

Supplemental information

Setdb2 Regulates Inflammatory Trigger-Induced Trained Immunity of Macrophages Through Two Different Epigenetic Mechanisms

This file includes Supplemental figures S1-S6, figure legends and Table S1

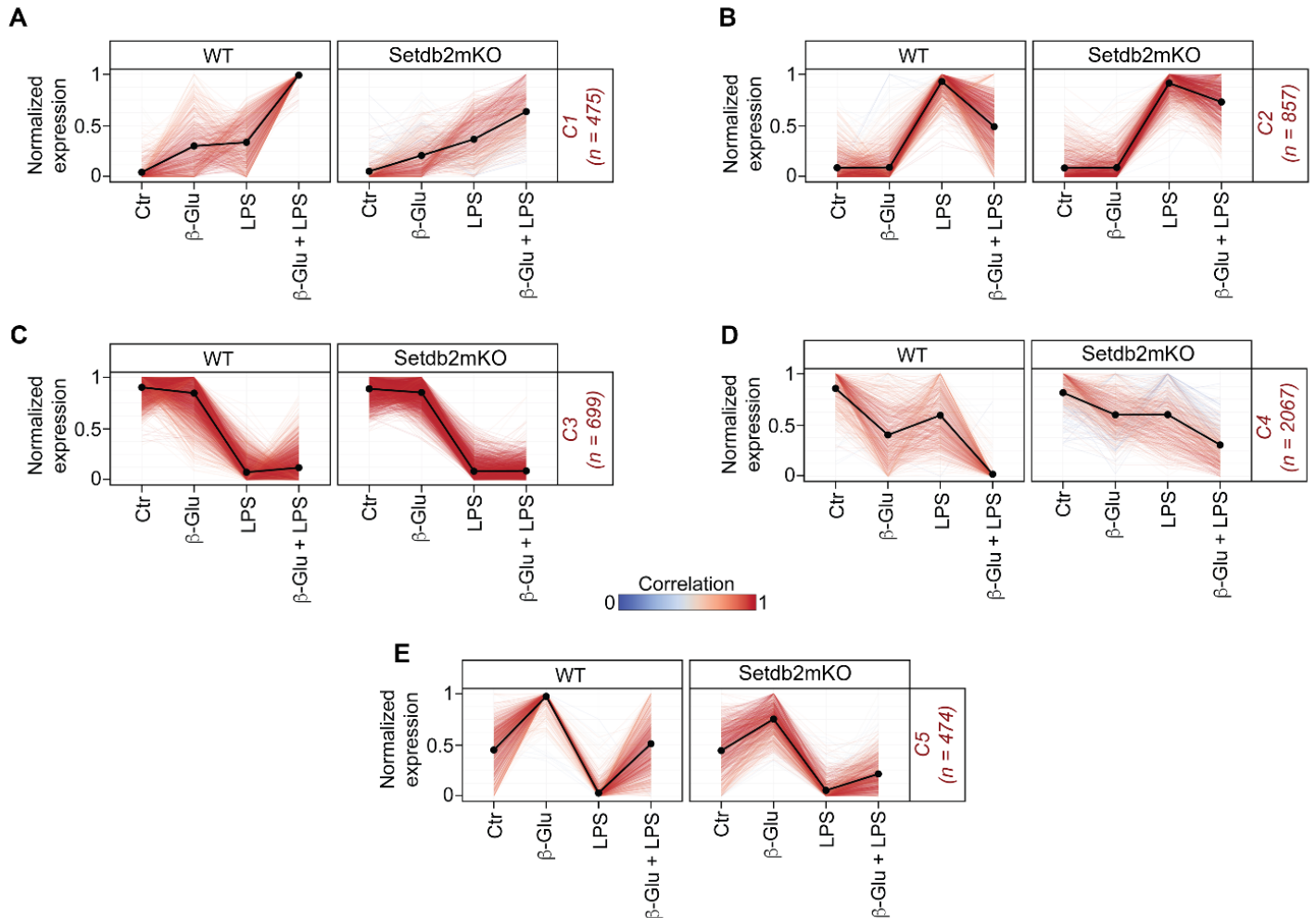


Figure S1. Hierarchical clustering analysis in β -glucan-induced trained immunity of BM-macrophages (Related to Figure 2)

(A-E) Line plots showing k-means ($k = 5$) and hierarchical clustering ($n = 3$) for genes expressed in untreated control, β -glucan alone, LPS alone, and β -glucan followed by LPS restimulated BM-macrophages from WT ($n = 3$) and Setdb2mKO mice ($n = 3$).

Statistical analysis for RNA-seq data was performed using DESeq2 with its default settings. Data are represented as mean \pm SD. 'n' represents biological replicates of each strain and treatment type. p-value was calculated using two-way ANOVA with Tukey post-hoc test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, ns, not significant change.

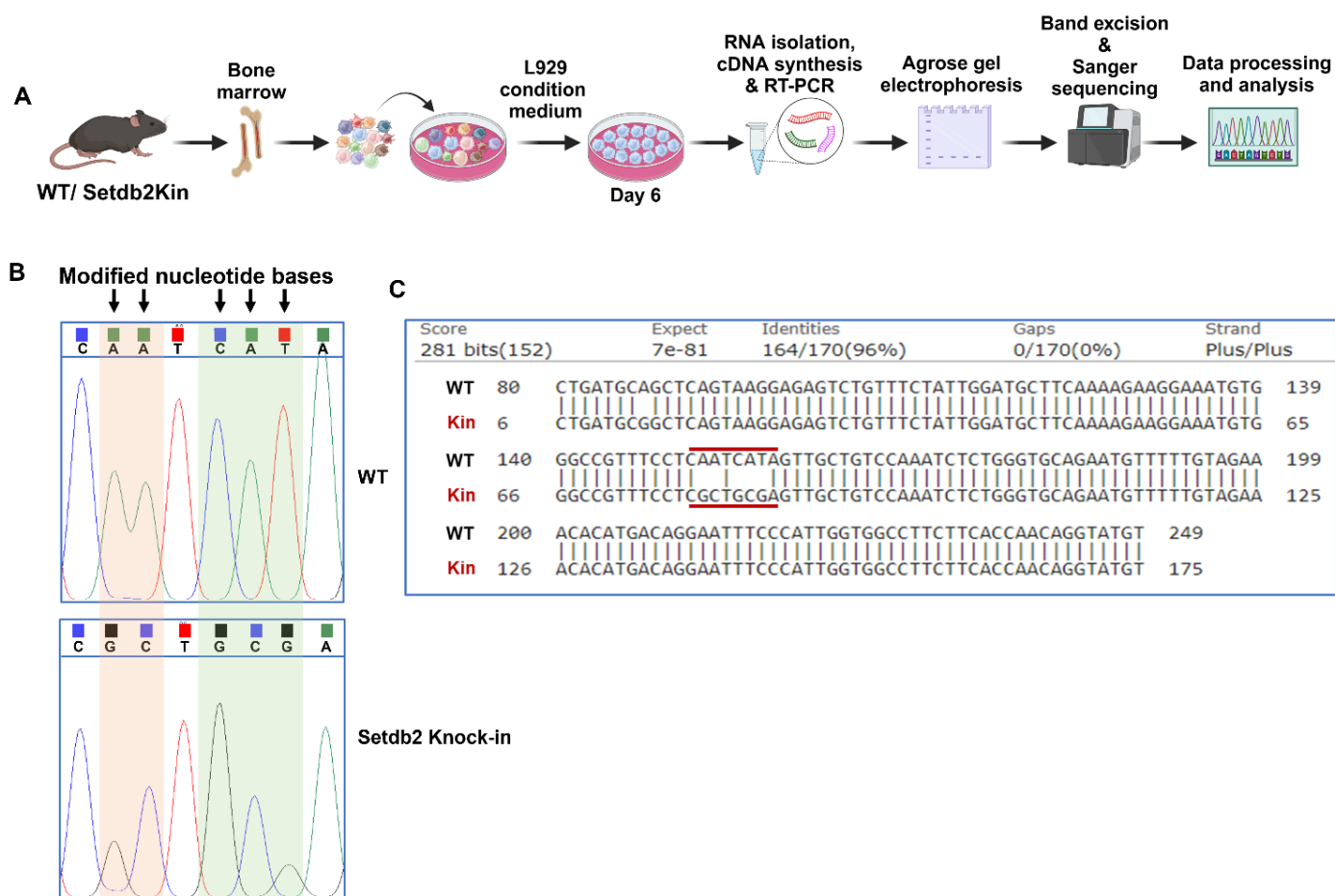


Figure S2. Setdb2Knock-in validation through sanger sequencing in BM-macrophages (Related to Figures 4 and S6)

(A) Work flow of Setdb2Kin confirmation. Bone marrow cells were isolated from WT and Setdb2Kin mice and differentiated with L929 condition medium for 6 days. Total RNA was isolated followed by cDNA synthesis. RT-PCR was performed with sequence specific primers (Table-S1) and products were visualized through agarose gel electrophoresis. Bands of interest were excised from the gel followed by purification and sanger DNA sequencing.

(B) Sequence chromatograms depicting targeted nucleotide bases in WT (top), and Setdb2Kin (bottom).

(C) Blast search display showing targeted nucleotide sequences in WT and Setdb2Kin mice.

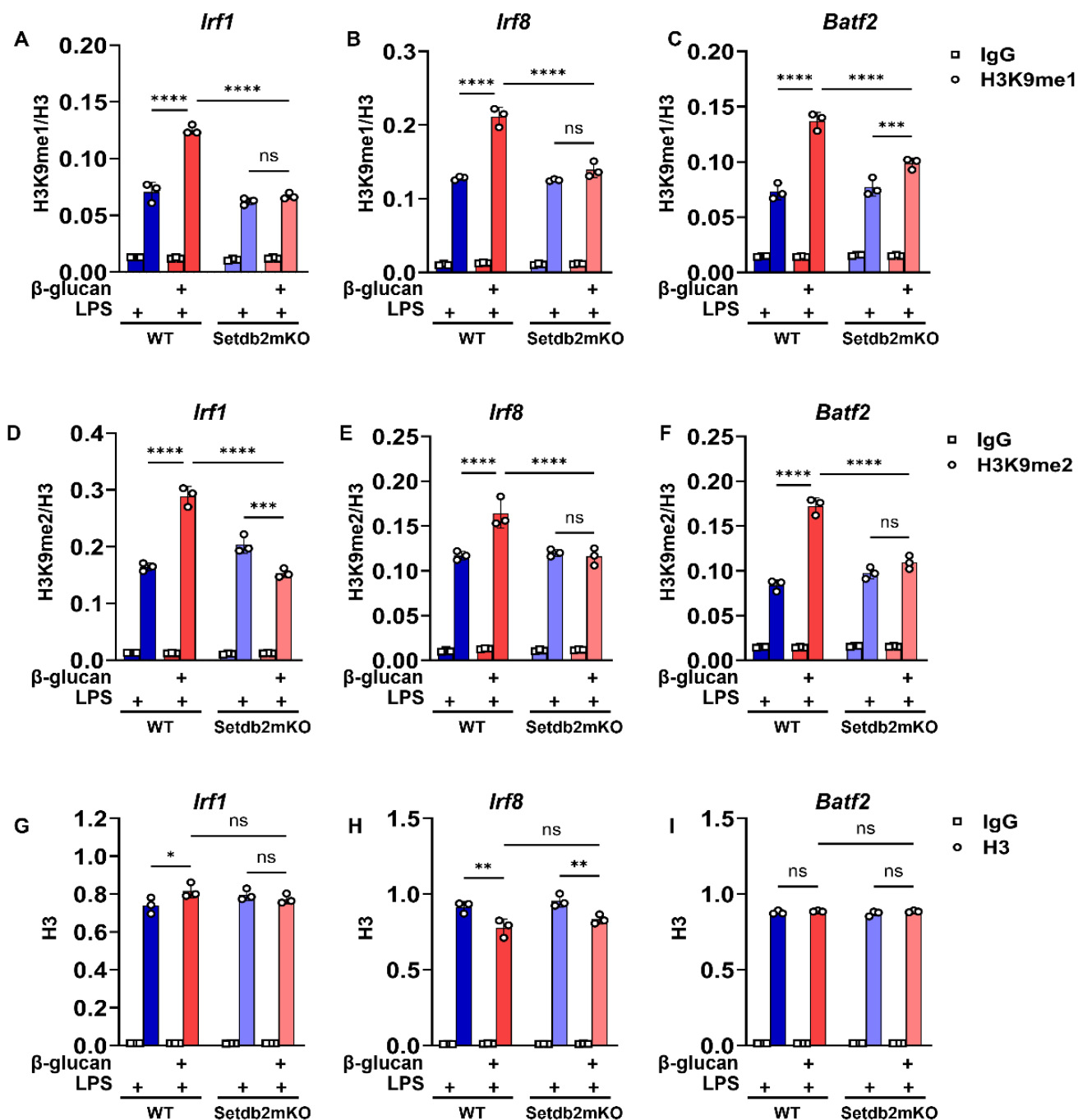
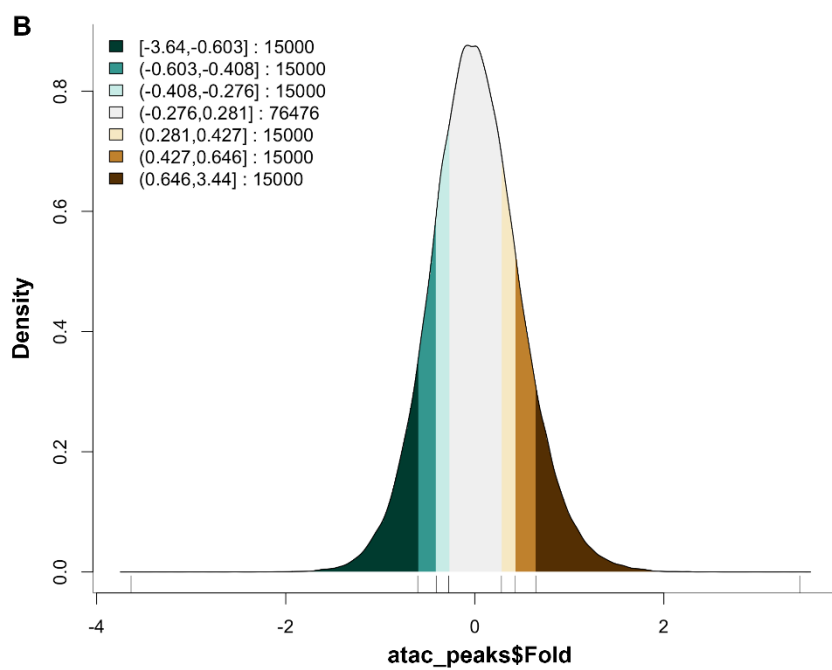
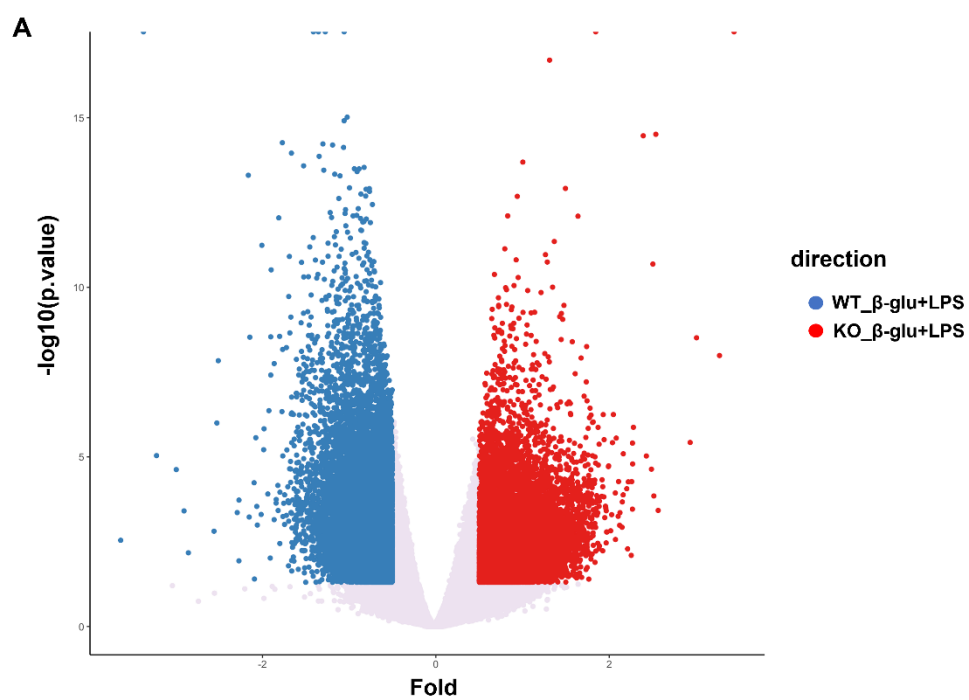


Figure S3. H3K9me levels in β -glucan trained WT and Setdb2mKO BM-macrophages (Related to Figure 5)

Bone marrow cells were treated as detailed in the legend of Figure 5 followed by ChIP-qPCR analysis.

(A-C) H3K9me1 (D-F) H3K9me2 and (G-I) H3 levels at *Irf1*, *Irf8*, and *Batf2* promoters in LPS alone or β -glucan followed by LPS restimulated BM-macrophages from WT (n = 3) and Setdb2mKO mice (n = 3).

Data are represented as mean \pm SD. 'n' represents biological replicates of each strain and treatment type. p-value was calculated using two-way ANOVA with Tukey post-hoc test for multiple comparisons. *p < 0.05, **p < 0.01, and ***p < 0.001, ns, not significant change.



C

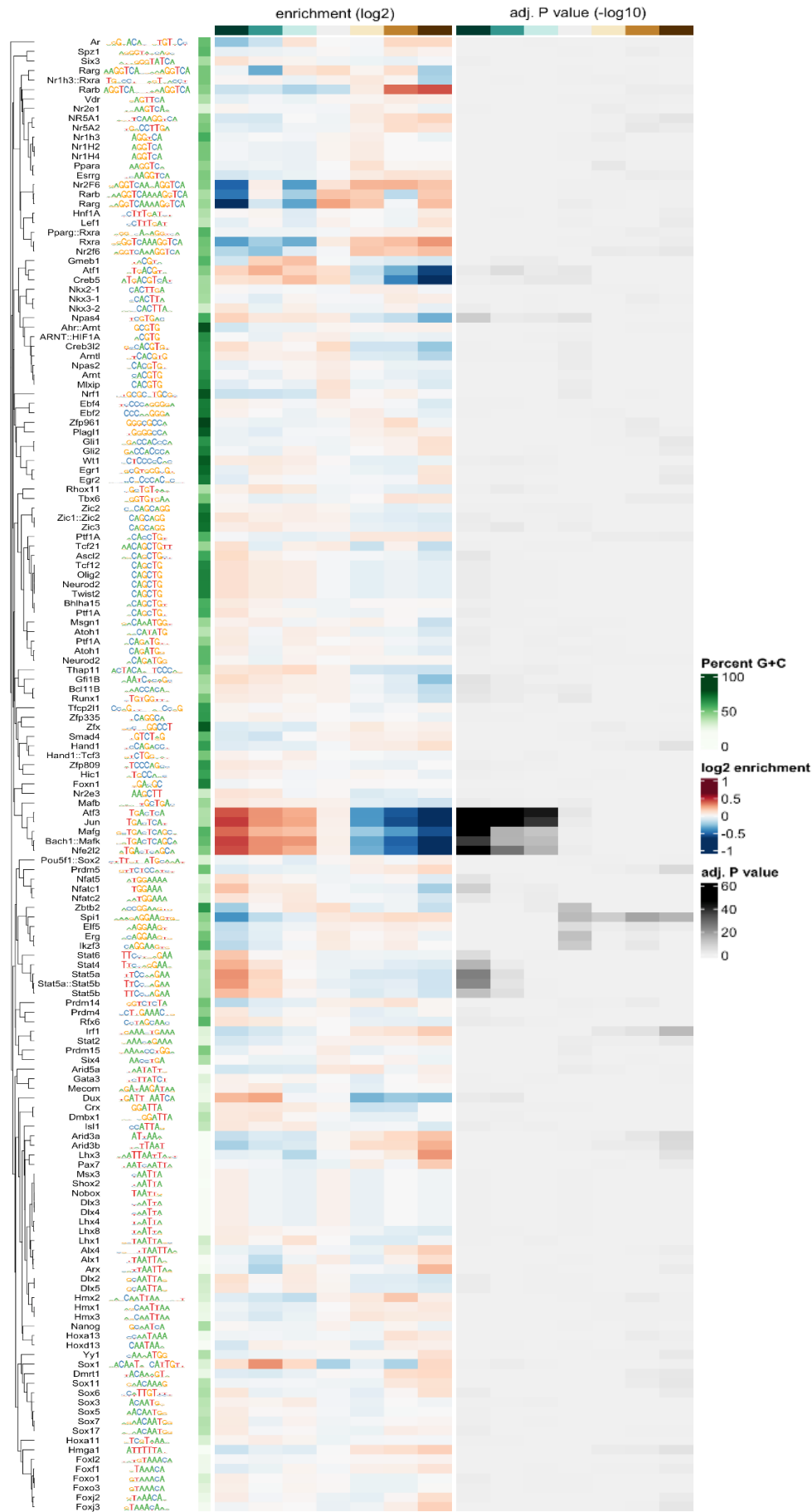
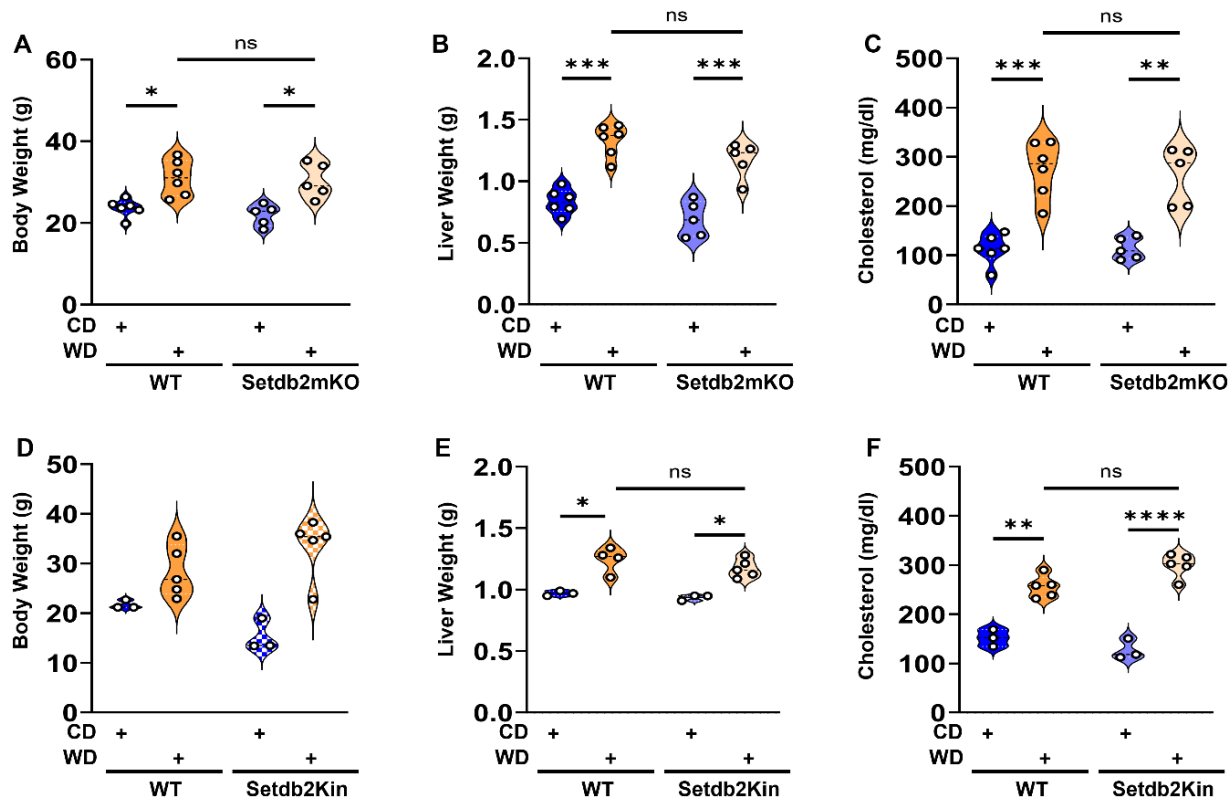


Figure S4. ATACseq analysis in β -glucan trained WT and Setdb2mKO BM-macrophages (Related to Figure 5)

Bone marrow cells were treated with 5 μ g/mL β -glucan (training stimulus) or culture medium for 24 h, cultured for 5 days, and restimulated with 100ng/mL LPS (secondary challenge) or culture medium for 6 h on day 6, followed by ATACseq profiling and analysis.

(A) Volcano plot representing differential peaks in samples from BM-macrophages treated with β -glucan followed by LPS stimulation from WT (n = 3) and Setdb2mKO mice (n = 3). (B, C) Top enriched JASPAR motifs of differential peaks stratified by fold-change using MonaLisa (MOTif aNALysis with Lisa) plot.

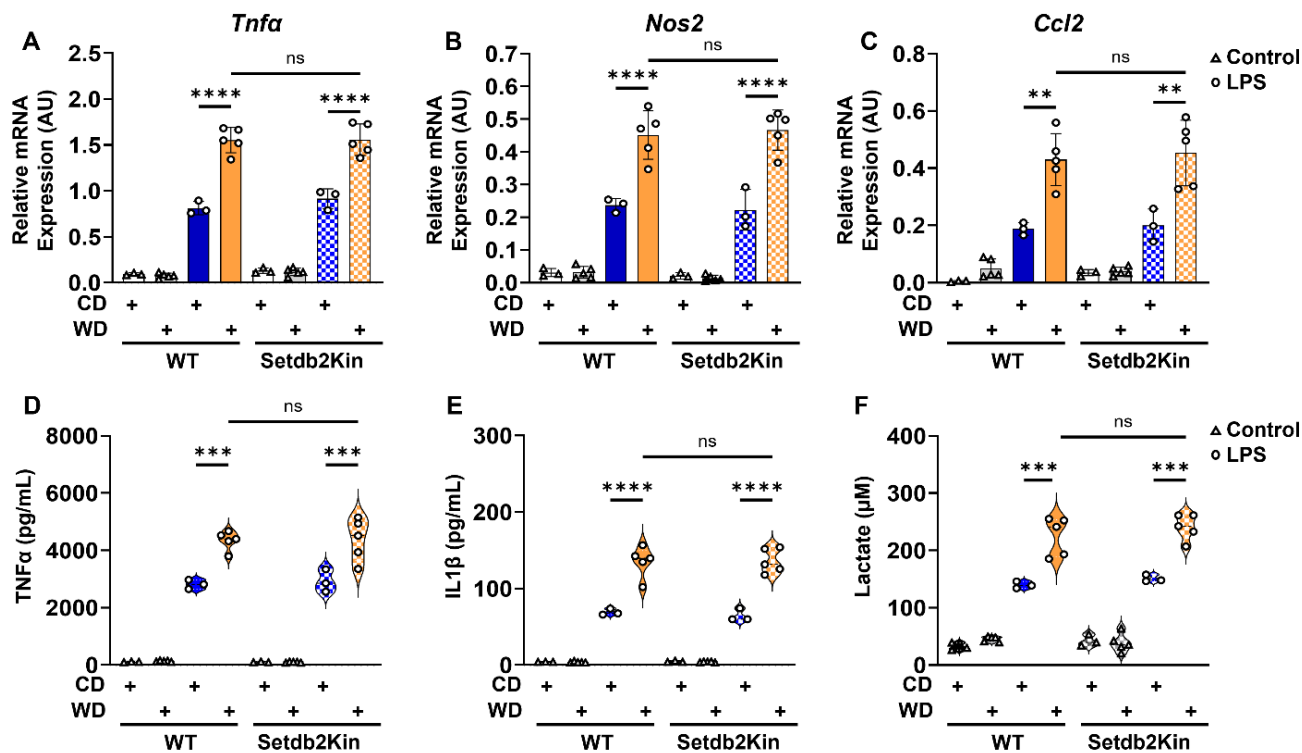
For ATAC-seq data, differential peak accessibility was analyzed using DiffBind with DESeq2, employing default parameters. Data are represented as mean \pm SD. 'n' represents biological replicates of each strain and treatment type. p-value was calculated using two-way ANOVA with Tukey post-hoc test for multiple comparisons. *p < 0.05, **p < 0.01, and ***p < 0.001, ns, not significant change.

**Figure S5. Whole body assessment of control vs 'Western-diet' fed WT, Setdb2mKO and Setdb2Kin mice (Related to Figure 6 and S6)**

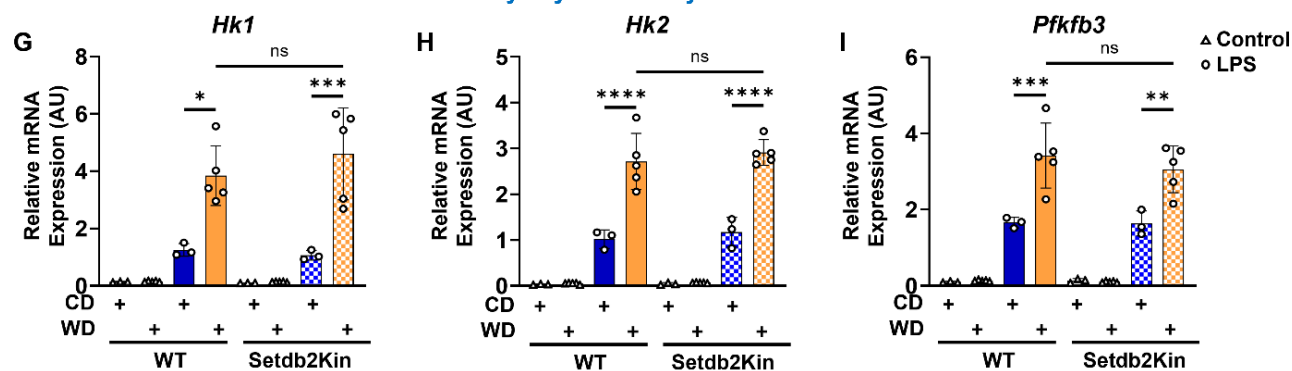
Mice were fed with western-diet or control-diet (training) for 20 weeks followed by estimation of body weight, liver weight and serum total cholesterol levels.

(A) Whole body weight (B) liver weight (C) and serum total cholesterol levels in control diet and western diet fed WT vs. Setdb2mKO mice. Control diet WT (n = 6), western diet WT (n = 6), control diet Setdb2mKO (n = 5), and western diet Setdb2mKO mice (n = 5).

(D) Whole body weight (E) liver weight (F) and serum total cholesterol levels in control diet and western diet fed WT vs. Setdb2Kin mice. Control diet WT (n=3), Control diet Setdb2Kin (n=3), Western diet WT (n=5), and Western diet Setdb2Kin mice (n=5). Data are represented as mean \pm SD. 'n' represents biological replicates of each strain and treatment type. p-value was calculated using two-way ANOVA with Tukey post-hoc test for multiple comparisons. *p < 0.05, **p < 0.01, and ***p < 0.001, ns, not significant change.



Cluster-1 "Glycolytic Pathway" Genes



Cluster-2 "Interferon Pathway" Genes

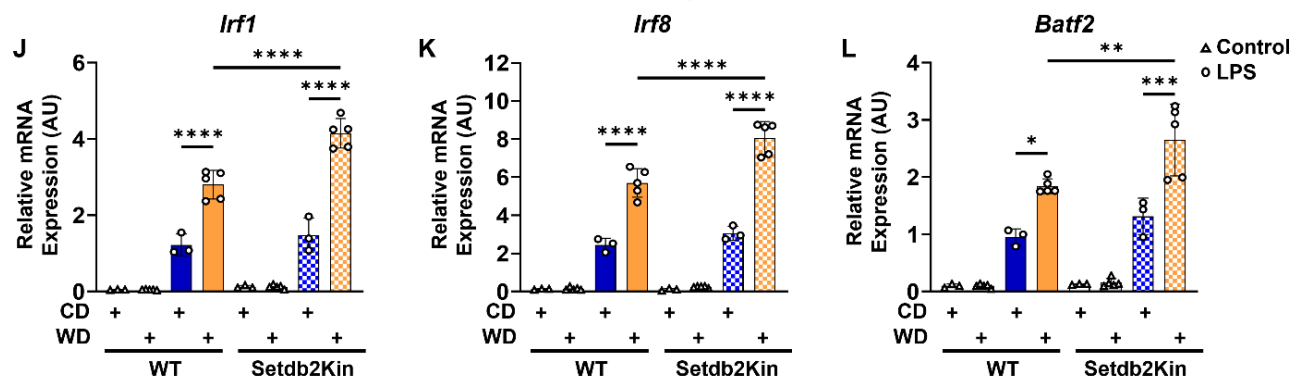


Figure S6. 'Western-diet triggered innate immune memory in BM-Monocytes from Setdb2Kin mice (Related to Figure 6 and S5)

CD11b⁺ BM-monocytes were isolated from control-diet or western-diet fed WT or Setdb2Kin mice followed by stimulation with 100ng/mL LPS or culture medium *in vitro* for 6 h.

(A) mRNA expression of *Tnfa* (B) *Nos2*, and (C) *Ccl2*, (D) Pro-inflammatory cytokine levels of TNF α , and (E) IL-1 β , (F) Extracellular lactate levels, (G) mRNA expression of *Hk1* (H) *Hk2*, (I) *Pfkfb3* (J) *Irf1* (K) *Irf8*, and (L) *Batf2* in control diet, western diet, control diet followed by *in vitro* LPS restimulation, and western diet followed by *in vitro* LPS restimulated BM-monocytes isolated from WT and Setdb2Kin mice.

Control diet WT (n=3), Control diet Setdb2Kin (n=3), Western diet WT (n=5), and Western diet Setdb2Kin mice (n=5), from one of the three independent experimental group. Data are represented as mean \pm SD. 'n' represents biological replicates of each strain and treatment type. p-value was calculated using two-way ANOVA with Tukey post-hoc test for multiple comparisons. *p < 0.05, **p < 0.01, and ***p < 0.001, ns, not significant change.

Table S1

Primer sequence for qPCR	
Gene	Sequence 5'-3'
<i>Tnfa</i> Forward:	CCTCACACTCAGATCATCTTCT
<i>Tnfa</i> Reverse:	GCTACGACGTGGGCTACAG
<i>Nos2</i> Forward:	CCAAGCCCTCACCTACTTCC
<i>Nos2</i> Reverse:	CTCTGAGGGCTGACACAAGG
<i>Ccl2</i> Forward:	CCCGTAAATCTGAAGCTAA
<i>Ccl2</i> Reverse:	CACACTGGTCACTCCTACAGAA
<i>Setdb2</i> Forward:	AACAAATCAAGTGCGGTTCC
<i>Setdb2</i> Reverse:	TTCAAGGACAGTGGGGTTTC
<i>Hk1</i> Forward:	AGGGCGCATTACTCCAGAG
<i>Hk1</i> Reverse:	CCCTGTGGGTGTCTTGTGTG
<i>Hk2</i> Forward:	TGATCGCCTGCTTATTACGG
<i>Hk2</i> Reverse:	AACCGCCTAGAAATCTCCAGA
<i>Pfkfb3</i> Forward:	CCCAGAGCCGGGTACAGAA
<i>Pfkfb3</i> Reverse:	GGGGAGTTGGTCAGCTTCG
<i>Irf1</i> Forward:	ATGCCAATCACTCGAATGCG
<i>Irf1</i> Reverse:	TTGTATCGGCCTGTGTGAATG
<i>Irf8</i> Forward:	CGGGGCTGATCTGGGAAAT
<i>Irf8</i> Reverse:	CACAGCGTAACCTCGTCTTC
<i>Batf2</i> Forward:	AGCGAGCTGCTGACTGAGA
<i>Batf2</i> Reverse:	CGCCTTACTGGTGTGCTTCT
<i>L32</i> Forward:	ACATTTGCCCTGAATGTGGT
<i>L32</i> Reverse:	ATCCTCTTGCCCTGATCCTT
Primer sequence for ChIP-qPCR	
Gene	Sequence 5'-3'
<i>Irf1</i> Forward:	TGCCTTGTACTTCCCCTTCG
<i>Irf1</i> Reverse:	GCCGCGAAGAAATCTAAACAC
<i>Irf8</i> Forward:	GCAAAAGTGATTTCTCGAAAGA
<i>Irf8</i> Reverse:	GCTTTTATAGATGGGGCGGG
<i>Batf2</i> Forward:	CAGCCCATGGTTTtaggagaaa
<i>Batf2</i> Reverse:	AGAGGTTCACTCTGGTCCTG
Primer sequence for 3C-qPCR	
Gene	Sequence 5'-3'
<i>Gapdh</i> Taqman probe	TATCCCTCCTCGGAACCTGAGAGC
<i>Gapdh</i> Forward:	GCAGCCTGGAAACCTGATAA
<i>Gapdh</i> Reverse:	GGGCTACAGTGGGTGAAAG
<i>Tnf</i> Taqman probe	ATTCCCAGGGCTGAGTTCATTCCC
Constant (<i>Tnf</i>)	GCTTGAGAGTTGGGAAGTGT
Test (<i>Tnf</i>) N1	CGGAGCCCTCCAGACAT
Test (<i>Tnf</i>) E1	CTGTAGCTAAATAGGACCTGAACA
Test (<i>Tnf</i>) E2	GGGTTGTCTGTTCTTGGGCA
Test (<i>Tnf</i>) N2	GAGGCCATAAAGGAGGGATT
<i>Ccl2</i> Taqman probe	CTGTGAAGGGTTCCAACCACTCC
Constant (<i>Ccl2</i>)	AAACTCACCTAGTGCTTACTCTG
Test (<i>Ccl2</i>) N1	CACCTTTTTATCAAGAGTCTGCTG
Test (<i>Ccl2</i>) E1	GCTGCCTCCTGTGCTAA
Test (<i>Ccl2</i>) N2	AAGACCACCTATCTAGTTACAAGTG
<i>Hk2</i> Taqman probe	CACGGAACACACGTCCCAACTCT
Constant (<i>Hk2</i>)	AGCCAATCAGCGCCTAGAG
Test (<i>Hk2</i>) N1	TGCTGACGATGGCGCTAA
Test (<i>Hk2</i>) E1	TCAGGTGGGAAATTCCTTCCTT
Test (<i>Hk2</i>) N2	GAGTTCTAGCCTAGAGCAGCA
Primer sequence for sanger sequencing	
Gene	Sequence 5'-3'
<i>Setdb2kin</i> Forward:	TGCCAAGTGAGAGGACAAAG
<i>Setdb2kin</i> Reverse:	ACATACCTGTTGGTGAAGAAGG