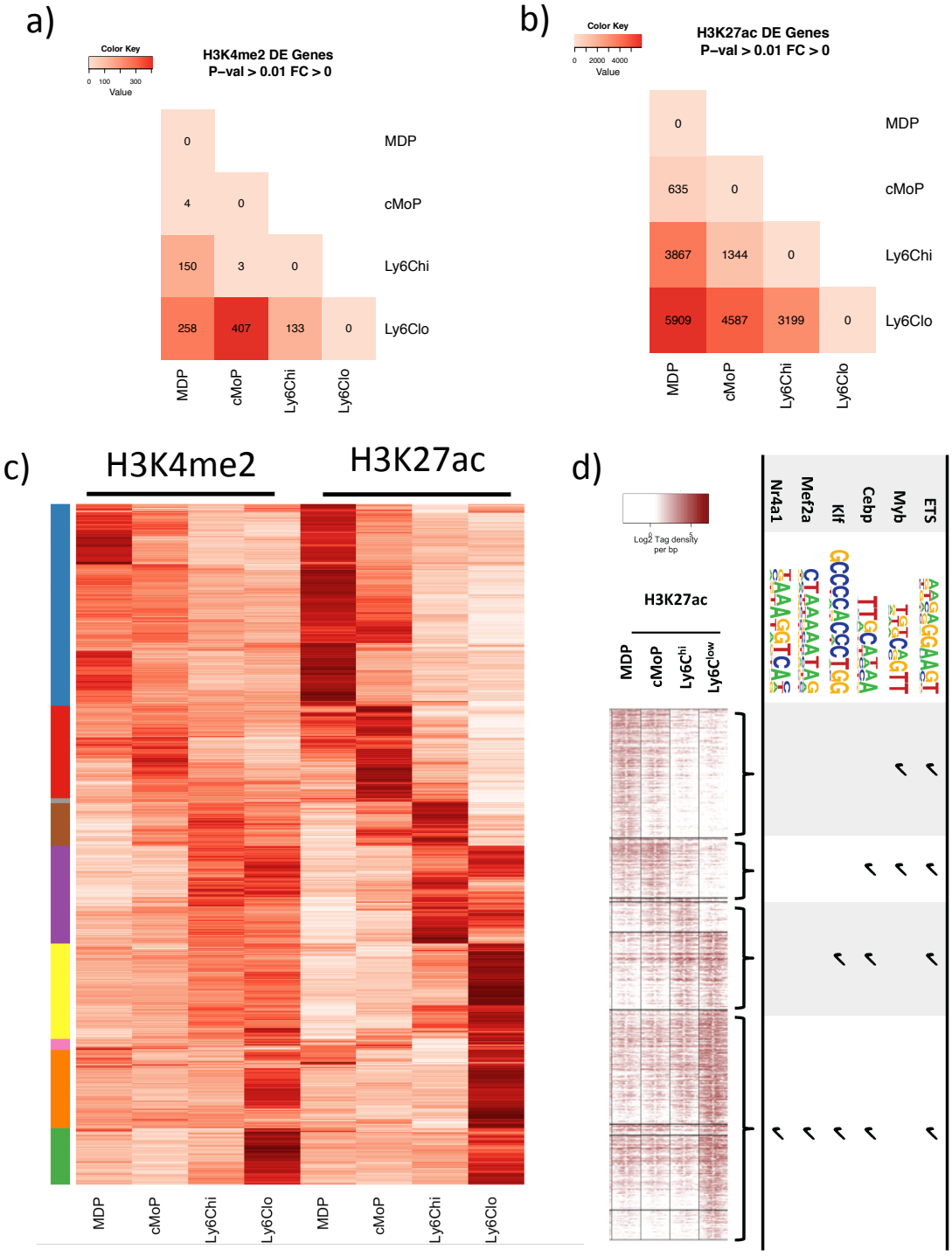


Figure S1, related to figure 1. a) Differentially bound H3K4me2 peaks (P,1*10^-5). In total 640 enhancer regions were found to be differentially regulated between all conditions. B) Differentially bound H3K27ac peaks (P,1*10^-5) . In total 9,879 enhancer regions were found to be differentially regulated between all conditions. c) Hierarchical clustering of DE enhancer regions. Bands on the left show clusters used to define groups in Figure 1c. d) Presence/ absence of *de novo* motif instances in clusters associated with MDP, MDP/cMoP, Ly6C^{hi}/ Ly6C^{low} Mo, or Ly6C^{low} Mo only, as defined in Figures 1c and S1c. Only motif instances with a significance p–value 1e-40 are shown, alongside the representative motif identified by HOMER..

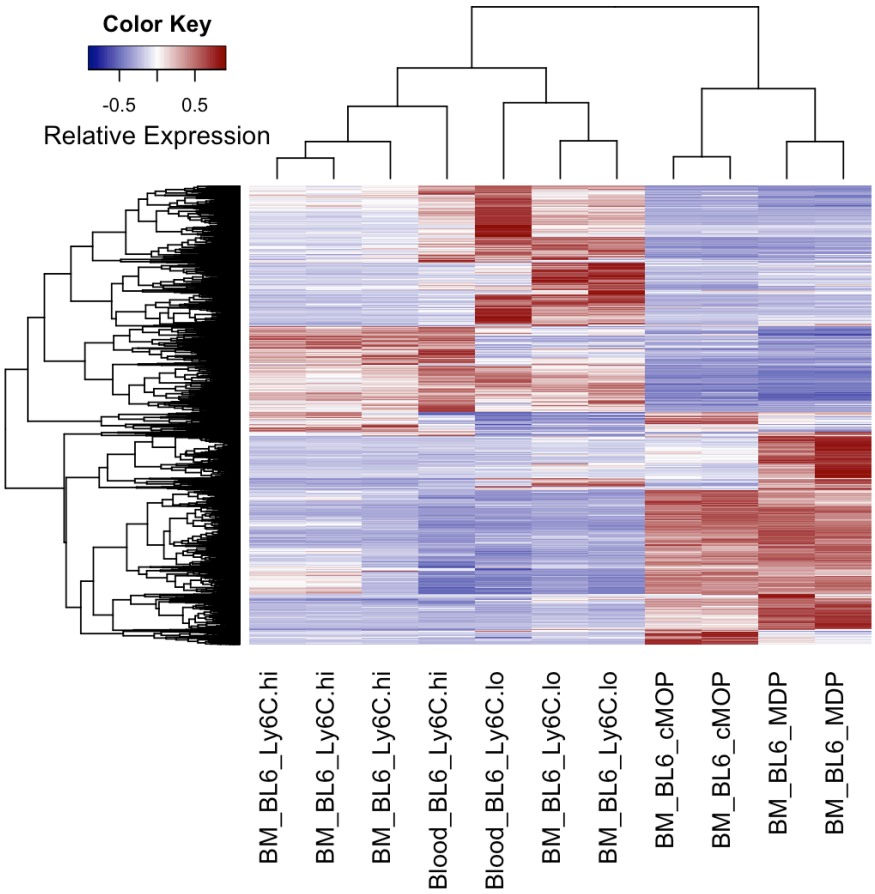


Figure S2, related to figure 1. Gene expression profiles Differential gene expression pairwise comparisons in mRNA-Seq data.

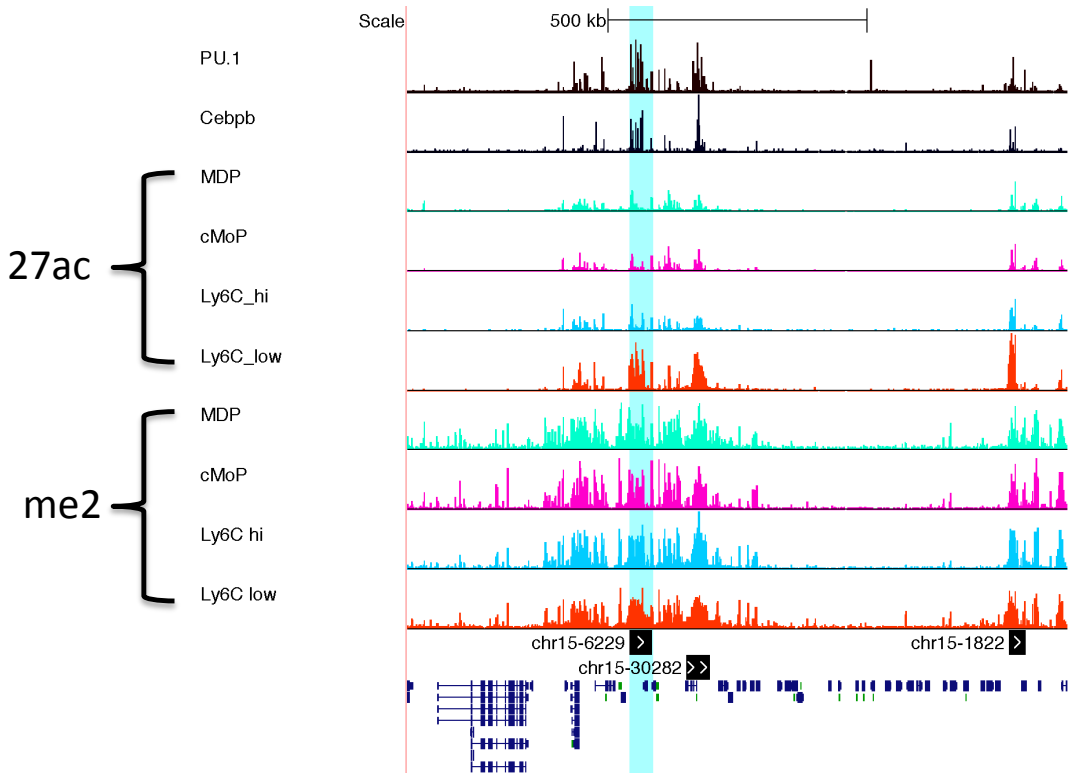


Figure S3, related to figure 2. UCSC genome browser screenshot of *Nr4a1* and surrounding region. The *Nr4a1*-associated super-enhancer (*Nr4a1se*) is highlighted in blue. Super-enhancer predictions for Ly6C^{low} monocytes are shown in black directly above the gene prediction track.

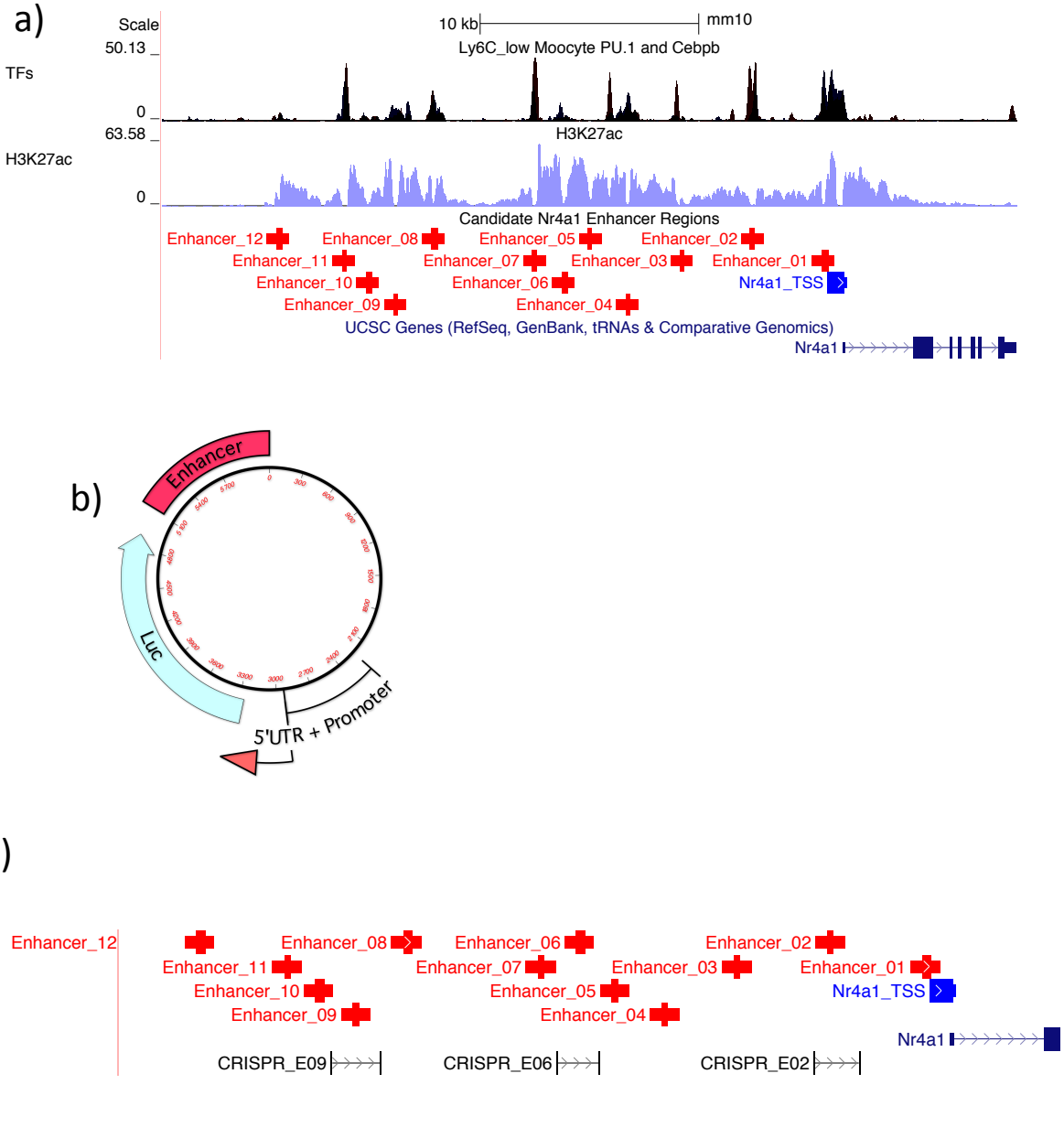
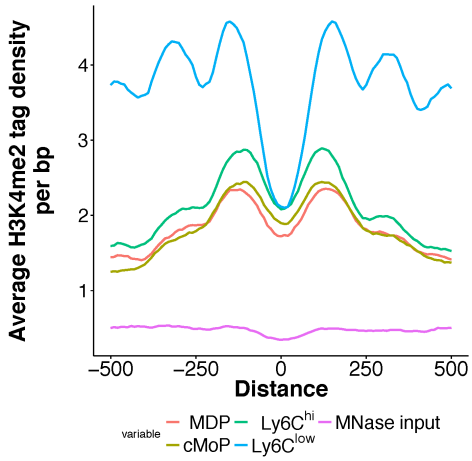


Figure S4, related to figure 3. a) UCSC genome browser screenshot showing positions of CRISPR sgRNA sites for E2, E6 and E9 domain-deficient mice. The TFs track shows superimposed PU.1 and CEBPb transcription factor binding profiles in Ly6C^{low} Mo. b) Schematic of pGL4.10 luciferase reporter vector containing *Nr4a1* 300bp of *Nr4a1* promoter upstream of the TSS and the 5' UTR sequence. c) sgRNA design.

a)



b)

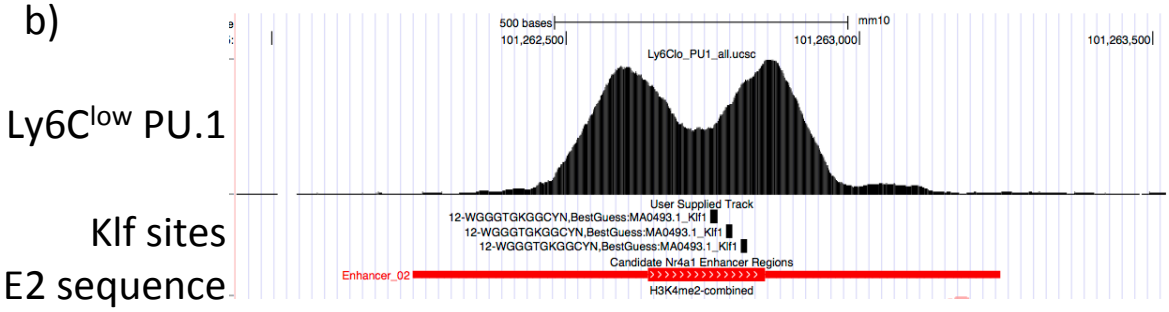
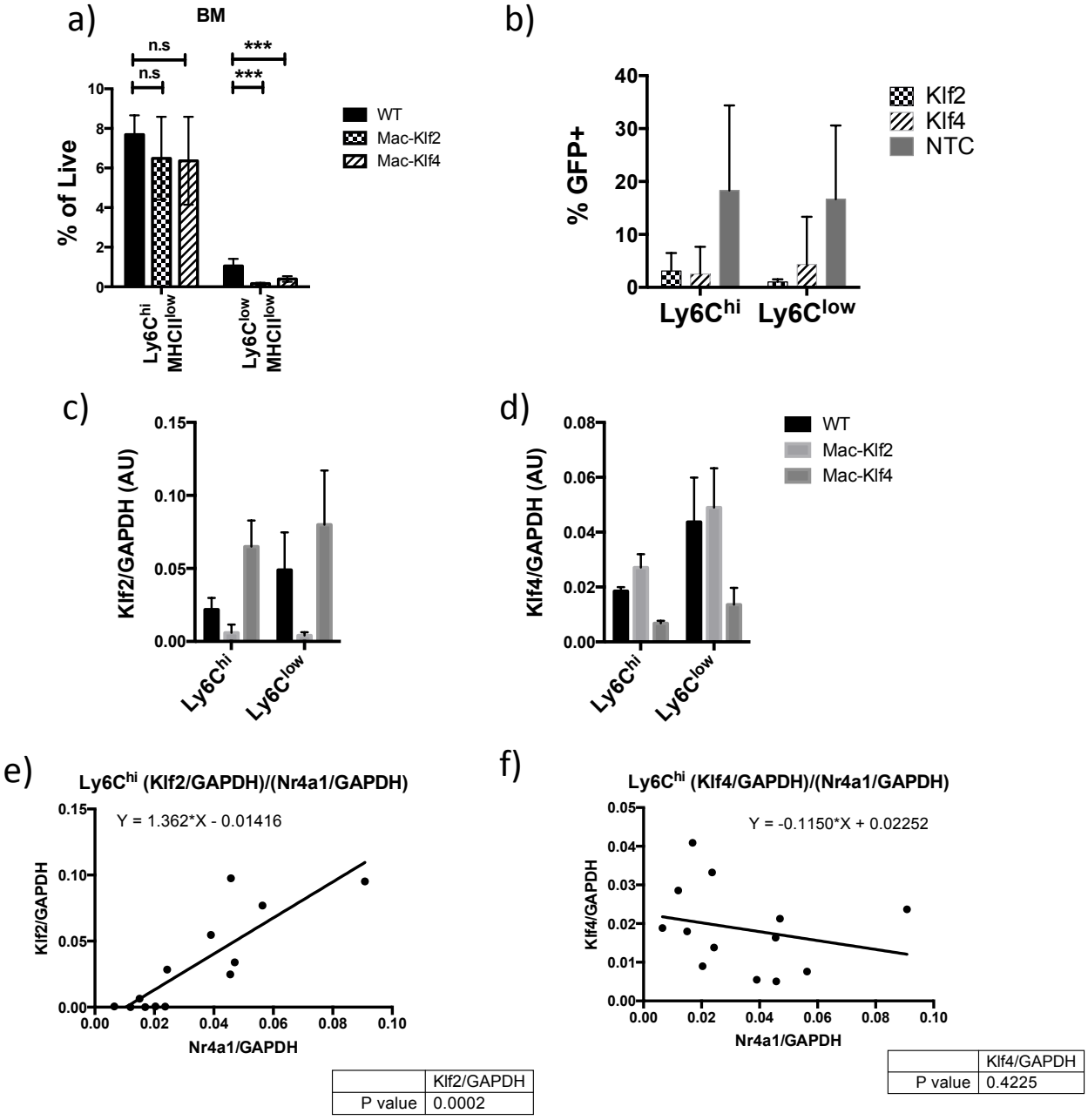


Figure S5, related to figures 5 and 6. a) mRNA expression levels of transcription factors belonging to family of over-represented motifs present in Ly6C^{low} monocyte enhancer regions based on RNA-Seq data. b) Klf motifs (identified using HOMER) present in the *Nr4a1* E2 enhancer sub-sequence.



	Klf2/GAPDH
P value	0.0002

	Klf4/GAPDH
P value	0.4225

Figure S6, related to figure 6. a) Flow cytometric analysis of Mo subset frequencies in the bone marrow of Mac-Klf2 and Mac-Klf4 mice. b) Frequencies of GFP+ cells transduced with GFP and shRNA expressing retrovirus targeting *Klf2*, *Klf4*, or non-targeting control (NTC) sequence. c, d) *Klf2* (c) and *Klf4* (d) mRNA expression in blood monocyte subsets sorted from Mac-Klf2 and Mac-Klf4 mice. e,f) *Klf2* (e) and *Klf4* (f) mRNA expression levels correlated against *Nr4a1* mRNA expression in Ly6C^{hi} Mo.

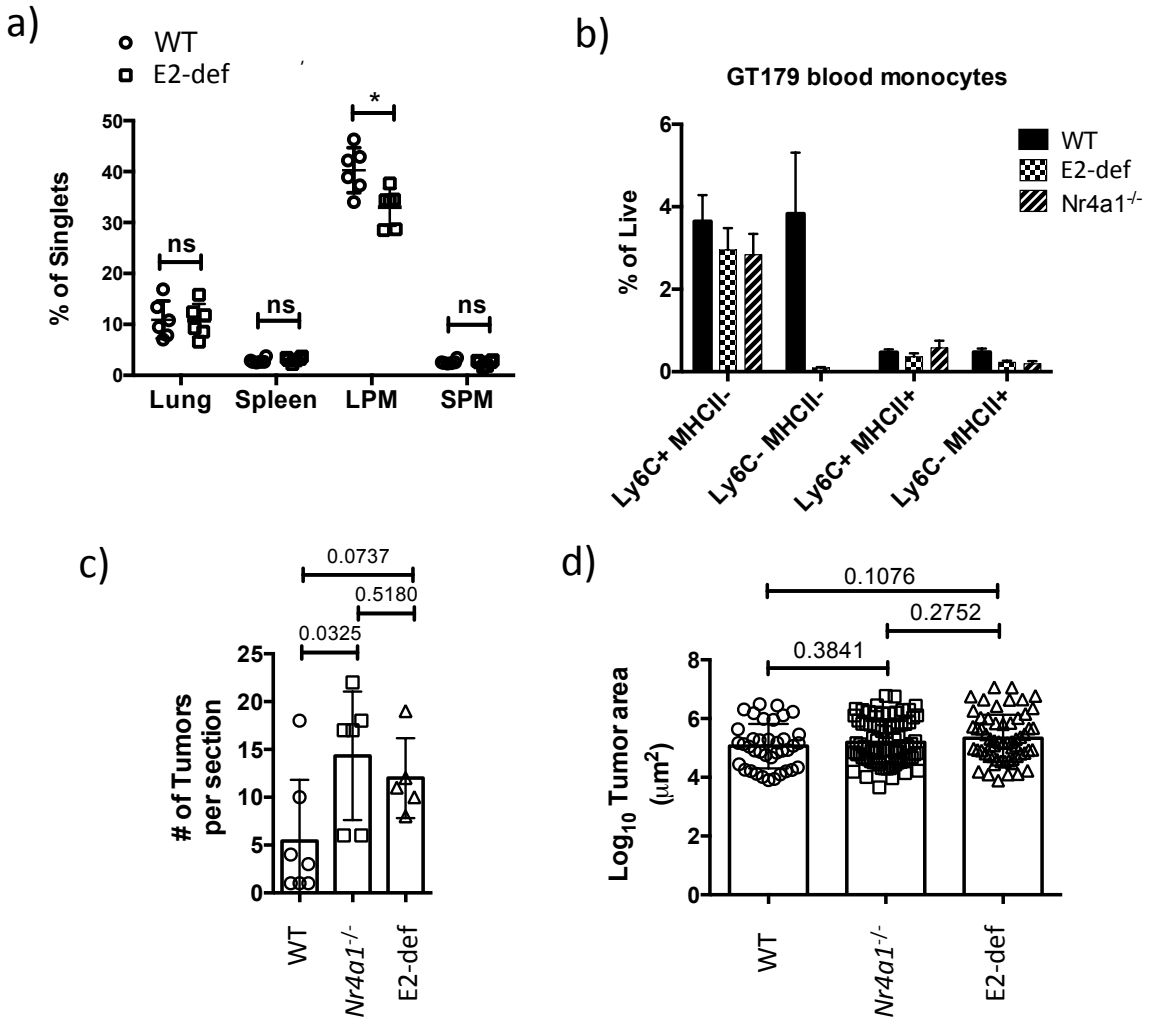


Figure S7, related for figure 7. a) Tissue macrophage subset frequencies in WT and E2 domain-deficient mice as measured by FACS. B) Blood monocyte subset frequencies in WT, *Nr4a1*$^{-/-}$ and E2 domain-deficient mice 18 days after injection with 300,000 B16F10 melanoma cells. c) Quantification of histological sections for B16F10 tumors d) Size distribution of individual tumors within lungs of mice relating to figures 7d. Statistics for c) and d) performed using students t-test, p values are shown.

Materials and Methods

Cell preparation and isolation

Bone marrow monocytes were harvested from femurs, tibias and hip bones of 8-12 week old male C57BL/6 mice (strain 006664) obtained from the Jackson laboratory (Bar Harbor, ME). Prior to FACS staining cells were subject to a brief (3 minute) red blood cell lysis (RBC lysis buffer, EBiosciences) at room temperature. All FACS staining was performed in FACS buffer (DPBS + 10% FCS + 2mM EDTA). Prior to surface staining FC receptors were blocked with anti-CD16/32 (clone 93) for 30 minutes. Surface staining was performed for 30 minutes in a final volume of 500ul for FACS sorts and 100ul for regular flow cytometry. Surface staining was performed using Live/Dead Yellow (Thermo Fisher) and antibody combinations in accordance with the gating schemes in the figures. Lineage positive cells were identified using pooled APC conjugated anti-CD3, CD19, Ly6G and NK1.1 (clones145-2C11, 1D3, 1A8 and PK136 respectively). Additional cell surface markers used were CD117-PE-Cy7 (ACK2), CD115 PE or BV421 (clone AFS98), Ly6C APC-Cy7 or PerCP-Cy5.5 (clone HK1.4), CD135-PE (clone A2F10.1), CD11c-FITC (clone N418), F4/80 PE-Cy7 (clone BM8), CD11b FITC (clone M1/70) and MHCII-BV605 (clone M1/70). All antibodies were obtained from Biolegend (San Diego, CA). Samples were washed twice in at least 200ul FACS buffer before acquisition. Cells were sorted using a FACS Aria II (BD biosciences) and conventional flow cytometry using an LSRII (BD biosciences). All flow cytometry was performed on live cells.

ChIP and sequencing library preparation

ChIP assays for histone modifications were performed as previously described (Gilfillan et al., 2012). To obtain sufficient numbers of MDPs bone marrow from 10 mice were pooled and sorted. For each ChIP assay 500,000 FACS isolated cells were immediately washed with PBS and resuspended in MNase digestion buffer (50mM Tris pH 8.0, 1mM CaCl2, 0.2% Triton X-100). Cells were then digested in to mononucleosomal fragments using micrococcal nuclease (MNase, Affymetrix, CA). Enzymatic digestion was quenched by addition of 1/10 volume stop buffer (110mM Tris pH 8.0, 55mM EDTA), samples briefly sonicated using a Bioruptor (Diagenode, Belgium) and then adjusted to RIPA buffer conditions by adding an equal volume of 2x RIPA buffer (280mM NaCl, 1.8% Triton x100, 0.2% SDS, 0.2% Sodium Deoxycholate, 5mM EGTA). Immunoprecipitations were performed in a final volume of 100 µl. 2 µg of anti-H3K4me2 (Millipore 07-030) or 2µg of anti-H3K27ac (Diagenode C15410196) were added and incubated overnight with rotation at 4 °C. Antibody-antigen-DNA complexes were recovered by incubating for 2 hours with 10 µl Protein A Dynabeads previously washed in RIPA buffer (10mM Tris pH 8.0, 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.2% SDS, 0.2% Sodium Deoxycholate). Complexes were then washed 5x in ice cold RIPA buffer and 1x in LiCl wash buffer (10mM Tris pH 8.0, 250mM LiCl, 1mM EDTA, 0.5% Igepal CA-630, 0.5% Sodium deoxycholate,), each wash was performed in 200 µl at 4 °C for 5 minutes with rotation. All above buffers were supplemented with 1x Protease inhibitor Cocktail, 1mM PMSF and 5mM Sodium Butyrate (Sigma). Beads were subject to a final wash in 200 µl ice cold TE buffer (Invitrogen) without protease inhibitors and eluted in 100 µl 1% SDS-TE buffer at 37 °C for 20 minutes. Finally, protein was treated with 2 µl proteinase K (Ambion) for 1h at 55 °C and DNA purified using a ChIP Clean & Concentrate column (Zymo, Irvine, CA) eluting in 30 µl final volume.

ChIP for PU.1 and C/EBPb were performed as previously described (Gosselin et al., 2014). 500,000 sorted cells were washed in PBS and immediately fixated for 9 minutes at room temperature in 1% methanol free formaldehyde in PBS (ThermoFisher Scientific). Fixation was quenched by addition of 1/20 volume 2.625M glycine solution and cells were washed twice with PBS. Cell pellets were then snap frozen in a dry ice/methanol bath and stored at -80oC until needed. Nuclei were enriched by resuspending cell pellets in 10mM HEPES pH 7.9, 85mM KCl, 1mM EDTA, 0.5% Igepal CA-630 and incubating on ice for 10 minutes.

Nuclei were harvested by spinning at 3000 g for 10 minutes, and lysed in 130 µl lysis buffer (10mM Tris pH 8.0, 100mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.1% Sodium Deoxycholate, 0.5% N-lauroylsarcosine). DNA was transferred in to Covaris micro tubes (Covaris, MA) sheared into 150-600bp fragments using a Covaris E220 (14 mins, duty cycle 3%, 100 cycles per burst). Final volume was adjusted to 200 µl and 22 µl 10% Triton X-100 added, cell debris was then cleared by spinning at maximum speed for 5 minutes. 20 µl of protein A dynabeads pre-conjugated to 3ug anti PU.1 or C/EBPb (sc-352x and sc-150x respectively, Santa Cruz Biotechnology, CA) were added to each chromatin preparation and incubated with rotation for 2h at 4 °C. Antibody-antigen-DNA complexes were then washed in 3x WBI (20mM Tris pH 7.4, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2mM EDTA,), 3x LiCl WB (10mM Tris Ph 7.4, 250mM LiCl,, 1% Triton X-100, 0.7% Sodium Deoxycholate, 1% Igepal CA-630) and 1x TET (TE, 0.2% Tween-20) buffer. All wassh volumes were 200 µl. DNA was eluted in 1% SDS-TE for 30 minutes at 37 °C, NaCl was added to a final concentration of 300mM and corsslinking reversed overnight at 65 °C. Finally, protein was treated with 2 µl proteinase K (Ambion) for 1h at 55 °C and DNA purified using a ChIP Clean & Concentrate column (Zymo, Irvine, CA) eluting in 30 µl final volume.

ChIP-Seq libraries were prepared using an initial 0.3 - 5 ng DNA using the ThruPlex-FD kit (Rubicon Genomics, MI) in accordance with the manufacturer's guidelines. RNA-Seq libraries were prepared using the Tru-Seq v2 library preparation kit (Illumina, La Jolla, CA) in accordance with the manufacturer's instructions.

RNA-Seq differential expression analysis

Sequencing libraries were sequenced using an Illumina HiSeq at the LIAI sequencing core facility. RNA-Seq libraries were sequenced as paired-end 50 base reads, and ChIP-Seq libraries were sequenced as single-end 50 base reads. Illumina BCL files were converted into FASTQ format using bcl2fastq (v1.8.4, Illumina).

Paired end RNA-Seq reads were mapped to the mouse mm10 reference genome using RNA-STAR v2.3.0 (Dobin et al., 2013) compiled with gene models derived from the Ensembl v73 genome annotation set. Gene counts were quantitated with the same annotation set using featureCounts (Liao et al., 2013). Differential expression analysis was performed using edgeR v3.4.2 (Robinson et al., 2010). Variance calculations were computed using the common dispersion method and sequencing depth differences adjusted using the 'relative log expression' method. Differential expression was computed against all pairwise comparisons and an FDR (Benjamini-Hochberg) corrected P-value threshold of 1e-5 applied for calling statistical significance. RPKM calculations were computed in edgeR using gene lengths derived from featureCounts. Publicly available RNA-Seq data from Levin *et al* were obtained from GEO GSE63340 (Levin *et al.,* 2014) and processed as above.

ChIP-Seq peak calling, differential binding and UCSC genome browser visualization

ChIP-Seq reads were mapped to the mm10 reference genome using Bowtie (v , Langmead et al., 2009). Homer (v4.4, Heinz et al., 2010) was used for ChIP-Seq peak calling.

Enhancer regions were defined by merging biological replicates for H3K4me2 libraries and running the findPeaks algorithm with 'style -histone' against the MNase-treated DNA input control. H3K4me2 profiles defined by each cell type were then aggregated using mergePeaks to give the final H3K4me2 peak set. These peaks were assigned to target genes using HOMER, and enhancers defined as H3K4me2 peaks whose centers were >2.5kb from the nearest annotated transcription start site. Enhancer activity was measured by quantitating H3K4me2 and H3K27ac within these defined enhancer regions. Differences in sequencing depth were accounted for by normalizing to an effective library size of $1x10^7$

reads per lane. Transcription factor ChIP-Seq peaks were called using HOMER by running the findPeaks algorithm with the 'style -factor' flag using CH2O treated input DNA control as background sequence. Transcription factor peaks were called independently for each lane and merged using mergePeaks prior to quantification of tags within peaks using the annotatePeaks command in HOMER. Visualization was performed at the UCSC genome browser using bigWig files generated in HOMER.

ChIP-Seq data for Lavin *et* al (2014) were obtained using GEO accession GSE63339 and processed as above. Human ChIP-Seq data were accessed from GEO accession SRP015328 and mapped to the human hg19 reference genome using the protocols outlined above. The bigWig file of human monocyte DNase-Seq was downloaded from the ENCODE project at (https://www.encodeproject.org/files/ENCFF000TAU/@@download/ENCFF000TAU.bigWig) and visualized directly in the UCSC genome browser.

Microarray data

Human monocyte Klf2 expression was obtained from NCBI GEO Profiles under accession GDS4219, data shown are for probe ID 219371_s_at, which is representative of the three probes available for Klf2 on this array type in this experiment.

Differential enhancer profiling, hierarchical clustering and ChIP-Seq visualization

Differential histone and transcription factor binding were determined using edgeR. To identify patterns of enhancer usage between MDP, cMoP, Ly6C^{hi} and Ly6C^{low} monocytes we employed a very permissive threshold for differential expression, this was to identify any weak signals in the data arising from these closely related cell types. Statistical significance was computed between all pairwise comparisons of cell types using an FDR corrected (Benjamini-Hochberg) p value threshold of 0.01 without the application of a fold-change cutoff. Variance was estimated using the common dispersion estimate by treating all conditions as pseudoreplicates of a single group. The final set of differentially enriched (DE) enhancers represents the union of all differentially bound enhancers determined by H3K4me2 or H3K27ac in any pairwise comparison.

In order to perform hierarchical clustering, matrices of tag counts for H3K4me2 and H3K27ac for the set of DE enhancers were obtained. Each matrix was independently mean centered and variance stabilized (with respect enhancers, row mean / row SD). The normalized matrices were then concatenated and subject to hierarchical clustering using a euclidean distance and complete linkage clustering approach using the base functions in R. Based upon visual inspection, the results the tree was cut into nine clusters, the ordering of these clusters was used to order the final histogram figure (Figure 1c). Within each cluster enhancers were then ranked according to their 'peak score' as determined by HOMER, this ranking was used to order rows within clusters in Figure 1c. All PU.1 peaks overlapping each DE enhancer were extracted and H3K4me2/H3K27ac tag counts calculated +/-1kb from each PU.1 peak center using HOMER's annotatePeaks with option '-ghist'. The final results were plotted using the R package gplots.

UCSC genome browser tracks were visualized using track hubs generated with the HOMER program makeBigWigHub.pl.

Differential PU.1 peak calling between Ly6C^{hi} and Ly6C^{low} Mo

A complete monocyte PU.1 peak set was obtained by concatenating the PU.1 peaks defined in both Ly6C^{hi} and Ly6C^{low} Mo as described in *'ChIP-Seq peak calling, differential binding and UCSC genome browser visualization'*. Tag counts for PU.1 libraries were counted within this peak set for both monocyte populations and differential binding an analysis was performed

in edgeR using the same procedures as for differential histone binding. For PU.1 peaks an FDR corrected (Benjamini-Hochberg) p-value cutoff of 1e-5 and log2 fold change cutoff of 3 were applied.

Enhancer cloning

All specific cloning details for individual sequences are in table S1. The *Nr4a1* 5'UTR and 650 bases of promoter sequence were cloned into the KpnI and SalI sites of a minimal pGL4.10 luc2 reporter vector. This vector, termed pGL4.10-TSS was used as the host vector for all enhancer clones. All enhancer sequences were isolated from genomic DNA by PCR using the primers detailed in Table 1. For PCR using Phusion High-fidelity polymerase (NEB, MA) 10ul 5x HF buffer, 1ul 10mM dNTP, 2.5ul F primer (10uM), 2.5ul R primer (10uM) 2ul mouse genomic DNA (250ng), 1.5ul DMSO, 30ul H2O and 0.5ul Phusion polymerase were used per reaction. PCR was performed using the following protocol at the indicated annealing temperature: - 1x 98oC 30s; 30x 98oC 10s, anneal 30s, 72oC 30s; 72oC 10min. For PCR using LA Taq GC buffer (Clontech, Mountain View, CA) 25ul GC buffer I, 8ul dNTP, 2.5ul F primer (10uM), 2.5ul R primer (10uM), 2ul DNA (250ng), 9.5ul H2O and 0.5ul LA-Taq were used per reaction. PCR was performed as per the manufacturer's instructions using the annealing temperatures in Table 1 and 1-minute extension time. PCR products were purified by gel extraction using Zymoclean Gen DNA recovery columns (Zymo, Irvine, CA). All cloning was performed at 1:4 vector: insert molar ratio using NEB reagents (Ipswich, MA) and clones were validated by PCR sequencing. PCR was performed using a MyCycler (BioRad, Irvine, CA)

Transfection and overexpression experiments

RAW264.7 macrophages were obtained from ATCC and maintained at low passage (<15) in DMEM +(10%FCS, 1% Pen/Strep, 1% L-glut). Transfection studies were performed using 0.3ug pGL4.10 luciferase reporter and 0.2ug cDNA or 0.5ug pGL4.10 luciferase reporter only, in addition 0.04ug pmaxGFP (Lonza, Basel) and 0.01ug pTK Renilla (ThermoFisher, Waltham, MA) luciferase control were added. 0.55ug of plasmid DNA was complexed at 8:1 ratio with lipofectamine LTX as per manufacturer's guidelines and 100ul per well given to RAW.264.7 cells at ~70% confluency and activity measured typically 12-16 hours later using the Dual Luciferase Reporter Assay (Promega, Madison, WI) on Sirius luminometer (Titerkek-Berthold, Pforzheim, Germany).

shRNA virus BMT experiments

PLAT-E packaging cells were grown to 60-70% confluency in DMEM (Gibco, +10%FCS, 1% L-glut, 1 μg/ml puromycin, 10 μg/ml blasticidin) in 10 cm dishes at 37°C in 10% CO2. Pools of three UltramiR (TRANSomiC technologies, Huntsville, AL) retroviral vectors (LMN) for Klf2 (ULTRA-3224750, ULTRA-3224748, ULTRA-3224747), Klf4 (ULTRA-3224770, ULTRA-3224769, ULTRA-3224768) and Non-targeting shRNA control were transfected into PLAT-E cells using JetPrime transfection in accordance with the manufactuer's guidelines. 7 μg total DNA was transfected per 10 cm plate. 1 hour prior to transfection media was replaced with antibiotic free media, cells were incubated overnight. The following were replaced with fresh 10 ml DMEM, without antibiotics. The following morning the retroviral supernatant was harvested and used for transduction of bone marrow stem cells (below). 8 ml fresh DMEM was added to the cells and the last step repeated the following day.

Murine stem cells were isolated from bone marrow by negative selection using the EasySep Mouse Hematopoietic Progenitor Isolation Kit (StemCell technologies, Vancouver, BC) in accordance with manufacturer's guidelines. Cells were resuspended in 2.5ml of STEMSPAN SFEM media (StemCell Technologies, Vancouver) containing 200 ng/ml rmSCF, 40 ng/ml rhIL-6 and 20 ng/ml rmIL-3 in 6 well plates previously coated for 2 hours with 2ml of 25

µg/ml human fibronectin and washed once with PBS. Bone marrow stem cells from one mouse were split between 2 wells and an equal volume (1.25ml) retroviral supernatant was added to each well. Cells were then spun at 850g for 1h at 37°C and incubated for 22h at 37°C in 10% CO_2. A second batch of retrovirus was added the following day and spin transduction repeated. Following the second transduction cells were harvested and transferred via retroorbital injection into lethally irradiated C57BL/6 host mice (2x 600 Rads, 2-3 hours apart). Bone marrow was allowed to reconstitute for 6 weeks prior to analysis.

CRISPR mouse generation

sgRNA sequences were designed using the http://crispr.mit.edu web tool. To minimize off-target effects we only considered sgRNAs with a score >70. For each enhancer deletion two pairs of sgRNAs flanking the target region were designed that were at least 50 base pairs apart. sgRNA templates were ordered as Ultramers from IDT (Coralville, IA) as a chimeric T7 promoter sequence, variable crRNA (see Table S2 for individual sequence information) and invariable tracrRNA. In order to promote robust transcription from the T7 promoter the first two bases of each sgRNA sequence were substituted for guanines. dsDNA templates for each sgRNA were generated by PCR using the Pfu Ultra II polymerase (Agilent Technologies, Santa Clara, CA). PCR reactions were cleaned using ChIP Clean & Concentrate columns (Zymo, Irvine, CA) and used as input for *in vitro* transcription using the MEGAscript T7 kit (Thermo Fisher, Waltham, MA) in accordance with the manufacturer's instructions. RNA was cleaned using the MEGAclear kit (Thermo Fisher) in accordance with the manufacturer's guidelines.

For embryo injections 0.5 day fertilized embryos were collected from 3-4-week-old superovulated C57BL/6 females (Harlan, WI) by injecting 5.0 I.U each of PMSG (Sigma Aldrich) and hCG (Sigma Aldrich). Embryos were transferred into M2 medium (Millipore) and injected into the cytoplasm with sgRNA mixed at 25ng/ul each along with 50ng/ul Cas9 mRNA (GeneArt CRISPR Nuclease mRNA, Thermo Fisher) to give a final 150ng/ul RNA in IDTE buffer. These injected embryos were cultured in an incubator in KSOMaa medium (Zenith) in a humidified atmosphere of 5% CO2 at 37C over night. The embryos were implanted at 2-cell stage into recipient pseudo pregnant ICR female mice. Embryo injections were performed at the University of California, San Diego transgenic core facility.

CRISPR mouse breeding and genotyping

Founder mice were screened for the presence of one or two altered alleles using a PCR strategy that flanks the expected mutation. PCRs were designed such that the wild type product is 2 - 3kb and the respective deletion allele around 1kb shorter. Primer sequences and PCR details are in table S3. PCRs were carried out using the specified kits in accordance with the manufacturer's guidelines, with primer extension and annealing temperatures stated in the table. Founder animals were crossed with C57BL/6 mice obtained from the Jackson laboratory (strain 000664) to obtain heterozygous F1 animals that were interbred via sibling mating to generate *Nr4a1se_2*$^{-/-}$, *Nr4a1se_6*$^{-/-}$, and *Nr4a1se_9*$^{-/-}$ mice. Observed phenotypes were present in homozygous null mice derived from at least three independent founder mice for each strain. For the *Nr4a1se_2*-/- strain we also confirmed by Sanger sequencing that the downstream DNA sequence from the deletion into the Nr4a1 first intron was not modified

LPS challenge

Male mice aged 8-15 weeks old were intraperitoneally injected with $2.5*10^6$ EU/kg (equivalent to 2.5mg/kg) Ultrapure LP-EB (Invivogen) in PBS. Mice were age and sex matched in all experiments. Prior to injection LPS was sonicated for 5 minutes at room

temperature in a bath sonicator (FS20H, Fisher Scientific). Mice were monitored three times daily for the first 72 hours and twice daily thereafter.

B16F10, histology and microscopy quantification

300,000 B16F10 melanoma cells were intravenously injected by tail vein injection into recipient mice as previously described (Hanna *et al* 2015). 18 days following injection mice were sacrificed, lungs were filled with zinc buffered formalin and stored in the same buffer before embedding into paraffin blocks. Sections were cut at 4 μm, adhered to positively charged slides and dried over night. Sections were dewaxed with Slide Brite, rehydrated and stained with hematoxylin (Thermofisher), differentiated with acid alcohol, blued with Scott's water and stained with eosin (Thermofisher). Slides were scanned with ZEISS AxioScan Z1 slide scanner using 10x/0.3NA or 20x/0.8NA objective.

Klf mice

For *Lys2*^{Cre} *Klf2*$^{flox/flox}$ and *Lys2*^{Cre} *Klf4*$^{flox/flox}$ studies bone marrow (femur, tibia and hip bone) and Cre negative littermate controls were harvested, cleaned and shipped overnight on wet ice in BMM (RPMI, 1% Hepes, 1% Anti/Anti (Gibco), 1% BME (Gibco), 1% NEAA (Gibco), 1% Sodium Pyruvate (Gibco), 10% FCS). Bone marrow was harvested by scraping bones clean and immersing in 70% ethanol for 10 seconds before extracting marrow by centrifugation at 5,900g for 15 seconds in a 1.5ml eppendorf tube. Bone marrow was immediately resuspended in room temperature sterile PBS and injected into lethally irradiated recipient mice (2 x 600 rads) by retroorbital injection at a ratio of 3:1 (recipient to donor). Mice were allowed to reconstitute for at least 6 weeks prior to sacrifice and analysis.

Table S1, related to experimental procedures. Details for cloning *Nr4a1* enhancer candidates into PGL4.10 luciferase reporter

Plasmid	Enh_F_primer_seq	Enh_R_primer_seq	PCR protocol	Anneal temp (oC)	Digetion	Com cells
pGL4_Nr4a1_E12	ATATGGATCCCAATGTTGGGTCTCTTTCTCAATTAGTTGC	ATATGTCGACAAATCGCTGTGGTTTGAATGCCA	Phusion HF	72	Bamh1 + SalI	Top10 OneShot
pGL4_Nr4a1_E11	ATATGGATCCGATTGATGTGGGAGGCCAGGGTT	ATATGTCGACGTGTGCACTACCATGTCCAGCATG	Phusion HF	72	Bamh1 + SalI	NEB Stable
pGL4_Nr4a1_E10	ATATGGATCCAACAAAAGCAAAACACTGTTTCATTAGCGG	ATATGTCGACAGGGAGAACCAAGCTACCCAGGA	Phusion HF	72	Bamh1 + SalI	NEB Stable
pGL4_Nr4a1_E09	ATATGGATCCCCTAGACTGGAGTTAATGACGGTCGTGA	ATATGTCGACAGAAAGATTACCCACAAATCAAAACCAGGGCT	LA Taq GC 1	66	Bamh1 + SalI	Top10 OneShot
pGL4_Nr4a1_E08	ATATGGATCCGTATATGAGTACACTGTAGCTCTTTTCAGACACAC	ATATGTCGACGGACAGCTTAAAGAGACAGGCTGAGAT	Phusion HF	70.4	Bamh1 + SalI	NEB Stable
pGL4_Nr4a1_E07	ATATGGATCCCCCAGAGCACGGCTAAGGGGT	ATATGTCGACCATCCTGTCCTGAGCAAGCCCTT	LA Taq GC 1	66	Bamh1 + SalI	Top10 OneShot
pGL4_Nr4a1_E06	ATATGGATCCTTCATGAGACATTATACCATCTCACATCT	ATATGTCGACTGAGGGATTCATCCATGCAGA	LA Taq GC 1	60	Bamh1 + SalI	Top10 OneShot
pGL4_Nr4a1_E05	ATATGGATCCGGCTCAGAAGAAAGACAGTGTACGGTG	ATATGTCGACGTTTGTTTGTTTTGTTTTTTCGAGACAGGGTTTCT	LA Taq GC 1	66	Bamh1 + SalI	Top10 OneShot
pGL4_Nr4a1_E04	ATATGGATCCGACAGGCGATGGGATAAGACACCTG	ATATCTCGAGTCTGCAATCCCAGTGTGTCAGGAGAC	Phusion HF	72	Bamh1 + XhoI	NEB Stable
pGL4_Nr4a1_E03	ATATGGATCCAGGGAGGCAGTGTGGGCTGAA	ATATGTCGACGTCTAGCTACCTCCATGAAACTCTGCACC	Phusion HF	72	Bamh1 + SalI	Top10 OneShot
pGL4_Nr4a1_E02	ATATGGATCCCATGGGACCTGGCCAGGTTTCA	ATATGTCGACATTCTCCCTCCATATATACATCTGTTCTATCGACAG	Phusion HF	72	Bamh1 + SalI	Top10 OneShot
pGL4_Nr4a1_E01	ATATGGATCCGGCTGGCAGCAGAAATCGGGAA	ATATGTCGACCGACCAGGAGGAGGGGGTGTT	Phusion HF	72	Bamh1 + SalI	Top10 OneShot
pGL4_Nr4a1_TSS	ATATGGTACCGAAGGCCAGAGTGCCTGTCC	ATATGAGCTCTCCCACTCCCTGTGGCCG	Phusion HF	68.5	KpnI + SacI	Top10 OneShot

sgRNA name	Enhancer region	Score	Guide sequence (genomic)	crRNA sequence (inc G substitution for T7 promoter)
E02_US_1	E02	80	GTGAACTGAACTCCCCACCG	GTGAACTGAACTCCCCACCG
E02_US_2	E02	79	GCGCTGAGATATATGAATGC	GCGCTGAGATATATGAATGC
E02_DS_1	E02	82	GGGCGGGGCGGTTCCTGATT	GGGCGGGGCGGTTCCTGATT
E02_DS_2	E02	78	GCAGCAGGGTCAGCGTGAAC	GCAGCAGGGTCAGCGTGAAC
E06_US_1	E06	88	TGCTTAGGCACGGTAGTCAT	GGCTTAGGCACGGTAGTCAT
E06_US_2	E06	75	TCTGGTCTGGTCACTACAAA	GCTGGTCTGGTCACTACAAA
E06_DS_1	E06	83	GTGATCTAACACACCCCCCT	GTGATCTAACACACCCCCCT
E06_DS_2	E06	80	GGGTTTGGGGCTAGTGTAAT	GGGTTTGGGGCTAGTGTAAT
E09_US_1	E09	73	GGGGTTTGACCTGAGCCATC	GGGGTTTGACCTGAGCCATC
E09_US_2	E09	72	GAGCTTTTGGTGTCTTGACC	GAGCTTTTGGTGTCTTGACC
E09_DS_1	E09	73	GGAGGGGTTAACTAACCCAC	GGAGGGGTTAACTAACCCAC
E09_DS_2	E09	72	GGATCAATAACTACTTGGCT	GGATCAATAACTACTTGGCT
E04_07_US_1	E04_E07	77	TAGCCATCTCCCAGTCAAGC	GAGCCATCTCCCAGTCAAGC
E04_07_US_2	E04_E07	76	ATGGACCCTTACTCCCAAAT	GTGGACCCTTACTCCCAAAT
E04_07_DS_1	E04_E07	94	GAGGTGAAGGGTCCCAATCG	GAGGTGAAGGGTCCCAATCG
E04_07_DS_2	E04_E07	77	CTGCGTTTTAAGCCTTATAA	GTGCGTTTTAAGCCTTATAA

Table S2, related to experimental procedures. crRNA design and sequences. sgRNAs were ordered as ultramers composed of an upstream T7 promoter (green), crRNA (red) and invariant downstream tracrRNA sequence (blue) as shown:

TAATACGACTCACTATAGGTGAACTGAACTCCCCACCGGTTTTAGAGCTAGAAATAGCAAGTTA
AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTT

Mouse	F primer	R primer	Polymerase kit	anneal (oC)	extend (min)	WT product size	KO product size
Nr4a1se_9	GCATCTCTGCTCCCCACTTT	CAGTAAGCCACCTTGAGCCA	NBE Phusion HF	68	3	2.5kb	1kb
Nr4a1e_6	GGCTCCCAGTGTGACCTTTT	CCTGAACGCCTGAGCTAACA	LA Taq GCI (Clontech)	58	2	1.8kb	500bp
Nr4a1se_2	CTGAGGCTCCTTATCGGGGA	CTGAATGCCCAAAACGCACC	NBE Phusion HF	68	3	2.6kb	1kb

Table S3, related to experimental procedures. PCR details for enhancer knockout mouse genotyping.