Supplemental information

Neutrophils Resist Ferroptosis and Promote Breast Cancer Metastasis through Aconitate Decarboxylase 1

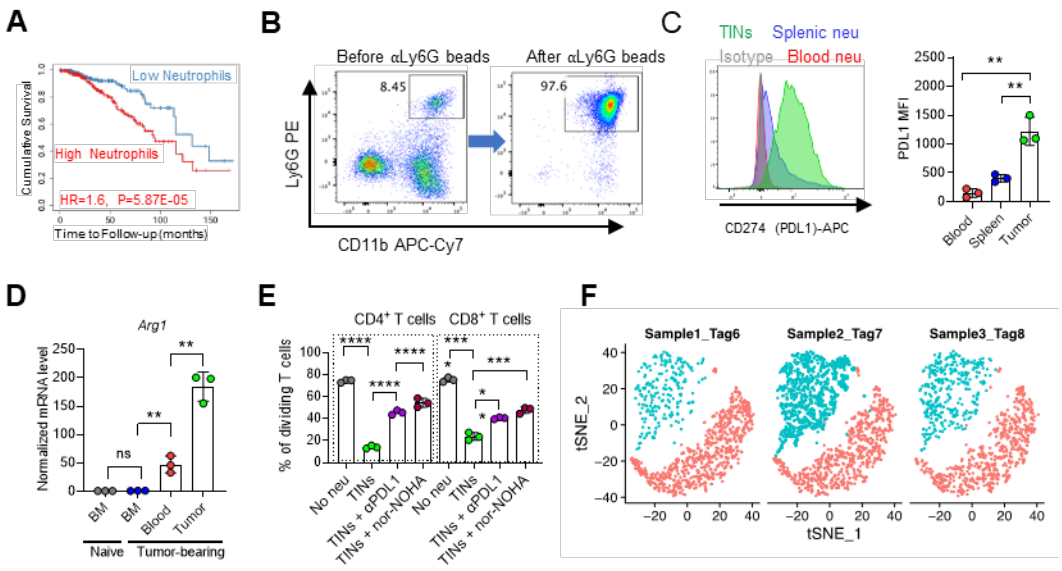

Figure S1. Neutrophils in the mammary TME employ PD-L1 and Arg1 to suppress CD4$^+$ and CD8$^+$ T cell proliferation. Related to Figure 1.
(A) Kaplan-Meier plot for neutrophil high (top 25%) and low (bottom 25%) groups of the breast cancer TCGA cohort (n=1100), generated by TIMER2.0. P value calculated based on Cox Proportional Hazard Model. **(B)** Representative flow cytometry result to show neutrophil percentage in CD45$^+$ cells from mammary tumors before and after purification with αLy6G magnetic microbeads. **(C)** Flow cytometry to assess PD-L1 expression in neutrophils isolated from blood, spleen, and tumor of E0771-bearing mice (n=3). **(D)** qRT-PCT to assess *Arg1* expression in neutrophils isolated from blood, spleen and tumor of E0771-bearing mice (n=3). **(E)** Flow cytometry to assess proliferation of CFSE-labelled T cells co-cultured for 48h with TINs with or without αPDL1 antibody at 10ug/ml or arginase inhibitor nor-NOHA at 300uM (n=3). **(F)** t-distributed stochastic neighbor embedding (t-SNE) plots colored by samples multiplexed with hashtag antibodies. For **C**, **D** and **E**, data represent mean ± s.e.m.; ns, not significant, **P<0.01, ***P<0.001, ****P<0.0001, unpaired two-tailed Student's t-test.

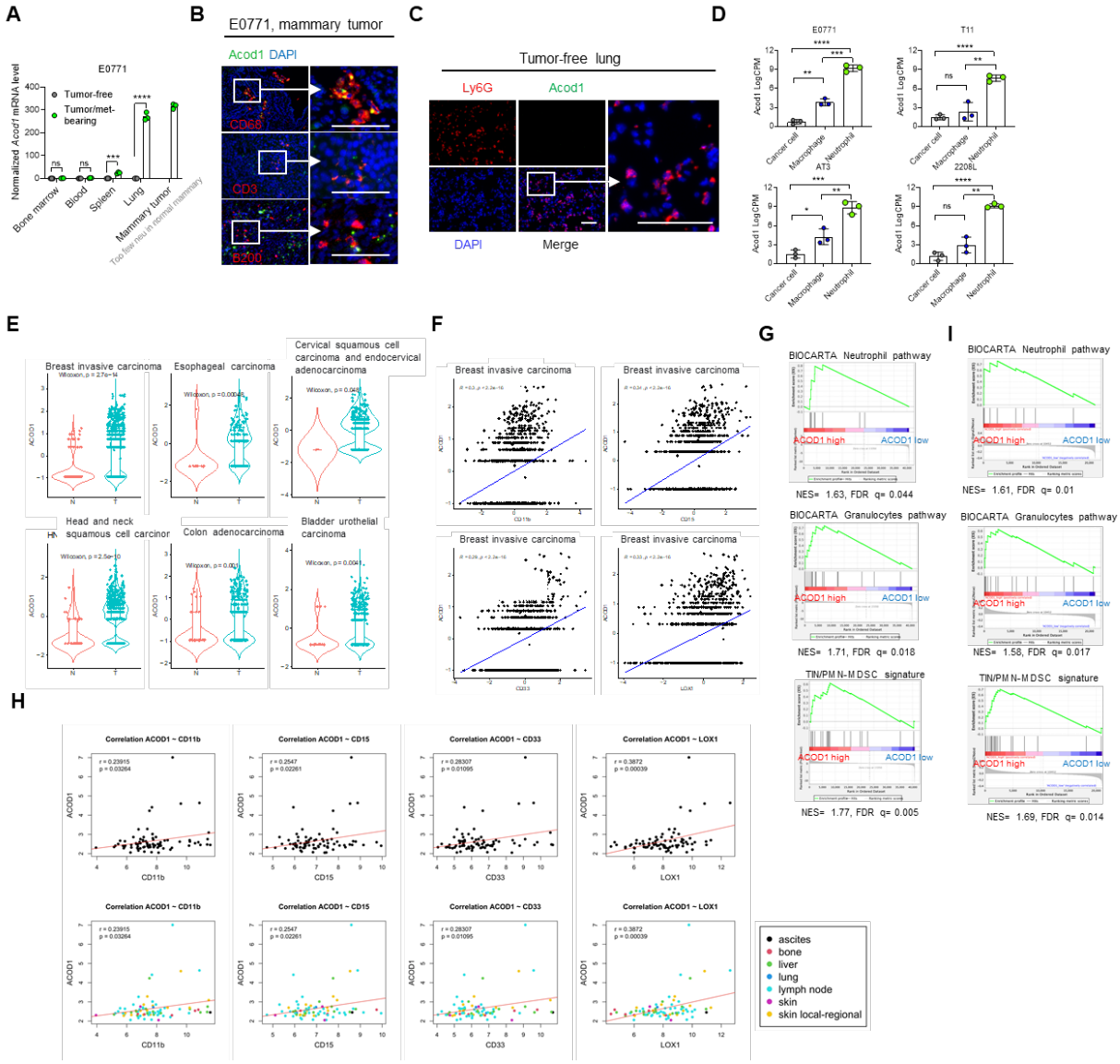

Figure S2. Acod1 is highly expressed in TINs in mouse and human breast cancer. Related to Figure 2.

(A) qRT-PCR to assess *Acod1* expression in neutrophils isolated from tumor-free and tumor-bearing C57BL/6 mice (n=3). **(B)** Immunofluorescence co-staining of Acod1 and immune cell markers in E0771 primary tumors. Staining markers: CD68 for macrophages, CD3 for T cells, B220 for B cells. Scale bar 100μm. **(C)** Immunofluorescence co-staining of Ly6G and Acod1 in tumor-free lung of naïve C57BL/6 mice. Scale bar 100μm. **(D)** Normalized RNA expression of *Acod1* in cancer cells, tumor-infiltrating macrophages and TINs of 4 murine mammary tumor models (E0771, T11, AT3, 2208L) with n=3 for each sample type. Data were reanalyzed from Kim et al. (GEO GSE104765) and shown as mean ± s.e.m.; ns, not significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, unpaired two-tailed Student's t-test. **(E)** *ACOD1* expression was significantly higher in tumors (T) than normal (N) in various TCGA datasets (six are shown). Statistical test method and P values are labeled. **(F)** Correlation of *ACOD1* with neutrophil markers CD11b (ITGAM), CD15 (FUT4) and CD33 and PMN-MDSC marker LOX1 (OLR1) in breast

invasive carcinoma TCGA dataset. Pearson correlation coefficient (R) and P values are labeled. **(G)** GSEA plots showing the enrichment of neutrophil, granulocyte and PMN-MDSC gene signatures for breast invasive carcinoma TCGA cases grouped as ACOD1-high compared with ACOD1-low cases (detailed method is described in Methods). Normalized enrichment score (NES) and false discovery rate (FDR) are labeled. **(H)** Analysis of bulk transcriptomics dataset GSE46141 showing the correlation of *ACOD1* with neutrophil markers CD11b (ITGAM), CD15 (FUT4) and CD33 and PMN-MDSC marker LOX1 (OLR1) in combined metastatic BC samples. Pearson correlation coefficient (R) and P values are labeled. **(I)** GSEA plots showing the enrichment of neutrophil, granulocyte and PMN-MDSC gene signatures for metastatic BC samples grouped as ACOD1-high compared with ACOD1-low. Normalized enrichment score (NES) and false discovery rate (FDR) are labeled.

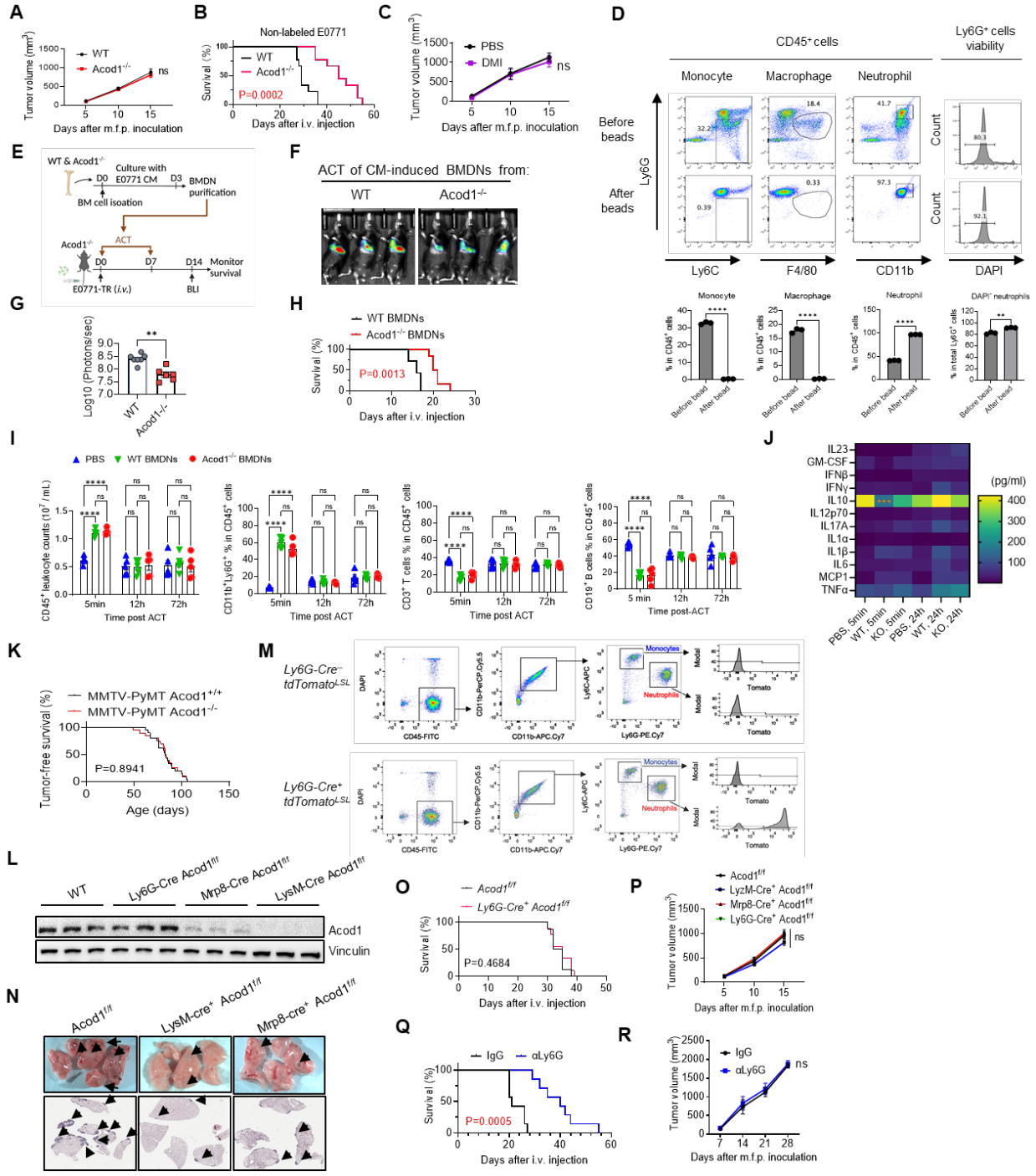

Figure S3. Acod1 loss did not affect primary tumor growth in BC models. Related to Figure 3.

(A) E0771 mammary tumor growth in WT and Acod1$^{-/-}$ cohorts (n=18/genotype). **(B)** Survival curves of WT and Acod1$^{-/-}$ mice injected with non-labeled E0771 cells for lung metastasis development (n=9 for each). **(C)** E0771 mammary tumor growth in two WT cohorts treated with PBS or DMI (100mg kg^{-1}, daily *i.p.*) (n=14/group). **(D)** Flow cytometry to characterize myeloid cell percentages and neutrophil viability in E0771-CM-cultured bone marrow cells before and after

Ly6G-microbead purification. Cells were gated from $CD45^+$ singlets. Monocytes: $Ly6C^+Ly6G^-$, macrophage: $F4/80^+$ $Ly6G^-$, neutrophil: $CD11b^+$ $Ly6G^+$. $DAPI^-$ populations were gated as viable cells. **(E)** ACT of WT or $Acod1^{-/-}$ BMDNs cultured with E0771 CM into $Acod1^{-/-}$ cohorts with *i.v.* injection of E0771-TR. **F-G,** Representative BLI images and quantification (WT n=7, $Acod1^{-/-}$ n=6) at day 14 after *i.v.* injection of E0771-TR. (**H)** Survival curves (WT n=7, $Acod1^{-/-}$ n=6). **(I)** Total leukocyte counts and subset percentages (T, B, neutrophils) in peripheral blood at the indicated time points after ACT of neutrophils. Two-way ANOVA Tukey's multiple comparisons test, ns, not significant, ***P<0.001. **(J**) Serum cytokine levels from peripheral blood at the indicated time points after ACT of neutrophils (n = 5 each condition), measured with Mouse Inflammation Panel (Biolegend LEGENDplex). Two-way ANOVA Dunnett's multiple comparisons test with PBS-5min as the control, the only significant condition was labeled ***P<0.001. **(K)** Tumor-free survival curves for *MMTV-PyMT Acod1*$^{+/+}$ (n=21) and *MMTV-PyMT Acod1*$^{-/-}$ (n=20). **(L)** Western blot of Acod1 expression in neutrophils isolated from lung metastases from WT, *Ly6G-cre*$^+$ *Acod1*$^{fl/fl}$, *Mrp8-Cre*$^+$ *Acod1*$^{fl/fl}$, and *LysM-Cre*$^+$ *Acod1*$^{fl/fl}$ mice. (**M)** Flow cytometry of tdTomato expression in neutrophils and monocytes in peripheral blood of *Ly6G-Cre*$^-$ *tdTomato*^{LSL} and *Ly6G-Cre*$^+$ *tdTomato*^{LSL} mice. **(N)** Representative photographs and HE staining of lungs in *Acod1*$^{f/f}$, *LysM-cre*$^+$*;Acod1*$^{f/f}$ and *Mrp8-cre*$^+$*;Acod1*$^{f/f}$ cohorts. Arrows denote metastases. (**O)** Survival curves of *Acod1*$^{f/f}$ (n=8) and *Ly6G-Cre*$^+$ *Acod1*$^{f/f}$ *mice* (n=8) after E0771 *i.v.* injection. **(P)** E0771 primary tumor growth in *Acod1*$^{f/f}$ (n=8), *Ly6G-Cre*$^+$ *Acod1*$^{f/f}$ (n=8), *Mrp8-Cre*$^+$ *Acod1*$^{f/f}$ (n=9), and *LysM-Cre*$^+$ *Acod1*$^{l/f}$ mice (n=9). **(Q)** Survival curve of WT mice i.v. injected with E0771-TR and treated with IgG and αLy6G antibody (100μg, *i.p.* once every 3 days). (**R)** E0771-TR mammary tumor growth in WT mice treated with IgG and αLy6G antibody (100μg, *i.p.* once every 3 days). For **A**, **C**, **P** and **R**, data are mean ± s.e.m., ns, not significant, based on Mann-Whitney test (**A, C, R**) or ANOVA test (**P**) of each time point. For **D**, **P<0.01, ****P<0.0001, unpaired two-tailed Student's t-test. For **B**, **H, K, O** and **Q,** log-rank test with P values labeled.

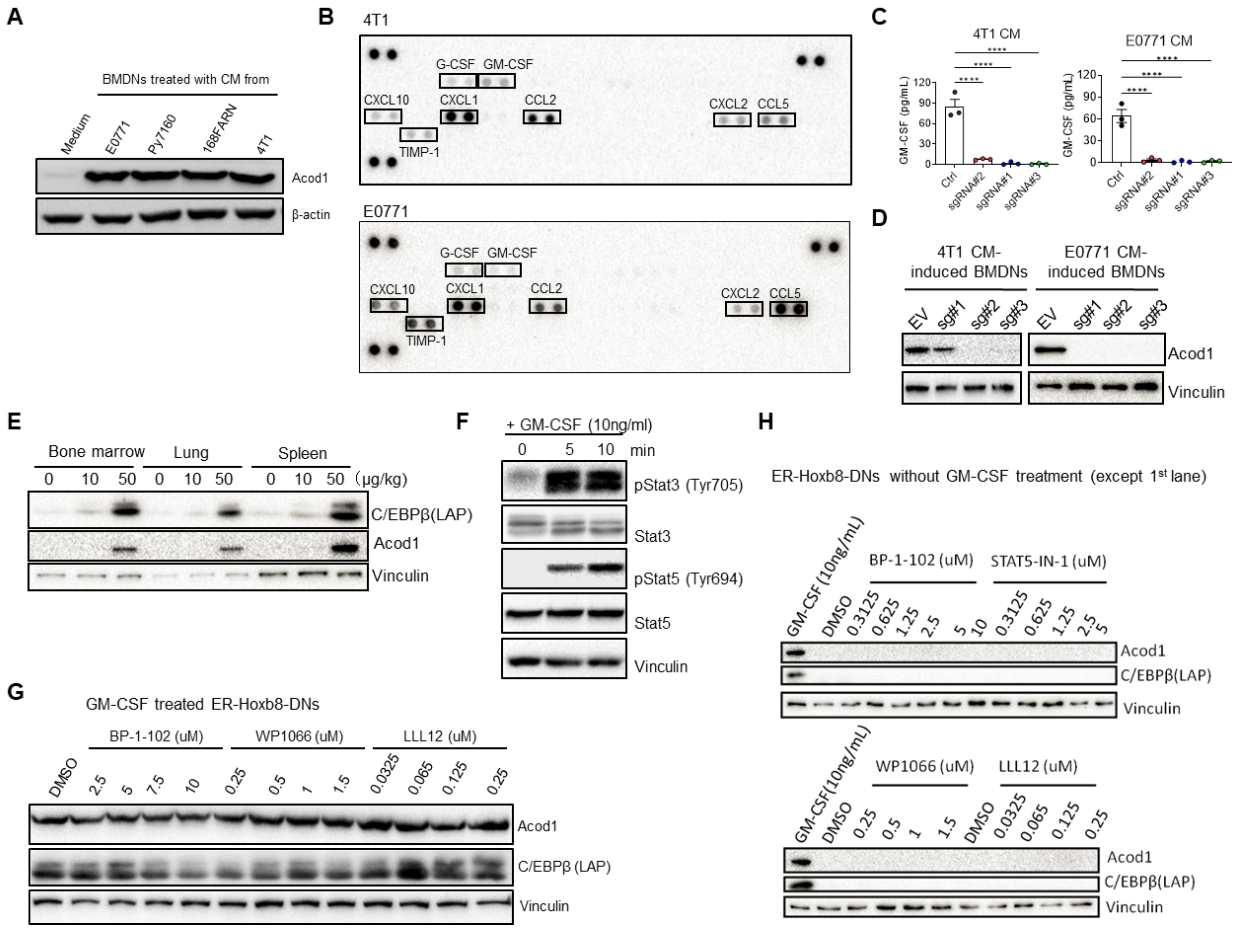

Figure S4. Tumor-secreted GM-CSF induces Acod1 in neutrophils through the STAT5-C/EBPβ axis. Related to Figure 4.

(A) Western blot to examine Acod1 expression in BMDNs treated with CM of murine mammary cell lines (E0771, Py7160, 168FARN and 4T1). (**B)** Proteome profiler mouse cytokine array to detect cytokines in CM of 4T1 and E0771 cells. (**C)** ELISA to detect concentration of GM-CSF in conditioned medium of 4T1 and E0771 cells with *Csf2* knockout by CRISPR/Cas9 (three sgRNA designs). ****P<0.0001, one-way ANOVA test with Dunnett's multiple comparisons correction, n=3. **(D)** Western blot of Acod1 in BMDNs induced by CM from 4T1 and E0771 with and without *Csf2* knockout. (**E)** Western blot to examine C/EBPβ and Acod1 expression in neutrophils isolated from mice treated with recombinant murine GM-CSF (10μg/kg and 50μg/kg, daily *i.v.* for 3 days). **(F)** Western blot to examine Stat3/5 and phosphorylated Stat3/5 for GM-CSF treated ER-Hoxb8-DNs at different time points. (**G-H)** Western blot of Acod1 and C/EBPβ (LAP) for ER-Hoxb8-DNs treated with **(G)** or without **(H)** GM-CSF (10ng/ml) and STAT3 or STAT5 inhibitors. Western blot results were representative of at least three independent experiments showing consistent patterns.

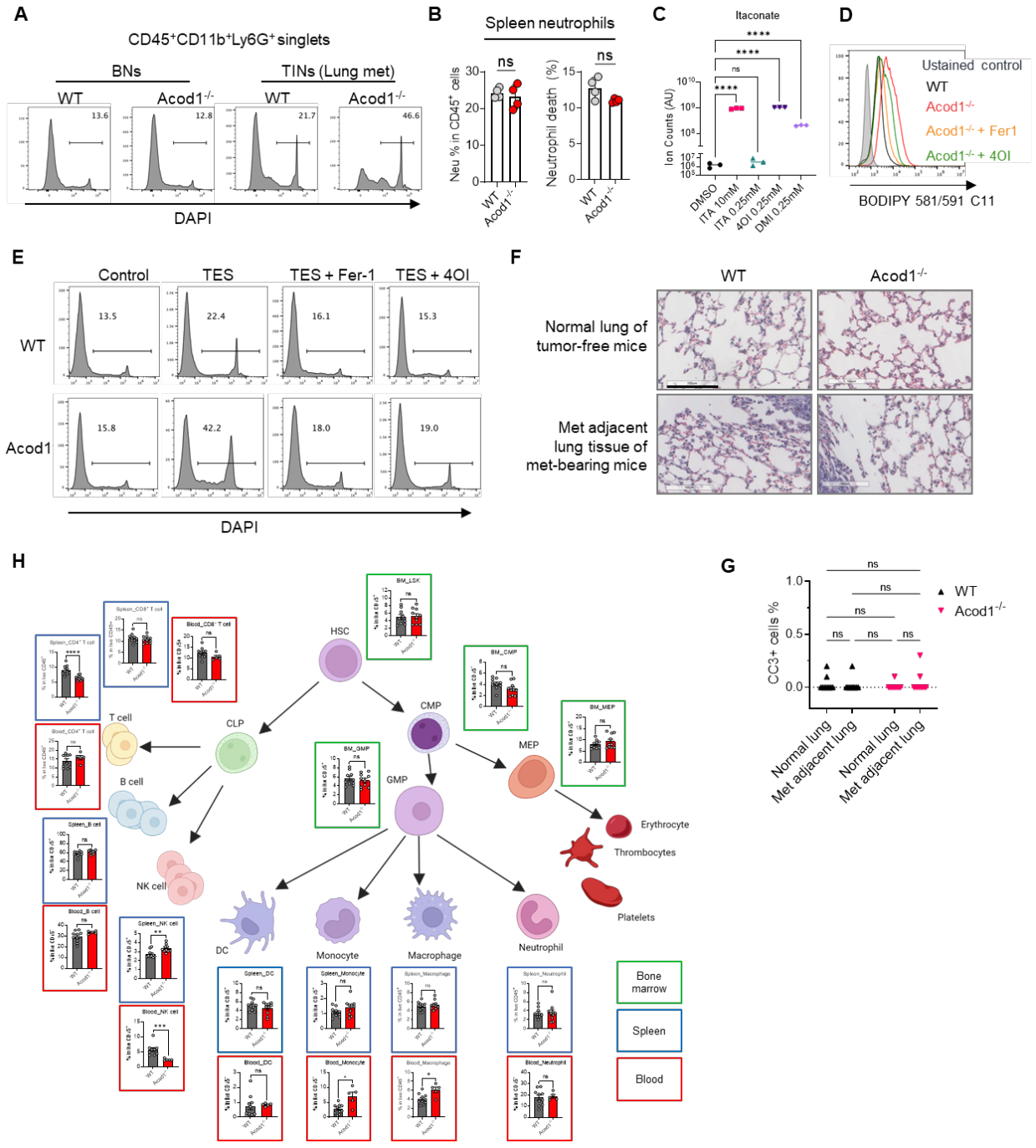

Figure S5. TIN viability is compromised by Acod1 loss and recovered by Fer-1 or 4OI treatment. Related to Figure 5.

(A) Representative flow cytometry histograms of the cell viability marker DAPI (low concentration) for BNs and TINs in E0771 lung metastasis-bearing WT and Acod1$^{-/-}$ mice. DAPI^{high} cells were considered dead. **(B)** Frequency and viability of CD11b^{+} Ly6G^{+} cells in the spleen of E0771-TR metastasis-bearing WT and Acod1$^{-/-}$ mice (n=4). **(C)** Quantification of intracellular itaconate in Acod1$^{-/-}$ BMDNs (activated with E0771 CM) treated with itaconate ITA (10mM, 0.25mM), 4OI (0.25mM) or DMI (0.25mM) for 12 hours. **(D)** Representative flow

cytometry histograms of lipid peroxidation measured with BODIPY 581/591 C11 for BMDNs treated with TES in the absence or presence of Fer-1 (10 μM) or 4OI (0.25mM). **(E)** Representative flow cytometry histograms of the cell viability marker DAPI ($DAPI^{high}$ considered dead.) for BMDNs treated with TES in the absence or presence of Fer-1 (10 μM) or 4OI (0.25mM). **(F-G)** H&E staining and cleaved Caspase-3 immunohistochemistry of the normal lung and metastasis-adjacent lung in both WT and $Acod1^{-/-}$ mice (n=10). Scale bar 100μm. **(H)** Flow cytometry quantification of hematopoietic cells in bone marrow, blood and spleen in tumor-free WT and $Acod1^{-/-}$ mice (n=5~10). Gating strategy: B cell ($CD45^+CD19^+$), NK cell ($CD45^+$ $NK1.1^+$), $CD8^+$ T cells ($CD45^+CD3^+CD8^+$), $CD4^+$ T cell ($CD45^+CD3^+CD4^+$), neutrophil ($CD45^+CD11b^+Ly6G^+$), monocyte ($CD45^+Ly6G^-Ly6C^+$), DC ($CD45^+MHCII^+CD11c^+$), LSK ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$), CMP ($Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+FcgR^-$), GMP ($Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^+FcgR^+$), MEP ($Lin^-Sca\text{-}1^-c\text{-}Kit^+CD34^-FcgR^-$). In **B**, **C** and **H**, data are mean ± s.e.m., ns, not significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, unpaired two-tailed Student's t-test. In **G**, ns, not significant, two-way AVOVA with Sidak's multiple comparisons test.

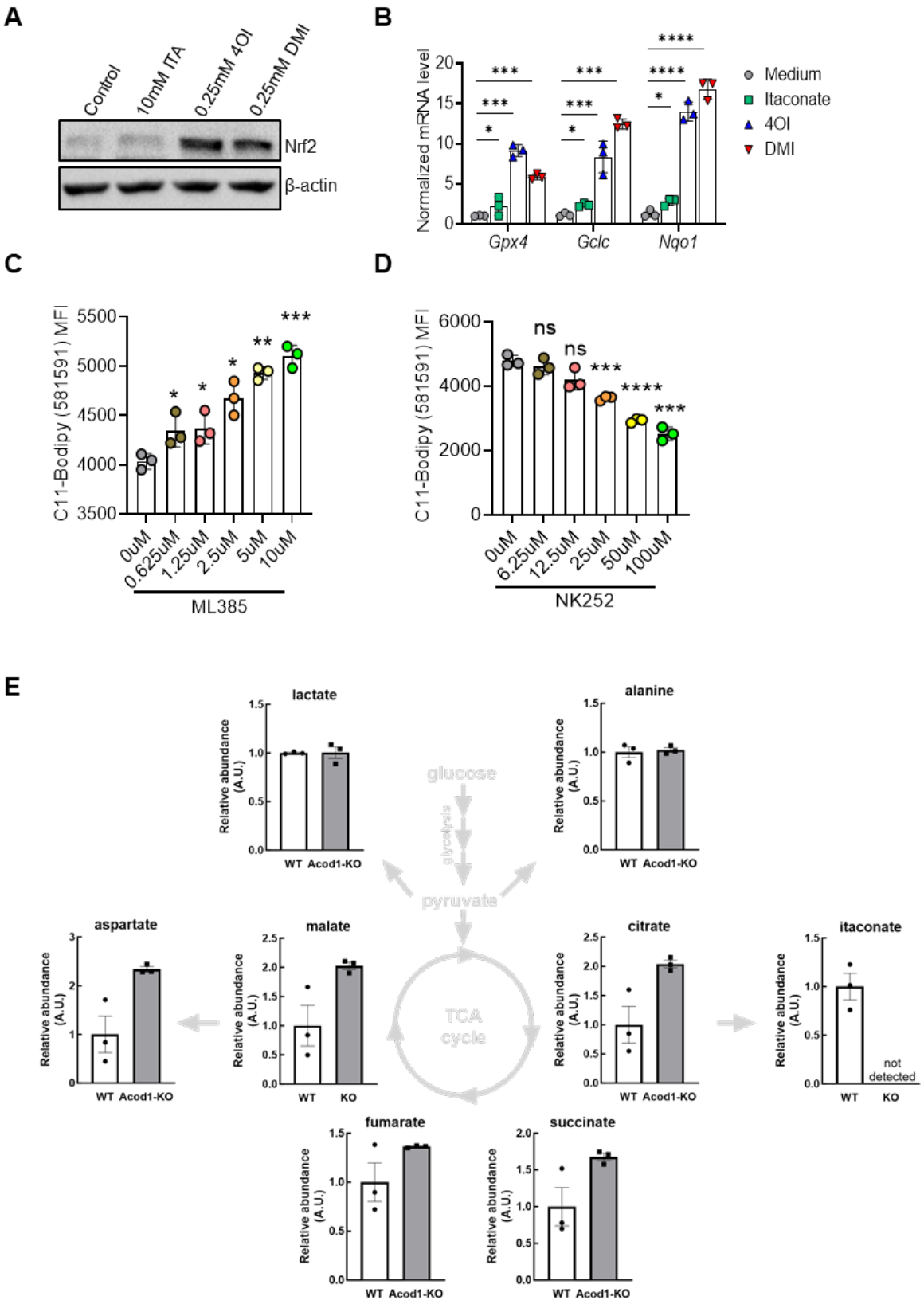
A
Control
10mM ITA
0.25mM 4OI
0.25mM DMI
Nrf2
β-actin
B
Normalized mRNA level
Medium
Itaconate
4OI
DMI
Gpx4
Gclc
Nqo1
C
C11-Bodipy (581591) MFI
0uM
0.625uM
1.25uM
2.5uM
5uM
10uM
ML385
D
C11-Bodipy (581591) MFI
ns
ns
0uM
6.25uM
12.5uM
25uM
50uM
100uM
NK252
E
lactate
alanine
glucose
glycolysis
pyruvate
aspartate
malate
citrate
itaconate
TCA cycle
not detected
fumarate
succinate
Relative abundance (A.U.)
WT
Acod1-KO
KO

Figure S6. Effect of itaconate derivatives and Nrf2 modulators on BMDNs. Related to Figure6. **(A)** Immunoblot of Nrf2 in WT BMDNs primed with E0771 CM for 48 hours and then treated with itaconate or its derivatives 4OI and DMI for 16 hours. (**B)** qRT-PCR to measure the expression of representative Nrf2-related genes in WT BMDNs primed with E0771 CM for 48 hours and then treated with itaconate or its derivatives 4OI and DMI for 16 hours. (**C**) Flow cytometry to measure lipid peroxidation (BODIPY 581/591 C11) in WT BMDNs cultured with E0771 CM for 48 hours and then primed with E0771 TES for 16 hours in the presence of ML385 at different concentrations. (**D)** Flow cytometry to measure lipid peroxidation (BODIPY 581/591 C11) in WT BMDNs cultured with E0771 CM for 48 hours and then primed with E0771 TES for 16 hours in the presence of NK252 at different concentrations. **(E)** Relative pool size of lactate, alanine, TCA cycle intermediate, and cataplerotic compounds itaconate and aspartate from targeted GCMS metabolomics in WT BMDNs and Acod1$^{-/-}$ BMDNs stimulated with E0771 conditioned medium (n=3/genotype). Data represent mean ± s.e.m.; ns, not significant, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, unpaired two-tailed Student's t-test. Experiments were repeated three times with consistent results.

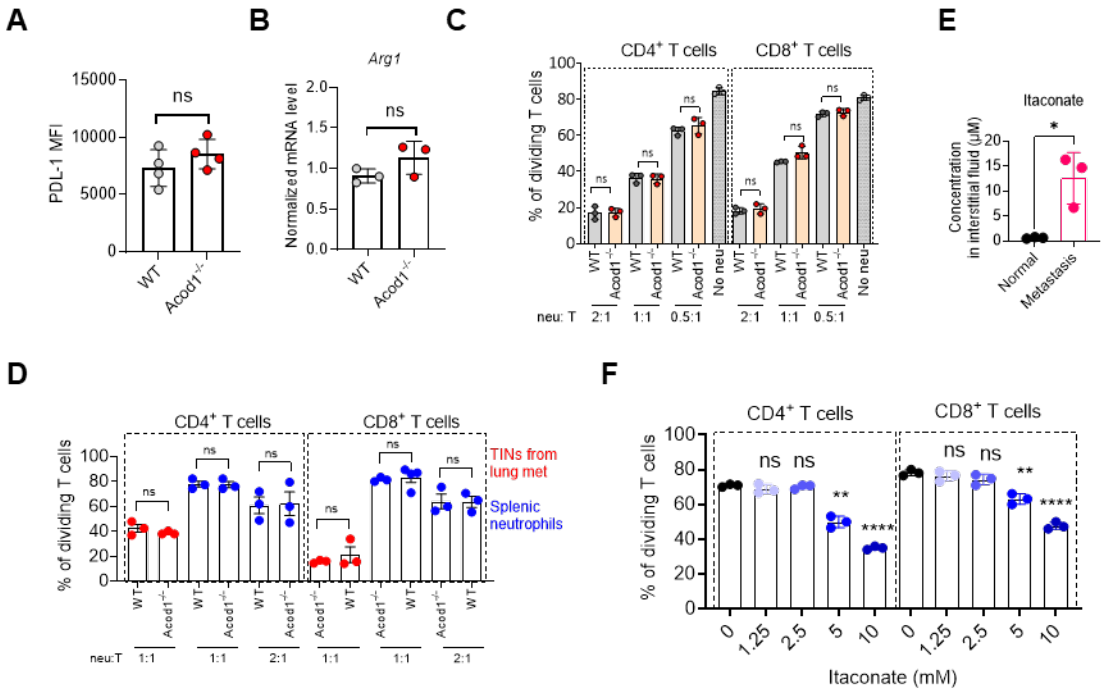

Figure S7. Direct effect of Acod1^{+} and Acod1^{-} TINs as well as exogenous itaconate on T cell proliferation. Related to Figure 7.

(A) Flow cytometry to detect PD-L1 expression of TINs isolated from E0771 lung metastases from WT and Acod1$^{-/-}$ cohorts (n=4 for each genotype). **(B)** qRT-PCR of *Arg1* expression in TINs isolated from E0771 lung metastases from WT and Acod1$^{-/-}$ cohorts (n=3 for each genotype). **(C)** Percentages of proliferating αCD3/αCD28-stimulated T cell subsets cocultured with TINs isolated from E0771 lung metastases in WT and Acod1$^{-/-}$ mice, measured at different neutrophil: T ratios (n=3 for each condition). **(D)** Percentages of proliferating αCD3/αCD28-stimulated T cell subsets cocultured with neutrophils isolated from spleen and lung of E0771 lung metastases-bearing WT and Acod1$^{-/-}$ mice (n=3 or 4 for each condition). **(E)** Concentration of itaconate in lung interstitial fluid extracted from normal lung or E0771 metastasis-bearing lung, measured with liquid chromatography–mass spectrometry (LC–MS). **(F)** Percentages of proliferating αCD3/CD28-stimulated T cell subsets in presence of itaconate at a concentration gradient (n=3 for each condition). Data represent mean ± s.e.m.; ns, not significant, *P<0.05, **P<0.01, ****P<0.0001, unpaired two-tailed Student's t-test. Experiments were repeated three times with consistent results.