Univerzita Karlova v Praze
1. Lekářská Fakulta

Charles University in Prague
First Faculty of Medicine

Autoreferát disertační práce

Summary of the Ph.D. Thesis

Role chromatin remodelačné ATPázy Smarca5 v krvetvorbě a vývoji červených krvinek

Role of Smarca5 (Snf2h) chromatin remodeling ATPase in hematopoietic development and erythropoiesis

Mgr. Juraj Kokavec

Školitel/Supervisor: **Prof. MD. Tomáš Stopka, Ph.D.**

Praha, 2017

CONTENT

ABSTRAKT

Jaderný protein Smarca5 (Snf2h) je jeden z nejvíce konzervovaných chromatin remodelačních faktorů a molekulárních motorů u savců. ATPáza Smarca5 (a ostatní příbuzní zástupci proteinové nadrodiny SWI/SNF2) společně se svými vazebnými partnery vytváří několika podjednotkové remodelační komplexy, jež posouvají, odstraňují nebo přináší histonové oktamery na DNA za účelem regulace trankripce, replikace, oprav poškození DNA a jiných nepostradatelných buněčných funkcí. Ve své disertační práci se zabývám studiem myšího modelu delece genu *Smarca5* na počátku definitivní krvetvorby. Jedinci s delecí genu *Smarca5* v hematopoetické kmenové buňce (aktivní promotor Vav1) umírají během pozdního vývoje *in utero* v důsledku závažné anémie. Ve fetálních játrech těchto experimentálních zvířat jsme pozorovali akumulaci krvetvorných kmenových a progenitorových buněk (HSPCs) a inhibici jejich dozrávání do erythroidní a myeloidní buněčné řady. Popsané poruchy vývoje byly doprovázeny dysplastickými změnami na úrovni proerytroblastů a poruchami buněčného cyklu na přechodu z G2 do M fáze u bazofilních erytroblastů. Dále jsme pozorovali, že nedostatečná funkce Smarca5 zvyšuje hladinu tumor supresorového proteinu p53 v erythroidních progenitorech, aktivaci jeho transkripčních cílů a k postranslačním modifikacím, jež jsou specifické pro poškození DNA - fosforylace ($S18^{Phos}$) a acetylace s CBP/p300 ($K382^{Ac}$). Nepostradatelnou funkci proteinu Smarca5 v erytropoéze jsme dále potvrdili využitím myšího modelu delece genu v již determinovaných erytroidních buňkách. Experimentální jedinci s delecí Smarca5 vázanou na aktivní promotor genu Epor (receptor pro erythropoietin) vykazovali podobné fenotypové změny jako jedinci s delecí v hematopoetické kmenové buňce včetně závažné embryonální anémie. In vitro studium buněčných kultur odvozených z těchto zvířat potvrdilo sníženou schopnost erythroidních progenitorů proliferace a maturace do diferenciovanějších stádií. Z popsaných dat usuzujeme, že Smarca5 hraje nepostradatelnou roli jak v časné krvetvorbě, tak i ve vývojově pozdějším vývoji červených krvinek.

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 (S-18) second with CBP/p300 (K382Ac), 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.

INTRODUCTION

Chromatin remodeling

Chromatin is a highly organized structure made of DNA, proteins and RNA molecules whose main purpose is to compress, protect and reinforce DNA in the eukaryotic nucleus and provide a stage for regulation of gene expression and DNA replication. Nucleosome is a basic structural unit of chromatin composed of a DNA macromolecule wrapping around the histone octamer composed core heterotetramer of H3 and H4 histones and two peripheral H2A/H2B heterodimers. Individual nucleosomes are separated by a short connecting linker DNA, which is occupied by linker histone H1 and additional DNA-binding proteins e.g. HP1 and Hmgb1. These are indispensable for higher degrees of chromatin compaction and contribute to barrier hardly accessible to other DNA-binding proteins, transcription factors and replicative machinery, alike. Chromatin, however is not a rigid structure, but a structural organization moldable by enzymatic posttranslational modifications of protruding N-terminal tails or globular domains of core histones, as well as H1 linker histone.

The ATP-dependent chromatin-remodeling complexes on the other hand are molecular motors which disrupt non-covalent histone-DNA interactions using chemical energy stored in ATP to facilitate either nucleosome octamer removal, destabilization, histone variant replacement, sliding or *de novo* deposition in a process called as **nucleosome assembly** (Cairns, 2007). The functional core of these enzymes is the DExH-box ATPase/helicase domain, an ancient protein fold whose appearance dates back to first prokaryotic organisms. We currently recognize several groups of the chromatin remodeling ATPases, most notable subfamilies are Imitation Switch (ISWI), Switch/Sucrose non-fermenting (SWI/SNF), Chromodomain-helicase-DNA-binding (CHD) and Inositol-requiring 80 (INO80), first described in yeast studies. Mammalian Smarca5 and Smarca1 proteins belong to the Imitation Switch (ISWI) subfamily of SNF2/RAD54 superfamily of chromatin remodeling ATPase/helicases. They are characterized by two C-terminal HAND domains, a SANT (Swi3, Ada2, N-CoR, TFIIIB) and one SLIDE (SANT-like ISWI) domains, which are involved in substrate recognition (Boyer et al., 2004; Grune et al., 2003) and interaction with their non-catalytic heterodimerizing partners. These accessory proteins contain additional domains that increase the functional diversity of ISWI ATPases (He et al., 2008).

Mammalian hematopoiesis

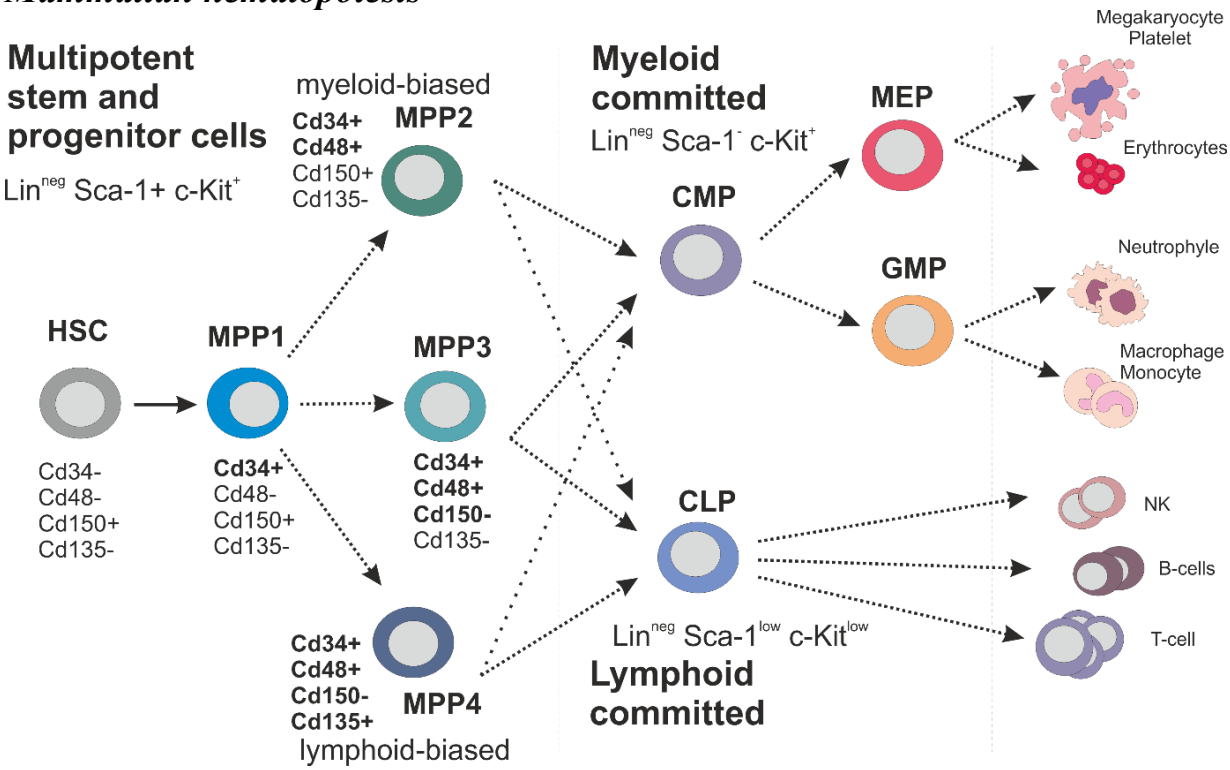

Figure 1: The hematopoietic tree: Classification of hematopoietic stem cells and progenitors upon surface marker expression (adapted from Cabezas-Wallscheid et al., 2014) MPP = multipotent progenitors, CMP = common myeloid progenitors, CLP = common lymphoid progenitors, MEP = megakaryocytic/erythroid progenitors, GMP = granulocyte/macrophage progenitors.

Erythroid lineage specification is the result of concerted activity of multiple transcription factors. The promotion of **megakaryocytic/erythroid lineage** from multipotent hematopoietic stem cells (HSCs) is accompanied by sweeping changes in transcription regulatory networks. Lmo2, Ldb1 and Tal1/Scl indispensable for HSCs are required also for promotion of erythroid lineage (Wadman et al., 1997). Three transcription factor are first employed for yolk sac erythropoiesis, where Lmo2 acts with Tal1/Scl to regulate RBC development and with Ldb1 to maintain erythroid precursors in an immature state. However, the most notable change in the regulatory landscape, is the replacement of Gata2 (hematopoietic TF) for Gata1 (erythroid TF) as dominant GATA factor, referred to as a "**GATA switch**" in promoting **erythroid lineage development** (Grass et al., 2003; Martowicz et al., 2005). Gata1 and Gata2 bind overlapping sets of genes, but often act oppositely on their target genes, and as result many HSC specific genes promoted by Gata2 are silenced by Gata1. During erythroid differentiation Gata1 displaces Gata2 to become the dominant GATA factor to sustain *Tal1/Scl* expression. Gata1 inhibits proliferation by inhibiting *c-Kit* expression and mediates cell cycle arrest in G1. This is antagonistic to c-Kit signaling which promotes c-Myc-sustained proliferation of erythroid progenitors.

The core erythroid transcription regulatory complex consist of Gata1, Tal1/Scl, Lmo2, Ldb1, Tcf3/E2A, Fli1, Zfpm1 and is required for activation of *Klf1* transcription and inhibition of hematopoietic stem cell genes in erythroid progenitors. During transition from hematopoietic transcription program, Gata2 is replaced by Gata1 in the complex and target specificity of the complex changes. *Gata1* mutant mice die early during embryonic development (E10.5 and E11.5) due to developmental arrest in proerythroblast stage and decreased survival of committed erythroid precursors (Fujiwara et al., 1996). Initially, *Gata1* is expressed in HSCs at very low levels, its expression increases as cells adopt myeloid fate and culminates in proerythroblast stage of erythroid development. Another powerful regulator of erythroid fate is corepressor Rcor1/CoREST, which promotes erythropoiesis by repressing HSC and/or progenitor genes, as well as genes and signaling pathways that lead to myeloid cell fate (e.g. Csf2r). *Rcor1-/-* embryos are anemic and die in late gestation around E16.5 as definitive erythroid cells from arrest at the transition from proerythroblast to basophilic erythroblast (Yao et al., 2014). Immediately upon erythroid commitment Gata1 activates the transcription factor Klf1/Eklf (Crossley et al., 1994); Welch et al., 2004) along with many other erythroid-specific genes. *Klf1*-/- embryos die of severe anemia by E16.5 due to defective definitive erythropoiesis. Klf1 is required for adult β-globin chain (*Hbb-b1* and *Hbb-b2*) expression (Perkins et al., 1995; Tewari et al., 1998).

Main aspects of erythroid lineage development is balancing the **proliferation vs. differentiation** and controlling survival of maturating red blood cells. Erythropoietin (Epo) and its receptor (Epor) are also essential for RBC production and deliver key signals for erythroid cell maturation and survival (Richmond et al., 2005). They do so through multiple signaling pathways, including JAK/STAT, to induce anti-apoptotic factor *Bcl2l1/Bcl-X_L* and *Gata1* expression (Socolovsky et al., 2001). *Stat5a/5b*-double KO mice are anemic, as they have reduced FL erythropoiesis, however some may survive into adulthood and fully recover (Socolovsky et al., 1999). On the other hand, both *Epo*- and *Epor*-knockout mice die between E13.5-E15.5 due to diminished yolk sac as well as definitive FL erythropoiesis but not progenitor specification as normal numbers of committed erythroid BFU-E and CFU-E progenitors are found in the fetal livers (Wu et al., 1995). In contrast, Jak2-/- embryos die before E13.0 as virtually no yolk sac and FL erythropoiesis can be observed after no BFU-E and CFU-E progenitors are generated in either hematopoietic organ (Neubauer et al., 1998). The repression of *c-Kit* and its downstream substrates by Gata1 inhibits cell proliferation during erythroid maturation (Munugalavadla et al., 2005).

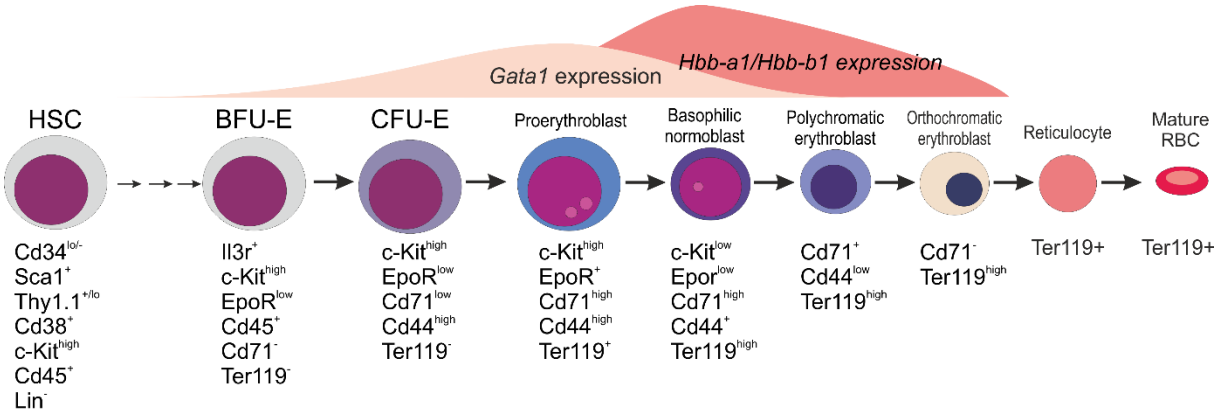

Figure 5: Schematic view of erythroid lineage development, proceeding from immature HSCs to fully maturated red blood cells.

ISWI chromatin remodeling during mammalian development

The two ISWI ATPases Smarca5 and Smarca1 play indispensable roles during mammalian development. Our laboratory has previously shown that Smarca5 is required for early embryonic development as *Smarca5*-/- mice die before gastrulation due to defective inner cell mass specification (Stopka and Skoultchi, 2003). *Smarca5* is also abundantly expressed in reproductive system during ovarian follicular genesis, especially in the hyperplastic granulosa cells before ovulation (Pepin et al., 2007) and in testes (Thompson et al., 2012). During spermatogenesis, *Smarca5* expression is required for epigenetic reprogramming of germ cells in pachytene stage of spermatocyte differentiation (Chong et al., 2007). Smarca5 is also required for the activation of αA-crystallin gene during lens differentiation (Yang et al., 2006). *Smarca5* is coexpressed with its homologue *Smarca1* also in neural tissues, where Smarca5 is predominantly associated with proliferating neurons, while Smarca1 is associated with terminally differentiated neurons (Lazzaro and Picketts, 2001). The disruption of chromatin structure in deficient neural cell line nuclei lead to an observation that Smarca5 *de facto* serves as a deposition cofactor for H1 linker histone and thus indirectly regulates chromatin density in the neural cell progenitors (Alvarez-Saavedra et al., 2014). *Smarca5* is predominantly expressed in highly proliferating tissues of the hematopoietic system. This is most evident in the case of the fetal liver at peak erythropoiesis E13.5-E15.5, when the FL output is the highest and many progenitor cells populations are expanding. *Smarca5* expression is associated with actively dividing HSPCs, however upon differentiation, *Smarca5* expression sharply decreases. We observed this phenomenon in adult mice after experimentally induced hemolytic anemia (Stopka et al., 2000), when after a brief expansion of erythroid progenitor populations *Smarca5* expression in BM and spleen is decreased. In addition, the antisense inhibition of *SMARCA5* expression in cultured human leukemic CD34+ progenitors suppress cytokine-induced erythropoiesis (Stopka and Skoultchi, 2003), thus establishing Smarca5 as an important regulator of proliferation and differentiation of hematopoietic progenitors.

HYPOTHESIS

Our laboratory has previously shown an indispensable role of Smarca5 in proliferation of human and mouse hematopoietic progenitors. However, the early lethality of homozygous mutants, prevented further studies of *Smarca5* deficiency during hematopoietic development (Stopka and Skoultchi, 2003; Stopka et al., 2000). In order to further elaborate on these initial observations we designed an inducible model for *Smarca5* deletion in mouse. We hypothesized, that because of high *Smarca5* expression in fetal liver, its loss of function should at first instance affect fetal liver hematopoietic development. However, we could not identify the exact stage when this requirement would be most critical as mammalian hematopoiesis is a complicated multistage process involving both proliferation and differentiation. Thus we decided to induce early pan-hematopoietic (Vav1-iCre) and later more lineage restricted erythroid (Epor-iCre) loss and observe the phenotypic outcome in the two diametrically opposing conditions. Initially, the specific role of Smarca5 in the maintenance of hematopoietic cells was unknown, thus we used this set of different models to get further insight into cellular functions of Smarca5 in hematopoietic tissues and pave the road to further advance our understanding of Smarca5 function in cell.

MATERIALS AND METHODS

Flow cytometry and FACS

The erythroid development and hematopoietic development in fetal liver (FL) was assessed using differentiation-associated surface markers Cd71, Ter119, Cd45, c-Kit antigens and LSK SLAM cocktails based on published protocols (Kiel et al., 2005; Pop et al., 2010). Hematopoietic stem / erythroid cell proliferation in the fetal liver was analyzed using the *in vivo* BrdU staining. Pregnant females from timed mating (at E13.5) received 200µl of BrdU (10 mg/ml in PBS) intraperitoneally. FLs were stained with the Lineage+ cocktail, Sca1, c-Kit, Cd48 and Cd150 antibodies or Cd71, Ter119, Cd45. BrdU incorporation was detected using the APC BrdU Flow Kit.

Mouse Colony-Forming Cell Assays

Colony-forming cell assays used Methocult M3434 optimized for murine hematopoietic cell growth (StemCell Technologies) at cell density of $4x10^4$/well in 5% CO_2 and humidified atmosphere at 37ºC for duration of 12 days. Colonies were scored at days 3/4 (CFU-E) and 11 (BFU-E, CFU-GEMM, CFU-GM, CFU-G, CFU-M) after seeding.

Microarray data analysis and expression assays

For expression analysis c-Kit progenitors were isolated from E14.5 FLs and magnetically sorted by AutoMACS cell sorter. For microarray analysis we used Affymetrix GeneChip Mouse Genome 430 2.0 Array with 45101 probes. cDNA was amplified in 8 µl 1x SYBR-Green Master Mix reaction (Applied Biosystems). Amplifications consisted of 40 cycles of 10 seconds at 95°C, 20s at 60°C and 30s at 72°C. Expression data was normalized to average expression of *Hprt1* and *Gapdh*.

Western blot

For western protein lysates of E14.5 FLs were prepared in Radio Immunoprecipitation Assay (RIPA) buffer supplemented with protease and phosphatase inhibitors.

TUNEL assay, histology, cytology, Caspase-3 staining

Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) assay was done according to the DeadEnd™ fluorometric TUNEL system (Promega, USA) following the manufacturer's instructions. Cytology was performed on cytospun cells & stained by Giemsa-May-Grunwald. Histology was performed on 4% paraformaldehyde-fixed material that was embedded in paraffin. Cleaved Caspase-3 in E16.5 embryos was detected utilizing 1:1000 dilution of antibody (Abcam, ab52293).

Isolation and cultivation of fetal liver-derived erythroid progenitors (FL-EP)

Fetal liver-derived erythroid progenitors (FL-EP) were isolated from E12.5 control *Smarca5*$^{flox5/flox5}$ and *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Cre-Esr1* embryos harboring a 4-hydroxytamoxifen (4-OHT) inducible estrogen receptor/Cre-recombinase fusion transcript upon the control of human β-actin promoter (Tg(CAG-cre/Esr1^{tax})^{5Amc} (Hayashi and McMahon, 2002). FL-EP cultures were maintained by full or partial medium changes in cell culture incubator (set to 37°C, 5% CO2) (Dolznig et al., 2005).

RESULTS

Smarca5 is required for definitive hematopoiesis.

Our previous study using conventional *Smarca5* knockout (*Smarca5*$^{\Delta 5\text{-}9}$) model has shown that *Smarca5*-null mouse embryos die shortly after implantation (Stopka and Skoultchi, 2003). To study the role of Smarca5 in the adult definitive hematopoiesis, we have developed a conditional model of *Smarca5* deletion in mouse (Fig.3A) and utilized a transgene that expresses iCre recombinase driven by the *Vav1*-promoter in all hematopoietic tissues including lymphoid, myeloid and erythroid progenitors (de Boer et al., 2003). *Vav1-iCre* transgenic mice were bred with *Smarca5*$^{+/\Delta 5\text{-}9}$ mice to produce the *Smarca5*$^{+/\Delta 5\text{-}9}$ *Vav1-iCre* males, which we mated with *Smarca5*$^{flox5/flox5}$ females. We initially observed *Smarca5*$^{flox5/\Delta 5\text{-}9}$ *Vav1-iCre* mice are not born, while other genotypes (*Smarca5*$^{flox5/+}$, *Smarca5*$^{flox5/+}$*Vav1-iCre*, and *Smarca5*$^{flox5/\Delta 5\text{-}9}$) were produced in the expected numbers. The lack of *Smarca5*$^{flox5/\Delta 5\text{-}9}$ *Vav1-iCre* mice in the litter of *Smarca5*$^{+/\Delta 5\text{-}9}$ *Vav1-iCre* and *Smarca5*$^{flox5/flox5}$ mice prompted us to perform analysis also *in utero*. Therefore, we examined embryos generated in timed mating between days E11.5-18.5. Indeed, we have observed that all *Smarca5*$^{flox5/\Delta 5\text{-}9}$ *Vav1-iCre* embryos were pale. The phenotype has full penetrance and is evident as early as E13.5. The anemia is readily observable by E14.5 (Fig. 3B). Mutant embryos have significantly smaller fetal liver (FL) (Fig. 3C) which is paler and consequently has decreased cellularity (Fig. 3D).

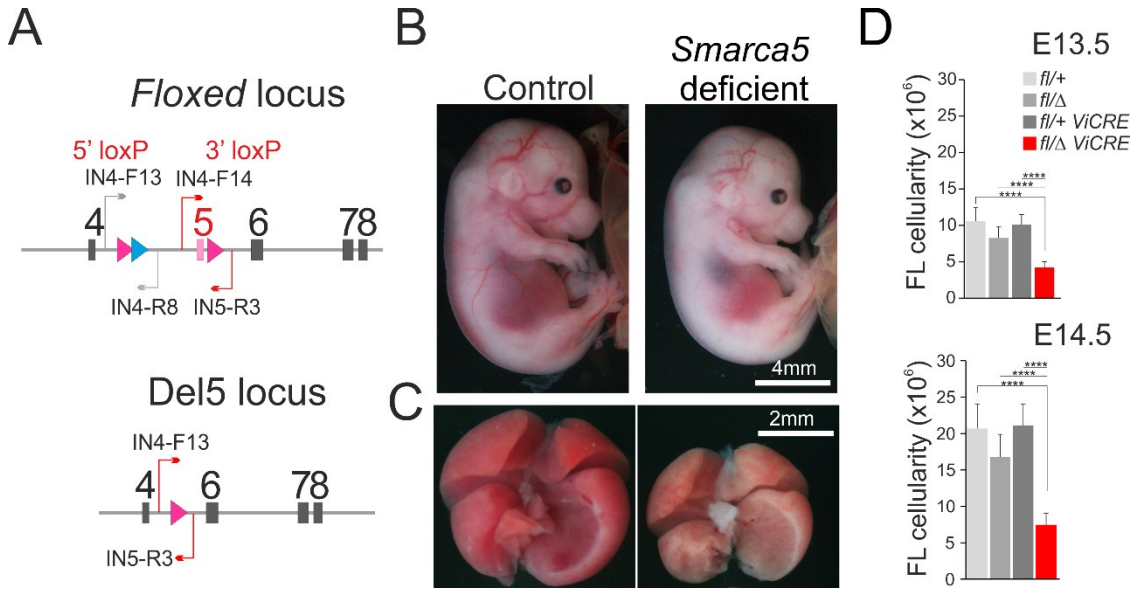

Figure 3: *Smarca5* deletion (Vav1-iCre) results in anemia. **(A)** Genetic modification of mouse Smarca5 locus (exon 5 is highlighted, *loxP* and *frt* sites are indicated as red and blue triangles, respectively) **(B)** Phenotypic appearance of control *Smarca5*$^{flox5/+}$ and Smarca5 deficient *Smarca5*$^{flox5/\Delta 5\text{-}9}$ *Vav1-iCre* (right) sibling embryos at E15.5 and **(C)** E15.5 FLs of the same genotypes. **(D)** FL cellularity (E13.5 and E14.5) of control *Smarca5*$^{flox5/+}$, *Smarca5*$^{flox5/\Delta 5\text{-}9}$ and mutant *Smarca5*$^{flox5/\Delta 5\text{-}9}$ *Vav1-iCre* embryos. Two-tailed Student's T-test ($p < 0.00001$ = ****).

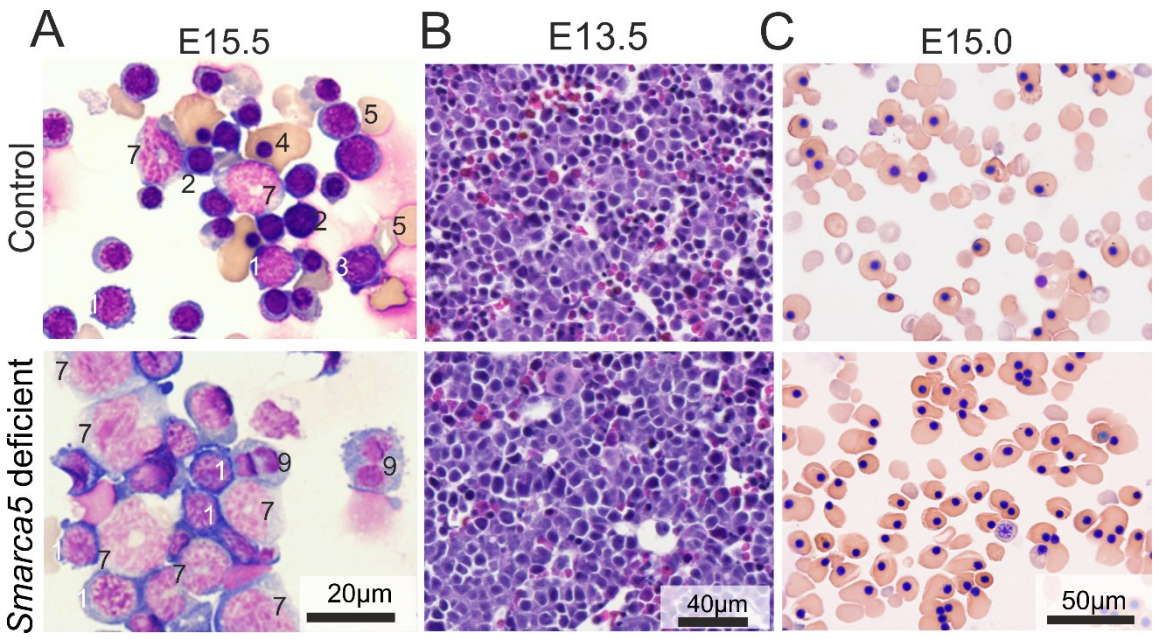

Figure 4: Cytological examination of fetal liver (FL) and peripheral blood (PB). (A) May-Grünwald-Giemsa stained cytospined fetal liver cells at day E14.5 fetal liver and **(B)** peripheral blood cells around E15.0. Cell subtypes: (1) proerythroblast, (2) basophilic normoblast, (3) polychromatic n., (4) orthochromatic n., (5) reticulocyte, (6) erythrocyte, (7) myeloid precursor, (8) embryonic erythrocyte, and (9) atypical double-nucleated cell. **(C)** Hematoxylin & Eosin (H&E) staining of E13.5 fetal liver of control (*Smarca5*$^{+/flox5}$) and mutant sibling (*Smarca5*$^{flox5/\Delta5\text{-}9}$*; Vav1-iCre*) embryo.

The pale color and decreased size of fetal liver in mutants prompted further examination of fetal liver structure. Cytological examination between E12.5 and E15.5 revealed profound lack of maturing erythroid cells in *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Vav1-iCre* mutants while control FLs contained a full set of maturing erythroid stages. Most evident was the lack of small rounded basophilic erythroblasts. These cells represent the majority of erythroid progenitors in wild-type FL (Fig.4A). The *Smarca5*$^{flox5/\Delta5\text{-}9}$ Vav1-iCre FLs lack the typical organization of hepatic cells around major veins, erythropoietic clusters and basophilic erythroblast scattered between hepatoblasts. The result is the disruption of acini and depletion of maturing erythroid cells in favor of larger immature hematopoietic cells. Furthermore, mutant FLs frequently displayed dysplastic changes such as atypical and often binucleate proerythroblasts (Fig.4B). Arrested erythroid development in the mutant FLs was reflected also in the peripheral blood (PB). Blood smears from E14.5 embryos showed decreased numbers of definitive (non-nucleated) erythrocytes (Fig.4C).

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

The early activity and specificity of *Vav1-iCre* transgene makes it a handful tool to study early hematopoietic development in mouse (de Boer et al., 2003). To distinguish between individual progenitor cell populations in E13.5 FL we used flow cytometry. HSPCs are highly c-Kit+ expressing cells, which at the same time are negative for lineage determinants designated as LK or ($Lin^-c\text{-}Kit^+$). The surface expression of Sca1 antigen is used to distinguish between LS-K ($Lin^-Sca1^-c\text{-}Kit^+$) and LSK ($Lin^-Sca1^-c\text{-}Kit^+$).

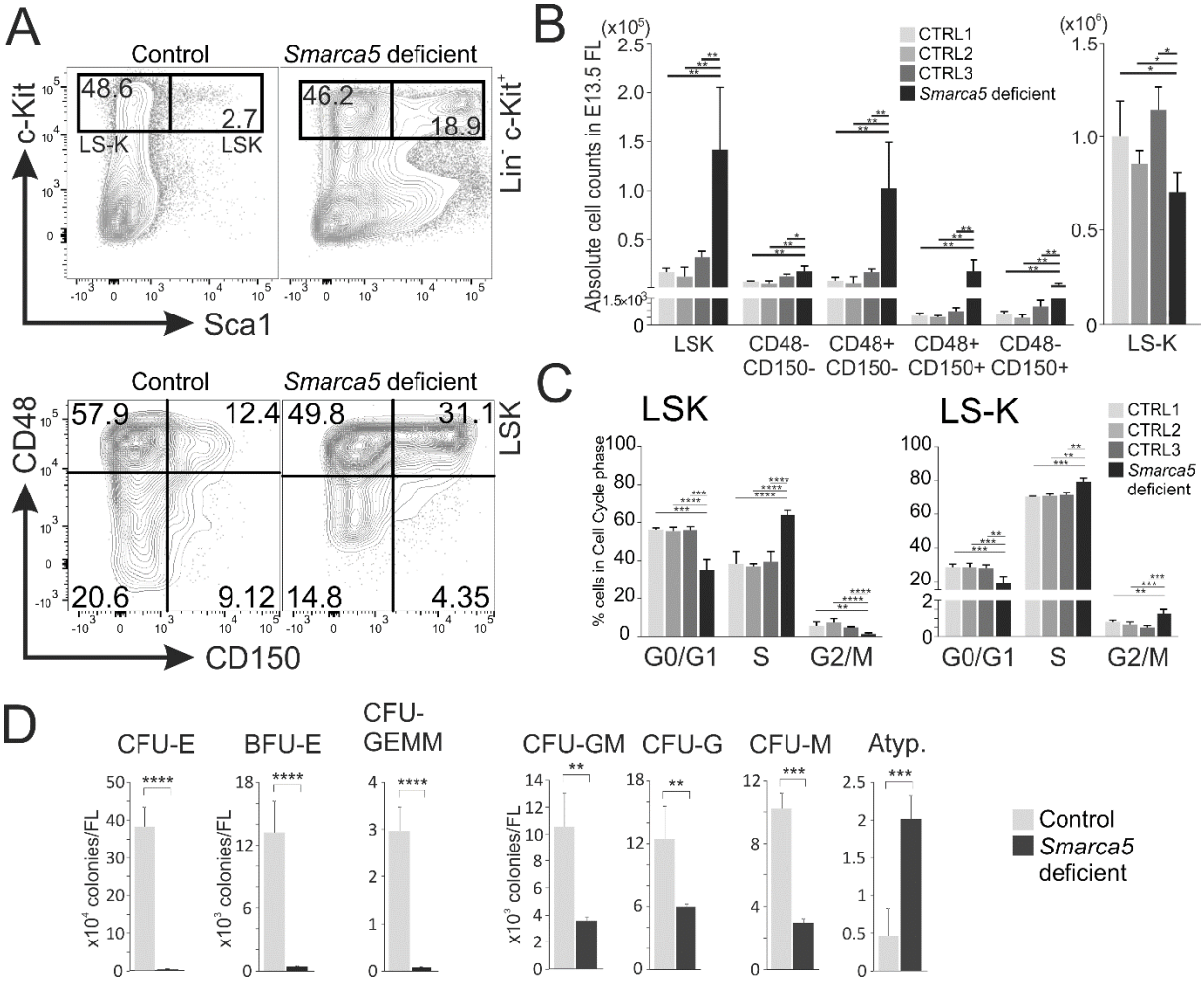

Figure 5: *Smarca5* deletion (Vav1-iCre) disturbs early hematopoiesis. (A) Flow cytometry analysis of control (*Smarca5*$^{flox5/+}$ *Rosa26*$^{eYFP/+}$ *Vav1-iCre*) and mutant *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Rosa26*$^{eYFP/+}$ *Vav1-iCre* E13.5 FL with relative proportions of Lin- Sca1+ c-Kit+ (LSK) and $Sca1^+$ or Lin- Sca1- c-Kit+ (LS-K) cells within Lin-$c\text{-}kit^+$ FL cells. 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*$^{flox5/+}$ *Rosa26*$^{eYFP/+}$; 2. *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Rosa26*$^{eYFP/+}$; 3. *Smarca5*$^{flox5/+}$ *Rosa26*$^{eYFP/+}$ *Vav1-iCre*; and mutant *Smarca5*$^{flox5/\Delta5\text{-}9}$ *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*$^{flox5/+}$ *Rosa26*$^{eYFP/+}$ *Vav1-iCre* and mutant *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Rosa26*$^{eYFP/+}$ *Vav1-iCre* embryos scored by day 9 according to standard procedures. Two-tailed Student's T-test ($p < 0.05$ = *, $p < 0.001$ = **, $p < 0.0001$ = ***, $p < 0.00001$ = ****).

However, only LSK cells contain the true multipotent progenitors. In contrast, LS-K cells include fast cycling and more differentiated progenitors of myeloid, early lymphoid or erythroid lineages. Based on expression of SLAM-specific (CD48, CD150) antigens, we can distinguish four distinct multipotent progenitor (MPP) FL stem cell populations within LSK. The most primitive group of multipotent FL progenitors is defined as MPP1 or LSK $CD48^{-}$ $CD150^{+}$). The latter are myeloid-biased MPP2 (LSK $CD48^{+}CD150^{+}$), myelo-lymphoid MPP3 (LSK $CD48^{+}CD150^{-}$) and erythromegakaryocytic-biased (LSK $CD150^{-}CD48^{-}$) progenitors (Kiel et al., 2005). The Ery/Mk-biased LSKs represent the short-term repopulating and stress-responsive MPPs (Oguro et al., 2013). The coexpression of Cd34 and Flt3/CD135 also defines progenitors with lymphoid potential (MPP4) (Cabezas-Wallscheid et al., 2014); however, we have not addressed these in the present study.

Initially, we have observed that upon deletion of *Smarca5* in E13.5 embryos, there was a relative increase of Sca1+ cells in mutant FLs. This phenomenon was also reflected as a relative increase of LSK cells (from 2.7 to 18.9, Fig.5A). In absolute numbers the increase of LSK was at least 5-fold (Fig.5B). Conversely, the absolute numbers of LS-K cells have slightly decreased (Fig.5B). Further analysis using SLAM antigens revealed that there was at least 20-fold increase in myeloid-biased MPP2 ($LSK^{+}CD48^{+}CD150^{+}$) and 5-fold increase in myeloid/lymphoid MPP-3 ($CD48^{+}CD150^{-}$). In the case of MPP1 (LSK $CD48^{-}CD150^{+}$) and Ery/Mk-biased LSKs ($CD150^{-}CD48^{-}$) the increase was moderated (Fig.5B). The relative proportions of MPP cells were disturbed slightly; however, we have observed that there was a net increase in Cd48+ c-kit+ progenitors (Fig.5C), suggesting that *Smarca5* loss is less detrimental for myeloid-biased progenitors in mutant FLs. To gain insight into the cell cycle dynamics of the accumulated HSCPs in mutant FL we performed *in vivo* labeling in E13.5 embryos. We have found that the percentage of the LSK cells in S-phase was increased relatively to controls (50% increase), whereas the proportion of these cells in G0/G1 and G2/M phases was decreased (Fig.5C). This results show that FL progenitors in mutant embryos respond to *Smarca5* deletion apparently by increasing their proliferation activity. Nevertheless, the committed progenitors within the $LS^{-}K$ compartment – the population of latter FL progenitors – additionally exhibit a significantly increased proportion of blocked cells in G2/M phase (Fig.5C), suggesting that these progenitors normally enter the cell cycle but become inhibited/blocked in their proliferation. This eventually leads to depletion of a progenitor pool available for production of mature blood cells.

The FL-derived progenitors from control E13.5 embryos formed both erythroid (BFU-E, CFU-E) (Fig.15A), myeloid (CFU-GM, CFU-GG, CFU-M), and multi-potential (CFU-GEMM) colonies; however, the colony formation ability of the *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Vav1-iCre* FL progenitor cells was severely reduced (Fig.5D). These results indicate that the number of earliest FL HSPCs in *Smarca5*-deficient embryos was increased due to excessive cycling but further differentiation to make mature blood cells is blocked.

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

In the approach to identify lineage-specific or other maturation-associated genes in mutant HSPCs, we compared the expression levels of 39,000 transcripts in E15.5 mutant (*Smarca5*$^{\Delta5/\Delta5\text{-}9}$ *Vav1-iCre*) and control FL-derived and magnetically sorted Kit+ progenitors by hybridization to Affymetrix GeneChip Mouse Genome 430 2.0 Array with 45,101 probes. The majority of differentially expressed genes belong to Gene Ontology (GO) categories linked to response to stress, cell cycle regulation, DNA damage, DNA repair, programmed cell death (Fig.6A).

One of the most differentially expressed GO categories in arrays belong to the p53-signaling pathway. This signaling pathway is activated by wide range of unfavorable stimuli, including DNA damage, lack of available nutrients for growth, tissue inflammation or hypoxia (Fig.18). The chromatin integrity plays a vital role in ensuring cell survival, therefore we concentrated to p53-signaling and DNA-damage response (DDR) pathways ranking between the highest GO biological categories. When the cellular damage becomes irreparable, severely damaged cells undergo programmed cell death. One of the initial steps involve cleavage of Caspase-3 (Casp3) proenzyme by upstream effector into two heterodimeric (p12 and p17) components of active enzyme, which we detect by immunohistochemistry in E16.5 FLs (Fig.19A). The data show that the mutant FLs activated Caspase-3 while the control FLs showed very low frequency of Caspase-3-positive cells.

To address whether DNA damage response pathways (DDR) were certainly activated upon *Smarca5* deletion in early hematopoietic progenitors, we tested the protein expression of activated checkpoint kinases Chk1 and Chk2 in E13.5 FL. However, we could not find any indications that these kinases were phosphorylated at residues S345 (Chk1) or T68 (Chk2) (Fig.20A). However, the absence of these modifications does not exclude other means of p53 activation; therefore, we tested different posttranslational modifications of p53 linked to DDR, including the protein expression levels of p53 and phosphorylation at Serine 18 (S18^{Phos}).

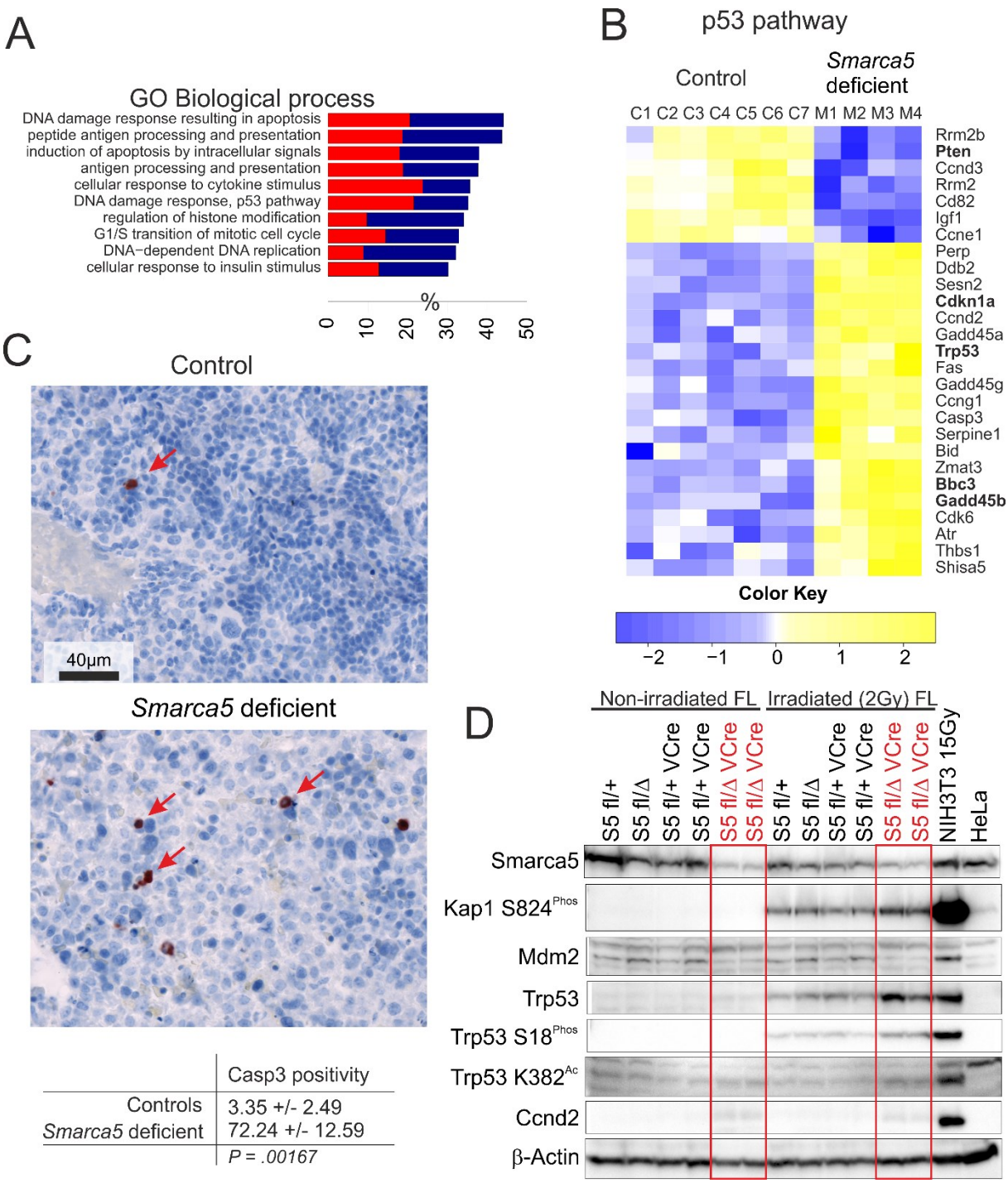

Figure 6: *Smarca5*-deleted progenitors express DNA damage mRNAs. (A) The 10 most significantly enriched categories in GO Biological Process database with FDR corrected significance below p=0.05. The x-axis represents a share of differentially regulated genes in a category. Downregulated genes are represented as blue and upregulated as red. **(B)** Heatmap of differentially regulated genes of the p53-signaling pathway. Upregulation (yellow), dowbregulation (blue), scale indicates the fold change. Mutants: *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Vav1-iCre c-kit*+ samples (N=4); controls (N=7). **(C)** IF for cleaved Caspase-3 in the E16.5 FL. Caspase-3 positivity per 1 mm^2 is shown. Arrows indicate positive signals. **(D)** Western blotting of FL lysates of *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Vav1-iCre* (within red rectangles) and contols, either irradiated (2Gy) or not irradiated. Irradiated NIH3T3 (15Gy) or HeLa cells were used as controls.

Phosphorylated S15 was previously associated with DDR activation in human cell lines and is equivalent to mouse S18 (Ullrich et al., 1993). As expected expression of p53 is almost undetectable in non-irradiated controls (Fig. 6D). Neither have we detected any p53 S18 phosphorylation. Nevertheless, irradiated controls had both increased p53 expression and $S18^{Phos}$ modification, proving that the approach works well for irradiated FL cells. Interestingly, in both non-irradiated and irradiated FL cells the expression of p53 and $S18^{Phos}$ modification was higher than respective controls, suggesting a genuine activation of p53 in *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Vav1-iCre* mutant FLs. Interestingly, both irradiated as well as non-irradiated mutants had reciprocally decreased levels of Mdm2 protein, an E3 ligase responsible for p53 degradation. To complement the image, we also checked the acetylation of p53 on Lysine 382 (pTp53 $K382^{Ac}$). The acetylation of this residue is mediated by either p300 or CBP (Crebbp) histone acetylases (Sakaguchi et al., 1998) and is indispensable for transcriptional activity of p53 protein. One the transcriptional target of p53 we detected in E13.5 FL was cyclin D2 (Ccnd2), again upregulated only in mutant FLs. Another modification we detected was the phosphorylation of Kap1 – E3 SUMO-protein ligase and pleiotropic regulator of chromatin structure – at Serine 824 (S824) by ATM/ATR upon DNA damage. ATM-induced phosphorylation on Ser-824 represses sumoylation leading to the de-repression of expression of a subset of genes involved in cell cycle control and apoptosis in response to genotoxic stress (e.g. Gadd45a and Cdkn1a/p21). We observe that the phosphorylation is present only in irradiated samples, which reinforces the role of Kap1 in DDR to ionizing radiation. Interestingly, the irradiated mutants have higher expression of this modification as the irradiated controls. We also detected decreased levels of *Smarca5* expression in *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Vav1-iCre* mutants, confirming that the observed modification are all consequence of *Smarca5*-loss of function.

In addition to p53-signaling pathway we also looked at the upregulation of hypoxia activated HIF1 signaling, it has been described that both p53 and HIF-1 pathways interact and use the histone acetylases p300/CBP to promote their transcriptional regulatory function (Obacz et al., 2013; Schmid et al., 2004; Zhou et al., 2015). As the previous results suggest a complex pattern of modifications leading to the activation of p53 signaling pathway we hypothesized that the absence of Smarca5 might actually inflict DNA damage in mutant cells. Therefore, we investigated the colocalization of γH2AX foci and p53BP1 and using fluorescent microscopy. The simultaneous localization of these proteins is a prerequisite for DNA repair and appears soon after DNA damage in affected cells. However, we could detect neither γH2AX foci nor localized expression of p53BP1 in *Smarca5-null* or control FLs, unlike in irradiated wild type thymi, which served as controls.

Smarca5 deficiency leads to perturbations in cell cycle progression and inhibition of erythroid differentiation.

Although *Smarca5*-deficient HSCs are able to differentiate into MPPs, the ability of these progenitors to mature into committed hematopoietic cells is limited. To investigate the underlying causes leading to the block of the erythroid terminal differentiation, we analyzed erythroid cell maturation within the developing FL by flow cytometry (Pop et al., 2010).

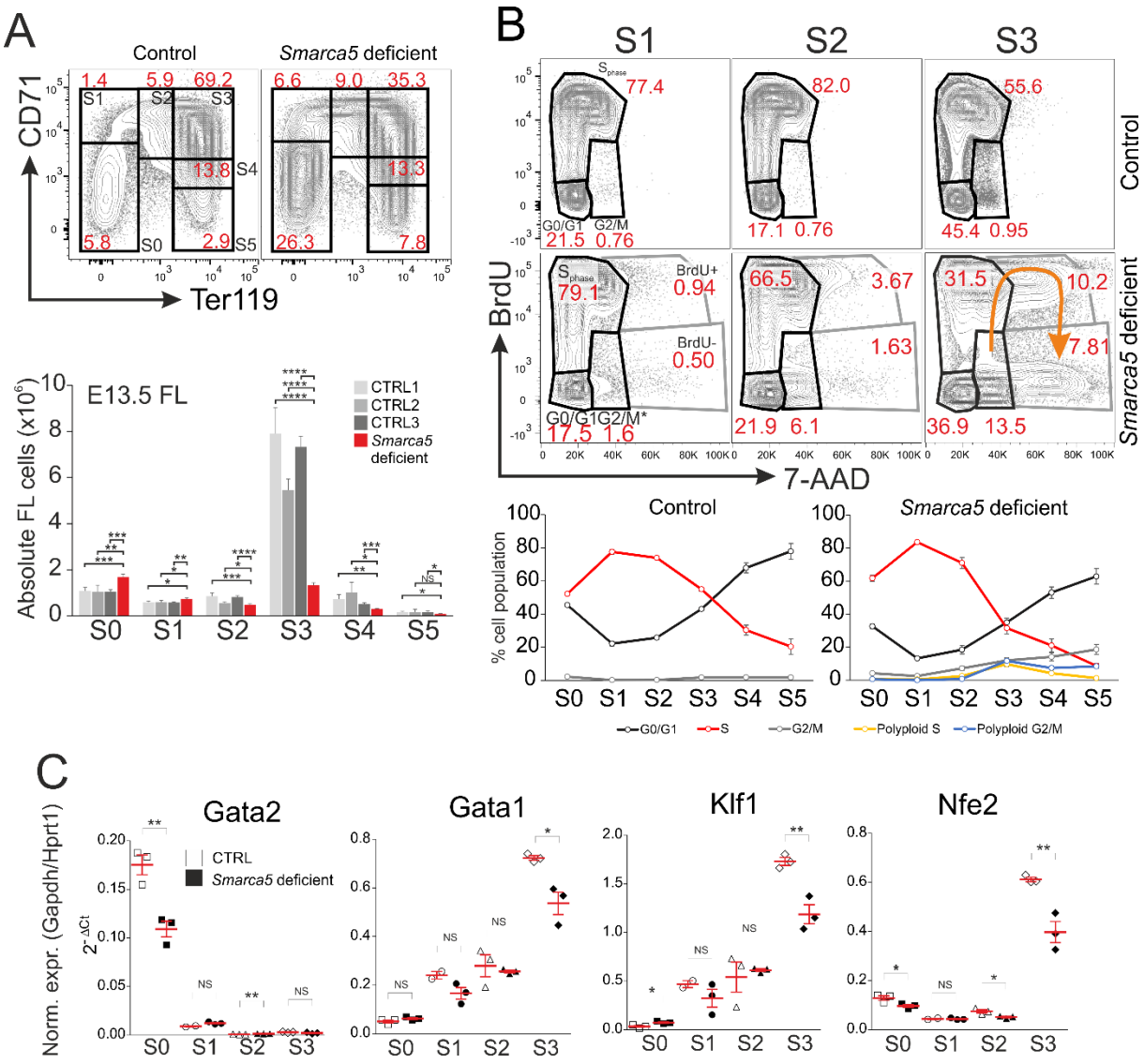

Figure 7: Smarca5 deletion (Vav1-iCre) results in blockade of erythropoiesis. (A) Flow cytometric analysis of Ter119 and CD71 expression in the E13.5 FLs. Relative (upper) and absolute (bottom) cell numbers in the E13.5 FL. Mutant: *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Vav1-iCre*. Controls: *Smarca5*$^{flox5/+}$; *Smarca5*$^{flox5/\Delta5\text{-}9}$; *Smarca5*$^{flox5/+}$ *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 E13.5 FLs. **(C)** qPCR expression of selected transcription factors from eYFP+ S0-S3 FACS-sorted cell populations in E14.5 FL. Controls: *Smarca5*$^{flox5/+}$ *Rosa26*$^{eYFP/+}$ *Vav1-iCre* (n=3) and mutants: *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Rosa26*$^{eYFP/+}$ *Vav1-iCre* (n=3). Statistics: two-tailed Student's T-test ($p < 0.05$ = *, $p < 0.001$ = **, $p < 0.0001$ = ***, $p < 0.00001$ = ****).

For analysis of erythroid development we quantified early multipotential progenitors (S0), committed erythroid (S1), proerythroblasts (S2), basophilic erythroblasts (S3), orthochromatophilic to polychromatophilic erythroblasts (S3-S4) to reticulocytes and mature red cells (S5) in E13.5 FLs. We confirm a high proportion of progenitors (S0 population, 26% vs 6% in controls) and loss of differentiated and more mature cell progeny in *Smarca5*$^{flox5/\Delta 5\text{-}9}$ *Vav1-iCre* mutants. Mutant also have higher proportion of erythroid progenitors S1 (7% *vs.* 1%) and S2 (9% *vs.* 6%). However, the most pronounced is the decrease of S3 cells or basophilic erythroblasts from 69% to 35% and all the subsequent erythroid cell types (Fig. 7A). Cell numbers of S0 rise on average by 50%. In contrast to early progenitor is the depletion of erythroid S3 population, were we observed a sharp 5-fold decrease in cell content (Fig.7A). We have reasoned that the defective erythroid differentiation might be due to impaired proliferation of erythroid progenitors. We used *in vivo* BrdU labeling in E13.5 embryos and tested cell cycle dynamics of FL cells by flow cytometry. We observed that in the mutant FLs there was a decreased proportion of cells in the S-phase within the S3-S5 populations and a significant increase of cells in G2/M phase in S2 through S5 populations. However, the most distinctive feature in mutants was the presence of polyploid cells in G2/M phase of S3 erythroid cells (Fig.7B).

As cell cycle dynamics/proliferation and differentiation of erythroid progenitors is intimately interconnected, we postulated that in addition to the observed disruption of cell cycle in mutants, there might also be an altered expression key erythroid transcription factors. In order to probe this hypothesis we assessed mRNA expression levels several hematopoietic genes. We observed a reduced expression of *Gata2* in S0 population, the GATA transcription factor in HSPCs biased towards megakaryocytic/erythroid progenitors (MEP). *Gata1*, *Klf1*, *Nfe2* had reduces mRNAs in the mutant S3 cells (Fig.7C). We also examined the expression levels of few erythroid target genes, including *Epor*, *Alas2*, embryonic and adult globins. Expression of adult definitive hemoglobin chain mRNAs (*Hba-a1* and *Hbb-b1*) and the erythroid-specific genes *Alas2* and *Epor* was reduced in the mutant FLs. Interestingly, the expression levels of embryonic α-like (*Hba-x*) and β-like (*Hbb-y* and *Hbb-bh1*) globin chain mRNAs was increased, indicating a developmental delay of embryonic switching in the mutants (not shown).

To gain further insight into possible mechanisms involving defective erythroid proliferation and premature cell death we analyzed the expression levels of several cell cycle associated and DNA damage response mRNAs identified previously in the microarray analysis of Kit+ cells (Figure 8). We used quantitative RT-PCR to determine the expression levels of these genes in the eYFP+ E14.5 FL-derived S0-S3 cell stages. We found that the expression of the G1/S and G2/M cyclin A2 *(Ccna2)*, was downregulated during erythroid differentiation.

Similarly, the expression G1/S inhibitor *Cdkn2d/p19* was downregulated in erythroid S3 population. In contrast, the expression levels of the G1/S-specific cyclins D2 (*Ccnd2*) and E1 (*Ccne1*) were upregulated in the mutant cells. In addition, p53 target and constitutively expressed G1/S hematopoietic cyclin gene *Ccng1* was upregulated in progenitors (S0), but downregulated in erythroid cells (S1-S3) (Fig.21A). The centromere-kinetochore complex component *Cenpf* required during G2/M was downregulated in mutant S3 cells (Fig.8A).

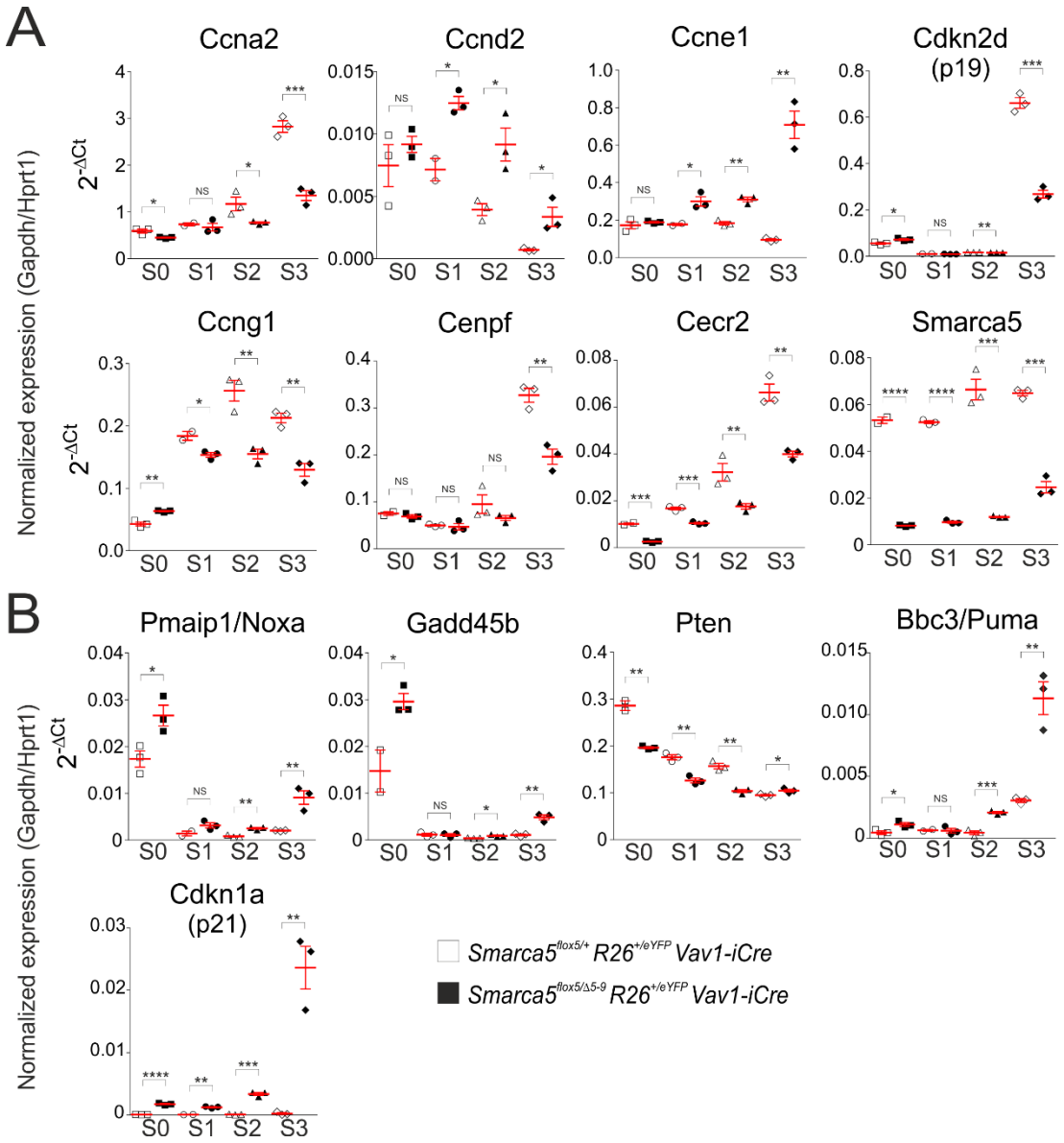

Figure 8: Expression of p53 target and cyclin mRNAs in *Smarca5*-null definitive erythrocytes. (A) qPCR expression of selected cyclin, CDK inhibitor p19 mRNAs as well as centromere-kinetochore complex protein *Cenpf*, chromatin remodeling factor subunit *Cecr2* and *Smarca5* in the eYFP+ S0-S3 FACS-sorted cells in E14.5 FL. **(B)** qPCR expression validation of selected p53-signaling pathway mRNAs identified in the expression arrays in the sorted E14.5 erythroid lineage cells. Controls: *Smarca5*$^{flox5/+}$ *Rosa26*$^{eYFP/+}$ *Vav1-iCre* (n=3) and mutants: *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Rosa26*$^{eYFP/+}$ *Vav1-iCre* (n=3). Statistics: two-tailed Student's T-test ($p < 0.05$ = *, $p < 0.001$ = **, $p < 0.0001$ = ***, $p < 0.00001$ = ****).

The four p53 target mRNAs assayed (*Pmaip/Noxa*, *Gadd45b*, *Cdkn1a*/*p21* and *Bbc3/Puma*) were all increased, whereas *Pten* phosphatase mRNA was decreased in mutant cells undergoing differentiation, suggesting persistent activation of p53 signaling during terminal erythroid development in mutants and accounting for increased cell cycle arrest and incidence of programmed cell death throughout hematopoietic development in *Smarca5* absence (Fig.8B)

Smarca5 is required for proliferation and survival of erythroid progenitors.

After exploring the effects of general *Vav1-iCre*-mediated hematopoietic deletion of *Smarca5*, which resulted in wide range of proliferative and differentiation defects in ranging from HSPCs, MPPs to erythroid cells, we decided to concentrate solely on adult definitive erythroid lineage. To address the effects of *Smarca5* deficiency in erythroid cells, we utilized iCre recombinase-expressing transgenic model $Epor^{eGFP:iCre}$ (Heinrich et al., 2004), an *Epor-iCre* knock-in, which expresses iCre recombinase from a native *Epor* locus, in fully committed erythroid progenitors and their progeny, and has an additional low eGFP fluorescent reporter activity.

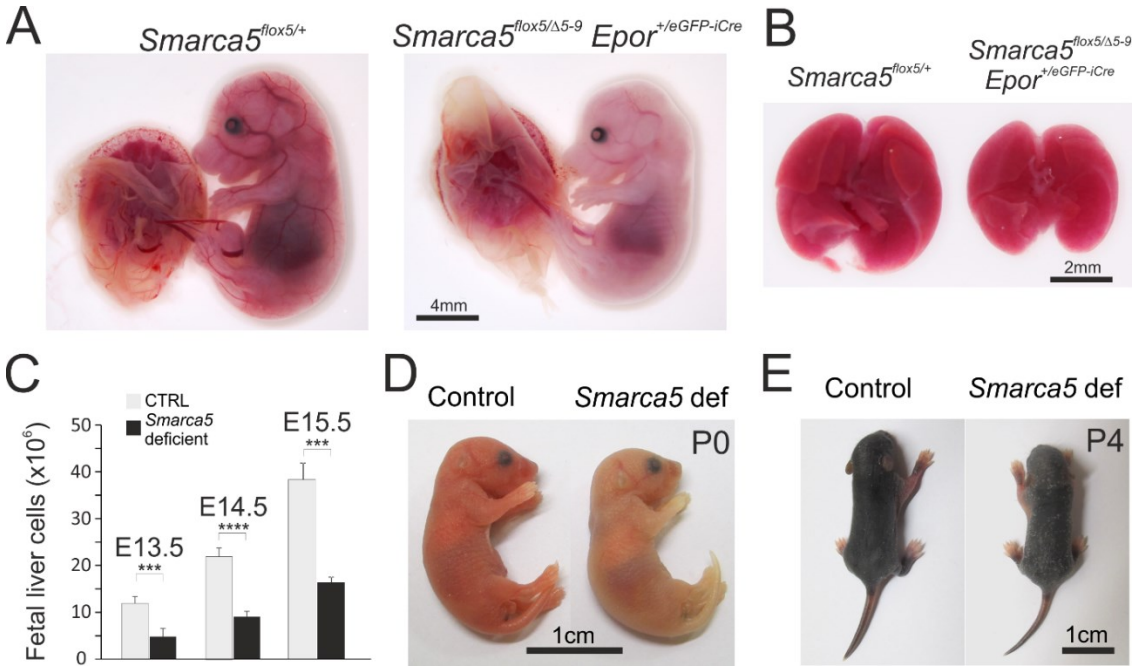

Figure 9: *Smarca5* deletion in erythroid lineage disrupts adult definitive FL erythropoiesis. **(A)** Phenotypic appearance of E15.5 embryos of control $Smarca5^{flox5/+}$ and mutant $Smarca5^{flox5/\Delta5\text{-}9}$ $Epor^{+/eGFP:iCre}$ embryos **(B)** Comparison of wild-type and mutant fetal livers at E15.5. **(C)** Average FL cellularity of indicated genotypes in E13.5 to E15.5 fetal livers. **(D)** Phenotypic appearance of $Smarca5^{flox5/+}$ and $Smarca5^{flox5/\Delta5\text{-}9}$ $Epor^{+/eGFP:iCre}$ newborns and **(E)** postnatal day 4 (P4) siblings. Statistics: two-tailed Student's T-test ($p < 0.0001$ = ***, $p < 0.00001$ = ****).

We have initially observed that fewer *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Epor-iCre* mice have grown to adulthood. However, closer examination of litters from *Smarca5*$^{+/\Delta5\text{-}9}$ *Epor-iCre* and *Smarca5*$^{flox5/flox5}$ crosses between postnatal days P0 and P9, showed that the majority of newborn *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Epor-iCre* pup die soon after birth. Newborn mutants were pale and displayed a variable degree of growth delay and failure to thrive (Fig. 9D,E). Additional examination of litters from timed matings (E12.5-E18.5) demonstrated that *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Epor-iCre* embryos had reduced size and 2.5-fold reduced cellularity of the FL (Fig. 9A-C), a phenotype closely reminiscing that of *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Vav1-iCre* embryos.

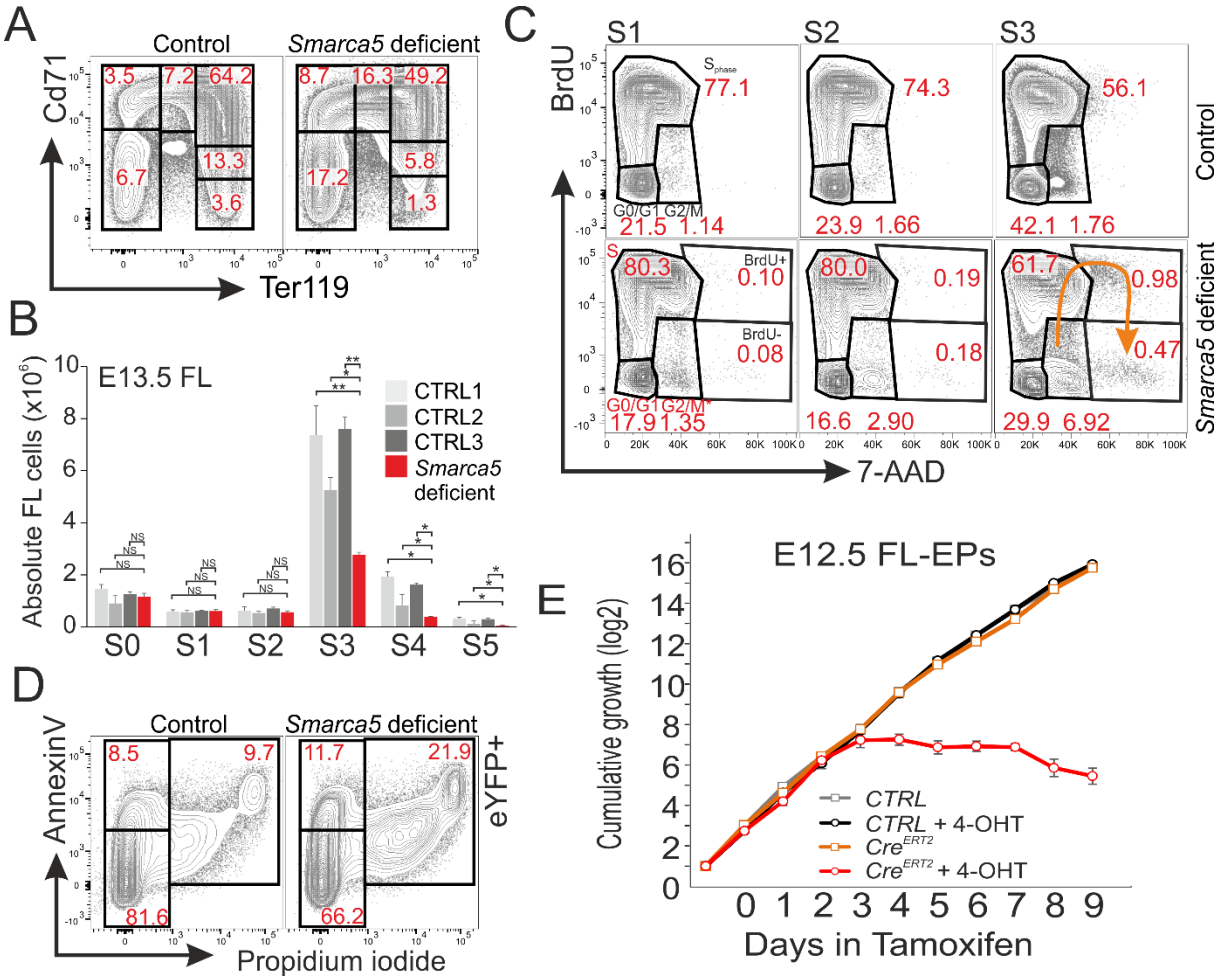

Figure 10: *Smarca5* deletion in erythroid lineage disrupts erythroid differentiation. **(A)** Flow cytometric analysis of control (*Smarca5*$^{flox5/+}$ *Rosa26*$^{eYFP/+}$ *Epor*$^{+/eGFP:iCre}$) and mutant (*Smarca5*$^{flox5/\Delta5\text{-}9}$ *Rosa26*$^{eYFP/+}$ *Epor*$^{+/eGFP:iCre}$) FLs at E13.5 FL. **(B)** Absolute cell numbers in E13.5 FL in S0-S5 erythroid differentiation stages. Controls: 1) *Smarca5*$^{flox5/+}$; 2) *Smarca5*$^{flox5/\Delta5\text{-}9}$; 3) *Smarca5*$^{flox5/+}$ *Epor*$^{+/eGFP:iCre}$ **(C)** BrdU labeling of S1-S3 erythroid populations from control (*Smarca5*$^{+/flox5}$) and mutant (*Smarca5*$^{flox5/\Delta5\text{-}9}$ *Epor*$^{+/eGFP:iCre}$) E13.5 FLs. **(D)** AnnexinV and propidium iodide staining in the eYFP+ cell fraction of E13.5 FL. **(E)** Cumulative growth curve (*y*-axis log2 of 10^6 cells) of primary FL-derived erythroid cells (FL-EPs) prepared from E12.5 control (*Smarca5*$^{flox5/flox5}$) and 4-hydroxytamoxifen-inducible (4-OHT) (*Smarca5*$^{flox5/\Delta5\text{-}9}$ *Cre-Esr1*^{ERT2}) embryos and grown in serum-free *in vitro* conditions with or without 4-OHT. CTRL: *Smarca5*$^{flox5/flox5}$; CRE^{TX}: *Smarca5*$^{flox5/\Delta5\text{-}9}$ *CAG-Cre*^{ERT2}. Two-tailed Student's T-test ($p < 0.05$ = *, $p < 0.001$ = **, $p < 0.0001$ = ***).

Flow cytometric analysis of E13.5 FL revealed that erythroid maturation was reduced in S3 population and onward in S4 and S5 (Fig. 10A). We have also seen a corresponding relative increase in progenitor (S0-S2) populations, but the absolute cell numbers remained the same. On the other hand, the population S3, most abundant in control FL and mainly made up by basophilic erythroblasts, was 3-fold reduced in *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Epor-iCre* FL (Fig. 10B). *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Epor-iCre* FLs produced less BFU-E, CFU-E and CFU-GEMM colonies in colony formation assays, although these colonies had normal appearance. Mutant FL had also normal cytology and morphology and all erythroid maturation stages were present. We have observed a slight increase in BrdU incorporation within S3 and S4 populations and reduction of cell in G0/G1 phase of cell cycle, consistent with activation of cell cycle proliferation. We also found a small percentage of polyploid cells (Fig.10C). Nevertheless, the overall observed defects were not as severe as in the *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Vav1-iCre* FLs, even though the degree of anemia is comparable. We therefore tested the survival of erythroid cells by flow cytometry. Accordingly, we detected increased annexinV staining in the FL suggesting that the decreased survival of erythroid progenitors is the main reason for the observed phenotypes (Fig. 10D). No change in expression of *Gata2*, *Gata1*, *Klf1*, *Ldb1*, *Zfpm1* or *Nfe2* was observed, leading to the conclusion that erythroid specification is not affected in *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Epor-iCre* embryos. Instead, it might be related to terminal differentiation as *Lmo2*, *Cbfa2t3*, *Runx1t1* and *Zbtb7a* involved in maturation were dysregulated. We also tested the mRNA expression of several cell cycle and survival genes, however no major changes in expression of these was observed. Only notable exception was a decreased expression of G2 to M cyclin *Ccna2* and increased expression of *Cdkn1a/p21* and *Ccng1*, a p53 targets, consistent with decreased survival, and cell cycle arrest in basophilic erythroblasts.

To determine whether the perceived defects caused by *Smarca5* loss in the erythroid progenitors are cell autonomous, we derived fetal liver erythroid progenitors (FL-EPs) from E12.5 inducible *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Cre-Esr1*^{TM} embryos and cultured them under conditions that favor proliferation of immature erythroid progenitors for duration of several days (Dolznig et al., 2005). We observed that growth of FL-EP cultures halted quite abruptly 72 hours after addition of 4-OHT (Fig.10E), while controls maintained the exponential growth. These findings indicate that Smarca5 is required for proliferation of erythroid progenitors in a cell autonomous manner.

DISCUSSION

In this study, using an early definitive hematopoiesis specific strain Vav1-iCre (de Boer et al., 2003), we show how the *Smarca5* loss affects hematopoietic development in the fetal liver. The deletion of *Smarca5* is initiated before E9.5 in the early definitive hematopoietic progenitors soon after their specification from hemogenic endothelium and formation of hematopoietic clusters in the principal hemogenic organs of definitive hematopoiesis – placenta and aorta-gonads-mesonephros (AGM). Because of the ubiquitous nature of *Vav1* expression, a GDP/GTP nucleotide exchange factor for Rho/Rac GTPases, in hematopoietic lineage; *Smarca5* deletion occurs in all descendant lineages, lymphoid, myeloid and early erythroid, in prenatal as well as postnatal life – in highly proliferating hematopoietic stem and progenitor cell (HSPC) populations of fetal liver, adult bone marrow and spleen.

We have observed that *Smarca5-null* embryos in this model develop visible anemia, soon after the colonization of FL by hematopoietic progenitors originating from external sites. FL is not involved in the *de novo* generation of hematopoietic progenitors, instead it serves as transient niche which supports and accommodates incoming progenitors and stimulates their rapid expansion by proliferation. Concurrently, FL promotes differentiation of erythroid lineage which is required for maintenance of fetal growth. As of E12.5 the FL is being colonized with hematopoietic progenitors and the erythropoiesis is ongoing, however soon erythroid maturation diminishes and embryos by E13.5 become visibly anemic. Both placenta and FL are paler, sign of reduced erythropoiesis, affected embryos die *in utero* by E17.5.

Upon closer examination we found that mutant FLs lack maturated stages of erythrocyte development and instead are enriched in progenitors. Interestingly, the size of c-Kit progenitor population in mutants and controls is comparable, nonetheless, mutants have much more LSK cells, which is the FL population enriched in multipotent HSPCs. The LS-K cells are enriched for more lineage-restricted progenitors. However, as the colony forming assays show, the remaining FL progenitor pool was defective, as we have observed only a minimal growth besides of complete lack of erythroid-containing CFU-GEMM colonies in methylcellulose colony-forming assays. The remaining colonies were mostly myeloid-like and showed no signs of erythroid differentiation. Not surprisingly, when we tested the repopulating capability of mutant E13.5 FL by transplantation into irradiated hosts, no detectable reconstitution was observed at 12 days after irradiation (not shown), indicating a profound defect in HSC function, further corroborating the limited growth of mutant FL in colony forming assays.

The LSK stem cells in the mutant E13.5 FL are overwhelmingly **myeloid-biased multipotent progenitors** (MPPs) (Lin$^-$ Sca1$^+$ Kit$^+$ CD48$^+$ CD150$^+$) or **balanced-MPP** (Lin$^-$ Sca1$^+$ Kit$^+$ CD48$^+$ CD150$^-$) with both myeloid and lymphoid potential (Kiel et al., 2005). Interestingly, this is well in line with the notion that myeloid specification is somewhat less affected in *Smarca5* mutants. We assume that these progenitors fail to properly differentiate and instead accumulate upon lack of *Smarca5*. Milder phenotype could be attributed to the Smarca5's role as a repressor of myeloid lineage transcription factor Sfpi1/PU.1 (Dluhosova et al., 2014) during FL hematopoiesis and the loss of *Smarca5* expression would therefore benefit myeloid differentiation at cost of other alternative lineage choices. *Smarca5* deficiency already affected cell cycle progression at the level of HSCs and MPPs, where the loss of *Smarca5* was reflected in the increased proportion of LSK cells entering S-phase. Within the LS$^-$K cells, proliferation was markedly reduced coincidently with G2/M phase accumulation.

At midgestation, RBC production is the major function of FL. Accordingly, the most evident phenotype of the *Smarca5$^{flox5/\Delta5\text{-}9}$ Vav1-iCre* embryos is anemia. Mutant FL had only slightly decreased populations of erythroid-biased progenitors, and proerythroblasts, however all consecutive erythroid maturation stages were diminished or completely lost, pointing towards a **proerythroblast-to-basophilic arrest** as primary cause of anemia. This phenotype is consistent with the previous finding, where antisense inhibition of SMARCA5 human CD34+ progenitors and K562 cells led to an inhibition of early erythroid differentiation and downregulation of the GATA1 target *HGB/β-globin* expression (Stopka and Skoultchi, 2003). Loss of *Hbb-b1* expression and maturation arrest is also observed in erythroid-specific transcription factor *Gata1*- (Fujiwara et al., 1996) or *Klf1*-deficient mice (Perkins et al., 1995). The phenotype of *Klf1*-/- mice is particularly interesting as the homozygous mutants die relatively late at E16.5, a time point very similar to the lethality of *Smarca5*-deficient embryos, which die around E17.5. This also raises a question whether Smarca5 is directly involved in transcriptional regulation by these TFs during erythroid differentiation. In effect, Smarca5 and Gata1 were previously shown to physically interact in the murine erythroleukemia (MEL) cell line (Rodriguez et al., 2005), which may be used to mimic proerythroblast to basophilic erythroblast transition. Deficiency of Rcor1/CoREST, which promotes erythropoiesis by repressing HSC and/or progenitor and myeloid lineage determination genes, causes premature embryonic lethality around E16.5 (Yao et al., 2014). Similarly to our model, the definitive erythroid cells in *Rcor1* mutants become arrested at the transition from proerythroblast to basophilic erythroblast stages.

It is well established, that cell cycle and differentiation are tightly coordinated during erythroid development (Pop et al., 2010). Thus, it is possible that the defective erythroid maturation might result either from indispensable role of Smarca5 in promoting DNA replication/cell cycle progression, facilitation of transcription regulation by erythroid transcription factor network (consisting of TFs Gata1, Lmo2, Ldb1, Tal1/Scl, Tcf3 and Klf1, Nfe2) or both at the same time. The deficiency of some erythroid transcription factors (e.g. Gata1, Klf1, Zbtb7a) or lack of survival signals indeed may cause decreased survival of mature erythroid cells, based on dysregulation of Tal1, Lmo2, Gata1, Nfe2, Klf1, Runx1t1/ETO, Cbfa2t3/ETO2, we think that the broad deregulation of the transcription regulatory network and resulting cell death and cell cycle arrest of *Smarca5*-deficient erythroblasts is likely attributed to disruption of basic cellular processes. For comparison, the *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Epor-iCre* embryos which in several instances do survive into adulthood, are similar to models of inefficient erythropoiesis (such as Stat5a-/-5b-/-, Socolovsky et al., 1999) and although FL erythropoiesis is initially severely affected, it does improve as the animals age. The *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Epor-iCre* mutants did not show a dysregulation in the heptad of transcription factor which were dysregulated in *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Vav1-iCre* embryos. Note that exception is Lmo2, which seem to functions in DNA replication independently of it role in erythroid transcriptional regulation (Sincennes et al., 2016). The additional dysregulated transcription factor we have identified is a KRAB-zinc finger transcriptional repressor *Zbtb7a*, which was previously described as and Klf1 target which promotes erythroid cell survival by inhibition of Bim proapoptotic factor. Zbtb7a-/- embryos died between E15.5 and E16.5 due to maturation arrest and increased cell death at the polychromatophilic to orthochromic erythroblast stage (Maeda et al., 2009).

Mechanistically however, the underlying cause for premature lethality of *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Vav1-iCre* embryos may be more complicated as other contributing factors are involved. One of those is the role of Smarca5 in cell proliferation. At least three of the known Smarca5 complexes are involved proliferation/replication (ACF, CHRAC and WICH) (He et al., 2006) (Sugimoto et al., 2011), not disregarding the role of many more in **DNA damage response (DDR)**, highlighting the possibility that defective chromatin remodeling during replication or replicative DNA damage might be the main reason behind observed phenotype. Notion of defective proliferation is based on observation of profound changes in cell cycle dynamics of hematopoietic and erythroid progenitors. Especially, the proliferation of basophilic erythroblasts; the most abundant erythroid cell subtype in normal fetal liver erythropoiesis was

perturbed in *Smarca5* mutants. While, in controls 80% of S2 (late proerythroblasts/early basophilic erythroblasts) and 60% S3 cells (basophilic erythroblasts) were in S phase of cell cycle in mutant this number was significantly lower (66 and 35%) indicative of proliferation defects. 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.

The highly proliferating cells such as HSPCs, MPPs, erythroid cells and thymocytes are prone to DNA damage by **replicative stress**. Protection against replicative stress is provided by the licensing of thousands of origins in G1 phase of cell cycle that are not necessarily activated in the subsequent S-phase. These 'dormant' replication origins provide a backup in the presence of stalled forks and may confer flexibility to the replication program in specific cell types during differentiation. Recent report by Sincennes *et al.* show, that a LIM-domain protein Lmo2, which is a member of the HSC and erythroid-lineage transcriptional-regulatory core complex (containing Tal1/Scl, Ldb1, Tcf3/E2A, Lmo2 and Gata2/Gata1) is recruited to DNA replication origins by interaction with three essential replication enzymes: DNA polymerase delta (POLD1), DNA primase (PRIM1), and minichromosome 6 (MCM6). The authors, also show that Lmo2 alone is sufficient to generate replication origins *in vitro*, thus concluding that Lmo2 can directly promote DNA replication and G1-S progression in hematopoietic stem cells and erythroid cells (Sincennes et al., 2016). They also show that Lmo2 directly interacts with Baz1a/Acf1, a subunit of a Smarca5 containing chromatin remodeling complex ACF, with a tantalizing possibility that Smarca5 might promote DNA replication in erythroid cells also through interaction with Lmo2.

The gene expression arrays in E15.5 FLs revealed that overwhelmingly transcripts from DNA replication, repair and p53-signaling pathways were differentially expressed in *Smarca5*$^{flox5/\Delta5\text{-}9}$ *Vav1-iCre* progenitors. Although, we could not confirm the Atm/Atr pathway activation of Chk1/Chk2 upon *Smarca5* deficiency, we identified two p53 modifications: p53 S18^{Phos} (previously associated with DNA damage, Ullrich et al., 1993) and p53 K382^{Ac} acetylation mediated by histone acetylases p300 and CREBBP/CBP, indispensable for transcriptional activity of p53 (Sakaguchi et al., 1998), supporting at least some degree of p53-signaling pathway activation. The ATM-induced phosphorylation of heterochromatin protein HP1-interacting SUMO-protein ligase Kap1 on Ser-824 upon DNA damage prevents Kap1 auto-SUMOylation and thus relieves a subset of cell cycle control, apoptosis and DDR involved

genes (e.g. Gadd45a and Cdkn1a/p21) from transcriptional repression. These events provide an alternative way for direct and fast p21 expression upregulation apart from a principal p53-signaling pathway upon DNA damage. Although this modification was not detected in non-irradiated mutants, we detected an increased Kap1 phosphorylation in irradiated mutants vs. controls, indicating that mutants are probably more sensitive to DNA damage immediately after ionizing radiation occurs. Our data however also show that there is no permanent source of γH2AX/P53BP1 foci associated with DNA damage in mutant cells, highlighting a possibility of a more complex mechanism upstream the p53-signaling pathway activation. Interestingly, p21 could be also activated by hypoxia via transcription factor Hif1 (Carmeliet et al., 1998) supporting the possibility that both p53 and p21 activation could be augmented by severe hypoxia as also documented by activation of the Hif1 mRNA targets (Fig. S3D). However, the effects of prolonged hypoxia on cell survival and p53 response are somewhat controversial (Zhou et al., 2015).

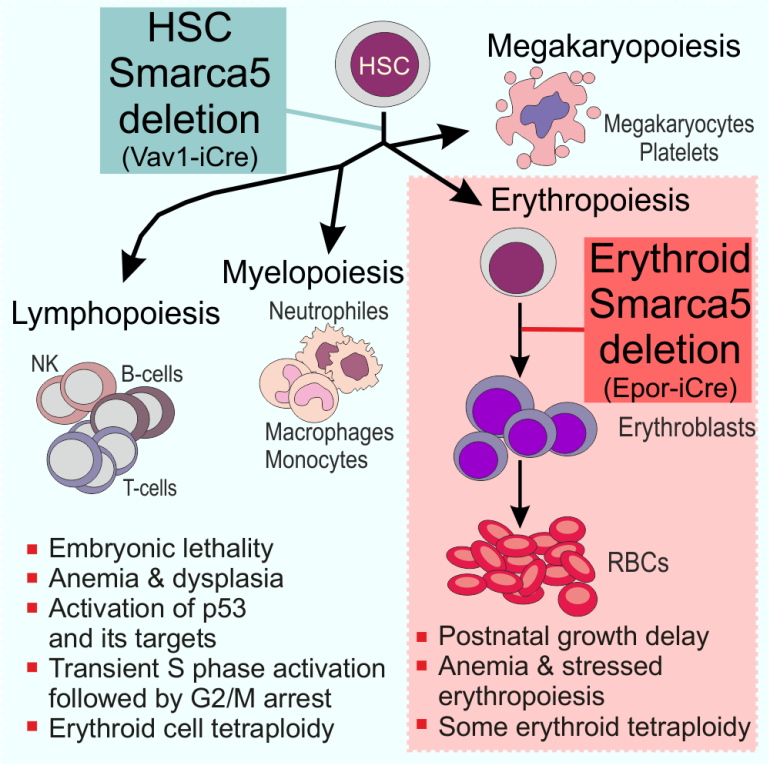

Figure 37: Smarca5 requirement during mouse hematopoietic development. Hematopoietic stem cells (HSCs) lacking *Smarca5* temporarily activate proliferation followed by G2/M arrest during erythroid and myeloid differentiation leading to fatal anemia with tetraploid erythroblasts and p53 activation. Erythroid-specific deletion of *Smarca5* resulted in milder phenotypes: anemia and tetraploidy.

CONCLUSIONS

ISWI ATPase Smarca5 (Snf2h), a remodeler of nucleosomal structure and position, is required for maturation of hematopoietic progenitor cells. *Smarca5* deficiency disturbs proliferation and differentiation of hematopoietic LSK and LS-K progenitors and result a specific loss of lymphoid-biased progenitors and downregulation of lymphoid transcription program in FL HSCs. Instead, increased commitment towards myeloid development is observed.

Hematopoietic stem cells (HSCs) lacking *Smarca5* temporarily activate proliferation followed by G2/M arrest effectively suppressing further development into mature erythroid and myeloid cells leading to fatal anemia. The dysregulation of the cell cycle on progenitor level leads to cessation of differentiation of MPPs, their accumulation and halted erythroid maturation causing severe anemia. Even though, the signaling cascade upstream p53 activation in not clear, Trp53 appears to be a mediator of the proliferation blockade and programmed cell death in the *Smarca5* mutants mainly by upregulation of cyclin-dependent kinase inhibitor p21 and other targets facilitating apoptosis.

To complement the hematopoietic model of conditional deletion of *Smarca5*, we also studied its role during erythroid development. Similarly, the deletion of *Smarca5* by erythroid lineage specific *Epor-iCre* transgene resulted in anemia, albeit initially severe, *Smarca5* deletion in erythroid lineage (downstream to HSC) was partially compatible with postnatal survival. FL was diminished in size and cellularity; and only minor changes in maturation were observed, indicating changes compatible with inefficient erythropoiesis resulting. *Smarca5* deficiency again led to perturbations of cell cycle and inhibition of erythroid differentiation accompanied by increased expression of Cdkn1a/p21 cyclin-dependent kinase inhibitor in basophilic erythroblasts (BEB). Interestingly, also these basophilic erythroblasts demonstrated polyploidization, however in much lower extent.

REFERENCES

Alvarez-Saavedra, M., De Repentigny, Y., Lagali, P. S., Raghu Ram, E. V., Yan, K., Hashem, E., Ivanochko, D., Huh, M. S., Yang, D., Mears, A. J., et al. (2014). Snf2h-mediated chromatin organization and histone H1 dynamics govern cerebellar morphogenesis and neural maturation. *Nature communications* **5**, 4181.

Boyer, L. A., Latek, R. R. and Peterson, C. L. (2004). The SANT domain: a unique histone-tail-binding module? *Nature reviews. Molecular cell biology* **5**, 158-163.

Cabezas-Wallscheid, N., Klimmeck, D., Hansson, J., Lipka, D. B., Reyes, A., Wang, Q., Weichenhan, D., Lier, A., von Paleske, L., Renders, S., et al. (2014). Identification of regulatory networks in HSCs and their immediate progeny via integrated proteome, transcriptome, and DNA methylome analysis. *Cell stem cell* **15**, 507-522.

Cairns, B. R. (2007). Chromatin remodeling: insights and intrigue from single-molecule studies. *Nature structural & molecular biology* **14**, 989-996.

Carmeliet, P., Dor, Y., Herbert, J. M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., et al. (1998). Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* **394**, 485-490.

Chong, S., Vickaryous, N., Ashe, A., Zamudio, N., Youngson, N., Hemley, S., Stopka, T., Skoultchi, A., Matthews, J., Scott, H. S., et al. (2007). Modifiers of epigenetic reprogramming show paternal effects in the mouse. *Nature genetics* **39**, 614-622.

Crossley, M., Tsang, A. P., Bieker, J. J. and Orkin, S. H. (1994). Regulation of the erythroid Kruppel-like factor (EKLF) gene promoter by the erythroid transcription factor GATA-1. *The Journal of biological chemistry* **269**, 15440-15444.

de Boer, J., Williams, A., Skavdis, G., Harker, N., Coles, M., Tolaini, M., Norton, T., Williams, K., Roderick, K., Potocnik, A. J., et al. (2003). Transgenic mice with hematopoietic and lymphoid specific expression of Cre. *European journal of immunology* **33**, 314-325.

Dluhosova, M., Curik, N., Vargova, J., Jonasova, A., Zikmund, T. and Stopka, T. (2014). Epigenetic control of SPI1 gene by CTCF and ISWI ATPase SMARCA5. *PloS one* **9**, e87448.

Dolznig, H., Kolbus, A., Leberbauer, C., Schmidt, U., Deiner, E. M., Mullner, E. W. and Beug, H. (2005). Expansion and differentiation of immature mouse and human hematopoietic progenitors. *Methods in molecular medicine* **105**, 323-344.

Fujiwara, Y., Browne, C. P., Cunniff, K., Goff, S. C. and Orkin, S. H. (1996). Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 12355-12358.

Grass, J. A., Boyer, M. E., Pal, S., Wu, J., Weiss, M. J. and Bresnick, E. H. (2003). GATA-1-dependent transcriptional repression of GATA-2 via disruption of positive autoregulation and domain-wide chromatin remodeling. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 8811-8816.

Grune, T., Brzeski, J., Eberharter, A., Clapier, C. R., Corona, D. F., Becker, P. B. and Muller, C. W. (2003). Crystal structure and functional analysis of a nucleosome recognition module of the remodeling factor ISWI. *Molecular cell* **12**, 449-460.

Hayashi, S. and McMahon, A. P. (2002). Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. *Developmental biology* **244**, 305-318.

He, X., Fan, H. Y., Garlick, J. D. and Kingston, R. E. (2008). Diverse regulation of SNF2h chromatin remodeling by noncatalytic subunits. *Biochemistry* **47**, 7025-7033.

He, X., Fan, H. Y., Narlikar, G. J. and Kingston, R. E. (2006). Human ACF1 alters the remodeling strategy of SNF2h. *The Journal of biological chemistry* **281**, 28636-28647.

Heinrich, A. C., Pelanda, R. and Klingmuller, U. (2004). A mouse model for visualization and conditional mutations in the erythroid lineage. *Blood* **104**, 659-666.

Kiel, M. J., Yilmaz, O. H., Iwashita, T., Yilmaz, O. H., Terhorst, C. and Morrison, S. J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* **121**, 1109-1121.

Lazzaro, M. A. and Picketts, D. J. (2001). Cloning and characterization of the murine Imitation Switch (ISWI) genes: differential expression patterns suggest distinct developmental roles for Snf2h and Snf2l. *Journal of neurochemistry* **77**, 1145-1156.

Maeda, T., Ito, K., Merghoub, T., Poliseno, L., Hobbs, R. M., Wang, G., Dong, L., Maeda, M., Dore, L. C., Zelent, A., et al. (2009). LRF is an essential downstream target of GATA1 in erythroid development and regulates BIM-dependent apoptosis. *Developmental cell* **17**, 527-540.

Martowicz, M. L., Grass, J. A., Boyer, M. E., Guend, H. and Bresnick, E. H. (2005). Dynamic GATA factor interplay at a multicomponent regulatory region of the GATA-2 locus. *The Journal of biological chemistry* **280**, 1724-1732.

Munugalavadla, V., Dore, L. C., Tan, B. L., Hong, L., Vishnu, M., Weiss, M. J. and Kapur, R. (2005). Repression of c-kit and its downstream substrates by GATA-1 inhibits cell proliferation during erythroid maturation. *Molecular and cellular biology* **25**, 6747-6759.

Neubauer, H., Cumano, A., Muller, M., Wu, H., Huffstadt, U. and Pfeffer, K. (1998). Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. *Cell* **93**, 397-409.

Obacz, J., Pastorekova, S., Vojtesek, B. and Hrstka, R. (2013). Cross-talk between HIF and p53 as mediators of molecular responses to physiological and genotoxic stresses. *Molecular cancer* **12**, 93.

Oguro, H., Ding, L. and Morrison, S. J. (2013). SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. *Cell stem cell* **13**, 102-116.

Pepin, D., Vanderhyden, B. C., Picketts, D. J. and Murphy, B. D. (2007). ISWI chromatin remodeling in ovarian somatic and germ cells: revenge of the NURFs. *Trends in endocrinology and metabolism: TEM* **18**, 215-224.

Perkins, A. C., Sharpe, A. H. and Orkin, S. H. (1995). Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. *Nature* **375**, 318-322.

Pop, R., Shearstone, J. R., Shen, Q., Liu, Y., Hallstrom, K., Koulnis, M., Gribnau, J. and Socolovsky, M. (2010). A key commitment step in erythropoiesis is synchronized with the cell cycle clock through mutual inhibition between PU.1 and S-phase progression. *PLoS biology* **8**.

Richmond, T. D., Chohan, M. and Barber, D. L. (2005). Turning cells red: signal transduction mediated by erythropoietin. *Trends in cell biology* **15**, 146-155.

Rodriguez, P., Bonte, E., Krijgsveld, J., Kolodziej, K. E., Guyot, B., Heck, A. J., Vyas, P., de Boer, E., Grosveld, F. and Strouboulis, J. (2005). GATA-1 forms distinct activating and repressive complexes in erythroid cells. *The EMBO journal* **24**, 2354-2366.

Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W. and Appella, E. (1998). DNA damage activates p53 through a phosphorylation-acetylation cascade. *Genes & development* **12**, 2831-2841.

Schmid, T., Zhou, J., Kohl, R. and Brune, B. (2004). p300 relieves p53-evoked transcriptional repression of hypoxia-inducible factor-1 (HIF-1). *The Biochemical journal* **380**, 289-295.

Sincennes, M. C., Humbert, M., Grondin, B., Lisi, V., Veiga, D. F., Haman, A., Cazaux, C., Mashtalir, N., Affar el, B., Verreault, A., et al. (2016). The LMO2 oncogene regulates DNA replication in hematopoietic cells. *Proceedings of the National Academy of Sciences of the United States of America* **113**, 1393-1398.

Socolovsky, M., Fallon, A. E., Wang, S., Brugnara, C. and Lodish, H. F. (1999). Fetal anemia and apoptosis of red cell progenitors in Stat5a-/-5b-/- mice: a direct role for Stat5 in Bcl-X(L) induction. *Cell* **98**, 181-191.

Socolovsky, M., Nam, H., Fleming, M. D., Haase, V. H., Brugnara, C. and Lodish, H. F. (2001). Ineffective erythropoiesis in Stat5a(-/-)5b(-/-) mice due to decreased survival of early erythroblasts. *Blood* **98**, 3261-3273.

Stopka, T. and Skoultchi, A. I. (2003). The ISWI ATPase Snf2h is required for early mouse development. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 14097-14102.

Stopka, T., Zakova, D., Fuchs, O., Kubrova, O., Blafkova, J., Jelinek, J., Necas, E. and Zivny, J. (2000). Chromatin remodeling gene SMARCA5 is dysregulated in primitive hematopoietic cells of acute leukemia. *Leukemia* **14**, 1247-1252.

Sugimoto, N., Yugawa, T., Iizuka, M., Kiyono, T. and Fujita, M. (2011). Chromatin remodeler sucrose nonfermenting 2 homolog (SNF2H) is recruited onto DNA replication origins through interaction with Cdc10 protein-dependent transcript 1 (Cdt1) and promotes pre-replication complex formation. *The Journal of biological chemistry* **286**, 39200-39210.

Tewari, R., Gillemans, N., Wijgerde, M., Nuez, B., von Lindern, M., Grosveld, F. and Philipsen, S. (1998). Erythroid Kruppel-like factor (EKLF) is active in primitive and definitive erythroid cells and is required for the function of 5'HS3 of the beta-globin locus control region. *The EMBO journal* **17**, 2334-2341.

Thompson, P. J., Norton, K. A., Niri, F. H., Dawe, C. E. and McDermid, H. E. (2012). CECR2 is involved in spermatogenesis and forms a complex with SNF2H in the testis. *Journal of molecular biology* **415**, 793-806.

Ullrich, S. J., Sakaguchi, K., Lees-Miller, S. P., Fiscella, M., Mercer, W. E., Anderson, C. W. and Appella, E. (1993). Phosphorylation at Ser-15 and Ser-392 in mutant p53 molecules from human tumors is altered compared to wild-type p53. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 5954-5958.

Wadman, I. A., Osada, H., Grutz, G. G., Agulnick, A. D., Westphal, H., Forster, A. and Rabbitts, T. H. (1997). The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *The EMBO journal* **16**, 3145-3157.

Wu, H., Liu, X., Jaenisch, R. and Lodish, H. F. (1995). Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. *Cell* **83**, 59-67.

Yang, Y., Stopka, T., Golestaneh, N., Wang, Y., Wu, K., Li, A., Chauhan, B. K., Gao, C. Y., Cveklova, K., Duncan, M. K., et al. (2006). Regulation of alphaA-crystallin via Pax6, c-Maf, CREB and a broad domain of lens-specific chromatin. *The EMBO journal* **25**, 2107-2118.

Yao, H., Goldman, D. C., Nechiporuk, T., Kawane, S., McWeeney, S. K., Tyner, J. W., Fan, G., Kerenyi, M. A., Orkin, S. H., Fleming, W. H., et al. (2014). Corepressor Rcor1 is essential for murine erythropoiesis. *Blood* **123**, 3175-3184.

Zhou, C. H., Zhang, X. P., Liu, F. and Wang, W. (2015). Modeling the interplay between the HIF-1 and p53 pathways in hypoxia. *Scientific reports* **5**, 13834.