SUPPLEMENTAL INFORMATION

Dutchak PA et al., "Regulation of Hematopoiesis and Methionine Homeostasis by mTORC1 Inhibitor NPRL2"

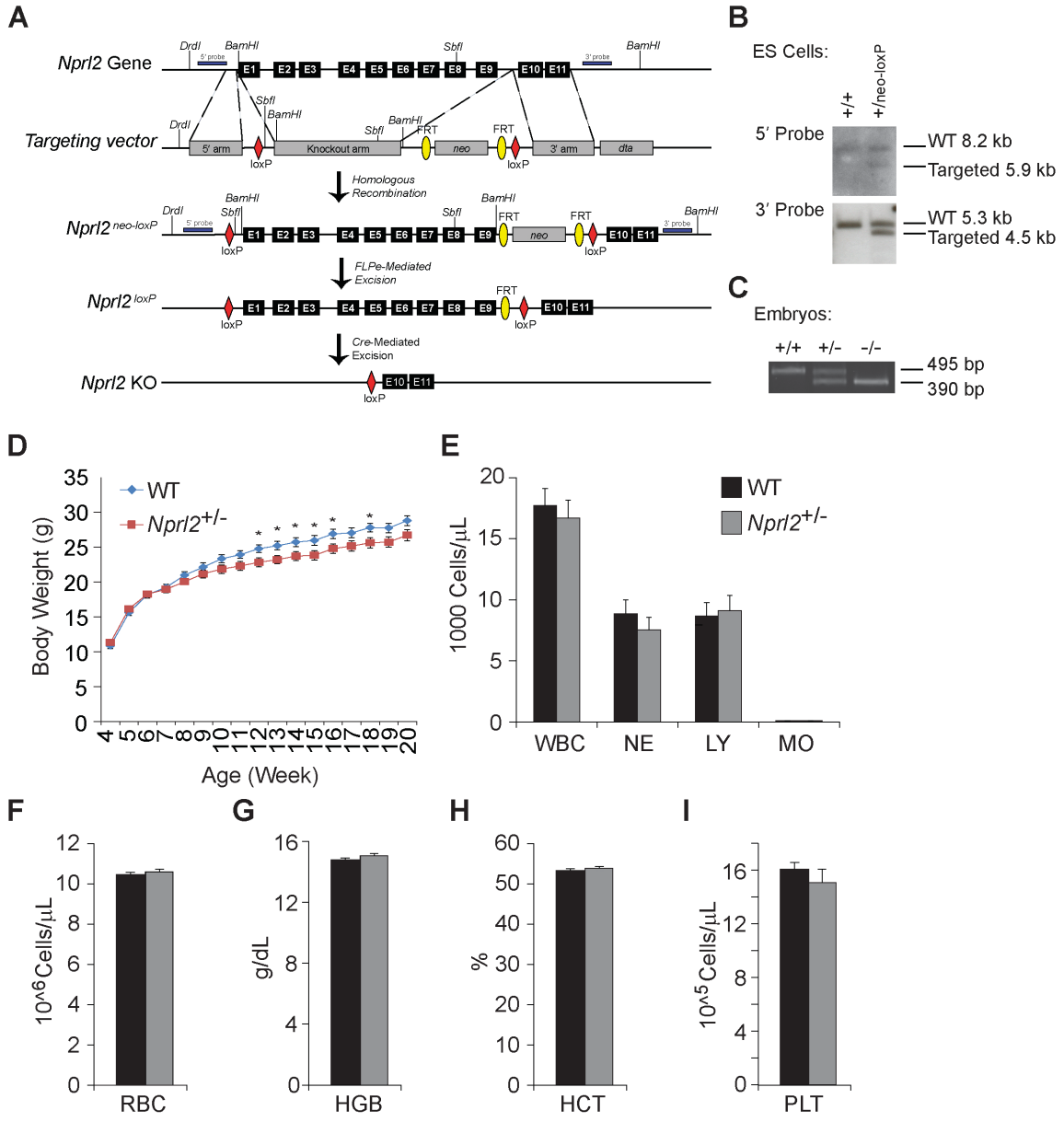

Figure S1 (related to Figure 1). (A) Schematic detailing the targeting strategy and construction of the NPRL2 KO mouse. (B) Southern blotting toward the 5' and 3' targeted regions of homologously recombined ES cells. (C) Genotyping PCR from genomic DNA of WT, *Nprl2*$^{+/-}$ and *Nprl2*$^{-/-}$ embryos. (D) Growth Curve of WT and *Nprl2*$^{+/-}$ male mice. (E-I) Complete blood cell analysis with differential quantitation of (E) white blood cells (WBC), neutrophils (NE), leukocytes (LY), monocytes (MO), (F) red blood cells (RBC), (G) hemoglobin (HGB), (H) hematocrit (HCT) and (I) platelets (PLT), from 20-week old mice. (black=WT, grey=*Nprl2*$^{+/-}$; n=5/7, respectively). *p<0.05

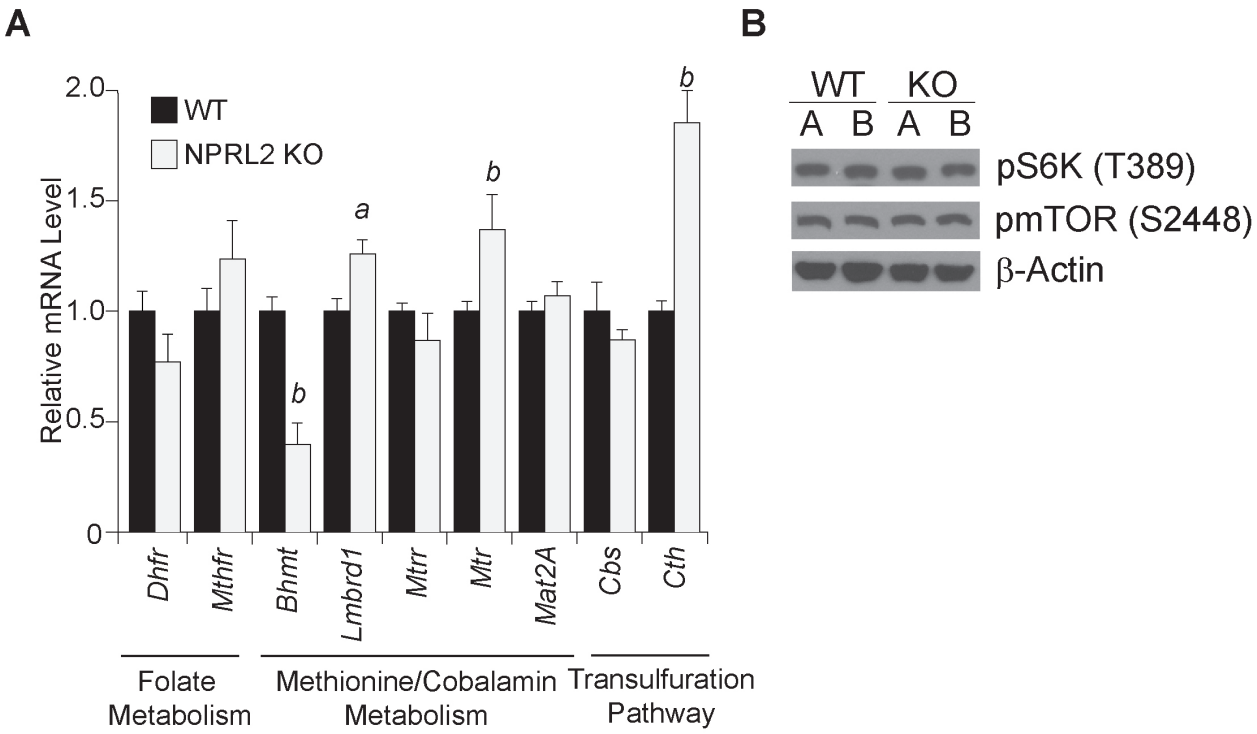

Figure S2 (related to Figures 2 and 3). (A) Quantitative RT-PCR of mRNA levels of genes involved in the methionine:folate cycle, including: *Dhfr*, *Mthfr*, *Bhmt*, *Lmbrd1*, *Mtrr*, *Mtr* and *Mat2a*, and the transsulfuration pathway, including: *Cbs* and *Cth* in the liver of E12.5 embryos (n=3/3). a, $p < 0.05$ vs WT; b, $p < 0.01$ vs WT. (B) Western blot analysis of pS6K and p-mTOR (S2448) from WT and NPRL2 KO embryonic livers at E12.5.

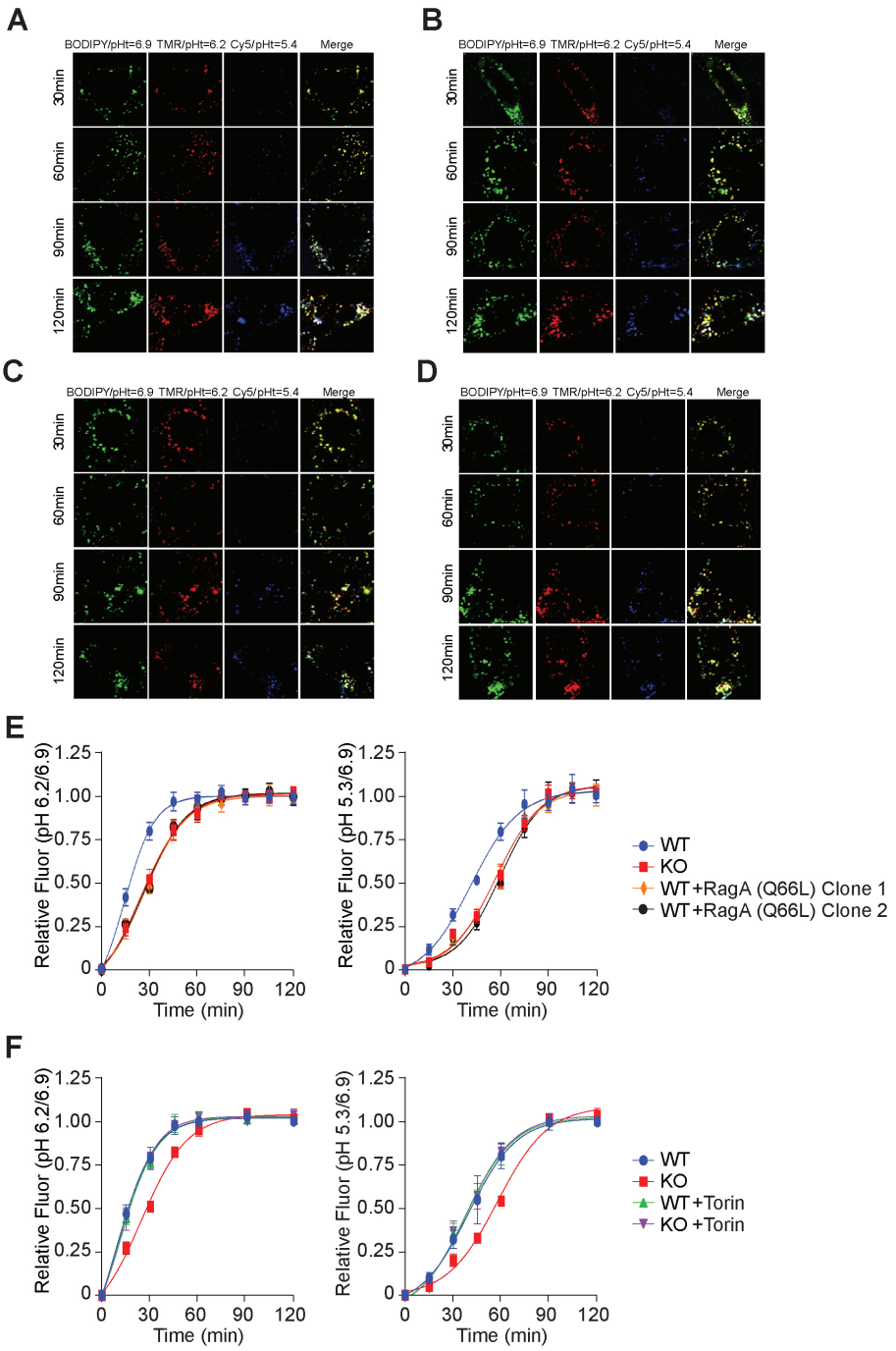

Figure S3 (related to Figure 2). (A-D) Representative confocal microscopy images of MEF cells treated with 100 ug/mL hybrid ultra pH-sensitive nanoprobes. (A) WT MEF cells in complete media, (B) WT MEF cells in EBSS media, (C) NPRL2 KO MEF cells in complete media, and (D) NPRL2 KO MEF cells in EBSS media. (E) WT, NPRL2 KO, and 2 independent clones of WT MEF cells overexpressing RagA (Q66L) were cultured in EBSS starvation media and 100 ug/mL hybrid ultra pH-sensitive nanoprobes. (F) WT and NPRL2 KO cells were incubated in complete media in the presence or absence of Torin1 for 1 h, followed by a switch to EBSS starvation media and 100 ug/mL hybrid ultra pH-sensitive nanoprobes. Confocal microscopy was used to determine the intracellular pH, as previously described.

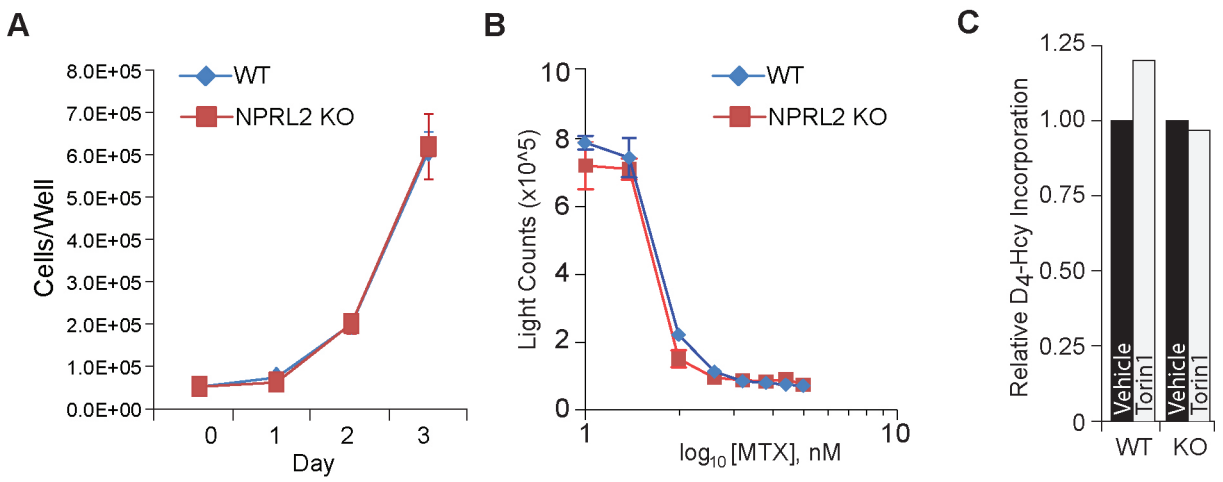

Figure S4 (related to Figure 3). (A) Growth curve of WT and NPRL2 KO MEF cells in complete DMEM with 10% FBS. Data represent average values from 3 independent experiments in triplicate +/- SEM. (B) Cell titer glo assays were used to determine viability of WT and NPRL2 KO MEF cells treated with increasing amounts of methotrexate (MTX) for 24 h. Data represent one experiment performed in triplicate; similar data was obtained from independent experiments. (C) WT and NPRL2 KO cells were incubated in methionine-deficient media (1% HI FBS) and 200 µM D_4-homocysteine in the presence or absence of 250 nM Torin1 for 4 h, synthesis of labeled methionine was determined by LC-MS/MS (n=3 pooled samples/group). Signal for WT and KO were normalized to their respective vehicle controls.

Supplemental Experimental Procedures

Animal Experiments

Mice were maintained in a 12/12 h light-dark cycle at a temperature of 72°F and fed standard chow (Harlan, #2016) *ad libitum*. All animal experiments were approved by the Institutional Animal Care and Research Advisory Committee of the University of Texas Southwestern Medical Center.

Generation of NPRL2 KO Mouse Embryonic Fibroblasts

Mouse embryonic fibroblasts (MEFs) were isolated from E12.5 embryos. Briefly, embryos were harvested, decapitated, and internal organs were removed. Tissues were minced in 0.25% trypsin-EDTA (Gibco®, #25200-056) and digested for 5 min at 37°C. Cell debris was cleared by centrifugation and isolated cells were plated in growth media consisting of Dulbecco's Modified Eagles Medium (Gibco®, #11965), 10% FBS (Gibco®, #10082-147) and 1X antibiotic/antimycotic (Gibco®, #15240-096). To generate stable cell-lines, cells were infected by lentivirus expressing the large-T antigen generated from HEK293T cells. Cells were propagated in growth media with 10% FBS, unless defined media compositions were utilized, as indicated in figure legends.

Histology and Embryology Imaging

E12.5 embryos were drop fixed in 4% paraformaldehyde for 48 h. Paraffin-embedded sections were prepared and stained with hematoxylin and eosin (H&E) for routine histology at the Molecular Pathology Core at the University of Texas Southwestern Medical Center.

LC-MS/MS Based Detection and Quantification of Metabolites from Embryos

Nprl2$^{+/-}$ mice were crossed, and E12.5 embryos were rapidly harvested, following decapitation of the dam. Two limb buds were isolated for genotyping purposes and embryos were immediately frozen in liquid nitrogen. Embryos were homogenized in ice-cold extraction buffer containing 80% methanol, 19.9% water, 0.1% formic acid and 0.2 mM tricine (pH 7.5) and incubated for 5 min in a methanol-dry ice mixture. Homogenates were vortexed, heated to 75°C for 3 min, cooled on ice, and centrifuged at 2000 g for 10 min at 4°C. Supernatants were collected, centrifuged at 20,000 g for 10 min and passed through a 0.2 µm PVDF 2 ml filtration tube (Grace). Samples were dried in a speed vacuum and stored at -80°C until processing. Extracted metabolites were measured using targeted LC-MS/MS methods. Briefly, a library of specified metabolites was constructed using commercial standards, and metabolites were detected using an AB SCIEX 3200 QTRAP triple quadrupole-linear ion trap mass spectrometer. This enabled targeted detection of metabolites by multiple reaction monitoring (MRM) after collision-induced fragmentation of the parent ion. Where possible, two MRMs per metabolite were included to maximize confidence. The area under each peak was quantitated using Analyst® Software, normalized against total ion count, and relative abundance was normalized to *Nprl2*$^{+/-}$ embryos for each litter.

Intracellular Vesicle pH Measurements

WT and NPRL2 KO MEF cells were cultured in DMEM with 10% FBS and treated with 100 µg/mL hybrid ultra-sensitive nanoprobes (Ma et al., 2014; Zhou et al., 2011) in DMEM without serum for 5 min. For

starvation experiments, cells were incubated in EBSS for 2 h and 100 μg/mL hybrid UPS nanoprobes were added pH directly to EBSS. Cells were washed with PBS for 3 times and maintained in DMEM with 10% FBS or EBSS, respectively. Cells were placed in a chamber mounted stage at 37°C in 5% CO_2 and imaged with a Nikon ECLIPSE TE2000-E confocal microscope. For data analysis, 2 to 5 cells were selected per time point per condition for each replicate. Percentage of fluorescent puncta per cell in BODIPY and TMR channels (R/G, representing acidified early endosomes), or BODIPY and Cy5 channels (B/G, representing acidified late endosomes or lysosomes) were calculated. Values are shown as mean ± standard deviation. Unpaired t-test was used to determine statistical difference between data sets.

Quantitative RT-PCR Analysis

Total RNA from cells and tissues were extracted using TRIzol® (Invitrogen), following the manufacturer protocol. Primers were selected to span exon junctions where possible. RNA extracts were subjected to DNase-treatment (Roche) and cDNA was synthesized from RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Quantitative RT-PCR reactions (10 μl) contained 25ng cDNA, 150nM of each primer and 5 μl of SYBR GreenER (Invitrogen). All reactions were performed in triplicate on the Applied Biosystems Prism 7900HT system and relative mRNA levels were calculated by the comparative threshold cycle method with U36B4 as the internal control. Primer sequences are listed in Table S2.

Western Blot Analysis

Cells were washed with PBS and re-suspended in homogenized in lysis buffer consisting of 150 mM sodium chloride, 50 mM sodium fluoride, 100 μM sodium orthovanadate (pH 10.0), 50 mM sodium pyrophosphate tetrabasic, 10 mM β-glycerophosphate, 5 mM EDTA, 5 mM EGTA, 10 mM HEPES (pH 7.4), 0.2% sodium deoxycholate and 0.5% Triton X-100 supplemented with complete anti-protease cocktail (Roche). Lysates were cleared by centrifugation and boiled in 6x Laemmli sample buffer. Proteins were separated by SDS-PAGE and western blots were performed using antibodies detailed in Table S4**.**

References:

Ma, X., Wang, Y., Zhao, T., Li, Y., Su, L.C., Wang, Z., Huang, G., Sumer, B.D., and Gao, J. (2014). Ultra-pH-sensitive nanoprobe library with broad pH tunability and fluorescence emissions. Journal of the American Chemical Society *136*, 11085-11092.

Zhou, K., Wang, Y., Huang, X., Luby-Phelps, K., Sumer, B.D., and Gao, J. (2011). Tunable, ultrasensitive pH-responsive nanoparticles targeting specific endocytic organelles in living cells. Angewandte Chemie *50*, 6109-6114.