

Iron-heme-Bach1 axis is involved in erythroblast adaptation to iron deficiency

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Supplementary Figure Legends

Figure S1. Heat maps showing the expression patterns of indicated genes of wild-type mice under the indicated conditions. (A) Heme biosynthetic enzymes. (B) Genes involved in cytoplasmic iron homeostasis. (C) Genes involved in mitochondrial iron and heme homeostasis.

Figure S2. Mitochondria accumulation in erythrocytes induced by iron deficiency is reversible. (A-B) Representative figures and cumulative results of FCM analysis of PB samples in wild-type mice under ND or LID-W for 7 weeks followed by 8 weeks ND (LID-W-ND). The panel in (A) shows CD71/Mito-G staining in Ter119-positive gated cells. The panel in (B) shows cumulative results of this analysis. Symbols in the beeswarm plot represent gender of mice: male (●), female (●).

Figure S3. Bach1 involved in the erythroblast adaptation for iron deficiency. (A) ROS accumulations in erythroblasts of wild-type (WT) or *Bach1*^{-/-} (B1KO) mice under LID-DW analyzed by DCFDA staining. (B) CD71/Mito-G staining in Ter119-positive gated cells of WT or B1KO mice under ND or LID-W. A part of this data was also shown in Figure 4C.

Supplementary Method

Hematological and serum biochemistry analyses

Peripheral blood samples were obtained from the retro-orbital vein in 3 weeks and/or 7 weeks and/or 12 weeks after starting ND or LID. Blood samplings were performed at the same timing for each condition and/or genotype. For complete blood cell counts, peripheral blood (PB) from the retro-orbital vein was analyzed by a hemocytometer (LC-152, HORIBA). Serum biochemical parameters were evaluated by Oriental Yeast Co. Ltd.

Measurement of total iron in Ter119-positive cells

Ter119-positive cells (2×10^6 cells) in BM were suspended in 100 μ L of 0.1% sodium dodecyl sulfate (Wako Pure Chemical Industries) and mixed with vortex. After the sonication, the sample was placed at room temperature for 2 hours and was centrifuged at 10,000 rpm for 5 minutes. The supernatant (95 μ L) and internal standard (Yttrium standard solution at final concentration of 1 ppm, Kanto Chemical) were mixed and total volume was adjusted to 2.0 mL with 0.1 M nitric acid. The amount of total iron was determined by inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista MPX, Varian, USA) with internal standard method.

Quantitative PCR (qPCR)

Total RNA from BM erythroblasts was prepared using an RNeasy Plus Micro kit (QIAGEN). Complementary DNA was synthesized by a SuperScript III First-Strand Synthesis System (Invitrogen). Sequences of the qPCR primers are described in *Online Supplementary Table S1*. The qPCR was performed using LightCycler Fast Start DNA Master SYBR Green I and LightCycler 1.5 or LCnano. Levels of mRNAs were normalized to the mRNA levels of *Actb* encoding β -actin.

Chromatin immunoprecipitation (ChIP)

Ter119-positive BM cells from control or iron deficient mice were cross-linked with 1% formaldehyde for 10 minutes at room temperature and neutralized with 0.125 M glycine. The nuclei lysates were sonicated to reduce DNA length using Sonifier (Branson, Danbury, CT, USA). Aliquots of each sample were kept as input DNA controls. The remaining samples were pre-cleared by using protein A sepharose (P3391; Sigma) in combination with normal rabbit serum, and were subsequently immunoprecipitated with anti-Bach1 antiserum A1-6¹ and an anti-MafK antibody (C-18; Santa Cruz Biotechnology). Normal rabbit serum was utilized as a negative control. Immunoprecipitated DNA was analyzed by SYBR Green fluorescence in 20 μ L reactions, and the amount of product was measured relative to a standard curve generated from titration of input chromatin. Analysis of dissociation-curves post-amplification showed

that primer pairs generated single products. Primer sequences were as described previously and listed in *Online Supplementary Table2*.^{1,2}

Expression profiling by microarray analysis

Total RNA extracted from erythroblasts subset II was amplified by the CyDye labeling method as described previously.³ Sure-Print G3 mouse GE microarray slides were used following the manufacturer's instructions (Agilent). Results were analyzed using the GeneSpring software program (Agilent) and interpreted using the Gene Set Enrichment Analysis methods (GSEA).^{4, 5} Data passed the filter on expression and detection was used. For clustering analysis, data filtered on error (CV < 50 percent) were analyzed by using hierarchical clustering algorithm on normalized intensity values. For GSEA, Geneset data were referenced by Molecular Signatures Database (MSigDB) in Broad Institute. ES (enrichment score), NES (normalized enrichment score), NOM p-val (nominal p-value), FDR q-val (false discovery rate q-value) and FWER p-val (familywise error rate p-value) were calculated by implementation of GSEA.

Post-bisulfite conversion adaptor tagging (PBAT) method

DNA was extracted from sorted erythroblasts distributed in subset II using the QIAamp DNA Micro kit (QIAGEN). Whole-genome bisulfite shotgun sequencing libraries were prepared from the genomic DNA (20 ng each) by the PBAT method⁶ and sequenced using a HiSeq2500 (Illumina) instrument and single-end 100-bp sequencing. Approximately 245-270 million reads were obtained for each sample, with an average sequencing depth of about 2.6. Sequencing reads were mapped to the mm10 genome to analyze methylation by bismark⁷ with mapping efficiencies of 57-58%. Bisulfite conversion rates determined from data of spike-in control DNA (unmethylated lambda phage DNA; purchased from Promega) were 99.4% for all samples.

References for Supplementary Information

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Figure S1

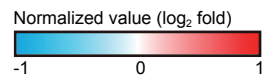
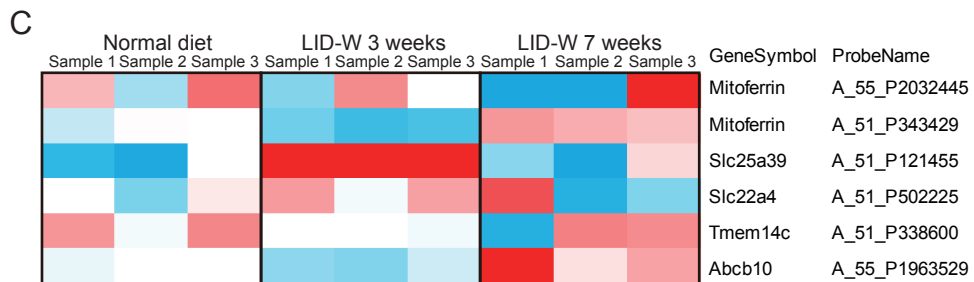
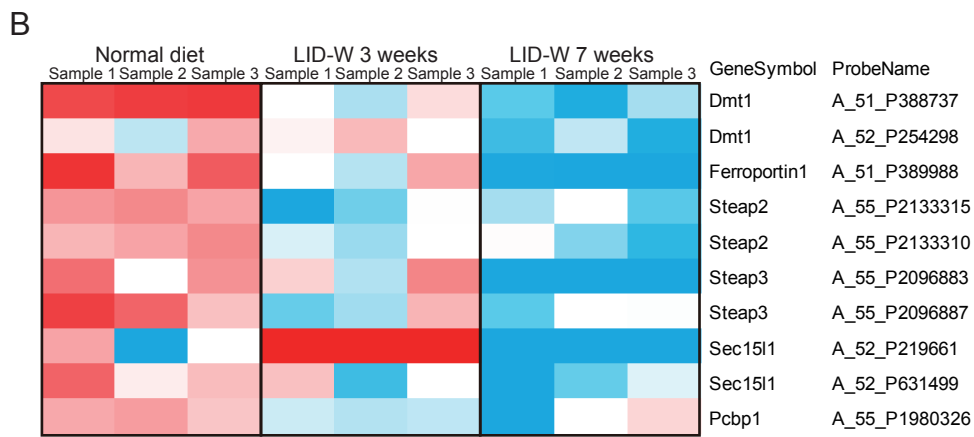
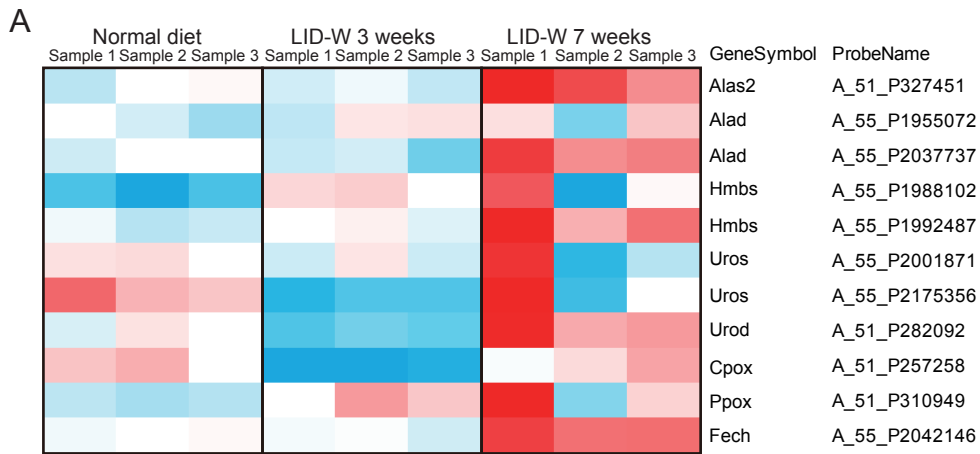
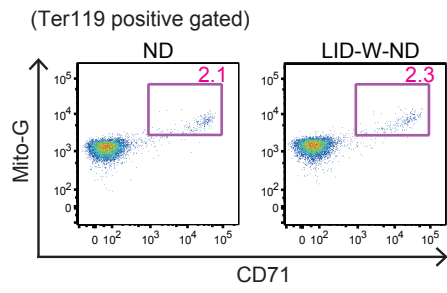


Figure S2

A



B

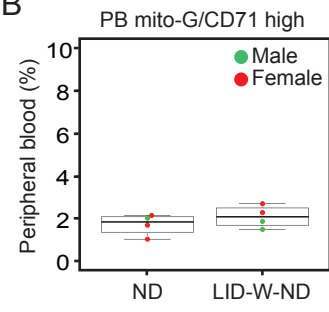


Figure S3

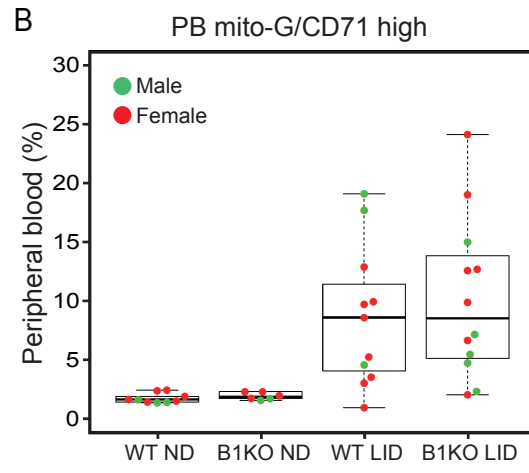
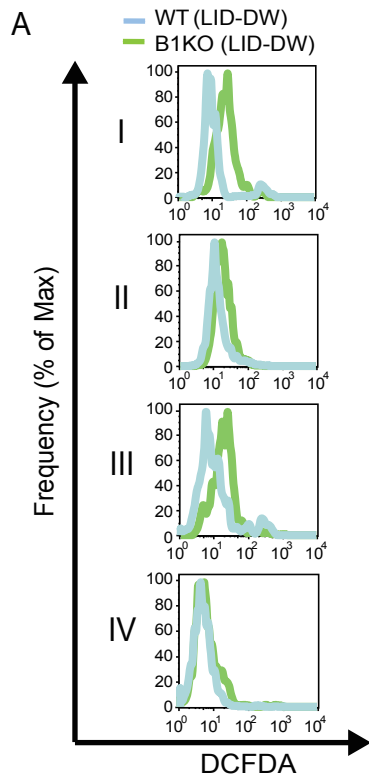


Table S1 : The sequences of real time PCR primers

Gene name	Primer F (5' - 3')	Primer R (5' - 3')
<i>Actb</i>	CGTTGACATCCGTAAAGACCTC	AGCCACCGATCCACACAGA
<i>Atg4d</i>	AGCGGAGTGGAAATCTGTGG	AAGTACAGTGAATGCCGGGG
<i>Gata1</i>	GGCCAAGAAGCGAATGATT	GGTTCACCTGATGGAGCTTGA
<i>Hba-a</i>	GTCACGGCAAGAAGGTCGC	GGGGTGAAATCGGCAGGGT
<i>Hbb-b</i>	GGAAAGGTGAACGCCGATGAA	GGGTCCAAGGGTAGACAACC
<i>Hmox1</i>	GGGTGACAGAAGAGGCTAAG	GTGTCTGGGATGAGCTAGTG
<i>Lmo2</i>	TACAAGCTGGGACGGAAATTGT	GGATCCGCTTGTCACAGGA

Table S2 : The sequences of real time PCR primers for quantitative ChIP analysis

Genomic regions	Primer F (5' - 3')	Primer R (5' - 3')
LCR HS2 ***	AGGGTGTGTGGCCAGATGTT	ACCCAGATAGCACTGATCACTCAC
<i>ey-globin</i> pro *	ATGACCTGGCTCCACCCAT	TCTTTGAAGCCATTGGTCAGC
<i>β-globin</i> pro *	TGAGCAAATGCGTTGCCA	AGCTCTGGGTACTCCCTCTGAATA
<i>α-globin</i> HS26 *	AGCAGACCACTGTGGGATCTATG	TGGCCTCTAGAACTCTGAGTCATG
<i>Hmox1</i> E1 *	TGAAGTTAAAGCCGTTCCGG	AGCGGCTGGAATGCTGAGT
<i>Hmox1</i> E2 *	GGGCTAGCATGCGAAGTGAG	AGACTCCGCCCTAAGGGTTC
<i>Hmox1</i> Pro *	AGGATAAGGGACACCGGTCAC	CAGAGTTTCCGCATACAACCA
<i>Alas2</i> intron8 **	CCTGCTCCTGCCTATCTAGTCATC	CCATGTTAGAGCCTTGCTTCTGT
<i>Hmox1</i> E2 **	GAGCTGTGCCTTTTCTGCTG	AGACCTCGGGACCGTGACT
<i>Hmox1</i> Pro **	GAGCAGAACCAGCCTGAACT	ACACAGTCGCTTGCCTGTC

* primers used for Figure 6C.

** primers used for Figure 6D.

Table S3: List of the genes whose expressions were significantly altered by LID as determined by volcano plot analysis

LID-W_3weeks_UP	
ProbeName	GeneSymbol
A_52_P108321	Ccdc71
A_51_P168695	Mast4
A_52_P204689	Map4k2
A_51_P204740	Cd34
A_55_P1955078	Igflr1
A_52_P438082	Cebpg
A_55_P2051429	
A_55_P2040490	
A_51_P330428	Eif4ebp1
A_51_P130095	Fcgr2b
A_52_P501049	
A_66_P120567	Anxa1
A_51_P519251	Nupr1
A_52_P365011	Fes
A_55_P1964648	Btla
A_51_P180176	Gm16223
A_52_P586004	Hk3
A_30_P01020724	
A_52_P116134	Aldh2
A_55_P2139886	Sft2d1
A_52_P116253	Atf2
A_55_P1992530	Clec4b1
A_55_P2051414	Axl
A_52_P409675	Wdr11
A_55_P1973543	Bbs10
A_52_P581326	Hcfc2
A_30_P01023722	
A_55_P2092310	Phgdh
A_52_P98232	Hira
A_51_P496845	Capza2
A_52_P383653	Cpne8
A_52_P545831	Tssc1
A_51_P313761	Shmt2
A_30_P01027171	
A_55_P2005555	Cellf2
A_55_P2100065	Fsd1l
A_66_P101835	Cd38
A_30_P01022804	
A_55_P1982454	Eps8
A_66_P102395	Elac2
A_55_P1988907	Eml4
A_52_P512329	Zfp219
A_55_P2070254	Pqbp1
A_51_P126067	Cd2
A_66_P101664	Scai
A_55_P2103206	Dusp8
A_30_P01022854	
A_51_P120093	Snx10
A_51_P142989	Zfp429
A_55_P2125049	Gm11127
A_55_P2121349	

LID-W_3weeks_DOWN	
ProbeName	GeneSymbol
A_30_P01025554	
A_55_P2018002	Abcg4
A_55_P2111005	Pafah1b1
A_52_P289213	Notch2
A_55_P1955242	
A_51_P195506	Csf1
A_55_P1971448	Snrpd1
A_30_P01030301	
A_55_P2140328	
A_55_P2032024	Uggt2
A_55_P2065074	Hdac9
A_55_P1955532	Tfr2
A_52_P580582	Nppa
A_55_P2102664	
A_51_P117115	Olfr53
A_55_P2102816	Rpl36a
A_55_P1964368	Kctd14
A_30_P01031282	
A_30_P01018376	
A_55_P2202184	4933400F21Rik
A_55_P1967478	Hbb-y
A_55_P2049597	Tpp2
A_55_P1979650	S100a1
A_55_P2081885	
A_55_P2149382	Gm3448
A_55_P1958103	
A_55_P1994388	Lsm14a
A_51_P111462	Arl15
A_55_P2039681	Gpr35
A_55_P2062632	Smc5
A_52_P136138	Fdft1
A_51_P302139	Mastl
A_55_P1981739	Donson
A_55_P2024327	Suv420h2
A_51_P198411	Grb2
A_55_P2151672	
A_55_P1952212	
A_55_P2036377	
A_30_P01026851	
A_55_P2060188	Hira
A_55_P2251082	Qk
A_55_P2145800	LOC102633883
A_55_P2041626	Ceacam12
A_55_P2002702	Ddx18
A_55_P1995404	Cd59b
A_30_P01030544	
A_55_P2067142	Fau
A_51_P361788	Vapa
A_55_P2163622	Rps27
A_51_P406077	1110001J03Rik
A_55_P2351300	A330094K24Rik

LID-W_7weeks_UP	
ProbeName	GeneSymbol
A_30_P01025554	
A_30_P01031558	
A_30_P01029765	
A_55_P2252586	3426406K10Rik
A_30_P01028667	
A_55_P2279572	C79015
A_30_P01024579	
A_55_P1958097	Eno1
A_55_P1955078	Igflr1
A_51_P104681	Krtap6-5
A_55_P2051429	
A_55_P2372228	A430104N18Rik
A_55_P2040490	
A_51_P397876	En2
A_55_P2049949	
A_30_P01022993	
A_55_P2138071	
A_55_P2165120	Cblb
A_30_P01026850	
A_51_P130095	Fcgr2b
(+)E1A_r60_a22	
A_55_P2032024	Uggt2
A_51_P116088	Rexo2
A_30_P01024186	
A_51_P519251	Nupr1
ERCC-00148_99	
A_55_P2129691	AB124611
A_51_P118132	Skil
A_55_P2102664	
A_30_P01028853	
A_30_P01022811	
A_55_P2246700	
A_55_P2117764	Vangl2
A_30_P01029403	
A_55_P2085979	Igf1
A_30_P01026324	
A_55_P1992530	Clec4b1
A_55_P2083454	Gm10844
A_30_P01022026	
A_55_P1979650	S100a1
A_52_P745	Acx3
A_51_P201480	Stat3
A_30_P01027826	
A_30_P01022480	
A_30_P01032336	
A_55_P1958103	
A_51_P462102	Gpr125
ERCC-00120_69	
A_30_P01024721	
A_30_P01022686	
A_55_P1984481	Mfn2

LID-W_7weeks_DOWN	
ProbeName	GeneSymbol
A_52_P108321	Ccdc71
A_55_P2018002	Abcg4
A_51_P385099	Tnf
A_55_P2041509	1110004F10Rik
A_51_P358243	Tstd3
A_52_P289213	Notch2
A_55_P2433218	Bloc1s6
A_30_P01031589	
A_55_P2110215	Ckap5
A_51_P204740	Cd34
A_51_P106952	Rbm26
A_55_P1952434	Tm2d1
A_55_P2186085	Atp11c
A_55_P1956687	Rab37
A_55_P2124736	Col14a1
A_55_P2145706	Thoc7
A_55_P2006255	Txnip
A_52_P142208	Akirin1
A_30_P01030301	
A_55_P2154480	Ctxn2
A_66_P115757	Mthfd2l
A_51_P125691	Cdca4
A_55_P2404808	2210406H18Rik
A_55_P1975456	Nfyb
A_55_P1964368	Kctd14
A_55_P2092958	Traf2
A_30_P01031282	
A_55_P2009943	Spen
A_30_P01024081	
A_30_P01020724	
A_55_P2457153	G2e3
A_55_P2400691	Arhgap21
A_30_P01025678	
A_52_P116134	Aldh2
A_55_P1991945	
A_55_P2107995	Psd
A_51_P232142	Thumpd3
A_55_P1969885	
A_30_P01032978	
A_55_P2049597	Tpp2
A_30_P01030305	
A_55_P2095899	2310057B04Rik
A_55_P2055419	Arid4a
A_66_P125209	Kif2c
A_51_P396917	Zfyve21
A_55_P1956832	Pip5k1b
A_55_P1994388	Lsm14a
A_51_P111462	Arl15
A_55_P2444515	Akap2
A_55_P2112235	Cdk5rap2
A_51_P302139	Mastl

A_55_P2058933	Kif1b
A_52_P221776	Kif12
A_52_P590661	Eef2k
A_55_P1992838	Socs2
A_52_P734742	Lrrc73
A_30_P01023787	
A_52_P229044	Slc20a2
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A_30_P01029138	
A_55_P2037689	
A_52_P157450	Abhd1
A_51_P454993	Tmcc2
A_52_P155708	Creg1
A_51_P509223	Arfgap1
A_52_P109319	E2f5
A_52_P418644	Plp2
A_55_P2082050	Ankrd12
A_52_P223524	Dennd6b
A_66_P100113	Dynl1a
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A_66_P123683	Nabp1
A_52_P601782	Mb21d1
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A_55_P2174863	Olfr766
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A_55_P2071536	
A_55_P2120254	Avpi1
A_55_P2021953	Olfr700
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A_51_P372172	Plod3
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A_30_P01021300	
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A_30_P01021682	
A_30_P01032129	
(+)E1A_r60_a135	
A_55_P1982454	Eps8
A_55_P1988907	Eml4
A_52_P512329	Zfp219
A_55_P2251082	Qk
A_55_P2126207	1600014K23Rik
A_30_P01031466	
A_55_P2009882	Ccbe1
A_52_P309890	Mcc
A_30_P01032030	
A_66_P101664	Scai
A_52_P84096	Pgd
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A_52_P37681	Fkbp9
A_51_P310398	Tk2
A_55_P1977318	Wbp11
A_55_P2023306	Pcdha9
A_55_P2044369	Hs2st1
A_55_P2109407	Rnase12
A_51_P183051	Upb1
A_52_P577208	Scfd2
A_55_P1979929	Prpc
A_55_P2028542	Becn2
A_52_P437201	Dna2
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A_51_P151586	Gsg2
A_55_P2162797	C1s1
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A_55_P1976734	Cacna2d2
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A_55_P2104917	Nphp1
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A_30_P01025929	
A_55_P2027930	Nfx1
A_55_P2043207	Magef1
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A_30_P01018583	
A_55_P2129886	Btnl5-ps
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A_52_P639229	Fgd4
A_51_P256246	Tspan13
A_51_P284946	Rnd3
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A_55_P2078459	Kpna2
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A_66_P136186	Wee1
A_55_P2077608	Hdac1
A_30_P01024333	
A_51_P431316	Ube2w
A_55_P2006668	Pi4kb
A_55_P2072543	Sin3b
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A_55_P2159994	Atf1
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A_55_P1955202	Gm10204
A_55_P1971828	Csnk2a1
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A_30_P01030767	
A_66_P111580	
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A_51_P509452	Tmlhe
A_30_P01027967	
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A_55_P2203996	Prdm10
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A_51_P432511	Lace1
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A_52_P158431	Tshz2

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A_52_P556099	Oip5
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A_55_P2147566	Tac2
A_55_P2125963	Gm13247
A_55_P2055463	Snapin
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A_30_P01029254	
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A_55_P2031681	Gstm6
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A_55_P2088018	Fhod3
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A_30_P01024368	
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A_30_P01019149	
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A_55_P2165969	Ncor1
A_55_P2175687	Tex16
A_30_P01025811	
A_55_P2065971	Setd3
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A_55_P2019014	Tmem120b
A_55_P2178491	Fam53b
A_55_P2014319	Armcx6
A_66_P110741	
A_52_P215829	Alg13
A_51_P206824	Hfe2
A_66_P133043	Mbd2
A_30_P01019519	
A_30_P01030606	
A_52_P577748	Lpxn
A_55_P2028504	Gm14957
A_51_P141164	Ddx50
A_55_P1966089	
A_55_P2009973	Ttc3
A_51_P406165	Nxpe2
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A_51_P345836	Prkra
A_66_P100420	Olfir1000
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A_55_P2073094	Trex2
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A_55_P2011950	
A_55_P2169064	Olfir1474
A_51_P301804	St3gal1
A_55_P2111735	Ppp1r2
A_51_P273203	Gpaa1
A_30_P01030325	
A_52_P555672	Vrk1
A_55_P2080130	Aim2
A_52_P5652	Sae1
A_52_P558087	Poc5
A_30_P01021036	
A_52_P266459	Ing2

Table S4. GSEA of BM erythroblast microarray data related to mitochondrial biology and Gata1 functions

	GS follow link to MSigDB	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val
1	MITOCHONDRIAL_INNER_MEMBRANE	64	0.58	1.50	0.000	0.057	0.000
2	MITOCHONDRIAL_MEMBRANE	83	0.53	1.46	0.000	0.057	0.044
3	MITOCHONDRIAL_MEMBRANE_PART	52	0.57	1.42	0.000	0.057	0.044
4	V\$GATA1_05	191	0.31	1.40	0.000	0.057	0.044
5	MITOCHONDRIAL_RESPIRATORY_CHAIN	24	0.63	1.39	0.000	0.085	0.097
6	V\$GATA_C	169	0.36	1.38	0.000	0.080	0.097