Supplementary Information

Phosphorylation of the Nuclear Receptor Co-repressor 1 by Protein Kinase B (PKB/Akt) Switches its Co-repressor Targets in the Liver

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Supporting Experimental Procedures

Animal Procedures and Biochemical Measurements. All mice were maintained in a temperature-controlled (23°C) facility with a 12-hr light/dark cycle and were given free access to food and water. Regular chow diet was obtained from UAR (Villemoison sur Orge, France). Mice were fed *ad libitum,* fasted 4-hr or 24-hr before harvesting blood for subsequent measurements, and tissues for RNA isolation, lipid measurements and histology. The tail vein injections were performed as described previously. Briefly, *NCoR1*$^{hep-/-}$ mice were injected 10 µg plasmid DNA in 200 µL of transfection mixture. After 6 hr, the mice were sacrificed and subjected to qRT-PCR. ipGTT was performed in animals that were fasted overnight. Glucose was administered by intraperitoneal injection at a dose of 2 g/kg BW. ITT was done in 4-hr fasted animals. Insulin was injected at a dose of 0.5 U/kg BW. Glucose quantification was done with the ACCU-CHEK® Aviva combo system (Roche Diagnostics, IN). Plasma insulin concentrations were measured using ELISA for mouse (Cristal Chem Inc., Downers Grove, IL). Biochemical analysis including triglycerides, free fatty acids, total cholesterol, LDL and HDL cholesterol were determined by enzymatic assays (Roche, Mannheim, Germany). EchoMRI-900 quantitative nuclear magnetic resonance (NMR) system (Echo Medical Systems, Houston, TX) was used for determination of lean mass and fat mass in conscious mice.

Mass spectrometry analysis. Gel lanes were cut into pieces and subjected to in-gel digestion with endoproteinase Glu-C or trypsin. For selective phosphopeptide

enrichment, TiO_2 Phosphopeptide Enrichment and Clean-up Kit (Thermo Fischer Scientific, Waltham, MA) was used. Enriched phosphopeptides were analyzed by nano-LC-MS/MS using an Orbitrap Elite mass spectrometer (Thermo Fischer Scientific) coupled to an ultraperformance LC (UPLC) system (Thermo Fischer Scientific Ultimate 3000 RSLC). Data analysis was performed with Proteome Discoverer (v. 1.3) and searches were performed with Mascot and Sequest against a mouse database (UniProt release 2013_01). Data were further processed, inspected and visualized using Scaffold 3 software (Proteome Software, Inc., Portland, OR, USA).

Generation of recombinant glutathione S-transferase (GST)–peptide fusion protein. To generate plasmids expressing the 1st (1453-1466 AA) and 2nd (2401-2415 AA) peptides of NCoR1, sense (5'-GGG GAA TTC GAA GTG GTC CAG AGC ACG A-3') and antisense (5'-TGC GGC CGC TCA AGA CTT GCT GGA AGA AAC ATC AG-3') primer for the 1st peptide and sense (5'-GGG GAA TTC GAG GCA CGG AGA GAT GAA G-3') and antisense (5'-TGC GGC CGC TCA GTC GTC ACT ATC AGA CAG TGT C-3') primer for the 2nd peptide were used. PCR was performed using the standard PCR condition (PrimeSTAR® HS Polymerase Takara Bio, Japan). The PCR fragments corresponding to the 1st and 2nd peptides were individually cloned into pGEX-5X-1 (Amersham Biosciences, Amersham, UK). *E. coli* BL21 was transformed with the recombinant constructs by heat shock and the expression of fusion proteins was induced with 1 mM IPTG at 37 °C for 3-hr. Cells were harvested by centrifugation, resuspended in 15 ml of phosphate buffered saline (PBS) including protease inhibitors cocktail (Roche) and disrupted by high

pressure (3000 psi) with a French Press (Spectronics Instruments, Rochester, NY, USA). Cell debris was removed by centrifugation at 10,000×g for 15 min at 4 °C. The supernatants were injected into a 1 ml GSTrap column (Amersham Biosciences). Bound protein was eluted with 50 mM Tris–HCl, 10 mM reduced glutathione, pH 8.0. Purified GST-1st and GST-2nd peptides were analyzed by 12% SDS-PAGE under reducing conditions with 0.1% Coomassie blue R250.

Plasmids and site-directed mutagenesis. The plasmids used in this study were generated using a human liver cDNA library (Invitrogen). To generate plasmids expressing HA-hLXRα, HA-hPPARα and HA-hERRα, the PCR products were amplified using primer sets flanking the entire coding region of each gene (LXRα-F-EcoRV: 5'-GGG GAT ATC CCA TGT CCT TGT GGC TGG GG-3'; LXRα-R-XhoI: 5'-TTT CTC GAG TCA TTC GTG CAC ATC CCA-3'; PPARα-F-NotI: 5'-GGG GCG GCC GCC CAT GGT GGA CAC GGA AAG C-3'; PPARα-R-ApaI: 5'-CCC GGG CCC TCA GTA CAT GTC CCT GTA GAT CTC C-3'; ERRα-F-EcoRI: 5'-GGG GAA TTC ATG TCC AGC CAG GTG GTG-3'; ERRα-R-XbaI: 5'-TCT AGA TCA GTC CAT CAT GGC CTC-3'). Each amplified PCR product was digested and ligated into pcDNA3-HA plasmid. pCMX-FLAG-mNCoR1 was generated as described. The site-directed mutagenesis in pCMX-FLAG-mNCoR1 vector to generate pCMX-FLAG-S1460E mNCoR1, pCMX-FLAG-S1460A mNCoR1 was performed according to the Stratagene QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) protocol by using the following primers: S1460E primers 5'-CGA GCC CGG CAC ACG GAA GTG GTG AGC TCT GG-3' and 5'-CCA GAG CTC ACC ACT TCC GTG TGC CGG GCT CG-3'; S1460A primers 5'-CGA GCC CGG CAC ACG GCA GTG

GTG AGC TCT G-3’ and 5’- CAG AGC TCA CCA CTG CCG TGT GCC GGG CTC G-3’.

Western Blot Analysis. Western blot analysis was performed according to standard methods with commercially available antibodies: Phospho-(Ser/Thr) Akt Substrate Antibody (Cell Signaling, #9611), Monoclonal anti-FLAG® M2-Peroxidase (HRP) (Sigma-Aldrich, A8952), anti-HA-Tag (6E2) mouse mAb (HRP Conjugate) (Cell Signaling, #2999), Mitoprofile Total OXPHOS Rodent WB Antibody Cocktail (Mitosciences/Abcam, Cambridge, MA, ab110413), anti-OxPhos Complex II 30 kDa subunit, mouse IgG2a, monoclonal 21A11 (Molecular Probes™, Eugene, Oregon, A21345), Anti-Nuclear Receptor Corepressor NCoR antibody (ChIP Grade, Abcam, ab24552), anti-NCoR1 antibody (Cell signaling, #5948) and anti-β-Actin Antibody (Cell Signaling, #4967).

Supporting Table 1. List of primer sets used for qRT-PCR.

Gene	Forward Primer	Reverse Primer
36B4	AGATTCGGGATATGCTGTTGG	AAAGCCTGGAAGAAGGAGGTC
Srebp1c	GGCACTAAGTGCCCTCAACCT	GCCACATAGATCTCTGCCAGTGT
Me1	GATGATAAGGTCTTCCTCACC	TTACTGGTTGACTTTGGTCTGT
Cpt1a	CTCCGCCTGAGCCATGAAG	CACCAGTGATGATGCCATTCT
mCad	CAACACTCGAAAGCGG	GCTGTTCCGTCAACTCAAAACTA
Sdhb	GGACCTATGGTGTTGGATGC	GTGTGCACGCCAGAGTATTG
Esrra	ACTGCCACTGCAGGATGAG	CACAGCCTCAGCATCTTCAA
Pygl	AAGGGTTTCCATGCCTGAG	CTATGGCTACGGCATTCGTT
G6pdx	GCCACCAGATGGTAGGATAGA	GTTGTACCAGGGTGATGCCT
Scd1	CCGGAGACCCCTTAGATCGA	TAGCCTGTAAAAGATTTCTGCAAACC
Elovl3	CCGCGTTCTCATGTAGGTCT	CTTAAGGCCCTTTTTGGAGG
Elovl5	CTGAGTGACGCATCGAAATG	CTTGCACATCCTCCTGCTC
Elovl6	AACTTGGCTCGCTTGTTCAT	CCAATGGATGCAGGAAAACT
Gpam	CAACACCATCCCCGACATC	GTGACCTTCGATTATGCGATCA
Acss2	GCTGAACTGACACACCTGGA	AACTTGGCGACAAAGTTGCT
Acss3	TCAATGTGCCGATCAATAGC	TACAAGCCCTGGACCAAAAC
Fasn	AGCTTCGGCTGCTGTTGGAAGT	TCGGATGCCTCTGAACCACTCACA
Plin2	CAATTTGTGGCTCCAGCTTC	CCCGTATTTGAGATCCGTGT
Fitm1	GTAGCCAGGAACACCACCAG	CTAGCCACGGCAACTTCTTC
Cidec	ATTGTGCCATCTTCCTCCAG	ATCATGGCTCACAGCTTGG
Ndufb3	TACCACAAACGCAGCAAACC	AAGGGACGCCATTAGAAACG
Ndufb5	CTTCGAACTTCCTGCTCCTT	GGCCCTGAAAAGAACTACG
Cycs	TCCATCAGGGTATCCTCTCC	GGAGGCAAGCATAAGACTGG
Cox5a	GAGCCCAAAATCATTGATGC	TGAGGTCCTGCTTTGTCCTT
Atp5g1	GCTGCTTGAGAGATGGGTTC	AGTTGGTGTGGCTGGATCA
Ncor1	CTGGTCTTTCAGCCACCATT	CCTTCATTGGATCCTCCATC
Rpl32	GGGAGCAACAAGAAAACCAA	TTGTGAGCAATCTCAGCACA
Srebp1a	GGCCGAGATGTGCGAACT	TTGTTGATGAGCTGGAGCATGT
Srebp2	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA
G6pc	CTGCAAGGGAGAACTCAGCAA	GAGGACCAAGGAAGCCACAAT
Uqcrfs1	ATGTGAAGCGACCCTTCCT	GGAAAAACGGACAGAAGCAG
Acox1	GCCCAACTGTGACTTCCATT	GGCATGTAACCCGTAGCACT
Ppara	CCTGAACATCGAGTGTCGAATAT	GGTTCTTCTTCTGAATCTTGCAGCT
Pparb/d	CTCTTCATCGCGGCCATCATTCT	TCTGCCATCTTCTGCAGCAGCTT
Pparg	ATGGGTGAAACTCTGGGAGATTCT	CTTGGAGCTTCAGGTCATATTTGTA
Esrrb	GGGAGCTTGTGTTCCTCATC	ATCTCCATCCAGGCACTCTG
Esrrg	GATGAGCCTCCTCCAGAGTG	TGCACAGCTTCCACATCTTC
Pgc1a	AAGTGTGGAACTCTCTGGAACTG	GGGTTATCTTGGTTGGCTTTATG
Pepck	CCACAGCTGCTGCAGAACA	GAAGGGTCGCATGGCAAA
hSREBP1c	ACAGTGACTTCCCTGGCCTAT	GCATGGACGGGTACATCTTCAA
hME1	GGGAGACCTTGGCTGTAATGG	TTCGGTTCCCACATCCAGAAT

Supporting Figure Legends

Supporting Figure 1. Motif scan graphic indicates Serine 1460 phosphorylation of mouse NCoR1 by Akt kinase with high stringency (http://scansite.mit.edu/).

Supporting Figure 2. (A-C) qRT-PCR analysis to evaluate mRNA levels of *Srebp1a, Me1, NCoR1* **(A)***, mCad, Esrra, Ndufb3* (**B**), *SREBP1c* and *ME1* (**C**) according to the sequence composition of NCoR1 phosphorylation site using indicated cell lines. HEK293T and Hep G2 were transfected with indicated plasmids (1 μg/well). (**D**) Co-immunoprecipitation assay using Flag-S1460E NCoR1 (SE) and HA-PPARα (1 μg/well per 6-well plate) in AML12 cells with or without Wy14643 (1 μM). (**E**) Western blot analysis to evaluate pS1460 NCoR1 and pAkt in primary hepatocytes from *NCoR1*$^{L2/L2}$ mice infected with either Ad-LacZ or Ad-Cre adenovirus grown in high glucose (25 mM) medium with or without a 10 min insulin treatment (100 nM). (**F**) qRT-PCR analysis to evaluate mRNA levels of *Me1*, *Elovl6, mCad* and *Ndufb5* according to the sequence composition of NCoR1 phosphorylation site in livers from 25 wk-old *NCoR1*$^{hep-/-}$ chow fed mice after naked DNA gene transfer through tail vein (n > 5/group). All data shown as mean ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Supporting Figure 3. Gene Set Enrichment Analysis using transcriptomes obtained using the GeneChip Gene 1.0 ST Array System (Affymetrix) in livers of 25 wk-old male *NCoR1*$^{hep-/-}$ chow fed mice (n=4/group). The gene set encompassing the

electron transport system (OxPhos) has nominal $p < 0.001$ and FDR $q = 0.035$.

Supporting Figure 4. (**A**) Validation of anti-NCoR1 antibody in liver lysates from *NCoR1*$^{hep+/+}$ and *NCoR1*$^{hep-/-}$ male mice injected with insulin (0.5 U/kg) or PBS after 4-hr fasting. To detect total NCoR1, polyclonal goat antibody provided by Dr. Ronald M. Evans (indicated as Evans) and commercially available polyclonal rabbit antibodies (indicated as Cell Signaling and Abcam) were used. PARP-1 was used as a loading control. (**B**) Western blot analysis showing specificity of anti-pS1460 NCoR1 Ab. HEK293T cells were transfected with plasmid DNA expressing either Flag-mNCoR1 or Flag-S1460A mNCoR1 (1 μg/well). After 48-hr, cells were exposed to 100 nM insulin for the indicated times. Subsequently, cell lysates were analyzed by immunoblotting for the levels and NCoR1 phosphorylation with an anti-pS1460 NCoR1 Ab.

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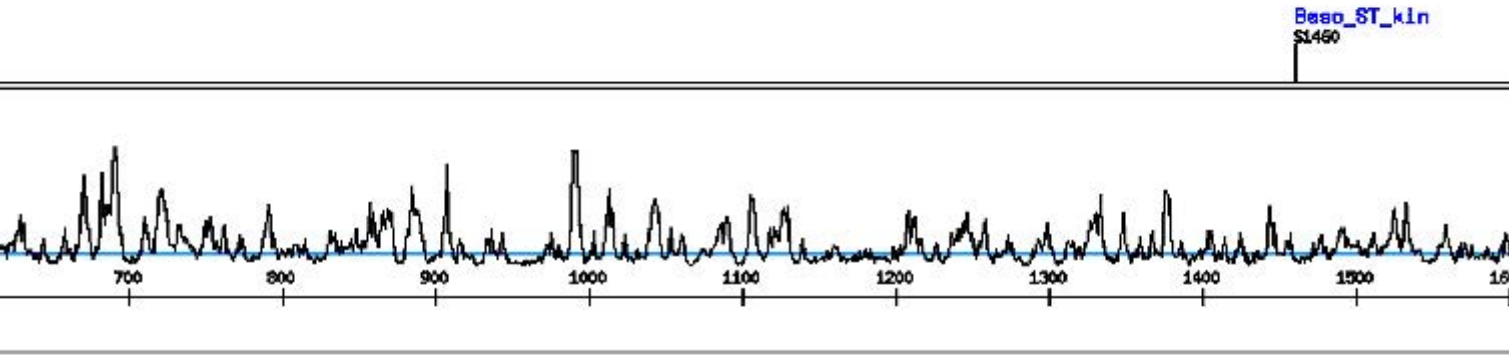
Show domains: No



Basophilic serine/threonine kinase group (Baso_ST_kin)
Akt Kinase Gene Card AKT1

Site	Score	Percentile	Sequence	SA
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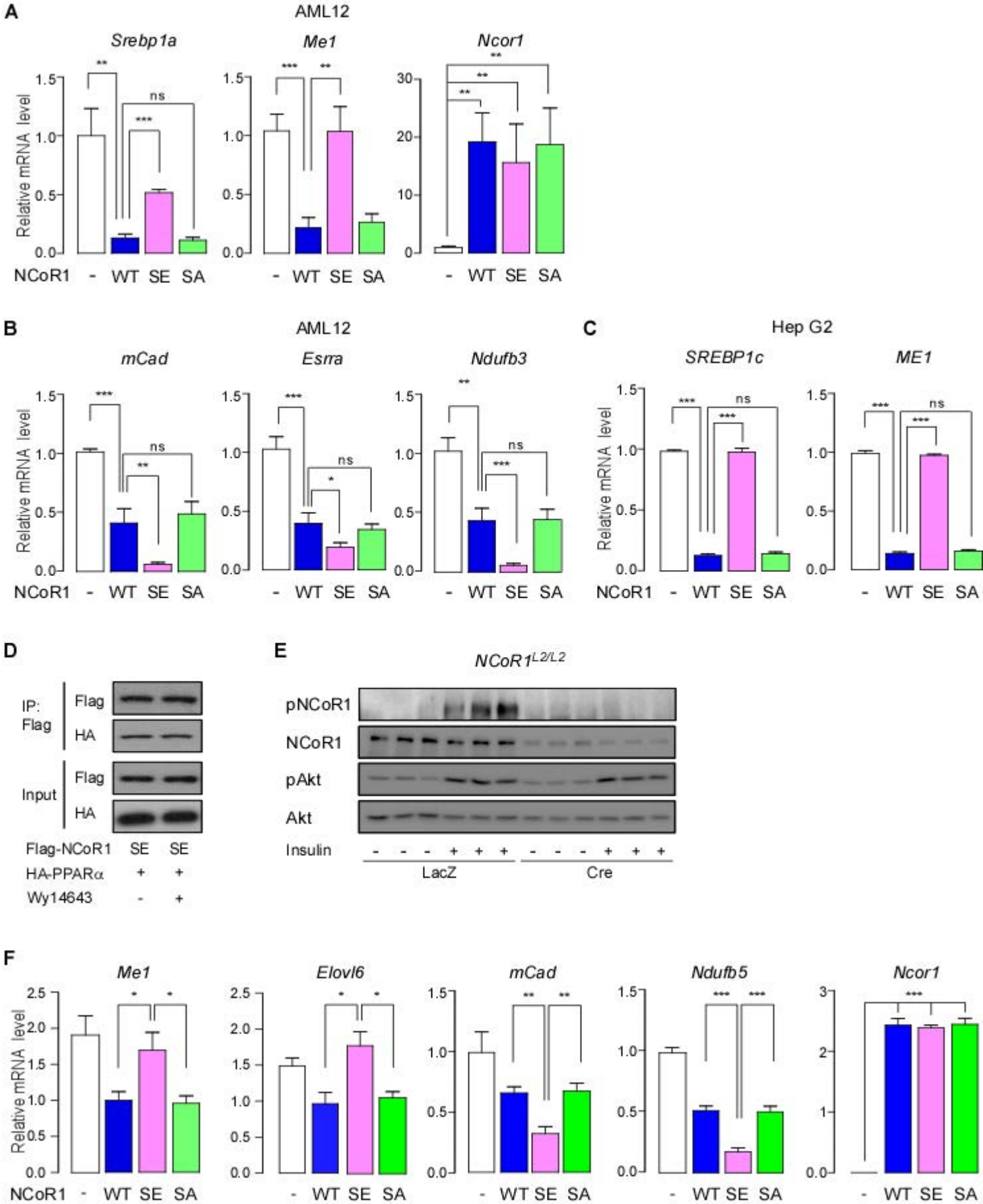
Repeat Scan at Medium Stringency

Repeat Scan at Low Stringency

Scan Another Protein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Hep G2
SREBP1c
ME1
D
IP: Flag
Flag
HA
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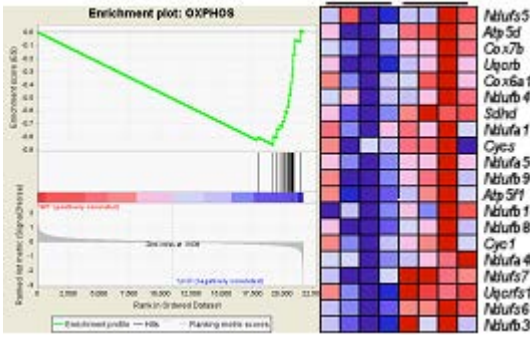
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Hits
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N CoM?
hep-/-
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Atp5d
Cox7b
Uqcrb
Cox6a1
Ndufb4
Sdhd
Ndufa1
Cycs
Ndufa5
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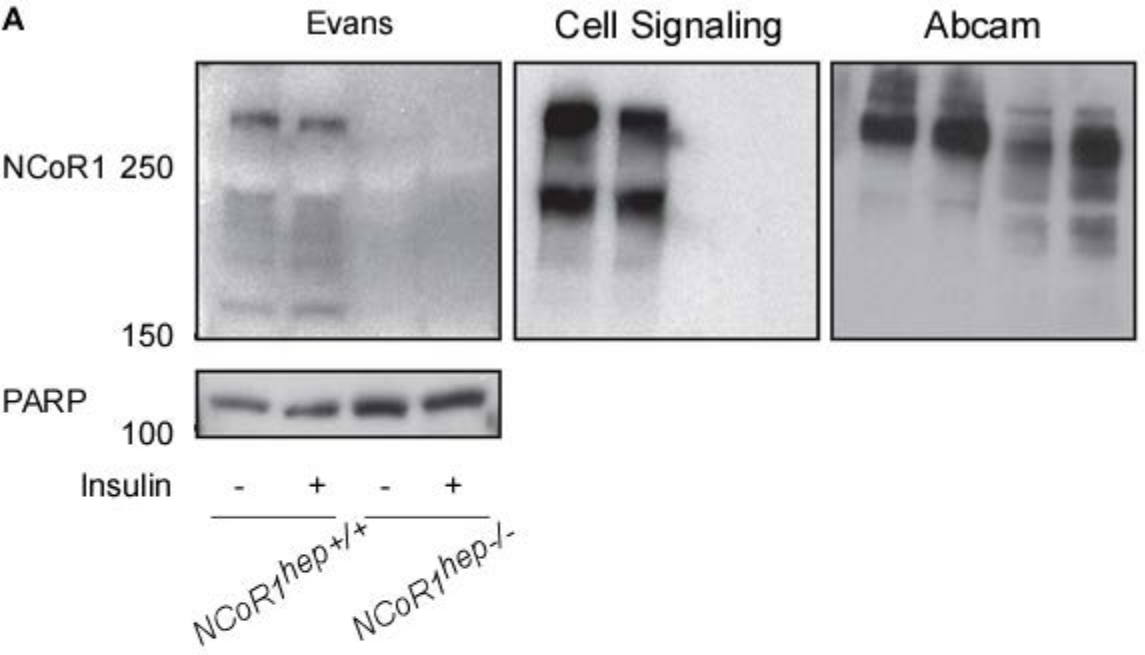
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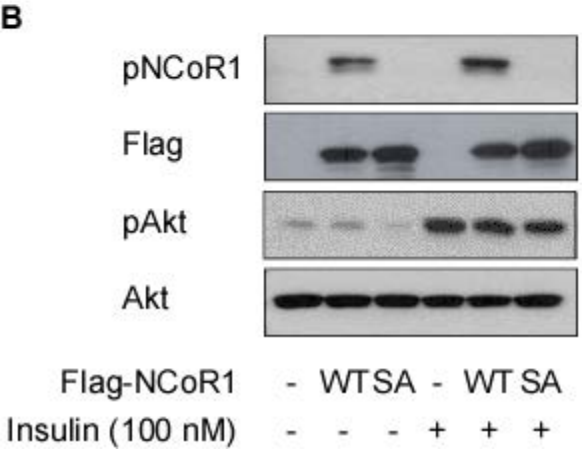
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Flag
pAkt
Akt
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