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Supplemental Information

GATA Factor-G-Protein-Coupled Receptor Circuit Suppresses Hematopoiesis

Xin Gao, Tongyu Wu, Kirby D. Johnson, Jamie L. Lahvic, Erik A. Ranheim, Leonard I. Zon, and Emery H. Bresnick

Figure S1

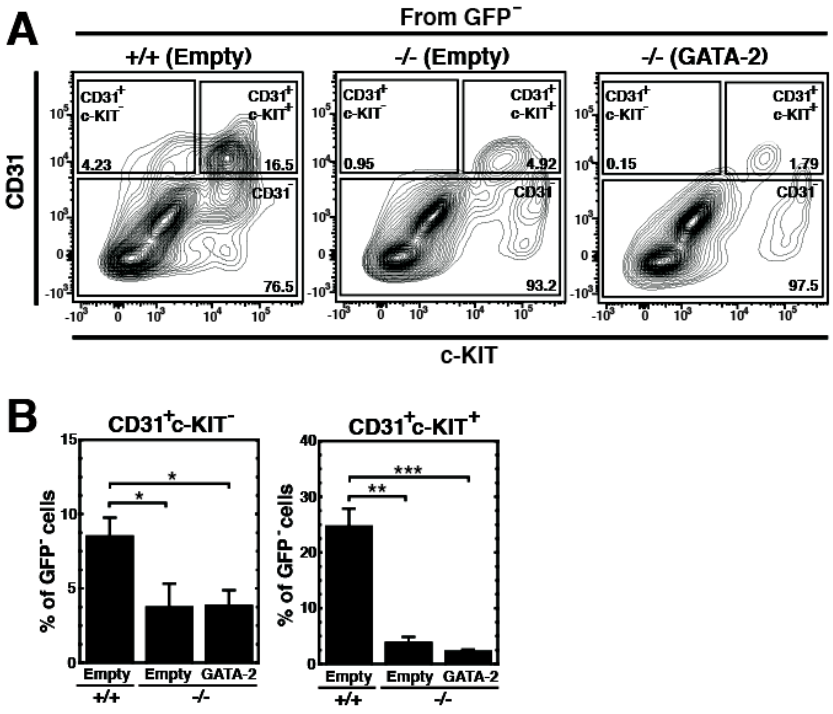

Figure S1. Related to Figure 1: Retroviral-mediated GATA-2 expression does not rescue CD31$^+$c-KIT$^+$ hematopoietic and CD31$^+$c-KIT$^-$ endothelial cells in the GFP$^-$ cell population. (A) Representative plots from flow cytometric analysis of CD31$^+$c-KIT$^+$ and CD31$^+$c-KIT$^-$ cell populations in GFP$^-$ cells after 96h of *ex vivo* culture. (B) Quantitation of flow cytometry data expressed as the percentage of CD31$^+$c-KIT$^-$ and CD31$^+$c-KIT$^+$ cells in GFP$^-$ cells (6 litters: +9.5$^{+/+}$-Empty [n=8 embryos]; +9.5$^{-/-}$-Empty [n=4 embryos]; +9.5$^{-/-}$-GATA-2 [n=6 embryos]). Error bars represent SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (two-tailed unpaired Student's t-test).

Figure S2

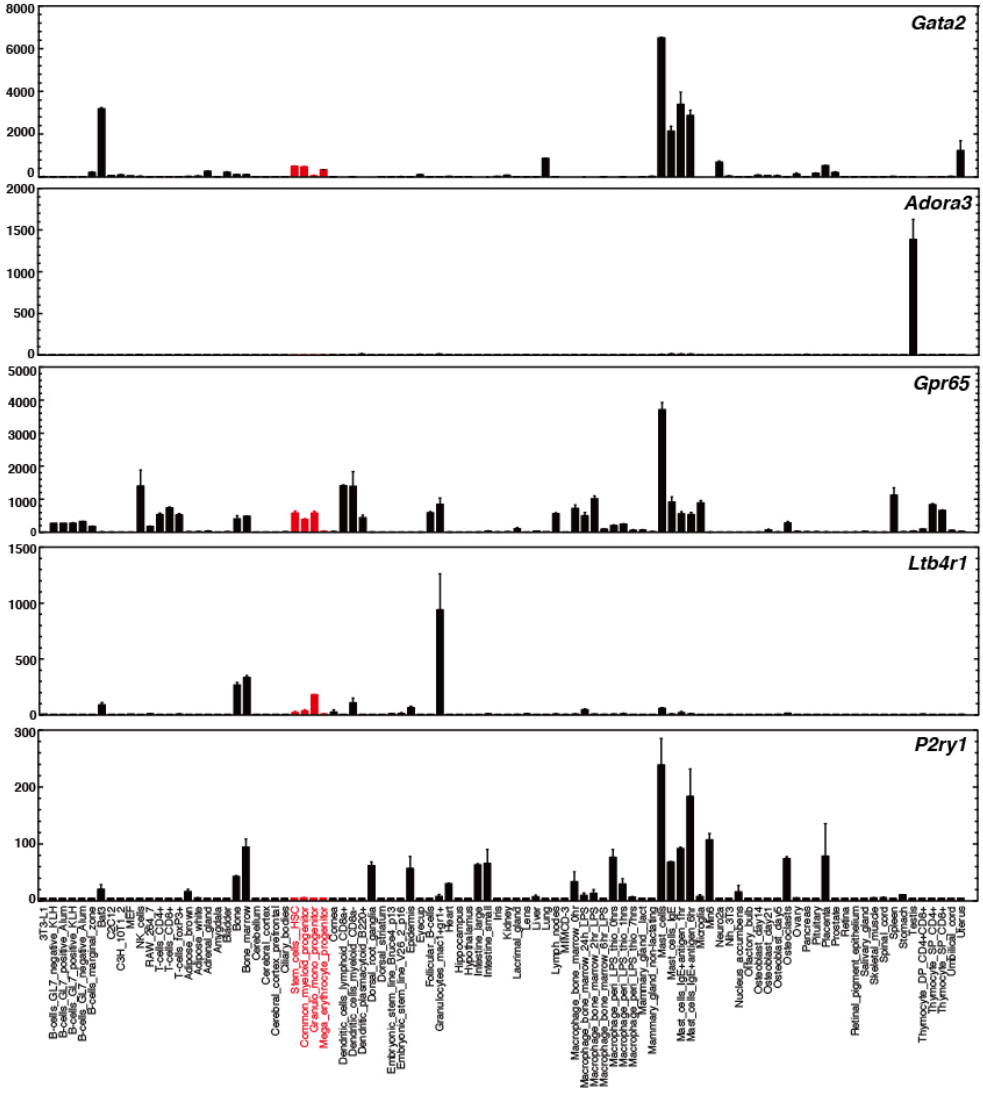

Figure S2. Related to Figure 3: Expression profiles from normal mouse tissues, organs, and cell lines (biogps.org).

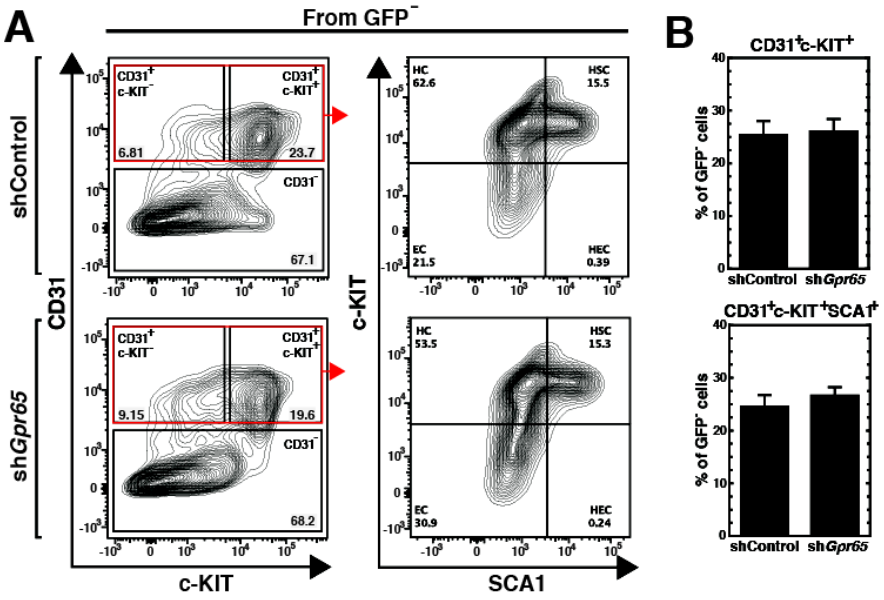

Figure S3. Related to Figure 4: Retroviral-mediated shGPR65 expression does not alter the CD31$^+$c-KIT$^+$ and CD31$^+$c-KIT$^+$SCA1$^+$ cell populations in the GFP$^-$ cell population. (A) Representative plots from flow cytometric analysis of CD31$^+$c-KIT$^+$ and CD31$^+$c-KIT$^+$SCA1$^+$ cell populations in GFP$^-$ cells after 96 h of culture. (B) Quantitative analysis of flow cytometry data expressed as the percentage of CD31$^+$c-KIT$^+$ and CD31$^+$c-KIT$^+$SCA1$^+$ cells in GFP$^-$ cells (Top: 9 litters: shLuc [n=22 embryos], sh*Gpr65* [n=26 embryos]; Bottom: 7 litters: shLuc [n=18 embryos]; sh*Gpr65* [n=22 embryos]).

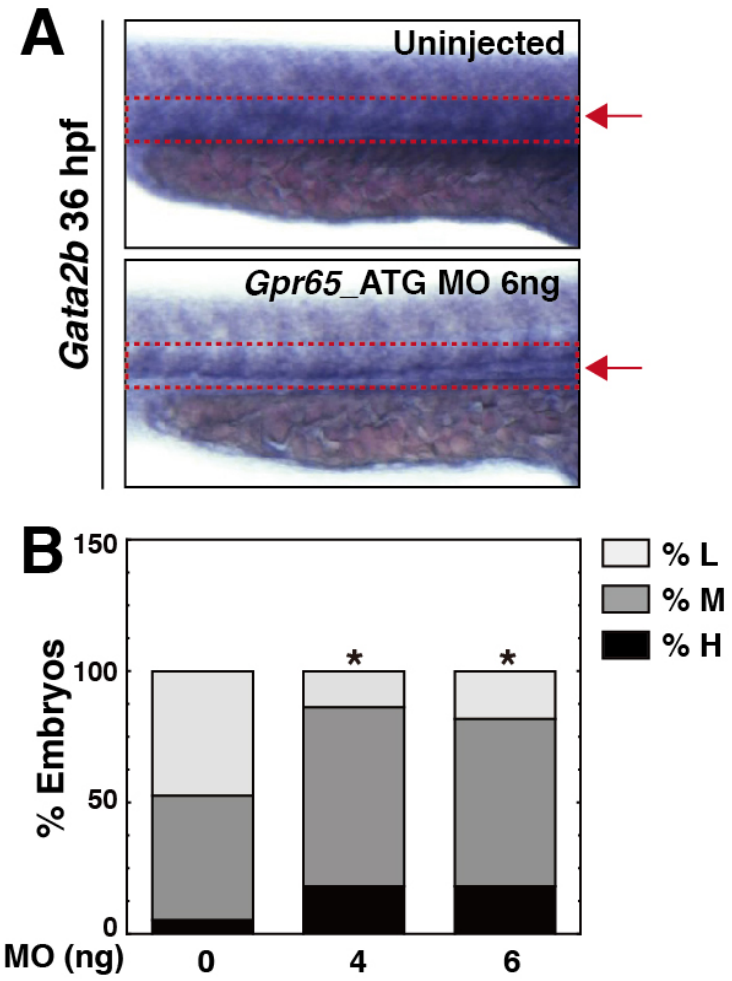

Figure S4. Related to Figure 5: GPR65 suppresses *Gata2* expression in the zebrafish embryo. (A) Representative images of in situ hybridization for *Gata2* at 36 hours post fertilization. The red rectangle illustrates the region enriched in hemogenic endothelium. (B) Quantitative analysis of in situ hybridization data expressed as percentage of embryos with high, medium, and low *Gata2* staining in total embryos (ATG_MO 0ng [n=104 embryos]; ATG_MO 4ng [n=22 embryos]; ATG_MO 6ng [n=40 embryos]). Error bars represent SEM. *, $P < 0.05$ (two-tailed unpaired Student's t-test).

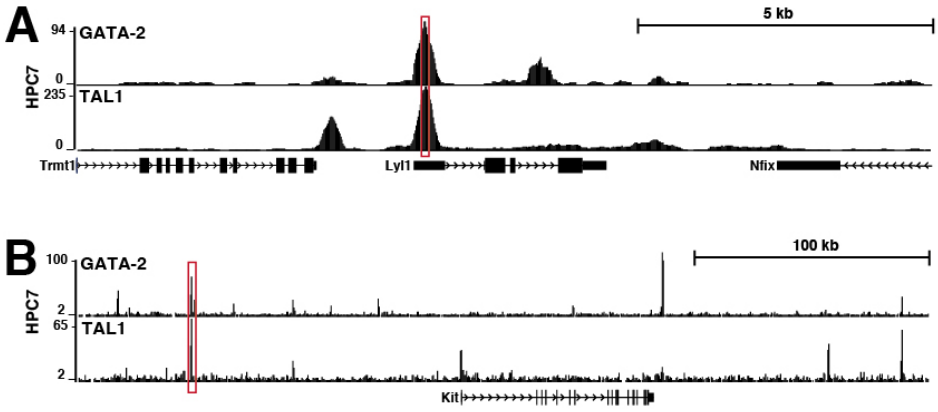

Figure S5. Related to Figure 6: ChIP-seq analysis of GATA-2 and TAL1 occupancy at Lyl1 (A) and KIT (B) loci in HPC7 cells. The red box depicts the amplified sequence from the real-time RT-PCR analysis of Figure 6K.

Supplemental Experimental Procedures

Primary erythroid precursor cell isolation. Primary erythroid precursors were isolated from E14.5 fetal livers using the EasySep negative selection Mouse Hematopoietic Progenitor Cell Enrichment kit (StemCell Technologies) (Hewitt et al., 2015; McIver et al., 2014). Fetal livers were dissociated by pipetting and resuspended at 5 x 10^7 cells/ml in PBS containing 2% FBS, 2.5 mM EDTA, and 10 mM glucose. EasySep Mouse Hematopoietic Progenitor Cell Enrichment Cocktail was added at 50 µg/ml supplemented with 2.5 µg/ml biotin-conjugated CD71 antibody (eBioscience). After 15 min incubation on ice, the cells were washed by centrifugation for at 1200 rpm at 4°C. Cells were resuspended at 5 X 10^7 cells/ml in PBS containing 2% FBS, 2.5 mM EDTA, and 10 mM glucose, and EasySep Biotin Selection Cocktail was added at 100 µg/ml. After 15 min incubation at 4°C, EasySep Mouse Progenitor Magnetic Microparticles were added at 50 µg/ml. After 10 min incubation at 4°C, cells were resuspended to 2.5 ml and incubated with a magnet for 3 min. Unbound cells were carefully transferred into 15 ml tube and used for subsequent experiments.

Cell culture. G1E-ER-GATA-1 cells were cultured with or without 1 µM estradiol as described (DeVilbiss et al., 2013). Fetal liver erythroid precursor cells were cultured and maintained at a density of 2.5 x 10^5 – 1 x 10^6 cells/ml in StemPro-34 (Gibco) supplemented with 10% nutrient supplement (Gibco), 2 mM L-glutamine (Cellgro), 1% penicillin/streptomycin (Cellgro), 100 µM monothioglycerol (Sigma-Aldrich), 1 µM dexamethasone (Sigma-Aldrich), 0.5 U/ml of erythropoietin, and 1%

conditioned medium from a KIT ligand-producing CHO cell line for expansion. Cells were cultured in a humidified incubator at 37°C and 5% carbon dioxide.

Retroviral infection: For concentrating retrovirus, the retroviral supernatant was mixed with the Retro-X Concentrator and rotated at 4°C for 1h. The mixture was then centrifuged at 1,500 g for 45 minutes to obtain a high-titer virus-containing pellet that was resuspended at 1% of the original volume in IMDM Media supplemented with 20% FBS [Gemini] and 1% penicillin/streptomycin [Cellgro].

Gpr65 shRNA sequence:

TGCTGTTGACAGTGAGCGAGCAGGTTAAGTTACATGGTATTAGTGAAGCCA

CAGATGTAATACCATGTAACTTAACCTGCCTGCCTACTGCCTCGGA

Quantitative real-time RT-PCR. Total RNA was purified from TRIzol (Invitrogen) according to manufacturers' instructions. cDNA was synthesized from 1 μg purified total RNA by Moloney murine leukemia virus reverse transcriptase (M-MLV RT). Real-time PCR was performed with SYBR green master mix (Applied Biosystems), and product accumulation was monitored by SYBR green fluorescence using either a StepOnePlus or Viia7 instrument (Applied Biosystems). Relative expression was determined from a standard curve of serial dilutions of cDNA samples, and values were normalized to 18S RNA expression. The sequences of primers used for RT-PCR and genotyping are provided below.

Colony assay. FACS-sorted $CD31^{+}$c-KIT^{+} cells from infected AGMs were plated in MethoCult M03434 complete media (StemCell Technologies) in a 35mm dish. After incubation in a humidified incubator at 37°C with 5% carbon dioxide,

colonies were visualized by microscopy and quantitated. Cells isolated from colonies were subjected to Wright-Giemsa staining.

Flow cytometry. Antibodies used for flow cytometry: APC-conjugated antibody CD31 (MEC13.3, Biolegend), PE-conjugated antibody c-KIT (2B8, eBioscience), PerCP-Cy5.5-conjugated antibody SCA1 (D7, eBioscience), APC-conjugated antibody Ter119 (TER119, eBioscience) and PE-conjugated CD71 (R17217, eBioscience) were used for flow cytometry and Fluorescence Activated Cell Sorting (FACS).

Dissociated cells from cultured AGM explants were resuspended in PBS containing 2% FBS and passed through 25 μm cell strainers to obtain single-cell suspensions prior to antibody staining. Fetal liver cells were washed with PBS once prior to antibody staining. Samples were analyzed on a FACSAria™ II cell sorter (BD Biosciences). Cells were gated on GFP to ensure retroviral expression. DAPI (Sigma-Aldrich) exclusion was utilized for live/dead discrimination. FACS-sorted cells were immediately processed for RNA isolation or colony assay.

Zebrafish morpholino knockdown. Detailed methodology is presented in supplemental experimental procedures.Wild-type zebrafish embryos were injected at the single cell stage with 0, 4, or 6 ng of *Gpr65*_ATG MO (MO sequence: CATCTCAAGGGAGCATAAGTGCGTC) or *Gpr65*_SP MO (MO sequence: TAAATCGACAACTCACCATAAGTGC). Embryos were grown to 36 hours post-fertilization. No defects in gross morphology or circulation were noted at the MO doses used. Embryos were fixed in formaldehyde and stained by *in*

situ hybridization with either a *Gata2b* probe or a *runx1/c-myb* probe mixture. Embryos were blindly scored as having either low, medium, or high AGM expression of *runx1/c-myb or Gata2b*. The *Gata2b* probe was generously provided by David Traver.

ChIP assay. Detailed methodology is presented in supplemental experimental procedures. Quantitative chromatin immunoprecipitation (ChIP) was conducted as described using antibodies specific to monomethylated H4K20 (Millipore), GATA-1, and Scl/TAL1 (DeVilbiss et al., 2015). Samples were analyzed by quantitative real-time PCR using either a StepOnePlus or Viia7 instrument (Applied Biosystems). The amount of product was determined relative to a standard curve generated from a serial dilution of input chromatin.

Primers.

+9.5 flanking forward: 5′-ATGTCCTTTCGGATCTCCTGCC-3′

+9.5 flanking reverse: 5′-GGTAAACAGAGCGCTACTCCTGTGTGTT-3′

18S rRNA forward: 5’-CGCCGCTAGAGGTGAAATTCT-3’

18S rRNA reverse: 5’-CGAACCTCCGACTTTCGTTCT-3’

Gata2 mRNA forward: 5′-GCAGAGAAGCAAGGCTCGC-3′

Gata2 mRNA reverse: 5′-CAGTTGACACACTCCCGGC-3′

Gpr65 mRNA forward: 5’-CAAGAGAAGCATCCCTCCAGAA-3’

Gpr65 mRNA reverse: 5’-TGTTTTTATTTTCACGCCGTTTG-3’

Gata2 primary transcript forward: 5’-GACATCTGCAGCCGGTAGATAAG-3’

Gata2 primary transcript reverse: 5’-CATTATTTGCAGAGTGGAGGGTATTAG-3’

MyoD promoter forward: 5′-GGGTAGAGGACAGCCGGTGT-3’

MyoD promoter reverse: 5′-GTACAATGACAAAGGTTCTGTGGGT-3’

Eif3k promoter forward: 5′-GTGATTTCCTTCCAGCAGTTGTAA-3’

Eif3k promoter reverse: 5′-CTCACGCTATTGGTCTCTTTTAAGTG-3’

Gata2 _9.5 Site _933 bp forward: 5′-CTTGCTGCTGGCTCTGAGAAC-3’

Gata2 _9.5 Site _933 bp reverse: 5′-AGTCCAGGGTCTTTTAAGGATAAATTC-3’

Gata2 _9.5 Site_480 bp forward: 5′-AACCTTCAAATGCAGACACTTCAC-3’

Gata2 _9.5 Site_480 bp reverse: 5′-GAATCCGCCAGAACGAAGAC-3’

Gata2_9.5 Site forward: 5′-GACATCTGCAGCCGGTAGATAAG-3’

Gata2_9.5 Site reverse: 5′-CATTATTTGCAGAGTGGAGGGTATTAG-3’

Gata2_9.5 Site_446 bp forward: 5′-GCCGAGGGAGTTCAGTGCTA-3’

Gata2_9.5 Site_446 bp reverse: 5′-AGCGCTACTCCTGTGTGTTCTTC-3’

Gata2_9.5 Site_880 bp forward: 5′-TCCTGGCGACTCCTAGATCCTA-3’

Gata2_9.5 Site_880 bp reverse: 5′-GAAAGCCCTGAGGAAGTTGGA-3’

Lyl1 Exon 1 forward: 5′-TCAGCATTGCTTCTTATCAGCC-3’

Lyl1 Exon 1 reverse: 5′-CGCAGAGGCCAGAGGATG-3’

Kit_114 kb forward: 5′-GCACACAGGACCTGACTCCA-3’

Kit_114 kb reverse: 5′-GTTCTGAGATGCGGTTGCTG-3’

Hdc mRNA forward: 5’-ACCTCCGACATGCCAACTCT-3’

Hdc mRNA reverse: 5’-CCGAATCACAAACCACAGCTT-3’

c-Kit mRNA forward: 5’-AGCAATGGCCTCACGAGTTCTA-3’

c-Kit mRNA reverse: 5’-CCAGGAAAAGTTTGGCAGGAT-3’

Lyl1 mRNA forward: 5’-AAGCGCAGACCAAGCCATAG-3’

Lyl1 mRNA reverse: 5’-AGCGCTCACGGCTGTTG-3’

c-Myb mRNA forward: 5’-CGAAGACCCTGAGAAGGAAA-3’

c-Myb mRNA reverse: 5’-GCTGCAAGTGTGGTTCTGTG-3’

Runx1 mRNA forward: 5’-TCACTGGCGCTGCAACAA-3’

Runx1 mRNA reverse: 5’-TCTGCCGAGTAGTTTTCATCGTT-3’

TAL1 mRNA forward: 5’-GAGGCCCTCCCCATATGAGA-3’

TAL1 mRNA reverse: 5’-GCGCCGCACTACTTTGGT-3’

Sfpi1 mRNA forward: 5’-GGCAGCGATGGAGAAAGC-3’

Sfpi1 mRNA reverse: 5’-GGACATGGTGTGCGGAGAA-3’