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Prospective isolation of radiation induced erythroid stress progenitors reveals unique transcriptomic and epigenetic signatures enabling increased erythroid output

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ABSTRACT

Massive expansion of erythroid progenitor cells is essential for surviving anemic stress. Research towards understanding this critical process, referred to as stress-erythropoiesis, has been hampered due to the lack of specific marker-combinations enabling analysis of the distinct stress-progenitor cells capable of providing radioprotection and enhanced red blood cell production. Here we present a method for the precise identification and *in vivo* validation of progenitor cells contributing to both steady-state and stress-erythropoiesis, enabling for the first time in-depth molecular characterization of these cells. Differential expression of surface markers CD150, CD9 and Sca1 defines a hierarchy of splenic stress-progenitors during irradiation-induced stress recovery in mice, and provides high-purity isolation of the functional stress erythroid burst-forming-units (stress-BFU-E) with a 100-fold improved enrichment compared to the state-of-the-art. By transplanting purified stress-progenitors expressing the fluorescent protein Kusabira Orange, we determined their kinetics *in vivo* and demonstrated that $CD150^{+}CD9^{+}Sca1^{-}$ stress-BFU-E provide a massive but transient radioprotective erythroid wave, followed by multi-lineage reconstitution from $CD150^{+}CD9^{+}Sca1^{+}$ multi-potent stem/progenitor cells. Whole genome transcriptional analysis revealed that stress-BFU-E express gene signatures more associated with erythropoiesis and proliferation compared to steady-state BFU-E, and are bone morphogenetic protein 4-responsive. Evaluation of chromatin accessibility through ATAC sequencing reveals enhanced and differential accessibility to binding sites of the chromatin-looping transcription factor CTCF in stress-BFU-E compared to steady-state BFU-E. Our findings offer a molecular insight into the unique capacity of stress-BFU-E to rapidly form erythroid cells in response to anemia and constitute an important step towards identifying novel erythropoiesis stimulating agents.

Introduction

Steady-state erythropoiesis is regulated mainly by changes in erythropoietin (EPO) levels that fine-tune survival and proliferation of erythroid colony-forming-units (CFU-E) and downstream precursor cells. In contrast, acute anemia induces a broader physiological response referred to as stress-erythropoiesis, which involves stimulation also of earlier progenitors to further increase the out-put of erythrocytes. This process is less characterized and mainly occurs in the murine spleen[1] after seeding of progenitors from the bone marrow (BM).[2,3] Stress-erythropoiesis is differentially regulated, including increased responsiveness to additional factors like hypoxia, corticosteroids and bone morphogenetic protein 4 (BMP4).[1,4-7] Importantly, stress-erythroid progenitors have the capacity to generate larger numbers of red blood cells than steady-state progenitors, and precise identification and enhanced understanding of their regulation are important steps towards discovering potential new erythroid-enhancing drugs for anemia treatment.

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While fluorescence-activated cell sorting (FACS)-based methods for fractionation of distinct erythroid progenitor cells in murine and human during steady-state[8-11] has enabled in-depth characterization of mechanisms regulating steady-state erythropoiesis,[11-15] the cells and mechanisms regulating stress-erythropoiesis remain poorly defined. To enable studies of stress-erythropoiesis we set out to identify novel marker-combinations separating and enriching for the early stress-progenitors mediating radioprotection and recovery from severe anemia. We previously demonstrated that fetal erythroid burst-forming-units (BFU-E) can be isolated as lineage-cKit$^+$CD71/CD24a^{low}Sca1$^-$CD34$^-$ with high purity from murine fetal liver, where erythropoiesis in many ways resemble stress-erythropoiesis.[16] Attempts by other groups to isolate adult stress-erythroid progenitors from spleens of anemic mice and *in vitro* cultures have shown stress-BFU-E to be lineage-cKit$^+$CD71/Ter119^{low}, and further enriched in the Sca1$^+$CD34$^-$CD133$^-$ fraction. However, very few of these cells possess BFU-E potential (0.1-0.2%). Furthermore, in the active debate on lineage potential of stem- and progenitor cells, genuine megakaryocytic/erythroid potential is often overlooked since mature erythrocytes and platelets are difficult to trace *in vivo* after transplantation. Hence, the identity of pure stress-BFU-E remains largely elusive.

Using a novel combination of surface markers together with the tracing marker Kusabira Orange which is expressed in all cells, we have developed a method for high purity fractionation of a hierarchy of multi-potent progenitors, stress-BFU-E, and stress-CFU-E within the lineage-cKit$^+$CD71/CD24a^{low} cells in spleen during irradiation-induced stress-erythropoiesis as well as in steady-state BM, and for the first time determined their kinetics and full differentiation potential *in vivo*. The formation of stress-BFU-E was highly dependent on functional BMP-signaling, and stress-BFU-E displayed enhanced expression of BMP-responsive genes, as well as gene signatures associated with erythropoiesis and proliferation compared to their steady-state counterpart. In addition, discrepancies in the epigenetic landscape were selectively enriched for putative binding sites for the chromatin-looping transcription factor CTCF. In conclusion, our findings provide high-purity isolation of both steady-state BFU-E and the stress-BFU-E mediating recovery from severe anemia, and offer molecular insight to and functional determination of the unique capacity of stress-BFU-E to rapidly form erythroid cells in response to anemia.

Methods

Mice and transplantations

All procedures involving mice were approved by the Animal Ethics Committee of Malmö/Lund, Sweden. Anemia was induced by lethally irradiating 8-12 week-old recipient mice (C57Bl/6; Ly5.2) with a split dose of 2x500 cGy, followed by transplantation of 2x10^6 unfractionated BM cells to rescue and trigger stress-erythropoiesis. Donor mice (B6SJL; Ly5.1) were either Kusabira Orange (KuO) positive or negative. Recipients were sacrificed on day 8 for analysis of stress recovery in the spleen.

For *in vivo* tracing, 500 multipotent progenitors (sMPP), 5,000 sBFU-E or 5,000 sCFU-E, all KuO$^+$, were FACS-sorted from day 8 stressed spleens and transplanted into lethally irradiated secondary recipients together with 105 unfractionated wild-type BM cells as support. Secondary recipients were bled at 1, 2 and 4 weeks, and sacrificed at 2 or 4 weeks post transplantation for analysis of lineage potential and kinetics in peripheral blood (PB), BM and spleen.

Flow cytometry

A complete description of all antibodies used is listed in the *Online Supplementary Materials and Methods*.

Hematopoietic progenitor assays

All colony assays were incubated at 37°C incubators in 5% CO2 with either 21% or 1-4% O_2 as indicated, and scored on day 4 (CFU-E) or 7-8 (BFU-E and mixed colonies).

RNA sequencing

Splenic stress- and BM steady-state progenitors were FACS-sorted in triplicates. Strand specific RNA-sequencing libraries were constructed using SMARTer Stranded Total RNA-Seq Kit v2 (Takara Bio) followed by sequencing on a HiSeq3000 (Illumina).

Assay for Transposase Accessible Chromatin sequencing

3,000 splenic stress- and BM steady-state BFU-E were FACS-sorted in triplicates for the assay of Transposase Accessible Chromatin (ATAC) library preparation and sequencing as described previously.[18] Libraries were subject to single-end sequencing on a NextSeq500 (Illumina).

Statistical analysis

For all statistical analysis, apart from RNA- and ATAC-sequencing for which the specifications are stated in the *Online Supplemental Materials and Methods*, statistical significance was calculated using ANOVA accounting for multiple comparisons, followed by Tukey's multiple comparisons test. One-way ANOVA was used for single time point analysis and Two-way ANOVA was used when measuring potential over time (Figure 2). $^*P\leq0.05$, $^{**}P\leq0.01$, $^{***}P\leq0.001$, $^{****}P\leq0.0001$.

A detailed description of all methods used is available online in the *Online Supplementary Materials and Methods*.

Results

CD150, CD9 and Sca1 identify a hierarchy of splenic stress-progenitors during irradiation-induced stress recovery

To identify the stress-progenitors involved in irradiation-induced anemia and stress recovery, we subjected mice to lethal irradiation followed by BM transplantation and analyzed recipient spleens on day 8 (Figure 1A) when the greatest expansion of stress-progenitors occurs.[3] Using this model, stress-progenitors were previously identified as Lin$^-$cKit$^+$CD71/Ter119^{low}, although at low frequencies (0.2%).[3] To further enrich for stress-erythroid progenitors we included CD150, known to mark megakaryocytic/erythroid progenitors during steady-state hematopoiesis.[9] All BFU-E potential in the cKit$^+$CD71^{low}/Ter119^{low} population resided in the CD150$^+$ fraction (Figure 1B-C). Extramedullary expansion of early erythroid progenitors during stress-erythropoiesis is reminiscent of fetal liver erythropoiesis. We therefore analyzed previously published mRNA expression data (GSE26086)[16] from E14.5 to E15.5 fetal liver erythroid progenitors to identify possible additional markers for further sub-division of adult stress-erythroid progenitors (*Online Supplementary Figure S1A*).

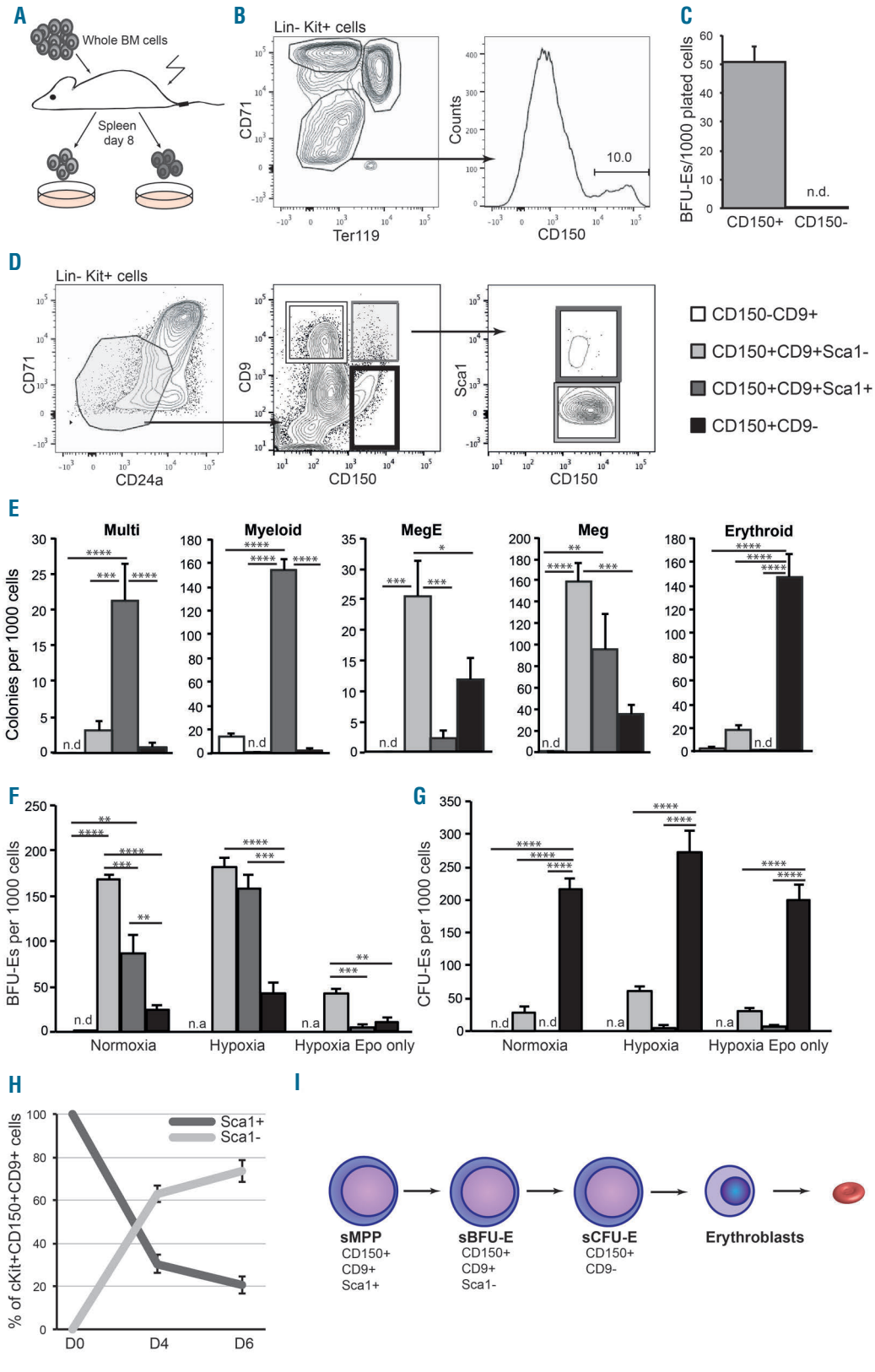

Figure 1. CD150, CD9 and Sca1 identify a hierarchy of splenic stress-progenitors during irradiation-induced stress recovery. (A) Stress-erythropoiesis was induced using lethal irradiation followed by transplantation of unfractionated bone marrow (BM), and splenic stress-progenitors were assessed on day 8 using colony assays. (B) Gating strategy and (C) BFU-E potential of FACS-sorted stress-progenitors from spleen day 8 based on CD150 expression (n=5). (D) Gating strategy for further fractionation of stress-progenitors within lineage-Kit$^+$ cells using CD150 and CD9 and Sca1. (D-G) Colony forming capacity of splenic day 8 stress-progenitors, FACS-sorted based on (D) CD150/CD9 (normoxia, n=4), and (E-G) CD150/CD9/Sca1 (n=7 for normoxia, n=4 for hypoxia) within the Lin-cKit$^+$CD71^{low}CD24^{low} fraction. The cells were incubated in normoxia (21% O2) or hypoxia (1% O2) as indicated, and scored on day 4 (CFU-E) or day 7-8 (mixed and BFU-E). (H) FACS sorted CD150$^+$CD9+Sca1$^+$ progenitors were cultured *in vitro* and analyzed at indicated time points for the formation of CD150$^+$CD9$^+$Sca1$^-$ cells (n=4). I) Expression pattern of novel surface markers within the Lin$^-$cKit$^+$CD71^{low}CD24^{low} fraction separating multi-potent stem/progenitor cells (sMPPs), stress-BFU-Es (sBFU-Es) and stress-CFU-E (sCFU-Es), defining a stress-progenitor hierarchy in spleen during irradiation-induced stress recovery, where Sca1 and subsequently CD9 are downregulated with increased differentiation. Data displayed as average ± standard error of the mean (SEM), n.d: not detectable, n.a: not applicable, *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.

As previously reported by us in fetal liver,[16] exclusion of mature erythroid precursor cells in spleen was facilitated by inclusion of CD24a as a negative selection marker (*Online Supplementary Figure S1B*). Of the surface markers tested (CD9, CD11a, CD34, CD48, CD63, and CD79b), only CD9 could further fractionate the BFU-E-containing $CD150^+$ population in stressed spleen (*Online Supplementary Figure S1C*). CD133 was used in a recent study together with CD34 to fractionate stress-progenitor potential of cultured $Sca1^+cKit^+CD71/Ter119^{low}$ cells.[17] However, neither CD34 nor CD133 could further fractionate $CD150^+$ stress-progenitors (*Online Supplementary Figure S1C* and *Online Supplementary Figure S2A*, respectively). To further discriminate putative multi-potent stress-progenitors from lineage restricted stress-BFU-E we also included Sca1, which in steady-state BM separates $Sca1^+$ hematopoietic stem cells (HSC) and $Sca1^-$ myelo-erythroid progenitors.[9] Analysis of $Lin^-cKit^+CD71^{low}/CD24a^{low}$ splenic stress-progenitors fractionated based on CD150/CD9/Sca1 expression (Figure 1D), clearly demonstrated that the majority of multi-lineage, megakaryocytic and BFU-E colony forming potential resided in the $CD150^+CD9^+$ population (Figure 1E-F). Stress-progenitors negative for CD9 gave rise to more mature erythroid colonies (Figure 1E, G), which was also true for $CD133^+$ cells (*Online Supplementary Figure S2B*), whereas $CD150^-$ cells mainly gave rise to myeloid colonies (Figure 1E). Only Sca1 expressing $CD150^+CD9^+$ $cKit^+$ $CD71^{low}/CD24a^{low}$ stress-progenitors (about 10%) gave rise to mixed and myeloid colonies (Figure 1E), while megakaryocytic/erythroid potential was retained in $Sca1^-$ stress-progenitors (Figure 1E-F). Stress-BFU-E are reported to form BFU-E colonies with Epo alone,[1] but require hypoxia and additional cytokines for maximum expansion.[3] Colony assays demonstrated that BFU-E-forming potential resided almost exclusively in the $CD150^+CD9^+$ population (Figure 1F). Importantly, as many as 21.4±2.2% of $CD150^+CD9^+$ cells gave rise to BFU-E

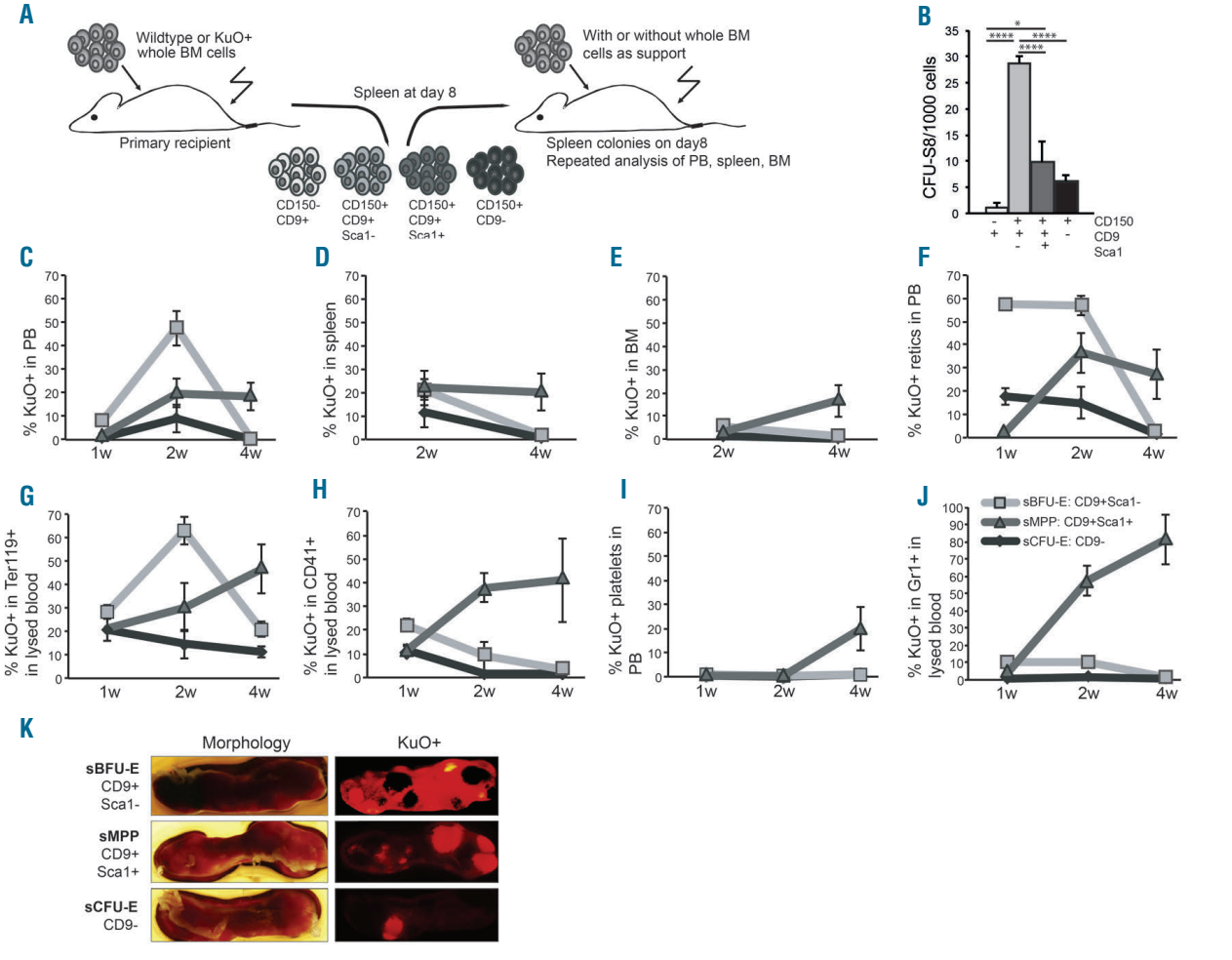

Figure 2. Stress-BFU-E provide a transient wave of primarily erythroid cells, followed by multi-lineage reconstitution from stress-MPP. (A) Stress-erythropoiesis was induced using lethal irradiation followed by transplantation of unfractionated bone marrow (BM) from (B) wild-type or (C-K) transgenic Kusabira Orange (KuO) mice. Splenic stress-progenitor populations were FACS-sorted on day 8, transplanted into lethally irradiated secondary recipients (B) without support to evaluate spleen colony formation (CFU-S8) (n=3-7; 600 cells per recipient without support) or (C-K) together with 105 unfractionated wild-type BM support cells to monitor their repopulation capacity *in vivo* over time in (C) peripheral blood (PB), (D) spleen and (E) BM, as determined by KuO fluorescence and FACS (n=6 at 1-2 weeks and n=3 at 4 weeks; 500 sMPP or 5,000 of each stress-erythroid progenitor per recipient, all with 105 wild-type support BM cells). (F-J) Contribution of sorted KuO+ progenitors in PB to (F) reticulocytes (whole PB), (G) $Ter119^+$ erythroid cells, (H) $CD41^+$ cells, (I) platelets (whole PB), and (J) Gr1+ myeloid cells in PB, as determined by FACS (after lysis of red blood cells if not stated otherwise). (K) Representative picture of whole spleens 2 weeks after transplantation assessing KuO contribution using epifluorescence microscopy and green filtered laser excitation. Data displayed as average ± standard error of the mean (SEM), *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001.

colonies, providing over 100-fold improved purity compared to the state-of-the-art (0.1-0.2%). Although both Sca1$^-$ and Sca1$^+$ cells formed BFU-E colonies when stem cell factor (SCF) was present, only Sca1- cells formed BFU-E in erythropoietin (Epo) alone (Figure 1F). Furthermore, Sca1$^-$ progenitors generally gave rise to larger colonies than Sca1$^+$ progenitors (*data not shown*). A notable 27.4±3.2% of CD150$^+$CD9$^-$ cells gave rise to CFU-E, which were mainly independent of hypoxia and SCF (Figure 1G). Although the number of colonies was not affected, hypoxia resulted in increased proliferation and larger colonies (*data not shown*). FACS-sorted

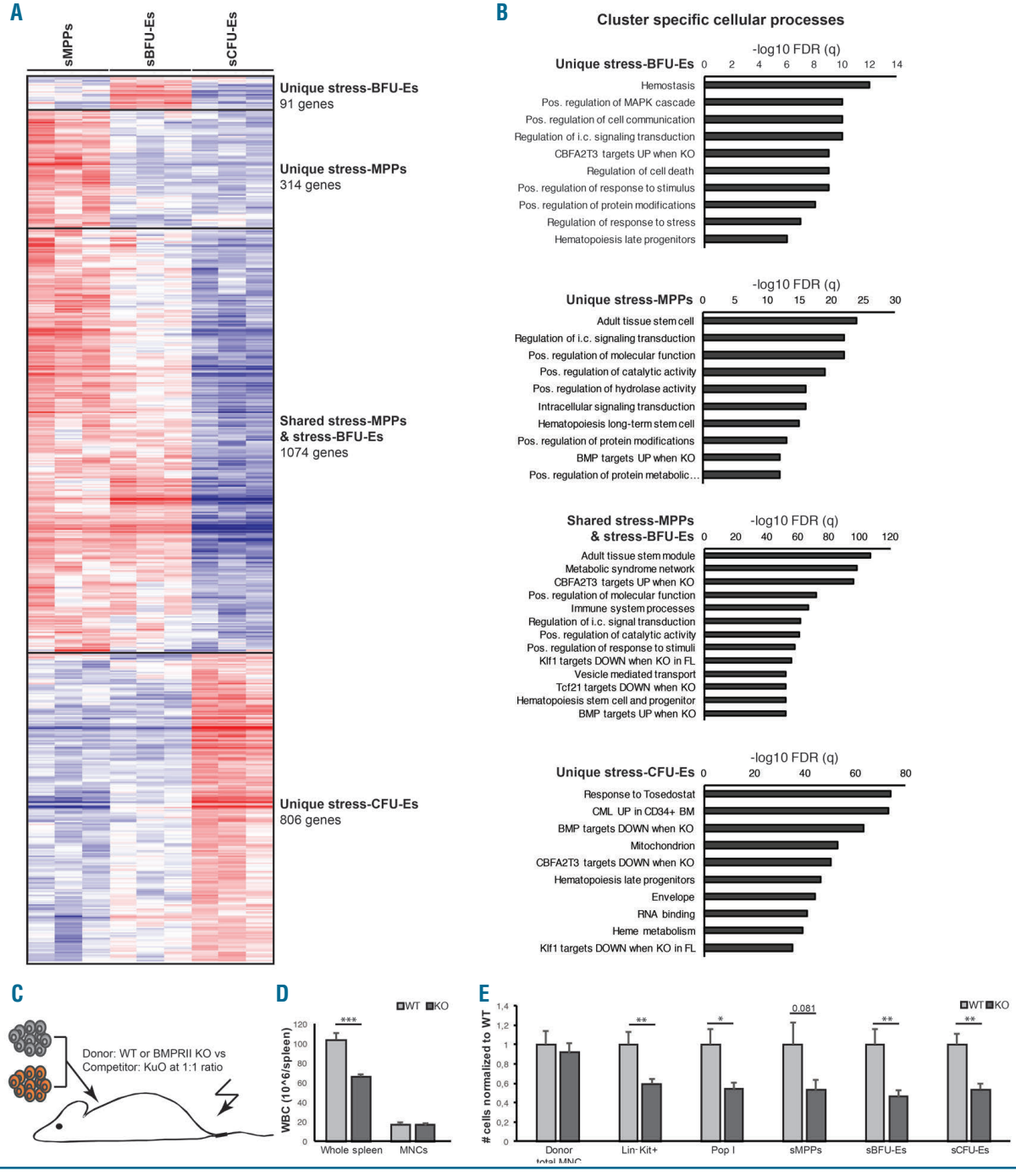

Figure 3. Stress-BFU-E and stress-CFU-E display opposing expression patterns of BMP- and CBFA2T3-responsive genes. (A) Unsupervised clustering of RPKM normalized data on genes with a significantly different expression between any of the samples (FDR<0.05, log$_2$ fold change>0.58) from RNA-sequencing of FACS sorted stress-progenitor populations as indicated (n=3, for further details see the Methods section). (B) Cellular processes for each of the clusters generated in (A), as analyzed by GSEA. For a complete gene lists for each cluster see the *Online Supplementary Table S1*. (C) Experimental outline of competitive transplantation of wild-type or BMP receptor II (BMPRII) deficient bone marrow against KuO$^+$ wild-type bone marrow. The stress recovery was analyzed in spleens of recipients on day 8 after transplantation (n=7 per group). (D) White blood cell count (WBC) per spleen before and after enrichment of mononuclear cells (MNC). (E) Detailed analysis of donor-derived (KuO-) MNC and stress-populations within MNC spleen cells as indicated. Data displayed as average ± standard error of the mean (SEM) of total number of cells/spleen (D) and normalized to wild-type (E), **P*≤0.05, ***P*≤0.01.

$CD150^+CD9^+Sca1^+$ progenitors gave rise to $CD150^+CD9^+Sca1^-$ in culture (Figure 1H), indicating that more restricted Sca1- progenitors arise from multi-potent Sca1+ progenitors. Taken together, differential expression of cell surface markers CD150, CD9 and Sca1 defines a hierarchy of splenic $Lin^-cKit^+CD71/CD24a^{low}$ stress-progenitors during irradiation-induced stress recovery in mice (Figure 1I). The stress-BFU-E, forming BFU-E colonies *in vitro* ($CD150^+CD9^+Sca1^-$, hereafter referred to as stress-BFU-E or sBFU-E), can be separated from multi-potent stress-progenitors ($CD150^+CD9^+Sca1^+$, hereafter referred to as stress-MPP or sMPP) and stress-CFU-E ($CD150^+CD9^-$, hereafter referred to as stress-CFU-E or sCFU-E) by Sca1 and CD9 expression respectively.

Stress-BFU-E provide a transient wave of primarily erythroid cells, followed by multi-lineage reconstitution from stress-MPP

To determine the kinetics and full *in vivo* potential of the identified stress-progenitors, we used a transgenic mouse that constitutively expresses the fluorescent protein Kusabira Orange (KuO) in all cells, including erythrocytes and platelets.[20] Lethally irradiated recipients were transplanted with BM from KuO mice, and KuO^+ splenic stress-progenitors isolated on day 8 were subsequently transplanted into secondary recipients, either without support cells to score spleen colony-forming-units day 8 (CFU-S8),[21] or together with 10^5 unfractionated wild-type BM support cells to monitor their *in vivo* repopulation capacity over time (Figure 2A). sBFU-E demonstrated the highest CFU-S8 potential (Figure 2B), indicative of robust short-term radio-protective capacity. Analysis of overall repopulation potential of sorted KuO^+ progenitors demonstrated

Table 1. Top 20 up-regulated genes in stress-BFU-E compared to steady-state.

Gene symbol	log2 fold change	-log10 FDR
Ctse	2,254	19,109
Myh10	1,542	7,858
Aldh1a1	1,529	5,762
Pop1	1,178	2,814
Tfrc	1,171	4,072
Psat1	1,133	3,136
Timm10b	1,123	2,505
Vkorc1	1,119	2,652
Sipa1	1,117	5,900
Tmem70	1,082	2,401
Usp14	1,063	6,477
Emilin2	1,062	3,012
Josd1	1,039	3,305
Psmb2	1,036	2,079
Suclg1	1,035	5,608
Trip13	1,014	2,078
Arap2	1,005	2,132
Tmem201	1,001	2,320
Mob1b	0,992	2,190
Atl2	0,984	3,323

The most highly up-regulated genes in sBFU-E compared to steady-state BFU-E were Ctse/Cathepsin E: an erythrocyte membrane aspartic proteinase previously described as a down-stream target of Foxo3 in erythroid regulation,[38] Myh10: a non-muscle myosin involved in cell division of erythroblasts and other cells,[39] Aldh1a1: a critical enzyme involved in metabolism of reactive oxygen species and retinoic acid shown to mark adult definitive erythroid fate in mouse and human blood development, and the transferrin receptor Tfrc/CD71.

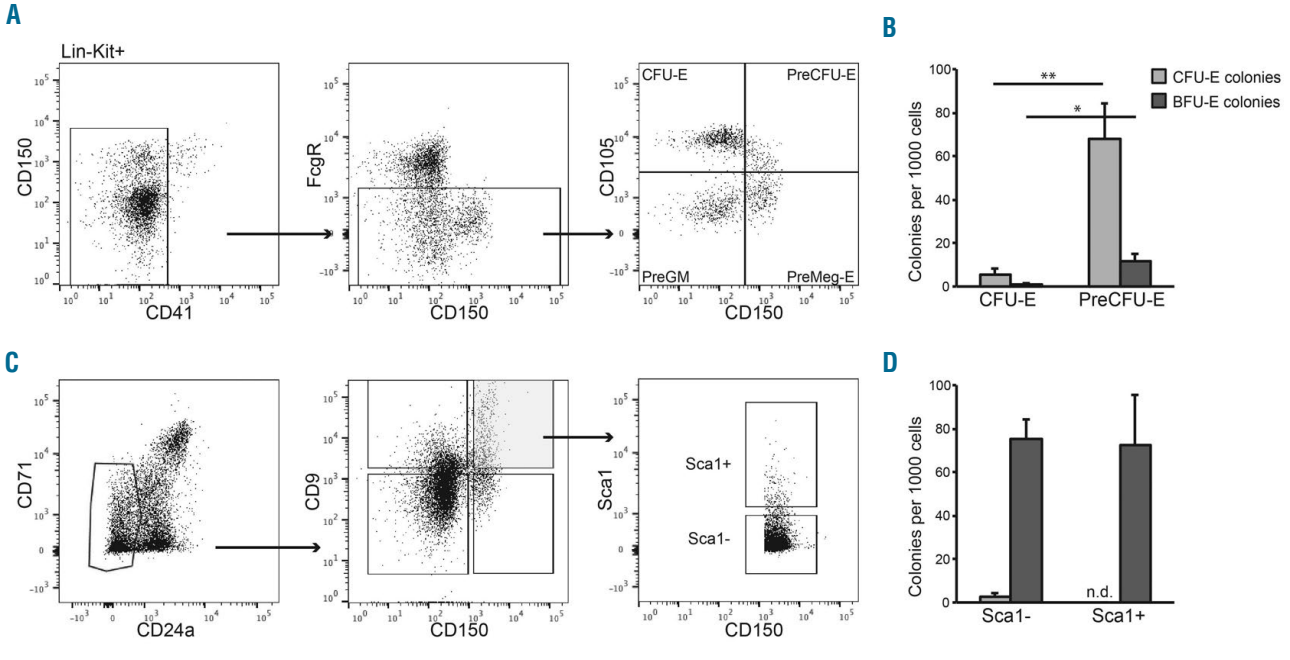

Figure 4. CD150 and CD9 mark BFU-E potential during steady-state erythropoiesis, providing improved identification of myelo-erythroid progenitors in the bone marrow. Gating strategy and colony forming potential (CFU-E; light grey, BFU-E; dark grey) of FACS sorted (A-D) steady-state bone marrow progenitors based on (A-B) classical fractionation using CD150 and CD1059, and (C-D) the new markers CD150/CD^9/Sca1. All cells were incubated in 4% O_2, and scored on day 4 (CFU-E) or day 7-8 (BFU-E). Data displayed as average ± standard error of the mean (SEM), n.d: not detectable, *$P \leq 0.05$, **$P \leq 0.01$.

that sBFU-E and sCFU-E provide a transient wave of reconstitution in PB and spleen, with limited contribution to BM hematopoiesis. In contrast, sMPP increased their output over time, and was the only population that efficiently populated the BM and still contributed to hematopoiesis at four weeks (Figure 2C-E).

At two weeks 47.4±7.6% of PB cells were KuO$^+$ in mice transplanted with sBFU-E, compared to 9.0±5.9% (P≤0.0001) for sCFU-E (Figure 2C). Analysis of the differentiation potential of transplanted stress-progenitors revealed that sBFU-E gave rise to 57.4±1.9% of reticulocytes compared to 17.5±3.5% (P≤0.0001) for sCFU-E, while reticulocytes from sMPP were barely detected (Figure 2F; PB 1 week). Furthermore, the initial overall formation of reticulocytes was 2-fold higher in mice transplanted with sBFU-E compared to sCFU-E (*Online*

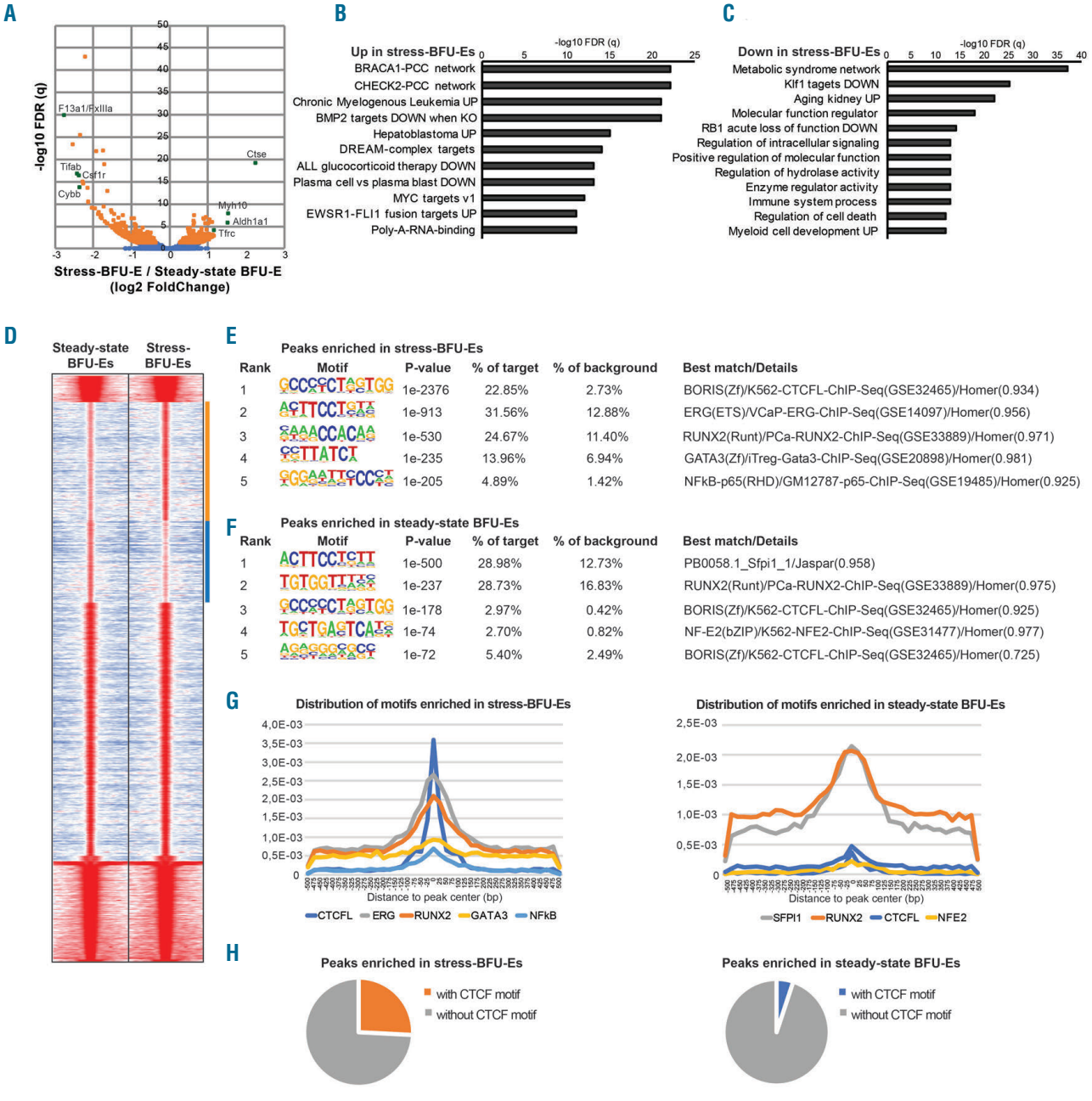

Figure 5. Compared to steady-state BFU-E, stress-BFU-E have enhanced expression of genes associated with BMP signaling, erythropoiesis and proliferation, and have enhanced and differential accessibility to CTCF binding sites. (A) RNA-sequencing was conducted on BFU-E FACS-sorted from steady-state bone marrow and day 8 stress spleens respectively (n=3). Significant differential expression was determined as -$\log_{10}$ FDR>2 and $\log_2$ fold change>0.58, for a full list of differentially expressed genes see the *Online Supplementary Table S2*. (B-C) Cellular processes for genes that were (B) up- and (C) down-regulated in stress-BFU-E compared to steady-state respectively, as analyzed by GSEA. (D) Fishbone heatmap outlining specific and shared ATAC-seq peaks in steady-state and stress BFU-E respectively. Each row represents one peak, and the color represents the intensity of chromatin accessibility. Peak files for heatmap were created in HOMER44 and grouped based on K-means clustering (3-clusters, 100-runs) performed in Cluster3, revealing clusters of peaks enriched in stress- (orange) and steady-state BFU-E (blue) respectively. (E-F) Motif analysis was performed on stress (E) and steady-state (F) enriched regions from peak lists extracted from panel D using findMotifsGenome.pl in HOMER. (G) Distribution of identified top motifs was investigated +/- 500 bp from corresponding peak centers. (H) Annotation of the CTCF motif within stress- (orange) and steady-state (blue) BFU-E enriched peaks respectively.

Supplementary Figure 3A), albeit from low levels, with virtually no contribution to reticulocytes from sMPP during the first week (*Online Supplementary Figure 3B*). sBFU-E also dominated the overall contribution to erythroid cells during the first two weeks of recovery (Figure 2G, $P \leq 0.0001$ and $P \leq 0.01$, respectively.). All cell populations gave rise to CD41$^+$ cells short after transplantation (Figure 2H), whereas only sMPP produced platelets, which displayed slower kinetics compared to the formation of erythroid cells (Figure 2I; $P \leq 0.0001$). In agreement with the colony formation ability *in vitro*, myeloid (Figure 2J) as well as lymphoid lineages (*Online Supplemental Figure S3C-D*) were mainly derived from sMPP.

The KuO$^+$ blood lineage distribution and repopulation kinetics in spleen after transplantation was largely reflected by that observed in PB (*Online Supplemental Figure S4*), while the BM had a considerably slower repopulation pattern (*Online Supplemental Figure S5*).

Morphological analysis of spleens at 2 weeks post transplantation revealed a strikingly different distribution of repopulating cells (Figure 2K). Progeny from sBFU-E was evenly distributed throughout the spleen with occasional KuO$^-$ clones possibly arising from the support BM. In contrast, sMPP gave rise to a limited number of large clones, consistent with spleen colonies formed by more primitive stem/progenitor cells (CFU-S12).[23] Taken together, *in vivo* tracing demonstrated clear separation of functionally distinct populations within cKit$^+$CD71^{low}/CD24a^{low} cells using the additional markers CD150, CD9 and Sca1, where sBFU-E mediated recovery from irradiation-induced acute anemia by providing a transient wave of erythroid cells in the PB and spleen, followed by multi-lineage reconstitution in the PB, spleen and BM from sMPP.

Table 2. Top 20 down-regulated genes in stress-BFU-E compared to steady-state.

Gene symbol	log2 fold change	-log10 FDR
F13a1	-2,754	29,923
Snord15a	-2,519	23,305
Tifab	-2,413	16,746
Csf1r	-2,358	16,322
Cybb	-2,344	13,756
Unc93b1	-2,321	25,436
Lgals1	-2,265	14,959
Cfl0	-2,250	14,576
Snord15b	-2,197	43,032
Rasa4	-2,127	13,580
Cd209a	-2,124	10,439
Klf4	-2,115	10,450
Lrp1	-2,024	9,143
Pik3r5	-1,939	8,927
Itsn1	-1,907	21,849
Ccr2	-1,860	7,775
Ly6c2l	-1,859	7,727
Trim47	-1,847	8,101
Nhsl2	-1,796	7,029
Irf8	-1,783	7,515

The genes most down-regulated in sBFU-E compared to steady-state included the coagulation factor *F13a1/FxIIIa* expressed by monocytes and megakaryocytes, *Tifab*; a del(5q) MDS gene known to regulate hematopoiesis and mediate immune signaling through the Toll-like receptor–TRAF6 pathways,[41] the Kit paralog *Csf1r*; essential for the survival of monocytes and macrophages,[42] and *Cybb/gp91-phox*; a heme-binding membrane glycoprotein that is a phagocyte respiratory burst oxidase component induced by inflammation.[43]

Stress-BFU-E and stress-CFU-E display opposing expression patterns of BMP- and CBFA2T3-responsive genes

To identify gene expression patterns associated with the distinct features of the radio-protective sBFU-E, RNA-sequencing was performed on FACS sorted stress-progenitor populations from day 8 spleens. Unsupervised clustering of genes with significant differential expression between any of the investigated populations revealed that relatively few genes were uniquely expressed by sBFU-E, about half of the genes differing between progenitor populations were shared between sMPP and sBFU-E (Figure 3A, gene lists for each cluster in the *Online Supplemental Table S1*), while the sCFU-E were clearly distinct from the other two populations. Gene set enrichment analysis (GSEA)[24] revealed that genes that were uniquely up-regulated in sMPP were associated with adult tissue stem cells and long-term hematopoietic stem cells (Figure 3B), while genes up-regulated in sBFU-E were associated with MAPK signaling, response to stress and stimulus, and more mature hematopoietic progenitors (Figure 3B). Interestingly, both sMPP and sBFU-E expressed BMP- as well as CBFA2T3 target genes known to be up-regulated in response to Bmp or Cbfa2t3 inactivation respectively (Figure 3B). sCFU-E on the other hand, uniquely expressed genes correlating with late progenitors, heme metabolism, and BMP- and CBFA2T3-responsive genes reported to be down-regulated in response to Bmp or Cbfa2t3 inactivation (Figure 3B).

The differential expression of BMP-responsive genes in sCFU-E and more primitive stress-progenitors is well in line with previous findings showing that effective generation of erythroid stress-progenitors is BMP-dependent. To explore the functional importance of BMP-signaling for the generation of stress-progenitor populations, BM cells deficient in BMP receptor II from conditional knock out mice (BmprIIfl/fl Vav-Cre) or wild-type littermate controls (BmprIIfl/fl Cre-negative) were competitively transplanted at a 1:1 ratio with KuO$^+$ wild-type BM, and analyzed for stress recovery contribution in the spleen on day 8 (Figure 3C). Mice transplanted with BMPRII deficient BM had smaller spleens and a 36% reduction in cells/spleen (Figure 3D), despite the fact that 50% of the transplanted BM cells were wild-type. This difference was no longer apparent after lymphoprep-enrichment of mononuclear cells, indicating that spleens of mice transplanted with 100% wild-type cells contained more erythroid cells (Figure 3D). Accordingly, BMPRII-deficient BM displayed a substantially decreased potential to form stress-progenitors from the stage of lineage negative cKit$^+$ progenitors (59.6±5,0% of wild-type), with the most prominent reduction observed in the number of sBFU-E (46.4±6.7% of wild-type) (Figure 3E, see Figure 1C for gating). Hence, in agreement with previous studies, our prospectively isolated stress-progenitor populations are dependent on BMP signaling for a full stress-response.

CD150 and CD9 mark BFU-E potential during steady-state erythropoiesis, providing improved identification of myelo-erythroid progenitors in the BM

Mapping transcriptional profiles of the splenic stress-progenitor populations (see Figure 3) to transcriptional pro-

files of steady-state progenitors from BM as recently defined by single-cell transcriptomics and fate assays,[15] demonstrated that splenic stress-progenitors map closely with their steady-state BM counterparts (*Online Supplementary Figure S6*). The most widely used protocol for delineating myelo-erythroid progenitors in steady-state BM nicely demonstrates that BFU-E potential resides in the $Lin^{-}cKit^{+}CD150^{+}CD105^{+}$ "Pre-CFU-E" fraction,[9] also confirmed in our hands (Figure 4A-B). However, since the "Pre-CFU-E" population contains relatively few BFU-E progenitors (Figure 4B and Pronk *et al.*),[9] a specific cell population possessing the BFU-E potential remains poorly defined. We therefore asked if CD9 could be used to also enrich for steady-state BFU-E. Progenitor populations were FACS-sorted from steady-state BM using the same marker-combinations as in stressed spleen ($CD150^{+}CD9^{+}Sca1^{-}$), and plated for erythroid colony formation. The FACS profile of steady-state BM was very similar to that of stressed spleen (Figure 1C and Figure 4C), and both $Sca1^{-}$ and $Sca1^{+}$ $CD150^{+}CD9^{+}$ BM cells efficiently gave rise to BFU-E colonies (BFU-E/CFU-E colony formed from $CD150^{+}CD9^{+}Sca1^{-}$: 30.0, frequency: 7.5±0.9%, Figure 4C-D), representing a 45-fold improved BFU-E/CFU-E ratio compared to previously used marker combination. In contrast, $CD150^{+}CD9^{+}$ cells were hardly present in spleens from steady-state mice, and of these only a few comprised BFU-E-forming potential (BFU-E/CFU-E colony formed from $CD150^{+}CD9^{+}Sca1^{-}$: 10.8, frequency: 1.0±0.4%; *Online Supplementary Figure S7*). Furthermore, steady-state BM progenitors gave rise to highly proliferative BFU-E colonies, whereas steady-state spleen progenitors resulted in much smaller BFU-E colonies (*data not shown*). In conclusion, CD150 and CD9 expression mark BFU-E potential, both during steady-state in the BM and in the spleen during acute anemia.

Compared to steady-state BFU-E, stress-BFU-E have enhanced expression of genes associated with BMP signaling, erythropoiesis and proliferation

To investigate prospective mechanisms giving stress-BFU-E their unique capacity to rapidly produce large numbers of erythroid cells in response to anemia, BFU-E ($CD150^{+}CD9^{+}Sca1^{-}$) were sorted from steady-state BM and day 8 stressed spleens and analysed for transcriptional differences using RNA-seq. Analysis demonstrated a large overlap between stress- and steady-state BFU-E, with only 277 genes being differentially expressed (Figure 5A, full list of differentially expressed genes in the *Online Supplemental Table S2*). Gene set enrichment analysis demonstrated that sBFU-E expressed higher levels of genes associated with BMP and glucocorticoid signaling, proliferation, maturation block, and erythropoiesis (Figure 5B and *Online Supplementary Figure S8*) with several erythropoiesis-related genes among the most highly up-regulated genes in stress- compared to steady-state BFU-E (Table 1). Steady-state BFU-E on the other hand retained gene signatures associated with myeloid cell development and immune response (Figure 5C), which was also reflected by the genes most down-regulated in stress- compared to steady-state BFU-E (Table 2).

Strong prevalence for the binding motif of chromatin-looping transcription factor CTCF under ATAC-seq peaks enriched in stress-BFU-E

To define potential differences in active regulatory DNA elements across the genome, we performed the assay for transposase-accessible chromatin sequencing (ATAC-seq) analysis[18] on the same stress- and steady-state BFU-E populations as used for transcriptional profiling. ATAC-seq peaks (open chromatin regions) were detected using ENCODE ATAC-seq pipeline (*https://github.com/kundaje-lab/atac_dnase_pipelines*). K-means clustering demonstrated a relatively large overlap in chromatin availability between the two populations, with some of the peaks being enriched in stress- (orange) or steady-state (blue) BFU-E (Figure 5D and *Online Supplementary Figure S9*). The absolute majority of peaks with differential accessibility were located at distal elements (>1,000 bp away from the transcription start site) with only 5% situated around the promoter regions in both stress- and steady-state BFU-E, indicating that transcriptional differences between stress- and steady-state BFU-E are likely to be regulated by distal chromatin interactions.

To identify DNA-binding factors with differential chromatin availability in stress-erythropoiesis we performed motif analysis using peak files extracted from the heatmap clusters. This detected the transcriptional regulator CTCF (CCCTC-binding factor, CTCFL; testis-specific paralog) as the most significantly enriched DNA-binding factor in stress-BFU-E peaks followed by ERG (Figure 5E), whereas steady-state BFU-E peaks most significantly enriched for SFBPI1 and RUNX2 (Figure 5F). Notably, only for CTCF was the motif distributed closely around the peak center (Figure 5G). CTCF is known to regulate the formation of chromatin loops by binding together strands of DNA, and also constitutes a primary part of insulators by blocking the interaction between enhancers and promoters (reviewed by Phillips *et al.*).[29] Interestingly, CTCF has been shown to mark active promoters and boundaries of repressive chromatin domains in primary human erythroid cells,[30] and LDB1-CTCF enhancer looping has recently been shown to underlie activation of a substantial fraction of erythroid genes.[31] Furthermore, CTCF has been shown to regulate growth and erythroid differentiation of human myeloid leukemia cells (K562 cell line).[32] Although both steady-state and stress-BFU-E enriched for the CTCF motif, the frequency was considerably higher in sBFU-E, where 24,3% of the enriched peaks marked a CTCF motif, compared to 8,5% in steady-state BFU-E (Figure 5H). In conclusion, while the transcriptional and chromatin landscape of stress- and steady-state BFU-E display a high degree of similarity, distinctions in gene expression patterns and in the epigenetic landscape might underlie the unique capacity of sBFU-E to rapidly make large numbers of erythroid cells in response to anemia.

Discussion

The identity of stress-erythroid progenitors has remained largely elusive, and their precise identification is important to understand the mechanisms governing recovery from anemia. Previous studies have identified stress-progenitors to be $cKit^{+}CD71^{low}/Ter119^{low}$,[14] and after culture the BFU-E potential is found in the $CD34^{-}CD133^{-}$ fraction of the $Sca1^{+}cKit^{+}CD71^{low}/Ter119^{low}$ population. However, only a small fraction of these cells (0.12-0.2%) gave rise to BFU-E colonies. Here we demonstrate that the differential expression of the surface markers CD150, CD9 and Sca1 provide fractionation and definition of a

hierarchy of functionally diverse splenic stress-progenitors during irradiation-induced recovery, providing 100-fold improved enrichment of stress-BFU-E compared to the current state-of-the-art.

Analysis of megakaryocytic-erythroid differentiation kinetics has been hampered by the lack of markers expressed by mature erythrocytes and platelets. By transplanting sorted stress-progenitor populations from transgenic mice expressing Kusabira Orange in all cells including erythrocytes and platelets,[20] we determined their kinetics and full differentiation potential *in vivo*. Although sBFU-E gave rise to megakaryocytic colonies *in vitro* and $CD41^+$ cells *in vivo*, only sMPP produced platelets *in vivo*, demonstrating the importance of *in vivo* experiments and the ability to trace all mature cell types for defining progenitor potential. In agreement with previous studies,[33] this shows that CD41 is not specific for the megakaryocytic lineage.

We further demonstrate that splenic sBFU-E provide a massive but transient erythroid wave, followed by multilineage reconstitution from sMPP. Harandi *et al.*[14] previously suggested that $cKit^+CD71^-Ter119^-$ cells were erythroid restricted.[14] However, using the same starting population of cells and *in vivo* reconstitution we now demonstrate that these cells contain both multi-potent progenitors ($CD150^+CD9^+Sca1^+$) and more erythroid restricted BFU-E ($CD150^+CD9^+Sca1^-$) and CFU-E ($CD150^+CD9^-$). The same group also proposes that stress-BFU-E are $Sca1^+$, whereas we show that $Sca1^+$ stress-progenitors are multi-potent with the capacity to give rise to more restricted Sca- sBFU-E. Sca1 is known to mark multi-potent HSC, and that Sca1 as a single surface marker can differentiate between multilineage and BFU-E potential is well in line with cellular hierarchy mapping in steady-state hematopoiesis.[34] Interestingly, CD9 has recently been reported to be expressed in murine HSC with MegE differentiation bias.[34] While Pronk *et al.*[9] have identified that CD150 marks "PreCFU-E" colony-forming potential in the BM during steady-state, we for the first time describe a FACS method for identifying progenitors with BFU-E potential, which resides in the $cKit^+CD71^{low}/CD24a^{low}CD150^+CD9^+$ population. This demonstrates that these markers can be used to enrich for both steady-state BFU-E and stress-BFU-E.

BMP-regulation of stress-erythropoiesis has previously been described by Paulson and colleagues, whereas we have shown that steady-state erythropoiesis remains unaffected by disruption of canonical BMP-signaling. Investigation of the functional importance of BMP-signaling in our system revealed that transplantation of BMP-deficient BM resulted in an impaired stress-response with substantially smaller spleens and decreased potential to form stress-progenitors, despite 50% of the transplanted BM cells being wild-type. In addition, genes upregulated in sBFU-E compared to their steady-state counterpart were associated with gene sets activated downstream of the BMP signaling pathway.[33] Within the stress-erythroid progenitor populations, sCFU-E expressed the same set of BMP-responsive genes to a higher degree than sBFU-E and sMPP. Taken together, these results model stress-erythroid progenitors in general and sCFU-E in particular as BMP-responsive cells. sCFU-E also displayed the highest expression of a set of genes that is down-regulated in response to inactivation of Cbfa2t3,[26] a transcriptional co-repressor that promotes degradation of hypoxia regulating protein Hif1a,[35] regulates Gata1-target genes critical for erythroid differentiation,[36] and maintains an erythroid-specific genetic program in progenitors primed for rapid activation when terminal erythroid differentiation is induced.[37] Stress-erythropoiesis is severely impaired in mice lacking Cbfa2t3.[26] Collectively, this makes Cbfa2t3 an interesting target to study further in stress-erythropoiesis regulation.

Analysis of active regulatory DNA elements across the genome using ATAC-seq revealed that sBFU-E displayed a stronger prevalence for the binding motif of chromatin-looping transcription factor CTCF compared to steady-state BFU-E which are less efficient in producing erythrocytes. CTCF has previously been implicated in marking active promoters in primary human erythroid cells,[30] and LDB1-CTCF enhancer looping underlies activation of a substantial fraction of erythroid genes.[31] In accordance, ectopic expression of CTCF in K562 cells promotes erythroid differentiation whereas CTCF knock-down significantly inhibits differentiation into the erythroid lineage.[32] Taken together, chromatin accessibility of CTCF is the most striking epigenetic difference between stress- and steady-state BFU-E, suggesting a role for CTCF-dependent mechanisms in stress-BFU-Es.

In conclusion, combining novel surface markers with the KuO tracing mouse, we have for the first time defined a cellular hierarchy of stress-progenitors, separating sMPP from sBFU-E and more mature sCFU-E based on their kinetics and differentiation potential *in vivo*. We further demonstrate that sBFU-E express gene signatures more associated with erythropoiesis and proliferation compared to steady-state BFU-E, and have enhanced and differential accessibility to CTCF binding sites. Our findings open up the field for further mechanistic and functional studies of how stress-erythropoiesis is regulated, and how sBFU-E contribute during recovery of erythroid disorders. Since the mechanisms regulating stress-erythropoiesis may be targeted for treatment of anemia, this is an important step towards identifying and studying novel erythropoiesis stimulating agents.

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