Smarca5 (Snf2h) is required for Proliferation of Hematopoietic Stem Cells Differentiating into Erythroid and Myeloid lineages.

JURAJ KOKAVEC1,2, TOMAS ZIKMUND1, FILIPP SAVVULIDI1, VOJTECH KULVAIT1, WINFRIED EDELMANN2, ARTHUR I. SKOULTCHI2* AND TOMAS STOPKA1*

Key words. Erythroid differentiation • Fetal liver erythropoiesis • Smarca5 • ISWI • Hematopoietic stem and progenitor cells • Cell cycle progression • p53 pathway • Hypoxia

ABSTRACT

The Imitation Switch (ISWI) nuclear ATPase Smarca5 (Snf2h) is one of the most conserved chromatin remodeling factors. It exists in a variety of oligosubunit complexes that move DNA with respect to the histone octamer to generate regularly spaced nucleosomal arrays. Smarca5 interacts with different accessory proteins and represents a molecular motor for DNA replication, repair and transcription. We deleted Smarca5 at the onset of definitive hematopoiesis (Vav1-iCre) and observed that animals die during late fetal development due to anemia. Hematopoietic stem and progenitor cells (HSPCs) accumulated but their maturation towards erythroid and myeloid lineages was inhibited. Proerythroblasts were dysplastic while basophilic erythroblasts were blocked in G2/M and depleted. Smarca5 deficiency led to increased p53 levels, its activation at two residues, one associated with DNA damage (S18 Phos) second with CBP/p300 (K382 Ac), and finally activation of the p53 targets. We also deleted Smarca5 in committed erythroid cells (Epor-iCre) and observed that animals were anemic postnatally. Furthermore, 4-OHT-mediated deletion of Smarca5 in the ex vivo cultures confirmed its requirement for erythroid cell proliferation. Thus, Smarca5 plays indispensable roles during early hematopoiesis and erythropoiesis.

SIGNIFICANCE STATEMENT:

ISWI chromatin remodeling ATPase Smarca5 is a highly conserved chromatin-remodeling factor that is expressed in hematopoietic tissues especially stem and progenitor cells. There exist several oligosubunit complexes containing Smarca5 as a catalytic subunit that were previously shown to actively regulate nucleosomal structure and position during DNA replication, repair and transcription. Genetic inactivation of Smarca5 specifically in murine definitive hematopoietic cells leads to the developmental blockade marked by unique proliferative defects such as tetraploidy and erythroid dysplasia. The Smarca5 gene is not mutated in cancer, however its expression is enhanced in acute myeloid leukemia and aggressive solid tumors. This may be exploited for development of future therapies for targeting the growth and repopulation of human malignancies.
Nuclear DNA is carefully packaged into chromatin, a compact nucleoprotein complex that regulates many genomic activities. Imitation SWItch (ISWI) proteins are SNF2-like ATPases that facilitate chromatin assembly and remodeling through their DEAD/H helicase domains located close to their N-termini. Their C-termini contain SANT and SLIDE domains that recruit a variety of bromodomain-containing factors that recognize epigenetically modified histones. ISWI proteins are integral units of several oligo-subunit complexes that establish regularly spaced nucleosomes during DNA replication, cooperate with lesion-sensor proteins in DNA repair, and facilitate or inhibit transcriptional outcomes mediated by all three types of RNA polymerases (reviewed in Erdel and Rippe [1]).

The relatively large spectrum of Smarca5 activities is enabled by structural differences in the non-catalytic subunits that provide distinct contributions to sense the state of the DNA adjacent to nucleosomes [2]. Vertebrate ISWIs are represented by two homologues: Smarca5 (Snf2h) and Smarca1 (Snf2l). While Smarca5 is expressed in stem and progenitor cells as well as in undifferentiated tumor cells, Smarca1 is expressed only at later stages suggesting that Smarca5 may be required at early stages of development when cell fate is being decided [3]. The blood system and especially erythroid cells are the most highly Smarca5-expressing tissues.

Hematopoietic stem cells (HSCs) possess great potential to self-renew throughout life and to give rise to several types of multipotent progenitors (MPPs), which then differentiate along myeloid or lymphoid pathways to produce sufficient amounts of the various mature specialized blood cells. Lineage-specific transcription factors cooperate with additional factors and are often involved in epigenetic modification that are necessary to promote differentiation of self-renewing stem cells. Transcriptional regulation of early hematopoiesis has been reported to involve the SWI/SNF2-like proteins. For example, a hypomorphic mutation of the murine Brg1 ATPase results in anemia, E14.5 lethality and a blockade at the polychromatophilic erythroblast stage [4]. Our previous work suggested that Smarca5 is also involved in regulation of hematopoiesis. Inhibiting its levels in human CD34+ progenitors suppresses cytokine-induced erythropoiesis in vitro [5]. Conventional knock-out of murine Smarca5 is lethal very early in embryonic development—long before primitive hematopoiesis is established [5], thus preventing a determination of its role in hematopoiesis. In this manuscript we describe new conditional Smarca5 knock-out mouse model and use it to determine how loss of Smarca5 affects hematopoiesis. Our results show that loss of Smarca5 disrupts definitive hematopoiesis in the fetal liver, causing anemia due to defects in proliferation and differentiation of both hematopoietic stem and multipotent progenitor cells. Smarca5 also is required for proliferation and survival of fully committed erythroid progenitors.

METHODS

**Generation of Smarca5 knock-out mice and cells.**

The Smarca5 targeting construct contained three 129Sv-derived murine genomic DNA fragments: i) the S’ homology arm (1.5kb Pmel-HindIII containing exon4), ii) the targeted HindIII-HindIII region (~1 kb with exon5 surrounded by loxP sites (floxed); deletion of which would create a frame shift), and iii) the 3’ homology arm (4.5 kb HindIII-HindIII containing exons 6-8) (Fig. 5A). The construct was electroporated into WW6 ES cells and 2 of 12 independent clones were injected into C57B1/6 blastocysts as described recently [6]. Detection of the targeted allele was determined by PCR amplification of a 3’loxP-containing fragment followed by cleavage at a unique (AscI) restriction site (Fig. S1B). The floxed Smarca5 allele can be detected by conventional PCR (Fig. S1C). Germline deletion using Zp3-Cre transgene [7] produced Smarca5<sup>+/Δ5</sup> heterozygous mice that displayed reduced Smarca5 protein levels. While the Smarca5<sup>Δ5/Δ5</sup> mice were fertile and viable, the progeny of Zp3-Cre-dependent germline inactivation recapitulated the early perimplantation lethality as described previously in Smarca5<sup>5-9/5-9</sup> mice [5]. Thus, deletion of exon5 results in a null allele (Fig. S1E). Methods for Fig. S1C are provided in the Supporting Information (SI). The Institutional Ethical Board and law on GMO approved mouse handling. Mouse E12.5 FL-derived Smarca5<sup>Δ5/Δ5</sup> Cre-Esr1 erythroid progenitors (FL-EPs) or Smarca5<sup>Δ5/Δ5</sup> were cultivated [8] and treated by 1μM 4-hydroxytamoxifen (4-OHT) (Tg(CAG-cre/Esr1tax)5Amc<sub>Asc1</sub>) [9] or diluent (ethanol) alone.

**Cell cycle, clonogenic, and apoptosis assays**

*In vivo* BrdU staining for 1 hour was analyzed by flow cytometry (see SI) on BD Canto II flow analyzer and analyzed by FlowJo software (TreeStar Inc., Ashland, OR, USA). CFU assay utilized Methocult M3434 (StemCell Technologies, Vancouver, BC, Canada). 4x10<sup>4</sup> FL cells were plated. Apoptosis assay utilized immunofluorescence (IF) of cleaved Caspase-3 and TUNEL assays (SI).

**Gene expression**

Cell staining by H&E on histology sections and cytology smears (May-Grünwald-Giemsa) are described in the SI section. Surface marker detection (FACS Canto II, BD Biosciences, San Jose, CA, USA) utilized conjugated antibodies and published protocols [10, 11]. The microarray analysis utilized Affymetrix GeneChip Mouse Genome 430 2.0 Array. Probes were accepted differentially expressed on FDR adjusted p-value p<0.01 (for more details see SI). Link to the expression data may be found in Gene Expression Omnibus database. Protocols and antibodies used for western blotting, immunofluorescence and flow cytometry are listed in the Supplement. Briefly, whole protein lysates from E13.5 or E14.5 FLs were prepared in RIPA buffer supplemented with
proteinase and phosphatase inhibitors. Blocking and staining was performed in TBS/0.1% Tween-20 with 5% milk or 3% BSA with antibody dilutions following manufacturer’s recommendations. Blots were visualized by ChemiDoc™ MP System (Bio-Rad, USA).

**RESULTS**

**Smarca5 is required for definitive hematopoiesis.**

We reported previously that *Smarca5* null mouse embryos die shortly after implantation [5]. To investigate the role of *Smarca5* in later development, we produced a conditional KO allele by inserting LoxP1 sites in introns 4 and 5 (Fig. S1A). The Cre-recombinase-mediated removal of exon5 in the *Smarca5* allele is predicted to result in a null allele due to removal of a portion of the catalytic ATPase domain, also creating a frameshift and premature stop codon. *Smarca5* mice were viable, fertile and developed normally. To study the role of *Smarca5* at the onset of definitive hematopoiesis, we utilized a transgene that expresses iCre recombinase driven by the Vav1-promoter. Vav1 is a target of the stem cell factor receptor (c-Kit) signaling [12] and is activated in definitive HSCs starting at embryonic day E10.5 [13]. *Vav1-iCre* transgenic mice were bred with *Smarca5* mice described previously [5] to produce the *Smarca5*/*Vav1-iCre* strain. This strain was then mated with *Smarca5*/*Vav1-iCre* mice. We observed that mice with the genotype *Smarca5*/*Vav1-iCre* are not born; other genotypes (*Smarca5*/*Smarca5*/*Vav1-iCre* and *Smarca5*/*Vav1-iCre*) were produced in the expected numbers (Table 1).

Analysis of embryos between days E11.5-18.5 indicated that all *Smarca5*/*Vav1-iCre* embryos are pale - the effect is apparent at E14.5 (Fig. S1F) and even more apparent at E15.5 (Fig. 1A). Mutant embryos display significantly smaller fetal livers (FLs) (Fig. 1B and S1G) and decreased FL cellularity (Fig. 1C). In contrast, FL development in *Smarca5*/*Vav1-iCre* mice is normal (Fig. 1B, middle panels), suggesting that the single *Smarca5* allele is not hypomorphic. By E18.5, all mutant embryos die in utero with subcutaneous swelling indicative of hemodynamic failure (Fig. S1H, Table 1). Cytologic examination of *Smarca5*/*Vav1-iCre* FLs between E12.5 and E14.5 revealed a lack of maturing erythroid cells, whereas control FLs contained all stages of maturing erythroid cells (Fig. S1I and 1D). The histologic examination of E13.5 and E16.5 mutant FLs confirmed that the *Smarca5*/*Vav1-iCre* FLs display disrupted structure of acinus and depletion of maturing erythroid cells in favor of immature hematopoietic cells (Fig. 1E and S1J). *Smarca5*/*Vav1-iCre* FLs frequently displayed dysplastic changes such as atypical and often binucleate proerythroblasts (Fig. 1D and S1I). Erythroid hypogenesis in the mutant FLs was reflected in the peripheral blood (PB) smears at E14.5 (Fig. 1F) and E15.5 (Fig. S1K) which showed decreased numbers of definitive (non-nucleated) erythrocytes. Instead, the PB contained large numbers of nucleated erythrocytes, indicating a defect in the embryonic-to-definitive erythropoiesis switch. Hematopoietic cells at E15.5 stage control FLs expressed *Smarca5*, but this was not observed in the mutant FLs by immunostaining (Fig. S1L). These results indicate that *Smarca5* loss abrogates definitive hematopoiesis within FL, leading to anemia and E18.5 lethality.

**Smarca5 deficiency disturbs proliferation and differentiation of hematopoietic LSK and LS K progenitors.**

Because Vav1-iCre is active at the earliest stages of definitive hematopoiesis [13], we could investigate the consequences of *Smarca5* deficiency in very early hematopoietic cells. We assessed hematopoiesis of the E13.5 FL via flow cytometry using stem cell (Sca1, c-Kit) and SLAM (CD48, CD150) antigens in lineage-negative FL cells as reported previously (Fig. S2A) [11, 14]. LSK cells are defined as Sca-1 and c-Kit-expressing cells which are depleted of lineage-positive marker expression while the LSK cells are those that lack expression of Sca-1 but are positive for c-Kit and negative for lineage markers. In absolute numbers, the mutants were greatly enriched in LSK cells but not in LSK (Fig. S2A and 2B). Further analysis using SLAM antigens revealed an increase in the absolute number of MPP cells including the earliest MPP-1 (LSK CD48 CD150), myeloid-biased MPP-2 (LSK CD48 CD150), MPP-3 (CD48 CD150) with myeloid-lymphoid potential, as well as the Ery/MK-biased LSKs (CD150 CD48) (Fig. 2B). The Ery/MK-biased LSKs represent the short repopulating stress-responsive MPPs [15]. In contrast to absolute numbers of the MPP cells, the relative numbers of MPP cells were not disturbed except for a ~2.5 fold increase in the myeloid-biased MPP-2 fraction (Fig. 2A, bottom). To gain insight into the cell cycling of the accumulated MPP populations, we utilized in vivo BrdU labeling in E13.5 embryos. We found that the percent of mutant E13.5 LSK cells in S-phase was increased relative to controls, whereas the percent of these cells in G0/G1 and G2/M phases was decreased (Fig. 2C, left). This phenomenon was observable for all analyzed MPP subpopulations (Fig. S2C). This suggests that *Smarca5* deletion induced proliferation of all types of the mutant LSK cells. In contrast, the mutant committed progenitors within the LS K compartment (together with less pronounced increase of S-phase) exhibited significantly increased proportion of blocked cells in G2/M phase suggesting that upon differentiation more *Smarca5*-negative cells became inhibited in proliferation.

To test the proliferative potential of committed FL-derived progenitors, we used a colony forming unit (CFU) assay in methylcellulose semisolid media. While FL-derived progenitors from control E13.5 embryos formed colonies of erythroid (BFU-E, CFU-E), myeloid (CFU-GM, CFU-GG, CFU-M), or mixed (CFU-GE-MM) po-
tential, formation of these colonies by the Smarca5flox5/Δ5-9 Vav1-iCre FL progenitors was severely reduced (Fig. 2D and S2D). The effect of Smarca5 deletion was more severe within the erythroid lineage, whereas formation of myeloid colonies was only partially affected. However, the surviving myeloid colonies displayed grossly atypical morphology. The atypical morphology in the mutants included dysplastic features such as cell size and cell shape differences and unevenness within one colony as well as atypical appearance and cellularity of individual colonies. To determine whether the atypical mutant myeloid colonies underwent Cre-mediated deletion, we utilized a mouse strain containing a Rosa26-STOP-eYFP reporter allele. Upon deletion of a transcription terminator signal by iCre-recombinase, this strain produces eYFP fluorescence detectable by flow cytometry [16]. Indeed, as indicated by positive eYFP fluorescence, these surviving colonies expressed iCre, suggesting that the atypical morphology is a result of Smarca5 gene deletion (Fig. S2D). In summary, these results indicate that the number of HSCs in Smarca5-deficient embryos is increased due to excessive cycling but their progeny, the MPPs, are blocked in their maturation.

**Smarca5 deficiency leads to induction of the p53 target mRNAs in hematopoietic progenitors.**

We sought insights into the maturation defect in the mutant progenitor population by investigating their gene expression profile. We compared the levels of 39,000 transcripts in mutant (Smarca5Δ5/Δ5-9 Vav1-iCre) and control FL-derived magnetically-sorted Kit+ progenitors from E15.5 embryos by hybridization to Affymetrix GeneChip Mouse Genome 430 2.0 Array with 45,101 probes. Many of the most significant, differentially expressed gene sets among GO Biological Process categories link to response to stress, DNA damage, repair & apoptosis, and also to hypoxia (Fig. 3A,B and S3A,D).

According to the DAVID annotation tool, many of the genes in the KEGG p53-signaling pathway are significantly, differentially expressed in Smarca5Δ5/Δ5-9 Vav1-iCre versus control progenitors (Fig. 3B). Changes in p53 pathway mRNAs are often associated with DNA damage and may result in apoptotic signaling-induced DNA fragmentation. To assess this, we used the TUNEL assay. The results showed that indeed mutant FLs exhibited a higher frequency of TUNEL-positive cells (Fig. S3B). In order to quantify the frequency of apoptotic cells in the mutants we determined the level of cleaved Caspase-3 in the FLs at E16.5 stage. The data show that the mutant FLs activated Caspase-3 while the control FLs showed very low frequency of Caspase-3-positive cells (Fig. 3C). This data is consistent with upregulation of Caspase-3 mRNA level in the mutant FL progenitor cells (see Fig. 3B).

To address whether by deleting Smarca5 at early progenitor stages the DNA damage response pathways (DDR) became activated, we tested DDR-dependent events including the levels of p53 and its S18 Phos modification which was previously associated with DDR activation and equal to S15 Phos observed in humans [17]. We also assessed acetylation of Lysine (K) 382 of p53 which is mediated by p300/CBP acetylases [18]. Firstly, we observed that the irradiated mutants expressed slightly higher level of p53 compared to controls and the level of its inhibitor Mdm2 was reciprocally decreased (Fig. 3D). Target of p53, Ccnd2, was also increased in the mutants. Secondly, we also detected an increase in two p53 modifications in the mutants: S18Phos and K382Ac (Fig. 3D). Additional control on the western blots included expression of Smarca5 that again confirmed that no gene product is made from the mutant allele. Next, to further study whether the Smarca5 mutants display a DNA damage we determined γH2AX foci and p53BP1 expression using fluorescent microscopy. However, neither of these two marks were positive in the Smarca5 mutants or control FLs unlike in the positive control (irradiated wild type thymus, Fig. S3E) supporting the possibility of more complex activation of p53. We conclude that Smarca5 deficiency activated p53 at two residues, one associated with DNA damage (S18Phos) and another with CBP/p300 (K382Ac).
The observed shift towards immature stages in the mutant could be due to defects in cell proliferation, cell differentiation or cell survival. To investigate cell proliferation, we used in vivo BrdU labeling and also scored the DNA content of the cells by flow cytometry after staining with 7-AAD. The E13.5 pregnant females were injected with BrdU and the FLs of the embryos were removed and analyzed for hematopoietic markers. We observed a marked decrease in the proportion of cells in S-phase in the mutant S3 population and a significant increase in cells in G2/M in the mutant S2 to S5 populations. Particularly noteworthy was a marked increase in the polyploidy of G2/M cells in the mutant S3 population (Fig. 4B) not seen in controls (Fig. S4C). Thus, Smarca5 deficiency leads to perturbations in cell cycling within the FL erythroid populations, including accumulation of cells with abnormal ploidy.

To assess the differentiation properties of the mutant cells, we used RT-PCR to analyze the mRNA levels of several key transcription factor genes involved in erythropoiesis. We utilized the Rosa26-STOP-eYFP allele to isolate eYFP-positive S0-S3 cells, which have undergone Vav1-iCre-mediated deletion. We observed a strong reduction of Gata2 mRNA in the mutant S0 cells, and significant reductions of Gata1, Klf1, and Nfe2 mRNAs in the mutant S3 cells (Fig. 4C). We also assessed the expression of erythroid target genes, including Epor, Alas2 and globins. Indeed, expression of definitive globin (Hba-a1 and Hbb-b1) mRNAs and the erythroid-specific Alas2 and Epor genes was significantly reduced in the mutants. Interestingly, expression of embryonic globin (Hba-x and Hbb-y) mRNAs was increased, indicating a developmental delay of erythropoiesis in the mutant (Fig. S4D). In addition, no compensatory increase of Smarca5 homolog Smarca1 was observed (Fig. S4D). To determine the survival properties of the mutant cells, we utilized AnnexinV and propidium iodide staining by flow cytometry and found that FL cells lacking Smarca5 contain an increased proportion of apoptotic (5% in controls vs. 7% in mutants) and necrotic (3 vs. 9%) cells (Fig. 4D).

To assess whether the DNA damage response pathway is involved in the shift to a more immature erythroid population in the mutant we measured the levels of several of the DNA damage response mRNAs identified in the microarray analysis of Kit+ cells (Fig. 3B, in bold). We used quantitative RT-PCR to analyze mRNA levels in the eYFP+ E14.5 FL-derived S0-S3 stages. We found that all of the assayed p53 target gene mRNAs (Pmaip/Noxa, Gadd45b, Cdkn1a/p21, and Bbc3/Puma) are increased in at least one of the erythropoietic populations from the mutant, whereas Smarca5 mRNA and Pten mRNA are decreased in all mutant populations cells undergoing erythropoietic development suggesting that erythroid cells maintained activation of p53 targets upon loss of Smarca5 (Fig. S4E). Thus, the induction of a DNA damage response pathway associated with Smarca5 deficiency, observed in mutant HSC/MPP cells, occurs also in Smarca5 deficient erythroid cells.

**Smarca5 is needed for proliferation and survival of erythroid progenitors.**

The results presented in the preceding sections indicate that Vav1-iCre-mediated Smarca5 deficiency leads to perturbations in cell cycling and inhibition of maturation of hematopoietic cells at several stages of development from LSK cells to committed erythroid progenitors. We next sought to determine whether Smarca5 is also required at later stages of erythroid development and whether the effects of Smarca5 deficiency on erythroid development are cell autonomous. To address the effects of Smarca5 deficiency specifically in erythroid cells, we utilized the Epor-iCre allele that is expressed only in fully committed erythroid progenitors and their progeny [19]. All genotypes from the mating of Smarca5 (Δ-5-9) Epor-iCre and Smarca5 (Δ-5-9) Epor-iCre were born including the Smarca5 (Δ-5-9) Epor-iCre mice. However, closer examination of pups surviving beyond P9 indicated that the mutants were underrepresented (Fig. S5A). Newborn Smarca5 (Δ-5-9) Epor-iCre pups were pale and displayed a variable degree of growth delay and failure to thrive (Fig. 5A). Additional study of embryos showed that indeed the E12.5-E18.5 Smarca5 (Δ-5-9) Epor-iCre embryos are anemic with reduced overall size andcellularity of the FL (Figs. S5B-D). Flow cytometric analysis of FL erythroid cells (S0-S5) showed a similar shift towards the more immature erythroid populations (S0-S1) in Smarca5 (Δ-5-9) Epor-iCre embryos (Fig. 5B), like that seen in Vav1-iCre mutant FLs. The S3-S5 populations were decreased in the mutants, although the reductions were not as large as seen in Smarca5 (Δ-5-9) Vav1-iCre FLs. This was consistent with the cytology examinations, that showed that the Smarca5 (Δ-5-9) Epor-iCre FLs were (unlike the Vav1-iCre mutants) comparably populated (to controls) by proerythroblasts and basophilic erythroblasts (Fig. S5E). Colony formation assays with Smarca5 (Δ-5-9) Epor-iCre FLs showed reduced BFU-E, CFU-E and CFU-GEEMM (Fig. 5E), although the reductions were again not as large as seen in Smarca5 (Δ-5-9) Vav1-iCre FLs. Using the Rosa26-STOP-eYFP allele we observed that Epor-iCre is active within the S1-S5 FL erythroid populations (Fig. S5H). Consistent with the expression of Epor in the definitive erythroid cells, no delay was noted in the embryonic-to-definitive hematopoiesis switch in Smarca5 (Δ-5-9) Epor-iCre FLs (Fig. S5F).

To investigate the cell proliferation and survival properties of FL erythroid cells that could account for an increase in the relative numbers of more immature populations (S0-S1) and a reduction in more mature cells (S3-S5) in Smarca5 (Δ-5-9) Epor-iCre FLs, we again utilized in vivo BrdU labeling and flow cytometry combined with 7-AAD, annexinV and propidium iodide staining. We observed a marked decrease in BrdU incorporation within the S3 compartment and an increase in polyploid cells, although these defects were not as severe as in the Smarca5 (Δ-5-9) Vav1-iCre FLs (Fig. 5C and 5I). Similarly to Vav1-iCre mutants there was evidence of increased apoptosis (8.5 vs. 12%) and necrosis (10 vs.
22%) in the Smarca5$^{\text{flox/\text{fl}}}$ Epor-iCre FLs compared to controls (Fig. 5D).

To determine whether the observed effect of Smarca5 deficiency in the erythroid compartment is cell autonomous, we employed the Cre-Esr$^{\text{TM}}$ allele [9] that promotes Cre-mediated inactivation upon addition of 4-hydroxytamoxifen (4-OHT). We isolated FLs from E12.5 Smarca5$^{\text{flox/\text{fl}}}$ Cre-Esr$^{\text{TM}}$ embryos and cultured them under conditions that promote proliferation of immature erythroid progenitors for several weeks [8]. Cells were cultured 24 hours before adding 4-OHT. We observed that growth of the cultures was halted quite abruptly 72 hours after addition of 4-OHT (Fig. 5F). These results indicate that Smarca5 is required in a cell autonomous manner for proliferation of erythroid progenitors.

**Discussion**

Smarca5 was previously shown to be an essential factor in early mammalian development [5]. In the current work, we have demonstrated that it is required for maturation of definitive HSCs and for completion of erythropoiesis. We observed that mutant Smarca5 HSC/MPPs accumulate in FLs (Fig. 2B) while the subsequent stages involving LSK cell proliferation becomes progressively blocked at early differentiation stage (Fig. 2C) and CFU-forming progenitors are not able to form blood colonies (Fig. 2D). This leads to anemia and fetal stage-lethality prior to birth. The phenotype is related to the Smarca5 expression, which is very high in early HSCs and proliferating progenitors and its down regulation occurs during the later stages of erythropoiesis [20].

The erythroid defect in the Smarca5 mutant mice involved a slowed onset of definitive hematopoiesis and defective proerythroblast-to-basophilic erythroblast maturation (Fig. 4) marked by reduced expression of Gata1 and its target genes. These observations resemble some properties seen in erythroid cells upon deletion of the erythroid-specific transcription factor Gata1 [21], suggesting the possibility that Smarca5 and Gata1 cooperate during erythroid differentiation. Indeed, Smarca5 and Gata1 were previously shown to physically interact in the proerythroblast/basophilic erythroblastic MEL cell line [22]. The fact that the Smarca5 mutant embryos die later (E18.5) vs previously shown erythroid gene knockouts (E13.5-E15.5) [23] could be caused by later onset of the Vav1 expression, (which starts at E10.5 [12]) and which is relatively later compared to erythroid genes (e.g. Epo and Epor expression start around E8.5 and anemic FLs are observed at E11.5). The defect in erythropoiesis in Smarca5 deficient mice also partly resembles phenotypic outcome of inactivating another chromatin remodeling factor, the SWI/SNF ATPase Brg1, that resulted in anemia and embryonic lethality [4], as well as dysregulation of the epigenetic state of the -globin locus [24, 25]. However, the phenotype of the Brg1 mutants (compared to the Smarca5 mutants) involved more differentiated erythroid cells and did not lead to significant accumulation of early progenitors [4]. The erythroid phenotype of Smarca5 deficiency is consistent with the previously reported SMARCA5 knockdown experiment that caused an inhibition of early erythroid differentiation and led to downregulation of -globin expression in human CD34+ progenitors and K562 cells [5]. Interestingly, compared to erythropoiesis the myelopoiesis in the Smarca5 mutant is affected less severely, consistent with SMARCA5 being a negative regulator of the myeloid transcriptional activator PU.1 [26]. Since downregulation of Gata1 does not directly induce apoptosis [27], we think that the cell death and cell cycle arrest of the Smarca5-deficient erythroblasts is rather a result of the loss of Smarca5 function in cellular pathways other than Gata1 promoted differentiation. This contention is also supported by the fact that downregulation of erythroid key regulators including Gata1, Gata2, Klf1, and Nfe2 in the FL kit+ cells and erythroid subpopulations S0-S3 from the YFP-positive fraction of Smarca5$^{\text{flox/\text{fl}}}$ Vav1-iCre mutants (Fig. 4C) was not observed in any of the erythroid fraction of the Smarca5$^{\text{flox/\text{fl}}}$ Epor-iCre mutants (Fig. S5J). This conclusion is also supported by the ex vivo proliferation experiment of Smarca5-deficient erythroid cells which showed that Smarca5 is required for proliferation of erythroid progenitors (Fig. 5).

Smarca5 deficiency affected cell cycle progression coincidently with tetraploidy and activation of a DNA damage response. Indeed, as early as the HSC/MPP stage the loss of Smarca5 was reflected in the increased proportion of LSK cells entering S-phase (Fig. 2). Furthermore, in the next developmental step, within the LSK cells, proliferation was markedly reduced coincidently with G2/M phase accumulation (Fig. 2). Gene expression analyses at the progenitor level revealed that the most significantly affected transcriptional programs in Smarca5-deficient progenitors belonged to stressed hematopoiesis pathways including the p53 target genes (Fig. 3). As expected, the p53 became activated at two residues: S18$^\text{phos}$ that was previously associated with DNA damage [17] and the K382 acetylation representing CBP/p300-mediated mark (Fig. 3D). In addition, another DNA damage mark, Kap1 S824$^\text{phos}$, became activated. Interestingly, the levels of Trp53 protein were increased while its inhibitor Mdm2 was reciprocally decreased in the mutants. However, we have not confirmed the Atm/Atr pathway activation of Chk1/Chk2 (Fig. S3C) upon Smarca5 deficiency. We conclude that Smarca5 deficiency activated two activating modifications of p53. Our data however also show that H2AX staining was not enriched in the mutants supporting possibility of more complex mechanism upstream the p53 activation.

Stressed hematopoiesis induced by genotoxic agents in HSC is often coupled with Gadd45a-mediated activation of MPPs followed by erythro-megakaryocytic and myelo-lymphoid differentiation [28]. However, this wave of coordinated proliferation and differentiation.
does not occur upon Smarca5 deficiency and instead the mutant cells are blocked from proliferation at the tetraploid state, which prevents further completion of cell differentiation. In addition, a significant proportion of the mutant cells underwent the p53-mediated apoptosis (Fig. 3), which further aggravated the anemic phenotype. Polyploidy was also previously shown to increase tolerance to mitotic errors [29] and may result in appearance of binucleate-like cells [30] that were also readily observable in the -mutant FLs. Activation of p53 and its program including induction of p21 as a consequence of polyploidy has been observed experimentally upon addition of microtubule assembly inhibitors [31]. Interestingly, p21 could be also activated by hypoxia via transcription factor HIF-1 [32] supporting the possibility that both p53 and p21 activation could be augmented by severe hypoxia [33] as also documented by activation of the HIF-1 mRNA targets (Fig. S3D).

Smarca5 deficiency thus disrupted the proliferation of hematopoietic stem cells differentiating into erythroid lineage, affecting its development, and causing severe anemia. Tp53 activation appears to be a mediator of the proliferation blockade and apoptosis in the Smarca5 mutants.

**CONCLUSION**

Smarca5 deficiency suppressed hematopoietic development and caused erythroid dysplasia and anemia coincident with activation of the p53 and its program. If Smarca5 is not available the cell cycle progression becomes progressively inhibited at the MPP level at G2/M phase leading to erythroid defect and severe anemia.

**ACKNOWLEDGMENTS**

Primary support: GAČR 16-05649S, AZV 16-27790A, KONTAKT II LH15170 (TS) and NIH GM116143, DK096266 (AIS), and GAUK 815316 (JK). Institutional (TS): UNCE 204021, PRVOUK P24, LQ1604 NPU II and CZ.1.05/1.1.00/02.0109. The authors thank to Ivan A. Kanchev, MVSc, DVM, Attila Juhasz and Marketa Pickova from Czech Centre for Phenogenomics at BIOCEV for their counseling and assistance with histology. We acknowledge the IMCF at BIOCEV, institution supported by the MEYS CR (LM2015062 Czech-Biolimaging).

**AUTHOR CONTRIBUTIONS**

J.K. experimental design, cloning, cell biology, flow cytometry and phenotype analysis, T.Z., genotyping and mouse colony, F.S. Fluorescence-activated cell sorting, R.M. and W.E. blastocyst injection and ES transfection, AIS and T.S. designed the experiments and co-wrote the manuscript. Conflict-of-interest disclosure: The authors declare no competing financial interests.

**REFERENCES**


De Santis Puzzonia M, Gonzalez L, Ascenzi S et al. Tetraploid cells produced by absence of substrate adhesion during cytokinesis are limited in their proliferation and enter senescence after DNA replication. Cell cycle. 2015;0.


Figure 1: Smarca5 deletion (Vav1-iCre) results in anemia. (A) Phenotypic appearance of control Smarca5\textsuperscript{floxed/+} (left), heterozygous Smarca5\textsuperscript{floxed/+} (middle), and Smarca5 deficient Smarca5\textsuperscript{floxed/-} Vav1-iCre (right) sibling embryos at E15.5. (B) E15.5 Fls of the same genotypes. (C) FL cellularity (E13.5 upper, E14.5 lower) of control Smarca5\textsuperscript{floxed/+}, Smarca5\textsuperscript{floxed/+} and mutant Smarca5\textsuperscript{floxed/-} Vav1-iCre embryos. (D) Cytology (May-Grünwald-Giemsa) of E14.5 FL and (E) histology (H&E) of E13.5 (top) and E16.5 Fls (bottom) (F) Cytology (May-Grünwald-Giemsa) of E14.5 peripheral blood. Cell subtypes: (1) proerythroblast, (2) basophilic normoblast, (3) polychromatic n., (4) orthochromatic n., (5) reticulocyte, (6) erythrocyte, (7) myeloid precursor, (8) embryonic erythrocyte, and (9) atypical double-nucleated cell. Similar results were obtained in at least six repeat experiments. Two-tailed Student’s T-test (p < 0.00001 = ****).
Figure 2: Smarca5 deletion (Vav1-iCre) disturbs early hematopoiesis. (A) Flow cytometry analysis of control (Smarca5^floxs/^-/-/Rosa26^eYFP/^-/-/Vav1-iCre) and mutant Smarca5^floxs/^-/-/Rosa26^eYFP/^-/-/Vav1-iCre E13.5 FL with relative proportions of Sca1+ or Sca1- cells within the c-Kit+lin- cells. Gating of Lin- and c-Kit cells, which are subdivided into Lin- Sca1- c-Kit+ (LS-K) and Lin- Sca1+ c-Kit+ (LSK) populations were enriched for hematopoietic progenitors. LSKs are further subdivided according to CD48 and CD150 expression. (B) Absolute numbers of LSK and LS-K cell populations in E13.5 FL determined as a number of CD48+ and/or CD150+ LSK events per total FL cells that were analyzed. Controls: 1. Smarca5^floxs/^-/-/Rosa26^eYFP/^-/-; 2. Smarca5^floxs/^-/-/Rosa26^eYFP/^-/-; 3. Smarca5^floxs/^-/-/Rosa26^eYFP/^-/-Vav1-iCre; and mutant Smarca5^floxs/^-/-/Rosa26^eYFP/^-/-Vav1-iCre. (C) Cell cycle progression in LSK and LS-K cell populations in E13.5 FL. (D) Colony forming assay of hematopoietic progenitors derived from E13.5 FL of Smarca5^floxs/^-/-/Rosa26^eYFP/^-/-Vav1-iCre and mutant Smarca5^floxs/^-/-/Rosa26^eYFP/^-/-Vav1-iCre embryos scored by day 9 according to standard procedures. Similar results were obtained in at least six repeat experiments. Two-tailed Student’s T-test (p < 0.05 = *, p < 0.001 = **, p < 0.0001 = ****, p < 0.00001 = ****).
Figure 3: Smarca5-deleted progenitors express DNA damage mRNAs. (A) The 10 most significantly enriched categories in GO Biological Process database with 20-500 member genes according to Fisher’s exact test and Z-test both with FDR corrected significance below p=0.05. The x-axis represents a share of differentially regulated genes in a given category. Downregulated genes are represented as blue and upregulated as red. (B) Heatmap of differentially regulated genes involved in the KEGG p53-signaling pathway. Upregulation (yellow), downregulation (blue), scale indicates the fold change. Mutants: Smarca5<sup>flox5/Δ5-9</sup> Vav1-iCre c-kit+ samples (N=4); controls (N=7). The genes in bold were also measured within the Fig. S4E. (C) IF for cleaved Caspase-3 in the E16.5 FL (n=7). Caspase-3 positivity per 1 mm<sup>2</sup> is shown. Arrows indicate positive signals. (D) Immunoblots of FL lysates of Smarca5<sup>flox5/Δ5-9</sup> Vav1-iCre (within red rectangles) and controls either irradiated (2Gy) or non-irradiated. Irradiated NIH3T3 (15Gy) or HeLa cells were used as controls. Similar results were obtained in at least two repeat experiments.
**Figure 4: Smarca5 deletion (Vav1-iCre) results in blockade of erythropoiesis.** (A) Flow cytometric analysis of Ter119 and CD71 expression in the E13.5 FLs. Relative (upper) and absolute (bottom) cell numbers in the E13.5 FL. Mutant: Smarca5flox5/5-9 Rosa26<sup>eYFP</sup>/+ Vav1-iCre. Controls: Smarca5flox5/Rosa26<sup>eYFP</sup>/+; Smarca5flox5/5-9 Rosa26<sup>eYFP</sup>/+, Smarca5flox5/Rosa26<sup>eYFP</sup>/+ Vav1-iCre. (B) Evaluation of erythroid cell cycle progression in S1-S3 stages by flow cytometry of BrdU/7-ADD stained E13.5 FL. Bottom: Cell cycle kinetics of progenitor and erythroid cell populations (S0-S5) in control and mutant E13.5 FLs. (C) qPCR expression of selected hematopoietic/erythroid transcription factors from eYFP+ S0-S3 FACS-sorted cell populations in E14.5 FL. Controls: Smarca5flox5/Rosa26<sup>eYFP</sup>/+ Vav1-iCre (n=3) and mutants: Smarca5flox5/5-9 Rosa26<sup>eYFP</sup>/+ Vav1-iCre (n=3). (D) AnnexinV and propidium iodide staining in the FL eYFP+ fraction of E13.5 FL. Similar results were obtained in at least six repeat experiments. Statistics: two-tailed Student’s T-test (p < 0.05 = *, p < 0.001 = **, p < 0.0001 = ***, p < 0.00001 = ****).
Figure 5: Smarca5 deletion (Epor-iCre) inhibits erythropoiesis. (A) Phenotypic appearance of Smarca5<sup>flox5/+</sup> and Smarca5<sup>flox5/5-9</sup> Epor<sup>iCre</sup> newborns and postnatal day 4 (P4) siblings. (B) Flow cytometric analysis of E13.5 FL cells. Control (Smarca5<sup>flox5/+</sup> Rosa26<sup>eYFP/+</sup> Epor<sup>iCre</sup>) and mutant (Smarca5<sup>flox5/5-9</sup> Rosa26<sup>eYFP/+</sup> Epor<sup>iCre</sup>) FLs. Absolute cell numbers in E13.5 FL in S0-S5 erythroid differentiation stages are shown for controls: 1) Smarca5<sup>flox5/+</sup> Rosa26<sup>eYFP/+</sup>; 2) Smarca5<sup>flox5/5-9</sup> Rosa26<sup>eYFP/+</sup>; 3) Smarca5<sup>flox5/+</sup> Rosa26<sup>eYFP/+</sup> Epor<sup>iCre</sup>; and mutants Smarca5<sup>flox5/5-9</sup> Rosa26<sup>eYFP/+</sup> Epor<sup>iCre</sup>. (C) BrdU labeling of the FL S1-S3 erythroid populations in Smarca5<sup>flox5/5-9</sup> Rosa26<sup>eYFP/+</sup> Epor<sup>iCre</sup> FLs and appearance of polyploid cells. (D) AnnexinV and propidium iodide staining in the eYFP+ cell fraction of E13.5 FL. Control: Smarca5<sup>flox5/+</sup> Rosa26<sup>eYFP/+</sup> Epor<sup>iCre</sup>; mutant: Smarca5<sup>flox5/5-9</sup> Rosa26<sup>eYFP/+</sup> Epor<sup>iCre</sup>. (E) CFU numbers from FL-derived progenitors E13.5 of the genotypes same as in Figure 5E (F) Cumulative growth curve (y-axis log2 of 10<sup>6</sup> cells) of primary FL-derived erythroid cells (FL-EPs) prepared from E12.5 control (Smarca5<sup>flox5/flox5</sup>) and 4-hydroxytamoxifen-inducible (4-OHT) (Smarca5<sup>flox5/5-9</sup> CAG-Cre<sup>ERT2</sup>) embryos and grown in serum-free in vitro conditions with or without 4-OHT. CTRL: Smarca5<sup>flox5/flox5</sup>; CRE<sup>ERT2</sup>: Smarca5<sup>flox5/5-9</sup> CAG-Cre<sup>ERT2</sup>. Similar results were obtained in at least six repeat experiments. Two-tailed Student’s T-test (p < 0.05 = *, p < 0.001 = **, p < 0.0001 = ***).
Table 1: Distribution of genotypes in utero at embryonic (E12.5-E18.5) and postnatal (P) stages in the progeny of Smarca5\textsuperscript{-/\textDelta 5-9}Vav1-iCre and Smarca5\textsuperscript{flox/flox} mice. A cross indicates non-viable embryos.

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Smarca5\textsuperscript{flox/+}</th>
<th>Smarca5\textsuperscript{flox/+} Vav1-iCre</th>
<th>Smarca5\textsuperscript{flox/Δ5-9}</th>
<th>Smarca5\textsuperscript{flox/Δ5-9} Vav1-iCre</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>E12.5</td>
<td>11 (28.9%)</td>
<td>10 (26.3%)</td>
<td>11 (28.9%)</td>
<td>6 (15.8%)</td>
<td>38</td>
</tr>
<tr>
<td>E13.5</td>
<td>92 (27.4%)</td>
<td>71 (21.1%)</td>
<td>82 (24.4%)</td>
<td>91 (27.1%)</td>
<td>336</td>
</tr>
<tr>
<td>E14.5</td>
<td>48 (23.5%)</td>
<td>55 (27.0%)</td>
<td>50 (24.5%)</td>
<td>51 (25.0%)</td>
<td>204</td>
</tr>
<tr>
<td>E15.5</td>
<td>15 (27.8%)</td>
<td>9 (16.7%)</td>
<td>15 (27.8%)</td>
<td>15 (27.8%)</td>
<td>54</td>
</tr>
<tr>
<td>E16.5</td>
<td>10 (23.8%)</td>
<td>12 (28.6%)</td>
<td>9 (21.4%)</td>
<td>11 (26.2%)</td>
<td>42</td>
</tr>
<tr>
<td>E17.5</td>
<td>4 (16.0%)</td>
<td>8 (32.0%)</td>
<td>7 (28.0%)</td>
<td>6 (24.0%)</td>
<td>25</td>
</tr>
<tr>
<td>E18.5</td>
<td>9 (21.4%)</td>
<td>10 (23.8%)</td>
<td>10 (23.8%)</td>
<td>12 (28.6%)</td>
<td>41</td>
</tr>
<tr>
<td>E12.5-E18.5</td>
<td>189 (25.5%)</td>
<td>175 (23.6%)</td>
<td>184 (24.9%)</td>
<td>192 (25.9%)</td>
<td>740</td>
</tr>
<tr>
<td>Postnatal</td>
<td>32 (37.2%)</td>
<td>30 (34.9%)</td>
<td>24 (27.9%)</td>
<td>0 (0.0%)</td>
<td>86</td>
</tr>
</tbody>
</table>
Graphical Abstract: Smarca5 requirement during mouse hematopoietic development. Hematopoietic stem cells (HSCs) lacking Smarca5 temporarily activate proliferation followed by G2/M arrest during erythroid and myeloid differentiation leading to fatal anemia with tetraploid erythroblasts and p53 activation. Erythroid-specific deletion of Smarca5 resulted in milder phenotypes: anemia and tetraploidy.