# SUPPLEMENTAL MATERIAL

# DP1 Activation Reverses Age-Related Hypertension via NEDD4L-Mediated T-bet Degradation in T Cells

**Running title:** DP1 and age-related hypertension

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## Supplemental Methods

### Reagents.

CHX was from Sigma-Aldrich (St. Louis, MO, USA). H89, Forskolin and BW245C were from Cayman Chemical (Ann Arbor, MI, USA). Lactacystin, mithramycin A, and MG132 were from Selleck Chemical (Houston, TX, USA). Phenylephrine (PE), acetylcholine (ACH), and sodium nitroprusside (SNP) were from Sigma-Aldrich (St. Louis, MO, USA). The TNFα neutralizing antibody etanercept was from AmGen (Thousand Oaks, CA, USA). Dihydroethidium (DHE) was from Santa Cruz (Santa Cruz, CA, USA).

### Isolation and preparation of $CD4^+$ single cell suspensions from spleen.

Mice were anesthetized and their spleens were isolated. The spleens were mechanically disrupted and then passed through a 70-μm sterile filter (Falcon, BD) and centrifuged at 300 × *g* for 10 min. After treatment with ACK lysing buffer, the pellet was resuspended in phosphate buffered saline (PBS). Total splenic leukocytes were stained and analyzed using flow cytometry. Mouse splenic $CD4^+$ T cells used for RNA, protein and PG assay were isolated using a negative selection kit (19852A, Stem cell, Vancouver, Canada).

### Flow cytometry of spleen, aortic and kidney leukocytes.

Spleens were directly crushed on 70-μm sterile filter to obtain single cell suspensions as above and were stained with various antibodies for further cell sorting using BD

FACSAria II. For aorta and kidney samples, tissues were mechanically dissociated and digested in HBSS containing 1.5mg/ml Collagenase II (Worthington, Lakewood, New Jersey), 1.5mg/ml Collagenase IV (Worthington), and 60U/ml DNase I (AppliChem, Lochem, Darmstadt) for 30 minutes at 37°C, with intermittent agitation. Tissue homogenates were filtered through 70-μm cell strainer to obtain single cell suspensions. Kidney samples were subjected to 36%/72% Percoll (17089102, GE, Marlborough, Massachusetts) gradient centrifugation before flow cytometric staining as reported[1]. Red blood cells were lysed before flow cytometric staining. Cells were stained with Fixable Viability Stain 780 (565388, BD Biosciences) before other required stainings. Samples were analyzed using BD FACSAria II. The following antibodies were used: CD45-FITC (553080, BD Biosciences), CD3-BV421 (562600, BD Biosciences), CD4-APC (553051, BD Biosciences), CD8-PE (553033, BD Biosciences).

**PG extraction and analysis**.

Sorted $CD4^+$ T cells from 2-month and 26-month-old mice were used for PG extraction. After centrifugation at 12,000 × *g* for 15 min at 4°C, 2 μl of an internal standard was added to the sample in 40 μl of 1 M citric acid and 5 μl of 10% butylated hydroxytoluene, and the sample was shocked with 1 ml solvent (normal hexane/ethylacetate, 1:1) for 1 min. The organic phase supernatant was collected after centrifugation at 6,000 × *g* /min for 10 min. The eluate was dried under nitrogen and dissolved in 100 μl of 10% acetonitrile in water. The prostanoid metabolites were

quantitated using liquid chromatography/mass spectrometry/mass spectrometry analyses. PG level was normalized to total protein concentration.

**Analysis of vasorelaxation**.

Superior mesenteric arteries (SMA) and thoracic aortas (TA) were dissected and mounted on a myograph system (Danish MyoTechnology, Aarhus, Denmark). The vessels were precontracted with phenylephrine (PE). Acetylcholine (ACH) dose-dependent response curves were plotted and the results were calculated as percentage of precontraction[2].

**Western blotting and co- immunoprecipitation (co-IP).**

The protein concentrations of spleen or cultured $CD4^+$ cells lysates were determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Equal quantities of proteins were denatured and resolved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels, transferred to nitrocellulose membranes, incubated with 5% skimmed milk for 1–1.5 h, and then incubated with primary antibodies overnight at 4°C. The primary antibodies were diluted as follows: HA-tag (1:1000; Cell Signaling), phospho-Sp1(T453) (1:1000; Abcam), Sp1 (1:1000; Cell Signaling), T-bet (1:500; Santa Cruz Biotechnology), NEDD4L (1:1000; Cell Signaling), phospho-Thr (1:1000, Cell Signaling), phosphor-Ser (1:1000, BD Biosciences). Actin (1:2000; Sigma-Aldrich) or GAPDH (1:2000; Cell Signaling) was used as loading controls. The membranes were then incubated in horseradish peroxidase (HRP)-labeled secondary antibody in

blocking buffer for 2 h at room temperature. The blots were developed using an enhanced chemiluminescence reagent (Thermo Scientific, Waltham, MA, USA).

For co-IP, the HEK-293T cells were lysed in IP lysis buffer supplemented with a protease inhibitor cocktail (P8340, Sigma). One milligram of the total lysate was incubated overnight with the indicated antibodies and protein A/G beads (sc-2003, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The immunocomplex was washed thrice, eluted with SDS loading buffer, and subjected to western blotting as mentioned above. Total protein (2%) was used as the input. For inhibition of proteasomal degradation, cells were incubated with 10 μM lactacystin for 6 h prior to cell lysis.

**Reconstitution of mNEDD4L.**

The mouse NEDD4L cDNA was subcloned into pcDNA3.1, and a hemagglutinin (HA) tag was added at the extracellular N-terminus. $CD4^+$ lymphocytes from $DP1^{-/-}$ mice were transiently transfected with the pcDNA3.1/NEDD4L plasmid or pcDNA3.1 empty vector using electrotransfection, according to the manufacturer's protocol.

**Luciferase reporter assays**.

EL4 cells were transfected with the empty vector or truncated NEDD4L reporter constructs together with pRL-TK renilla using a standard electrotransfection method. Then, the cells were lysed and luciferase activity was measured using the dual-luciferase reporter assay system according to the manufacturer's instructions

(Promega).

**Chromatin immunoprecipitation.**

Total $CD4^+$ lymphocytes ($2 \times 10^7$) were analyzed using enzymatic chromatin IP kit (agarose beads) per manufacturer's instructions (Cell Signaling). Briefly, the anti-Sp1 1:500 antibody (Cell Signaling) was used for ChIP (10 mg of crosslinked chromatin). The amount of *NEDD4L* in the Sp1-immunoprecipitated chromatin was detected using polymerase chain reaction (PCR) and normalized to the total amount of the gene present in 2% input chromatin of each immunoprecipitated sample.

**RNA extraction and quantitative reverse transcription PCR (qRT-PCR)**.

Total RNA samples from sorted cells or cultured $CD4^+$ cells were prepared using the Trizol reagent from Life Technologies (Carlsbad, CA, USA). Total RNA (1 μg) was reverse-transcribed to cDNA using a reverse transcription reagent kit from Takara (Dalian, China) according to the manufacturer's instructions. The resulting cDNA was amplified for 40 cycles. GAPDH RNA was amplified as an internal control. The primer sequences for PCR are summarized in Supplemental Table 2 and Supplemental Table 3.

**Histological analysis**.

Paraformaldehyde-fixed tissue samples were embedded in paraffin and 7-μm sections were stained with hematoxylin and eosin (H & E) or Sirius Red. For DHE

staining in tissues, the deparaffinized sections were washed in PBS for 5 min and then incubated with 100 nmol/l DHE in the dark at room temperature for 30 min. Finally, the slides were washed and mounted. Images were captured using an Olympus (FV1000) laser-scanning confocal microscope for further analysis.

**Enzyme-linked immunosorbent assay (ELISA)**.

Cytokine levels in serum were assayed using ELISA according to the manufacturer's instructions (R & D Systems).

## Supplemental Tables

### Supplemental Table 1. Characteristics of young and old volunteers

| Parameter | Young(n=47) | Old(n=48) | P value |
|---|---|---|---|
| **Age, y** | 26.14±0.34 | 66.2±1.08** | < 0.0001 |
| **SBP, mm Hg** | 107.90±1.51 | 124.6±1.92** | < 0.0001 |
| **DBP, mm Hg** | 69.60±0.79 | 78.94±1.21** | < 0.0001 |
| **Heart rate, bpm** | 79.27±1.36 | 78.21±1.31 | 0.7510 |
| **Total Cholesterol, mmol/L** | 4.72±0.14 | 5.21±0.14* | 0.0167 |
| **LDL, mmol/L** | 2.69±0.09 | 3.03±0.12* | 0.0306 |
| **HDL, mmol/L** | 1.33±0.04 | 1.40±0.05 | 0.2407 |
| **Triglycerides, mmol/L** | 1.25±0.11 | 1.70±0.15* | 0.0111 |
| **Creatinine, μmol/L** | 82.98±1.21 | 87.16±3.70 | 0.2609 |
| **Blood glucose, mmol/L** | 5.01±0.05 | 6.01±0.21* | < 0.0001 |
| **Urea，mmol/L** | 5.07±0.14 | 5.74±0.24* | 0.0164 |
| **Uric Acid，μmol/L** | 359.1±8.88 | 347.5±9.7 | 0.3705 |
| **IFNγ，pg/Ml** | 162.8±22.70 | 235.2±22.62* | 0.0280 |
| **TNFα，pg/Ml** | 318.0 ±31.08 | 481.3±55.29* | 0.0132 |
| **White blood cell count($10^9$/L)** | 6.40±0.15 | 6.15±0.23 | 0.3522 |
| **Neutrophil, %** | 55.33±0.96 | 59.21±1.16* | 0.0121 |
| **Lymphocyte, %** | 34.31±0.87 | 30.34±0.93** | 0.0025 |
| **Monocyte, %** | 7.48±0.23 | 7.53±0.24 | 0.8807 |
| **CD4/CD3,%** | 43.32±4.14 | 59.21±2.91** | 0.0039 |

| | | | |
|---|---|---|---|
| **CD8/CD3,%** | 47.02±4.02 | 31.67±2.50** | 0.0021 |

Male old volunteers versus young control. SBP indicates systolic blood pressure, DBP indicates diastolic blood pressure, HDL indicates high-density lipoprotein, and LDL indicates low-density lipoprotein. All the parameters are mean (SEM). Statistical analysis was performed using unpaired Student's t test or non-parametric Mann–Whitney U test for variables with non-normal distribution. *P<0.05, **P<0.01.

**Supplemental Table 2. Primers for real-time PCR analysis in mice**

| Gene | Sense | Anti-sense |
|---|---|---|
| DP1 | AACCTCTATGACATGCACAGGCG | AAGGCTTGGAGGTCTTCTGAGTC |
| DP2 | TCTCAACCAATCAGCACACCCGA | GATGTAGCGGAGGCTAGAGTTGC |
| TNFα | ACGGCATGGATCTCAAAGAC | CGGACTCCGCAAAGTCTAAG |
| IFNr | ATGAACGCTACACACTGCATC | CCATCCTTTTGCCAGTTCCTC |
| IL2 | TGAGCAGGATGGAGAATTACAGG | GTCCAAGTTCATCTTCTAGGCAC |
| IL4 | CTCGAATGTACCAGGAGCCA | TCGTTGCTGTGAGGACGTTT |
| IL5 | AGCAATGAGACGATGAGGCT | GTACCCCCACGGACAGTTTG |
| IL13 | CCTGGCTCTTGCTTGCCTTGG | TCTTGTGTGATGTTGCTCA |
| IL17F | TGCTACTGTTGATGTTGGGAC | AATGCCCTGGTTTTGGTTGAA |
| IL22 | ATGAGTTTTTCCCTTATGGGGAC | GCTGGAAGTTGGACACCTCAA |
| T-bet | AGCAAGGACGGCGAATGTT | GGGTGGACATATAAGCGGTTC |
| NEDD4L | CACGGGTGGTGAGGAATCC | GCCGAGTCCAAGTTGTGGT |
| Roquin | GGCTTGCCCATTTGACCAGA | TGCTTTGTATCTTCAACCCCAC |
| WWP1 | GGCAGTCTCAGCGGAATCAAT | GGTCCATAGGGGTCATTTTCTG |
| WWP2 | TTTGAGAAGTCCCAGCTTACCC | CTCCAGACCTTCAGATCCAAATG |
| NEDD4 | TCGGAGGACGAGGTATGGG | GGTACGGATCAGCAGTGAACA |
| Rettax | CCCCGTCCCATGTCTCTGT | CAAATTGTTCGTCCTGGGTTTTC |
| Cblb | GGTCGCATTTTGGGGATTATTGA | TTTGGCACAGTCTTACCACTTT |
| Smurf1 | AGCATCAAGATCCGTCTGACA | CCAGAGCCGTCCACAACAAT |
| Smurf2 | AAACAGTTGCTTGGGAAGTCA | TGCTCAACACAGAAGGTATGGT |

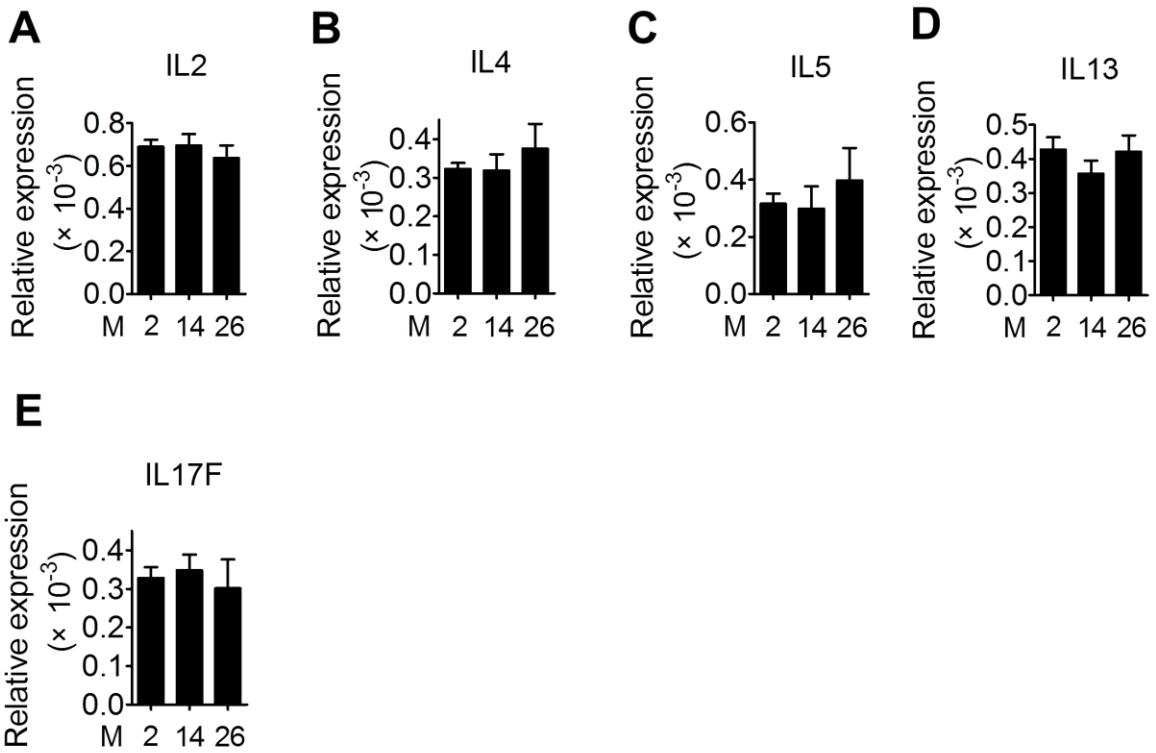
| | | |
|---|---|---|
| GRAIL | ATTCAAAGAGGCATCCAAGTCAC | TGCATTTCGTAATCTTCGAGCAG |
| Itch | TGGGTAGTCTGACCATGAAATCT | GGGGTAACAATAACTGTGAGGG |
| Rnf130 | CTCACGTTCCGCATCGACC | AAGGCGATCCACTGTTTGATATT |
| DUBA | AGTCCGGAACGTGAAGAGGT | TCAAACCAGTGTTCCTGCTG |
| SRSF1 | TCTCACGAGGGAGAAACTGC | CTGCTACGGCTTCTGCTACG |
| SRSF2 | CGCGCTCCAGATCAACCTC | CTTGGACTCTCGCTTCGACAC |
| USP2A | TACAGAATCGTCCCGCTACAC | CCCCTGTCACAGTCCAGAAT |
| USP2B | CGTCGTCCCCCAATGATGTG | GTGGCGCATATCTCTGGATCT |
| USP9X | TCCAACAGAATCAGACTTCATCG | TGGAAATGCAGGTTCCTCATCT |
| USP10 | AACCCACAGTATATCTTTGGCG | CCCTCACTAGGTTCGATGACTTC |
| USP15 | CCGTGGATGAAAACCTGAGTAG | TTCTCTTAGGCAGACAGGGATAA |
| USP18 | TTGGGCTCCTGAGGAAACC | CGATGTTGTGTAAACCAACCAGA |
| USP20 | TGGACTGCATAGGGGAGGTG | ACTGGCAGGTTCCCTTAGATT |
| USP21 | CCGAGTGGGAGCCAAGATAC | AAGGGGACCTCTAGGACGAGA |
| USP44 | ATGGATAGGTGCAAGCACGTT | GCTCTTGGATGTACTTCCCACAG |
| Perforin | CAAGGTAGCCAATTTTGCAGC | GTACATGCGACACTCTACTGTG |
| GAPDH | CCCTTATTGACCTCAACTACATGGT | GAGGGGCCATCCACAGTCTTCTG |

**Supplemental Table 3. Primers for real-time PCR analysis in human**

| Gene | Sense | Anti-sense |
|---|---|---|
| HPGDS | ACCAGAGCCTAGCAATAGCAA | AGAGTGTCCACAATAGCATCAAC |
| DP1 | CTGGGCAAGTGCCTCCTAAG | CAACGAGTTGTCCAATGCGG |
| DP2 | AAAAGGCTCGGGAAGGTTAAATG | ACCGGGGAACCAAGAGAGAG |
| TNFα | CCTCTCTCTAATCAGCCCTCTG | GAGGACCTGGGAGTAGATGAG |
| IFNγ | TGGTAACTGACTTGAATGTCCA | TCGCTTCCCTGTTTTAGCTGC |
| GAPDH | GGAGCGAGATCCCTCCAAAAT | GGCTGTTGTCATACTTCTCATGG |

## Supplemental Figures and Figure Legends

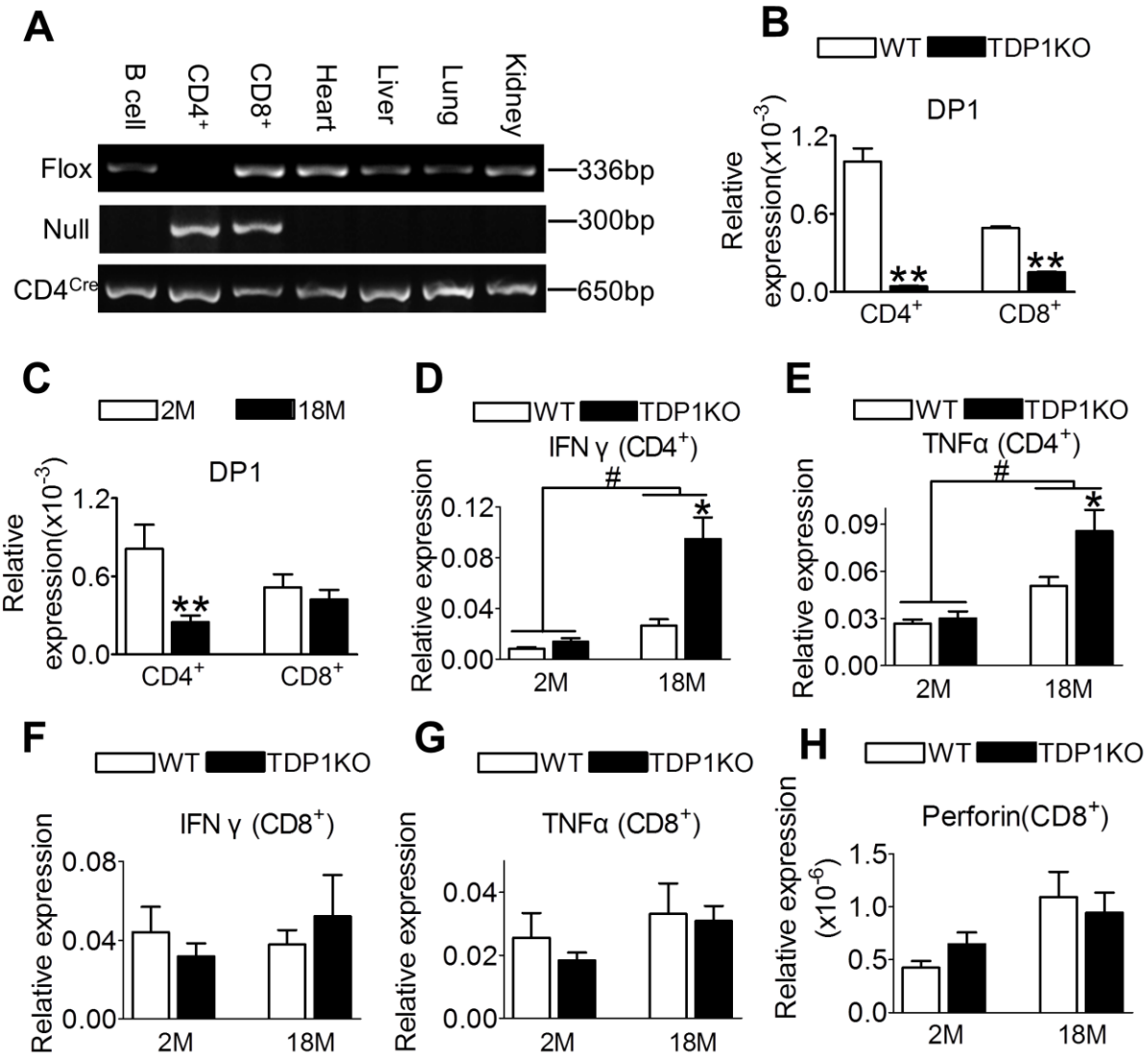
**Supplemental Figure 1**




**Supplemental Figure 1. Expression of T cell cytokines in CD4$^+$ cells from mice of different ages.**

(**A**-**E**) Indicated genes mRNA level were quantified (n = 6). Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Bonferroni post hoc test. IL indicates Interleukin; M, month.

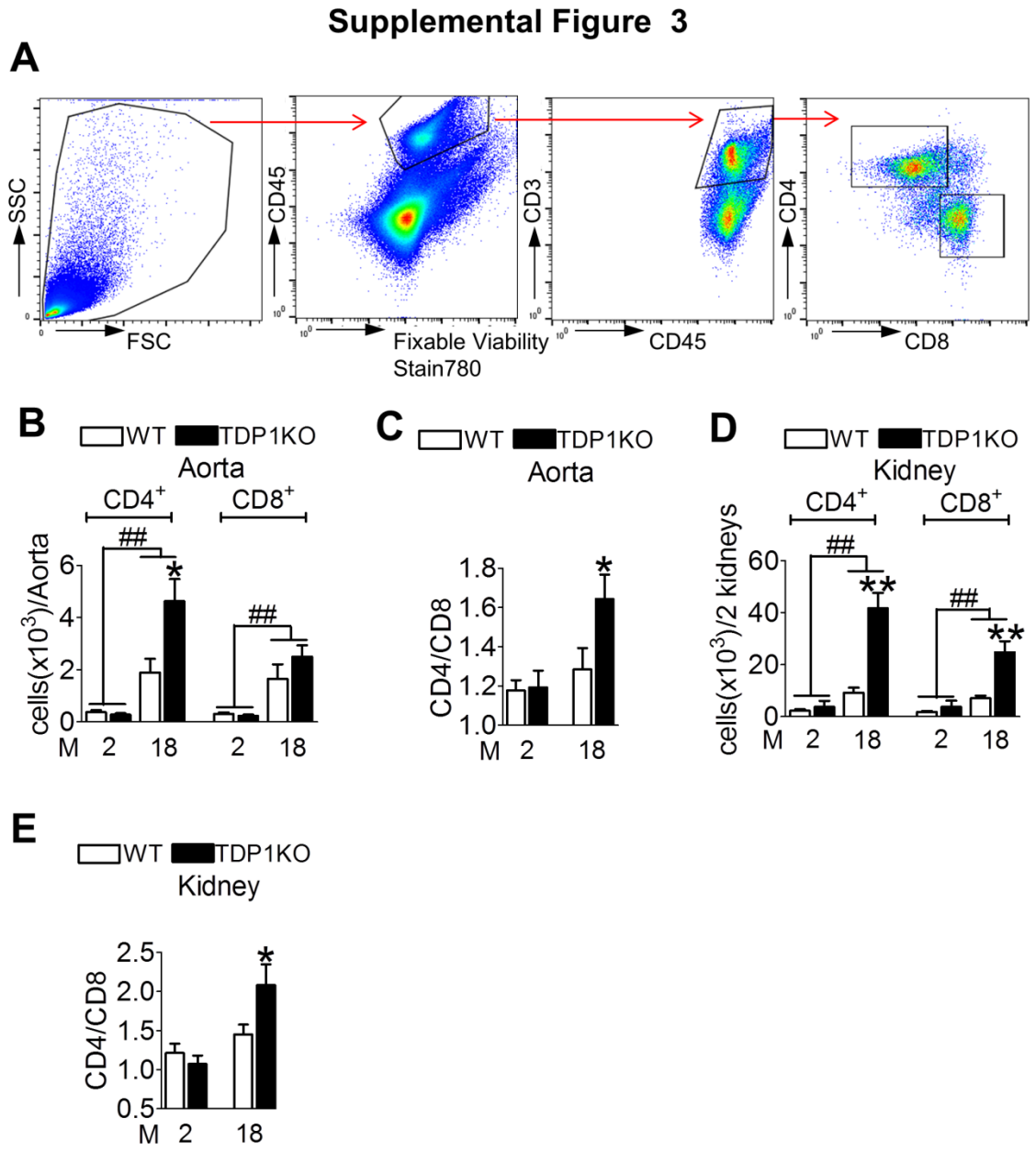
## Supplemental Figure 2




**Supplemental Figure 2. Characterization of TDP1KO mice.**

**(A)** PCR analysis of genomic DNA isolated from various tissues of TDP1KO ($DP1^{F/F}CD4^{Cre}$) mice. $CD4^+$, $CD4^+$ T cells; $CD8^+$, $CD8^+$ T cells. (**B**) QRT-PCR analysis of DP1 gene expression in $CD4^+$ and $CD8^+$ T cells isolated from spleens of WT and TDP1KO mice. $^{**}P < 0.01$ compared to WT(n = 6). (**C**) DP1 expression in $CD4^+$ and $CD8^+$ T cells from young and aged mice. $^{**}P < 0.01$ compared to WT(n = 6). (**D** and **E**)

Effect of DP1 deletion on IFN γ (**D**) and TNF α (**E**) expression in sorted $CD4^+$ T cells from young and aged mice. *$P$ < 0.05 compared to 18 months WT, $^{\#}P$ < 0.05 compared to as indicated (n = 4–6). (**F**-**H**) Effect of DP1 deletion on IFN γ (**F**), TNF α (**G**) and Perforin (**H**) expression in sorted $CD8^+$ T cells from young and aged mice (n = 4–8). Data are expressed as mean ± SEM. Statistical analysis was performed using two-tailed unpaired student's t-test (B-C) or two-way ANOVA followed by Bonferroni post hoc test (D-H). DP1 indicates Prostaglandin $D_2$ receptor subtype 1; WT, floxed control mice; TDP1KO, $CD4^+$ T cell-specific DP1-deficient; IFNγ, Interferin gama; TNFα, Tumor necrosis factor alpha; M, month.

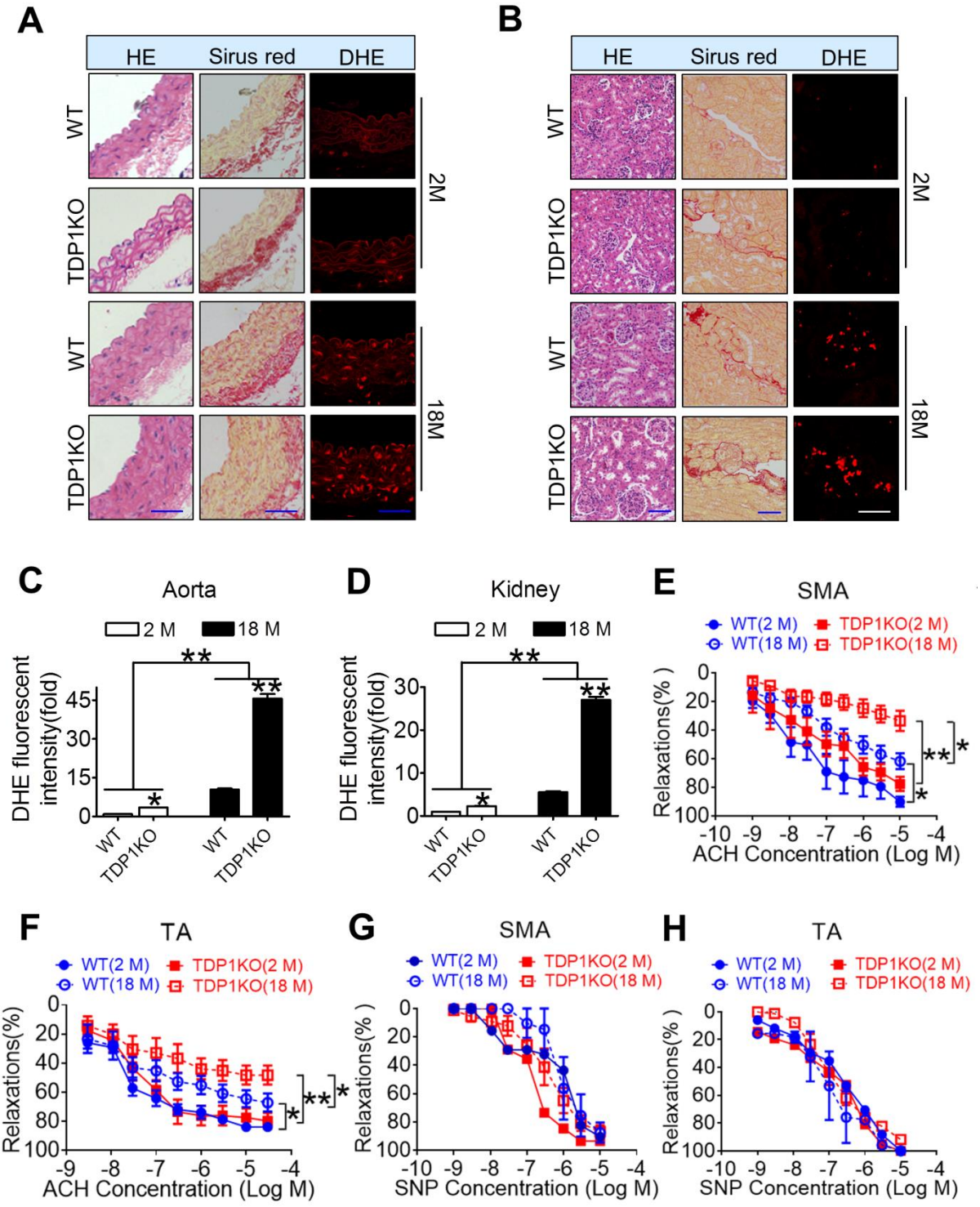



**Supplemental Figure 3. T cell DP1 deficiency increases CD4$^+$ T cell infiltration in aorta and kidneys in aged mice.**

(**A**) Gating strategy for flow cytometric analysis of T cell subsets in thoracic aorta and kidneys. Cell suspension was prepared and labeled with various antibodies. CD45 was used in conjunction with Fixable Viability Stain780 to identify CD45$^+$ 780$^{low}$ cells, which included live T cells. After doublet exclusion, T cells were selected as CD45$^+$

$CD3^+$, $CD4^+$ and $CD8^+$ T cell subsets were identified by corresponding antibodies. (**B**) Quantification of $CD4^+$ and $CD8^+$ T cells infiltrated in aorta from young and aged mice. **P* < 0.05 compared to 18 months WT, $^{\#\#}$*P* < 0.01 compared to as indicated (n=6-8). (**C**) Ratios of $CD4^+/CD8^+$ cells in thoracic aortas. **P* < 0.05 compared to 18 months WT (n=6-8). (**D**) Quantification of $CD4^+$ and $CD8^+$ T cells infiltrated in kidneys from young and aged mice. ***P* < 0.01 compared to 18 months WT, $^{\#\#}$*P* < 0.01 compared to as indicated (n=7-10). (**E**) Ratio of $CD4^+/CD8^+$ T cells in kidneys from aged mice. **P* < 0.05 compared to 18 months WT (n=7-10). Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post hoc test. SSC indicates side scatter; FSC, forward scatter; WT, floxed control mice; TDP1KO, $CD4^+$ T cell-specific DP1-deficient; M, month.

## Supplemental Figure 4

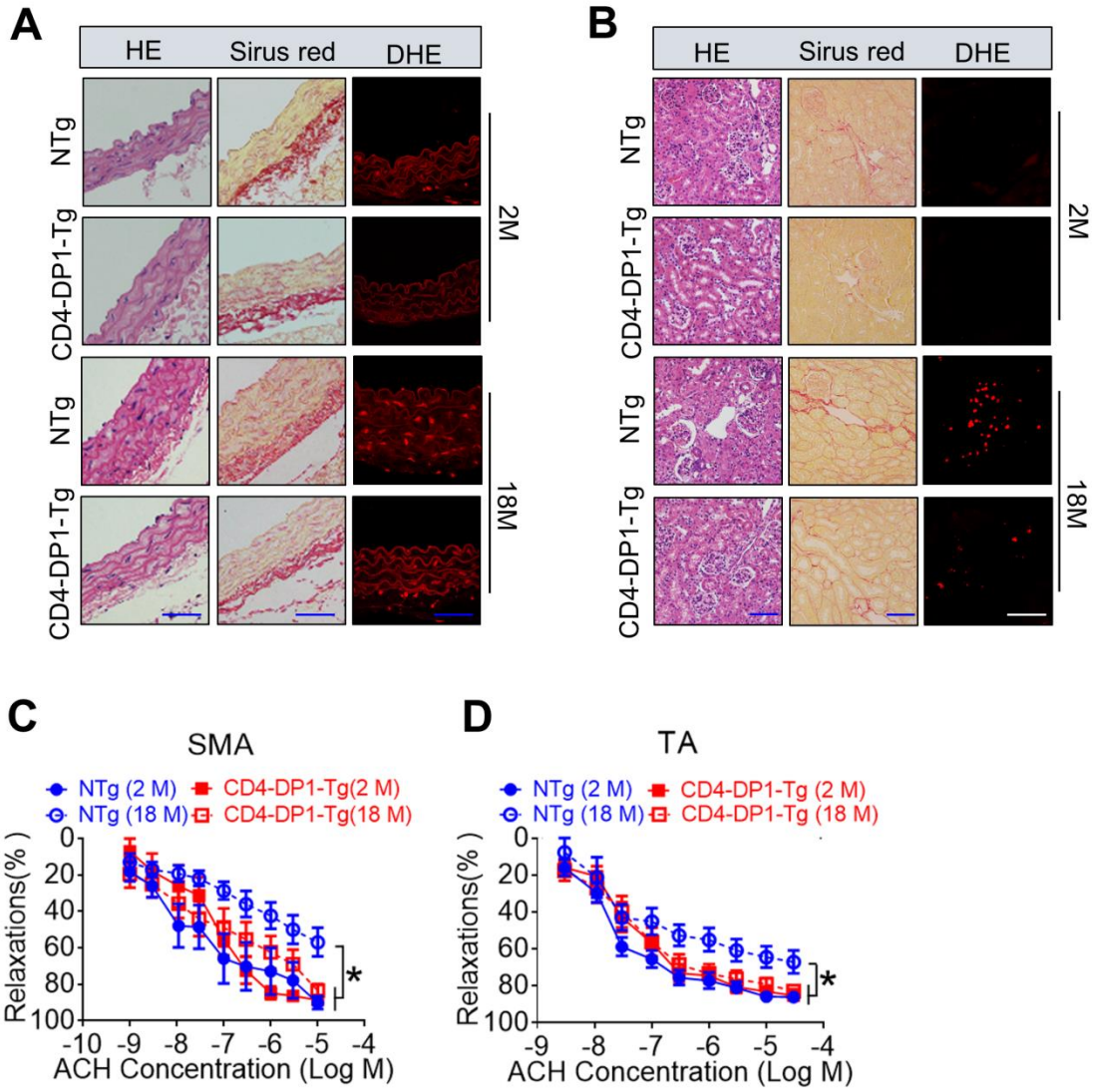



**Supplemental Figure 4. DP1 deficiency in T cells exacerbates age-associated increase in arterial thickness, fibrosis, superoxide production in aorta and**

**kidneys, and aging-induced endothelial dysfunction in vasculature.**

(**A** and **B**) Representative HE staining, Sirius Red staining, and immunofluorescence staining of DHE in aortic (**A**) and kidney (**B**) sections from young and aged TDP1KO mice. Blue scale bars, 50 μm; white scale bar, 10 μm. (**C** and **D**) Quantification of mean red DHE fluorescence intensity in aorta and kidneys. **P* < 0.05, ***P* < 0.01 compared to indicated (n = 4-5). (**E** and **F**) Acetylcholine (ACH) dose-response relaxation curves of superior mesenteric arteries (SMAs) and thoracic aortas (TAs) from young and aged TDP1KO mice. **P* < 0.05, ***P* < 0.01 compared to indicated (n = 4-6). (**G** and **H**) Endothelium-independent relaxation to sodium nitroprusside (SNP) in SMA and TA from young and aged TDP1KO mice (n = 3). Data are expressed as mean ± SEM. Data in C-H were analyzed using two-way ANOVA followed by Bonferroni post hoc test. HE indicates hematoxylin and eosin; DHE, dihydroethidium; WT, floxed control mice; TDP1KO, $CD4^+$ T cell-specific DP1-deficient; M, month; SMA, superior mesenteric arterie; ACH, acetylcholine; TA, thoracic aorta; SNP, sodium nitroprusside.

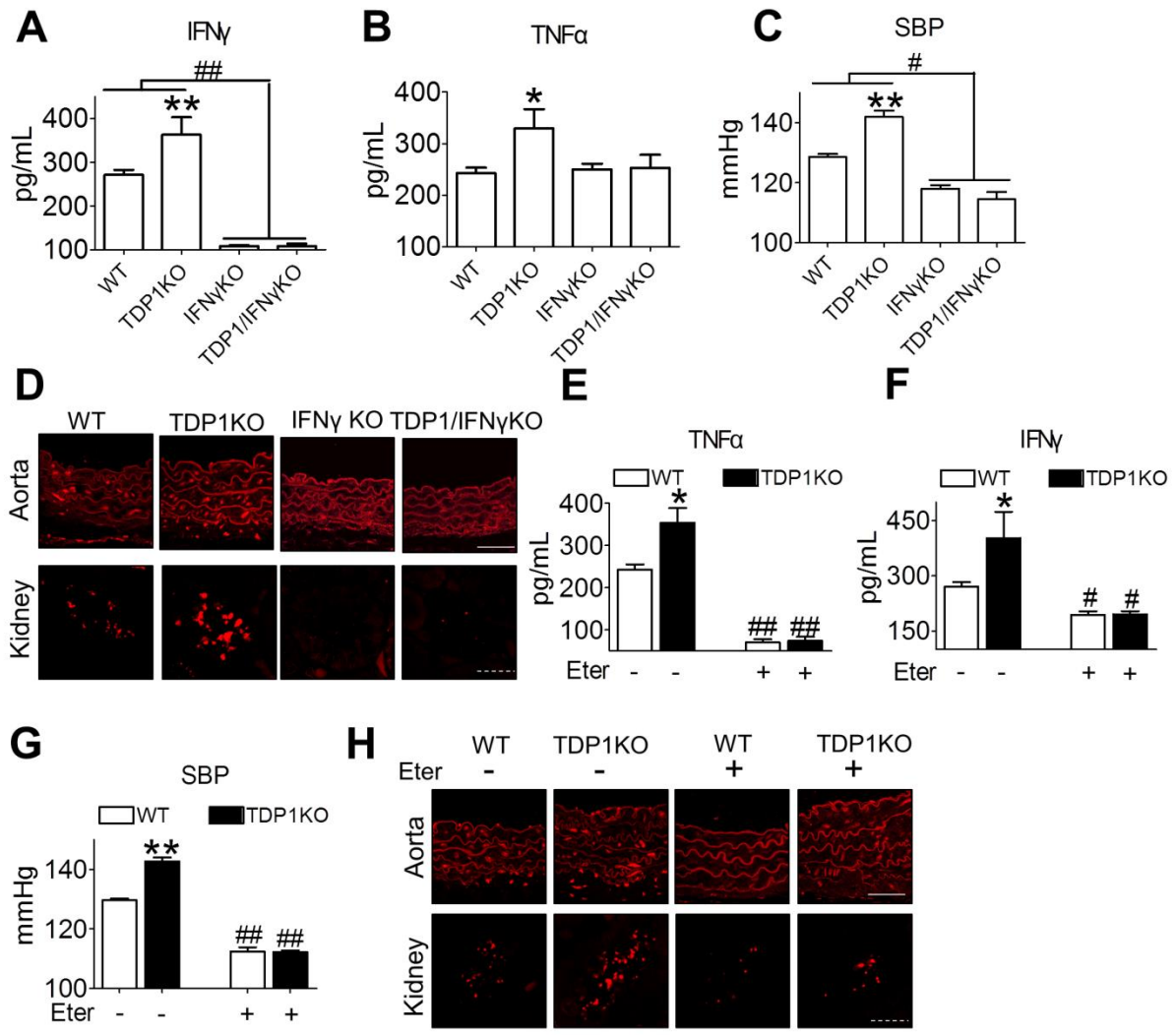
## Supplemental Figure 5




**Supplemental Figure 5. DP1 overexpression in T cells alleviates age-associated increase in arterial thickness, fibrosis and superoxide production in vascular system and kidneys, and aging-induced endothelial dysfunction in vasculature.** (**A** and **B**) Representative HE staining, Sirius Red staining, and immunofluorescence staining of DHE in aortic (**A**) and kidney (**B**) sections from young and aged CD4-DP1-Tg mice. Blue scale bars, 50 μm; white scale bar, 10 μm. (**C** and **D**) Acetylcholine (ACH) dose-response curves of SMAs and TAs from young and aged

CD4-DP1-Tg mice.*$P < 0.05$ compared to indicated (n = 4-6). Data are expressed as mean ± SEM. Data in C and D were analyzed using two-way ANOVA followed by Bonferroni post hoc test. HE indicates hematoxylin and eosin; DHE, dihydroethidium; NTg, not transgenic; Tg, transgenic; DP1, Prostaglandin $D_2$ receptor subtype 1; M, month; SMA, superior mesenteric arterie; ACH, acetylcholine; TA, thoracic aorta.

## Supplemental Figure 6

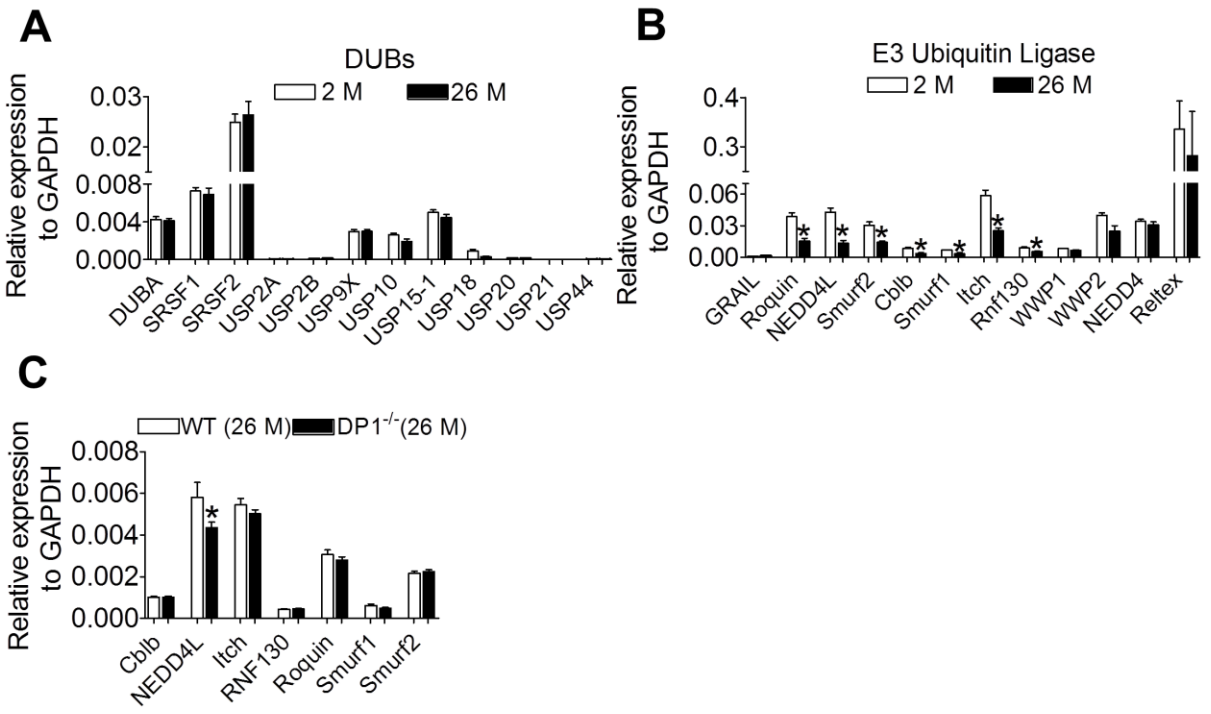



**Supplemental Figure 6. IFNγ deletion or TNFα neutralization ameliorates age-related hypertension in TDP1KO mice.**

(**A** and **B**) Effect of IFNγ deletion on serum levels of IFNγ and TNFα in aged TDP1KO mice (18-month-old). *$P$ < 0.05, **$P$ < 0.01 compared to WT, $^{\#\#}P$ < 0.01 compared to as indicated (n = 5-8). (**C**) Effect of IFNγ deletion on SBP in aged TDP1KO mice . *$P$ < 0.05 compared to WT, $^{\#}P$ < 0.05 compared to as indicated (n = 8-10). (**D**) Representative DHE staining of aortic and kidney sections from aged TDP1 and IFNγ

double KO mice (TDP1/ IFNγKO). Solid scale bar, 50 μm; dotted scale bar, 10 μm. (**E**-**G**) Effect of TNFα neutralization antibody etanercept (Eter) treatment on serum levels of TNFα, IFNγ, and SBP in aged TDP1KO mice (18-month-old) (n = 6-10). *$P$ < 0.05, **$P$ < 0.01 compared to WT. $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 compared to vehicle. (**H**) Representative DHE staining of aortic and kidney sections from Eter-treated 18-month-old TDP1KO mice. Solid scale bar, 50 μm; dotted scale bar, 10 μm. Data were analyzed using two-way ANOVA followed by Bonferroni post hoc analysis. WT indicates floxed control mice; TDP1KO, $CD4^+$ T cell-specific DP1-deficient; KO, knock out; IFNγ, Interferin gama; TNFα, Tumor necrosis factor alpha; SBP, systolic blood pressure; Eter, etanercept.

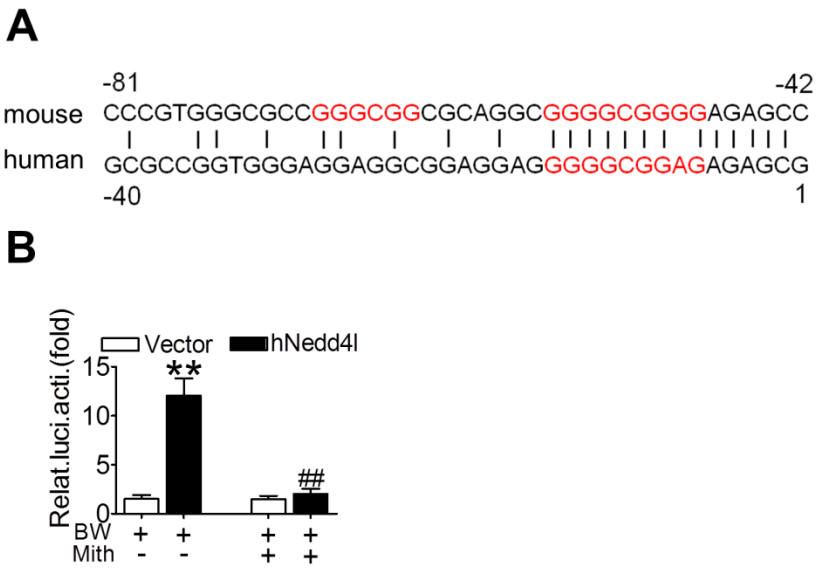
## Supplemental Figure 7




**Supplemental Figure 7. NEDD4L expression is down-regulated in CD4$^+$ T cells during aging.**

(**A** and **B**) Quantitative RT-PCR analysis of the mRNA levels of deubiquitinases (DUB) (**A**) and E3 ligases(**B**) in spleen CD4$^+$ T lymphocytes from WT mice of different ages, **P* < 0.05 compared to 2 months (n = 4-6). (**C**) Effect of DP1 deletion on E3 ligase expression in aged (26 months) WT and DP1$^{-/-}$ CD4$^+$ T cells. **P* < 0.05 compared to WT (n = 4-5). Data are expressed as mean ± SEM. Statistical analysis was performed using unpaired Student's t-test. GAPDH indicates glyceraldehydephosphate dehydrogenase; DUBs, deubiquitinating enzymes; M, month; WT, wild type; DP1$^{-/-}$, DP1 knock out.

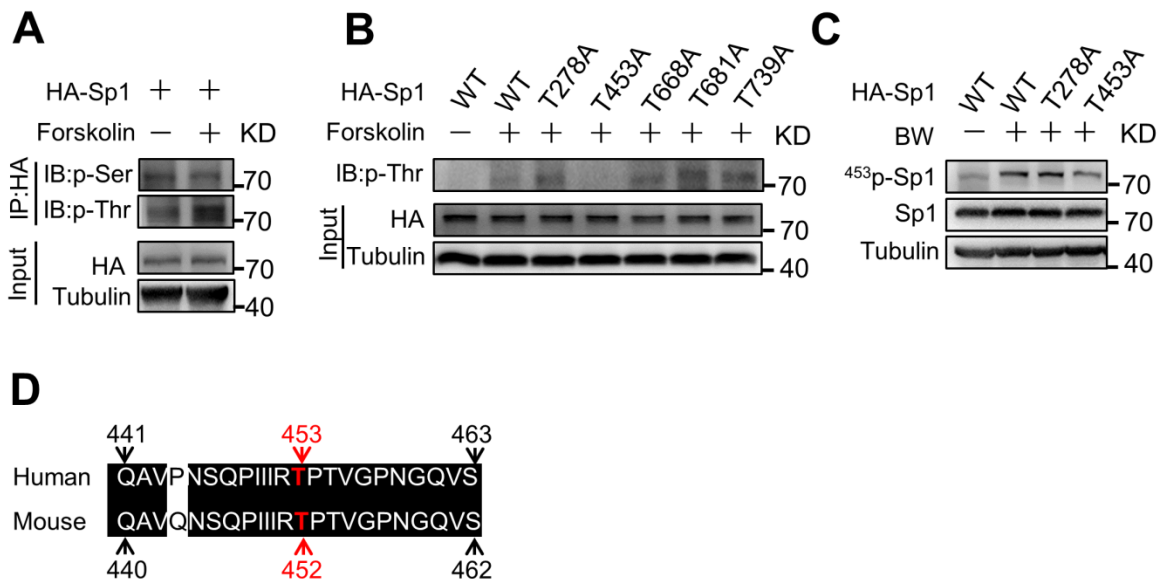
## Supplemental Figure 8




**Supplemental Figure 8. Effect of DP1 activation or Sp1 inhibition on human *NEDD4L* (h*NEDD4L*) promoter region-mediated luciferase activity in EL4 cells.**

(**A**) Alignment of mouse and human NEDD4L promoter region. Red sequences indicate the GC box. (**B**) Effect of BW245C (BW) and Sp1 inhibitor mithramycin A (Mith) on human predicted *NEDD4L* promoter region-mediated luciferase activity, $^{**}P < 0.01$ compared to vehicle. $^{\#\#}P < 0.01$ compared to without Mith (n = 3). Data are expressed as mean ± SEM. Statistical analysis was performed using two-way ANOVA followed by Bonferroni post hoc analysis. NEDD4L indicates neural precursor cell expressed developmentally down-regulated 4-like; hNedd4l indicates human NEDD4L; BW, BW245C, DP1 agonist; Mith, mithramycin A, the Sp1 selective inhibitor.

## Supplemental Figure 9




**Supplemental Figure 9. PKA phosphorylates Sp1 at Threonine (T) 453**.

(**A**) Effect of forskolin on phosphorylation of serine (S) or threonine (T) residues in human Sp1 protein in 293T cells. (**B**) Effect of T to A mutation on forskolin-induced human Sp1 phosphorylation in 293T cells. (**C**) Effect of DP1 agonist BW245C (BW) treatment on human Sp1 phosphorylation in 293T cells. (**D**) Alignment of the conserved threonine (T) site in mouse and human. HA indicates hemagglutinin; Sp1, specificity protein 1; IP, immunoprecipitation; IB,immune bloting; p-Ser, phosphorylated serine; p-Thr, phosphorylated threonine; WT, wild type; T278A, threonine mutanted to alanine; BW, BW245C, DP1 agonist.

## Supplemental References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**2. Hilgers RH, Kundumani-Sridharan V, Subramani J, Chen LC, Cuello LG, Rusch NJ, Das KC. Thioredoxin reverses age-related hypertension by chronically improving vascular redox and restoring enos function. *Sci Transl Med*. 2017;9:eaaf6094**