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Supplemental information

Severe Impairment of TNF Post-transcriptional Regulation Leads to Embryonic Death

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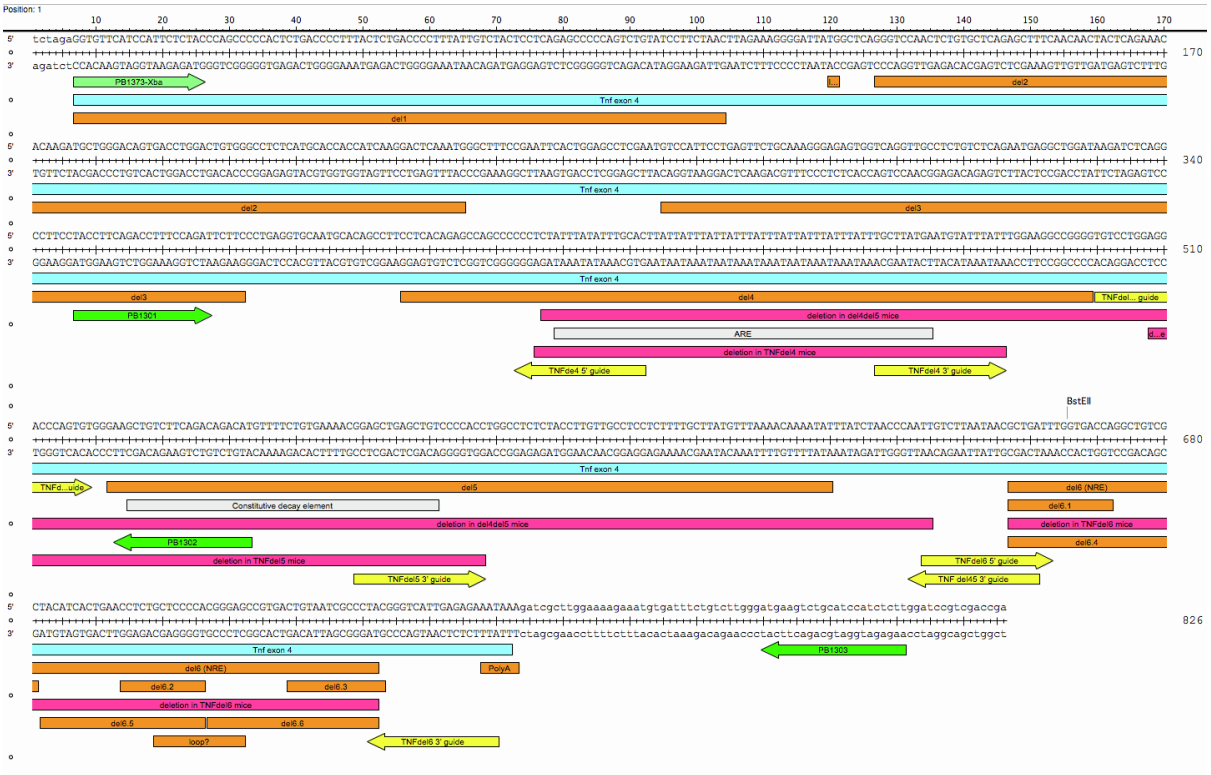

Figure S1. Summary of the deletions that were made in *Tnf* 3'UTR. Related to Figures 1, 2, 3 and 4. The blue box indicates the end of *Tnf* exon 4 (*Tnf* 3'UTR). Grey boxes indicate the ARE and the CDE. Orange boxes refer to the deletions we made in the GFP reporter system and are labelled del1-del6. (Please note that del6 also correspond to the region that we defined as the NRE). Deletions shown by boxes labelled 6.1 to 6.6 indicate smaller deletions within region 6 that were tested in conjunction with del4 to identify a particular motif within the NRE. Yellow arrows indicate the guide RNAs that were used to engineer the deletions in the mice (Please note that the 5' guide used to engineer TNFdel4del5 mice is the same that was used for TNFdel4 mice, marked as TNFdel4 5' guide.
Magenta boxes show the sequences that are actually deleted in each mouse strain, as verified by Sanger sequencing of individual alleles after the founder mice had been crossed with wt C57BL/6 mice (*Tnf* 3'UTR in TNFdel4del6 mice lacks exactly the sequences missing in TNFdel4 and TNFdel6 mice). Green arrows show the primers that are used to genotype mice.

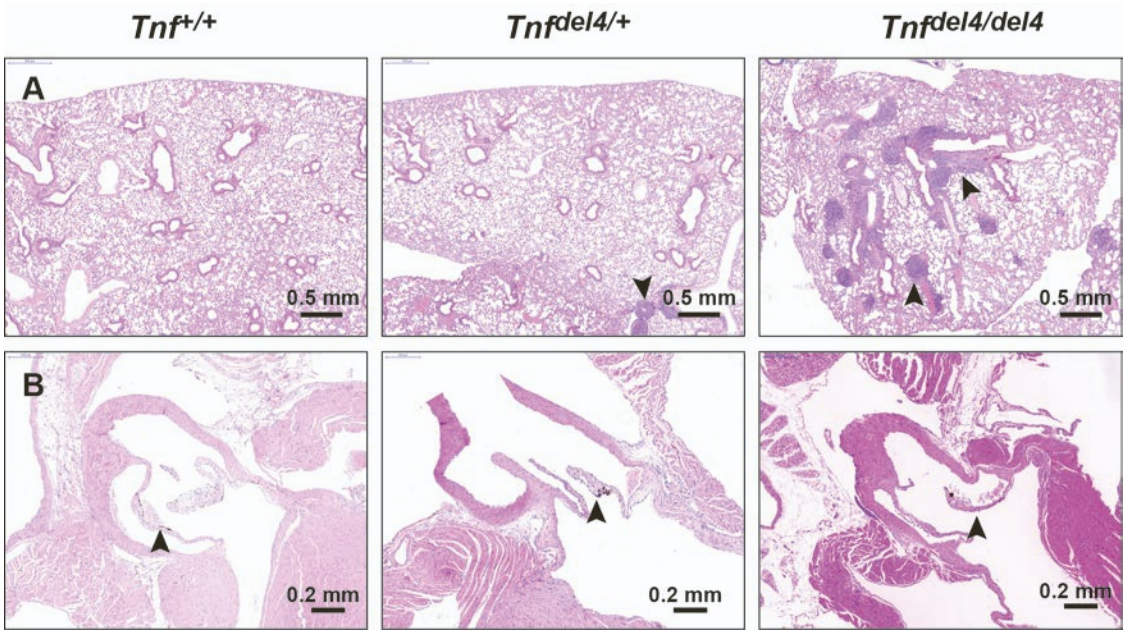

Figure S2. **Phenotype of TNFdel4 mice.** Related to Figure 2. (A) H&E sections showing the presence of iBALT in the lungs of *Tnf*$^{del4/+}$ and *Tnf*$^{del4/del4}$ mice. (B) H&E sections showing the absence of heart valve disease in 200 days-old *Tnf*$^{del4/+}$ and *Tnf*$^{del4/del4}$ mice.

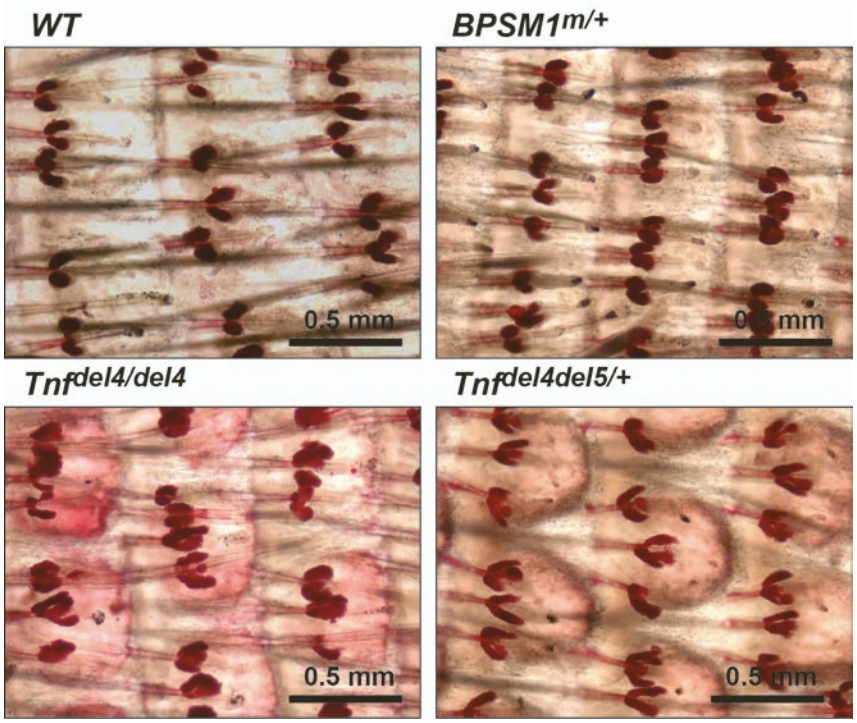

Figure S3. **Increased size of sebaceous glands in *Tnf*$^{del4/del4}$ and *Tnf*$^{del4del5/+}$ mice.** Related to Figure 2 and Figure 3. Oil red O staining of the tail skin shows the increased size and complexity of the sebaceous glands in 8 weeks-old *Tnf*$^{del4/del4}$ and *Tnf*$^{del4del5/+}$ mice.

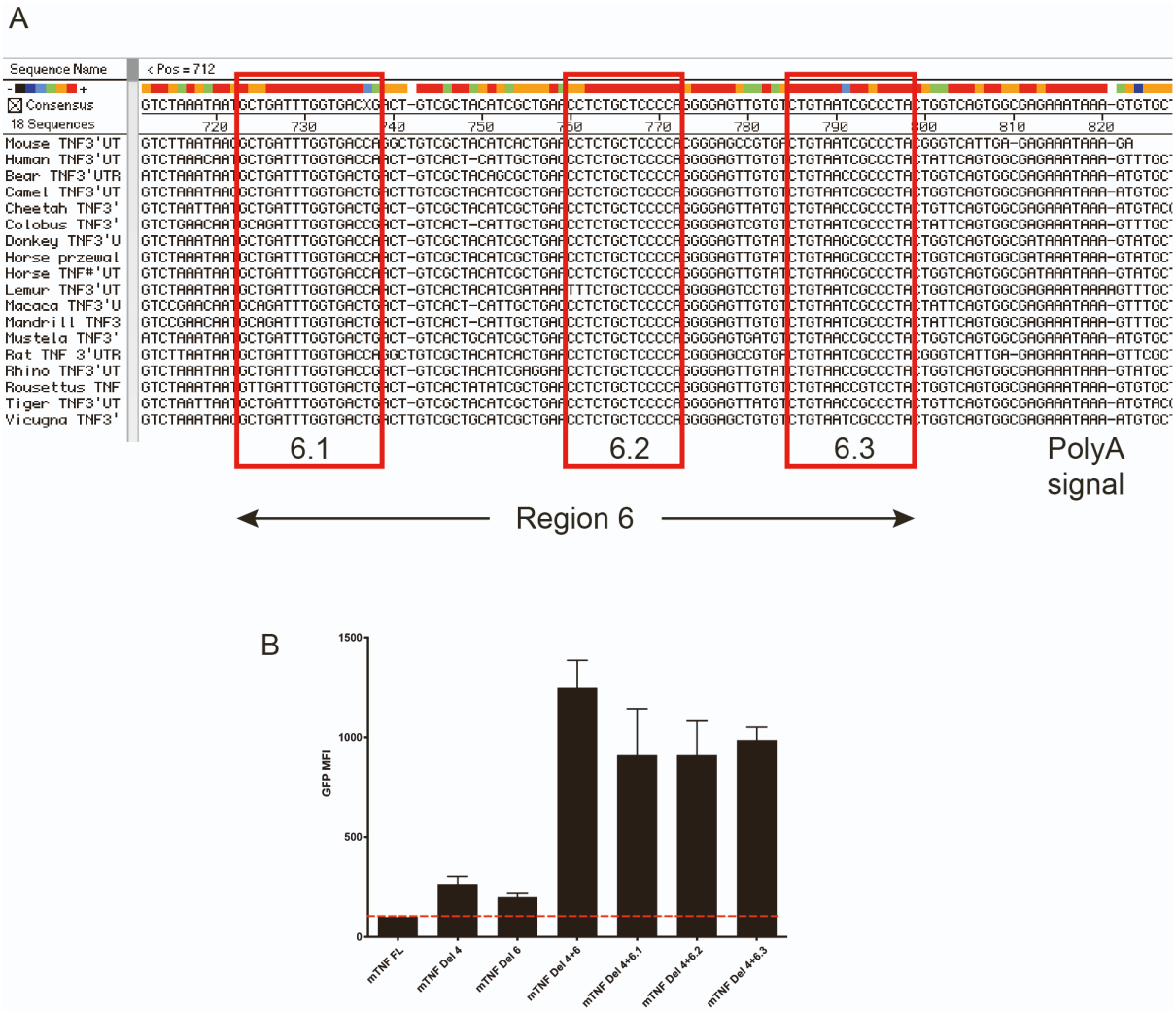

Figure S4. **Deletions of region 4 and conserved elements of region 6 demonstrate the cooperation between these two regions in *Tnf* regulation**. Related to Figure 1 and Figure S1. (A) Alignment of region 6 of *Tnf* 3'UTR from 18 species shows three conserved regions denoted 6.1, 6.2 and 6.3. (Sequences accession numbers: vicugna, XM_006215316.2; tiger, XM_007098808.2; rousettus, XR_001594076.1; rhino, XM_014782979.1; rat, NM_012675.3, mustela putorius, XM_004781005.2; mouse, NM_001278601.1; mandrill, XM_012000466.1; macaca, XM_005553562.2; lemur, XM_012741058.1; human, M10988.1; horse, XM_005603490.1; donkey, XM_014831604.1; colobus, XM_011944682.1; cheetah, XM_015082146.2; camel, XM_006178751.3; bear, XM_008711909.1). (B) GFP reporter constructs with deletion of regions 4+6.1, 4+6.2, 4+6.3 were designed to identify a motif within region 6 that could explain its regulatory effect (a potential stem loop indicated on Figure S1 would be destroyed by the 4+6.2 deletion). Data represent mean ± SEM of two independent experiments.

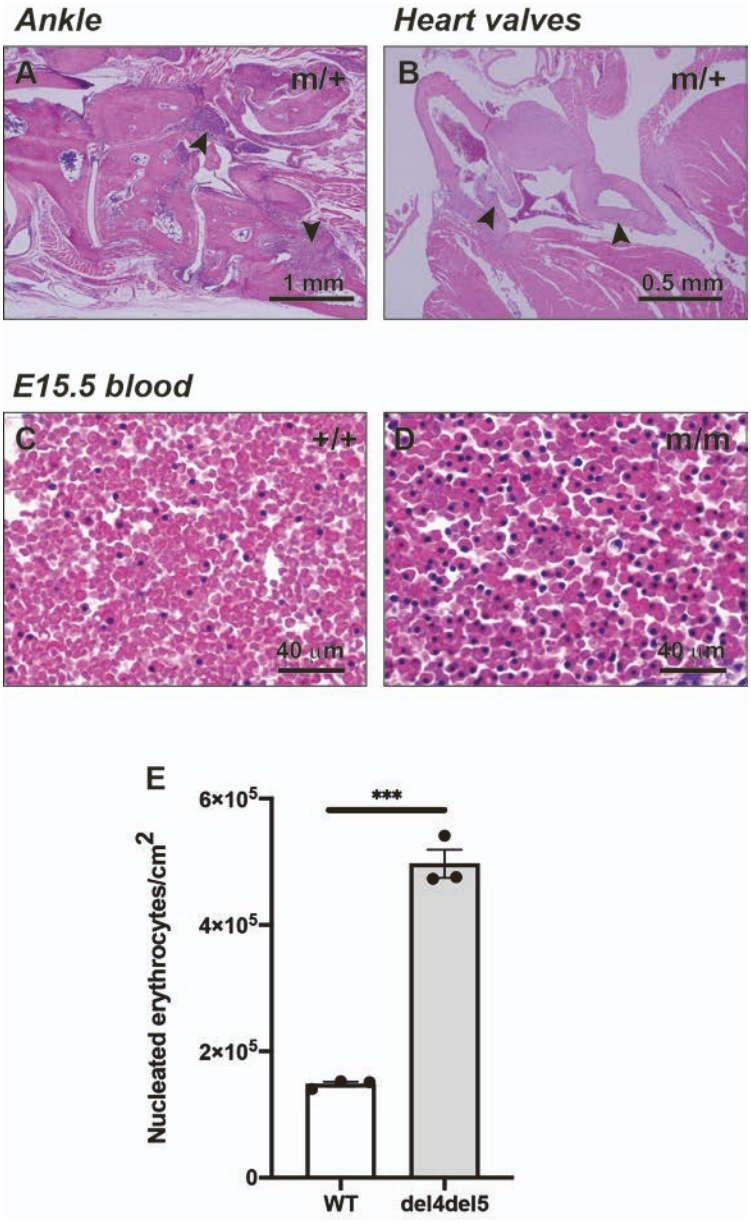

Figure S5. **Additional phenotypes in TNFdel4del5 mice.** Related to Figure 3. H&E sections arthritis (A) and heart valve disease (B). (C, D) H&E staining of blood of E15.5 embryos shows a large proportion of nucleated erythrocytes in homozygote TNFdel4del5 embryo (m/m, D) compared to a WT embryo at the same stage of development (+/+, C). (E) Quantification of nucleated erythrocytes in E15.5 WT (n=3) and TNFdel4del5 homozygote (n=3) embryos. Data are presented as mean ± SEM. Statistical significance was assessed by an unpaired Student's t test. ***, p=0.001.

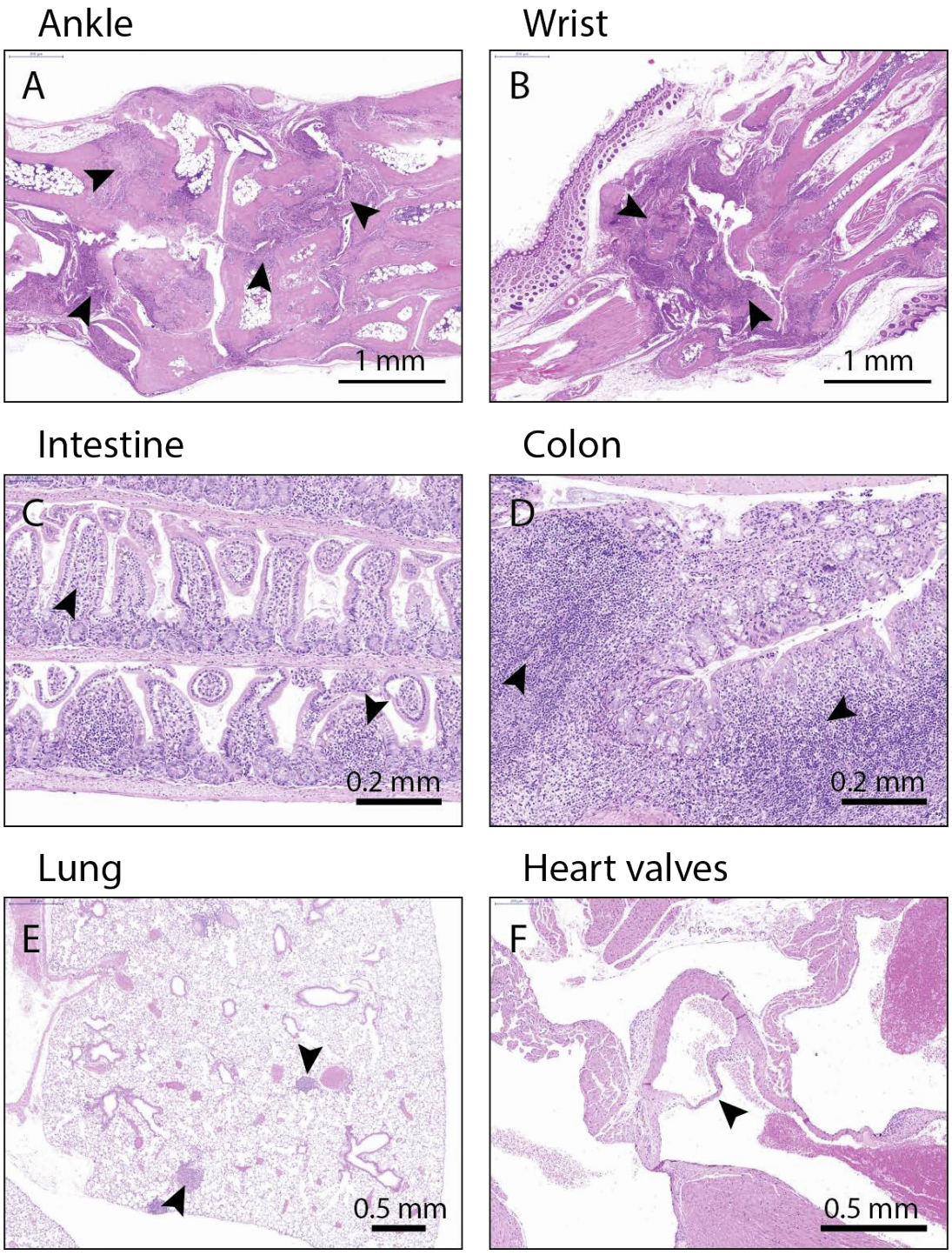

Figure S6. **Loss of one *Tnfr1* allele prevents the embryonic death of *Tnf*$^{del4del5/del4del5}$ mice.** Related to Figure 3. 100 days-old *Tnf*$^{del4del5/del4del5}$/*Tnfr1*$^{+/-}$ animals present with very severe arthritis in the ankles (A) and wrists (B), severe IBD (C, D), as well as iBALT in the lungs (E). Arrowheads indicate pannus tissue in (A) and (B), immune cell infiltration in (C) and (D), and iBALT in (E). 100 days-old *Tnf*$^{del4del5/del4del5}$/*Tnfr1*$^{+/-}$ animals do not develop heart valve (arrowhead) disease (F).

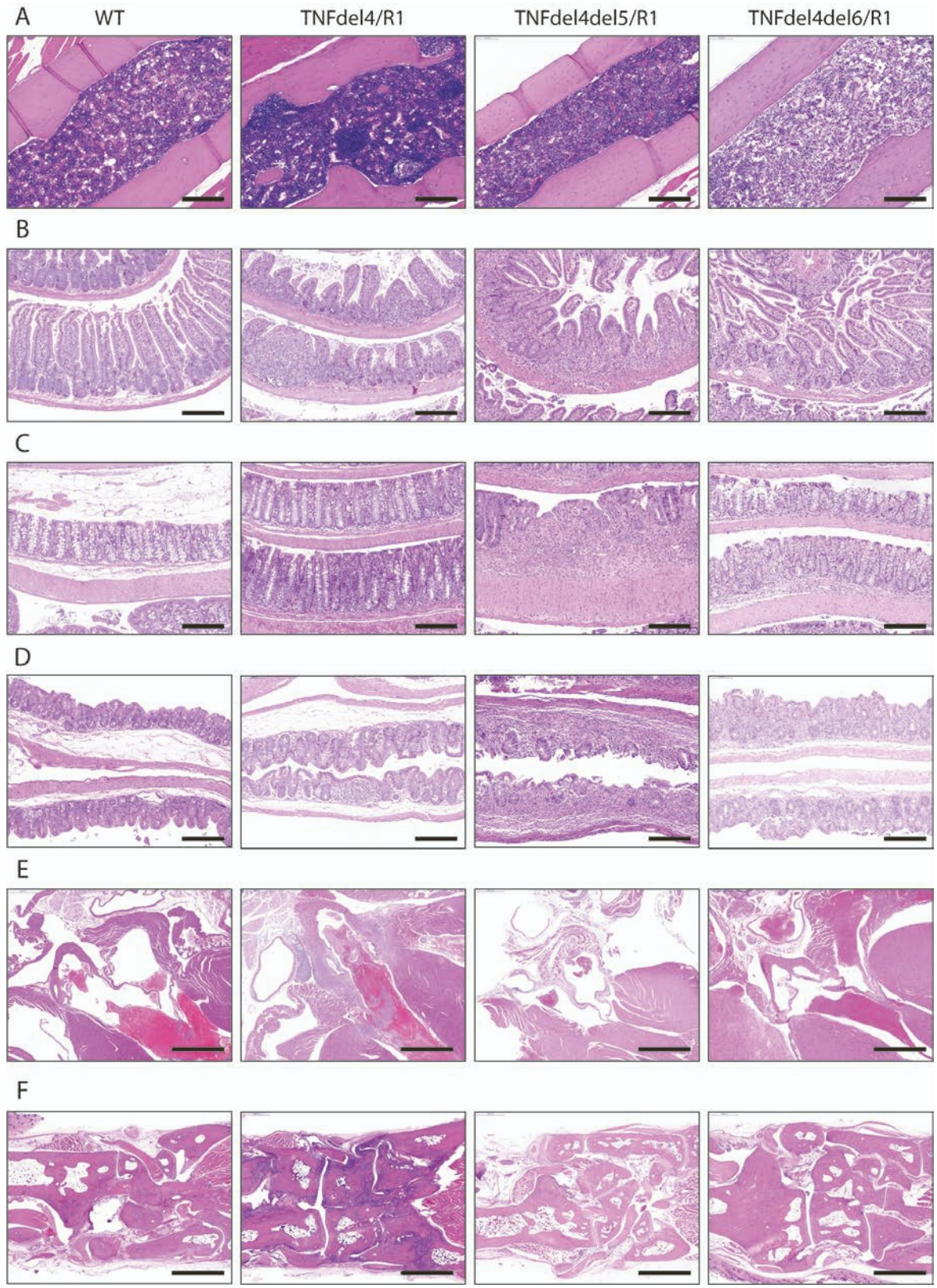

Figure S7. **Hematopoietic reconstitution.** Related to Figure 5. H&E-stained sections through bone (A), intestine (B), colon (C), caecum (D), heart valves (E) and ankle (F) of lethally irradiated WT C57BL/6 recipient mice transplanted with 1-2 10^6 bone marrow cells from WT, *Tnf*$^{del4/del4}$*/Tnfr1*$^{-/-}$ (TNFdel4/R1), *Tnf*$^{del4del5/del4del5}$*/Tnfr1*$^{-/-}$ (TNFdel4del5/R1) or *Tnf*$^{del4del6/del4del6}$*/Tnfr1*$^{-/-}$ (TNFdel4del6/R1) donors (n=5 recipients for each BM genotype). Recipients of WT and TNFdel4/R1 BM cells were examined 5 months post transplantation. Recipients of TNFdel4del5/R1 BM cells became sick and were examined 21 days

post transplantation. Recipients of TNFdel4del6/R1 BM cells failed to reconstitute their hematopoietic system and were examined 9 days post transplantation. Bars: 0.2mm in A, B, C and D; 0.5 mm in E and F.

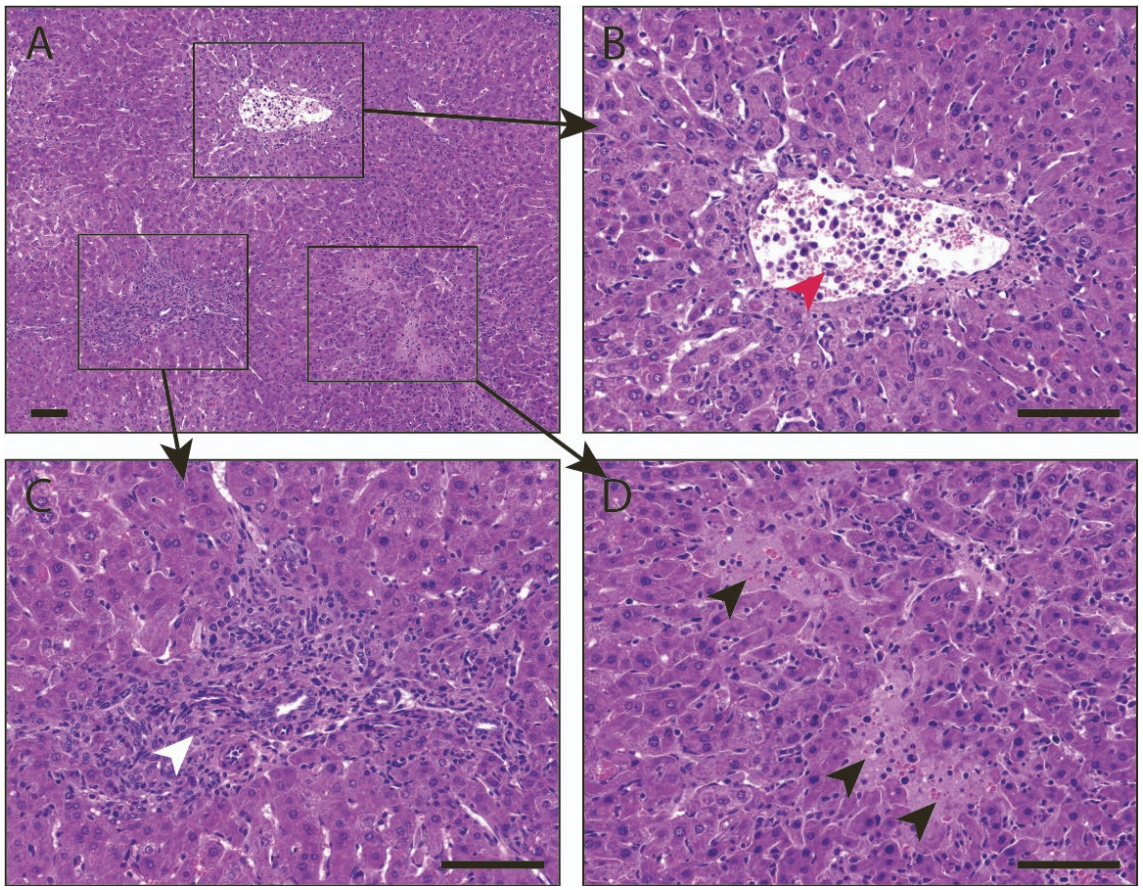

Figure S8. **Acute liver necrosis in the recipients of TNFdel4del6/R1 BM cells.** Related to Figure 5 and Figure S7. (A) H&E-stained section of the liver of a lethally-irradiated WT recipient of TNFdel4del6/R1 BM cells shows the presence of blood vessels filled with the remnants of dead hepatocytes (B, red arrowhead), perivascular infiltrates of immune cells (C, white arrowhead) and necrotic zones devoid of live cells (D, black arrowheads). n=5 recipients of TNFdel4del6/R1 BM cells. Bars: 100μm in all panels.

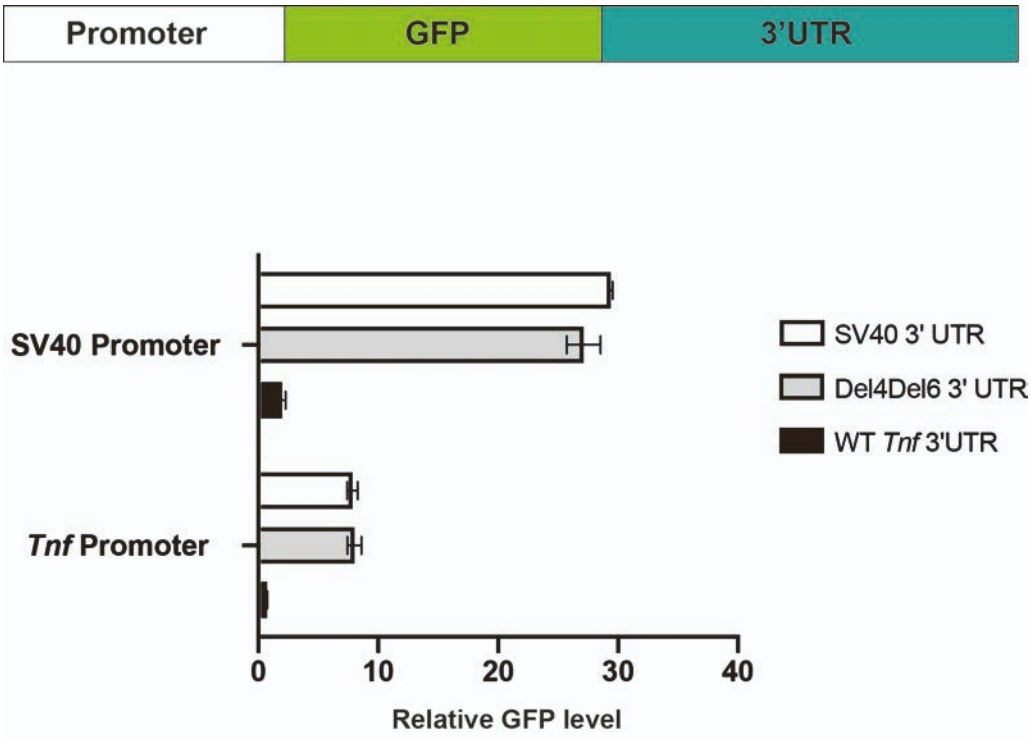

Figure S9. ***Tnf* promoter is constitutively active in HEK293 cells.** Related to Figure 1. Schematic representation of the GFP reporter constructs used to assay the activity of *Tnf* promoter in HEK293 cells and relative GFP levels following transient transfection of HEK293 cells. Data represent mean ± SD from 3 independent experiments.

Transparent Methods

Mice. All animal experiments were conducted with the approval of the Animal Ethics Committee of the Walter and Eliza Hall Institute. BPSM1 mice were the result of a spontaneous mutation. Deletions within *Tnf* 3'UTR were engineered with CRISPR/Cas9 technology {Wang, 2013 #22927} using the protocol described in {Kueh, 2017 #22912}. Guide RNAs used for each strain are shown in Figure S1. The deletion in each mutant allele was determined after crossing founder mice with wildtype C57BL/6 mice for one generation. Actual deletion present in each strain is shown in Figure S1 and Table S1. Conclusions were reached after examining at least ten mice of each genotype by histology. All mice were on the C57BL/6 genetic background. The age or developmental stage of all mice used is provided in the figure legends.

TNF 3'UTR reporter assays. GFP reporter constructs engineered as in (Lacey et al. 2015) contained an SV40 early promoter driving the expression of eGFP. Murine *Tnf* 3'UTR (WT, BPSM1-derived, or containing deletions of regions 1-6 as indicated) were inserted between the Xba1 and BamH1 sites (complete sequences upon request). HEK293 cells were transiently transfected using Fugene 6 (Promega) with GFP-*Tnf*3'UTR reporter constructs and a pGL3-mCherry control construct, and analyzed 3 days later using flow cytometry on a LSR IIW (BD Biosciences). GFP Mean fluorescence intensity was calculated on live mCherry-positive cells and compared to empty vector control.

TNF ELISA. Secreted TNF was measured from mouse serum using the TNF alpha mouse uncoated ELISA kit (ThermoFisherScientific) and read using the Chameleon plate reader (Hidex, Turku, Finland).

Bone marrow transplantation experiments. Wildtype C57BL/6 recipients were lethally irradiated (2x 550 rad) and injected with 1-2 x 10^6 BM cells.

Imaging

For routine histology, tissue samples were fixed in 10% neutral buffered formalin for 24 hours, paraffin embedded, and 5μm-thick sections were generated and stained with haematoxylin and eosin (H&E).

For cleaved-caspase 3 immunohistochemistry (IHC), unstained sections were dewaxed in xylene and subjected to antigen retrieval at low pH with boiling citrate buffer (97°C) for 30 minutes. Sections were then blocked from endogenous biotin and peroxidase, incubated with anti-CC3 (9661, Cell Signaling Technology, Danvers, MA) for 1 hour at room temperature followed by goat anti-rabbit biotinylated secondary (BA-100, Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. Finally, sections were stained using the HRP-Avidin-Biotinylated Complex (PK-6100, Vector laboratories, Burlingame, CA), developed with DAB peroxidase substrate (SK-4100, Vector laboratories, Burlingame, CA) and counterstained with haematoxylin.

Oil red O staining. Tail skin was removed using blade and forceps, cut in equal pieces and incubated in PBS/EDTA 5mM overnight at 37°C. Epidermal sheets were carefully separated from the dermis using forceps, fixed in 4% PFA for 20 minutes at room temperature, washed twice in PBS (5 min each), then washed in 60% isopropanol for 5 min. Samples were stained in Oil-red-O solution (0,5% Oil red O in isopropanol, then diluted 3:2 with H2O, and filtered) for 1hr at room temperature. Samples were gently washed twice in 60% isopropanol, then rinsed in PBS before mounting using a gelatin-based mounting media (in-house: 50% glycerol, 3.5% gelatin in H2O).

All slides were scanned on a 3DHISTECH Pannoramic Scan II scanner and analysed with Case Viewer 2.2.1 for Mac.

Light sheet microscopy imaging. E15.5 embryo paws were cleared using passive clarity (PACT; (23)). In brief, embryos were harvested and fixed overnight in 4% paraformaldehyde in PBS, pH 7.4. The paws were resected and immersed in PACT monomer solution containing 4% acrylamide (Biorad) and Polymerization Thermal Initiator VA044 (0.25% w/v final concentration; Wako) in PBS overnight at 4dC. The gel was set for 3hrs at 37°C and the tissue was moved into borate-buffered clearing solution (8% (wt/vol) SDS and 50mM sodium sulphite in 0.2M boric acid buffer, pH 8.5). Once cleared, the tissue was washed by repeatedly diluting the clearing solution (1:1) with borate-buffered wash solution (1% TritonX-100 SDS and 50mM sodium sulphite in 0.2M boric acid buffer, pH 8.5) to gradually remove the SDS. The paws were mounted in 1% low melting point agarose and immersed in EasyIndex solution (LifeCanvas) for refractive index matching.
The tissue was imaged using a Zeiss Z.1 light sheet microscope equipped with a 5x/0.16 detection objective. Blood vessels were detected using hemoglobin autofluorescence excited with the 405nm laser line and detected using a 500-545nm GFP band pass filter (24). Multiview data sets were acquired at 120-degree angles and fused using the Multiview Reconstruction plugin in FIJI (25). 3D reconstruction was performed in Imaris (Bitplane).

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was assessed by unpaired Student's t test.

Data and Software availability

The reference *Tnf* sequence is Genbank Accession: NM_013693.3, GI: 518831586. The sequences of the *Tnf* 3'UTRs found in our mutants have been deposited in Genbank: MW116778 (TNFdel4), MW116779 (TNFdel5), MW116780 (TNFdel6), MW116781 (TNFdel4del5), MW116782 (TNFdel4del6).

Mouse	Sequence deleted	Guide RNAs
TNFdel4	tctatttatatttgcacttattatttattatttatttattatttatttgcttatgaatgtatttatt	gtgcaaatataaatagaggg tgcttatgaatgtatttatt
TNFdel5	aggacccagtgtgggaagctgtcttcagacagacatgttttctgtgaaaacggagctgagct gtccccacc	tgtcctggaggacccagtgt ggagctgagctgtccccacc
TNFdel6	gctgatttggtgaccaggctgtcgctacatcactgaacctctgctccccacgggagccgtga ctgtaatcgcccta	ttgtcttaataacgctgatt atttctctcaatgacccgta
TNFdel4del5	ctatttatatttgcacttattatttattatttatttattatttatttgcttatgaatgtatttatttgg aaggccggggtgtcctggaggacccagtgtgggaagctgtcttcagacagacatgttttctg tgaaaacggagctgagctgtccccacctggcctctctaccttgttgcctcctcttttgcttatgttt aaaacaaaatatttatctaacccaatt	gtgcaaatataaatagaggg tcagcgttattaagacaatt
TNFdel4del6	tctatttatatttgcacttattatttattatttatttattatttatttgcttatgaatgtatttatt and gctgatttggtgaccaggctgtcgctacatcactgaacctctgctccccacgggagccgt gactgtaatcgcccta	gtgcaaatataaatagaggg tgcttatgaatgtatttatt and ttgtcttaataacgctgatt atttctctcaatgacccgta

Table S1. **Engineered deletions in *Tnf* 3' UTR.** Related to Figure 1 and Figure S1. This table lists the guide RNAs that were used to engineer the deletions in *Tnf* 3'UTR using CRISPR and the actual deletion in each mouse strain as determined by sequencing of the mutated alleles.