Figures and figure supplements

Neutrophil infiltration regulates clock-gene expression to organize daily hepatic metabolism

María Crespo *et al*

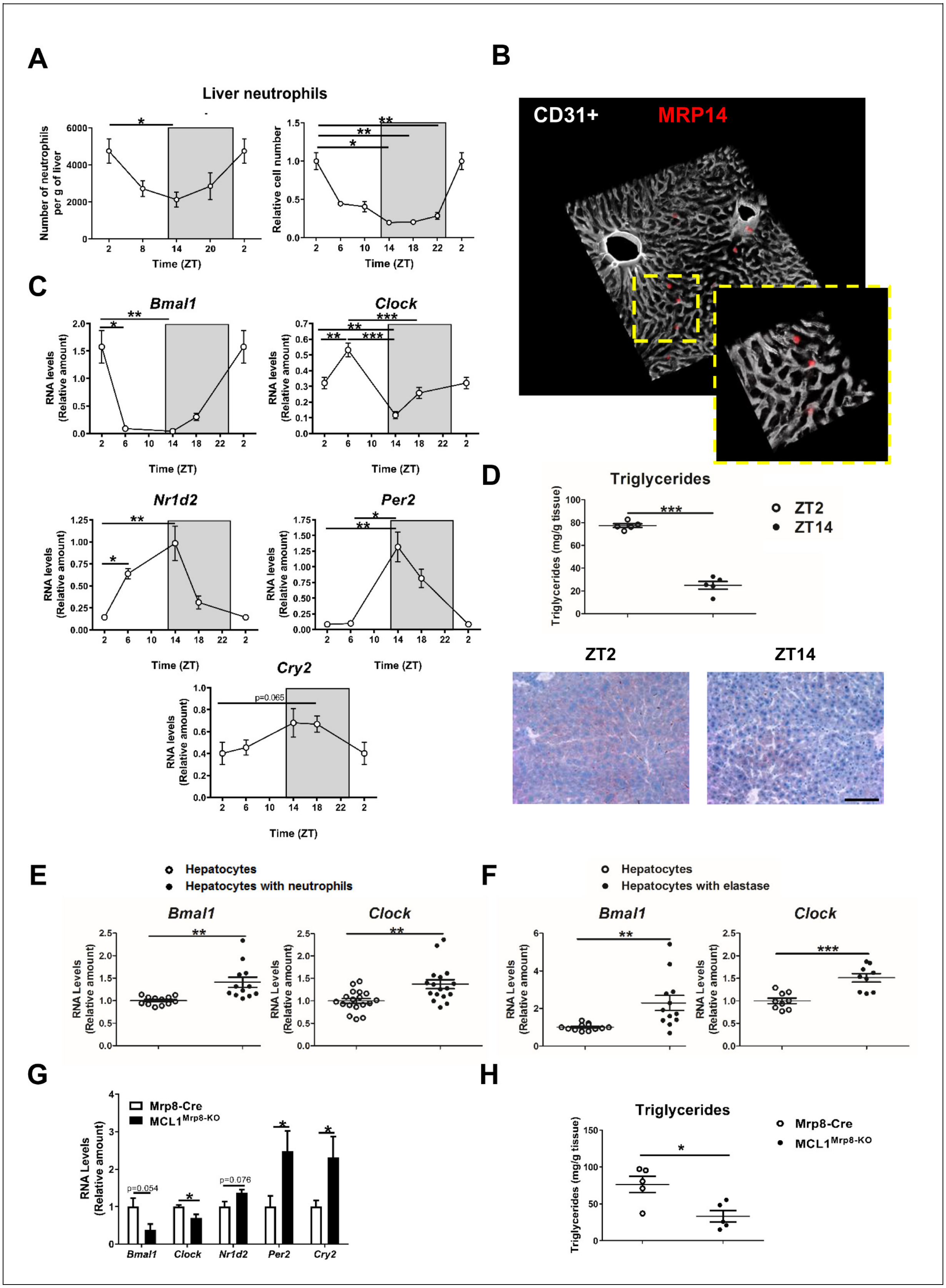

Figure 1. Neutrophil infiltration into the liver controls hepatic clock-gene expression. (**A**) Flow cytometry analysis of the CD11b$^+$Ly6G$^+$ liver myeloid subset, isolated from C57BL6J mice at the indicated ZTs. Left, CD11b$^+$Ly6G$^+$ liver myeloid subset analyzed at 6 hr intervals and normalized by the

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tissue weight. Right, percentage of $CD11b^+Ly6G^+$ population analyzed at 4 hr intervals and normalized to ZT2 (n = 5). (**B**) Representative 3-D image of liver section showing the distribution on infiltrated neutrophils. Livers were stained with anti-S100A9 (Mrp14) (red) and vessels were stained with anti-CD31 and anti-endomucin (grey). Sizes of the liver sections are 510 x 510 x 28 µm and 160 x 160 x 28 µm, respectively. (**C**) qRT-PCR analysis of circadian clock-gene and nuclear-receptor mRNA expression in livers from C57BL6J mice at the indicated ZTs (n = 5). (**D**) Liver triglycerides and oil-red-stained liver sections prepared from C57BL6J mice at ZT2 and ZT14. Scale bar, 50 µm (n = 5). (**E**) qRT-PCR analysis of clock-gene mRNA in hepatocyte cultures exposed to freshly isolated FMLP-activated neutrophils (n = 4-6 wells of 3 independent experiments). (**F**) qRT-PCR analysis of clock-gene mRNA in hepatocyte cultures treated with 5 nM elastase (n = 3-4 wells of 3 independent experiments). (**G**) qRT-PCR analysis of clock-gene and nuclear-receptor mRNA expression in livers from control mice (Mrp8-Cre) and neutropenic mice ($MCL1^{Mrp8\text{-}KO}$) sacrificed at ZT2 (n = 5). (**H**) Hepatic triglycerides detected in livers from control mice (Mrp8-Cre) and neutropenic mice ($MCL1^{Mrp8\text{-}KO}$) at ZT2 (n = 5). Data are means ± SEM from at least 2 independent experiments. *p<0.05; **p<0.01; ***p<0.005 (A, left panel) One-way ANOVA with Tukey's post hoc test. (A, right panel) Kruskal-Wallis test with Dunn's post hoc test. (**C**) One-way ANOVA with Tukey's post hoc test or Kruskal-Wallis test with Dunn's post hoc test. (D to H) *t-test* or Welch's test. ZT2 point is double plotted to facilitate viewing.

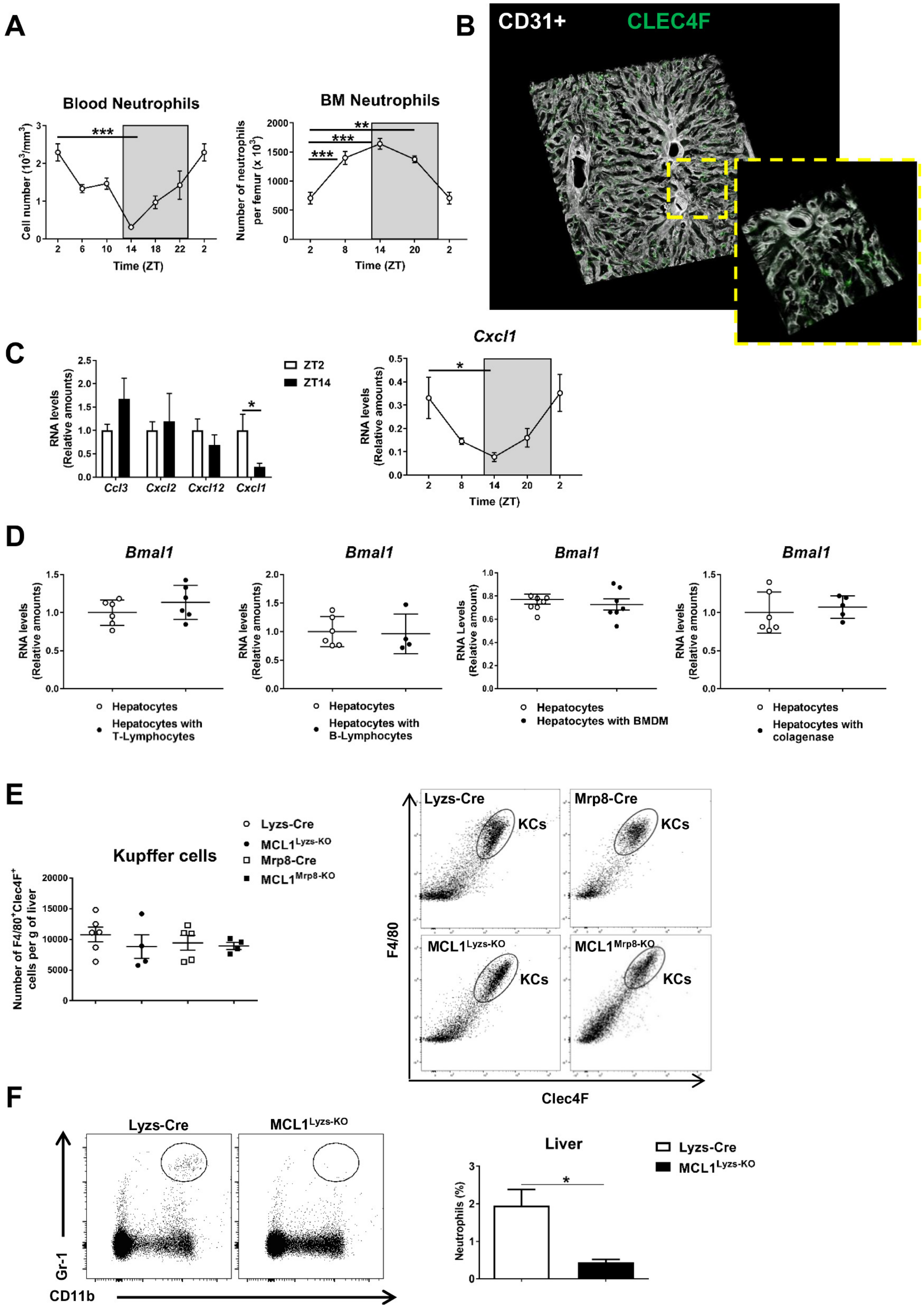

Figure 1—figure supplement 1. Neutrophils follow a circadian rhythm. (A) Left, circulating neutrophils quantified at 4 hr intervals in whole blood of C57BL6J mice. Right, flow cytometry analysis at 6 hr intervals of the CD11b$^+$Ly6G$^+$ myeloid subset in bone marrow from C57BL6J mice. ZT2 point is

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double plotted to facilitate viewing (n = 5). (**B**) Representative 3-D image of liver section showing the distribution of Kupffer cells. Livers were stained with anti-Clec4F (green) and vessels were stained with anti-CD31 and anti-endomucin (grey). Sizes of the liver sections are 510 x 510 x 28 μm and 160 x 160 x 28 μm, respectively (n = 5-7). (**C**) qRT-PCR of *Ccl3, Cxcl2, Cxcl12* and *Cxcl1* chemokines mRNA expression at ZT2 and ZT14 and qRT-PCR of *Cxcl1* mRNA expression at 6 hr intervals in livers from C57BL6J mice (n = 5). (**D**) qRT-PCR of *Bmal1* mRNA expression in hepatocyte cultures exposed to freshly isolated T-lymphocytes, B-lymphocytes or bone-marrow derived macrophages (BMDM) and 1 μM FMLP; *Bmal1* mRNA expression in hepatocyte cultures treated with 0.5 mg/mL collagenase (n = 3 wells of 2 to 3 independent experiments) (**E**) Left, flow cytometry analysis of number of liver Kupffer cells (KCs) in control Lyzs-Cre and MCL1$^{Lyzs\text{-}KO}$ mice and in Mrp8-Cre and MCL1$^{Mrp8\text{-}KO}$ mice normalized by tissue weight. Right, representative dot plots showing F4/80^{+}Clec4F^{+} population gated on total intrahepatic CD45^{+}CD11b^{+} leukocyte population (n = 4-6). (**F**) Flow cytometry analysis of the CD11b^{+} Gr-1^{high} liver myeloid subset isolated from control (Lyzs-Cre) and neutropenic (MCL1$^{Lyzs\text{-}KO}$) mice. The bar chart shows the CD11b^{+} Gr-1^{high} population as a percentage of the total intrahepatic CD11b^{+} leukocyte population (n = 7-10). Data are means ± SEM. *p<0.05; **p<0.01; ***p<0.005 (A, left) Kruskal-Wallis with Dunn's post-hoc test. (A, right) One-way ANOVA with Tukey's pots hoc test. (C, left) *t-test*. (C, right) Kruskal-Wallis with Dunn's post-hoc test. (**D**) *t-test*. (**E**) One-way ANOVA with Tukey's pots-hoc test. (**F**) *t-test*.

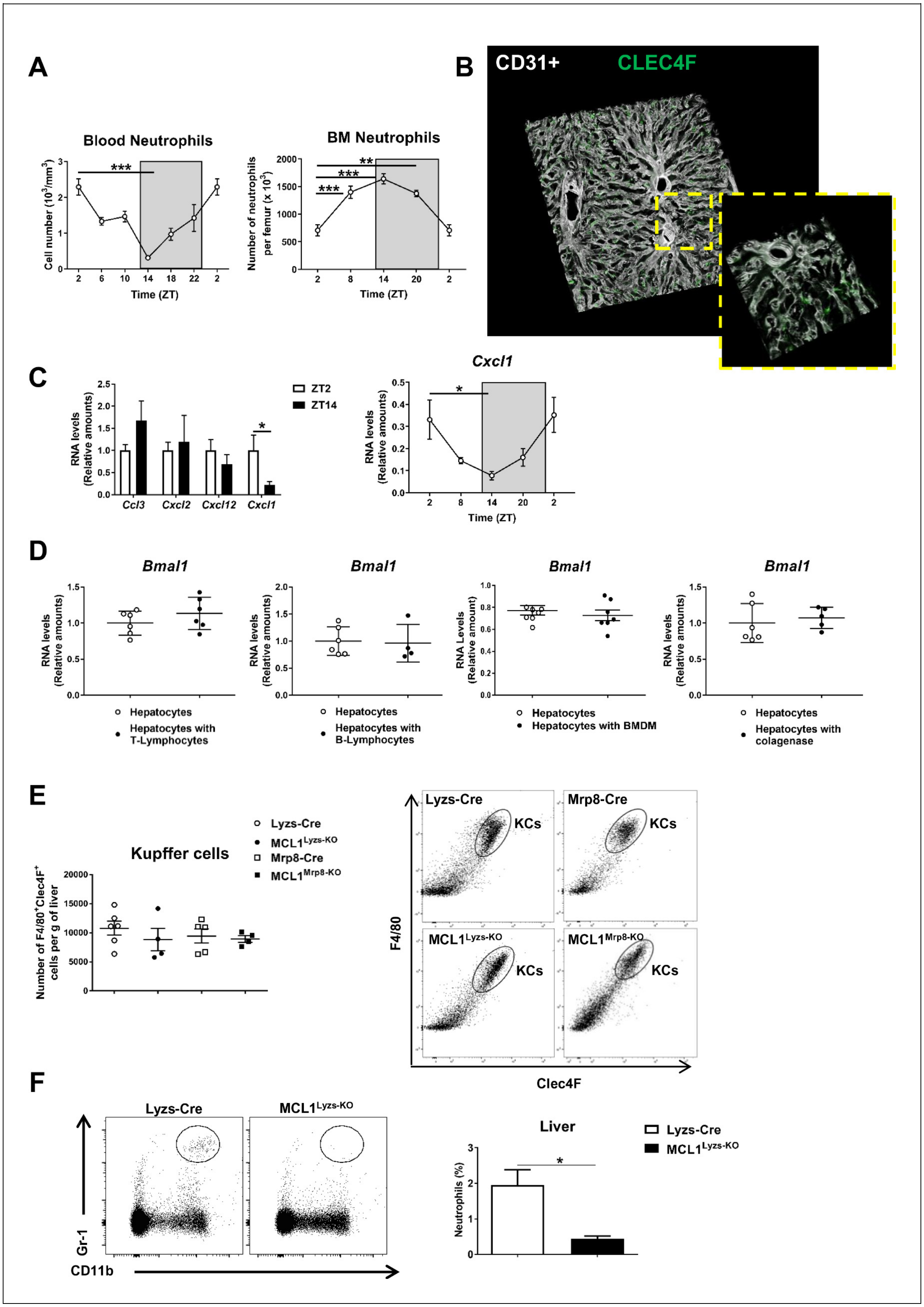

Figure 1—figure supplement 2. Neutrophil deficiency alters clock-gene expression. (A) Representative dot plots showing the decrease in the CD11b$^+$ Gr-1^{high} population in blood, bone marrow, and spleen from neutropenic mice (MCL1$^{Lyzs-KO}$) compared with control mice (Lyzs-Cre). Bar charts show

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the CD11b$^+$ Gr-1^{high} population as a percentage of the total CD11b$^+$ leukocyte population. (**B**) Blood levels of monocytes and neutrophils in control and neutropenic mice. (**C**) Myeloid cell populations in bone marrow and liver determined by flow cytometry and representative dot plots (CD11b$^+$ Gr-1^{neg} as macrophages, CD11b$^+$ Gr-1^{int} as monocytes and CD11b$^+$ Gr-1^{high} as neutrophils). (**D**) qRT-PCR of clock genes in the livers from control (Lyzs-Cre) and neutropenic (MCL1$^{Lyzs\text{-}KO}$) mice. ZT2 point is double plotted to facilitate viewing (n = 5-7). (**E**) Left, flow cytometry analysis of the CD11b$^+$ Ly6G$^+$ lung myeloid subset of control (Lyzs-Cre) and neutropenic (MCL1$^{Lyzs\text{-}KO}$) mice at the indicated ZTs (n = 4). Right, qRT-PCR analysis of *Bmal1* in lungs of control (Lyzs-Cre) and neutropenic (MCL1$^{Lyzs\text{-}KO}$) mice at the indicated ZTs (n = 4-6). Data are means ± SEM. *p<0.05; **p< 0.01; ***p<0.005. All tests are *t-test* or Welch's test.

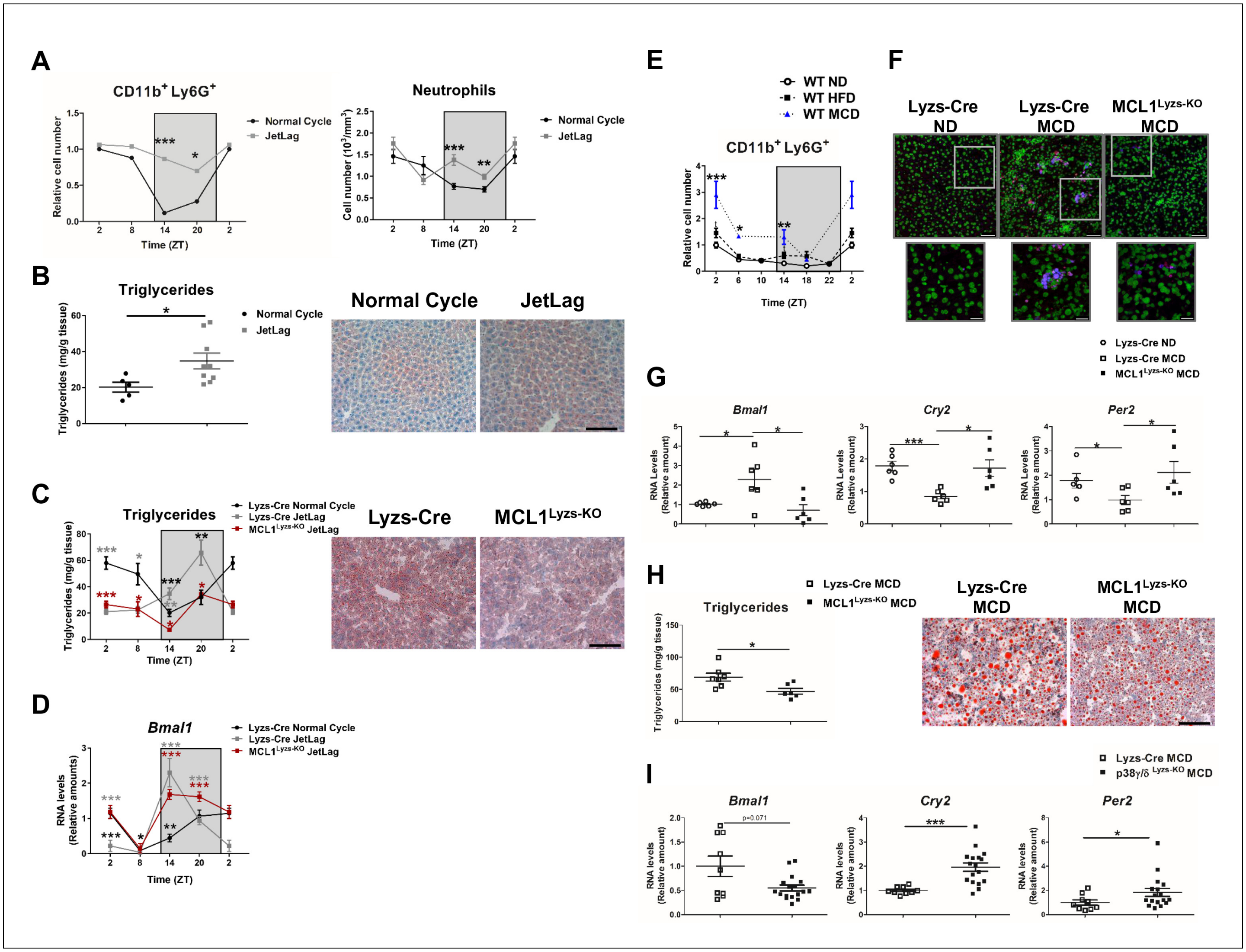

Figure 2. Increased hepatic neutrophil infiltration alters clock-genes expression and augments triglyceride content in the liver. (**A–D**) Control (Lyzs-Cre) (**A–B**) and control and neutropenic (MCL1$^{Lyzs-KO}$) mice (**C–D**) were housed for 3 weeks with a normal 12 hr: 12 hr light/dark cycle (Normal Cycle) or with the dark period extended by 12 hr every 5 days (JetLag). Samples were obtained at the indicated ZTs. (**A**) Left, flow cytometry analysis of the CD11b$^+$Ly6G$^+$ liver myeloid subset. Data represents the percentage CD11b$^+$Ly6G$^+$ normalized to Normal Cycle ZT2. Right, circulating neutrophils in whole blood. (n = 5-8). (**B**) Liver triglycerides and representative oil-red-stained liver sections at ZT14. Scale bar, 50 µm (n = 9-10). (**C**) Hepatic triglyceride content analyzed at 6 hr intervals, and representative oil-red-stained liver sections at ZT14. Scale bar, 50 µm (n = 4-6). (**D**) qRT-PCR analysis of *Bmal1* mRNA in livers. (n = 5-8). (**E**) Flow cytometry analysis of the CD11b$^+$Ly6G$^+$ liver myeloid subset isolated at 6 hr intervals from C57BL6J mice fed a ND, a HFD (8 weeks) or a MCD (3 weeks). The chart shows the CD11b$^+$Ly6G$^+$ population as a percentage of the total intrahepatic CD11b$^+$ leukocyte population normalized to ND group at ZT2 (n = 5 to 10). (**F–I**) Control mice (Lyzs-Cre) and neutropenic mice (MCL1$^{Lyzs-KO}$) or p38γ/δ$^{Lyzs-KO}$ were fed a ND or the MCD diet for 3 weeks and sacrificed at ZT2. (**F**) Representative images of the infiltration of neutrophils in the liver stained with anti-Mrp14 (blue) and anti-NE (red); nuclei with Sytox Green. Scale bar, 50 µm (Top) and 25 µm (Bottom). (**G**) qRT-PCR analysis of clock-gene expression in livers (n = 6). (**H**) Liver triglycerides and representative oil-red-stained liver sections. Scale bar, 50 µm (n = 7-6). (**I**) qRT-PCR analysis of clock genes in livers at ZT2 (n = 9-17). Data are means ± SEM from at least two independent experiments. *p<0.05; **p<0.01; ***p<0.005 (A to D) *t-test* or Welch's test. (**E**) Two-way ANOVA with Fisher's post hoc test; p<0.05 ND vs HFD; p<0.0001 ND vs MCD. *p<0.05; ***p<0.005 (G to I) *t-test* or Welch's test. ZT2 point is double plotted to facilitate viewing.

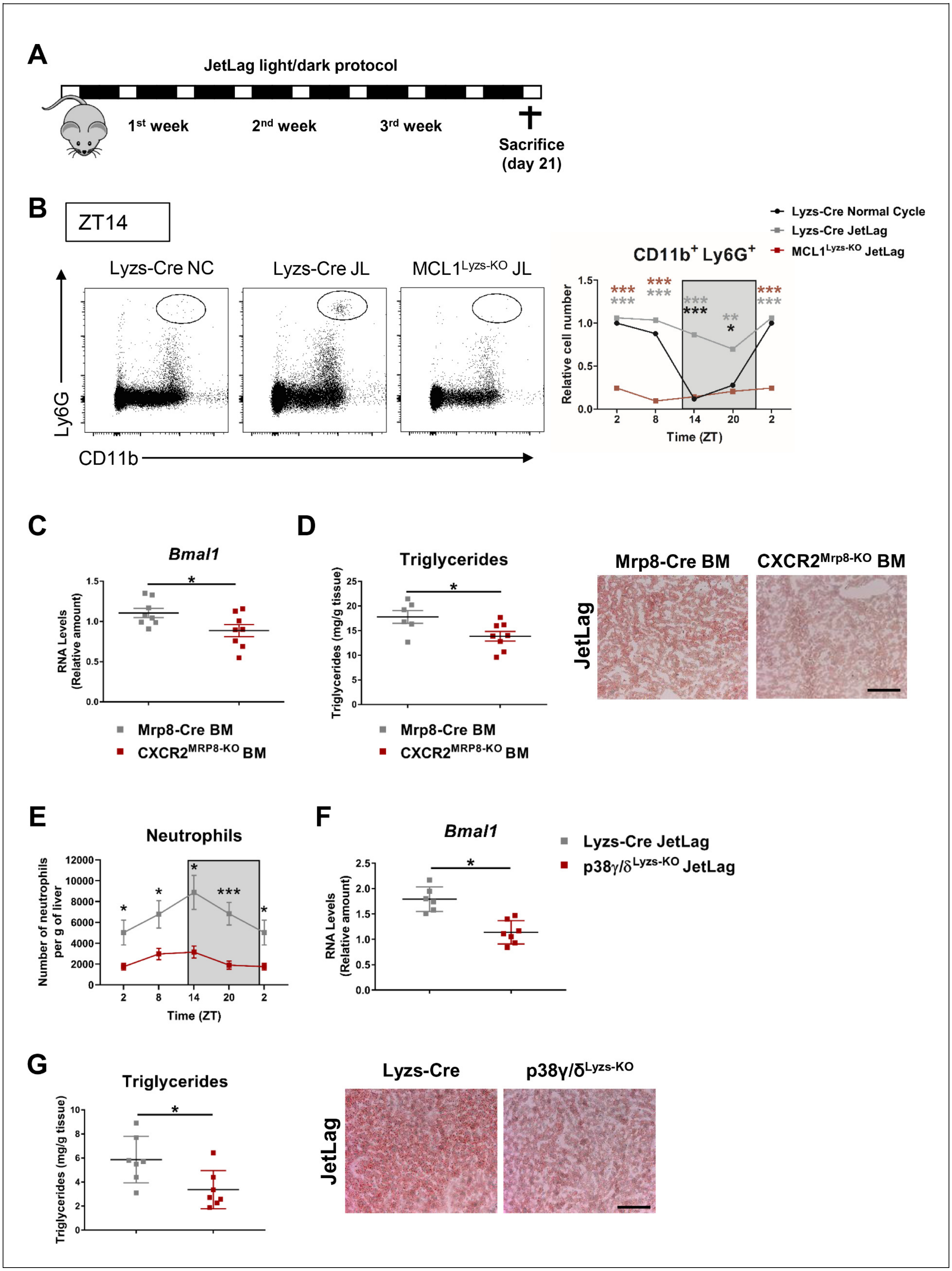

Figure 2—figure supplement 1. Defective neutrophil migration to the liver alters hepatic clock- gene expression and triglyceride content. (A) Schematic representation of JetLag protocol with stepwise increases in the dark period of 12 h12h every 5 days (B) Flow cytometry analysis of the

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$CD11b^+Ly6G^+$ liver myeloid subset isolated from control (Lyzs-Cre) and neutropenic ($MCL1^{Lyzs\text{-}KO}$) mice housed for 3 weeks under a 12 hr:12 hr light/dark cycle (Normal Cycle) or Jetlag. The bar chart shows the percentage of $CD11b^+Ly6G^+$ total intrahepatic $CD11b^+$ leukocyte population analyzed at 6-h intervals and normalized to Normal Cycle ZT2 (n = 5-7). Dot plots show $CD11b^+$ $Ly6G^+$ population at ZT14. (**C–D**) After bone -marrow (BM) reconstitution of irradiated WT mice using Mrp8-Cre (Mrp8-Cre BM) or $CXCR2^{Mrp8\text{-}KO}$ ($CXCR2^{Mrp8\text{-}KO}$) mice as BM donors, mice were housed for 3 weeks under JetLag (n = 6-8) (**C**) qRT-PCR analysis of *Bmal1* mRNA in livers at ZT14. (**D**) Hepatic triglyceride content and representative oil-red-stained liver sections at ZT14. Scale bar, 50 µm. (**E–G**) Control (Lyzs-Cre) and $p38\gamma/\delta^{Lyzs\text{-}KO}$ mice were housed for 3 weeks under JetLag (n = 6-7) (**E**) Flow cytometry analysis of the $CD11b^+$ $Ly6G^+$ liver myeloid subset analyzed at 6 hr intervals and normalized by the tissue weight. (**F**) qRT-PCR analysis of *Bmal1* mRNA in livers at ZT14. (**G**) Hepatic triglyceride content and representative oil-red-stained liver sections at ZT14. Scale bar, 50 µm. Data are means ± SEM. $^*p<0.05$; $^{**}p< 0.01$; $^{***}p<0.005$ All tests are *t-test* or Welch's test. ZT2 point is double plotted to facilitate viewing.

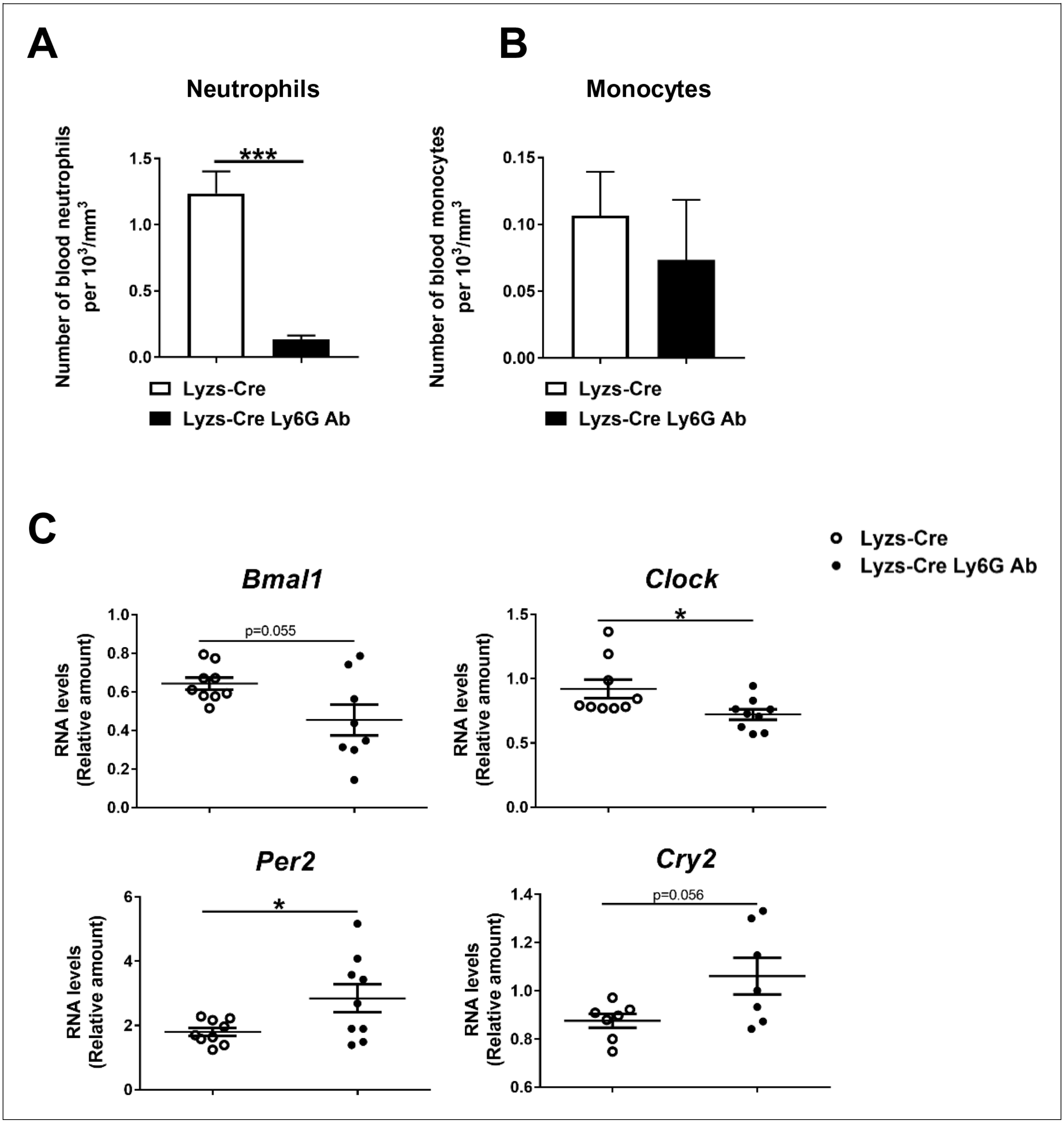

Figure 2—figure supplement 2. Neutrophil depletion alters hepatic clock-gene expression. (A-C) Osmotic minipumps containing saline or Ly6G antibody were implanted subcutaneously in Lyzs-Cre mice. These animals were fed with a MCD diet for 3 weeks and sacrificed at ZT2. (A-B) Blood levels of neutrophils and monocytes in Lyzs-Cre after 3 weeks of MCD diet treated or not with Ly6G antibody. (C) qRT-PCR of clock genes in the liver (n = 7-9). Data are means ± SEM. *p<0.05; ***p<0.005. All tests are *t-test* or Welch's test.

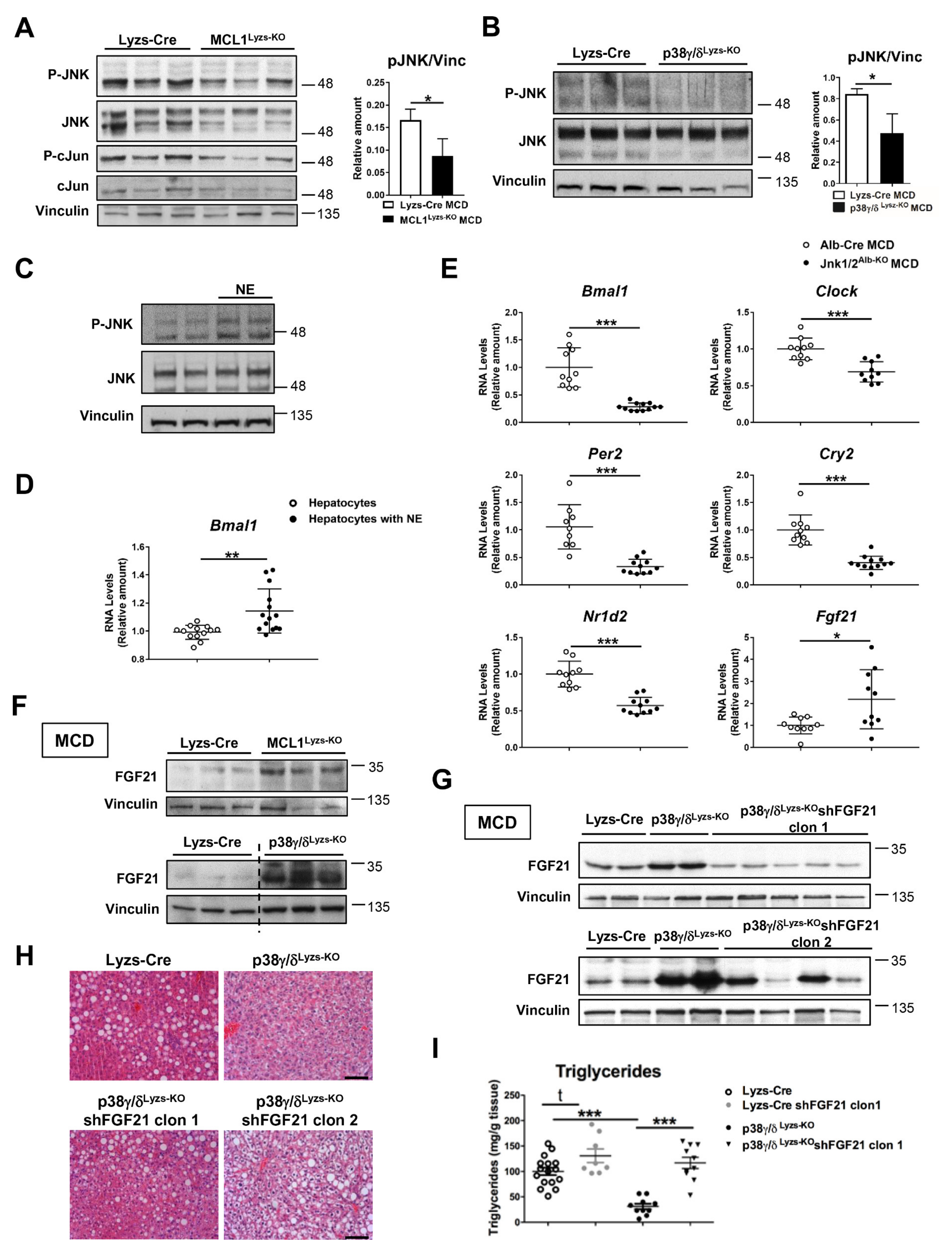

Figure 3. Diurnal regulation of liver metabolism involves neutrophil-mediated regulation of JNK and the hepatokine FGF21. Immunoblot analysis of JNK content and activation at ZT2 in liver extracts prepared from control (Lyzs-Cre) and neutropenic (MCL1$^{\text{Lyzs-KO}}$) mice fed a MCD diet for 3 weeks (**A**)

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or Lyzs-Cre and p38γ/δ$^{Lyzs\text{-}KO}$ mice after 3 weeks of MCD diet (**B**). Immunoblot analysis of JNK content and activation (**C**) and *Bmal1* RNA expression (**D**) in hepatocyte cultures exposed to NE for 2 hr (n = 14 wells of 3 independent experiments). Immunoblot quantification is shown in ***Figure 3—figure supplement 1D*** (**E**) qRT-PCR analysis of clock genes and *Fgf21* in livers from Alb-Cre, and JNK1/2$^{Alb\text{-}KO}$ mice after 3 weeks of MCD diet at ZT2 (n = 9-12). (**F**) Immunoblot analysis of FGF21 content in liver extracts prepared from control (Lyzs-Cre) and neutropenic (MCL1$^{Lyzs\text{-}KO}$) mice, or from Lyzs-Cre, and p38γ/δ$^{Lyzs\text{-}KO}$ mice after 3 weeks of MCD diet sacrificed at ZT2. Immunoblot quantification is shown in ***Figure 3—figure supplement 1I,J***. (**G–I**) Lyzs-Cre and p38γ/δ$^{Lyzs\text{-}KO}$ mice were injected with 2 shRNA independent clones targeting FGF21. Seven days after infection, mice were placed on the MCD diet and sacrificed after 3 weeks at ZT2. (**G**) Immunoblot analysis of FGF21 content in liver extracts prepared from Lyzs-Cre, p38γ/δ$^{Lyzs\text{-}KO}$, and p38γ/δ$^{Lyzs\text{-}KO}$ mice infected with FGF21 shRNA. Immunoblot quantification is shown in ***Figure 3—figure supplement 1K***. (**H**) Representative H&E-stained liver sections. Scale bar, 50 μm. (**I**) Hepatic triglyceride content at the end of the treatment period (n = 8-10). Data are means ± SEM from at least 2 independent experiments. *p<0.05; **p<0.01; ***p<0.005 (**A, B, D and E**) *t*-test or Welch's test. (**I**) One-way ANOVA with Bonferroni post hoc test or *t-test*.

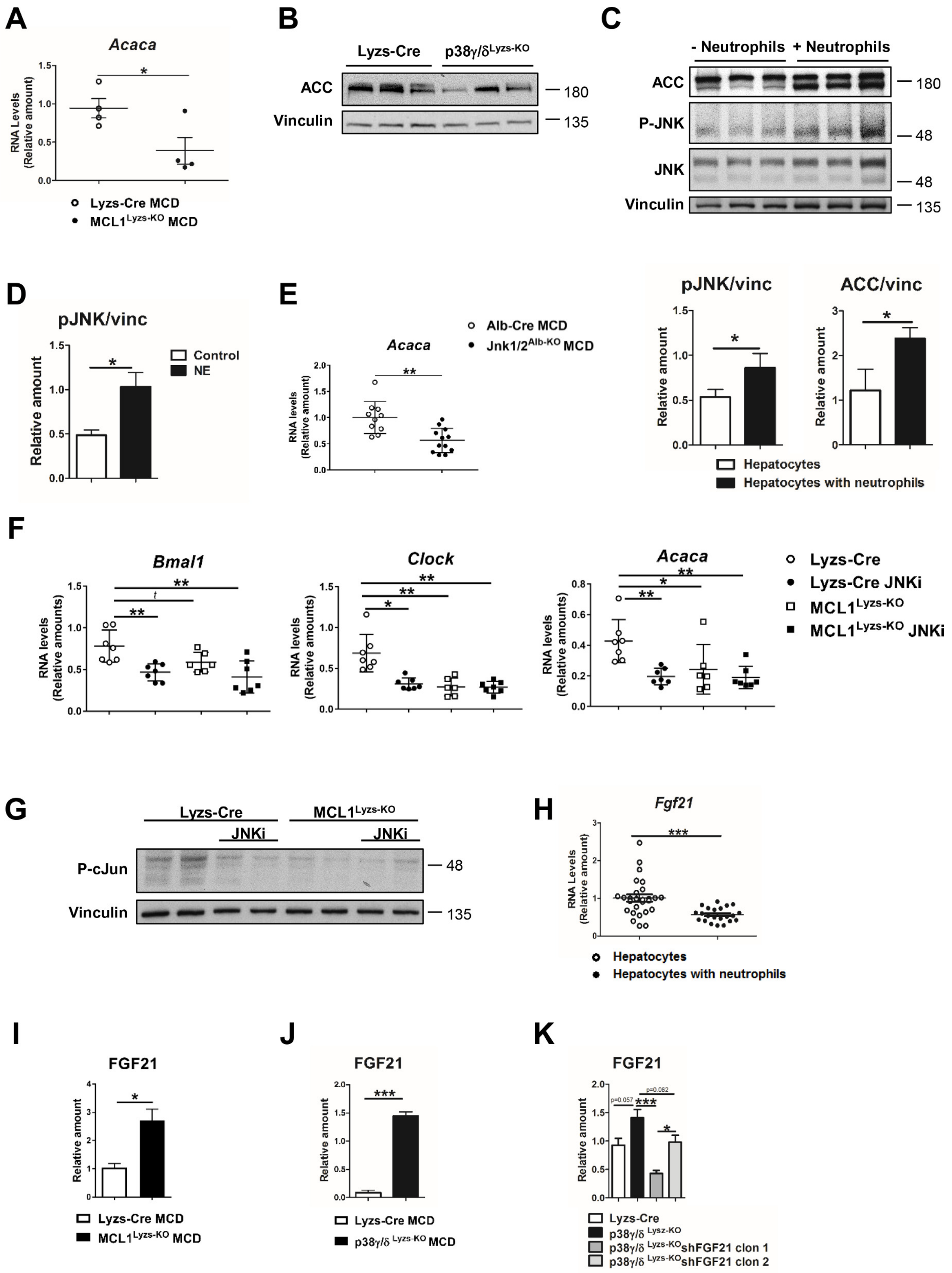

Figure 3—figure supplement 1. Neutrophils regulate hepatic metabolism and clock genes through JNK and FGF21. (**A**) qRT-PCR analysis of the metabolic gene *Acaca* in livers of control (Lyzs-Cre) and neutropenic (MCL1$^{Lyzs-KO}$) mice fed the MCD diet for 3 weeks (n = 4). (**B**) Immunoblot analysis

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of ACC content in livers from Lyzs-Cre and p38γ/δ$^{Lyzs\text{-}KO}$ mice at the end of the MCD diet. (**C**) Immunoblot analysis of ACC content and JNK content and activation in extracts prepared from hepatocyte cultures exposed to freshly isolated FMLP-activated for 1 h. Quantification is shown in the bottom panels. (**D**) Immunoblot analysis quantification of JNK content and activation in hepatocyte cultures treated with neutrophil elastase (NE) for 2 h. (**E**) qRT-PCR analysis of the metabolic gene *Acaca* mRNA expression from livers of Alb-Cre and JNK1/2$^{Alb\text{-}KO}$ mice fed a MCD for 3 weeks (n = 10-12). (**F**) qRT-PCR analysis of the clock genes *Bmal1* and *Clock* and the metabolic gene *Acaca* mRNA expression from livers of control and neutropenic mice treated with the JNK inhibitor SP600125. Mice were sacrificed at ZT2 (n = 6-7). (**G**) Immunoblot of c-Jun activation at ZT2 in livers from control and neutropenic mice treated with the JNK inhibitor SP600125. (H) qRT-PCR analysis of *Fgf21* mRNA expression in hepatocyte cultures exposed to freshly isolated FMLP-activated neutrophils 1 hr (n = 4 to 6 wells of 3 independent experiments). (I-K) Quantification of the immunoblot analysis of FGF21 content in extracts prepared from livers of control (Lyzs-Cre) and neutropenic (MCL1$^{Lyzs\text{-}KO}$) mice fed the MCD diet for 3 weeks (I), Lyzs-Cre, and p38γ/δ$^{Lyzs\text{-}KO}$ mice fed the MCD diet for 3 weeks (J**C**), and Lyzs-Cre and p38γ/δ$^{Lyzs\text{-}KO}$ mice injected with 2 shRNA independent clones targeting FGF21 and fed the MCD diet for 3 weeks (K) (n = 3). Data are means ± SEM. *p< 0.05; **p< 0.01; ***p<0.005 (**A–E**) *t-test.* (**F**) One-way ANOVA with Tukey's post hoc test, Kruskal-Wallis with Dunn's post hoc test or *t-test.* (H to J) *t*-test or Welch's test. (K) One-way ANOVA with Bonferroni post hoc test or *t-test.*

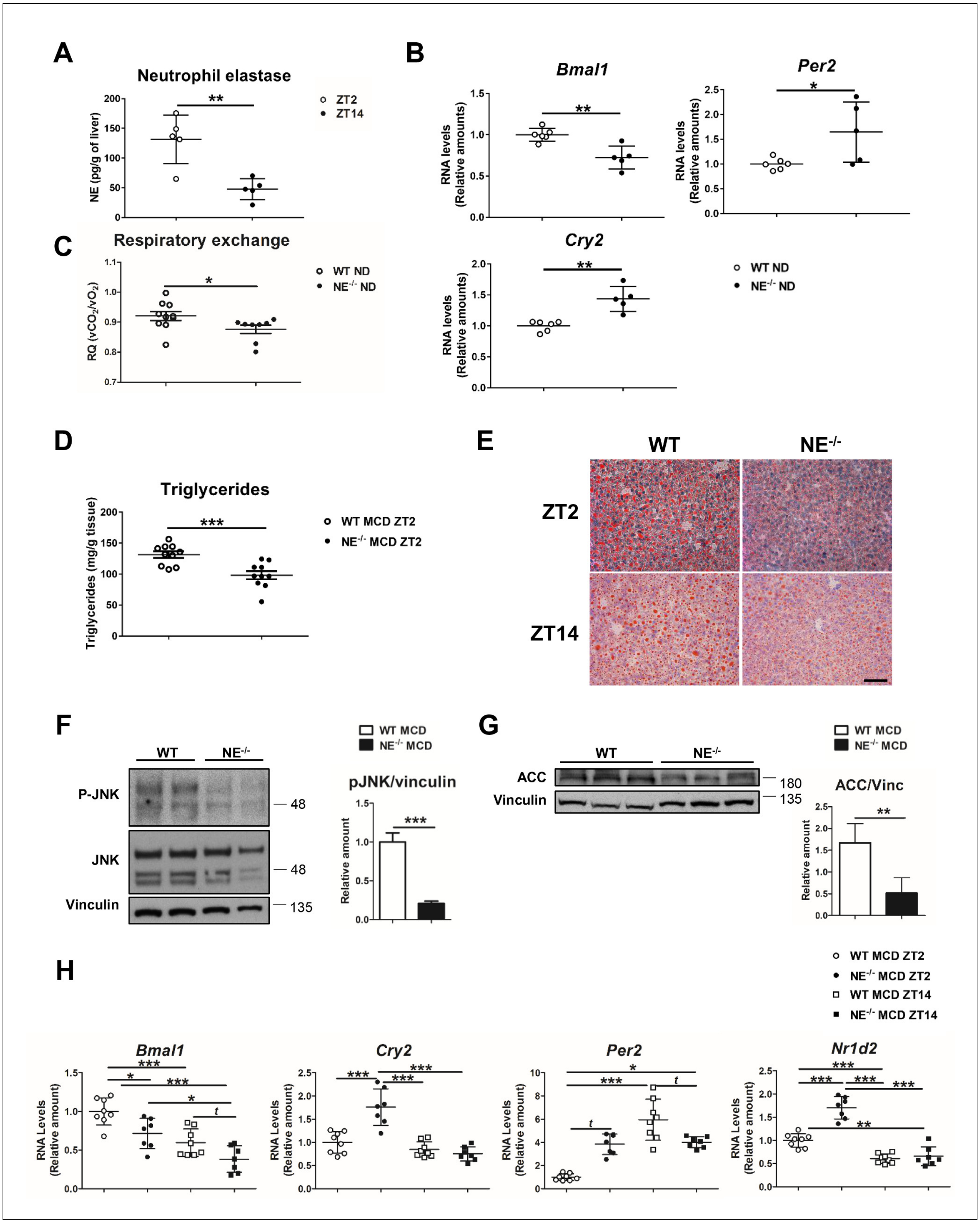

Figure 4. Elastase controls liver clock-gene expression modulating JNK activation. (**A**) Extracellular NE levels in livers from WT mice at ZT2 and ZT14. (**B**) qRT-PCR analysis of clock-genes and nuclear-receptor mRNA expression in livers from WT and NE KO mice (NE$^{-/-}$) at ZT2 (n = 5–6). (**C**) Respiratory

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exchange ratio of WT and NE$^{-/-}$ mice fed with ND. Results are from the lights-on period (n = 9). (**D–H**) WT and NE$^{-/-}$ mice were fed a MCD diet for 3 weeks and sacrificed at the indicated time. (**D**) Liver triglycerides at the end of the diet period. (**E**) Representative oil-red-stained liver sections. Scale bar, 50 μm (n = 10). (**F**) Immunoblot analysis and quantifications of JNK content and activation in liver extracts prepared from WT and NE$^{-/-}$. (**G**) Immunoblot analysis and quantification of ACC content in liver extracts from WT and NE$^{-/-}$ mice. (**H**) qRT-PCR analysis of clock-genes and nuclear-receptor mRNA expression in livers from WT and NE$^{-/-}$ mice at ZT2 and ZT14 (n = 7–8). Data are means ± SEM from at least two independent experiments. *p<0.05; **p<0.01; ***p<0.005 (A to G) *t*-test or Welch's test. (**H**) One-way ANOVA with to Tukey's post hoc test, *t-test* or Welch's test.

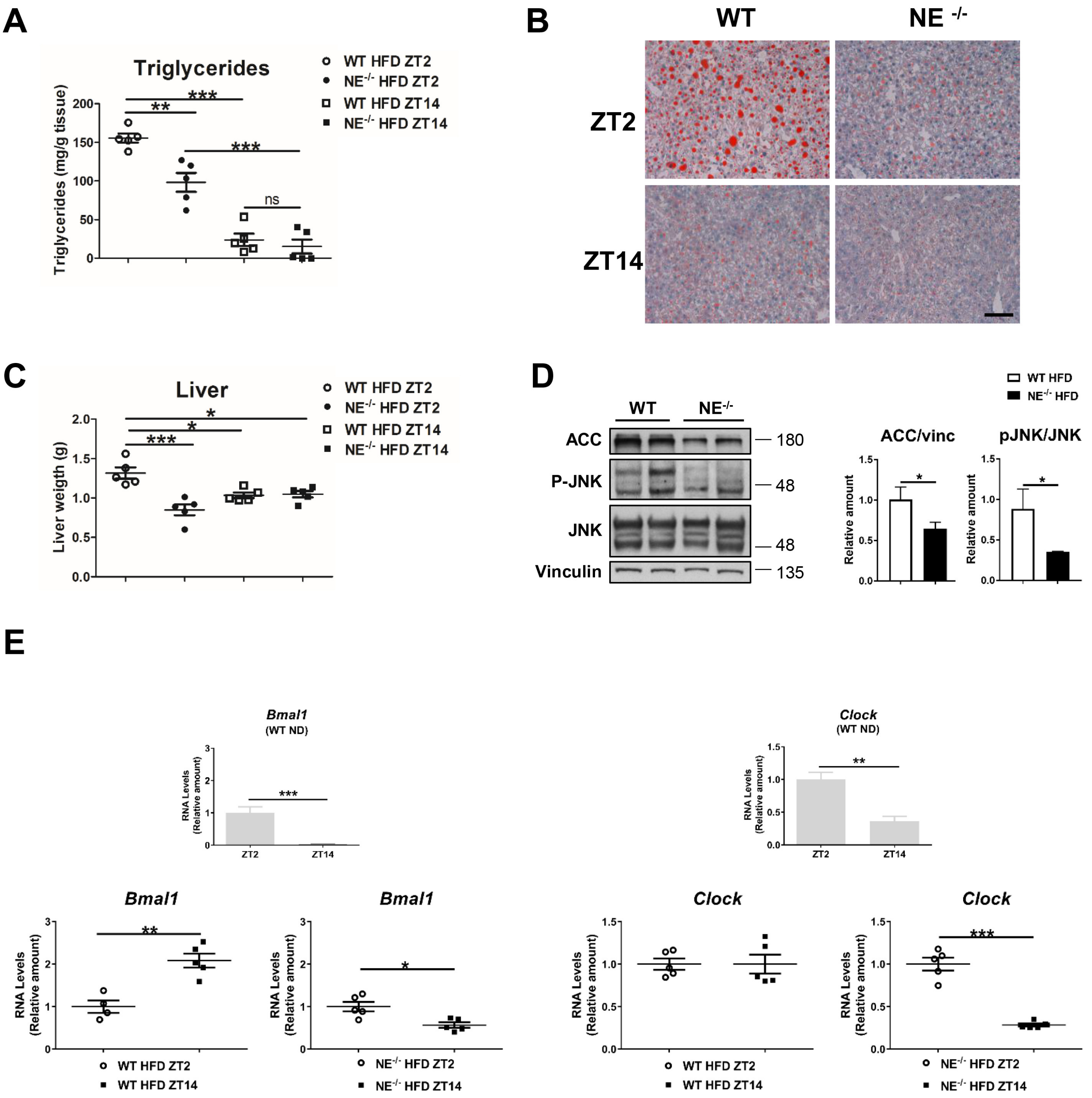

Figure 4—figure supplement 1. Neutrophil elastase regulates daily hepatic metabolism through JNK. NE$^{-/-}$ and control mice were fed a HFD for 8 weeks. (**A**) Liver triglycerides at the end of the diet period (n = 5). (**B**) Representative oil-red-stained liver sections. Scale bar, 50 µm. (**C**) Liver weight at the end of the treatment (n = 5). (**D**) Immunoblot analysis and quantifications of ACC content and JNK content and activation in liver extracts prepared from WT and NE$^{-/-}$ mice. (**E**) qRT-PCR analysis of clock-genes mRNA expression in livers from WT mice fed a ND (upper panels) and in WT and NE$^{-/-}$ mice fed a HFD (at ZT2 and ZT14 (bottom panels) at ZT12 and ZT14 (n = 5)). Data are means ± SEM from at least 2 independent experiments.*p<0.05; **p<0.01; ***p<0.005 (**A and C**) One-way ANOVA with Bonferroni post hoc test. (**D and E**) *t*-test or Welch's test.

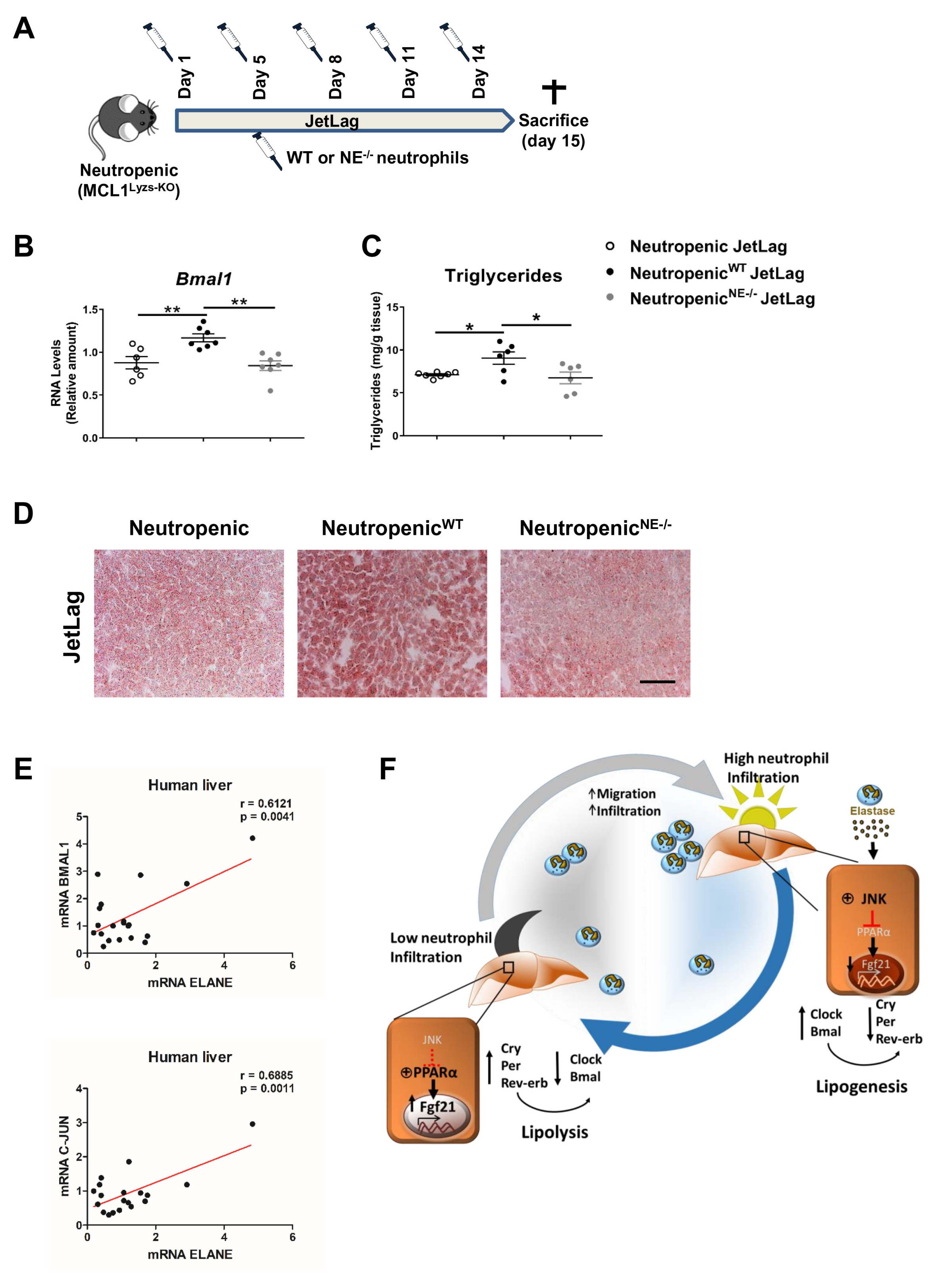

Figure 5. Neutrophil elastase reverses neutropenic mice phenotype through regulation of daily hepatic metabolism. (**A–D**) Neutropenic (MCL1$^{Lyzs\text{-}KO}$) mice were housed for 2 weeks with the dark period extended by 12 hr every 5 days (JetLag). Mice were infused with purified WT or NE$^{-/-}$ neutrophils.

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Samples were obtained at ZT14. (**A**) Picture describing the neutrophil infusion schedule during the JetLag protocol. (**B**) qRT-PCR analysis of *Bmal1* mRNA in livers. (**C**) Liver triglycerides and (**D**) representative oil-red-stained liver sections. Scale bar, 50 µm (n = 6-7). Data are means ± SEM. $*p<0.05$; t-test. (**E**) Correlation between mRNA levels of *BMAL1* and *ELANE* (r = 0.6141; p = 0.0052) or *JUN* and *ELANE* (r = 0.7362; p = 0.001105) in human livers. The mRNA levels of *JUN*, *BMAL1* and *ELANE* were determined by qRT-PCR. Linear relationships between variables were tested using Pearson's correlation coefficient (n = 23). (**F**) Circadian neutrophil infiltration regulates hepatic metabolism through elastase, JNK and FGF21. Data are means ± SEM. $*p< 0.05$; $**p< 0.01$; (B) One-way ANOVA with Tukey's pots hoc test. (**C**) *t-test* or Welch's test.