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Yap1 promotes proliferation of transiently amplifying stress erythroid progenitors during erythroid regeneration

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In contrast to steady-state erythropoiesis, which generates new erythrocytes at a constant rate, stress erythropoiesis rapidly produces a large bolus of new erythrocytes in response to anemic stress. In this study, we illustrate that Yes-associated protein (Yap1) promotes the rapid expansion of a transit-amplifying population of stress erythroid progenitors in vivo and in vitro. Yap1-mutated erythroid progenitors failed to proliferate in the spleen after transplantation into lethally irradiated recipient mice. Additionally, loss of Yap1 impaired the growth of actively proliferating erythroid progenitors in vitro. This role in proliferation is supported by gene expression profiles showing that transiently amplifying stress erythroid progenitors express high levels of genes associated with Yap1 activity and genes induced by Yap1. Furthermore, Yap1 promotes the proliferation of stress erythroid progenitors in part by regulating the expression of key glutamine-metabolizing enzymes. Thus, Yap1 acts as an erythroid regulator that coordinates the metabolic status with the proliferation of erythroid progenitors to promote stress erythropoiesis.

Steady-state erythropoiesis is a hierarchical process, which starts from self-renewing hematopoietic stem cells (HSCs) and involves differentiation of multipotent progenitor cells through lineage-committed erythroid progenitors, which give rise to a series of early and late erythroblasts, ultimately leading to the formation of erythrocytes. Steady-state erythropoiesis produces new erythrocytes at a relatively constant rate, which matches senescent red cell loss, maintaining the erythroid homeostasis. In contrast, anemic stress induces stress erythropoiesis, which rapidly generates a bolus of new erythrocytes much faster than steady-state erythropoiesis and is designed to maintain homeostasis until steady-state erythropoiesis can resume [1]. Most of what we know about stress erythropoiesis comes from the analysis of the murine model. Stress erythropoiesis is driven by signals that are distinct from the signals regulating steady-state erythropoiesis. They include bone morphogenetic protein 4 (Bmp4), Hedgehog (Hh), stem cell factor (Scf), growth and differentiation factor 15 (Gdf15), corticosteroids, and hypoxia [2–7]. Some of these signals are also known to regulate key events during development [8–11]. One of the most intensively studied signals that regulate tissue growth and organ size is the Hippo pathway, which is a kinase cascade that negatively regulates growth by inhibiting the transcriptional regulators Yes-associated protein (Yap1) and Taz [12,13]. On activation of the Hippo pathway, Yap1 becomes phosphorylated on serine residue,

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S127, by Lats kinase, leading to ubiquitination and proteasomal degradation of Yap1 protein in cytoplasm [14,15]. However, the absence of phosphorylation at Yap1 S127 results in translocation of Yap1 to the cell nucleus, where it forms a complex with TEAD family transcription factors to drive transcription of growth-promoting and anti-apoptotic genes [16].

Yap1 activity has been implicated in diverse tissue and organ systems. Previous work revealed that Yap1 activation promotes self-renewal of embryonic stem cells [12]. Yap1 has been reported to play pivotal roles in controlling cell cycle during tumor initiation and oncogenic proliferation, and the *Yap1* locus is often amplified in medulloblastomas [17,18]. The Hippo pathway restrains cardiomyocyte proliferation and regulates heart size in the developing mouse heart, where loss of Hippo control of Yap1 resulted in enlarged hearts and overgrown cardiomyocyte populations [19]. In adult cardiomyocytes, Hippo deficiency increases expression of Yap1 target genes that govern the regenerative proliferation and stress response, implying a regeneration-promoting role for Yap1 [20]. Furthermore, Yap1 is critical for intestinal regeneration following exposure to ionizing radiation as it promotes intestinal stem cell survival and induces a regenerative program [21,22]. As Yap1 mutations are embryonic lethal in mice at E8.5, the function of Yap1 in hematopoiesis is evolving [23]. Enforced expression of Yap1 does not alter hematopoietic stem cell homeostasis, and conditional knockout of both Yap1 and Taz in the adult hematopoietic system leads to mild anemia that manifests 6 months post deletion [24,25]. Little is known about the role of Yap1 in erythropoiesis. However, based on its role in other regenerating tissues, we propose that Yap1 may act in erythroid regeneration in response to anemic stress by promoting proliferation of and inhibiting differentiation of immature progenitors.

Recent work has also revealed an interplay between Yap1/Taz and metabolism, where nutrient availability controls Yap1/Taz activity, while Yap1 and Taz, in turn, regulate cellular metabolism [26]. Compelling evidence links Yap1 activation to enhanced glucose and glutamine metabolism, which is correlated with increased metabolic needs for sustained cell proliferation [27]. When cells actively incorporate glucose and route it through glycolysis, Yap1/Taz transcriptional activity is increased [28]. More recent studies revealed that Yap1/Taz activation modulates the amount of intracellular glutamate through expression of *SLC1A5*, the glutamine transporter, and glutaminase (*Gls1*), which encodes a key enzyme in the glutaminolysis pathway [29,30]. Previously, we reported that glutamine metabolism plays a key role in stress erythropoiesis by supporting the proliferation and differentiation of erythroid progenitors [31].

In this study, we found that Yap1 has a role in promoting the proliferation of transiently amplifying stress erythroid progenitors in vitro using a stress erythropoiesis culture system, and in vivo using erythroid short-term radioprotection after bone marrow transplant. Yap1 promotes proliferation in part through the upregulated expression of key enzymes governing glutamine metabolism.

Methods

The detailed experimental design and methods are described in the Supplementary Methods (online only, available at www.exphem.org).

Mice

C57BL/6 (CD45.2$^+$) mice were used as wild-type (WT) recipients in bone marrow transplants. C57BL/6, B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (CD45.1$^+$), C57BL/6-Tg(UBC-GFP)30Scha/J (GFP$^+$), B6.C-Tg (CMV-cre)1Cgn/J (CMVCre), and B6;129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT)/Nat}$/J (R26CreER) mouse strains were purchased from The Jackson Laboratory (Bar Harbor, Maine). C57BL/6-*Yap1*$^{flox/flox}$ (Yap1$^{F/F}$) mice were a generous gift from Dr. Duojia Pan [32]. Yap1$^{F/-}$ and Yap1$^{F/-}$;R26CreER$^+$ mice were obtained by breeding, as illustrated in Supplementary Figure E1A (online only, available at www.exphem.org). All mice were 8–12 weeks old when experiments were performed. The Institutional Animal Use and Care Committee at the Pennsylvania State University approved all procedures.

Microarray analysis and gene set enrichment analysis

Microarray data can be accessed via NCBI's Gene Expression Omnibus accession no. GSE122390. Gene expression profile was visualized by Transcriptome Analysis Console software (Affymetrix). The normalized data set was processed with gene set enrichment analysis (GSEA) to determine statistically differentially expressed gene sets in the two groups of SEPs. Gene sets derived from published gene signatures were described in our previous work [31]. GSEA was performed to investigate whether the proliferative signature of PKH26^{lo}CD133^{neg}Kit$^+$Sca1$^+$ (PKH26^{low}) cells was associated with elevated expression of YAP1/TAZ [33] and YAP1 [34], induced by YAP1 [35], and repressed by YAP1 [35].

Statistical analysis

Significance tests were performed with Student t test on two groups, or one-way analysis of variance (ANOVA) followed by Tukey comparison on three groups. A p value ≤ 0.05 was considered to indicate significance. Survival analysis was conducted with the Gehan–Breslow–Wilcoxon test. False discovery rate (FDR) p values are reported for GSEA.

Results

Yap1 expression is induced during stress erythropoiesis

To address the role of Yap1 in stress erythropoiesis, we first examined the expression of Yap1 in the spleen following bone marrow transplant (BMT). When WT donor cells were transplanted into lethally irradiated WT recipient mice, we observed elevated mRNA expression of Yap1 with a peak expression on days 2 and 4 during the erythroid recovery from BMT

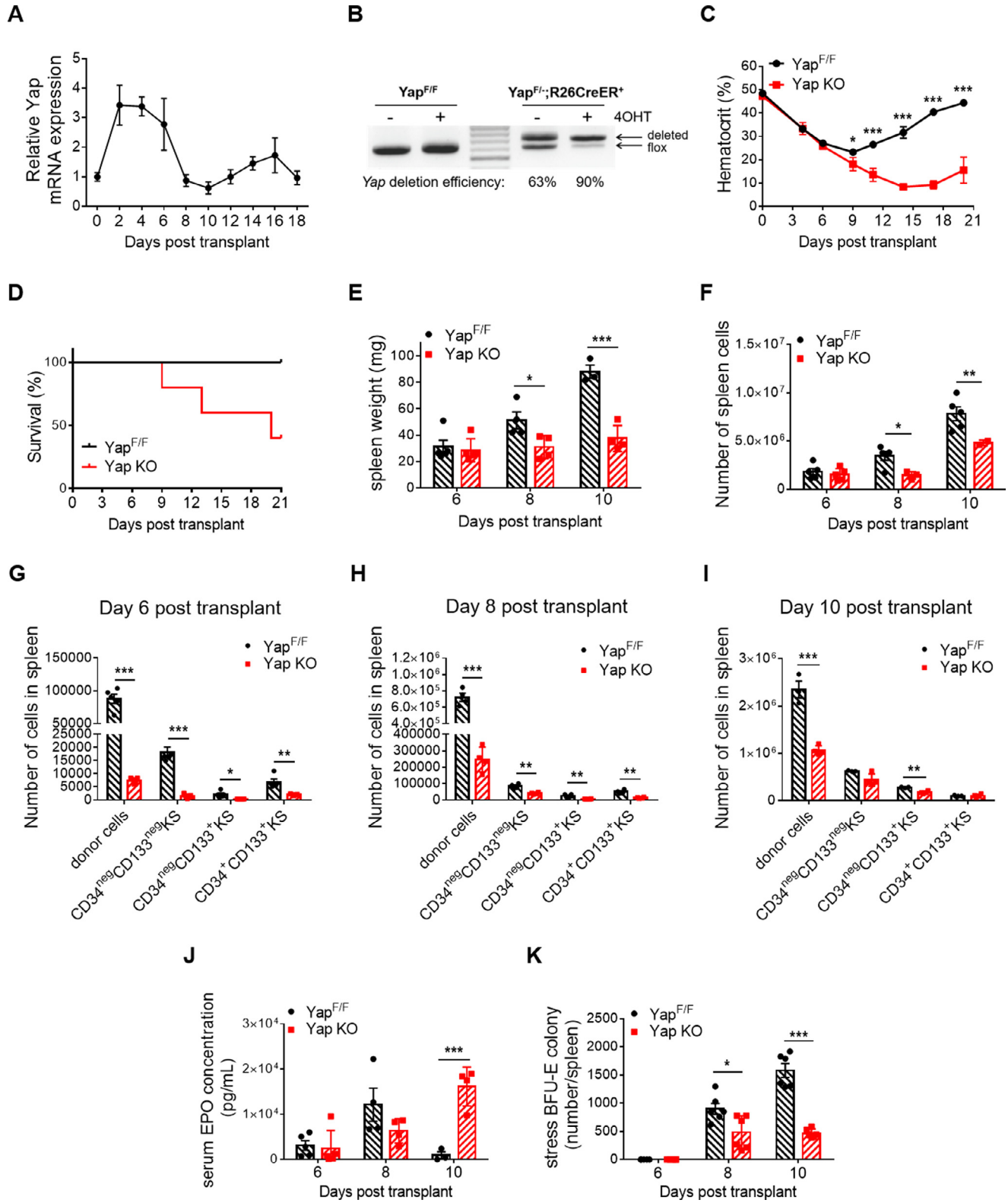


Figure 1. Yap1-depleted stress erythroid progenitors fail to provide short-term radioprotection following bone marrow transplant. **(A)** Analysis of Yap1 mRNA expression in the spleen of control (wild-type donor cells to wild-type recipients) bone marrow transplant recipients. Each time point reflects analysis of four mice. Data are expressed as the mean ± SEM. **(B)** Analysis of Yap1 deletion efficiency on 4OHT-treated bone marrow cells, which served as donor cells for bone marrow transplant. **(C–F)** Five hundred thousand Yap1$^{F/F}$ or Yap1 KO donor cells were transplanted to lethally irradiated wild-type recipients. Recovery was monitored and analyzed for 3 weeks following transplant. Spleens were isolated and analyzed at the indicated time points.

(Figure 1A). This peak expression of Yap1 corresponds to the enhanced expansion of early stress erythroid progenitors during the first phase (days 0–8) of erythroid recovery following irradiation and transplantation [1,36]. During this phase, many proliferation signals are activated, including growth differentiation factor 15 (Gdf15), bone morphogenetic protein 4 (Bmp4), and hedgehog (Hh), which are crucial for expansion of stress erythroid progenitor (SEP) populations and consequent survival of lethally irradiated mice [37].

Yap1 is required for the recovery from irradiation followed by bone marrow transplant

To assess the effects of Yap1 loss of function on stress erythropoiesis, we generated $Yap1^{F/}$;$R26CreER^{+}$ mice (Supplementary Figure E1A). We observed that mice carrying the one-allele deletion of *Yap1* are viable and fertile with no defects in steady-state erythropoiesis (Supplementary Figure E1B). Furthermore, these mice exhibited no defects in stress erythropoiesis as assayed in vivo or in vitro (Supplementary Figure E1C–F). By treating $Yap1^{F/-}$;$R26CreER^{+}$ bone marrow cells with 10 μmol/L 4-hydroxytamoxifen (4OHT) for 48 hours, the deletion efficiency of *Yap1* gene was >90% (Figure 1B). 4OHT-treated $Yap1^{F/-}$;$R26CreER^{+}$ cells are referred as Yap1 KO cells.

We next sought to assess the role of Yap1 in stress erythropoiesis using the BMT model. When the same number of $Yap1^{F/F}$ or Yap1 KO progenitor cells were transplanted into lethally irradiated WT recipient mice, Yap1 KO donor SEPs were unable to provide short-term radioprotection after BMT. Four of six recipient mice died within 21 days after receiving Yap1 KO donor cells, while surviving animals exhibited significantly slower recovery compared with the mice that received $Yap1^{F/F}$ cells (Figure 1C, D). Yap1 KO donor SEPs failed to expand their populations in the recipient splenic niche, resulting in non-enlarged spleens and fewer numbers of cells when compared with the control group (Figure 1E, F).

To isolate $Yap1^{F/F}$ and Yap1 KO donor cells in the spleens of recipient animals to further characterize different SEP populations, we utilized B6.SJL congenic $CD45.1^{+}$ recipient mice, which differ from the $CD45.2^{+}$ donor cells. We collected spleen cells at key time points post-BMT, days 6, 8, and 10, when erythroid recovery was at the transition from initial stage (days 0–8) to active recovery stage (days 8–16). The initial stage involves an expansion of the predominant SEP population that contains the most immature SEPs ($CD34^{+}CD133^{+}Kit^{+}Sca1^{+}$) [1,36,37]. The immature SEPs are replaced by more mature $CD34^{neg}CD133^{neg}Kit^{+}Sca1^{+}$ progenitors and stress burst-forming unit erythroid (BFU-E) progenitors during the active recovery stage, resulting in increased red blood cell numbers in peripheral blood [1,36,37]. At these key time points post-BMT, Yap1 KO donor SEPs failed to expand crucial SEP populations, resulting in significantly fewer $CD34^{neg}CD133^{neg}Kit^{+}Sca1^{+}$, $CD34^{neg}CD133^{+}Kit^{+}Sca1^{+}$, and $CD34^{+}CD133^{+}Kit^{+}Sca1^{+}$ cells (Figure 1G–I), suggesting that Yap1 regulates the proliferation of stress erythroid progenitors during recovery from BMT.

Erythropoietin (Epo), which is expressed by the kidney, is the signal that promotes the transition of progenitors from expansion to differentiation into lineage-committed progenitors that are capable of forming stress BFU-Es [1,36]. Serum Epo concentration of the control group peaked at posttransplant day 8, while mutation of Yap1 in donor SEPs significantly delayed Epo induction (Figure 1J). This observation is consistent with previous data indicating the converse: that in a model of sterile inflammation, increased Epo expression correlates with the expansion of SEP populations in the spleen, not with the anemia [38]. However, the mechanisms that define the relationship between SEP proliferation in the spleen and Epo expression in the kidney remain unknown. Taken together, these observations indicate that the ineffective expansion of SEP populations and delayed Epo production led to insufficient production of stress BFU-Es (Figure 1K), which resulted in delayed recovery and death of recipient mice transplanted with Yap1 KO donor cells.

Yap1 regulates the proliferation of transiently amplifying SEPs

We next assessed Yap1 function in our in vitro culture system. In addition to the induction of Yap1 expression during the recovery from BMT, Yap1 expression was elevated during in vitro culture of WT unfractionated bone marrow cells (Figure 2A). In the expansion phase, Yap1 expression peaked on day 2 before the proliferation of SEPs, which is characterized by exponential growth of SEPs on days 3 to 7. Differentiation of SEPs was induced in these cultures by adding Epo and switching the cultures to 2% O_2. We observed a second

(C) Hematocrit levels during the entire recovery period. Each time point reflects analysis of more than three mice. Data are expressed as the mean ± SEM. **(D)** Survival analysis of mice receiving $Yap1^{F/F}$ or Yap1 KO donor cells with the Gehan–Breslow–Wilcoxon test ($p = 0.0494$). **(E)** Spleen weights at key time points during recovery. **(F)** Analysis of cells in spleen on the indicated days after transplant. **(G–K)** Donor cells were $CD45.2^{+}$, and recipient mice were $CD45.1^{+}$. Blood and spleens were collected at posttransplant days 6, 8, and 10 for analysis. Analysis of donor cell proliferation, total $CD45.2^{+}$ donor cells, $CD34^{neg}CD133^{neg}Kit^{+}Sca1^{+}$ ($CD34^{neg}CD133^{neg}KS$) progenitors, $CD34^{neg}CD133^{+}Kit^{+}Sca1^{+}$ ($CD34^{neg}CD133^{+}KS$) progenitors, and $CD34^{+}CD133^{+}Kit^{+}Sca1^{+}$ ($CD34^{+}CD133^{+}KS$) progenitors in each spleen at day 6 **(G)**, day 8 **(H)**, and day 10 **(I)** after transplant. **(J)** Analysis of serum Epo concentration. **(K)** Number of stress BFU-Es in the spleen on the indicated days following transplant. Data are expressed as individual subject and the mean ± SEM. $*p < 0.05$. $**p < 0.01$. $***p < 0.001$.

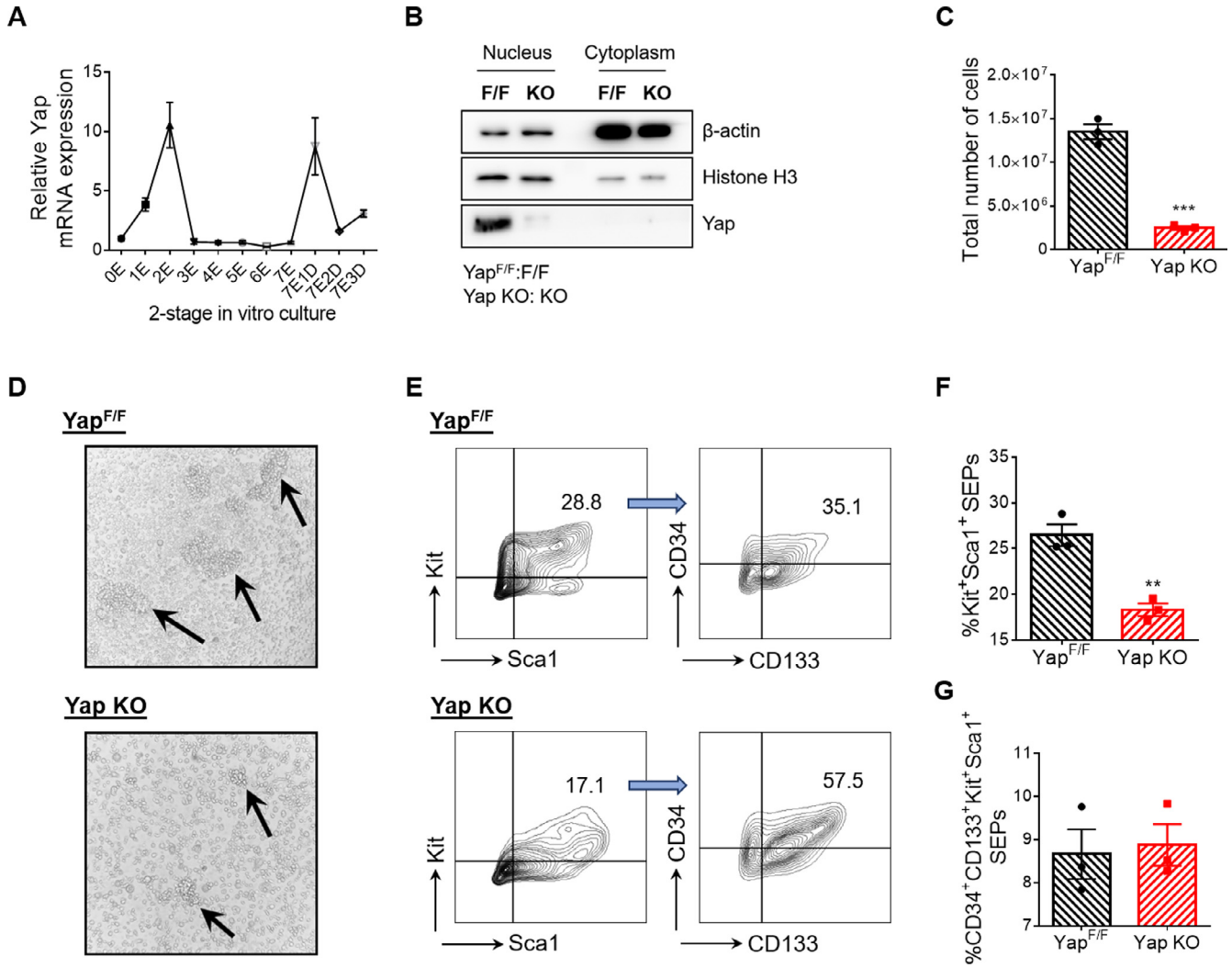


Figure 2. Mutation of Yap1 impairs expansion of stress erythroid progenitors in vitro. **(A)** Analysis of Yap1 mRNA expression in SEPs harvested from in vitro two-stage control culture, expansion culture **(E)** for 7 days followed by differentiation culture **(D)** for 3 days. Each time point reflects analysis of three mice. Data are expressed as the mean ± SEM. **(B–G)** Unfractionated bone marrow cells were isolated for expansion culture in SEEM medium. 4OHT was supplemented for the first 48 hours of culture. Cells were harvested after 5 total days of culture. **(B)** Analysis of Yap1 protein expression in cultured progenitors. Western blots were probed with anti-Yap1, anti-β-actin, and anti-histone H3 antibodies. **(C)** Quantification of total number of cells at the end of expansion culture. **(D)** Representative images of proliferating progenitors. Pictures were taken with 200 × magnification using a Nikon Eclipse TE300 microscope. **(E–G)** SEPs were collected and stained with fluorescent antibodies. **(E)** Representative flow cytometry analysis. Detailed gating strategy is illustrated in Supplementary Figure E1D (online only, available at www.exphem.org. **(F)** Percentage of Kit^+Sca1^+ progenitors. **(G)** Percentage of $CD34^+CD133^+Kit^+Sca1^+$ progenitors. Data are expressed as individual subject and mean ± SEM. $^{**}p < 0.01$. $^{***}p < 0.001$.

peak of Yap1 expression in the differentiation phase, which is consistent with the nature of SEP differentiation, where proliferation occurs along with the differentiation progression. To address the role of Yap1 during the comprehensive process of stress erythropoiesis, we first performed in vitro expansion culture comparing the growth of Yap1 KO bone marrow cells with of the $Yap1^{F/F}$ control. *Yap1* deletion was induced by supplementing $Yap1^{F/-}$;$R26CreER^+$ bone marrow cells with 4OHT for 48 hours during the expansion culture in stress erythropoiesis expansion media (SEEM) medium. The deletion efficiency was verified by Western blot on Yap1 protein in both cytoplasm and nucleus. 4OHT-treated $Yap1^{F/-}$;$R26CreER^+$ (Yap1 KO) cells exhibited a loss of Yap1 protein in the nucleus and cytoplasm (Figure 2B).

When *Yap1* was deleted during the expansion culture, the average total cell numbers decreased dramatically, compared with $Yap1^{F/F}$ control (Figure 2C). Proliferation of SEPs was largely impaired in Yap1 KO culture as reflected by lower percentages of Kit^+Sca1^+ cells and smaller clusters formed by growing cells

(Figure 2D–G). However, the percentages of immature $CD34^+CD133^+Kit^+Sca1^+$ SEPs remained unchanged when Yap1 KO cultures were compared with $Yap1^{F/F}$ cultures, indicating that actively proliferating cells are more sensitive to Yap1 activity (Figure 2F, G). To determine whether Yap1 KO in stromal cells affected SEP proliferation, we performed 1:1 co-culture of GFP^+ wildtype and Yap1 KO (GFP^{neg}) BM cells. When compared with WT (GFP^+) cells, Yap1 KO (GFP^{neg}) cells proliferated less (Supplementary Figure E2A, online only, available at www.exphem.org). They produced fewer Kit^+Sca1^+ SEPs, and although similar percentages of $CD34^+CD133^+Kit^+Sca1^+$ cells were observed, the total number of $CD34^+CD133^+Kit^+Sca1^+$ cells was significantly reduced (Supplementary Figure E2). Thus, Yap1 KO in the co-culture system led to dramatic reductions of Kit^+Sca1^+ progenitor cells and $CD34^+CD133^+Kit^+Sca1^+$ SEPs, consistent with the results of Yap1 KO cultures.

The decrease in total cell numbers and the decrease in the numbers of specialized SEP populations resulting from Yap1 mutation could be explained by impaired proliferation or increased cell death or apoptosis. To assess the possibility that reduced total cell number is a result of increased cell death and apoptosis, samples were collected from in vitro expansion cultures at days 5, 6, and 7. There were no significant differences in the percentages of living cells, which were marked as $Yellow^{neg}AnnexinV^{neg}$, when comparing Yap1 KO progenitors with $Yap1^{F/-}$ and $Yap1^{F/F}$ SEPs, suggesting that cell death and apoptosis are not major contributing factors to the Yap1-dependent defects in stress erythropoiesis (Supplementary Figure E3, online only, available at www.exphem.org).

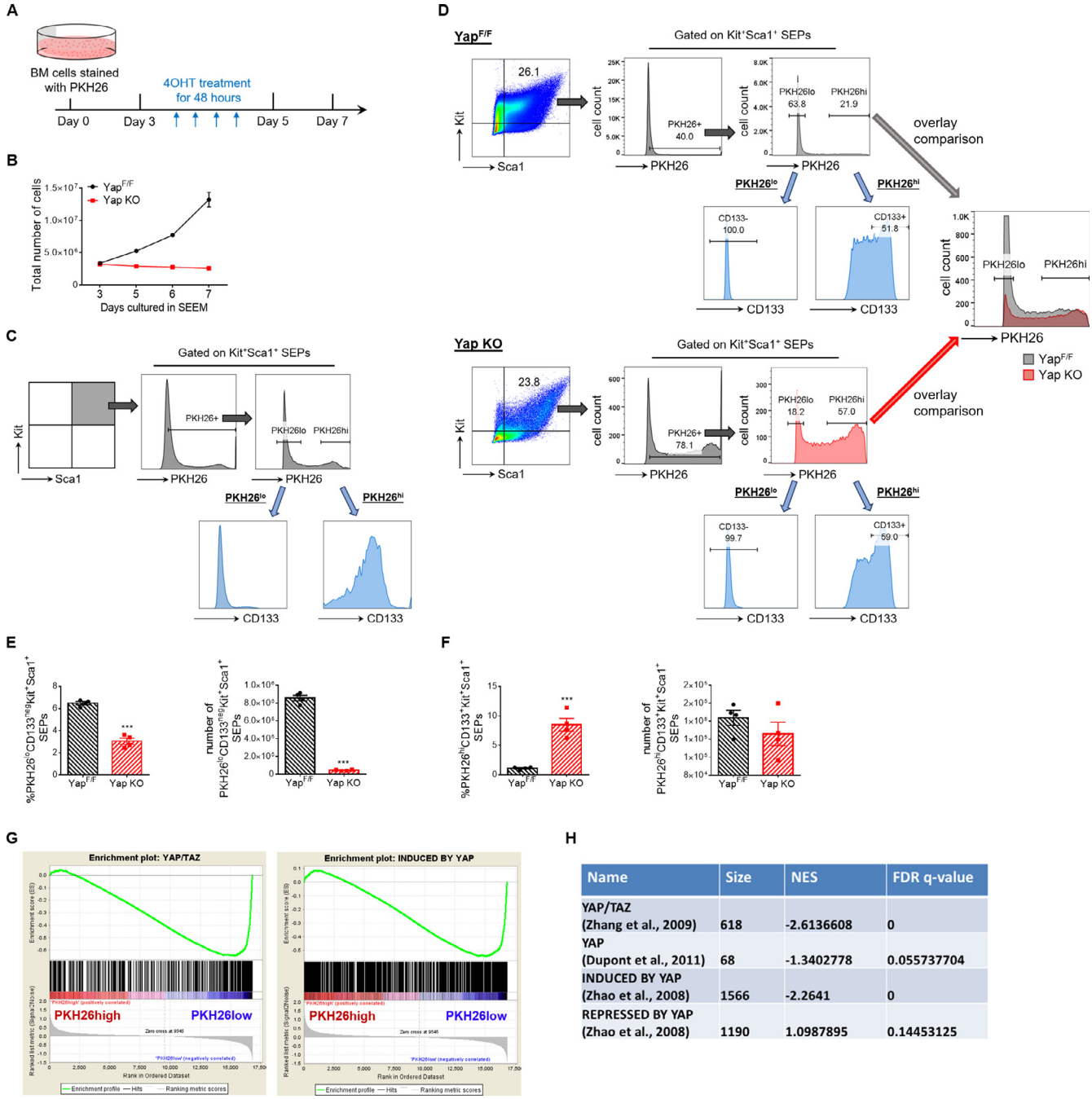
We further analyzed the role of Yap1 in regulating SEP proliferation by labeling cells with the membrane fluorescent dye PKH26 in a SEEM culture of bone marrow cells. We labeled Yap1 KO and $Yap1^{F/F}$ cells with PKH26 and followed the decrease in fluorescence as the cells proliferated (Figure 3A). Along the course of a 7-day culture, Yap1 KO cells failed to expand their populations (Figure 3B). The percentage and total number of transiently amplifying $PKH26^{lo}CD133^{neg}Kit^+Sca1^+$ ($PKH26^{low}$) SEPs were significantly reduced in Yap1 KO cultures (Figure 3C–E). Although the less proliferative $PKH26^{hi}CD133^+Kit^+Sca1^+$ ($PKH26^{high}$) SEPs made up a greater percentage of the SEP population, because of the decreased overall proliferation, the increased percentage corresponded to a similar number of $PKH26^{high}$ cells when compared with the control group (Figure 3D, F). This difference in proliferation was not due to differences in cell cycle progress as cell cycle analysis of $PKH26^{low}$ and $PKH26^{high}$ SEPs revealed no evidence of altered cell cycle profile of Yap1 KO cells when compared with $Yap1^{F/F}$ cells (Supplementary Figure E4, online only, available at www.exphem.org). GSEA of wild-type $PKH26^{low}$ and $PKH26^{high}$ SEPs revealed that the rapidly amplifying SEPs ($PKH26^{low}$) exhibited the YAP1/TAZ signature and were significantly associated with the expression of genes induced by YAP1 [33–35] (Figure 3G, H). Yap1 activity is negatively regulated by the Hippo pathway kinase cascade. We observed decreased expression of Hippo pathway core components in $PKH26^{low}$ SEPs, which is consistent with the role of Yap1 in promoting proliferation of this SEP population [14,39,40] (Supplementary Figure E5, online only, available at www.exphem.org). Taken together, our data indicate that Yap1 regulates the proliferation of transiently amplifying SEPs during stress erythropoiesis.

We next tested whether Yap1 plays a role during SEP differentiation. When deletion of *Yap1* was induced during the differentiation stage of in vitro culture, the total cell number was decreased compared with that of $Yap1^{F/F}$ control, but not as dramatically as in expansion culture (compare Figure 2C with Figure 4A). When we analyzed the effects on specific SEP populations, we observed a decreased percentage of Kit^+Sca1^+ SEPs in general. We also observed a decrease in the Kit^+Sca1^{neg} SEP population, which gives rise to stress BFU-Es and which further resulted in reduced production of stress BFU-Es from Yap1 KO culture (Figure 4B–E). Thus, Yap1 activity is required for the development of SEPs during stress erythropoiesis, as Yap1 promotes the proliferation of transiently amplifying SEPs in the expansion phase and the proliferation of differentiating SEPs in the presence of EPO during the differentiation phase.

Enhanced expression of Yap1 contributes to stress erythropoiesis

Functional studies have implicated Yap1 as a vital factor in stem cell maintenance and proliferation. Overexpression of Yap1 stimulates proliferation of stem and progenitor cells in many tissues [12,41–44]. Although a recent study concluded that enforced expression of Yap1 has no significant effects on murine hematopoiesis [24], we sought to investigate whether increased activity of Yap1 would benefit the growth of stress erythroid lineage cells. We evaluated the effect of Yap1 using an MSCV retroviral vector that expresses GFP to overexpress human YAP1 (hYAP1), $hYAP1^{WT}$, and constitutively activated $hYAP1^{S127A}$, which maintains hYAP1 protein localization to the nucleus and maintains its transcriptional activity.

When BM cells were transduced with hYAP1-expressing retrovirus during in vitro expansion culture and analyzed for progenitor cell surface markers, we observed increased percentages of Kit^+Sca1^+ SEPs in bone marrow cells transduced by $hYAP1^{S127A}$, indicating that active Yap1 promotes the proliferation of SEPs (Figure 5A). $hYAP1^{WT}$,



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Name	Size	NES	FDR q-value
YAP/TAZ (Zhang et al., 2009)	618	-2.6136608	0
YAP (Dupont et al., 2011)	68	-1.3402778	0.055737704
INDUCED BY YAP (Zhao et al., 2008)	1566	-2.2641	0
REPRESSED BY YAP (Zhao et al., 2008)	1190	1.0987895	0.14453125

Figure 3. Yap1 regulates proliferation of transiently amplifying SEPs. **(A)** Illustration of culture strategy. Unfractionated Yap1$^{F/F}$ or Yap1 KO bone marrow cells were labeled with PKH26 dye at the beginning of a 7-day culture in SEEM. The culture medium was supplemented with 4OHT at day 3, and the 4OHT treatment lasted 48 hours. After washing off 4OHT, bone marrow (BM) cells were continued to be cultured in fresh SEEM for another 2 days. **(B)** Cumulative cell counts along the SEEM culture. Data are expressed as the mean ± SEM. **(C)** Schematic of flow cytometry gating strategy. **(D)** Representative flow cytometry analysis. Overlay comparison indicates that expansion of the PKH26^{lo}Kit^{+}Sca1^{+} SEP population is largely impaired in Yap1 KO culture. **(E)** Percentage (*left*) and number (*right*) of PKH26^{lo}CD133^{neg}Kit^{+}Sca1^{+} (PKH26^{low}) cells. **(F)** Percentage (*left*) and number (*right*) of PKH26^{hi}CD133^{+}Kit^{+}Sca1^{+} (PKH26^{high}) cells. Data are expressed as individual subject and mean ± SEM. ***$p < 0.001$. **(G–H)** GSEA of sorted wild-type PKH26low and PKH26high cells on genes that are associated with YAP1 or YAP1/TAZ activity, induced by YAP1, and repressed by YAP1 according to published literature. **(G)** Enrichment plot of genes associated with YAP1/TAZ activity (*left*) and genes induced by YAP1 (*right*). **(H)** Table of GSEA results. Names of gene sets are listed with the size of each set. The normalized enrichment score (NES) was calculated for each gene set by comparing PKH26^{low} SEPs with PKH26^{high} SEPs. A positive score suggests that genes involved in the indicated pathway are highly correlated with/expressed in PKH26^{high} SEPs, while a negative score implies that the gene set is highly correlated with/expressed in PKH26^{low} SEPs. The false discovery rate (FDR) represents the estimated probability that a gene set with a given NES is a false-positive finding.

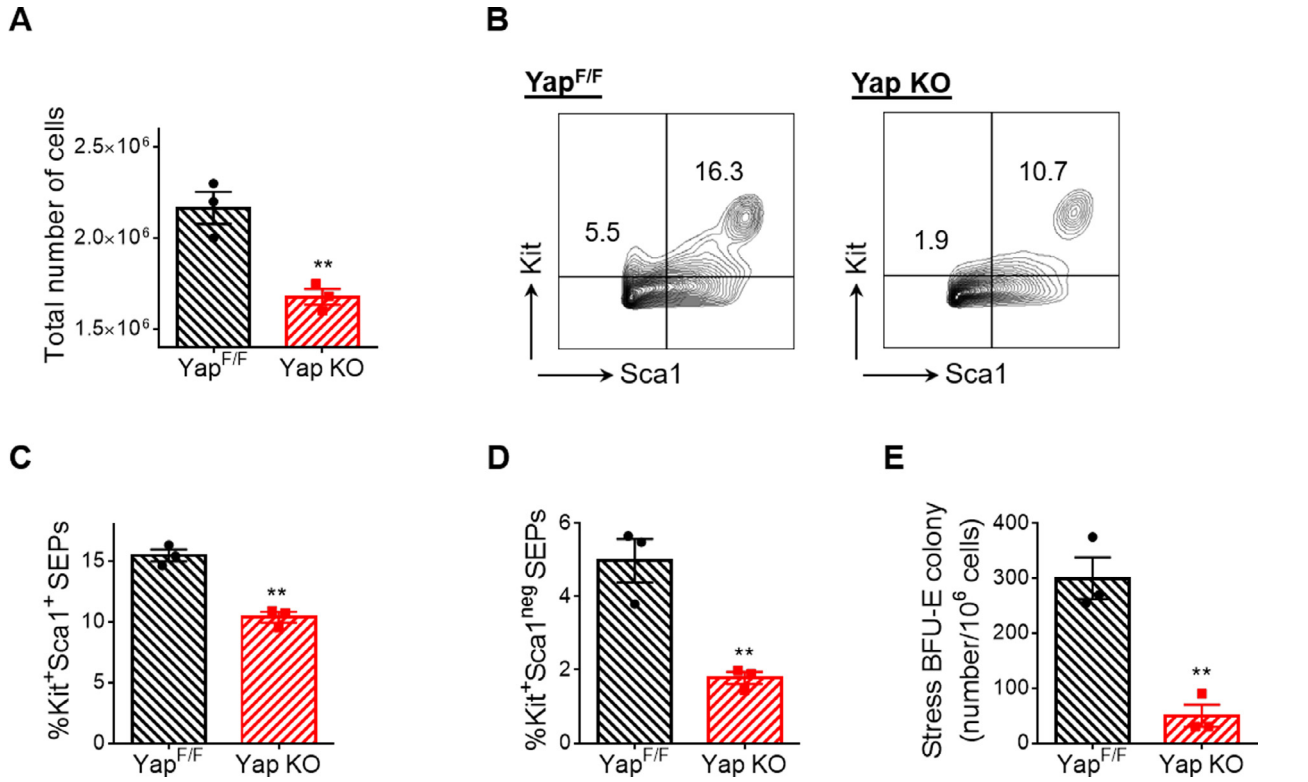

Figure 4. Yap1 mutation affects SEP proliferation during the differentiation phase. Unfractionated bone marrow cells were cultured in expansion medium for 7 days without addition of 4OHT, which was added for 48 hours at the beginning of differentiation culture. SEPs were collected for analysis after the course of two-stage culture. **(A)** Statistical analysis of total cell number after culture. **(B)** Representative flow cytometry analysis. **(C)** Percentage of Kit^+Sca1^+ SEPs. **(D)** Percentage of Kit^+Sca1^{neg} SEPs. **(E)** Number of stress BFU-E colonies under 2% O_2 culture condition. Data are expressed as individual subject and mean ± SEM. $^{**}p < 0.01$.

$hYAP1^{S127A}$, or vector control virus-transduced (GFP^+) SEPs were sorted from the cultures and transplanted to lethally irradiated WT recipients. Because of the limited numbers of cells that were transplanted into each recipient mouse, the recipients took longer (30 days) to fully recover from transplant following lethal irradiation compared with a control whole bone marrow transplant recovery progress, which usually takes 21 days (Figure 1C). Overexpression of $hYAP1^{S127A}$ stimulated an earlier turning point of recovery as demonstrated by higher hematocrit levels on day 18 post transplant. At this time point, mice receiving empty retroviral vector (Ctrl)- or $hYAP1^{WT}$-transduced cells reached their lowest hematocrit levels (Figure 5B). Animals that received cells with enforced expression of $hYAP1^{S127A}$ exhibited a modest, but significant improvement of erythropoietic reconstitution on days 21 and 24 following transplant (Figure 5B).

To investigate further whether Yap1 overexpression could rescue the proliferation of Yap1 KO stress erythroid progenitors in vitro, we transduced Yap1 KO BM cells with $hYAP1^{WT}$, $hYAP1^{S127A}$, or vector control viruses. Although there was a trend toward increased total cell numbers and percentages of Kit^+Sca1^+ SEPs when overexpressing $hYAP1^{WT}$ and $hYAP1^{S127A}$, the differences were not statistically significant, and the growth of cultured Yap1 KO cells was only partially rescued when compared with $Yap1^{F/F}$ cultures (Figure 5C–E).

Yap1 regulates genes involved in self-renewal and glutamine metabolism

Our data indicate that Yap1 regulates the proliferation of a transiently amplifying population of SEPs. Yap1 interacts with a number of transcription factors including the TEAD family members, β-catenin, and BMP-activated Smad1,5 [19,39,44]. The increase in cardiomyocytes in the developing heart is in part regulated by Yap1-dependent expression of Sox2, a stem cell transcription factor that promotes self-renewal via binding with TEAD2 [19,39,45,46]. Sox2 has been reported to facilitate regeneration in zebrafish lateral line neuromasts, spinal cord regeneration in *Xenopus* tadpoles, and murine skin repair [47–49]. Additionally, Yap1 enhances BMP/Smad1-mediated transcription of *Id1*, which functions as a negative regulator of terminal differentiation [44,50]. Sox2 and Id1 represent Yap1 target genes that could play a role in self-renewal and the inhibition of differentiation during the expansion of SEP populations. To assess the expression levels of Sox2 and Id1 on Yap1 mutation, we performed in vitro

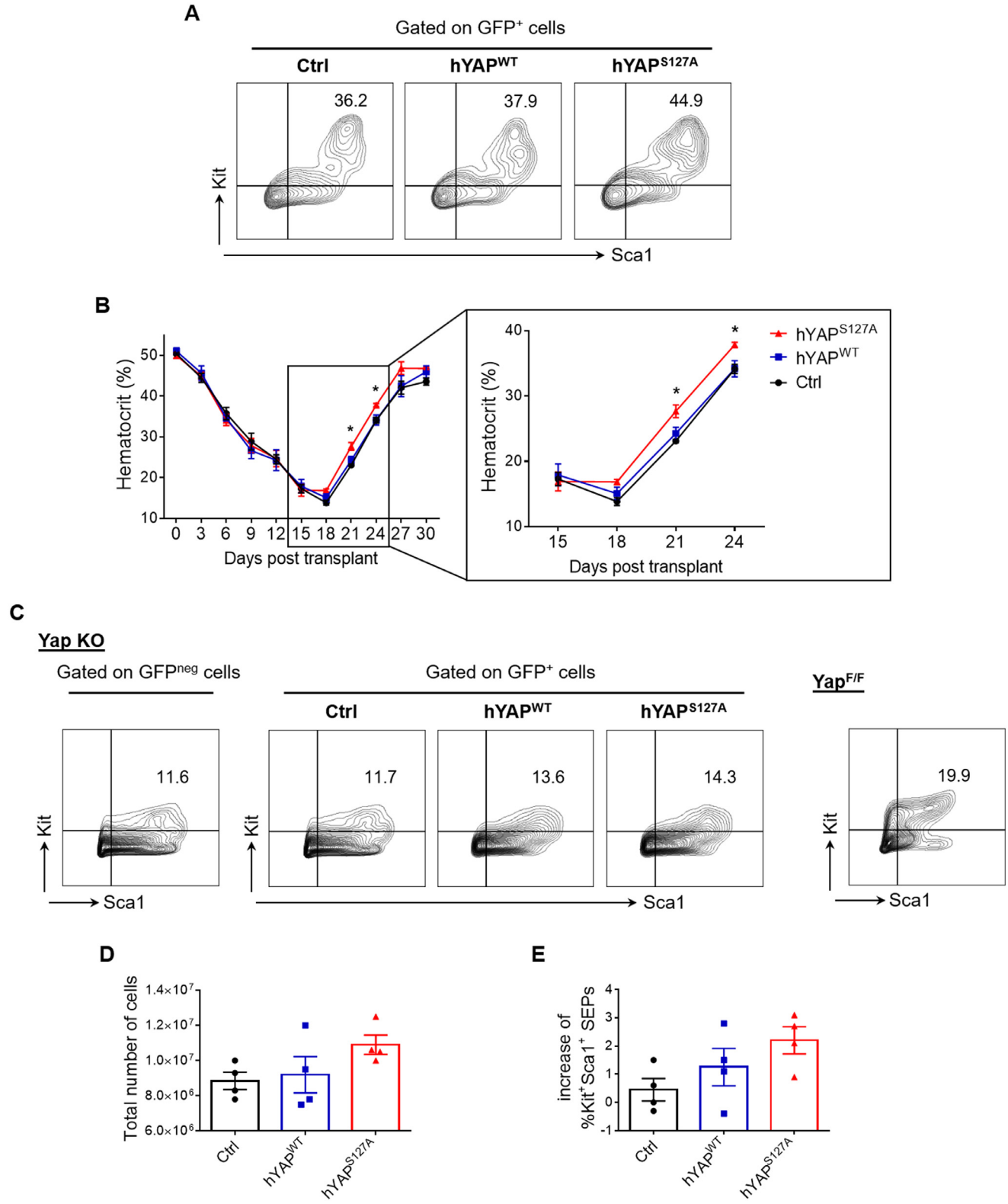

Figure 5. Enhanced expression of Yap1 promotes expansion of Kit^+Sca1^+ SEPs. **(A, B)** Retroviral transduction was performed on wild-type bone marrow cells during in vitro culture with SEEM for 7 days. Cultured cells were stained with fluorescent antibodies targeting SEP cell surface markers. The same numbers of sorted SEPs of each group were transplanted to lethally irradiated wild-type recipient mice. **(A)** Representative flow cytometry analysis of transduced progenitors. **(B)** Analysis of hematocrit levels following bone marrow transplant. Each time point reflects analysis of more than three mice. Data are expressed as the mean ± SEM. A one-way ANOVA followed by Tukey comparison was performed. $p = 0.055$ on day 18. $p = 0.016$ on day 21.

culture of bone marrow cells in expansion medium, SEEM. We observed reduced expression of both Id1 and Sox2 in sorted Yap1 KO Kit^+Sca1^+ SEPs after a course of 7-day expansion culture, suggesting that Yap1 regulates Sox2 and Id1 expression in proliferating SEPs (Figure 6A).

Cellular metabolism is an emerging regulatory node of stem cell proliferation and differentiation. Yap1 activation has been reported to enhance glucose and glutamine metabolism, which is correlated with increased metabolic needs to support cell proliferation [27]. Additionally, glutamine dependency of breast cancer cells is controlled by Yap1/Taz-activated expression of glutamine-utilizing transaminases, glutamic–oxaloacetic transaminase (Got1), and phosphoserine aminotransferase (Psat1) [51]. We first confirmed the importance of glutamine-metabolizing genes in promoting sustainable proliferation of $PKH26^{low}$ cells via microarray analysis and GSEA. Gls1, Myc, Got1, and Psat1 were highly expressed in rapidly proliferating $PKH26^{low}$ cells compared with $PKH26^{high}$ SEPs (Figure 6B and see [31]). As a key component of serine and glycine biosynthesis pathways, Psat1 expression was significantly higher in $PKH26^{low}$ SEPs. In addition, the genes involved in glycine, serine, and threonine metabolism were enriched in $PKH26^{low}$ progenitors (Figure 6B, C). Consistent with a role for Yap1 in this process, we observed altered expression of genes governing glutaminolysis, including Gls1, Myc, Got1, and Psat1, in Yap1 KO cultures (Figure 6D). Given the importance of enhanced glutamine metabolism to meet increased metabolic needs for sustained cell proliferation during stress erythropoiesis, these findings suggest that Yap1 functions as a regulator of metabolic enzymes required for the proliferation of stress erythroid progenitors.

As reduced expression of Gls1 led to insufficient glutamate availability for cell proliferation, we next investigated whether Yap1-mediated glutaminolysis could be rescued by the addition of 2 mmol/L glutamate to expansion medium during in vitro culture of Yap1 KO bone marrow cells. Glutamate supplementation significantly increased the total number of cells contained in Yap1 KO cultures, although the increase was not sufficient to fully restore the growth of Yap1 KO progenitors when compared with $Yap1^{F/F}$ cultures (Figure 6E, F). These data suggest that Yap1-mediated SEP proliferation acts in part through glutaminolysis.

Discussion

Stress erythropoiesis is a comprehensive process that involves hematopoietic stem cells committing to erythroid lineage, amplification of erythroid progenitor and precursors, and terminal differentiation to generate large numbers of erythrocytes. This process shares many regulatory pathways with tissue regeneration and repair. The Hippo signaling pathway plays an important role in tissue regeneration [52,53]. We observed that mutation of the downstream transcriptional effector, Yap1, largely impairs the proliferative capacity of stress erythroid progenitors in vivo and in vitro, leading to failures in erythroid regeneration and the re-establishment of homeostasis. Our analysis revealed that Yap1 mutation affected primarily proliferating progenitors, while the more immature stem-cell like stress erythroid progenitors, which undergo few cell divisions, were not sensitive to Yap1 mutation. Although the action of Yap1 in regulating apoptosis has been reported in many studies [15,54,55], we did not observe a significant apoptotic impact of Yap1 mutation on cultured SEPs (Supplementary Figure E3). Thus, we conclude that Yap1 promotes the proliferation of rapidly amplifying stress erythroid progenitors in response to anemic stress to re-establish erythroid homeostasis.

Our data indicate that Yap1 regulates the expression of Sox2 and Id1 in proliferating SEPs. These have been implicated in stem cell self-renewal and in the regeneration of damaged tissues [22,45]. Furthermore, recent findings have demonstrated an interplay between Yap1 and cellular metabolism. Yap1 phosphorylation is affected by cellular glucose levels [28,56–58]. Inhibition of glycolysis induced Yap1 phosphorylation and inactivation. In contrast, fatty acids and G-protein-coupled receptor ligands activate Yap1 [59,60]. Yap1 promotes glycolysis, lipogenesis, and glutaminolysis, suggesting that Yap1 serves as a regulatory node in coordinating energy status and nutrient availability with cell growth and tissue homeostasis [26,29,51,61]. We found that the expression of metabolic enzymes involved in glutaminolysis (Gls1, Myc, Got1, Psat1) is lost in Yap1 KO cultures (Figure 6D). The key role of Yap1 in regulating glutamine metabolism was underscored by the observation that supplementing Yap1 KO cultures with glutamate moderately increased total cell number and the number of amplifying $CD133^{neg}$-Kit^+Sca1^+ SEPs (Figure 6E, F), suggesting that Yap1 mediates proliferation of SEPs in part through regulation of Gls1 expression.

The $hYAP1^{S127A}$ group is significantly different from the control. $p = 0.035$ on day 24. The $hYAP1^{S127A}$ group is significantly different from the control. **(C–E)** Retroviral transduction on Yap1 KO bone marrow cells in expansion culture with SEEM medium. Progenitor cells were collected at the end of 7-day culture and stained with fluorescent antibodies. **(C)** Representative flow cytometry analysis of transduced Yap1 KO cells (GFP^+) compared with untreated $Yap1^{F/F}$ control cells. **(D)** Statistical analysis of total cell number after culture. **(E)** Analysis of increased percentage of Kit^+Sca1^+ SEPs. Increase in percentage is presented as (percentage of Kit^+Sca1^+ SEPs among GFP^+ cells) – (percentage of Kit^+Sca1^+ SEPs among GFP^{neg} cells). Data are expressed as individual subject and mean ± SEM. $*p < 0.05$.

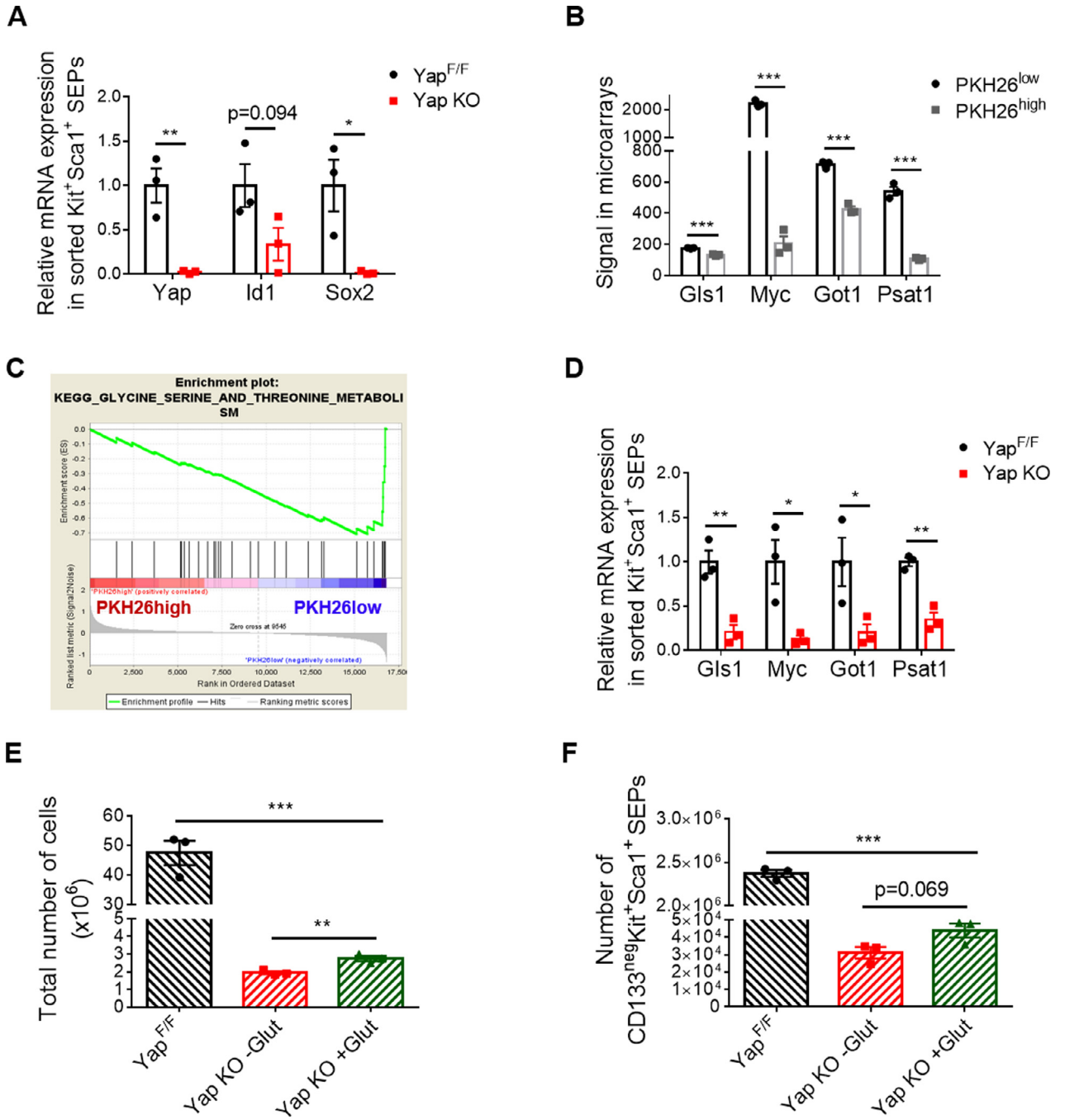

Figure 6. Yap1 regulates the expression of key metabolic enzymes associated with glutamine metabolism. **(A)** Kit^{+}Sca1^{+} progenitors were collected and sorted from Yap1$^{F/F}$ and Yap1 KO expansion cultures at day 7. Analysis of relative mRNA expression of genes in sorted Kit^{+}Sca1^{+} SEPs. **(B, C)** Analysis on sorted wildtype PKH26^{low} and PKH26^{high} cells. **(B)** Relative expression levels of genes in PKH26^{low} and PKH26^{high} SEPs according to microarray analysis. **(C)** Enrichment plot of genes associated with glycine, serine, and threonine metabolism. Detailed information about GSEA can be found in Figures 5 and 6. NES = −1.04. FDR q-value = 0.289. **(D)** Analysis of relative mRNA expression of genes associated with glutaminolysis in sorted Kit^{+}Sca1^{+} SEPs. **(E, F)** Unfractionated bone marrow cells were treated with 4OHT for 48 hours in expansion media before the addition of 2 mmol/L glutamate (Glut). **(E)** Statistical analysis of total number of cells after culture. **(F)** Analysis of absolute number of CD133^{neg}Kit^{+}Sca1^{+} progenitors. Paired *t* tests were performed in comparison of Yap1 KO −Glut with Yap1 KO +Glut and Yap1$^{F/F}$ with Yap1 KO +Glut. Data are shown as individual subject and the mean ± SEM. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$.

In summary, Yap1 mediates regenerative reconstitution of erythroid homeostasis following irradiation and bone marrow transplant by promoting the proliferation of transiently amplifying stress erythroid progenitors. This effect is in part regulated by transcriptional activation of key glutamine-metabolizing enzymes. Our

findings implicate a role for Yap1 in coordinating cellular metabolism with cell growth and tissue homeostasis restoration following erythroid stress.

Acknowledgments

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Conflict-of-interest disclosure

The authors have no relevant conflicts to disclose.

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Supplementary Data

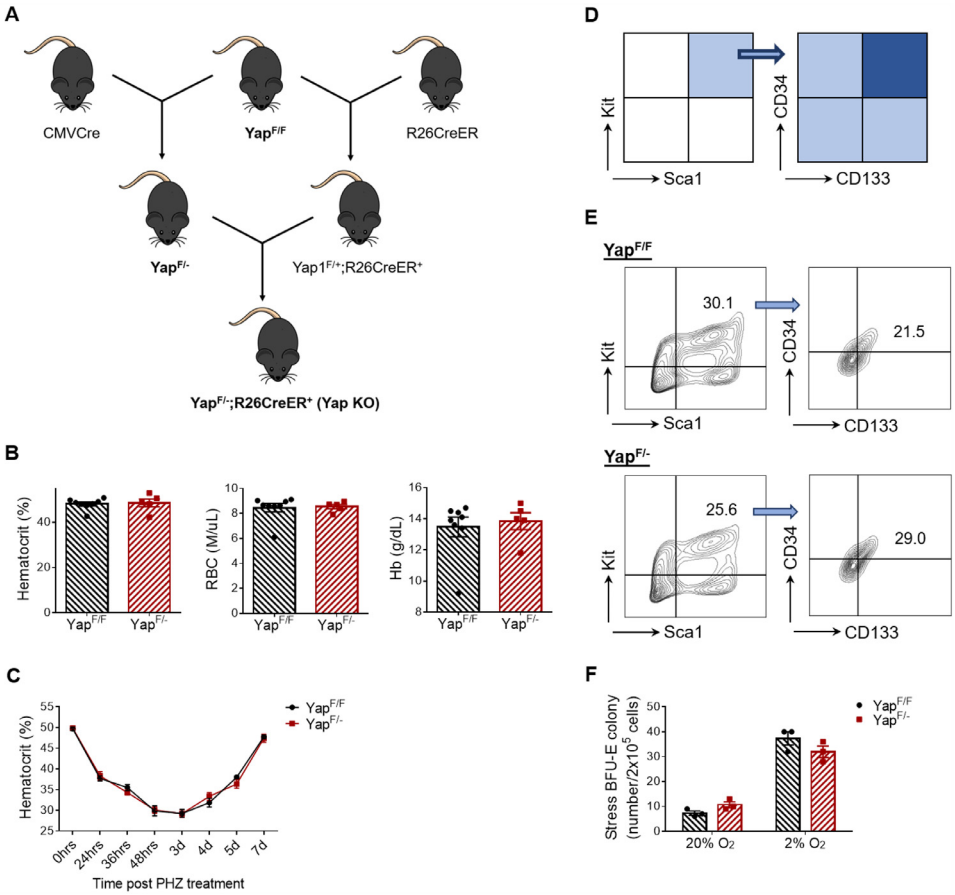
Supplemental methods and materials

Bone marrow transplant. Recipient mice were lethally irradiated at a dose of 950 cGy prior to transplantation. 5×10^5 donor cells were prepared in phosphate-buffered saline (PBS) buffer and transplanted to recipient mice via intravenous injection into the retro-orbital sinus[62].

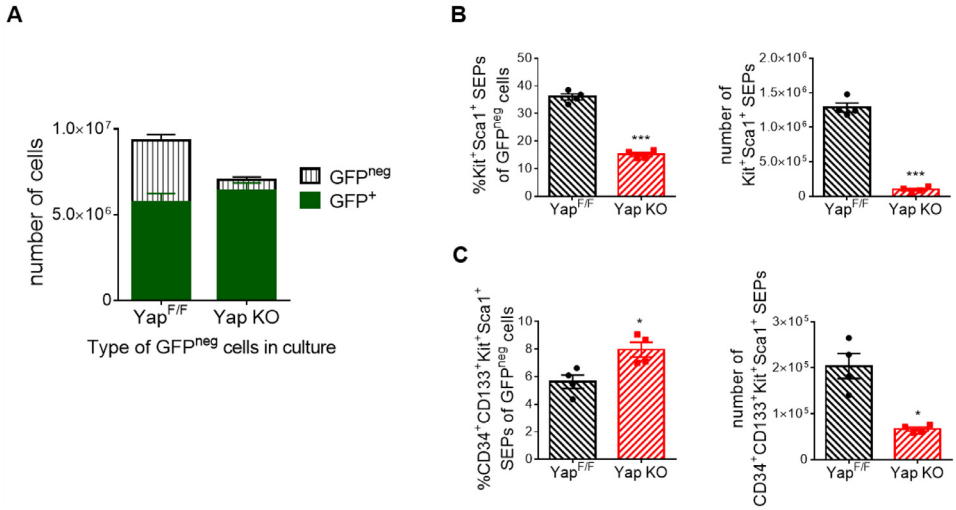
Phenylhydrazine challenge. Acute hemolytic anemia was induced by intraperitoneal injection of phenylhydrazine (Sigma-Aldrich) at a concentration of 100 mg/kg body weight to the mice. The recovery from PHZ challenge was monitored and analyzed in the following 7 days by blood collection at indicated time points[2].

Stress BFU-E assay. Splenocytes or cultured erythroid progenitors were plated in 2 mL M3334 methylcellulose media (StemCell Technologies) containing 3 U/mL EPO at a concentration of 1×10^5 cells/mL media. Cells were cultured at 20% O_2 in a 37°C incubator for 5 days to assay for mature stress BFU-Es. Colony assays that were performed in methylcellulose media containing EPO supplemented with BMP4 (15 ng/mL) and SCF (50 ng/mL) at the same cell concentration in 2% O_2 were used to assay for total stress BFU-E potentials[63,64].

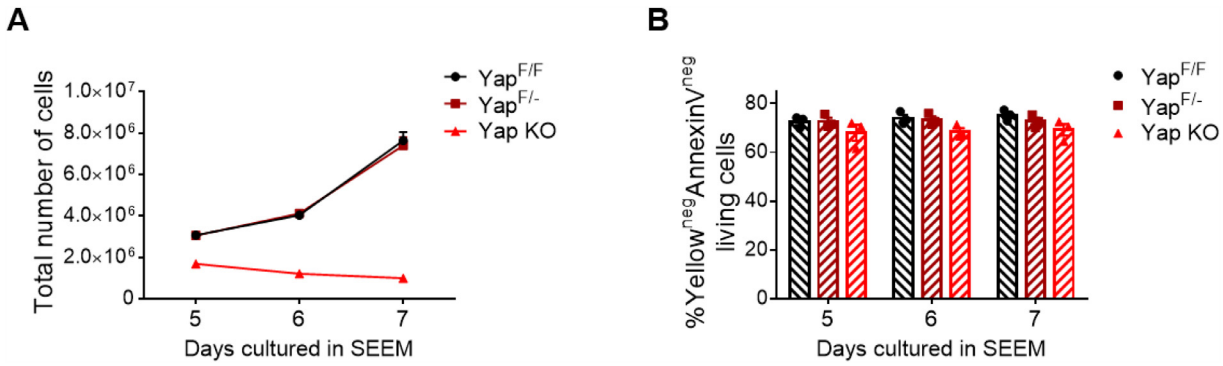
In vitro culture system[64,65]. For expansion culture, bone marrow cells were cultured in Iscove's Modified Dulbecco's Media (IMDM, Thermo Fisher Scientific) media supplemented with 1% (*m/v*) BSA, 0.0007% (*v/v*) β-mercaptoethanol, 10% (*v/v*) fetal bovine serum (FBS), 10 μg/mL insulin, 200 μg/mL transferrin, 20mM L-Glutamine, 15 ng/mL bone morphogenetic protein 4 (BMP4), 50 ng/mL stem cell factor



Supplementary Figure S1. One-allele deletion of *Yap1* does not alert homeostatic or stress erythropoiesis. (A) Breeding strategy to generate Yap1$^{F/-}$ and Yap1$^{F/-}$;R26CreER$^+$ mice. Yap1$^{F/-}$ mice were generated by crossing Yap1$^{F/F}$ mice with CMVCre mice that express a human cytomegalovirus minimal promoter during early embryogenesis. In order to maximize the effect of *Yap1* gene mutation, Yap1$^{F/-}$;R26CreER$^+$ mice were obtained by breeding Yap1$^{F/-}$ mice with the offspring of Yap1$^{F/-}$ and R26CreER cross. (B) Analysis of hematocrit levels (left), RBC counts (middle), and hemoglobin concentration (right). (C) Mice were injected with 100 mg/kg body weight phenylhydrazine (PHZ). The recovery from PHZ treatment was monitored and analyzed for seven days. Hematocirt levels were analyzed during the recovery period. Each time point reflects > 3 mice analyzed. Data are shown as the mean ± SEM. (D-F) Bone marrow cells were isolated and plated in expansion culture for 7 days. Non-adherent stress erythroid progenitors (SEPs) were collected and stained with fluorescent anti-Kit, anti-Sca1, anti-CD133 and anti-CD34 antibodies. (D) Flow cytometry gating strategy schematic. (E) Representative flow cytometry analysis. (F) Quantification of stress BFU-E colonies produced at 20% O_2 and 2% O_2 with addition of BMP4 and SCF. Data are shown as individual subject and the mean ± SEM.



Supplementary Figure S2. Effects of Yap1 mutation cannot be rescued by co-culturing with wildtype GFP$^+$ BM cells. Yap1$^{F/F}$ or Yap1 KO BM cells were co-cultured with GFP$^+$ BM cells (with wildtype genetic background) at 1: 1 ratio in expansion media for 7 days. Progenitor cells were collected for analysis. (A) Statistical analysis on number of GFP^{neg} and GFP$^+$ cells after culture. (B) Percentage (left) and absolute number (right) of Kit$^+$Sca1$^+$ SEPs. (C) Percentage (left) and absolute number (right) of CD34$^+$CD133$^+$Kit$^+$Sca1$^+$ SEPs. Data are shown as individual subject and the mean ± SEM. * $p<0.05$ and *** $p<0.001$.



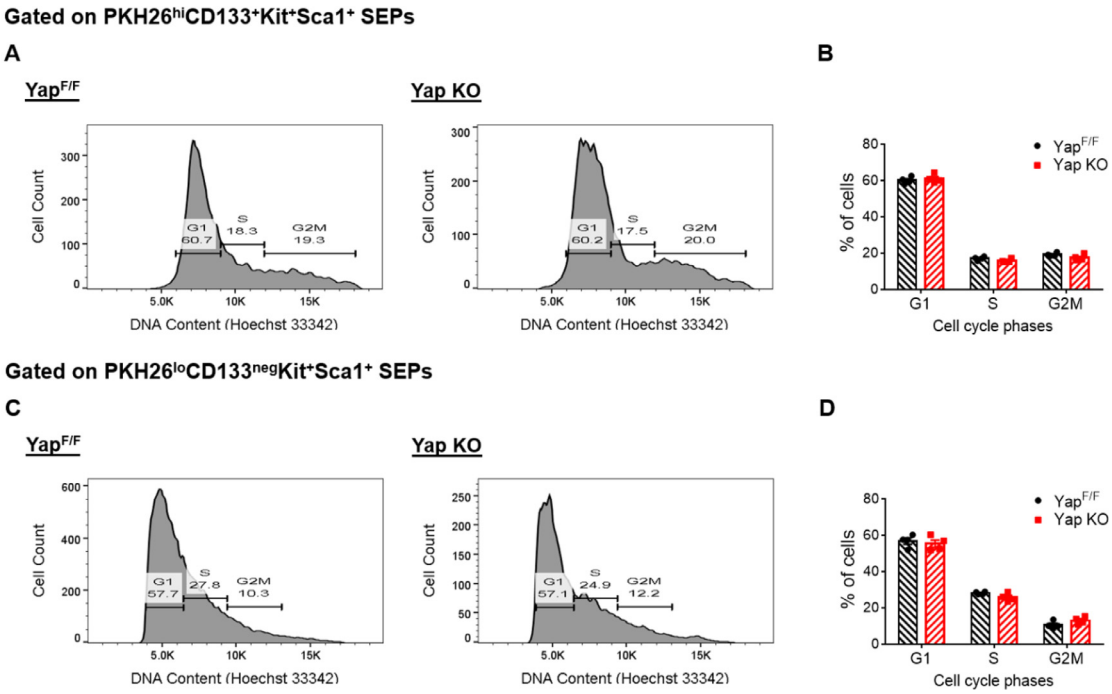
Supplementary Figure S3. Apoptosis is not a significant contributor to Yap1-mediated proliferation of SEPs. Unfractionated bone marrow cells were isolated and cultured in expansion media (SEEM) for 7 days. SEPs were collected and analyzed on day 5, 6 and 7. (A) Growth analysis as shown in total number of cells in the culture. Each time point reflects 3 mice analyzed. Data are shown as the mean ± SEM. (B) Living cells are characterized as negative of Yellow LIVE/DEAD dye and negative of Annexin V (Yellow^{neg}AnnexinV^{neg}). Percentage of living cells on indicated days during the culture. Data are shown as individual subject and the mean ± SEM. One-way ANOVA analyses were performed among three genotypes on each indicated time point. Day 5: p=0.315. Day 6: p=0.061. Day 7: p=0.130.

(SCF), 25 ng/mL sonic hedgehog (SHH), and 30 ng/mL growth-differentiation factor 15 (GDF15) for indicated period of time. Complete expansion media was named stress erythropoiesis expansion media (SEEM). Stress erythropoiesis differentiation media (SEDM) was made by supplementing 3 U/mL erythropoietin (EPO) to SEEM. SEDM cultures were incubated in hypoxia (2% O_2) for 3 days.

Analysis of serum Epo concentration. Concentration of erythropoietin (EPO) in mouse serum was quantified by ELISA Kit (MEP00B, R&D) following manufacturer's instruction. The serum EPO concentrations were calculated by utilizing the standard curve, which was generated by graphing OD_{405} readings and concentrations of standard samples.

Retroviral Transduction of cultured BM cells. Retroviral transduction was employed for enhanced gene expression in primary BM cells. Enforced expression of human YAP1 (hYAP1) was induced in primary BM cultures using GFP-expressing MIGR1 retroviral vector. hYAP1^{WT} stands for the construct of MIGR1 vector inserted with wildtype hYAP1 cDNA sequence (CDS: NM_001195044.1). hYAP1^{S127A} stands for the construct of MIGR1 vector inserted with hYAP1 cDNA sequence containing a nonsynonymous serine 127 to alanine (S127A) mutation. MIGR1 vector with no insertion was used as control (Ctrl).

Flow cytometry and cell sorting. Isolated spleen cells or cultured bone marrow cells were stained with conjugated fluorescent antibodies. For flow cytometry analysis, stained



Supplementary Figure S4. Yap1 promotes SEP transient amplification without alerting their cell cycle profiles. Unfractionated bone marrow cells were labeled with PKH26 dye at the beginning of a course of 7 day SEEM culture during which 4OHT was supplemented for 48 hours as described in Figure 3A. Harvested SEPs were stained with fluorescent antibodies as well as cell cycle dye, Hoechst 33342. (A-B) Representative flow cytometry analysis (A) and statistical view (B) of DNA content and cell cycle profile on PKH26^{hi}CD133^{+}Kit^{+}Sca1^{+} SEPs. (C-D) Representative flow cytometry analysis (A) and statistical view (B) of DNA content and cell cycle profile on PKH26^{lo}CD133^{neg}Kit^{+}Sca1^{+} SEPs. Data are shown as individual subject and the mean ± SEM.

Gene name	PKH26^{high} Avg (log2)	PKH26^{low} Avg (log2)	Fold change	FDR q-value	
Rassf1	**7.22**	6.84	1.3	0.0461	
Taok1	**11.5**	1.45	2.07	0.0009	
Mark1	**10.19**	9.64	1.47	0.0313	
Mark2	9.3	**9.7**	-1.32	0.0174	
Mark3	**10.74**	10.51	1.17	0.2053	
Mark4	**9.22**	8.71	1.42	0.0162	
Nf2/Merlin	7.76	**8.06**	-1.23	0.0371	
Sav1	**10.8**	10.08	1.64	0.0012	Core pathway of Hippo signaling
Mst1	**5.52**	5.07	1.37	0.0103	
Lats1	**10.15**	9.81	1.26	0.0196	
Lats2	**7.83**	6.55	2.43	0.0000992	
Mob1	**12.94**	11.99	1.94	0.0004	
Ajuba	6.57	**7.5**	-1.91	0.0016	
Rassf6	4.21	**4.9**	-1.61	0.0037	
Sik1	8.02	**8.15**	-1.1	0.5703	

Supplementary Figure S5. Gene expression profile of Hippo pathway components. Gene expression profiles come from microarray analysis on sorted wildtype PKH26^{high} and PKH26^{low} cells. Average expression levels of Hippo pathway components in PKH26^{high} and PKH26^{low} cells are presented in log2 scale. A positive fold change suggests that the gene is highly expressed by PKH26^{high} cells, while a negative number implies that the gene has high expression level in PKH26^{low} cells. The false discovery rate (FDR) q-value represents the estimated probability that a differentially expressed gene is a false positive finding. Genes that are negative regulators of Yap1 are highlighted by blue color, while genes that are positive regulators of Yap1 are highlighted by red color. Core pathway of Hippo signaling were emphasized.

Supplementary Table S1. Flow cytometry antibodies

Species	Marker	Fluorophore	Source
Mouse	CD34	Alexa Fluor 647	#560230, BD Biosciences
Mouse	CD133	PE/Cy7	#141210, BioLegend
Mouse	CD133	PE	#4325032, eBioscience
Mouse	Ly-6A/E (Sca1)	FITC	#108106, BioLegend
Mouse	CD117 (c-Kit)	Pacific Blue	#105820, BioLegend

Supplementary Table S2. qRT-PCR TaqMan probes

Gene	Identifier
Yap1	Mm01143263_m1
Id1	Mm00775963_g1
Sox2	Mm03053810_s1
Pcx	Mm00500992_m1
Ldha	Mm01612132_g1
Gls1	Mm01257297_m1
Myc	Mm00487804_m1
Got1	Mm00494698_m1
Psat1	Mm04932904_m1

cells were analyzed on a BD LSR Fortessa Cytometer (BD Biosciences) using FlowJo data analysis program. Cells were analyzed on a Beckman Coulter MoFlo Astrios cell sorter (Beckman Coulter) in order to collect specialized progenitor populations.

Protein isolation and Western blot. Bone marrow stress erythroid progenitors were collected from in vitro culture. In order to separate cytoplasmic proteins versus nuclear proteins, cells were first processed with cytoplasmic protein extraction buffer, which contains 10 mM HEPES, 10 Mm KCl, 1.5 mM $MgCl_2$ and 0.1% (*v/v*) NP-40, on ice for 15 minutes followed by centrifuging at 3,000 rpm for 10 minutes at 4°C. Supernatant was stored as cytoplasmic protein solution, while the nucleus pellet was further lysed with Pierce® RIPA buffer (89900, Thermo Fisher Scientific) to extract nuclear proteins. Western blots were probed with primary antibodies including anti-Yap1 (sc-376830, Santa Cruz Biotechnology) at 1:500 dilution, anti-Histone H3 (9715S, Cell Signaling Technology) at 1:2000 dilution, and anti-β-actin (sc-47778, Santa Cruz Biotechnology) at 1:2000 dilution for overnight incubation at 4°C. Secondary goat anti-rabbit and goat anti-mouse antibodies were used at 1:2000 dilution. The bands were visualized by SuperSignal West Pico PLUS Chemiluminescent Substrate western blotting detection reagent (34580, Thermo Fisher Scientific).

Supplementary References

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