Aging disrupts homeostatic circadian gene regulation and function in macrophages

Eran Blacher[1] *, Connie Tsai[1,2] *, Lev Litichevskiy[3,4,5], Zohar Shipony[6], Chinyere Agbaegbu Iweka[1], Kai Markus Schneider[3,4,5], Bayarsaikhan Chuluun[7], H. Craig Heller[7], Vilas Menon[8], Christoph A. Thaiss[3,4,5] # and Katrin I. Andreasson[1,9,10] #

Supplementary Materials

Methods

Animals

This study was conducted in accordance with National Institutes of Health (NIH) guidelines; protocols were approved by the Institutional Animal Care and Use Committee at Stanford University. Young (2-3 months old) and aged (20-22 months old) C57/BL6 male mice were entrained for 2 weeks to a 12-hour light/12-hour dark cycle with food and water available *ad libitum*.

Flow cytometry

Young and aged mice were terminally anesthetized (combination of 100 mg kg^{-1} ketamine and 10 mg kg^{-1} xylazine intraperitoneally) at 4 hours intervals over the course of 24 hours. Blood samples were collected retro-orbitally, diluted 1:4 in PBS w/o Ca^{2+}/Mg^{2+}, centrifuged over a Ficoll gradient to remove granulocytes and RBCs and washed once with PBS. Bone marrow cells were flushed from tibias and spleens were homogenized and filtered through a 40 μm strainer to remove large cellular debris. Single cell suspensions of bone marrow, blood and spleen samples were subjected to RBC lysis and washed with PBS. Cells were then stained with antibodies for 45 min on ice against CD45 (clone 30-F11, LOT: B247956), CD11b (clone M1/70, LOT: B253261), Ly6C (clone HK1.4, LOT: B268312), Ly6G (clone 1A8, LOT: B248965), CD115 (clone AF598, LOT: B265220), CD3 (clone 17A2, LOT: B304392), CD19 (clone 6D5, LOT: B196382) and F4/80 (clone BM8, LOT: B272101), all from BioLegend, analyzed on a BD-LSR II cytometer with FlowJo software, version 10.6.1.

Lipopolysaccharide (LPS)-induced sepsis

Mice were maintained in a controlled light/dark environment for 2 weeks before the LPS challenge. Mice were i.p. injected either at ZT0 or ZT12 with LPS derived from E. coli serotype 055:B5 (Sigma Aldrich) in sterile PBS at 25 mg/kg and monitored for one week. The probability of survival was calculated using the Kaplan–Meier method, and statistical analysis was performed using a log-rank test.

Macrophage Isolation

Peritoneal macrophages were harvested from anesthetized young and aged mice every 4 hours over the course of 24 hours by flushing the peritoneal cavity with 10 ml of ice cold PBS[1,2]. Miltenyi Cd11b magnetic microbeads were incubated with peritoneal macrophages for 10 minutes at 4˚C and then passed through a Miltenyi MS column according to the manufacturer's protocol to enrich for macrophages.

RNA-seq

Library Preparation: Samples were lysed in Invitrogen TRIzol reagent and RNA was isolated using Invitrogen PureLink RNA Micro columns. RNA samples were converted into Illumina libraries by using the Lexogen Quantseq Forward kit. Approximately 250 ng of total RNA was reverse transcribed using oligodT priming to bias fragments toward the 3'-end of RNA fragments. cDNA fragments were converted to double stranded cDNA and subjected to ligation of sequencing adapters and PCR amplification. All samples were pooled into a single group and sequenced on four lanes of Illumina HiSeq 2000 with the 50-bp single-end chemistry. RNA quality and quantity and sequencing quality did not differ significantly between age groups or time points.

RNA-seq Analysis: Fastq files of the raw RNA-seq reads were trimmed with Bbduk and then aligned to the mouse reference genome (mm10) with STAR using parameters as described in the Lexogen Quantseq protocol[3]. Reads were counted using HTSeq. For PCA analysis, counts were normalized using the "vst" procedure in DESeq2[4]. For evaluation of circadian rhythmicity using JTK-CYCLE, reads were library-size normalized and $\log_2$ transformed using the CPM command in R. Only genes with mean log2(CPM) expression > 3 were evaluated by JTK-CYCLE. KEGG pathway enrichment was performed using the "kegga" command in limma. oPossum was used to identify transcription factors enriched in the promoter regions of differentially rhythmic genes.

Quantitative Real-Time PCR:

RNA was isolated using Invitrogen TRIzol and purified with the Invitrogen Purelink RNA Micro columns or with a chloroform-phenol protocol. cDNA was generated from 300 ng of RNA by using the Applied Biosystems High Capacity RT kit. qRT-PCR was performed on the QuantStudio6 using Taqman primers for Arntl (Mm00500226_m1), Per2 (Mm00478099_m1), and Gapdh (Mm99999915_g1). Relative expression was measured using the $\Delta\Delta C_t$ method with Gapdh as endogenous reference.

Ex vivo **phagocytic activity**

Macrophage phagocytic activity was assessed using the Vybrant Phagocytosis Assay Kit (Thermo-Fisher Scientific) according to the manufacturer's instruction. Briefly, macrophages were isolated and cultured in 96-well black culture plates at 10^6 cells/well for 2 h. Culture medium was removed and fluorescein-labeled *Escherichia coli* BioParticles or PBS was added. After 2 h, supernatant was removed and 100 μl of trypan blue was immediately added to each well for 1 min to quench extracellular fluorescence. Excess of trypan blue dye was removed by aspiration. The plate was read on a SpectraMax M2e microplate reader (Molecular Devices; excitation: 480 nm and emission: 520 nm; bottom reading

with 50 flashes per well).

Transposase reaction, library generation, and sequencing

Peritoneal macrophages were isolated every 4 h over the course of 24 h from young and aged mice, were washed with cold PBS at 4°C and centrifuged at 500g for 5 minutes. The cell pellet was resuspended in the Tn5 transposase reaction mix [25 µL 2× TD buffer, 2.5 µL Tn5 transposase and 22.5 µL nuclease-free water] and incubated at 37°C for 30 min with mild agitation. DNA from the transposase reaction was purified using the DNA Clean & Concentrator Kit (Zymo, catalog no. D4014) and PCR amplified as described [4]. Fragment analysis was run on the PCR products using NGS Fragment 1-6000bp (Agilent) as a quality control step and to construct the library. Library was then sequenced on a Illumina HiSeq 4000 instrument as 2×100 mers.

Analysis of ATAC-seq data

Data processing: Fastq files were trimmed using Trim Galore! (version 0.6.4_dev, parameters: --stringency 5) and aligned to the mm10 genome using Bowtie2 (version 2.3.4.1, parameters: --very-sensitive-local --dovetail --no-discordant --no-mixed). The following reads were filtered out: reads aligning to the mitochondrial genome (samtools idxstats {in_path} | cut -f 1 | grep -v chrM | xargs samtools view -b {in_path} > out_path}), PCR duplicates (java -jar $PICARD_JAR MarkDuplicates I={in_path} O={out_path} M={out_metrics_path} REMOVE_DUPLICATES=true), and reads aligning to ENCODE blacklisted regions (samtools view -f 2 -q 20 -b {in_path} | bedtools intersect -v -abam - -b mm10-blacklist.v2.bed -wa > {out_path}).

Creating ATAC-seq count matrices: For each sample, ChrAccR (https://greenleaflab.github.io/ChrAccR/articles/overview.html) was used to count the number of fragments (transposase cut-site centered) within pre-selected genomic regions. We created two different count matrices: one in which fragments were counted within open chromatin regions as determined by a peak-calling algorithm, and one in which fragments were counted within the 4000 bp surrounding each transcription start site. Peaks were called using Genrich (version 0.6, all default parameters), which considers all replicates of the same condition together. Only peaks with q-value < 0.05 were retained, and these variable-width peaks were merged across all samples (bedtools version 2.2.9.2). For each merged peak, the summit of the constituent peak with the most significant q-value was identified. Around each of these summits, a 500 bp region was extracted, resulting in n=67,992 regions with a fixed width of 500 bp. For all but the DESeq2 analyses, these matrices were library-size normalized [counts per million (CPM)] and then $\log_2$ transformed using the CPM command in R[7].

Downstream analysis: PCA was performed on the library-size normalized and $\log_2$ transformed matrices. DESeq2 (version 1.28.1) was run using the following design formula: *~Time + Age + Time:Age*, with age as the main effect and time-point as a covariate. Genomic regions were associated with genes using GREAT[8].

Estimating TF binding motif enrichment: chromVAR[9] was used to quantify transcription factor activity in n=176 motifs from the JASPAR2018 database.

Study Population

The UKBioBank study is a large, multisite, community-based cohort study with the overarching aim of improving the prevention, detection, and treatment of a wide range of serious and life-threatening diseases. The study invited people aged 40 to 69 years to take part. All UK residents aged 40 to 69 years who were registered with the National Health Service and living up to 25 miles from 1 of the 22 study assessment centers were invited to participate. All participants gave informed consent for genotyping and data linkage to medical reports. Genotyping of the KLF4 variant rs2236599 was conducted in a total of 488,377 subjects. Ongoing inpatient hospital records beginning in 1996 were used to identify diagnoses according to the International Classification of Diseases, Tenth Revision (ICD-10) codes. The presence of the following ICD10 codes was evaluated: Infection with E coli (A04) and Overall infections (A00-A99). The UK Biobank receives death notifications (age at death and primary ICD diagnosis that led to death) through linkage to national death registries. End of follow-up was defined as death or end of hospital inpatient data collection in June 2020. Specific causes of death included death by infection (A00-A99) and death by E.coli infection (A04). Detailed information about the study is available at the UKBB website (www.ukbiobank.ac.uk). The study has been approved by the UKB Access Committee (Project #59657).

Statistical Analysis

3-5 biological replicates were used for each experiment. All of the data are presented as the mean ± SEM. All patterns of rhythmicity (e.g. in gene expression, FACS populations, phagocytic activity, and chromatin accessibility) were assessed using JTK_cycle[11], which was parametrized to look for rhythms of exactly 24 hours. An adjusted *p*-value cutoff of 0.2 was used across all analyses in the study except for KEGG pathway analysis (Fig. 2g-h) where an adjusted *p*-value cutoff of 0.1 was used to decrease the number of genes that pass FDR correction. Multiple hypothesis correction was performed with the Benjamini-Hochberg procedure.

Data availability

Transcriptomics data: GSE128830: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE128830

Token: ybqjocoontgtluf ; ATAC-seq data: in process.
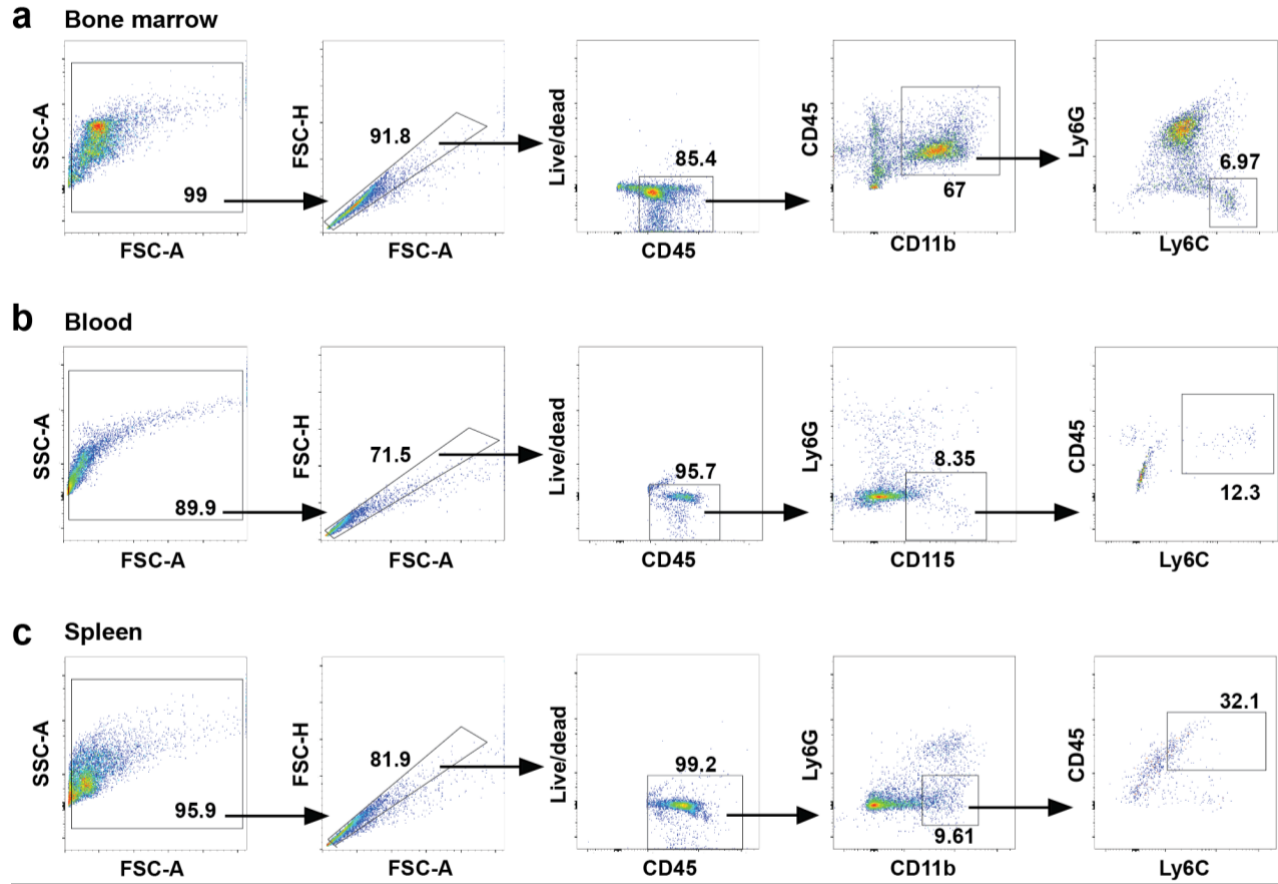
Supplementary Figure 1. Gating strategy for monocytes and macrophages in bone marrow, blood and spleen. Mononuclear cells from bone marrow (**a**) blood (**b**) and spleen (**c**) were gated for forward and side-scatter (FSC/SSC), doublets, and live/dead prior to identification of bone marrow (CD45$^+$CD11b$^+$Ly6G$^-$Ly6C$^+$), blood (CD45$^+$CD115$^+$Ly6G$^-$) and splenic (CD45$^+$CD11b$^+$Ly6G$^-$) monocytes and macrophages.
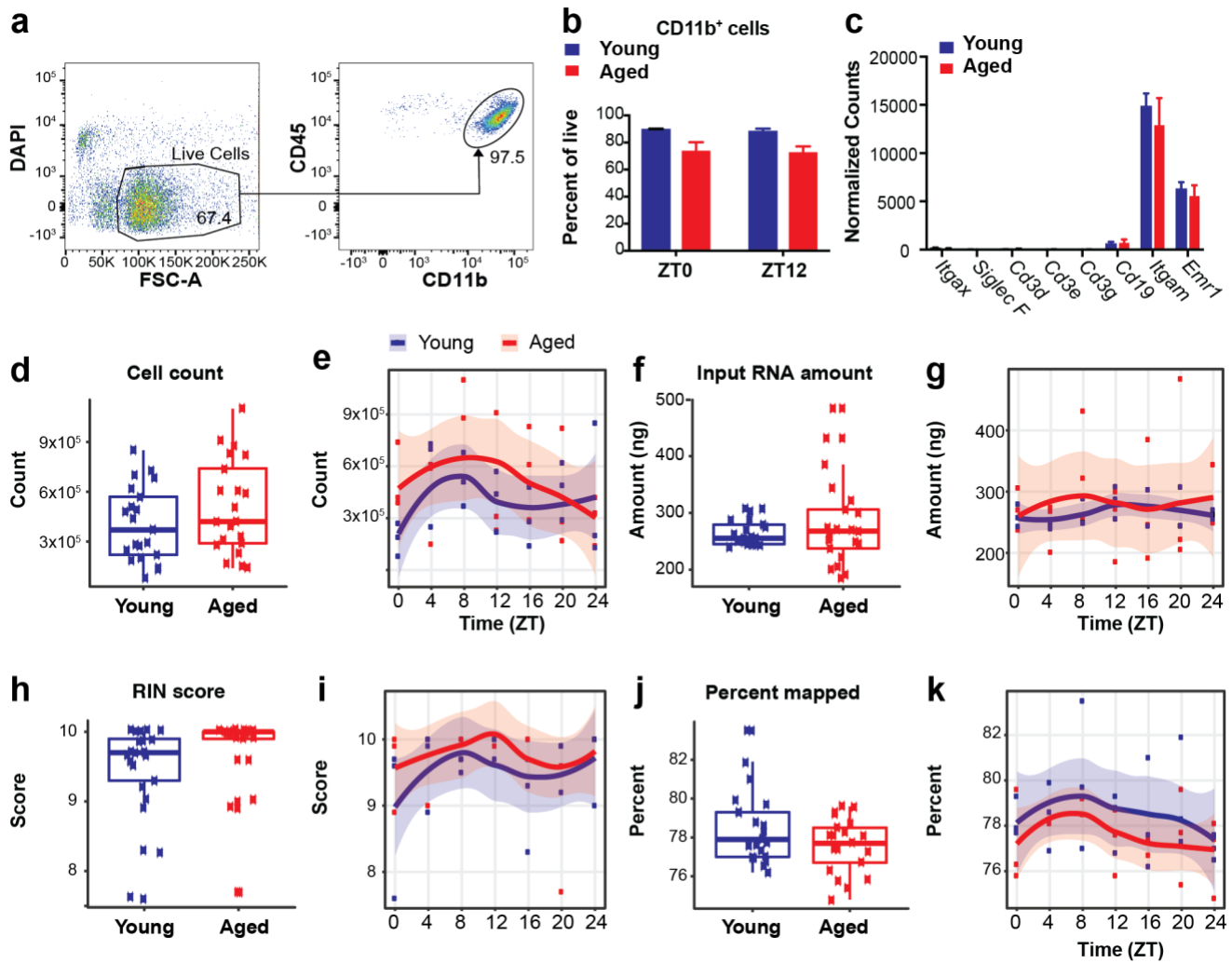
Supplementary Figure 2. Macrophage enrichment validation and RNA-seq quality control and analysis

a. Gating strategy for measurement of macrophage enrichment of samples at ZT0 and ZT12.

b. Percent of live Cd11b+ macrophages in young vs aged at ZT0 and ZT12 (n = 3, 2-way ANOVA age factor p=0.0065).

c. Expression levels of macrophage transcripts, *Itgam* and *Emr1* versus markers of dendritic cells (*Itgax*), eosinophils (*Siglec F*), T cells (*Cd3d, Cd3e, Cd3g*), and B cells (*Cd19*).

d-k. Pooled values (**d, f, h, j**) and time-dependent presentations (**e, g, i, k**) of sample total cell counts (**d-e**), input RNA quantity (**f-g**), RIN scores (**h-i**) and number of mapped reads (**j-k**) of circadian RNA-seq analysis of young and aged peritoneal macrophages.

8
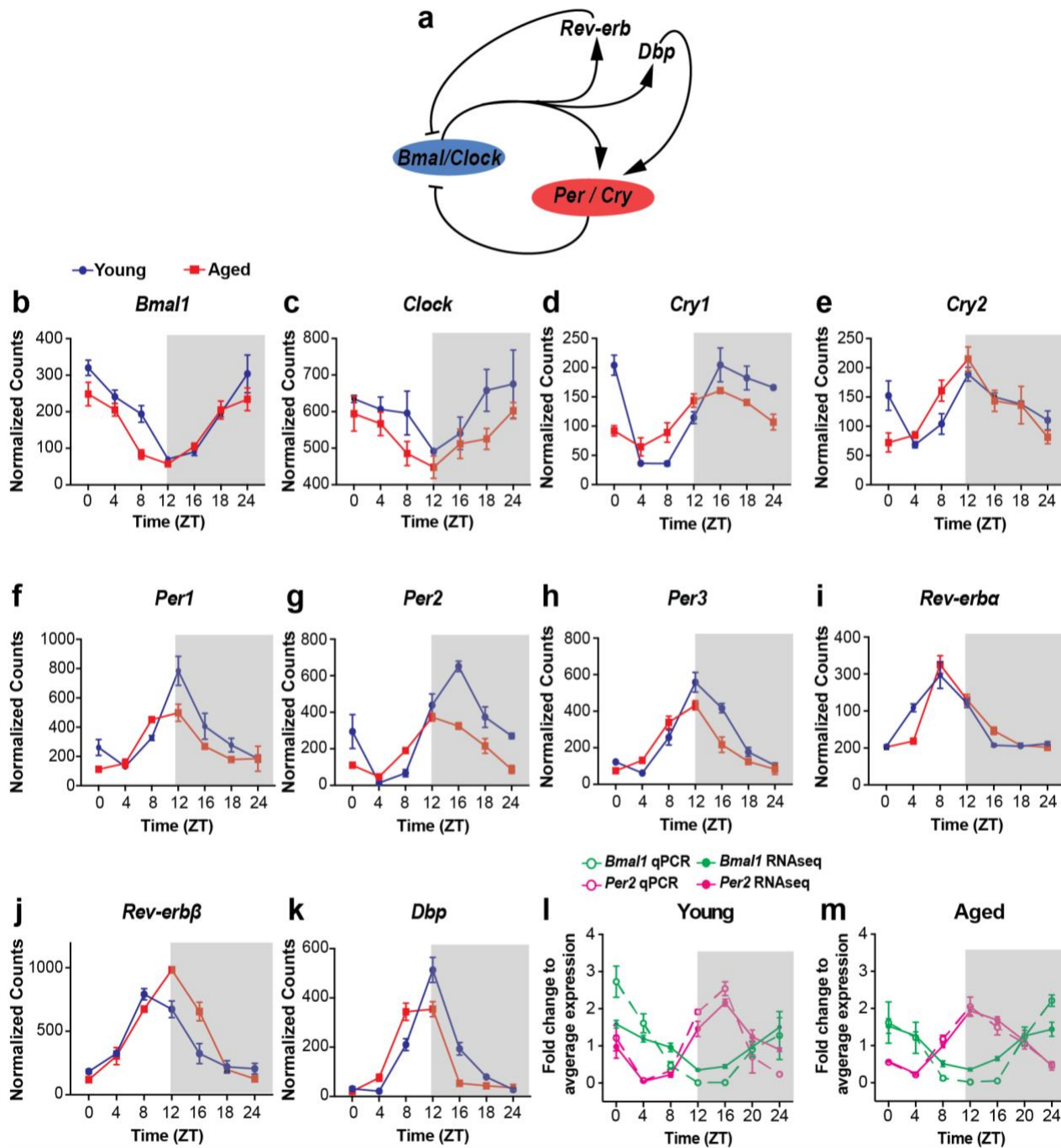
Supplementary Figure 3. The core circadian clock genes remain rhythmic in aged peritoneal macrophages.

**a.** Overview of circadian clock.

b-k, Individual representations of circadian expression levels measured by RNA-seq of the positive arm (**b-c**), negative arm (**d-h**) and supporting genes (**i-k**) of the core clock machinery. (**l-m**) qPCR validation of *Bmal1* and *Per2* expression in young and aged peritoneal macrophages. n=21 mice in each age group and n=3 in each time group.

9
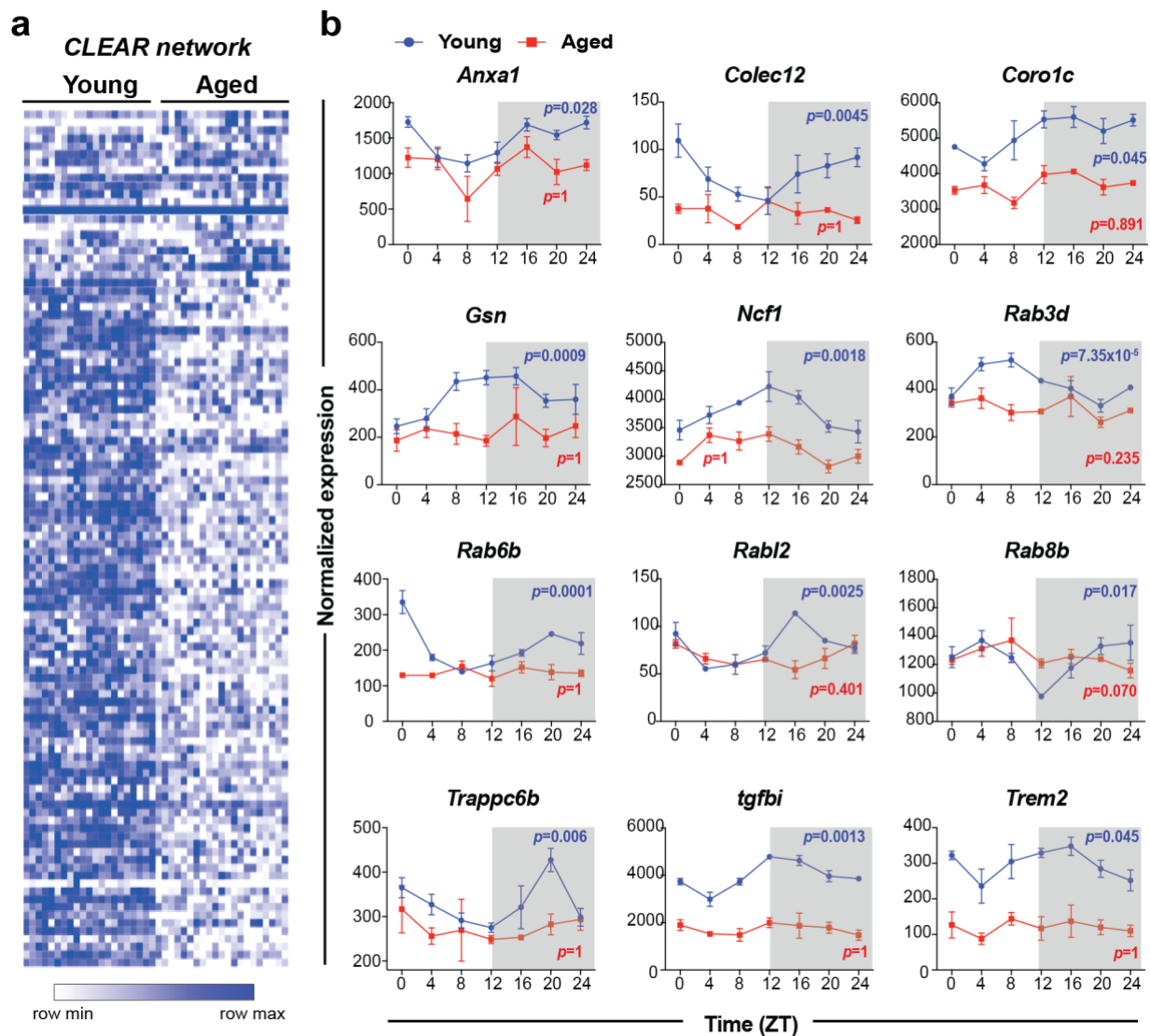
Supplementary Figure 4. Loss of circadian rhythmicity of phagocytosis gene expression in aged macrophages.

a. Heatmap of CLEAR network genes for all time points for each age group shows a decrease in overall gene expression in aged as compared to young peritoneal macrophages.

b. Circadian RNA-seq expression patterns of phagocytosis-related genes reveal loss of rhythmicity in aged peritoneal as compared to young macrophages. n=21 mice in each age group and n=3 in each time group. Indicated p-values were calculated by JTK_cycle.
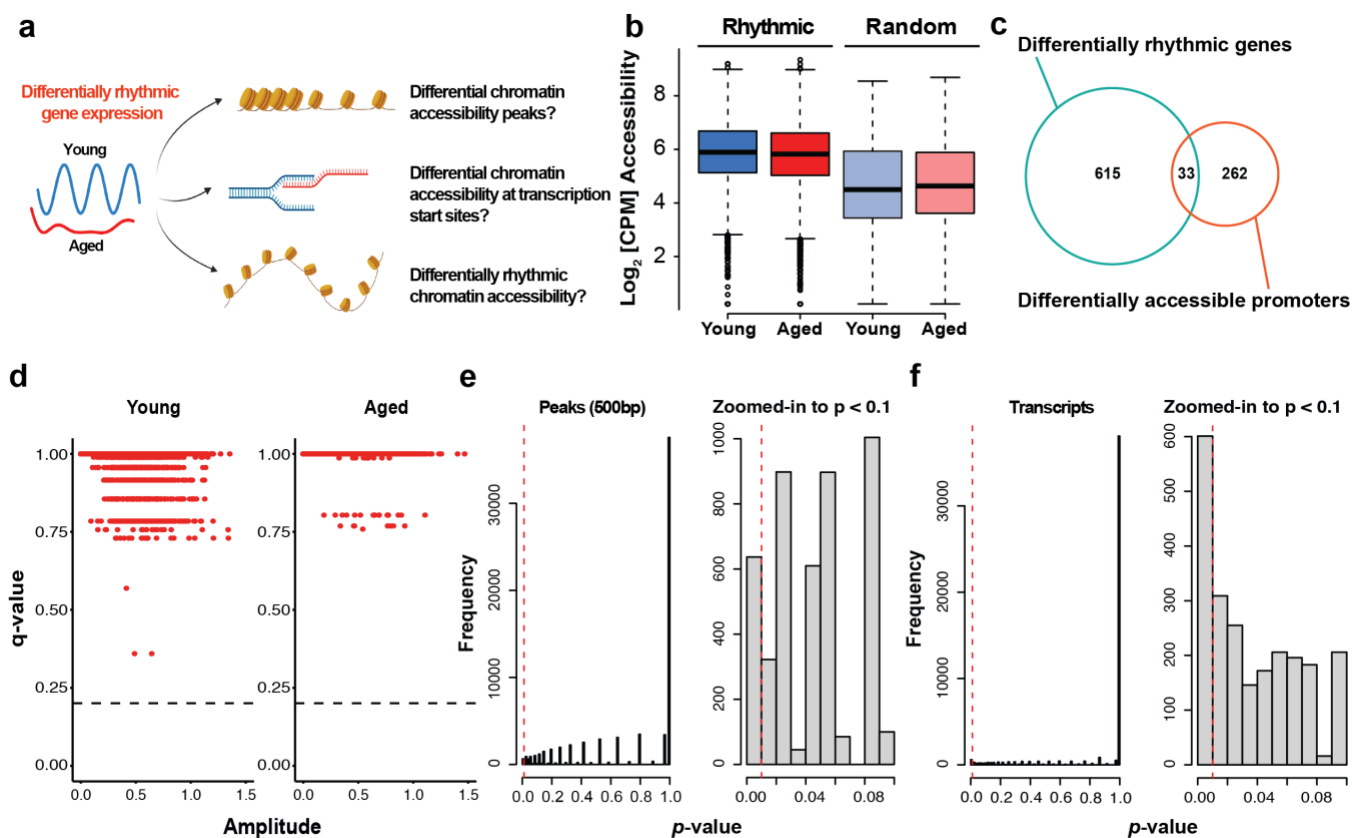
**Supplementary Figure 5. Chromatin accessibility of young and aged macrophages is not rhythmic.**

a. Schematic of investigated possible explanations for differentially rhythmic gene expression by chromatin accessibility.

b. Chromatin accessibility (as normalized and log$_2$-transformed values) of promoter regions of differentially rhythmic genes between young and aged peritoneal macrophages. Note that rhythmically expressed genes have higher chromatin accessibility compared to an equal number of randomly selected control genes.

c. Venn diagrams of the numbers of differentially rhythmic genes between the two age groups (RNA-seq) and the numbers of differentially accessible promoter regions assessed by ATAC-seq.

d, Scatterplots of amplitude and *q*-value for open chromatin peaks as assessed by JTK_cycle. No element shows *q*<0.2 (indicated by dotted line).

e, **f**, Distribution of unadjusted *p*-values for oscillations of open chromatin peaks (**e**) and transcripts (**f**) in young macrophages, zoomed-in to *p*<0.1, JTK_cycle.
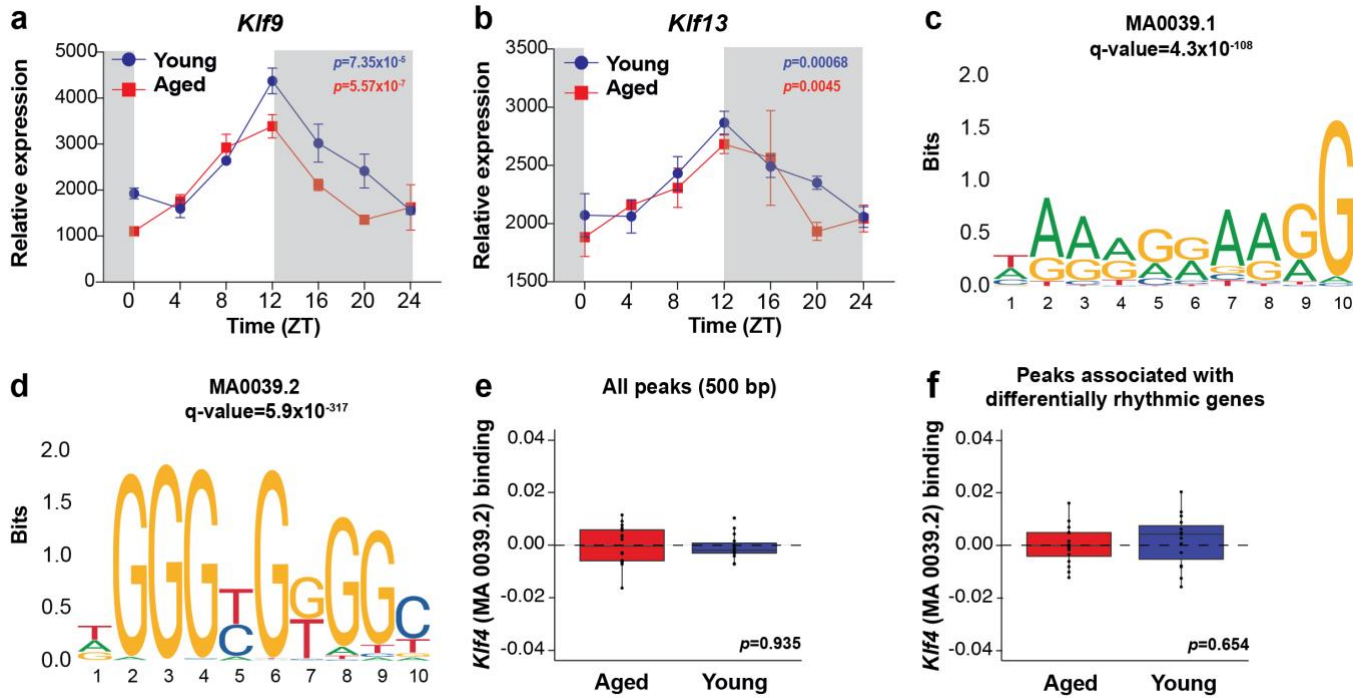
Extended Data Figure 6. Two distinct DNA binding motifs of Klf4.

a, **b**. Circadian expression levels measured by RNA-seq of KLF family members *Klf9* and *Klf13*. n=21 mice in each age group and n=3 in each time group. *p*-values determined by JTK_cycle.

c, **d**. Depiction of the two known KLF4 binding motifs, MA0039.1 and MA0039.2. *q*-values from *de novo* motif discovery on a KLF4 ChIP-seq experiment from ENCODE (https://factorbook.org/experiment/ENCSR265WJC/motif).

e. chromVAR[9] deviations within all 500 bp peaks indicating Klf4 binding by estimating accessibility within peaks sharing the MA0039.2 motif or annotation. *p*=0.935, two-sided Mann-Whitney U test.

f. chromVAR[9] deviations within peaks associated with differentially rhythmic genes indicating KLF4 binding by estimating accessibility within peaks sharing the MA0039.2 motif or annotation. *p*=0.654, two-sided Mann-Whitney U test.

All	Controls n=329757	TT n=15 537	p-Value (univariate)	p-value (multivariate)
Characteristics				
Age (years)	56.5±8.1	56.6±8.0	.20	
Women (%)	54	55	**.007**	
BMI (kg/m^2)	27.4±4.8	27.3±4.8	**.003**	
Survival (years)	10.9	10.9	.35	
Died (%)	6.0	6.1	.57	
Overall infections (A00-A99)(%)	4.5	4.6	.45	.40
Infection with E coli (A04) (%)[a]	0.01	0.04	**.003**	**.006**
≤65years	**Controls** n=266 771	**TT** n=12 593	**p-Value**	
Characteristics				
Age (years)	54.0±7.0	56.6±8.0	.20	
Women (%)	55	56	**.007**	
BMI (kg/m^2)	27.4±4.8	27.3±4.8	**.006**	
Overall infections (A00-A99) (%)	4.07	4.20	.051	.054
Infection with E coli (A04) (%)[b]	0.01	0.04	**.003**	**.021**
≥65years	**Controls** n=62 986	**TT** n=2 944	**p-Value**	
Characteristics				
Age (years)	66.9±1.5	66.9±1.5	.78	
Women (%)	50	51	**.24**	
BMI (kg/m^2)	27.6±4.8	27.5±4.8	**.18**	
Overall infections (A00-A99) (%)[c]	6.70	5.70	.020	**.021**
Infection with E coli (A04) (%)	0.01	0.03	**.52**	**.30**
Died	**Controls** n=19 791	**TT** n= 949		
Characteristics				
Age (years)	61.6±6.5	61.3±6.8	.24	
Women (%)	40	41	.51	
BMI (kg/m^2)	28.3±5.4	28.4±5.5	.56	
Death of Infection (A00-A99) (%)[d]	0.6	1.2	.052	**.047**

Supplementary Table 1: Comparison of baseline characteristics in participants with rs2236599_T non-carriers and homozygotes. Quantitative measures are expressed as mean with standard deviation or relative frequency (%) and their corresponding univariate p-values. Abbreviations: BMI, body mass index.

ICD10 codes are given in round brackets. All multivariate analyses were adjusted for age, sex and BMI. [a]aOR=3.2[1.2-8.1]; [b]aOR3.5[1.2-9.9]; [c]aOR=0.83[0.71-0.97]; [d]aOR=1.87[1.01-3.49].

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