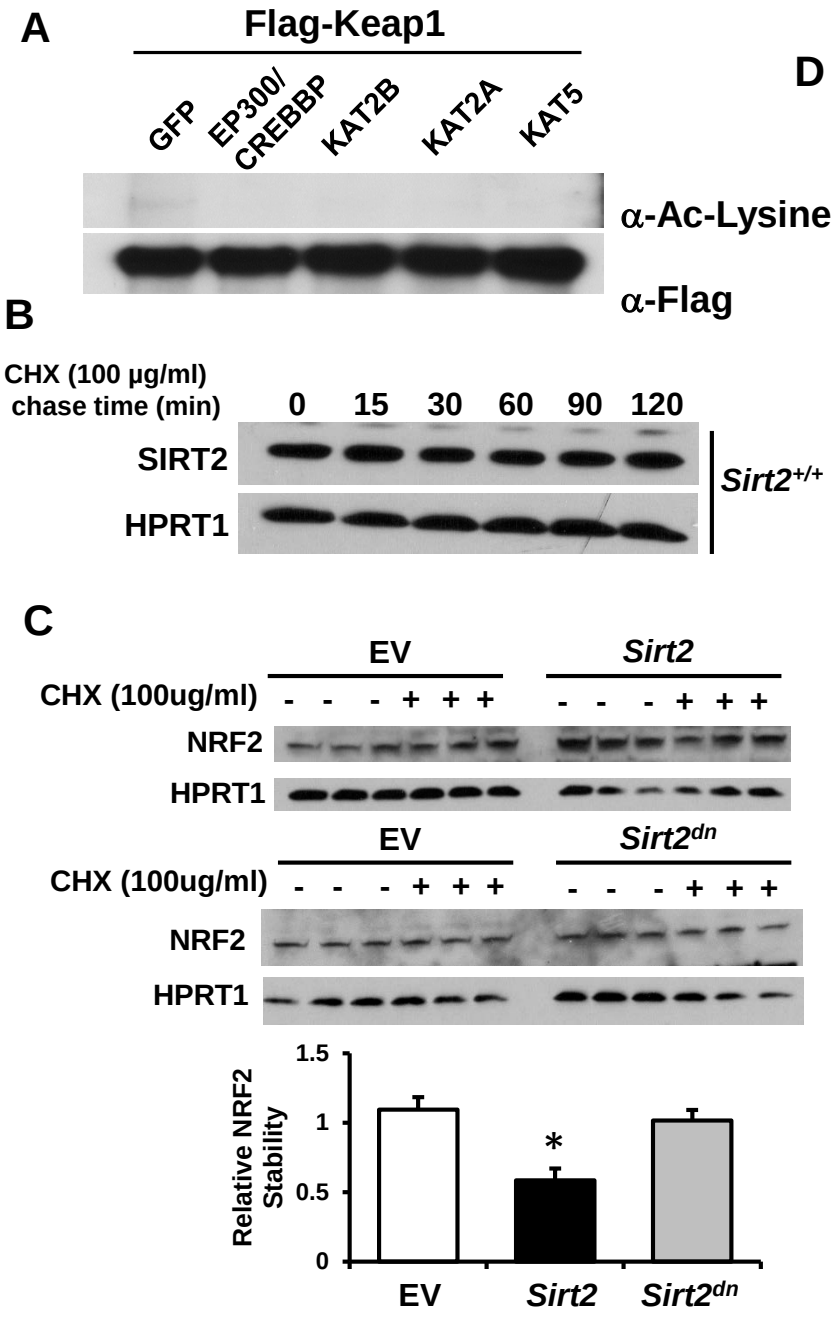
Supplemental Figure 5



Supplemental Figure 5. None-heme iron in Sirt2-/- MEFs and protein expression in cells transfected with WT and dominant-negative *Sirt2*.
(**A**) non-heme iron in *Sirt2*$^{+/+}$ MEFs infected with lenti-control shRNA or lenti-*Nrf2* shRNA (n=6). (**B**) Western blot of HEK293 cells transfected with *Nrf2* and WT or dominant negative *Sirt2* (corresponds to Figure 3H). Data are presented as mean ± SEM, **P* < 0.05 by Student's t test.

Supplemental Figure 6



D

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Cow             WPPGDPVQPLSSSQGNSAAARDSHCENAPKKEVPVSPGHRKTPFTKDKHSSRLEAHLTRD
Mouse           HSPGDTVQPLSPAQGHSAPMRESQCENTTKKEVPVSPGHQKAPFTKDKHSSRLEAHLTRD
Chicken         RFPDQVLPSVEP--GTQTPSLQRM--NTPKKDPPAGPGHPKAPFTKDKPSGRLEAHLTRD
Frog            PFTEDTYFSLSPFISHDTDPFEVKAHDSPAKEIPASPGYSKAPFAKDKSLNLQEARFTRD
Zebrafish       VYLNDGSQ--TPLSEKSSTEKQEMKLKNPKMEPAEASGHSKPPFTKDKLKKRSEARLSRD
Fly             SLPQGSGSLPRPQARDKKPL-------VATKTASKGASAGNSSSVGGNSSNLEEEHLTRD

                     462       472                                                  506 508
Human      458  ELRAKALHIPFPVEKIINLPVVDFNEMMSKEQFNEAQLALIRDIRRRGKNKVAAQNCRKR
Monkey          ELRAKALHIPFPVEKIINLPVVDFNEMMSKEQFNEAQLALIRDIRRRGKNKVAAQNCRKR
Cow             ELRAKALHIPFPVEKIINLPVEDFNEMMSKEQFNEAQLALIRDIRRRGKNKVAAQNCRKR
Mouse           ELRAKALHIPFPVEKIINLPVDDFNEMMSKEQFNEAQLALIRDIRRRGKNKVAAQNCRKR
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Frog            EQRAKVLNLPFTVEKIVNLPVDSFNEIMSKYQFNEAQLALIRDIRRRGKNKVAAQNCRKR
Zebrafish       EQRAKALQIPFTVDMIINLPVDDFNEMMSKHQLNEAQLALVRDIRRRGKNKVAAQNCRKR
Fly             EKRARSLNIPISVPDIINLPMDEFNERLSKYDLSENQLSLIRDIRRRGKNKVAAQNCRKR

                518                          543   548    555
Human      518  KLENIVELEQDLDHLKDEKEKLLKEKGENDKSLHLLKKQLSTLYLEVFSMLRDEDGKPYS
Monkey          KLENIVELEQDLDHLKDEKEKLLKEKGENDKSLHLLKKQLSTLYLEVFSMLRDEDGKPYS
Cow             KLENIVELEQDLDHLKDEKEKLLKERGENDKSLHLLKKQLSTLYLEVFSMLRDENGKPYS
Mouse           KLENIVELEQDLGHLKDEREKLLREKGENDRNLHLLKRRLSTLYLEVFSMLRDEDGKPYS
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Frog            KMENIVELETDLDTLKYEKEKLLAERGEYNNSLSQLKKNLGNLYMEVFNKLQDENGKPYS
Zebrafish       KLENIVGLEYELDSLKEEKERLMKEKSERSSNLKEMKQQLSTLYQEVFGMLRDENGKAFS
Fly             KLDQILTLEDEVNAVVKRKTQLNQDRDHLESERKRISNKFAMLHRHVFQYLRDPEGNPCS

                     596 598 599 603
Human      578  PSEYSLQQTRDGNVFLVPKSKKPDVKKN 605
Monkey          PSEYSLQQTRDGNVFLVPKSKKPDVKKN
Cow             PSEYSLQQTSDGNVFLVPKSKRPDIKKN
Mouse           PSEYSLQQTRDGNVFLVPKSKKPDTKKN
Chicken         PSEYSLQQTRDGNIFLVPKSRKAETKL
Frog            PQEYSLQQTKEGNIFLVPKTKKVSIKKE
Zebrafish       PNEFSLQHTADGTVFLVPRLKKTLVKNI
Fly             PADYSLQQAADGSVYLLPREKSEGNNTATAASNAVSSA
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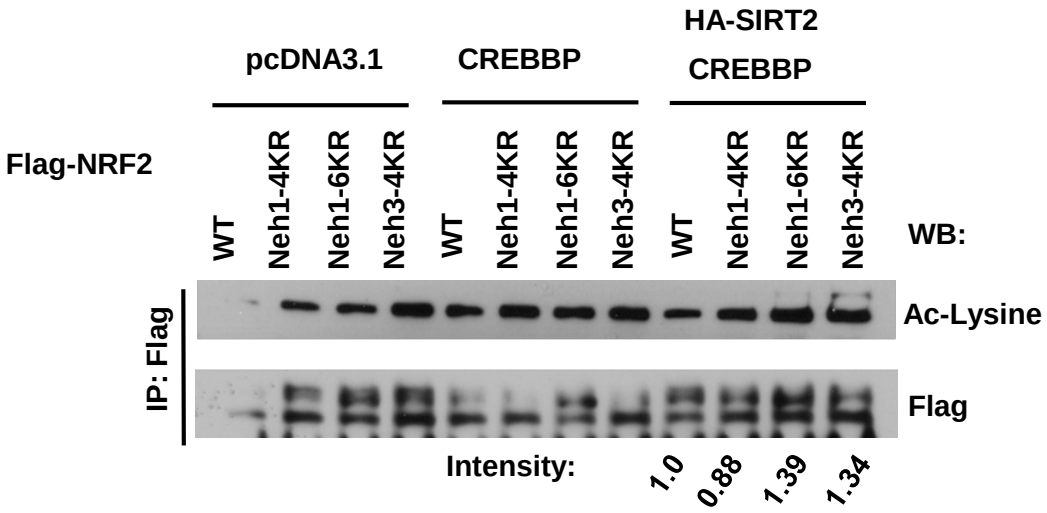
Supplemental Figure 6. Lysine residues (K) of overexpressed human *NRF2* that are acetylated in *Sirt2*$^{-/-}$ MEFs but deacetylated in SIRT2$^{+/+}$ MEFs.

(**A**) Co-IP experiments of HEK293T cells transfected with Flag-Keap1 and either green fluorescent protein (GFP, control) or different HATs including EP300/CREBBP, KAT2B, KAT2A, and KAT5, using an anti-Flag antibody to IP and anti-acetyl-lysine or anti-Flag antibodies as immunoblot antibody. (**B**) Time course of SIRT2 protein stability after treatment with 100 µg/ml of CHX in *Sirt2*$^{+/+}$ MEFs. (**C**) NRF2 stability in *Sirt2*$^{-/-}$ MEFs overexpressing WT *Sirt2* or *Sirt2*^{dn} (n=3). Data presented as mean ± SEM, * $p<0.05$ compared with EV by ANOVA with Bonferroni correction for multiple comparisons. (**D**) Protein sequence alignment of NRF2 across different species (using Kalign Tool). Sequences in shade show the most conserved region across species. All lysine residues of overexpressed human *NRF2* that are acetylated in *Sirt2*$^{-/-}$ MEFs but deacetylated in *Sirt2*$^{+/+}$ MEFs were highlighted in sequence number. Three different de-acetylation mimetic mutants by mutating lysine (K) to arginine (R) in the following three regions were made: 1) the first 4 lysines (443,445,462 and 472) in Neh1 (Neh1-4KR), 2) the remaining 6 lysine residues (506, 508, 518, 543, 548 and 555) in Neh1 (Neh1-6KR), 3) 4 lysine residues (596, 598, 599 and 603) in Neh3 (Neh3-4KR).

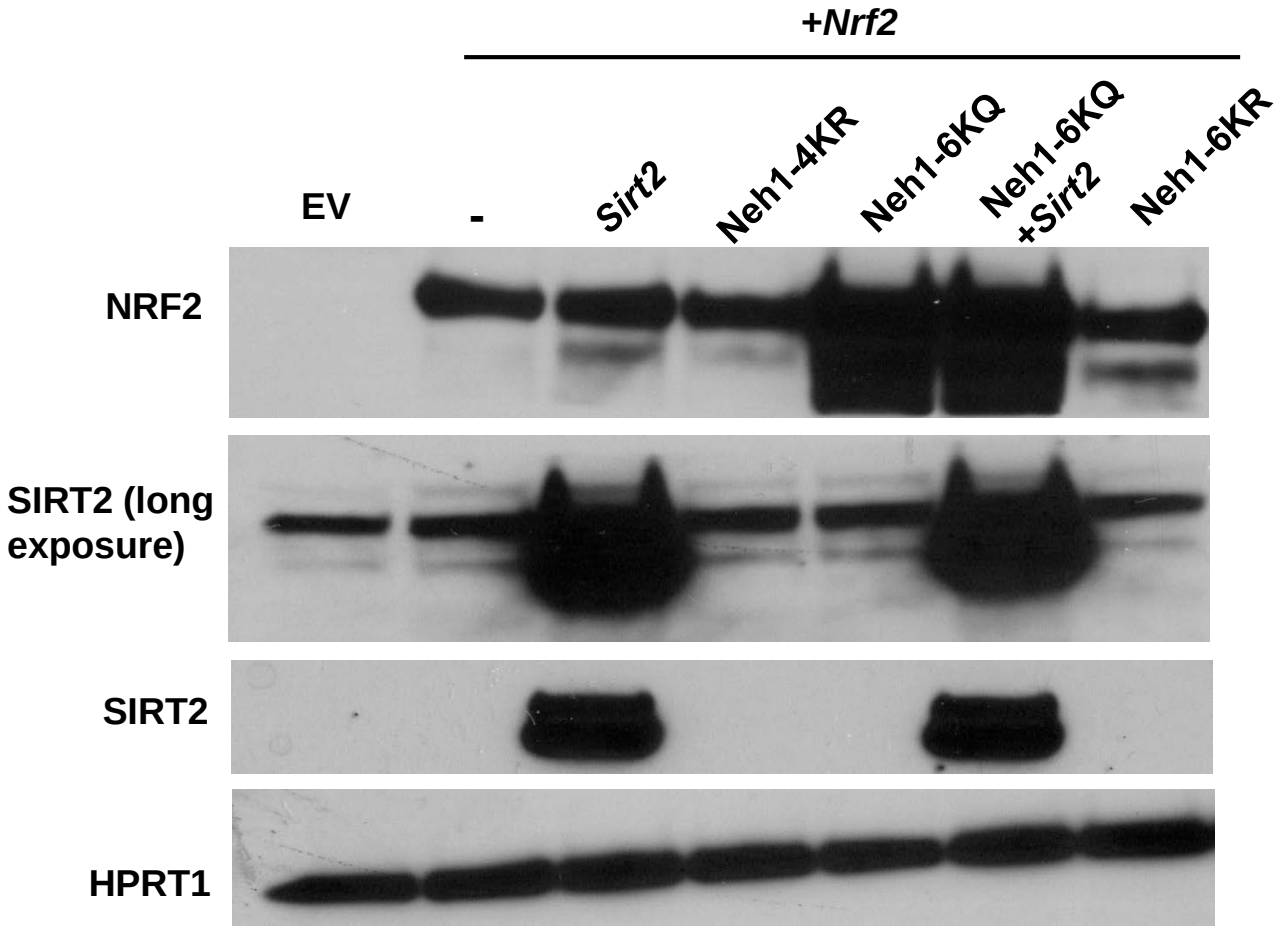
Supplemental Figure 7



Supplemental Figure 7. Repeat Western blot confirming that lysines 506 and 508 of NRF2 are targeted and deacetylated by SIRT2.

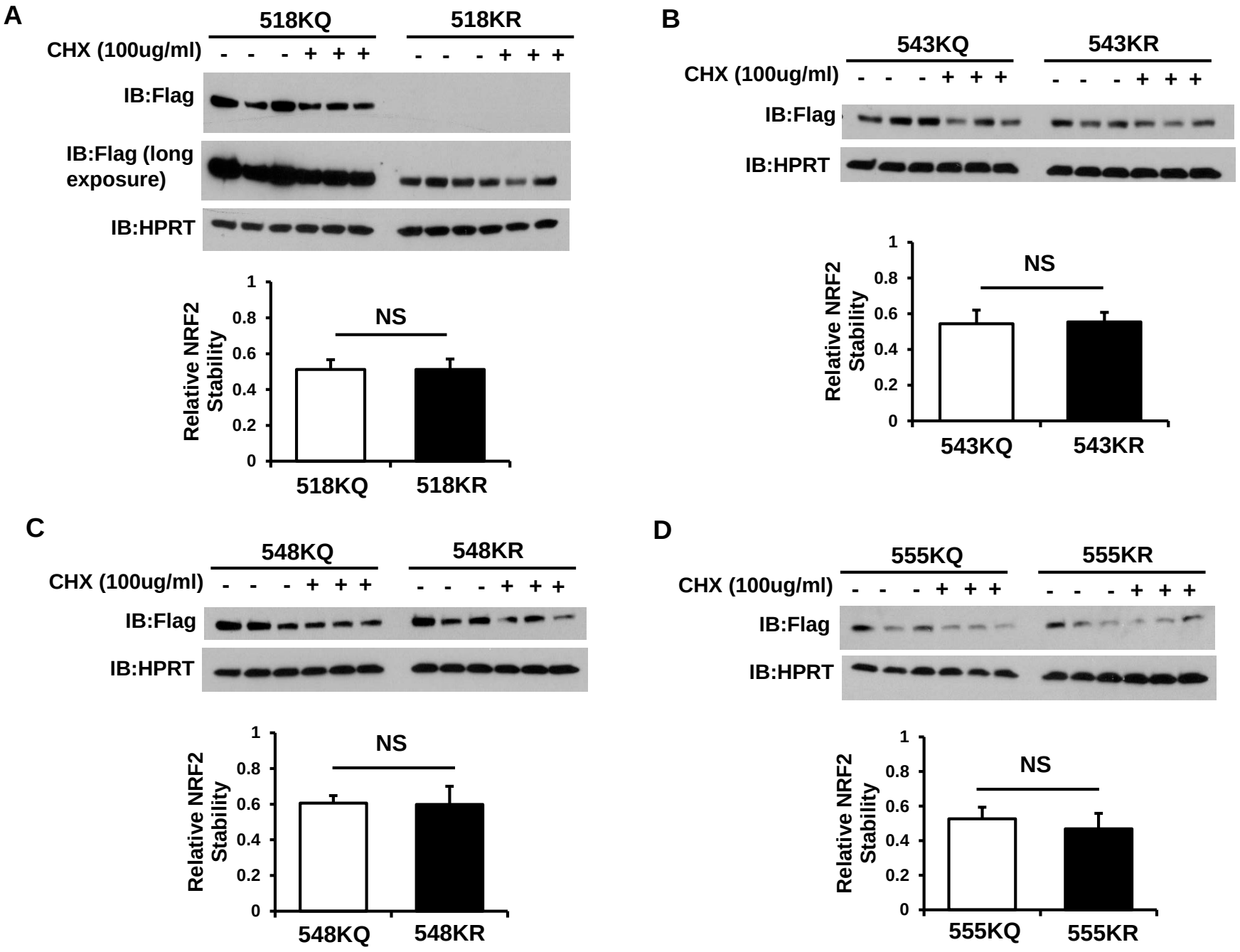
(**A**) Co-IP experiments in HEK293T cells overexpressing Flag-NRF2 or mutant plasmids, and CBP together with or without SIRT2 using an anti-Flag antibody to IP and anti-acetyl-lysine as immunoblot antibody. The numbers under the gel reflect the degree of protein acetylation in the presence of SIRT2 and CREBBP normalized to CREBBP only. DNA3.1 empty vector was used as control. This Western blot is consistent with results obtained in Figure 5A.

Supplemental Figure 8



Supplemental Figure 8. Western blot of HepG2 cells transfected with murine *FPN1* ARE-luciferase reporter construct along with NRF2 plasmid or NRF2 6KQ or 6KR mutants. This Western blot was performed to confirm overexpression of the constructs and corresponds to Figure 5B.

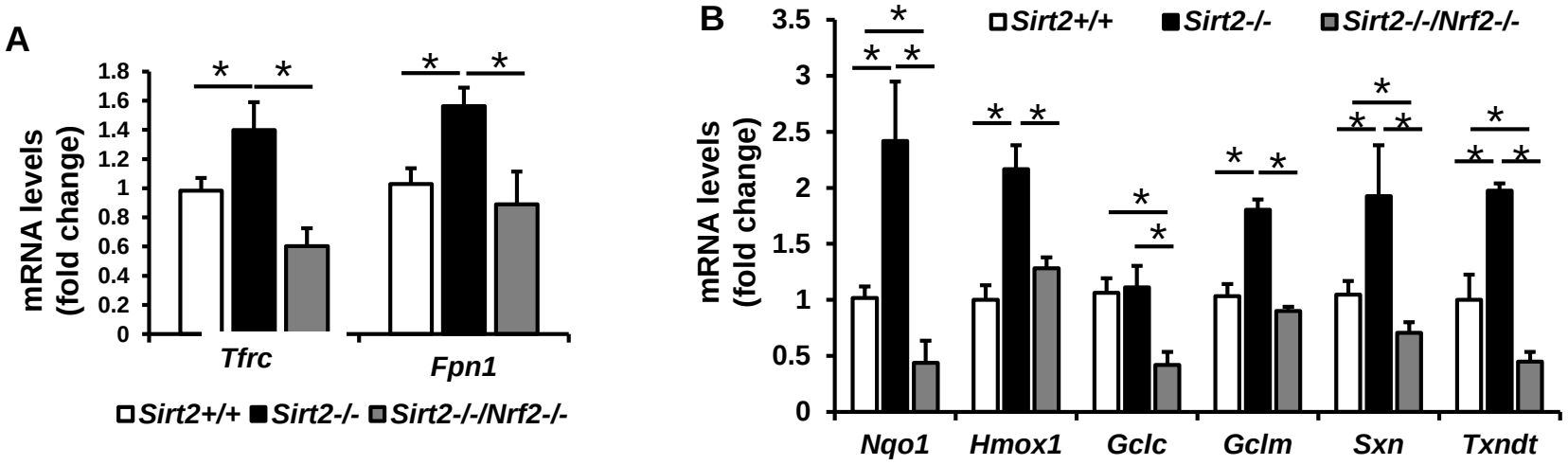
Supplemental Figure 9



Supplemental Figure 9. Protein stability of *NRF2* mutants with mutation from lysine (K) to either glutamine (Q) or arginine (R).

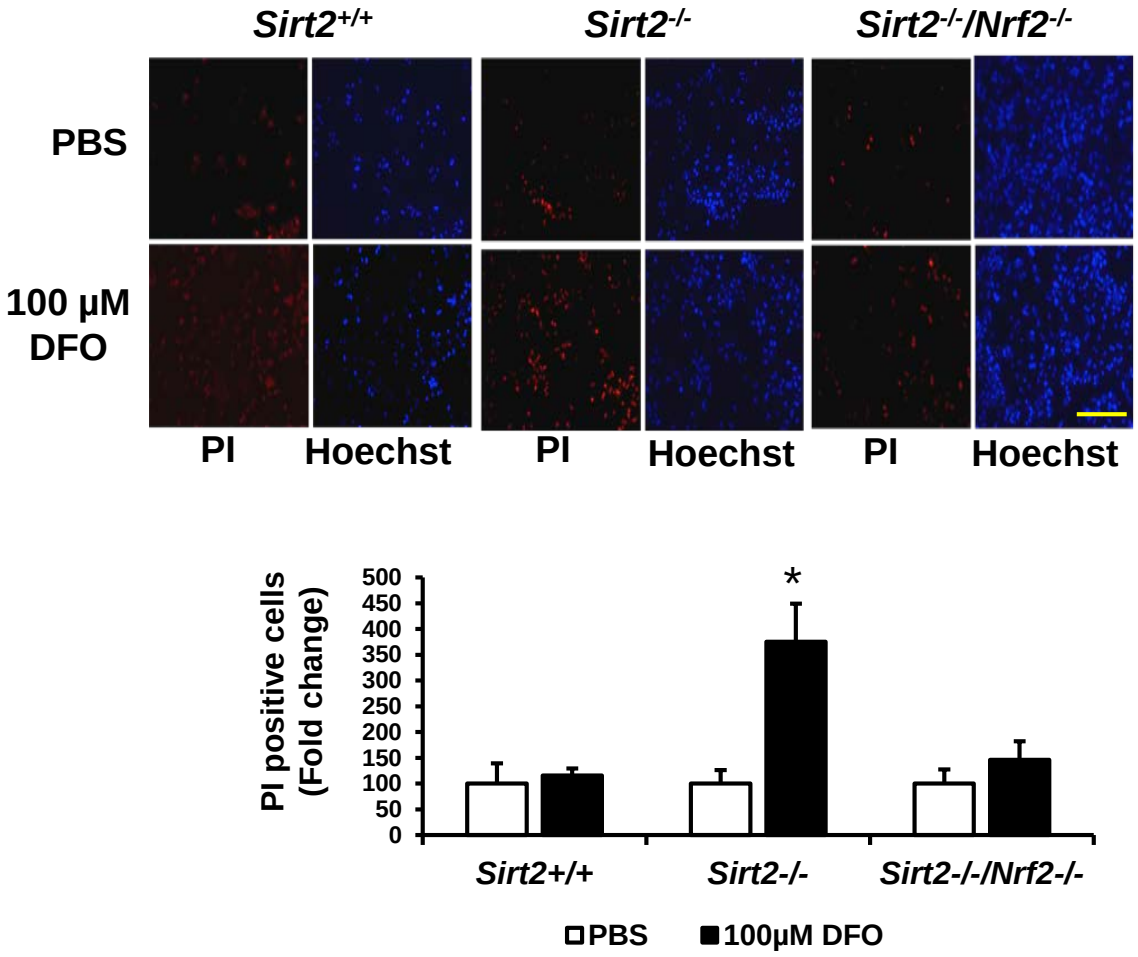
Time course of protein stability of overexpressed *NRF2* mutants with mutation at 518 (**A**), 543 (**B**), 548 (**C**), and 555 (**D**) and after treatment with cyclohexamide (100 µg/ml) in HepG2 cells. Data are presented as mean ± SEM, **P* < 0.05 by Student's t-test.

Supplemental Figure 10



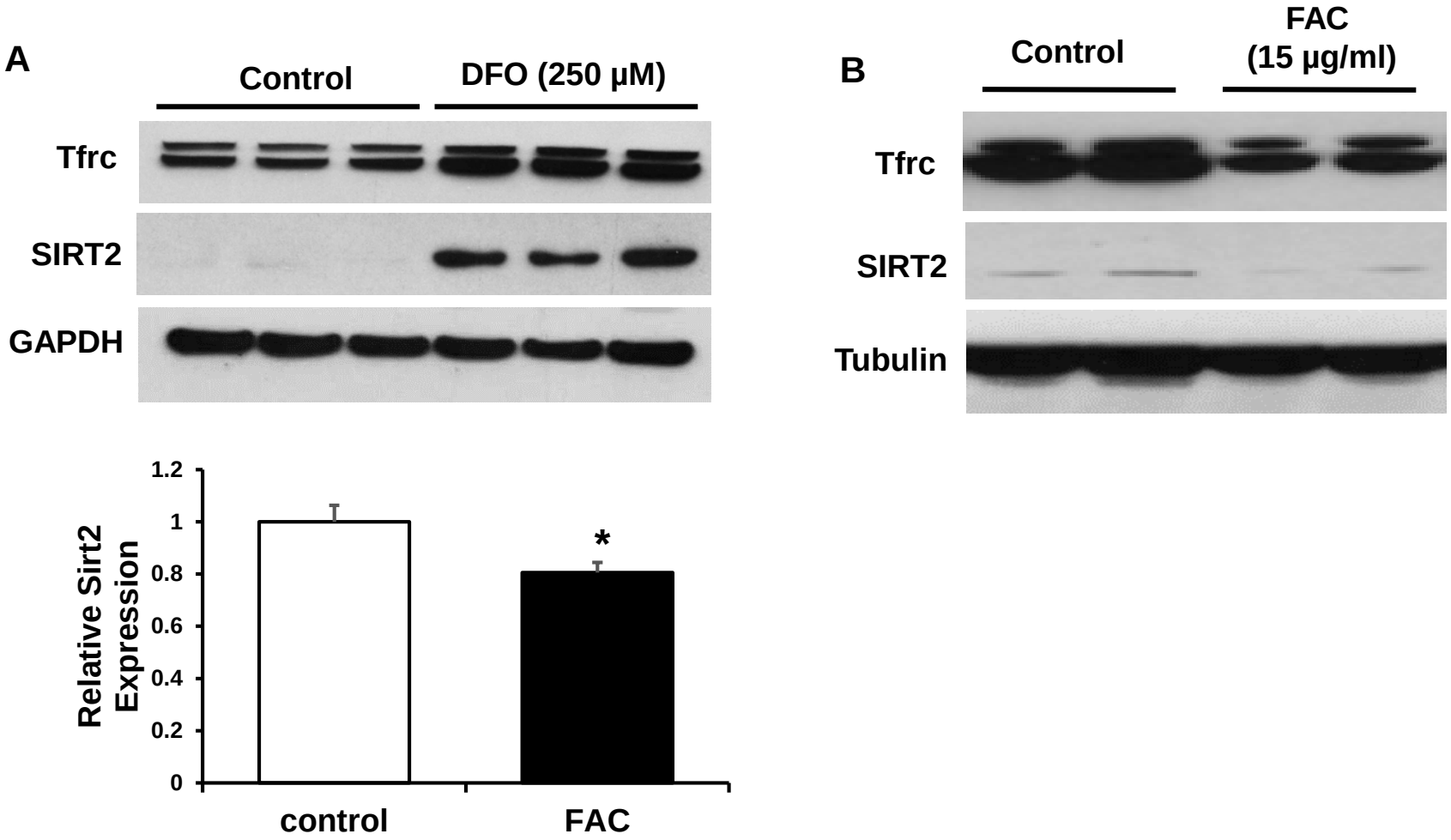
Supplemental Figure 10. SIRT2 regulates FPN1 and anti-oxidant genes in mouse livers.
(**A**) *Tfrc* and *Fpn1* mRNA in the livers of *Sirt2*$^{+/+}$, *Sirt2*$^{-/-}$ and *Sirt2*$^{-/-}$/*Nrf2*$^{-/-}$ mice (n=6). (**B**) Anti-oxidant gene expression in the livers of *Sirt2*$^{+/+}$, *Sirt2*$^{-/-}$ and *Sirt2*$^{-/-}$/*Nrf2*$^{-/-}$ mice (n=6). Data are presented as mean ± SEM. *$P < 0.05$ by ANOVA with Bonferroni correction for multiple comparisons.

Supplemental Figure 11



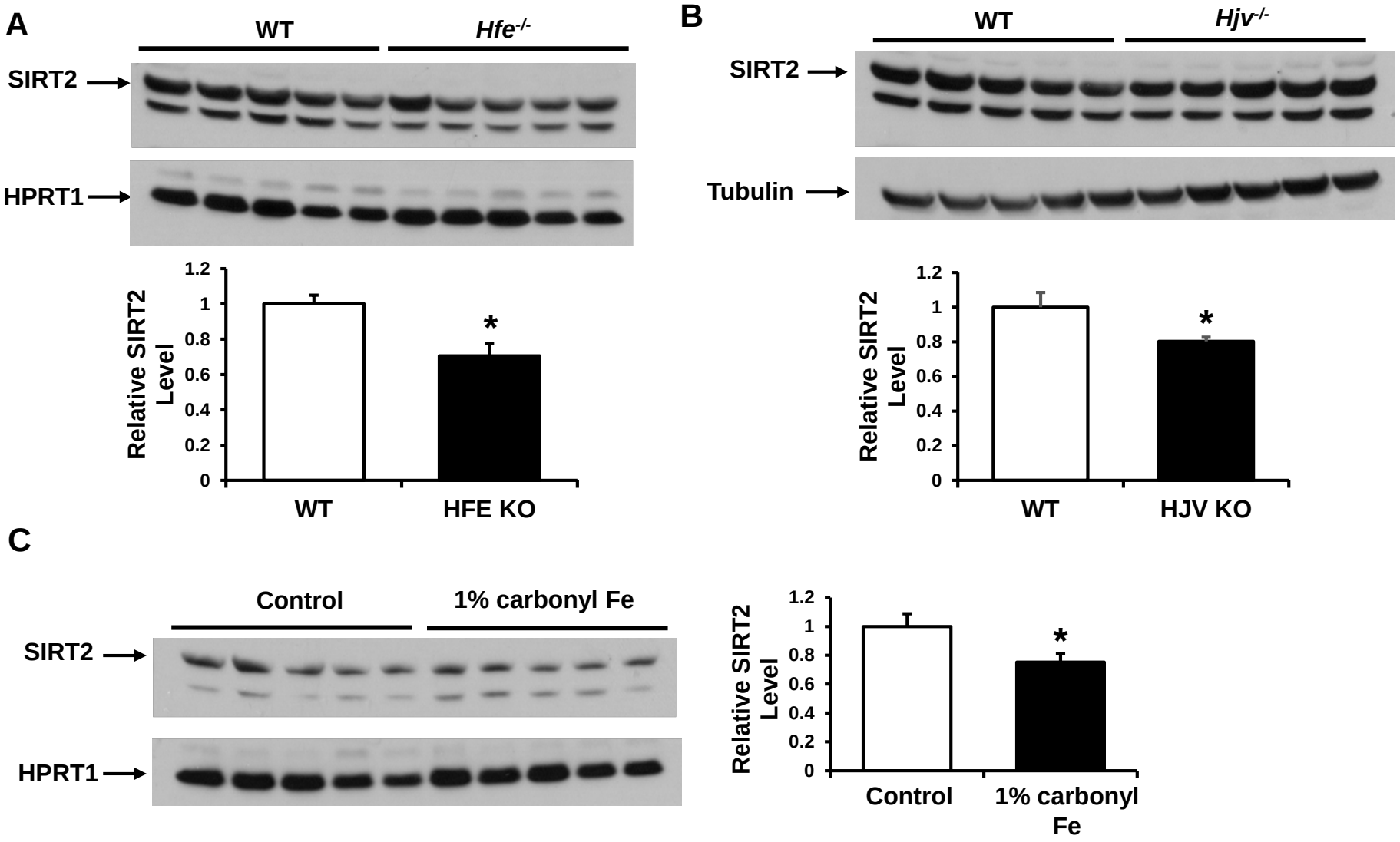
Supplemental Figure 11. *Nrf2* deletion reverses cell death in the livers of *Sirt2*$^{-/-}$ mice.
Cell death in primary hepatocytes of *Sirt2*$^{+/+}$, *Sirt2*$^{-/-}$ and *Sirt2*$^{-/-}$/*Nrf2*$^{-/-}$ mice measured by PI staining (*n* = 1 per genotype; cells cultured in 5-6 individual wells). The bar graph summary of the results are shown underneath the images. Data are presented as mean ± SEM, scale bar = 68µm. *$P < 0.05$ by Student's t-test.

Supplemental Figure 12



Supplemental Figure 12. SIRT2 levels are increased in iron deficiency and decreased with iron overload.
(**A**) SIRT2 expression on HepG2 cells treated with 250 μM deferoxamine (DFO). (**B**) SIRT2 expression on HepG2 cells treated with 15 μg/ml ferric ammonium citrate (FAC). (**C**) SIRT2 mRNA in HepG2 cells treated with 15 μg/ml FAC. n=3 for each group. Data are presented as mean ± SEM, *$P < 0.05$ by Student's t-test.

Supplemental Figure 13

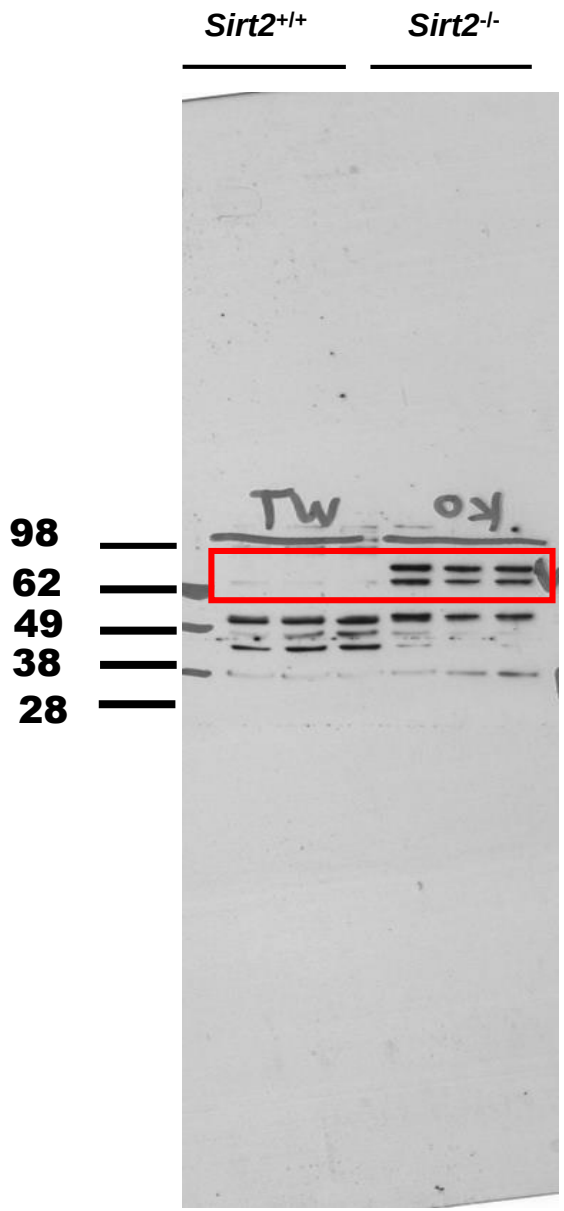


Supplemental Figure 13. SIRT2 levels in liver extracts from mouse models of iron overload.
Three different models of iron overload were studied: (**A**) *Hfe*$^{-/-}$ mice at 16 weeks of age, (**B**) *Hjv*$^{-/-}$ mice at 12 weeks of age, and (**C**) weanling mice fed an iron-loaded diet (1% carbonyl iron for 4 weeks). Data are presented as mean ± SEM, by Student's t-test.

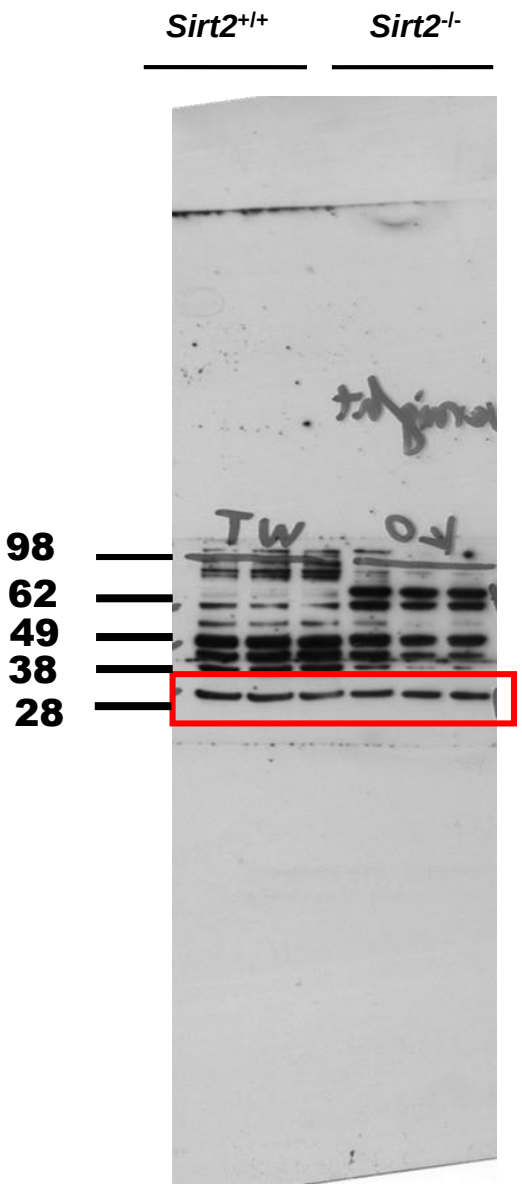
Original Western Blots

Full unedited gels for Figure 2B

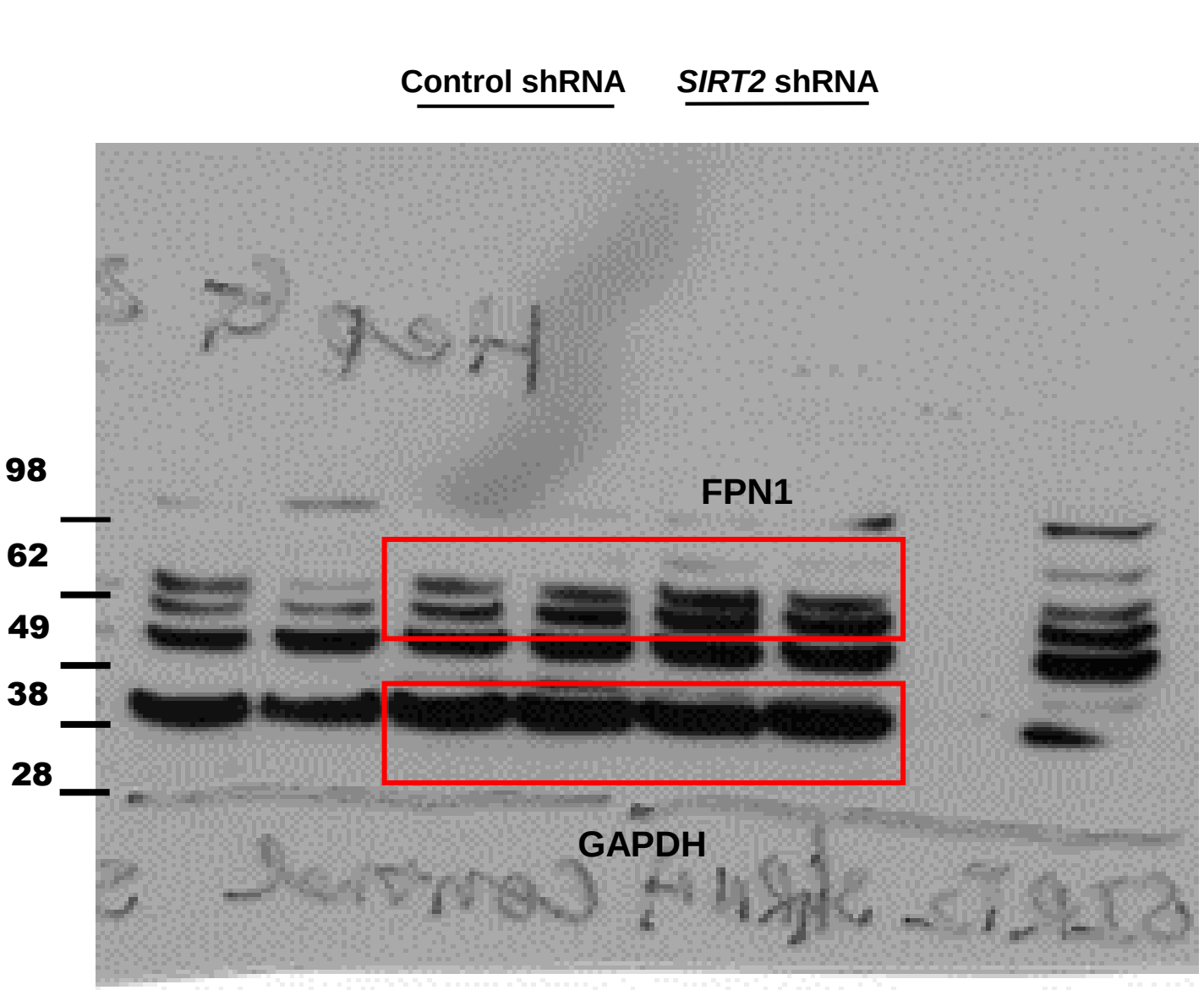
FPN1

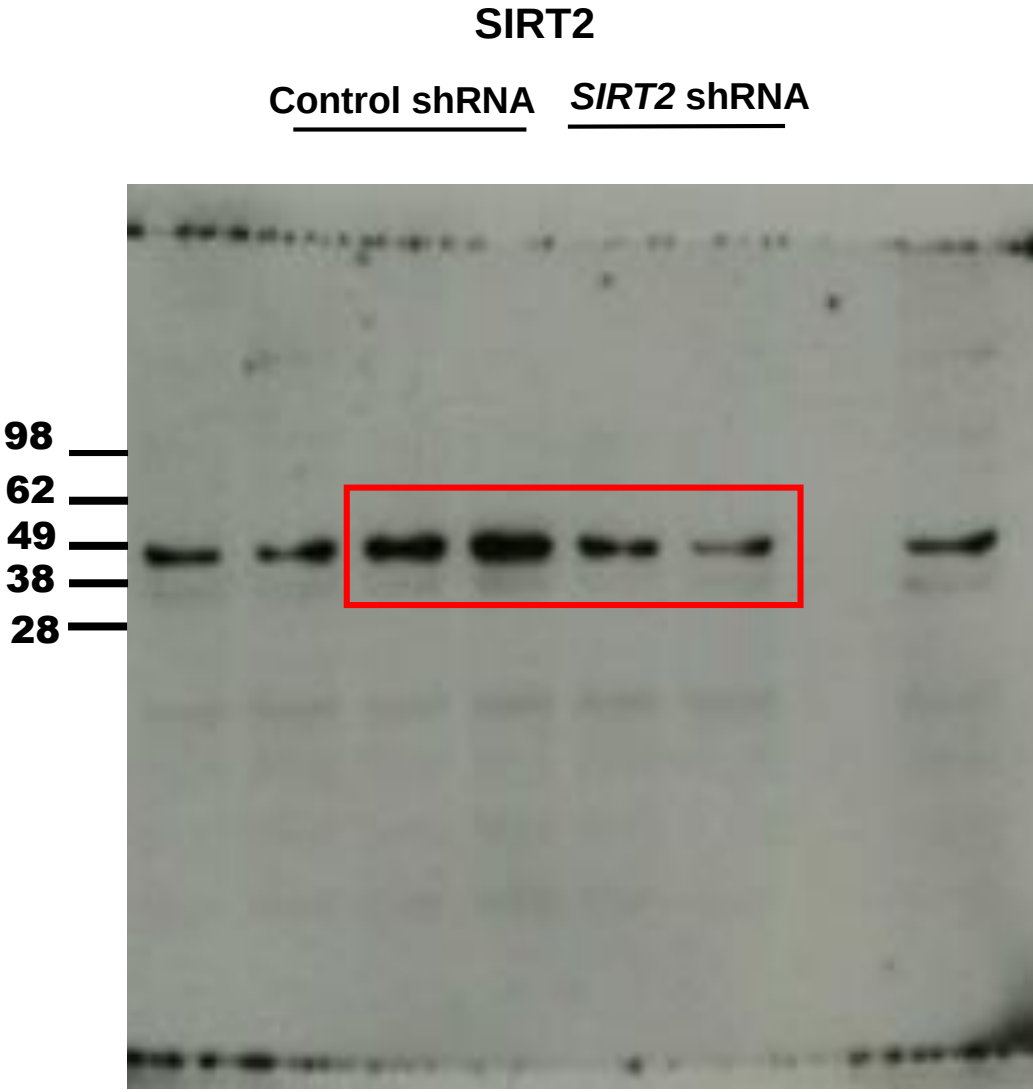


GAPDH



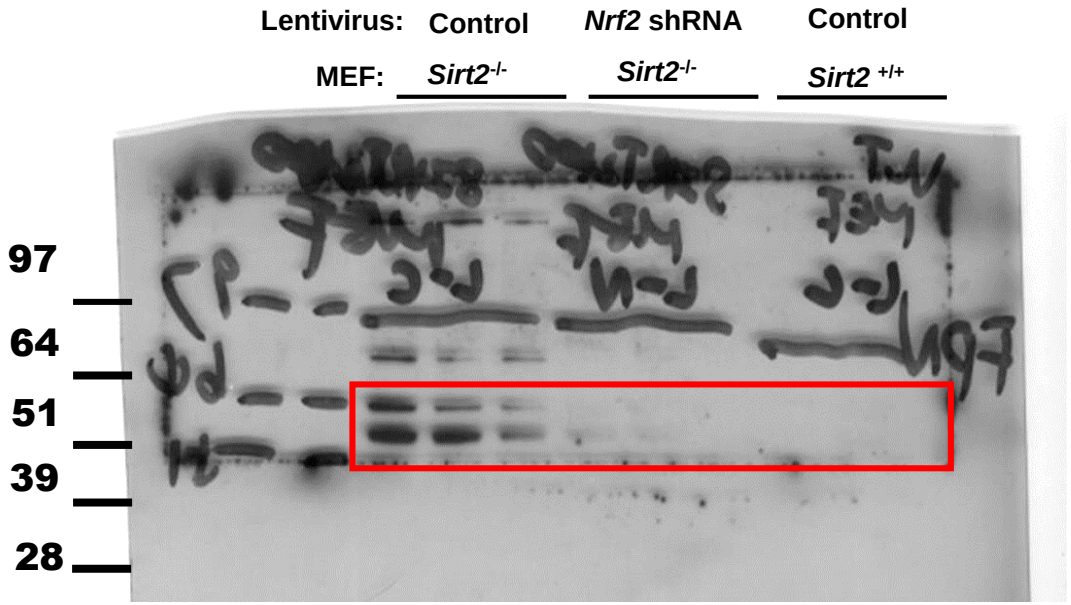
Full unedited gels for Figure 2C



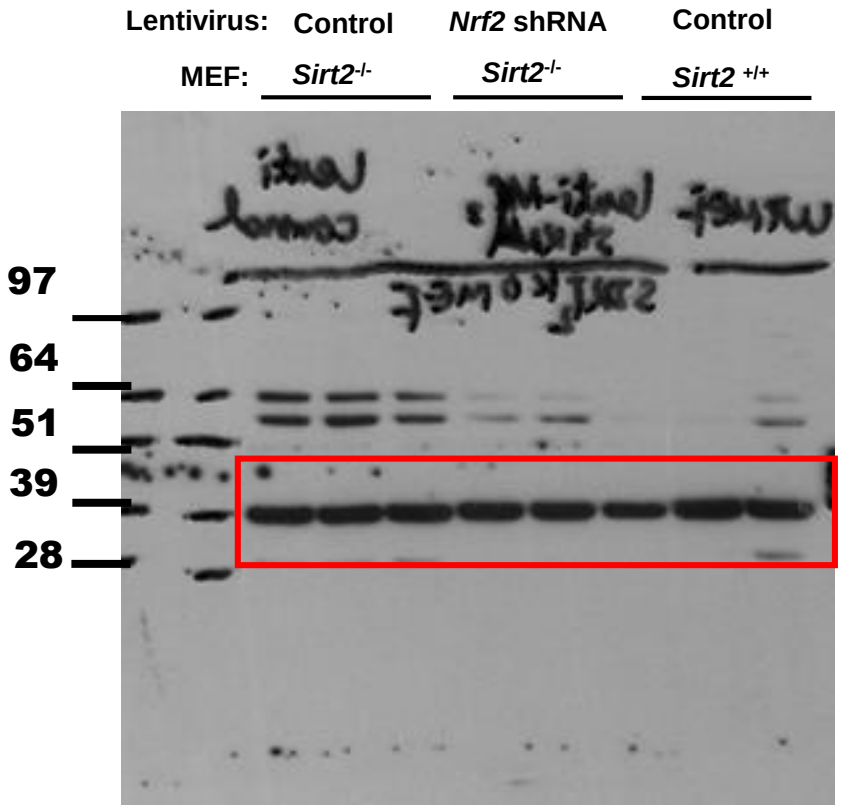


Full unedited gels for Figure 3B

FPN1



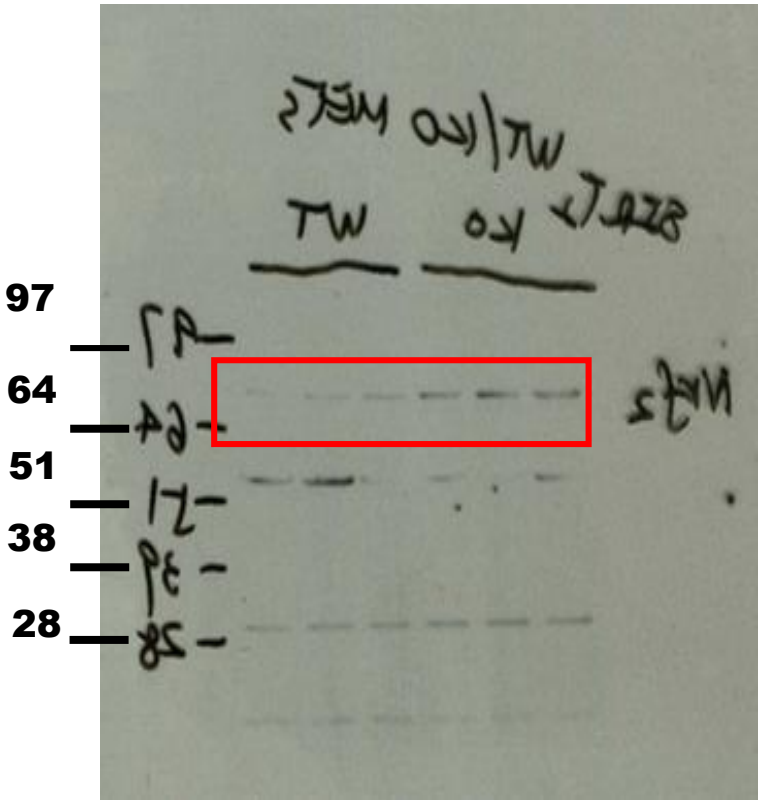
GAPDH



Full unedited gels for Figure 3C

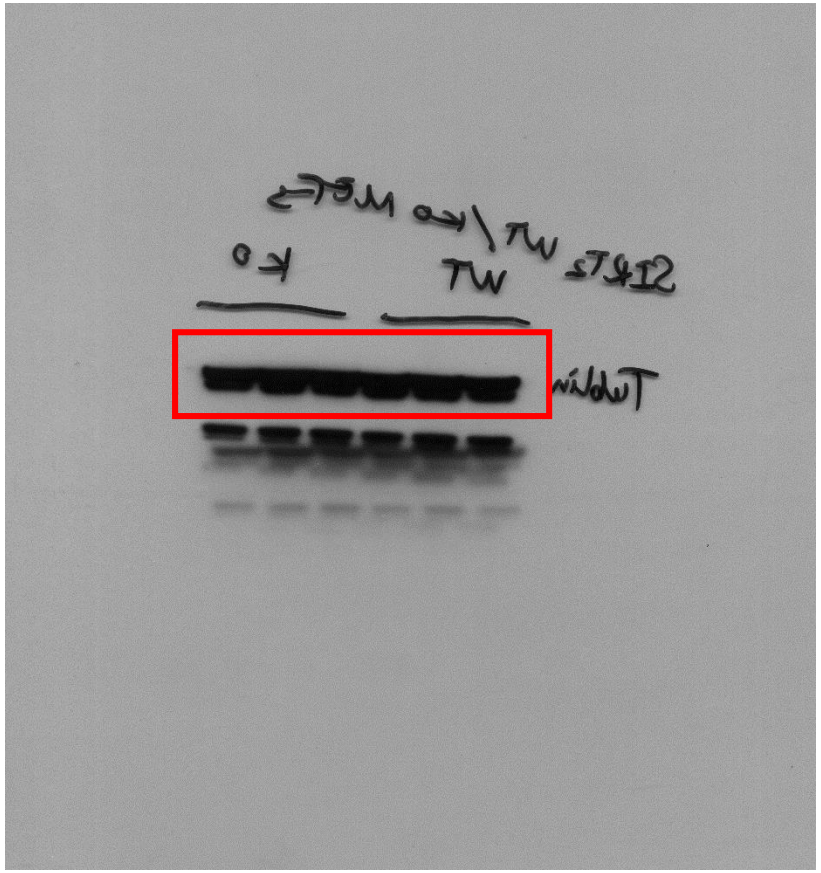
NRF2

Sirt2$^{+/+}$ *Sirt2*$^{-/-}$



Tubulin

Sirt2$^{+/+}$ *Sirt2*$^{-/-}$



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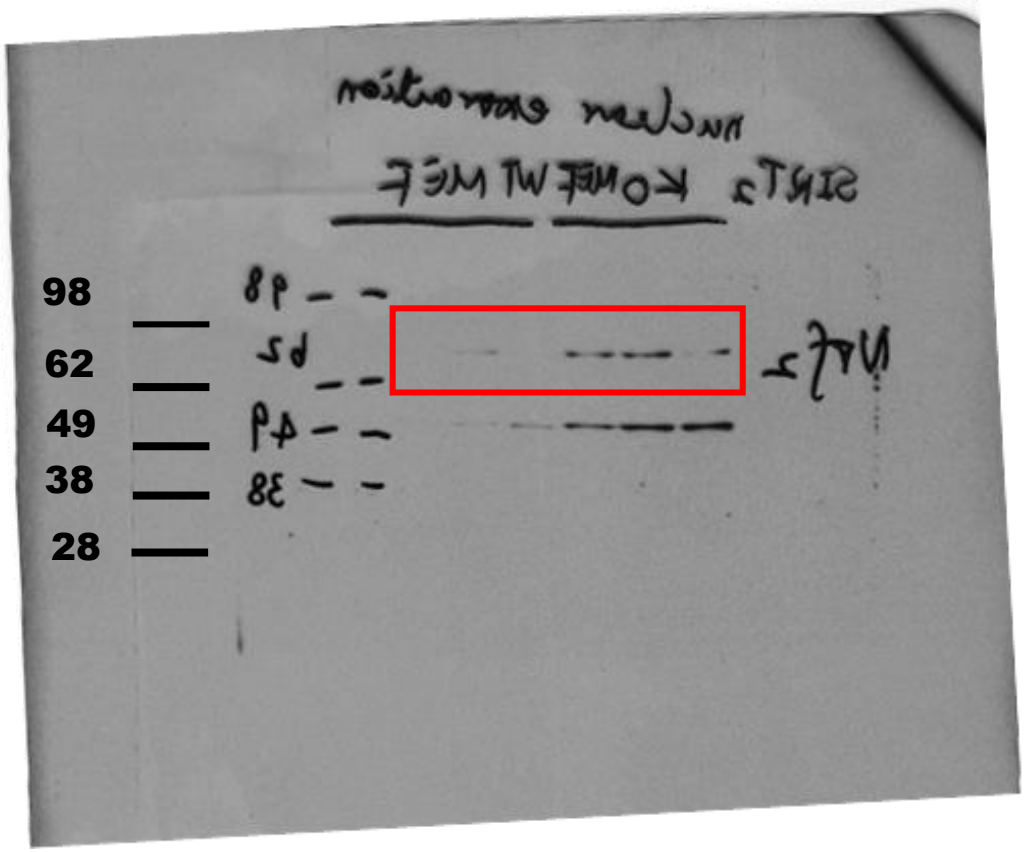
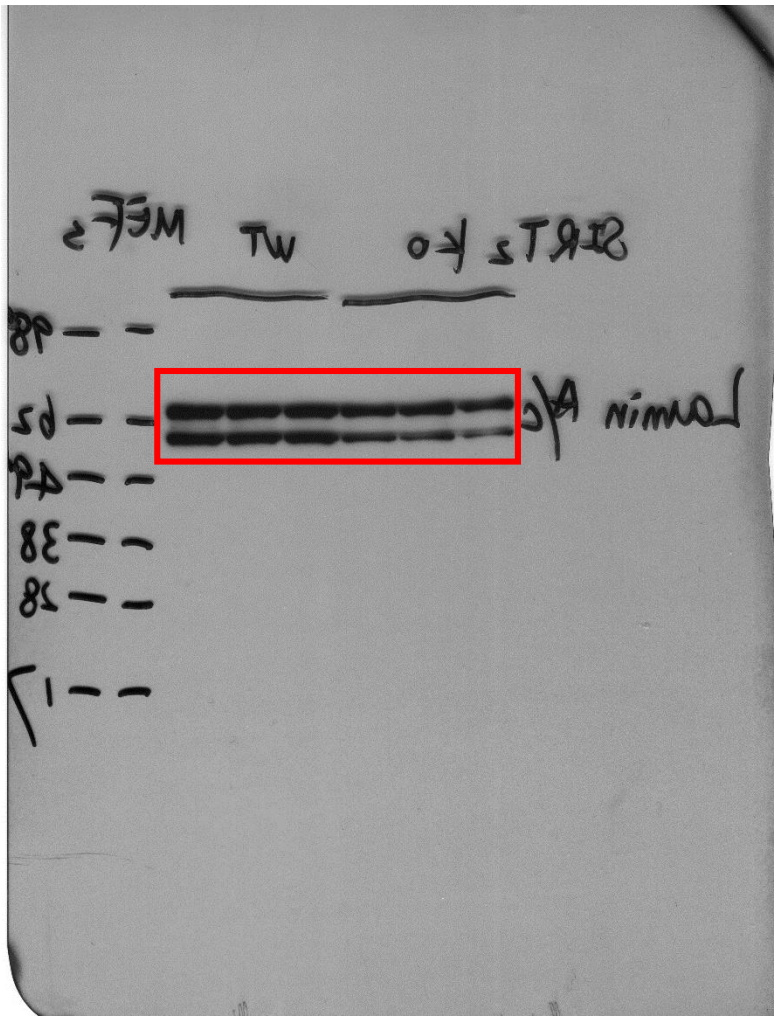
NRF2



Lamin A/C



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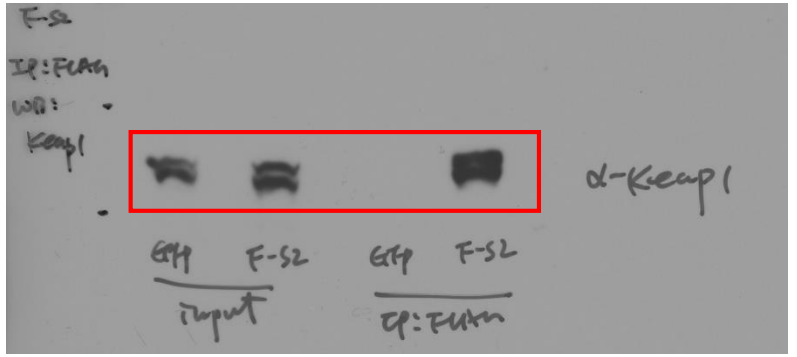
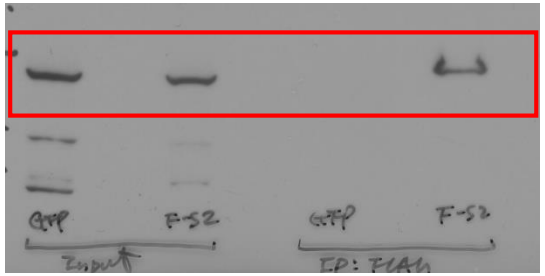
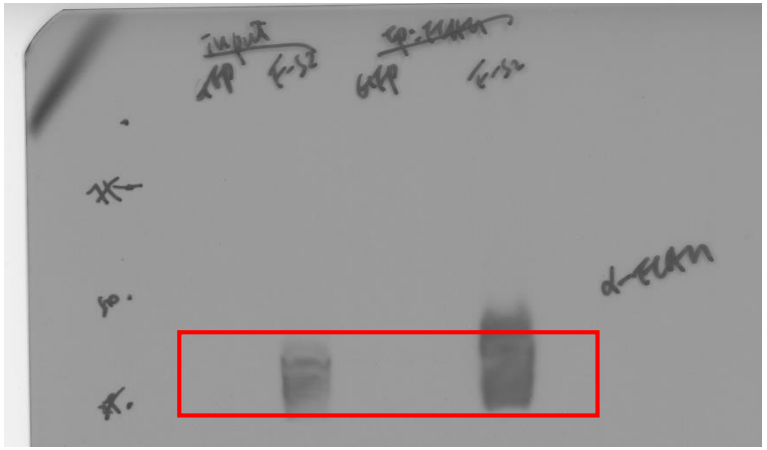
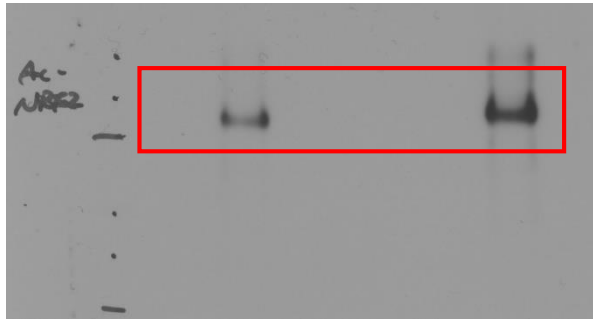
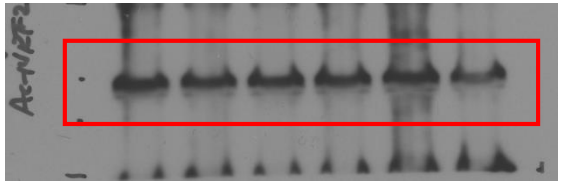




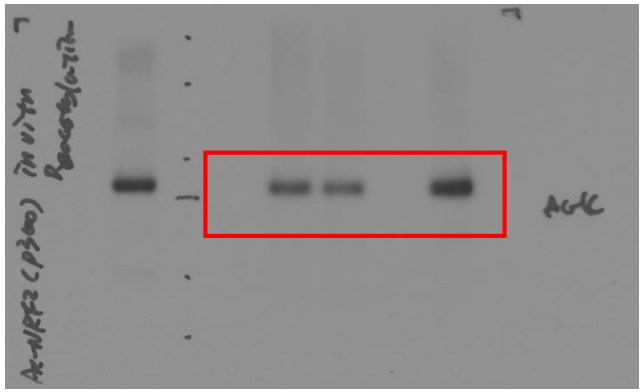


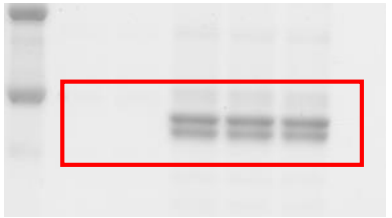
Full unedited gels for Figure 4B



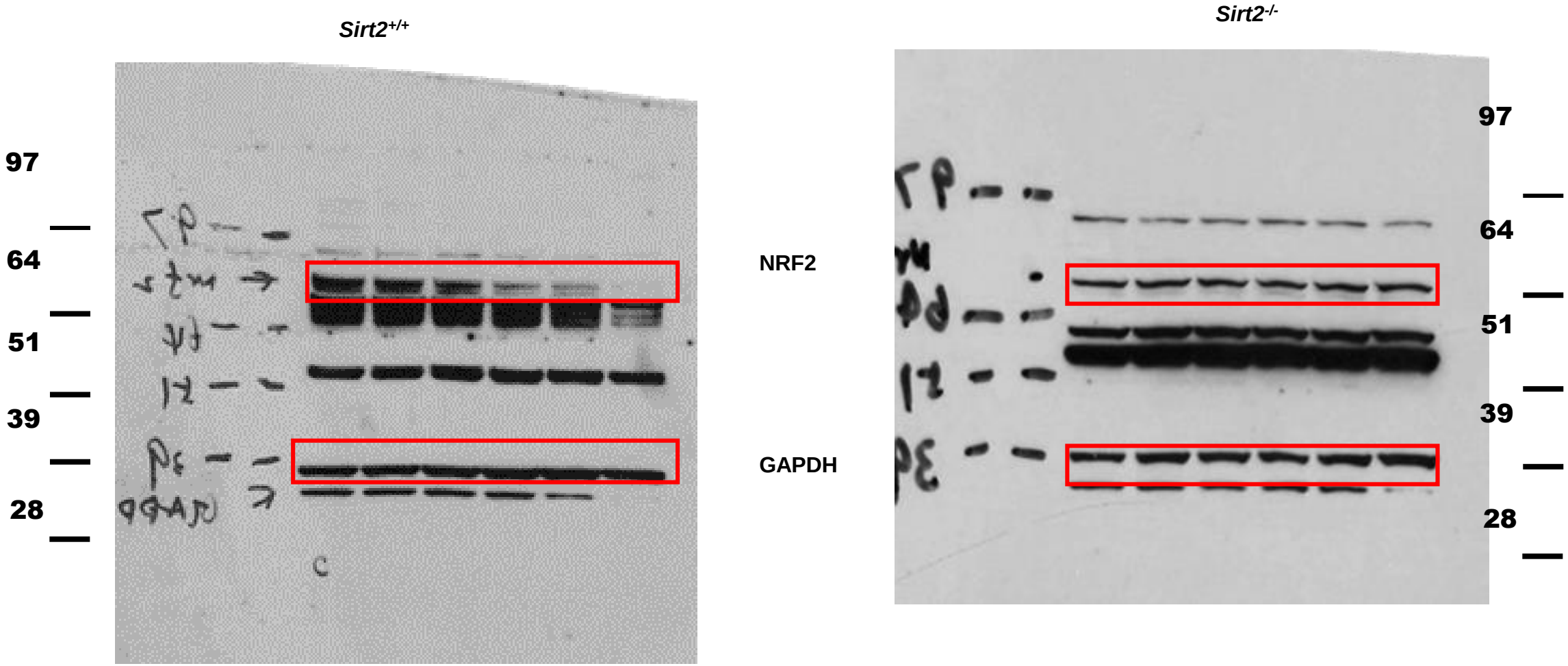


Full unedited gels for Figure 4C

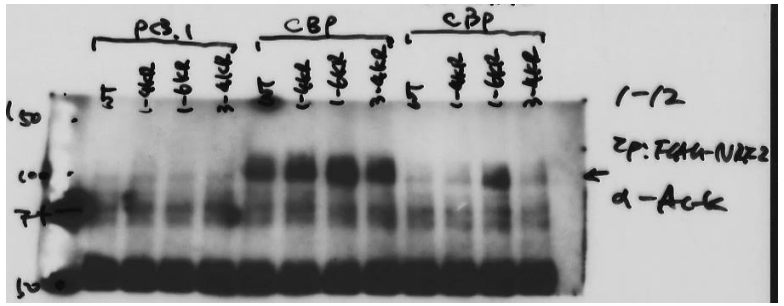


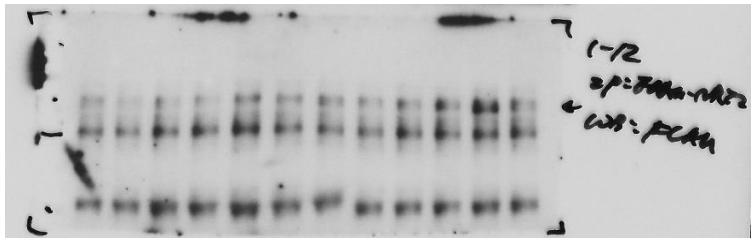


Full unedited gels for Figure 4D



Full unedited gels for Figure 5A





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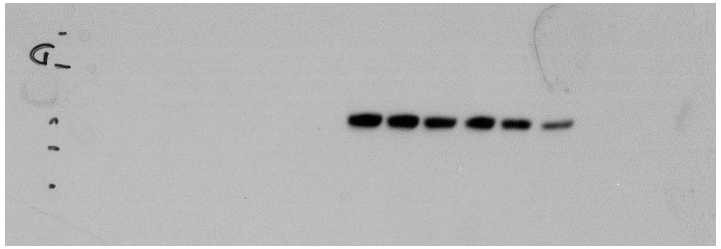
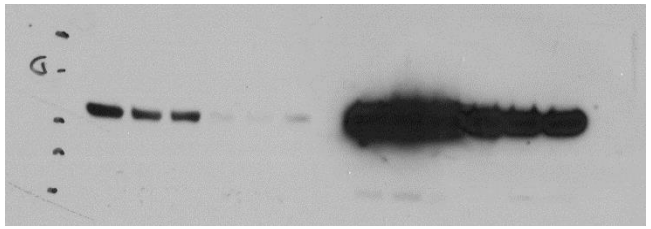
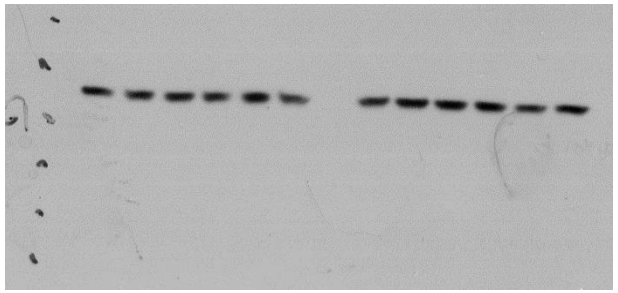
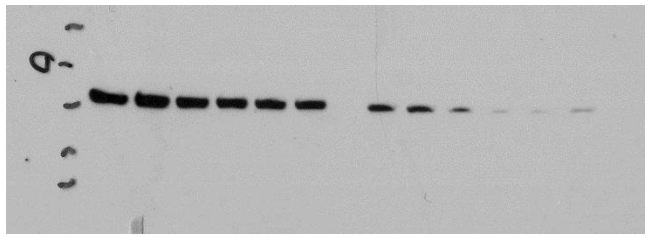
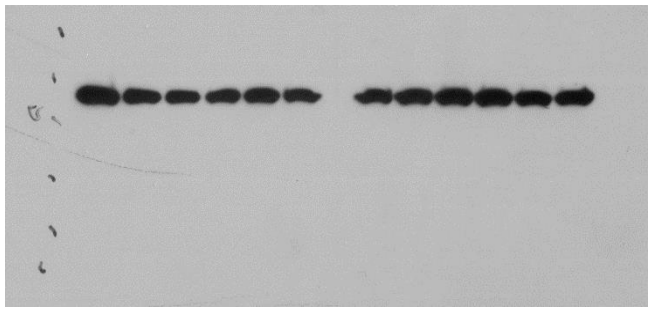


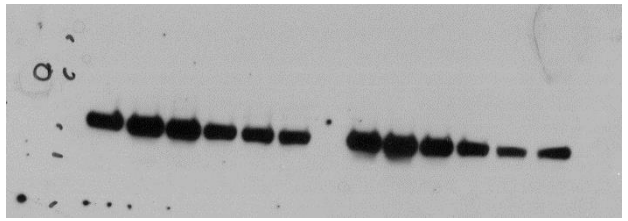


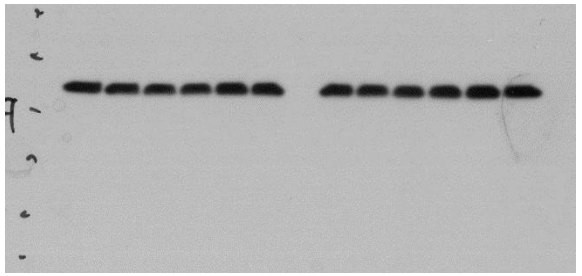


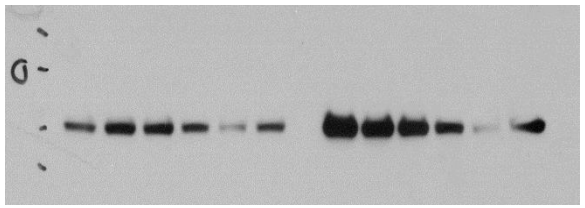


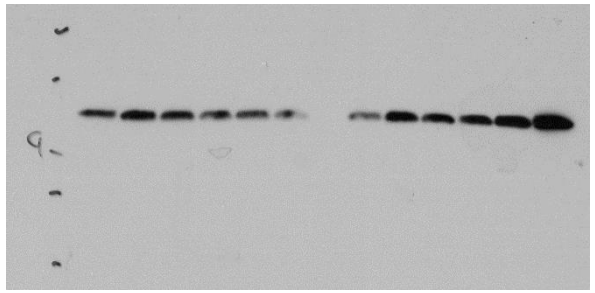


Full unedited gels for Figure 5E



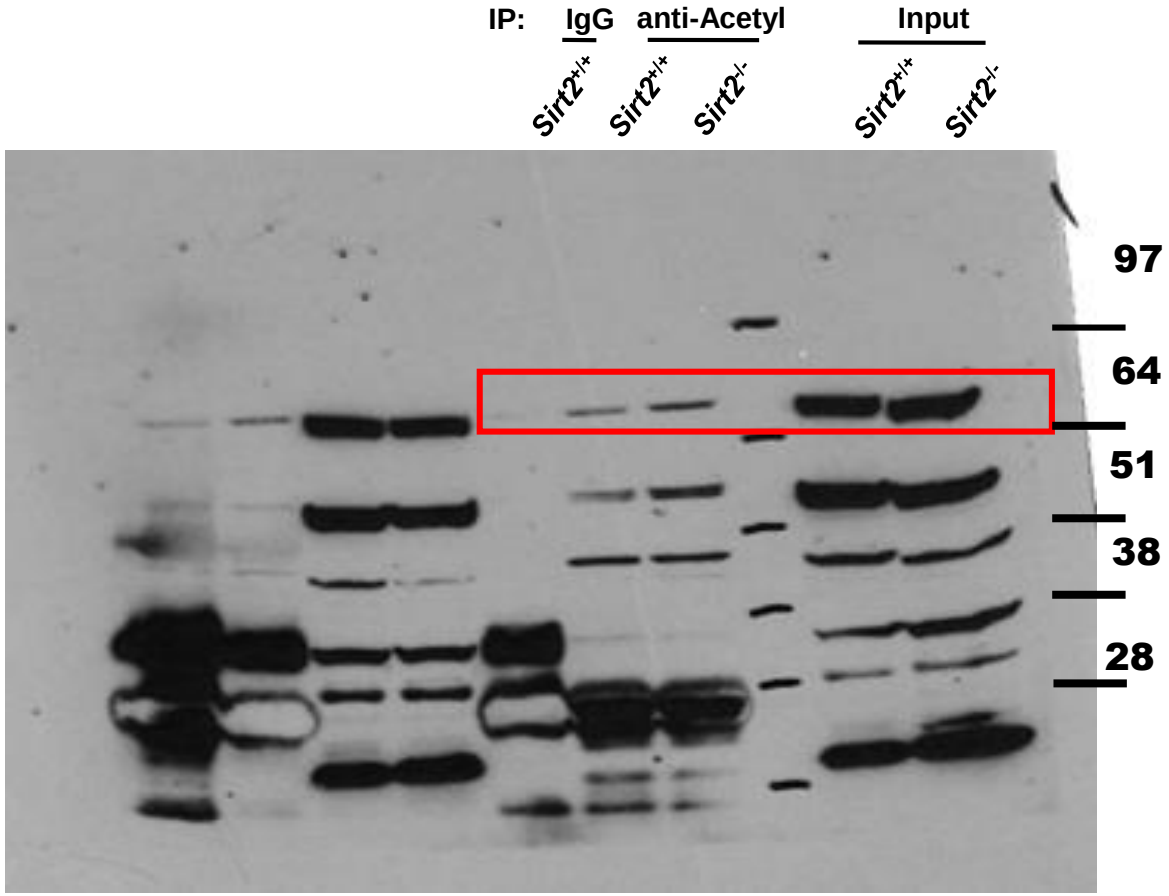






Full unedited gels for Figure 6A

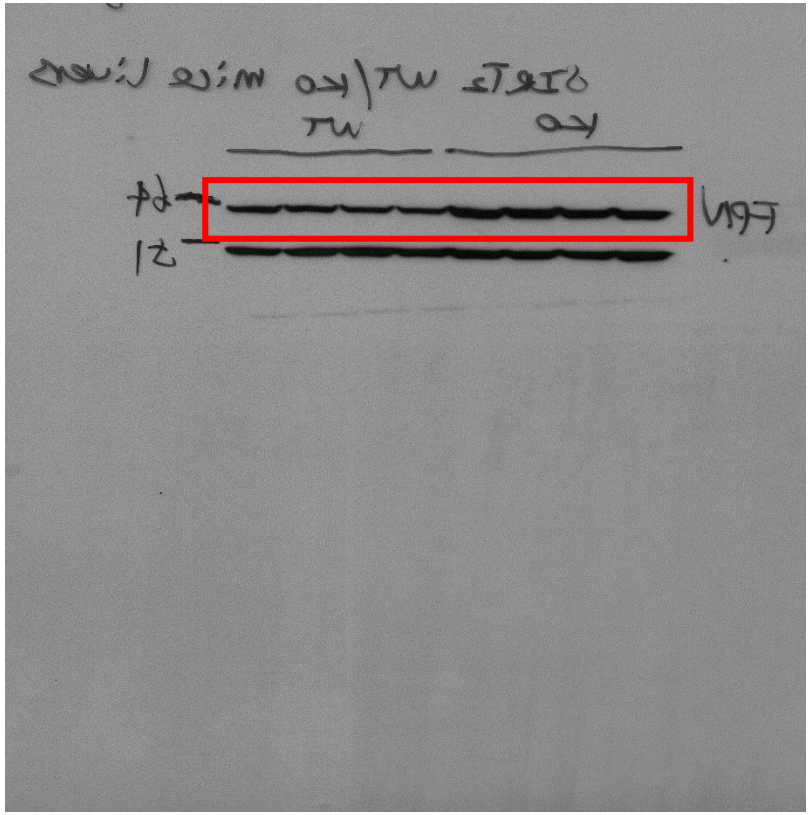
NRF2



Full unedited gels for Figure 6D

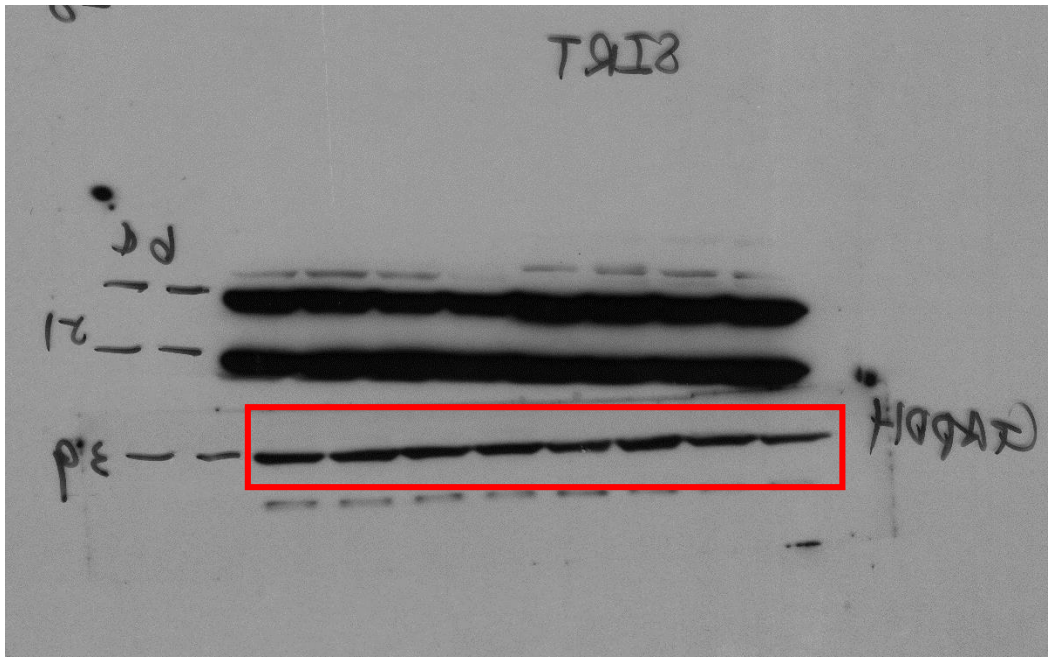
FPN1

Sirt2$^{+/+}$ *Sirt2*$^{-/-}$



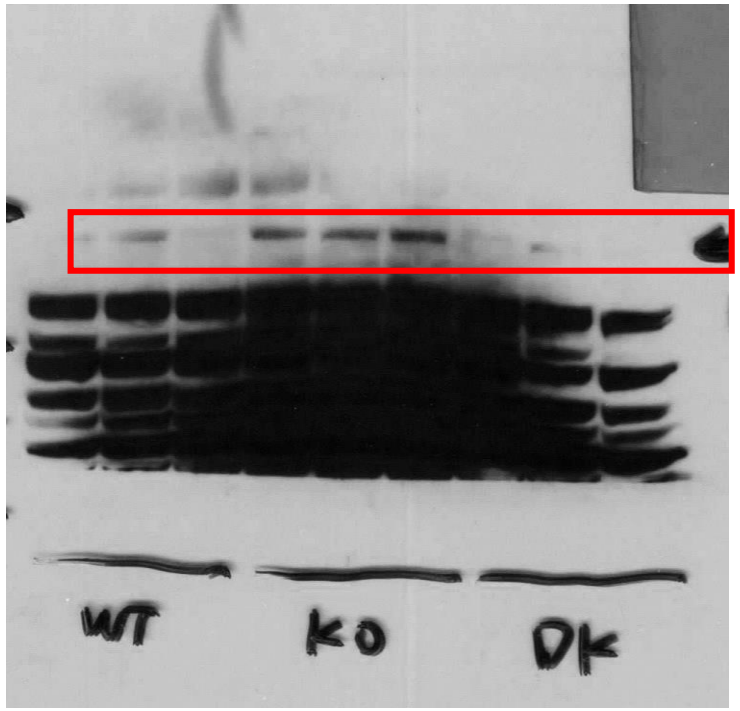
GAPDH

Sirt2$^{+/+}$ *Sirt2*$^{-/-}$

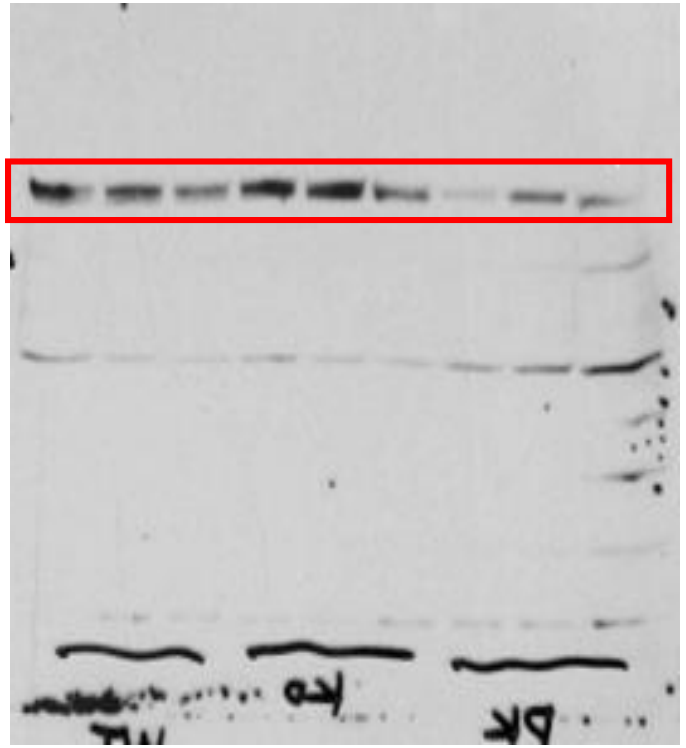


Full unedited gels for Figure 7B

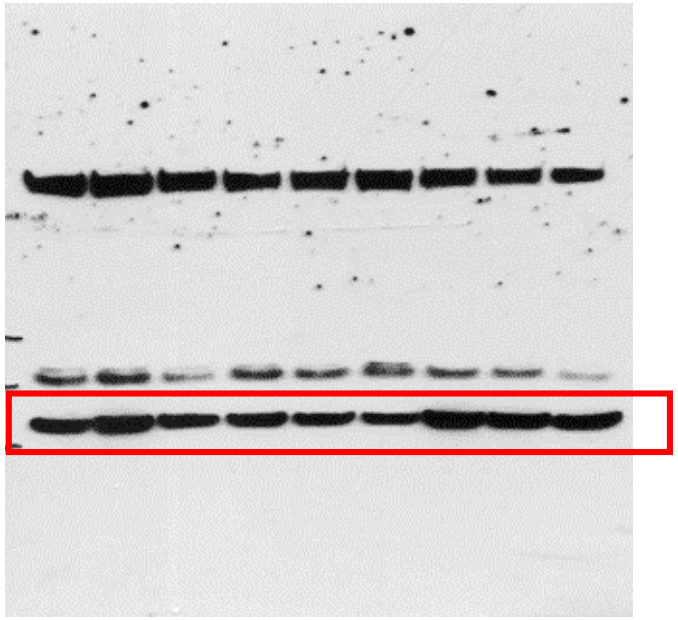
NRF2



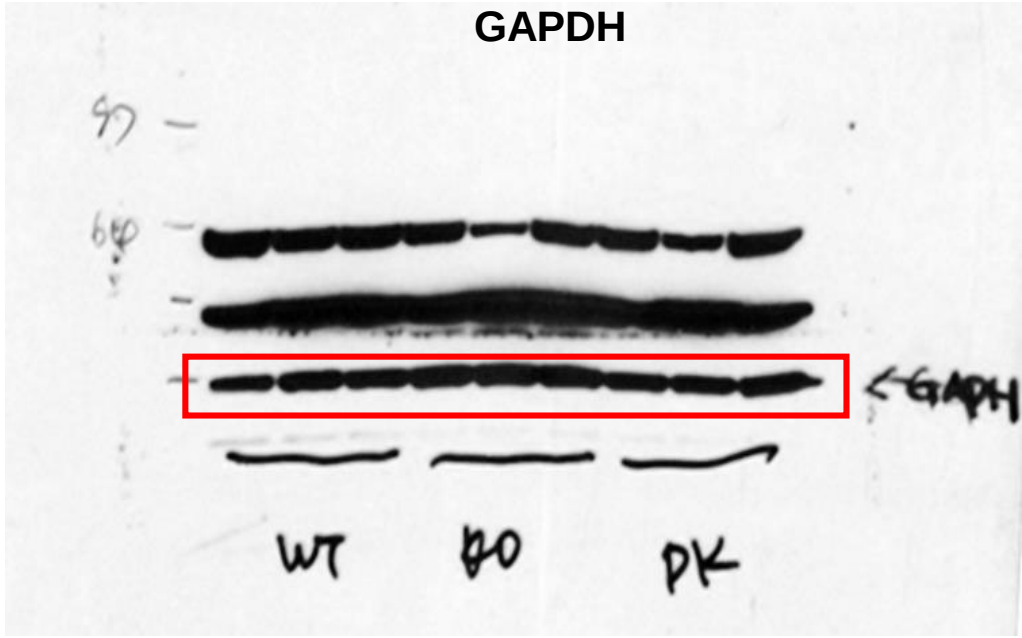
FPN1



FTL

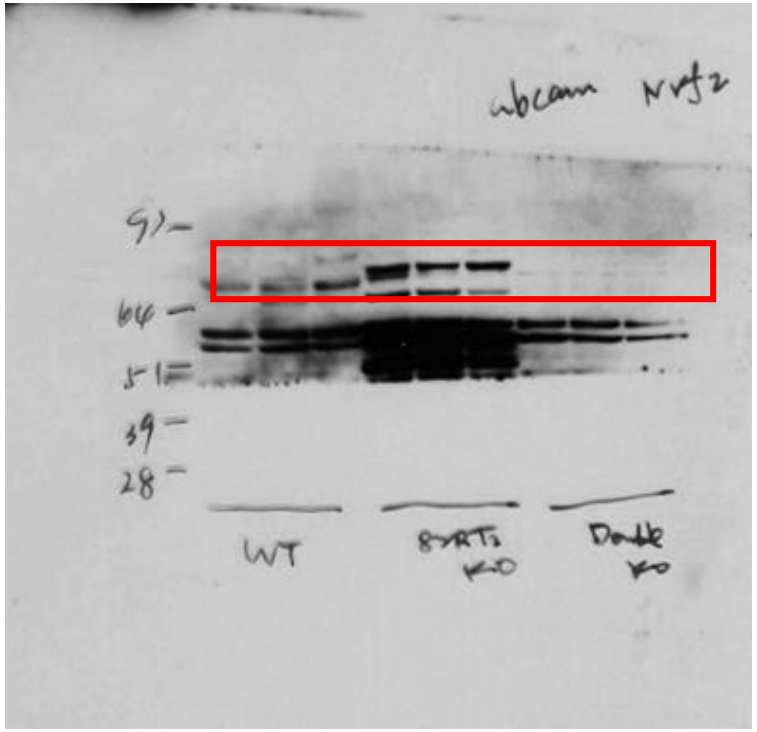


GAPDH

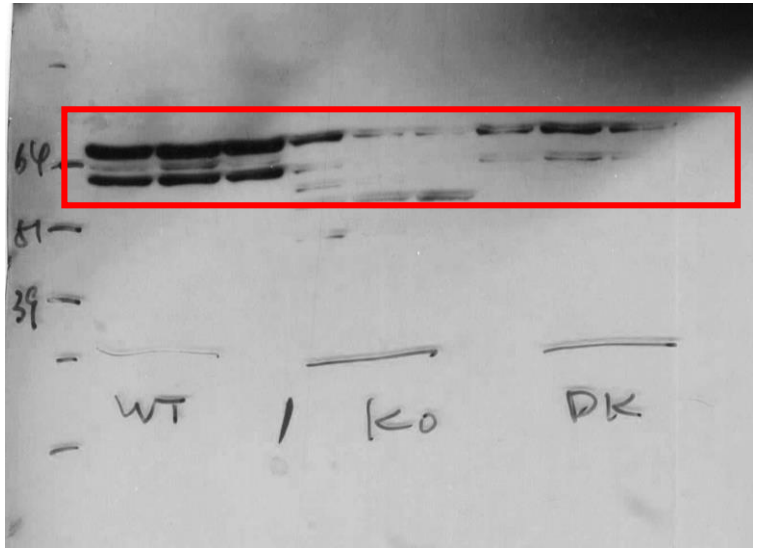


Full unedited gels for Figure 7C

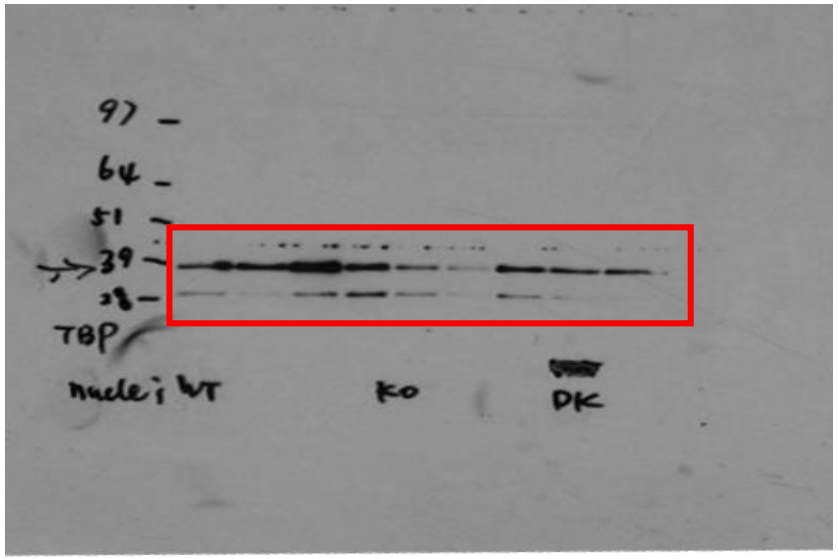
NRF2



Lamin A/C

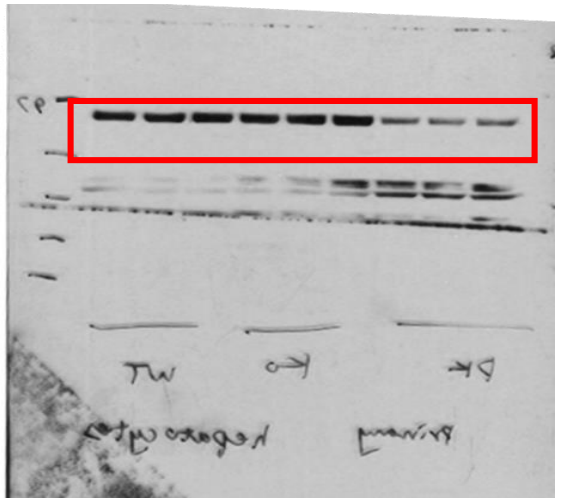


TBP

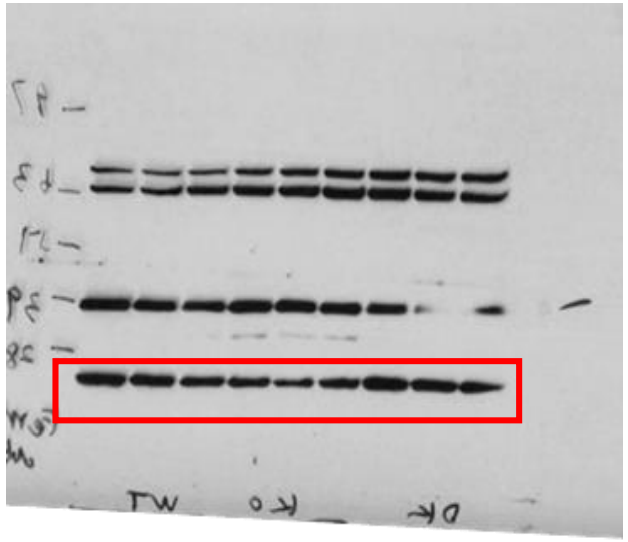


Full unedited gels for Figure 7D

TFRC1



FTL



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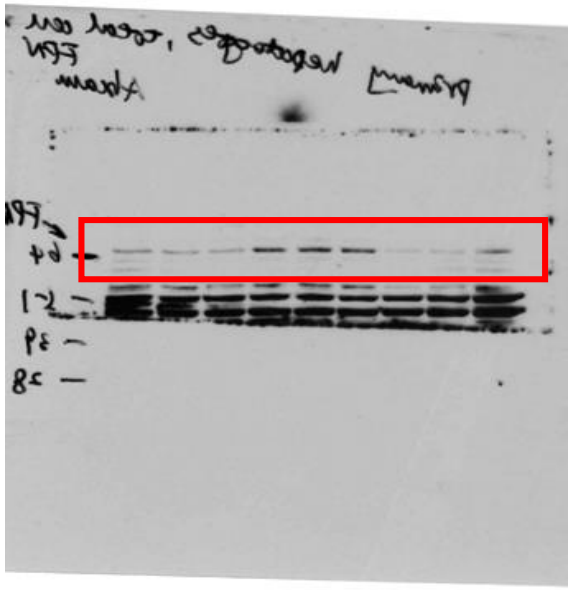
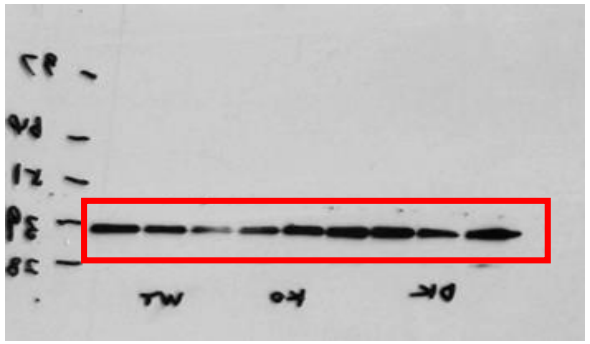
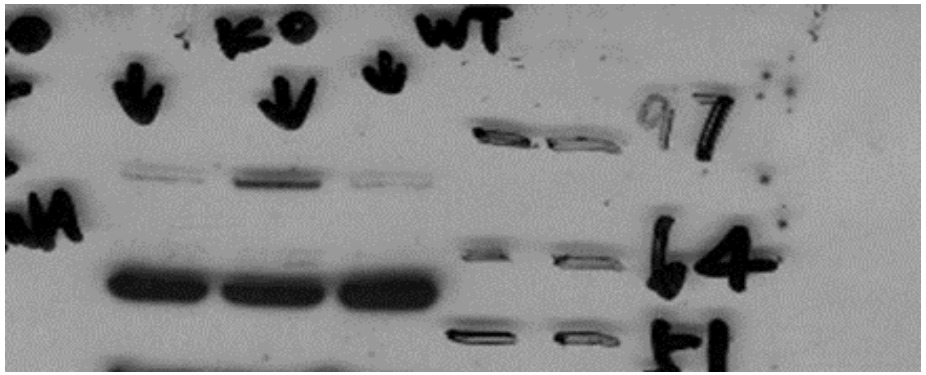
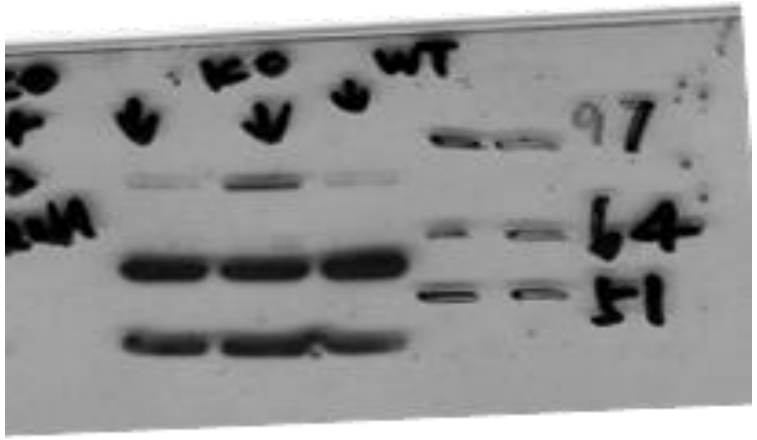


GAPDH

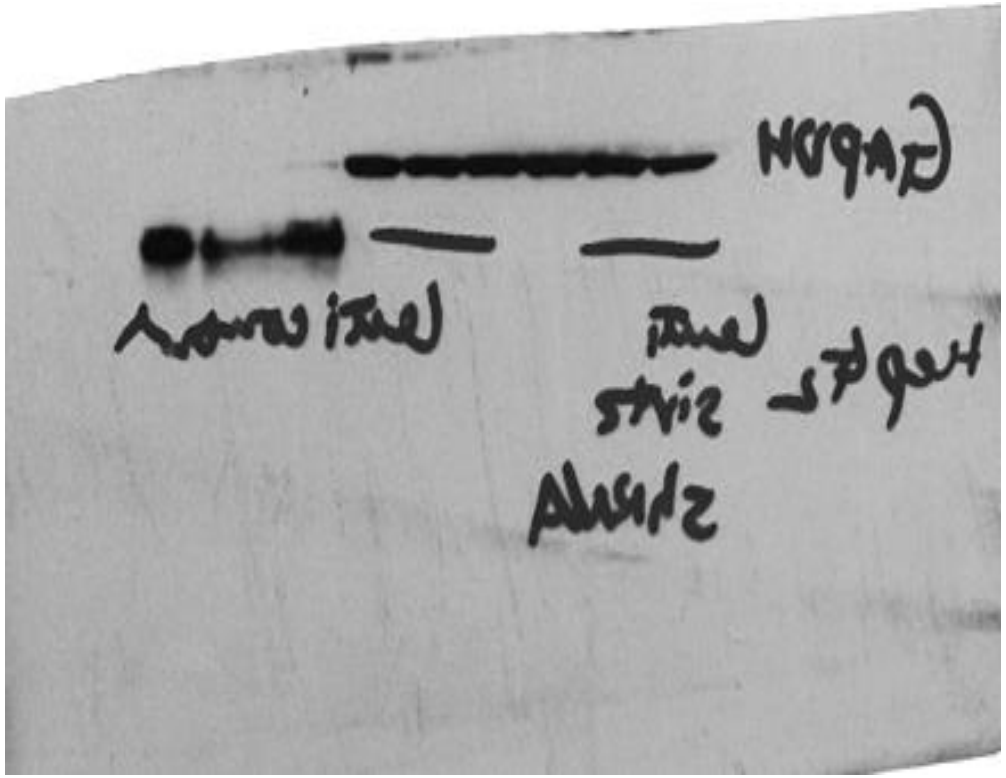


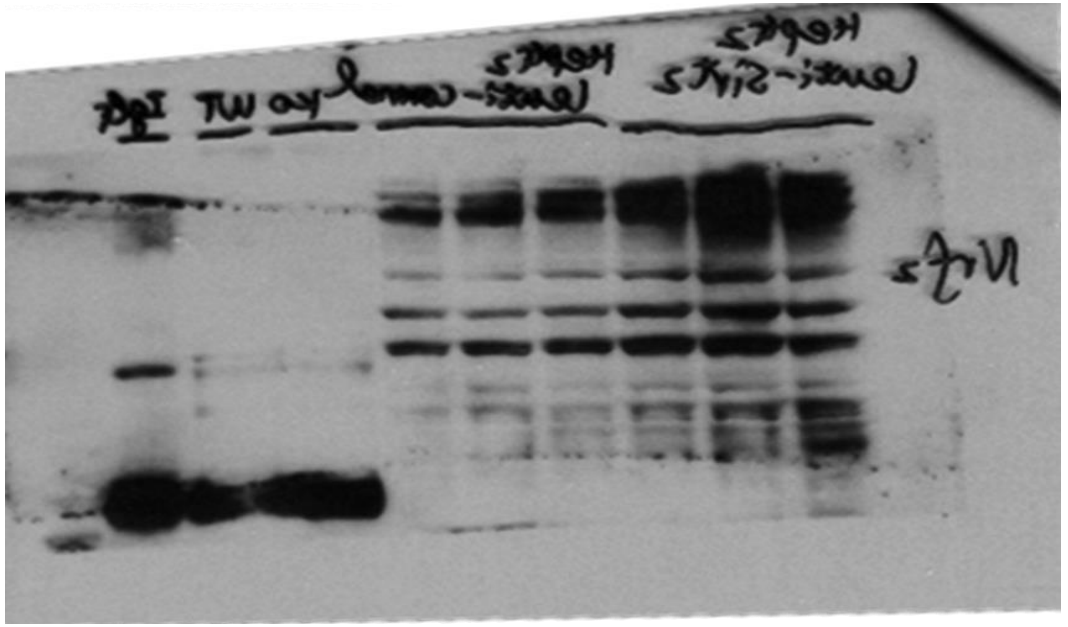
Full unedited gels for Supplemental Figure 3A





Full unedited gels for Supplemental Figure 3B





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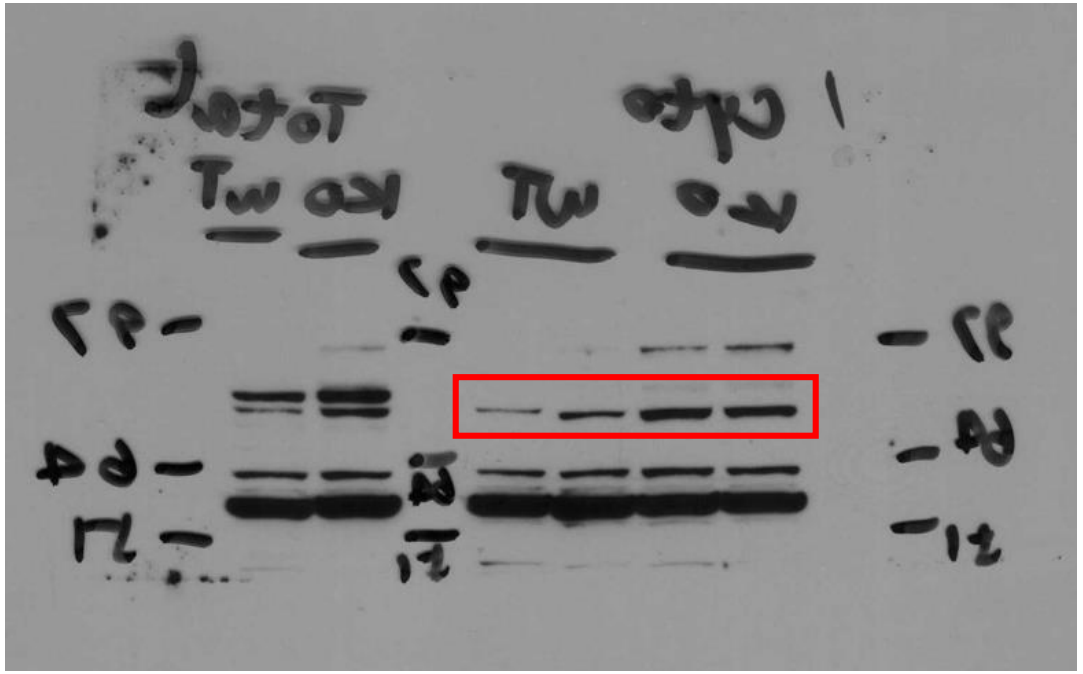

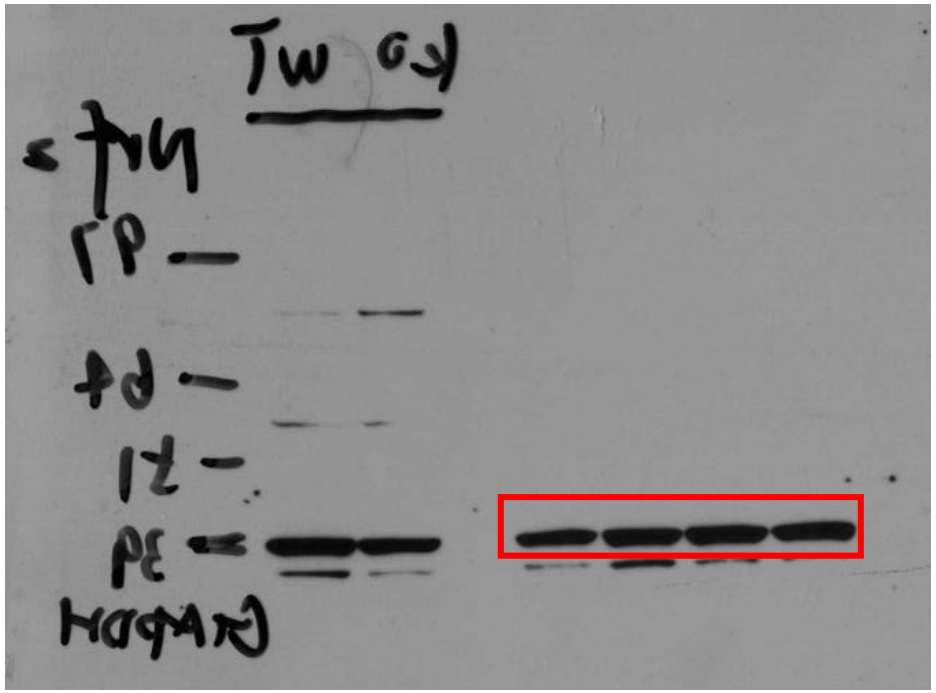

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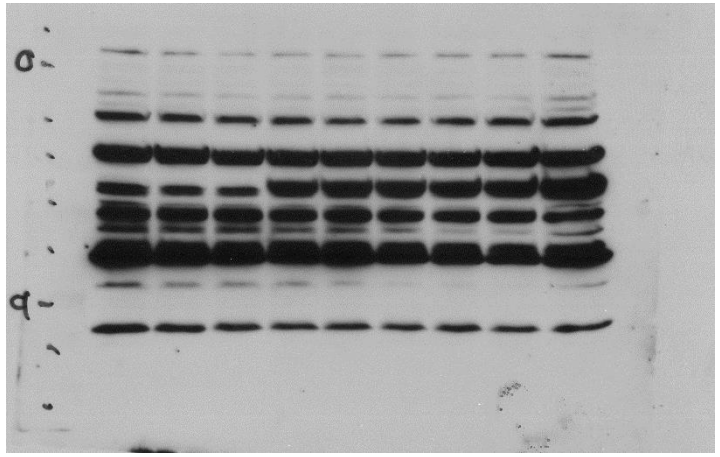

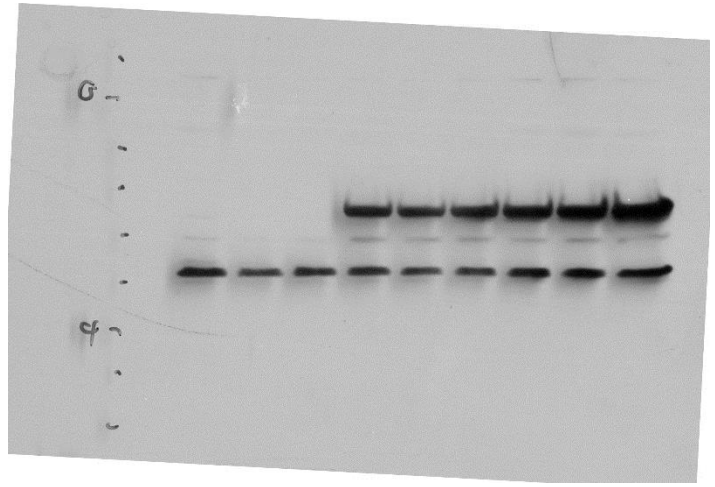

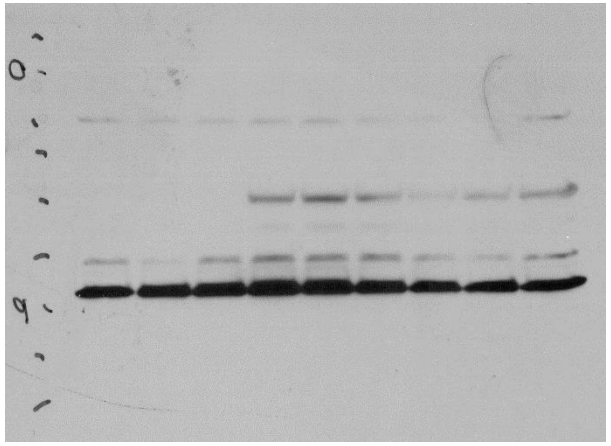

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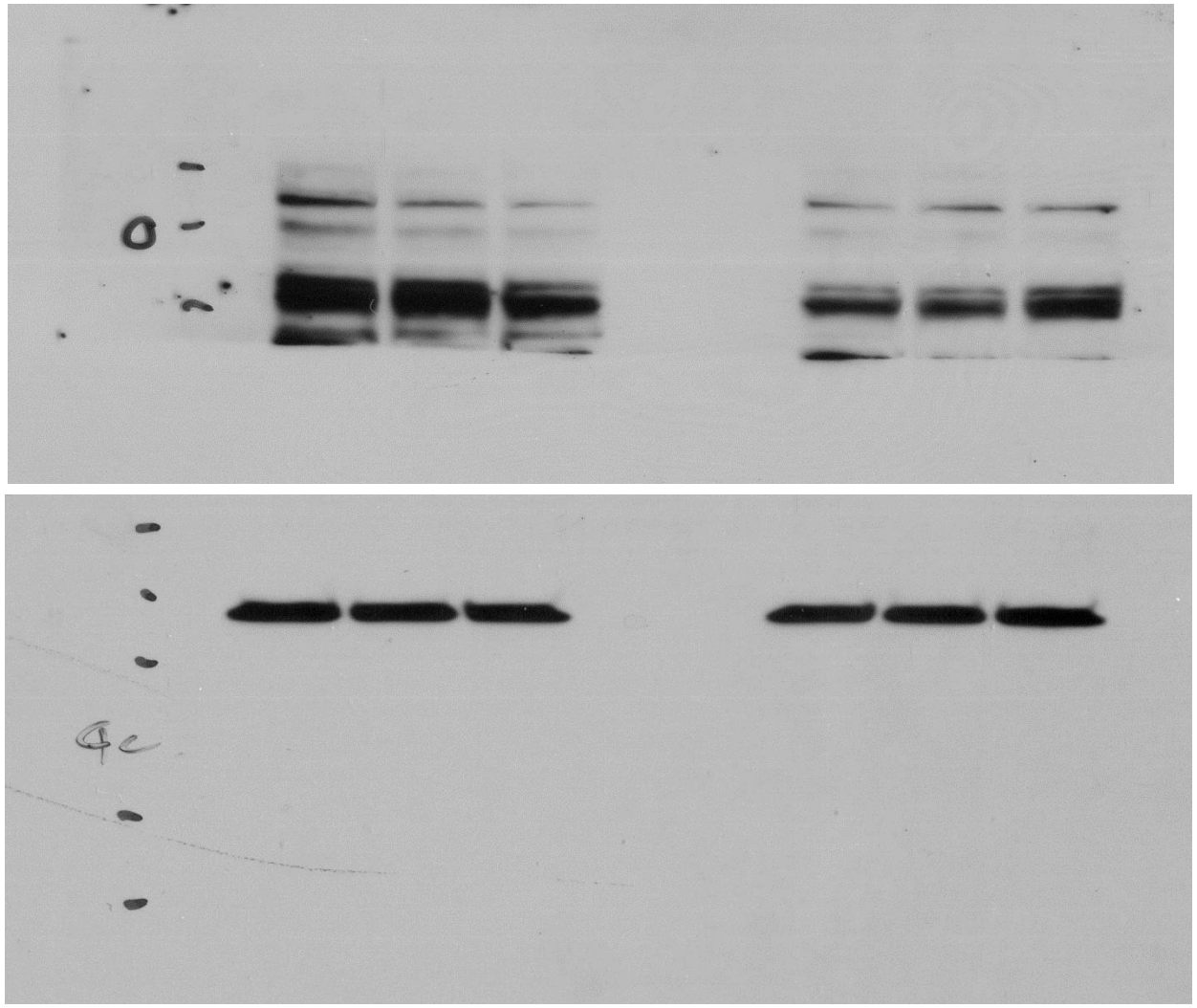

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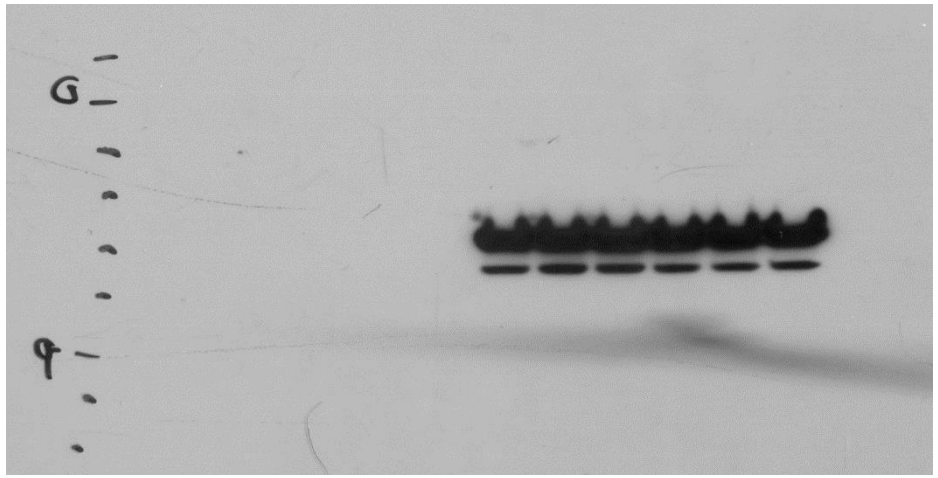
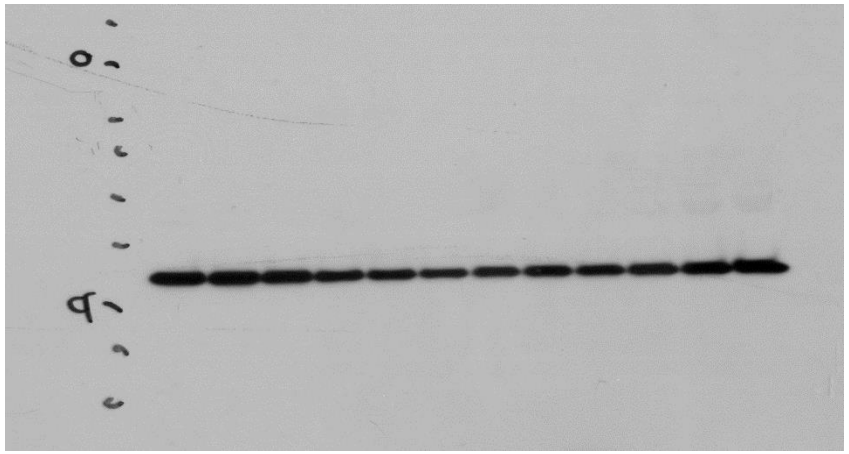
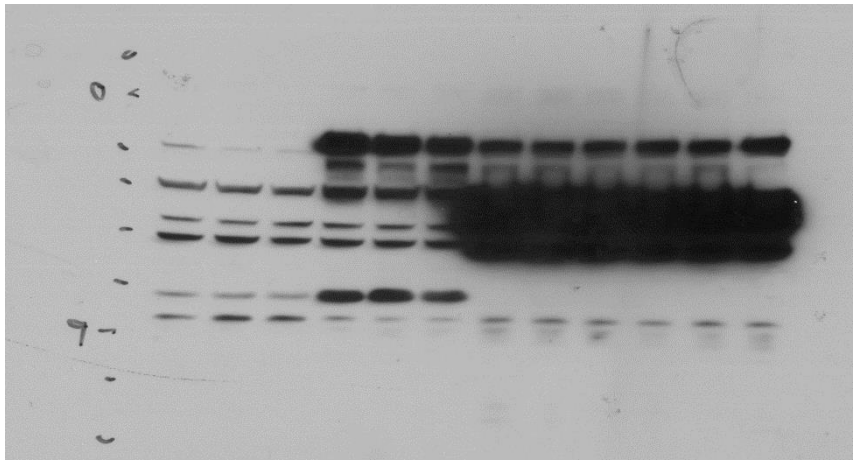
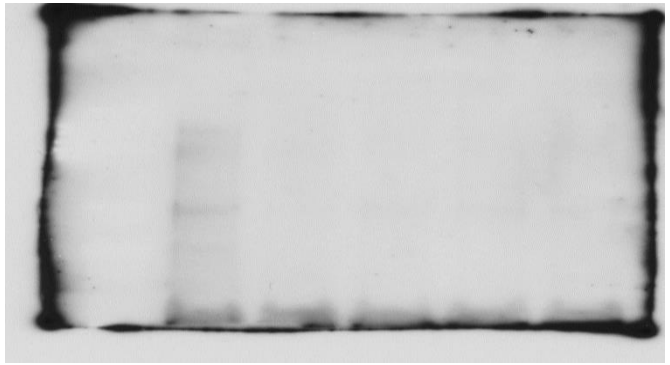
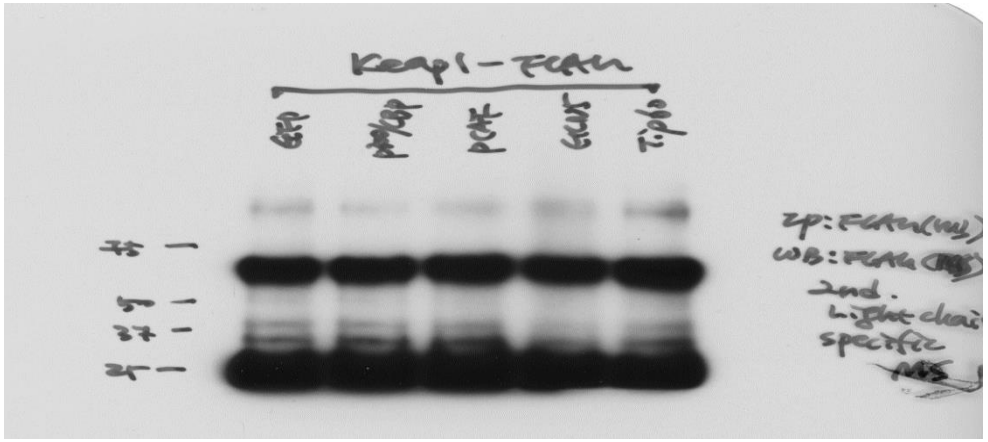






Full unedited gels for Supplemental Figure 6A





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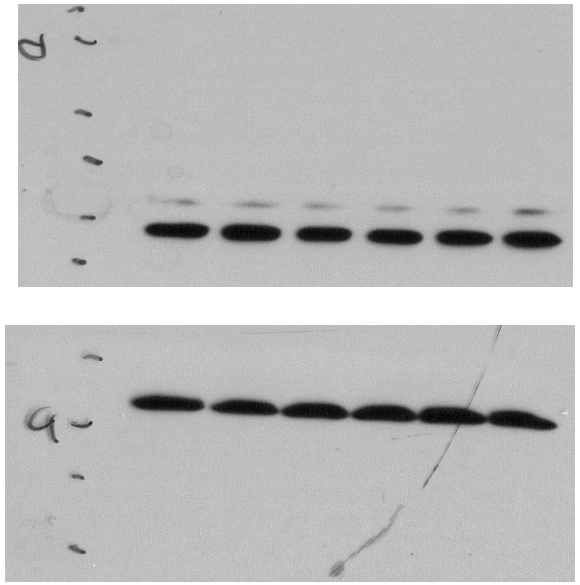
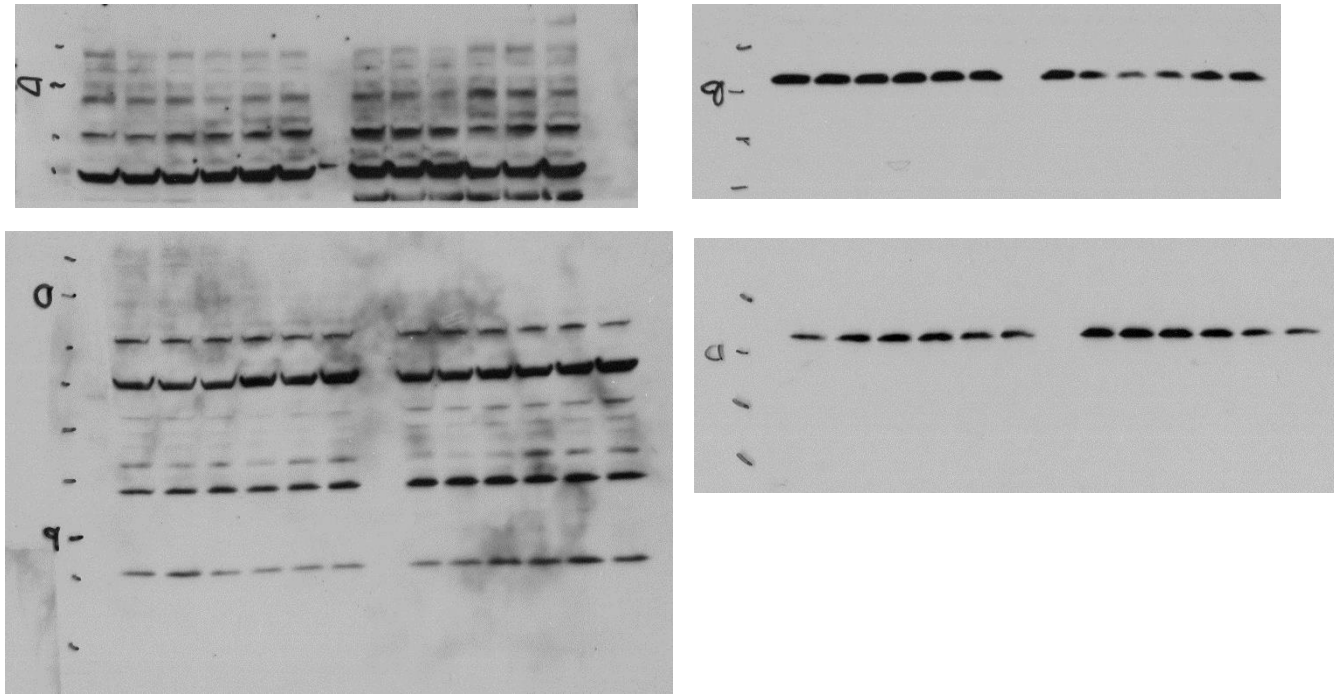
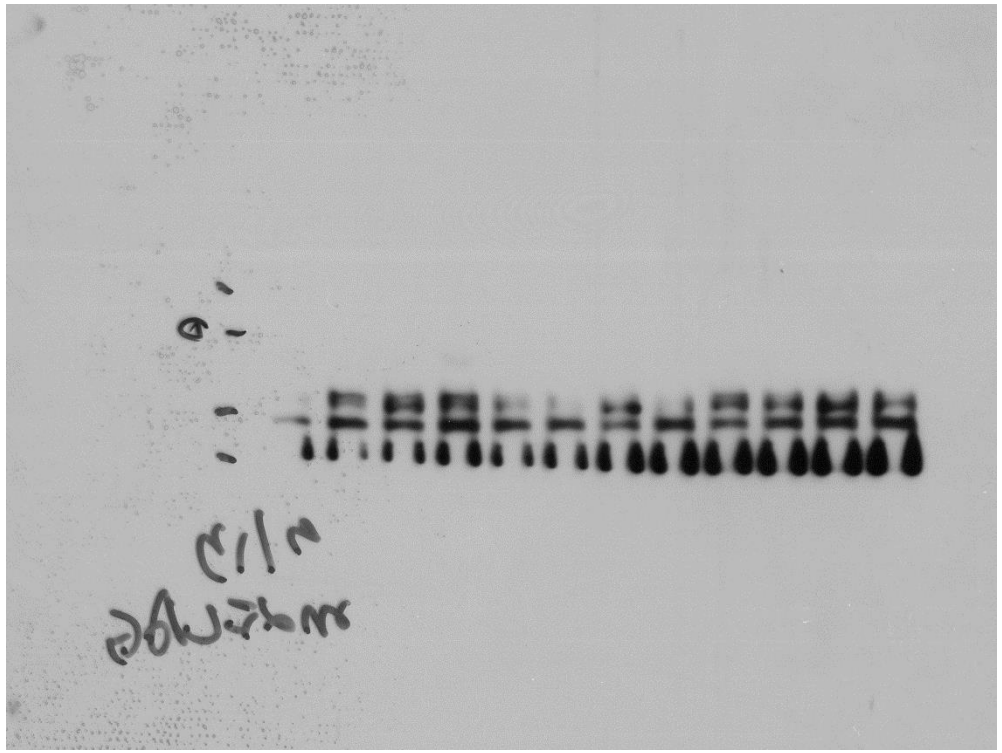
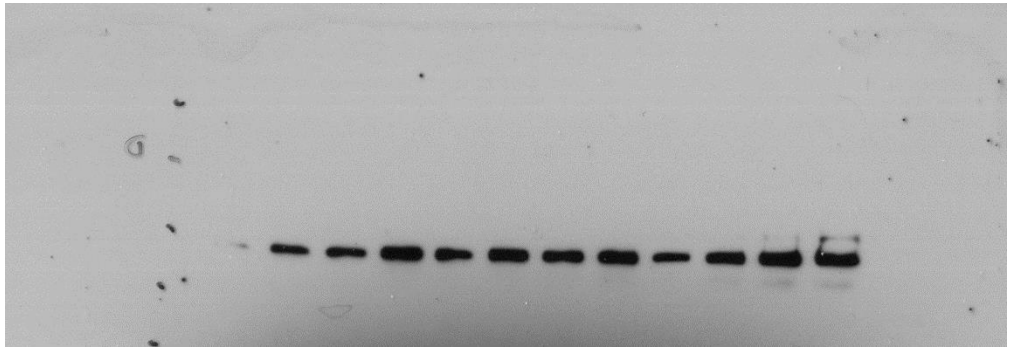


Full unedited gels for Supplemental Figure 6C

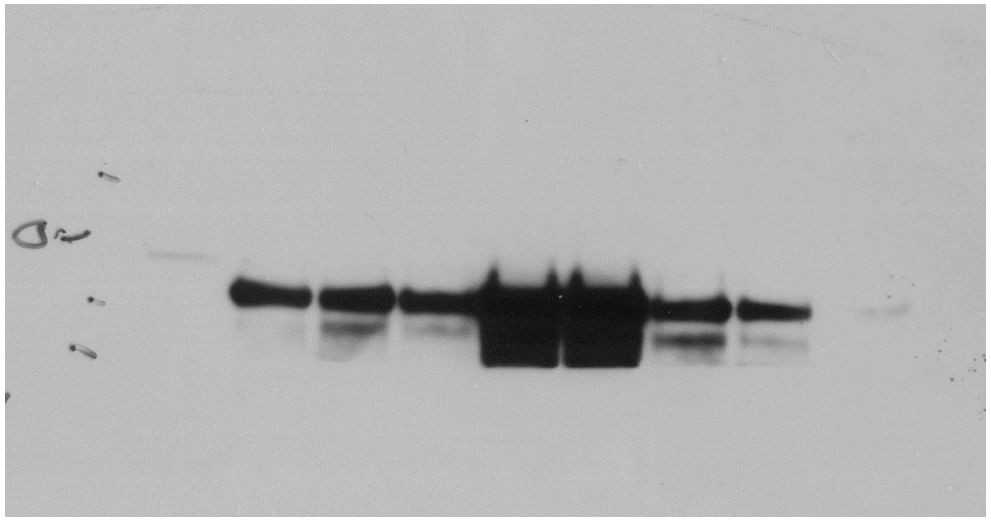


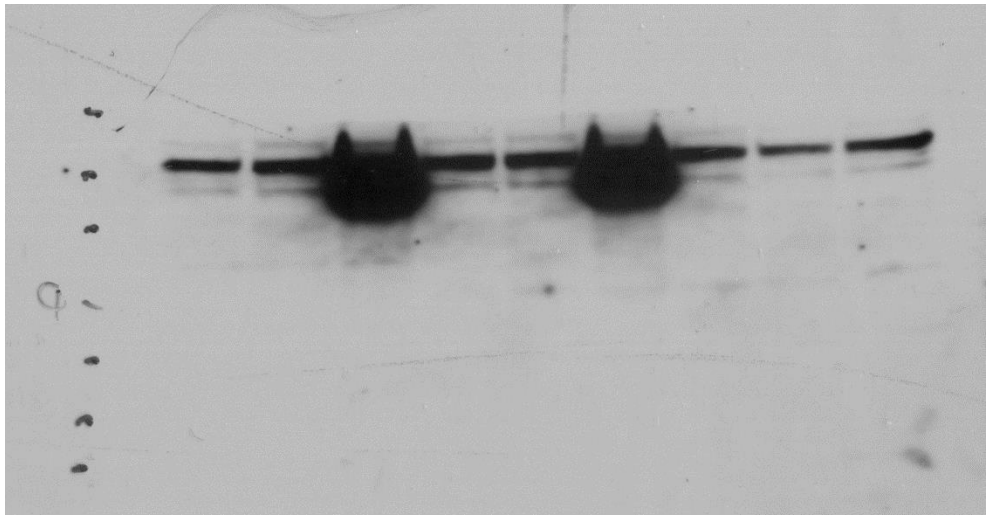
Full unedited gels for Supplemental Figure 7

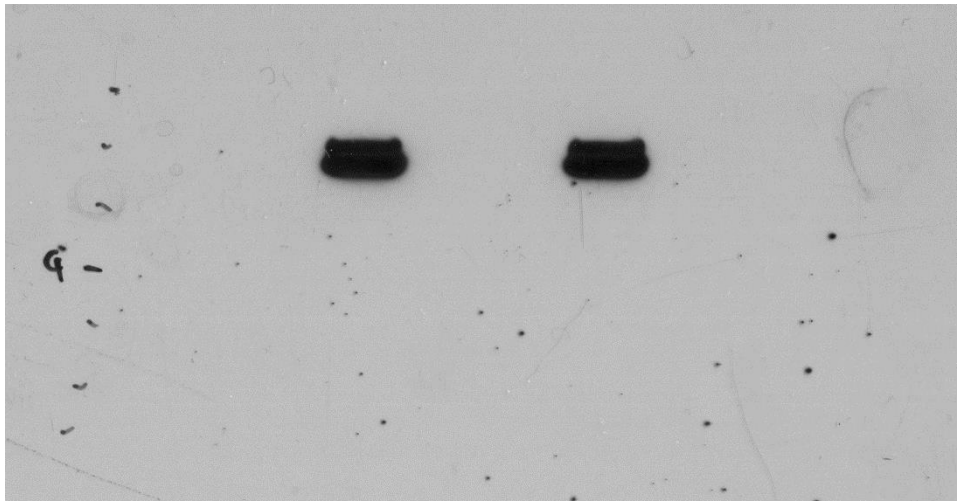


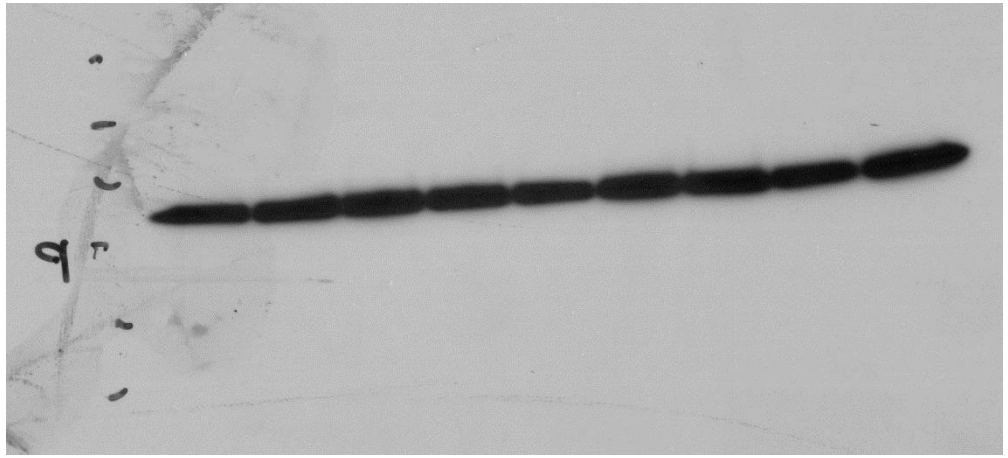


Full unedited gels for Supplemental Figure 8

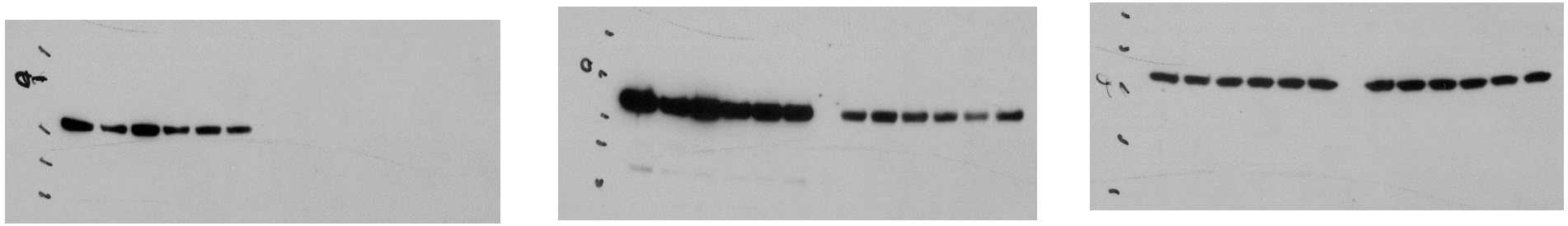




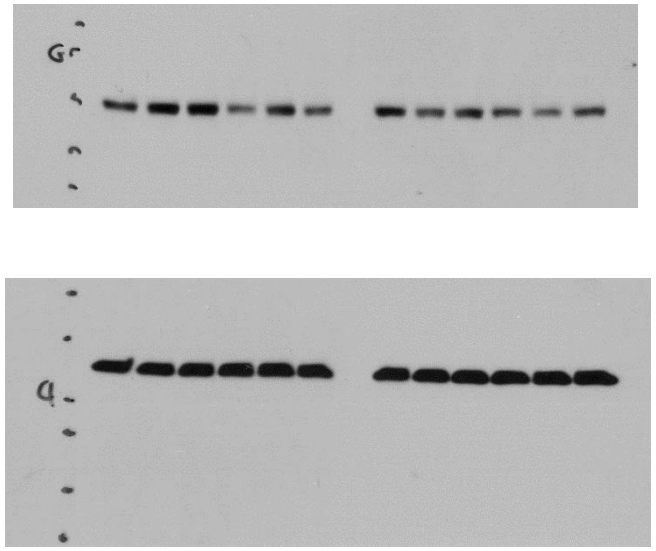




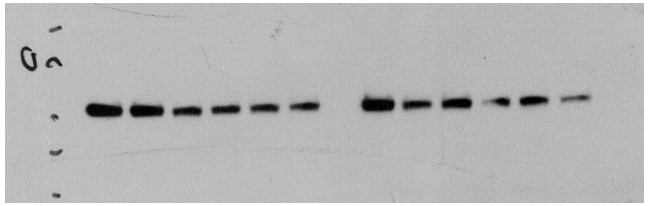
Full unedited gels for Supplemental Figure 9A

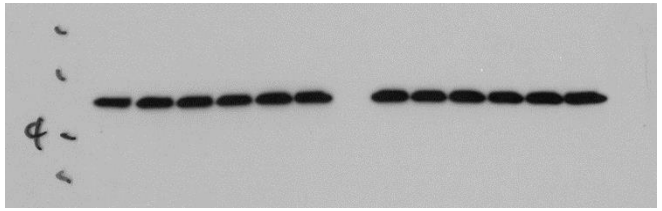


Full unedited gels for Supplemental Figure 9B

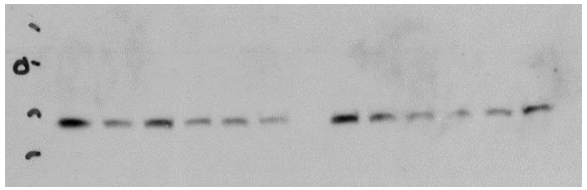


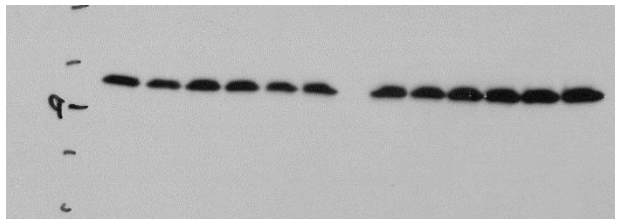
Full unedited gels for Supplemental Figure 9C



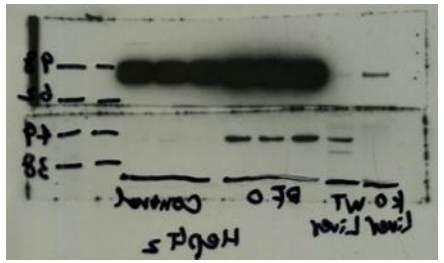


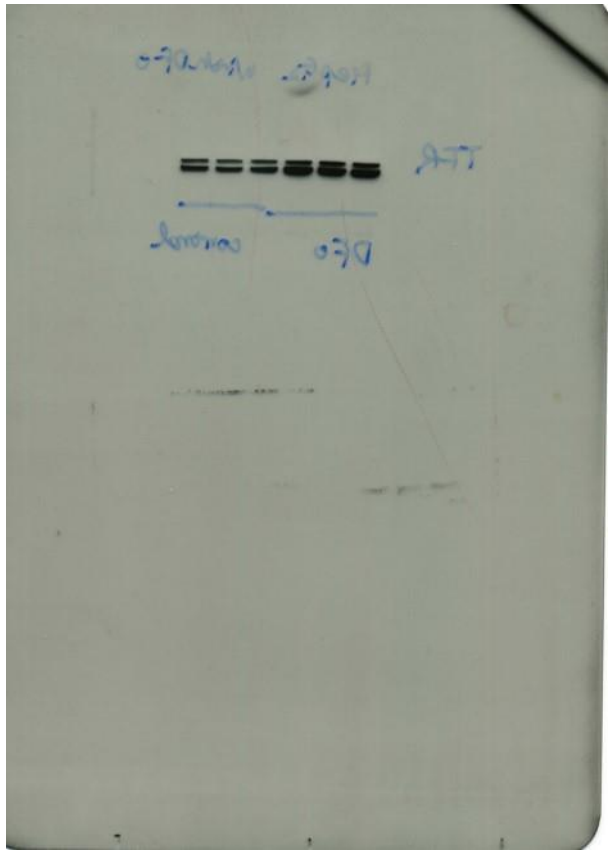
Full unedited gels for Supplemental Figure 9D

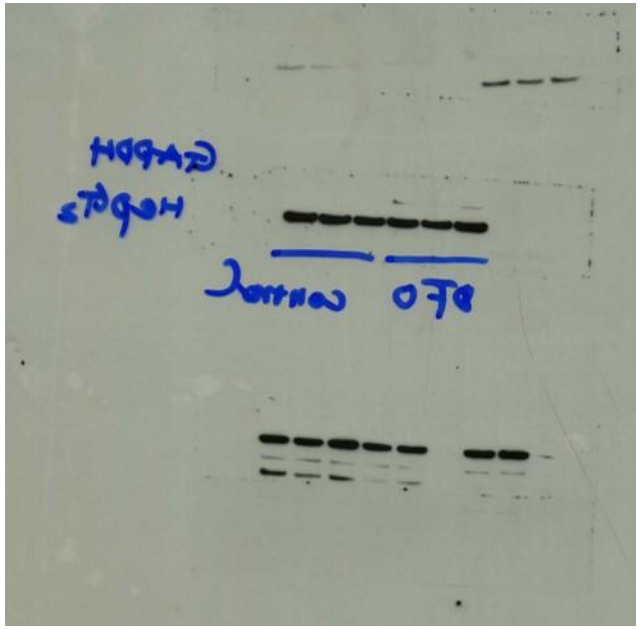




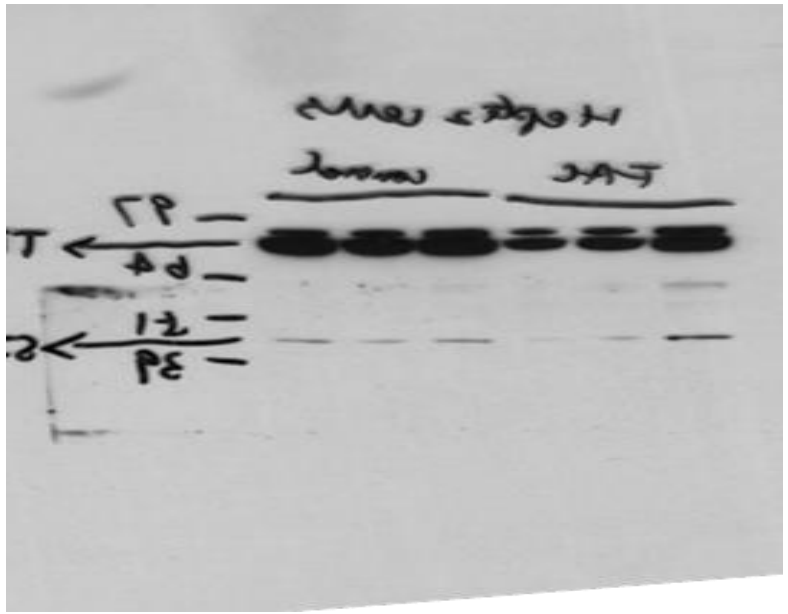
Full unedited gels for Supplemental Figure 12A

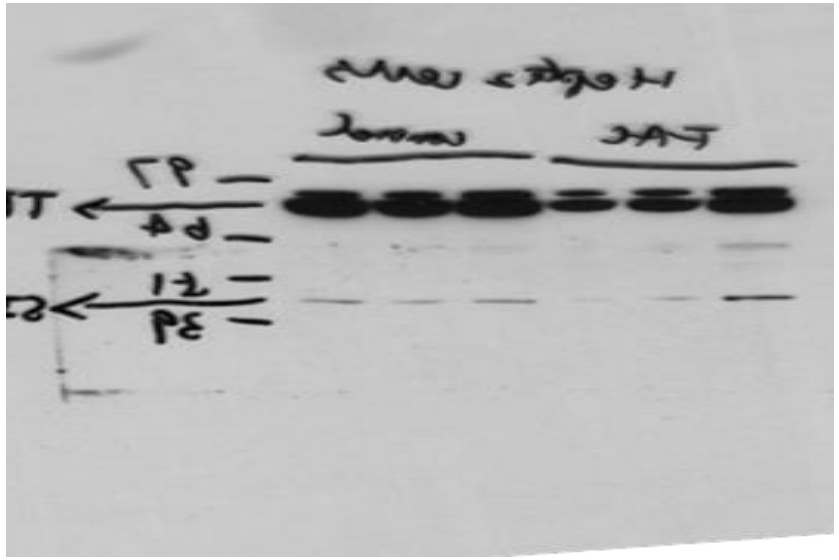


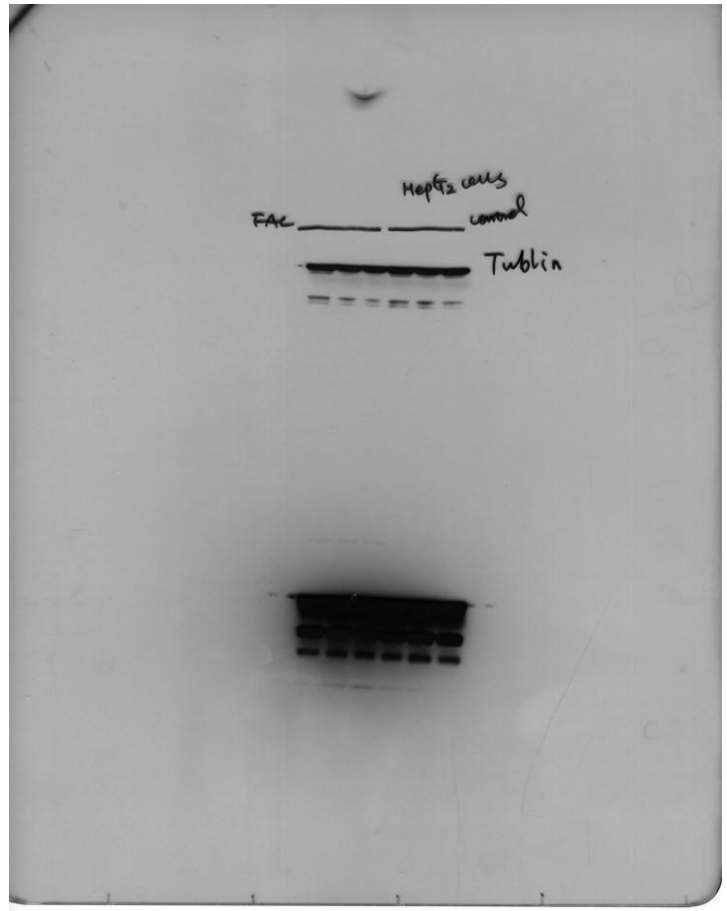




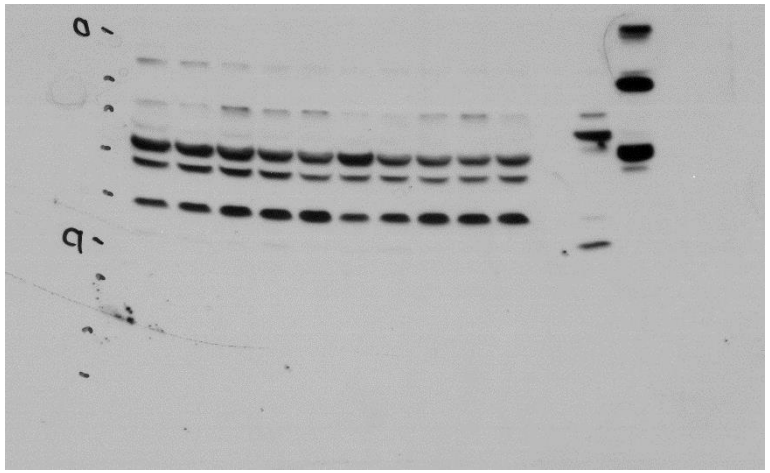
Full unedited gels for Supplemental Figure 12B

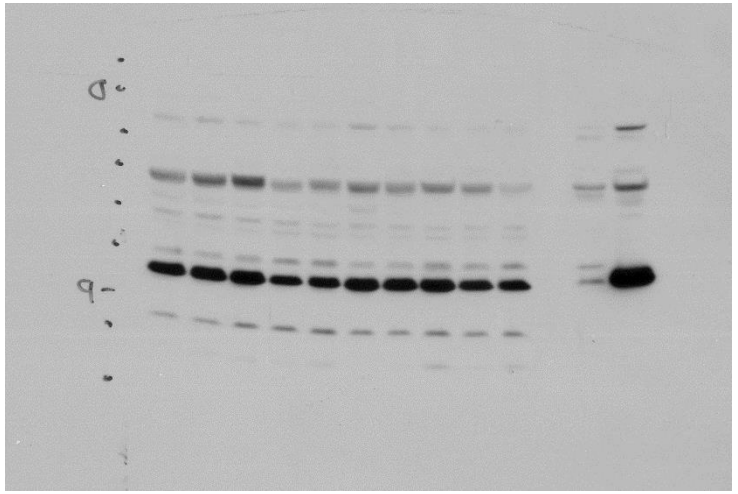




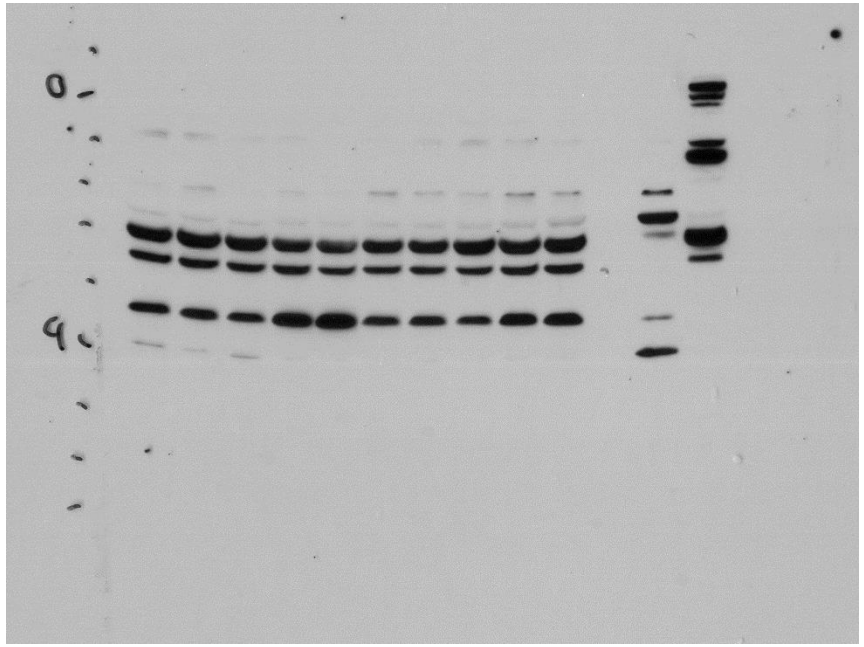


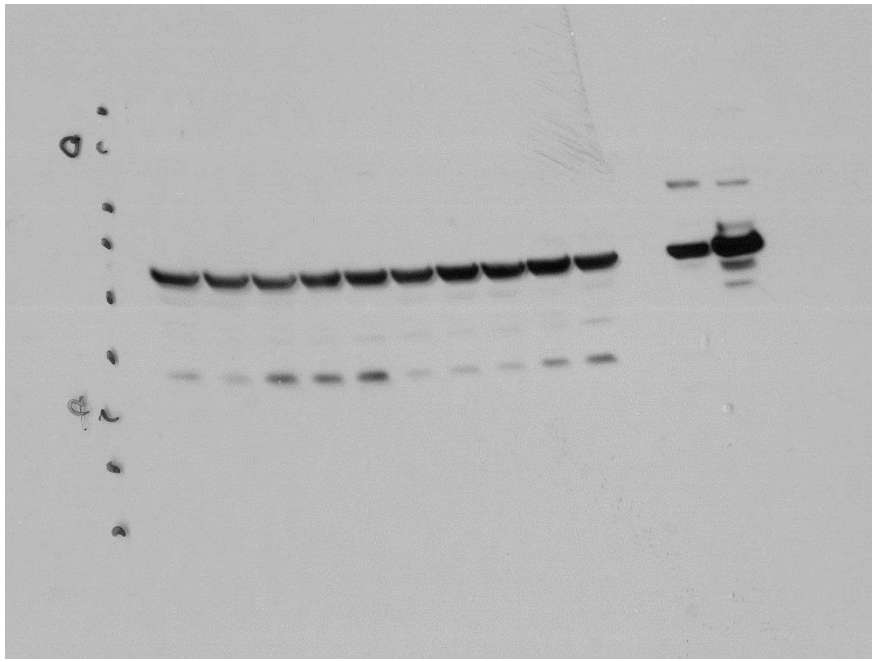
Full unedited gels for Supplemental Figure 13A





Full unedited gels for Supplemental Figure 13B





Full unedited gels for Supplemental Figure 13C