Geminin deletion increases the number of fetal hematopoietic stem cells by affecting the expression of key transcription factors

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ABSTRACT

Balancing stem cell self-renewal and initiation of lineage specification programs is essential for the development and homeostasis of the hematopoietic system. We have specifically ablated geminin in the developing murine hematopoietic system and observed profound defects in the generation of mature blood cells, leading to embryonic lethality. Hematopoietic stem cells (HSCs) accumulated in the fetal liver following geminin ablation, while committed progenitors were reduced. Genome-wide transcriptome analysis identified key HSC transcription factors as being upregulated upon geminin deletion, revealing a gene network linked with geminin that controls fetal hematopoiesis. In order to obtain mechanistic insight into the ability of geminin to regulate transcription, we examined *Hoxa9* as an example of a key gene in definitive hematopoiesis. We demonstrate that in human K562 cells geminin is associated with *HOXA9* regulatory elements and its absence increases *HOXA9* transcription similarly to that observed *in vivo*. Moreover, silencing geminin reduced recruitment of the PRC2 component SUZ12 to the *HOXA9* locus and resulted in an increase in RNA polymerase II recruitment and H3K4 trimethylation (H3K4me3), whereas the repressive marks H3K9me3 and H3K27me3 were reduced. The chromatin landscape was also modified at the regulatory regions of *HOXA10* and *GATA1*. K562 cells showed a reduced ability to differentiate to erythrocytes and megakaryocytes upon geminin silencing. Our data suggest that geminin is indispensable for fetal hematopoiesis and regulates the generation of a physiological pool of stem and progenitor cells in the fetal hematopoietic system.

KEY WORDS: Geminin, Fetal hematopoiesis, HoxA9, Transcriptional regulation, Cell cycle, Epigenetic regulation, Mouse

INTRODUCTION

The hematopoietic system is generated by a hematopoietic stem cell (HSC) population that maintains its multipotency and self-renewal capacity throughout life (Kondo et al., 2003). During hematopoiesis, mechanisms that control self-renewal, fate commitment and differentiation decisions are precisely balanced so as to prevent exhaustion of fetal and adult HSCs and maintain lifelong generation and replacement of blood cells and an effective response to

hematological stress and challenges (Beerman et al., 2010; Cheshier et al., 2007). The prevailing model of hematopoiesis supports the existence of long-term hematopoietic stem cells (LT-HSCs), which can provide long-term multipotent reconstitution of the hematopoietic system, and of short-term hematopoietic stem cells (ST-HSCs) or multipotent progenitors (MPPs), with the potential to generate all blood lineages but with reduced self-renewal capacity (Luc et al., 2007; Mebius et al., 2001; Morrison et al., 1995; Randall et al., 1996; Traver et al., 2001).

Even though it remains unclear how the balance between self-renewal of HSCs and fate commitment is controlled and how a decision for differentiation is specified at the molecular level, there is evidence suggesting that epigenetic modifications leading to the activation and repression of transcriptional programs participate in the regulation of these events (Bonifer et al., 2006; Surani et al., 2007). These transcriptional programs are controlled by a close coordination between transcription factors and chromatin states, both of which are regulated by extracellular signals. Indeed, protein complexes that modulate chromatin structure, including SWI/SNF and Polycomb, have been implicated in the differentiation of stem cell populations (Bernstein et al., 2006; Chambeyron and Bickmore, 2004; Mikkelsen et al., 2007).

Geminin is a protein that has been implicated in the regulation of cell proliferation and differentiation through interactions with the complexes that license chromatin for replication, transcription factors and epigenetic regulators (Karamitros et al., 2011; Kroll, 2007). It inhibits cell cycle progression through direct binding to Cdt1, a central replication-licensing factor (Petropoulou et al., 2008; Tada et al., 2001; Wohlschlegel et al., 2000). Furthermore, geminin has been proposed to antagonize Brg1 (Smarca4) chromatin remodeling activity in embryonic and neural stem cells, maintaining cells in a pluripotent/uncommitted state (Roukos et al., 2007; Seo et al., 2005; Yang et al., 2011). Moreover, its interaction with the Polycomb group complex has been shown to regulate fate commitment decisions in the early *Xenopus* embryo (Lim et al., 2011). Recent studies have extended the role of geminin in the regulation of development and homeostasis of the hematopoietic and immune systems (Karamitros et al., 2010b; Ohno et al., 2010; Ohtsubo et al., 2008; Shinnick et al., 2010). Geminin has been proposed to regulate erythrocyte and megakaryocyte generation in the adult hematopoietic system (Shinnick et al., 2010). We have previously shown that geminin is not required for intrathymic T cell commitment and proliferation, but is essential for the rapid divisions of peripheral T cells after T cell receptor activation *in vitro* or during homeostatic expansion *in vivo* (Karamitros et al., 2010a).

Here, we investigate the *in vivo* role of geminin in definitive hematopoiesis. Genetic ablation of geminin during development increases HSC numbers and blocks the generation of committed and differentiated cells, leading to severely impaired hematopoiesis and

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embryonic lethality. This phenotype is distinct from that reported for inactivation of geminin in the adult hematopoietic system, in which the population of definitive HSCs and progenitor cells was preserved (Shinnick et al., 2010). Transcriptomics analysis reveals that geminin is required for the expression of genes that are crucial for the maintenance of self-renewing HSCs and the generation of lineage-committed progenitor cells. To investigate further the mechanism by which geminin regulates the expression of key transcription factors we examined HoxA9, HoxA10 and Gata1 as key factors for the maintenance and differentiation of HSCs. Our data show that in K562 cells geminin binds to *HOXA9* regulatory regions and its absence reduces the recruitment of SUZ12, leading to an increase in epigenetic marks linked with transcriptional activation, accompanied by a respective decrease in the repressive marks examined. Similarly, changes in histone modifications at the *HOXA10* and *GATA1* genes were detected in K562 cells that were consistent with their upregulation and downregulation, respectively, in the absence of geminin.

RESULTS
Geminin ablation in HSCs leads to embryonic lethality
To examine the *in vivo* role of geminin in the self-renewal and differentiation of fetal hematopoietic stem and progenitor cells (HSPCs) we inactivated geminin in HSCs during embryogenesis. Mice homozygous for the floxed allele (Fl/Fl) were crossed with mice carrying one functional and one null allele of geminin and the Vav1:iCre transgene (Wt/koVav1:iCre) (de Boer et al., 2003; Karamitros et al., 2010b) (supplementary material Fig. S1A). Fl/koVav1:iCre mice were used as experimental subjects, while Fl/wt animals with or without the *Cre* transgene and Fl/ko littermates were used as controls. Ablation of geminin expression was confirmed by qPCR on RNA samples from LSK [Lin$^-$ c-Kit^{high} Sca1$^+$ (Lin refers to lineage marker; Sca1 is also known as Ly6a)] cells derived from fetal liver from Fl/koVav1:iCre embryos, revealing a reduction of more than 90% in geminin mRNA expression (supplementary material Fig. S1B).

Morphological examination at embryonic day (E) 13.5 revealed that Fl/koVav1:iCre embryos were paler and anemic with evident dysgenesis of fetal liver compared with control littermates. At E15.5, the fetal liver, which is the major hematopoietic organ, was significantly smaller and exhibited dyserythropoiesis (supplementary material Fig. S1C). Total fetal liver cellularity in Fl/koVav1:iCre embryos corresponded to 21% of that of the respective controls [control, 5.96×10^7 cells (*N*=16); Fl/koVav1: iCre, 1.29×10^7 cells (*N*=11); *P*<0.001, supplementary material Fig. S1D]. Necrotic tissue was observed in embryos lacking geminin expression at E17.5, suggesting that lethality was occurring around this stage, a likely result of insufficient oxygenation of the tissue due to the severe anemia, and we did not detect any viable mice lacking geminin expression in the hematopoietic system (supplementary material Fig. S1E). Our results indicate that ablation of geminin in HSCs results in severe defects in fetal liver hematopoiesis and leads to embryonic lethality.

Defective fetal liver hematopoiesis in the absence of geminin
In order to dissect the hematopoietic phenotype, the cellular composition of Fl/koVav1:iCre mice was analyzed in fetal liver at E15.5. The total number of Ter119 (Ly76)$^+$ cells was reduced by 90% in Fl/koVav1:iCre compared with control embryos [control, 3.54×10^7 (*N*=16); Fl/koVav1:iCre, 0.34×10^7 (*N*=11); *P*<0.001; Fig. 1A]. Similarly, the Ter119$^-$ population presented a 76%

reduction in Fl/koVav1:iCre embryos [control, 3.3×10^6 (*N*=16); Fl/koVav1:iCre, 0.79×10^6 (*N*=11); *P*<0.001; Fig. 1A]. Significant reductions were observed in myeloid and lymphoid lineage cells. More specifically, myeloid Gr1 (Ly6g)$^+$ and CD11b (Itgam)$^+$ fetal liver cells were reduced by 85% [control, 3.92×10^5 (*N*=16); Fl/koVav1:iCre, 0.566×10^5 (*N*=11); *P*<0.001] and 89% [control, 2.59×10^5 (*N*=16); Fl/koVav1:iCre, 0.275×10^5 (*N*=11); *P*<0.001], respectively, in Fl/koVav1:iCre embryos compared with control embryos (Fig. 1B). The number of B220 (Ptprc)$^+$ cells exhibited a 70% reduction [control, 6.7×10^4 (*N*=16); Fl/koVav1:iCre, 1.88×10^4 (*N*=11); *P*<0.001; Fig. 1B]. Reduction in the population of CD11c (Itgax)$^+$ cells in the fetal liver was also observed in embryos lacking geminin expression [control, 5.83×10^5 (*N*=16); Fl/koVav1:iCre, 3.29×10^5 (*N*=11); *P*=0.06; Fig. 1B]. Our data suggest that the generation of differentiated cells in the fetal hematopoietic system is severely compromised by the absence of geminin.

Geminin is required for the generation of committed hematopoietic progenitor cells during fetal development
We next examined whether geminin ablation preferentially affects the differentiation to specific hematopoietic lineages by further analyzing the phenotypic composition of the hematopoietic progenitor compartment. The expression of FcγRII/III [also known as Fcgr2b (CD32)/Fcgr3 (CD16)] and CD34 in hematopoietic progenitor cells (HPCs) was analyzed to quantify the common myeloid progenitor (CMP; Lin$^-$ c-Kit^{high} Sca1$^-$ FcγRII/III^{lo} CD34$^+$), granulocyte/monocyte progenitor (GMP; Lin$^-$ c-Kit^{high} Sca1$^-$ FcγRII/III^{high} CD34$^+$) and megakaryocyte/ erythrocyte progenitor (MEP; Lin$^-$ c-Kit^{high} Sca1$^-$ FcγRII/III^{lo} CD34$^-$) populations. Fetal liver of Fl/koVav1:iCre embryos exhibited a severe impairment in the generation of HPCs (Lin$^-$ c-Kit^{high} Sca1$^-$) and was almost devoid of CMPs [control, 7.04×10^4 (*N*=13); Fl/koVav1:iCre, 0.21×10^4 (*N*=5); *P*<0.001; Fig. 2A,B]. Similarly, the MEP and GMP subsets, which are the immediate progeny of CMPs, were dramatically reduced in the fetal liver of geminin-deficient embryos [MEP: control, 3.76×10^5 (*N*=13); Fl/ koVav1:iCre, 0.029×10^5 (*N*=5); *P*<0.001; GMP: control, 8.90×10^4 (*N*=13); Fl/koVav1:iCre, 0.028×10^4 (*N*=5); *P*<0.05; Fig. 2A,B). Moreover, the total number of common lymphoid progenitors (CLPs; Lin$^-$ Flt3$^+$ IL7Rα$^+$) was also reduced by 60% in the fetal liver of Fl/koVav1:iCre compared with control embryos (supplementary material Fig. S2). Our results suggest a more prominent function for geminin in fetal hematopoiesis, in contrast to a previous study in which no significant defects were observed in the generation of CMPs, MEPs or GMPs in the absence of geminin from the adult hematopoietic compartment (Shinnick et al., 2010).

To confirm reductions in the number of committed progenitors observed in the *in vivo* analysis we evaluated their proliferation and differentiation potential by clonogenic *in vitro* colony-forming assays. In the presence of SCF (Kitl), IL3, IL6, Flt3l, GM-CSF (Csf2), TPO and EPO, fetal liver cells obtained from control embryos gave rise to various types of colonies including CFU-GEMM, CFU-G, CFU-M, CFU-GM and BFU-E. By contrast, cells from Fl/koVav1:iCre embryos exhibited a severely reduced number of clonogenic progenitors (Fig. 2C). To further evaluate the capacity of geminin-deficient HPCs, a cytokine cocktail driving cell specification towards myeloid or erythroid differentiation was used. Geminin-deficient fetal liver HPCs exhibited a severe defect in the generation of CFU-G, CFU-M, CFU-GM and BFU-E colonies, demonstrating a virtual absence of functional myeloid and erythroid progenitors in line with the reduced numbers of
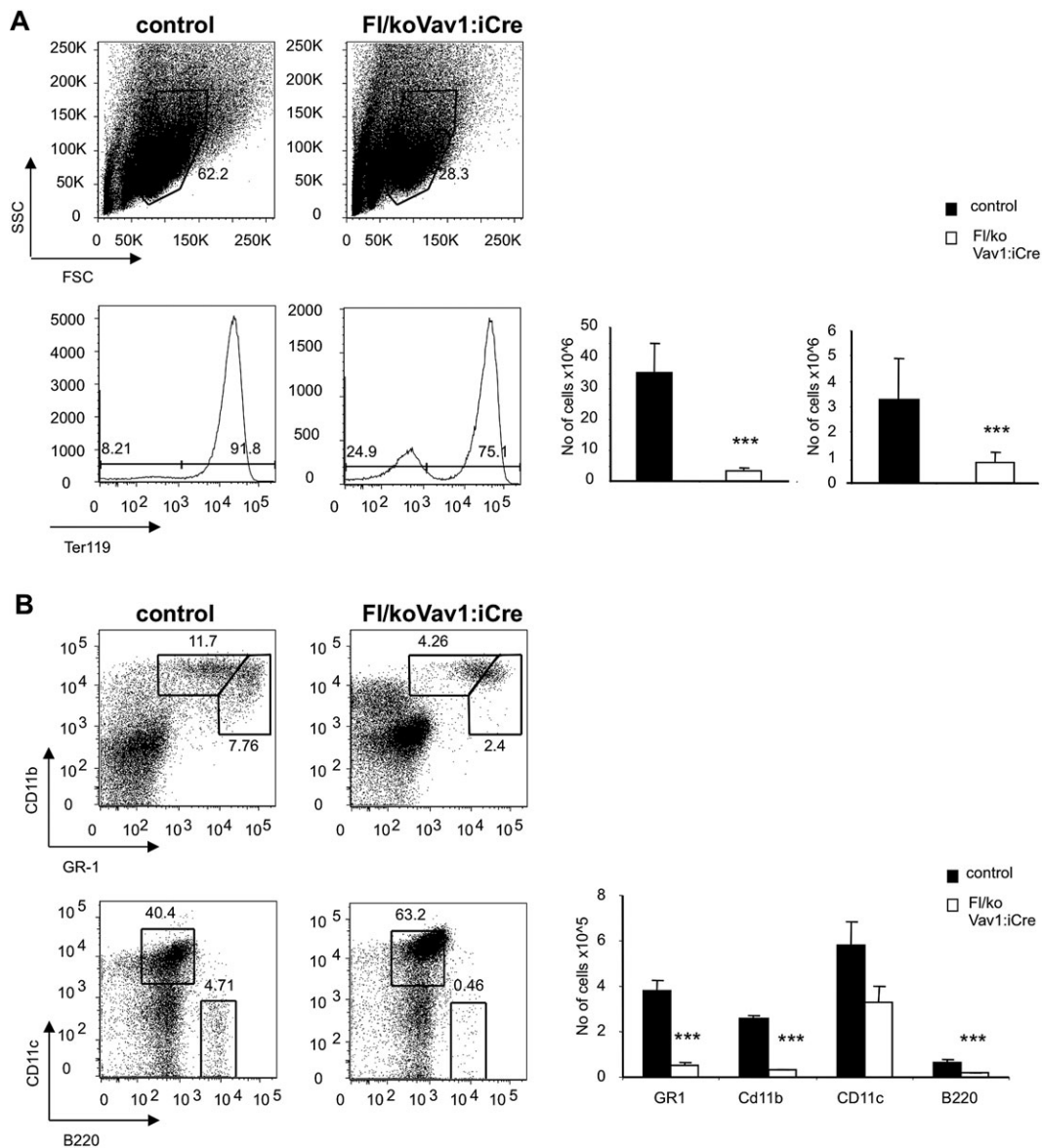
Fig. 1. Inactivation of geminin in fetal HSCs reduces the number of differentiated erythroid, myeloid and lymphoid cells. (A) Absolute numbers of Ter119$^+$ and Ter119$^-$ cells from livers of control (N=16) or Fl/koVav1:iCre (N=11) E15.5 mouse embryos. (B) Absolute numbers of myeloid and lymphoid lineage cells. Representative FACS analyses are shown. Values indicate the average number of cells; error bars indicate s.e.m. ***P<0.001 (two-tailed t-test).

phenotypically defined hematopoietic progenitors (Fig. 2D,E). Cell cycle assessment of fetal liver HPCs was determined by Ki67 and 7-amino actinomycin D (7-AAD) intracellular staining. In the absence of geminin, Lin$^-$ c-Kit^{high} Sca1$^-$ cells exhibited a reduced percentage in G1 phase and a small increase of cells in S/G2/M phase, indicating no gross defect in HPC proliferation *in vivo* (see Fig. 4).

In vivo and *ex vivo* analyses therefore suggested that lineage-committed progenitors that lack geminin expression were greatly reduced in number, possibly due to defects in their generation. Furthermore, geminin-deficient fetal HPCs also failed to generate differentiated progeny.

Accumulation of geminin-deficient fetal HSCs in the absence of further differentiation

Defects in mature blood cells and committed progenitor cells observed in fetal liver in the absence of geminin expression might

reflect a disturbed homeostasis in the HSC compartment. We therefore investigated the number of fetal liver HSCs in Fl/koVav1:iCre mice. Flow cytometric analysis revealed that Fl/koVav1:iCre fetal livers contained threefold more LSK (Lin$^-$ c-Kit^{high} Sca1$^+$) cells than the control mice [control, 1.76×10^4 (N=11); Fl/koVav1:iCre, 4.61×10^4 (N=9); P<0.05; Fig. 3A,C]. By contrast, the absolute number of HPCs (Lin$^-$ c-Kit^{high} Sca1$^-$) in the fetal liver of Fl/koVav1:iCre mice was reduced by >95% [control, 6.41×10^5 (N=11); Fl/koVav1:iCre, 0.141×10^5 (N=9); P<0.01], which is in accordance with the reduced numbers of committed progenitor cells observed in the absence of geminin (Fig. 3A,B).

To further validate that ablation of geminin can promote an increase in the HSC population we refined our analysis by investigating CD150 (Slamf1) SLAM family receptor expression, as previously shown to be present on fetal liver LT-HSCs (Kim et al., 2006). The absolute number of LSK CD150$^+$ CD48$^-$ fetal liver cells was increased by 60% in Fl/koVav1:iCre embryos when
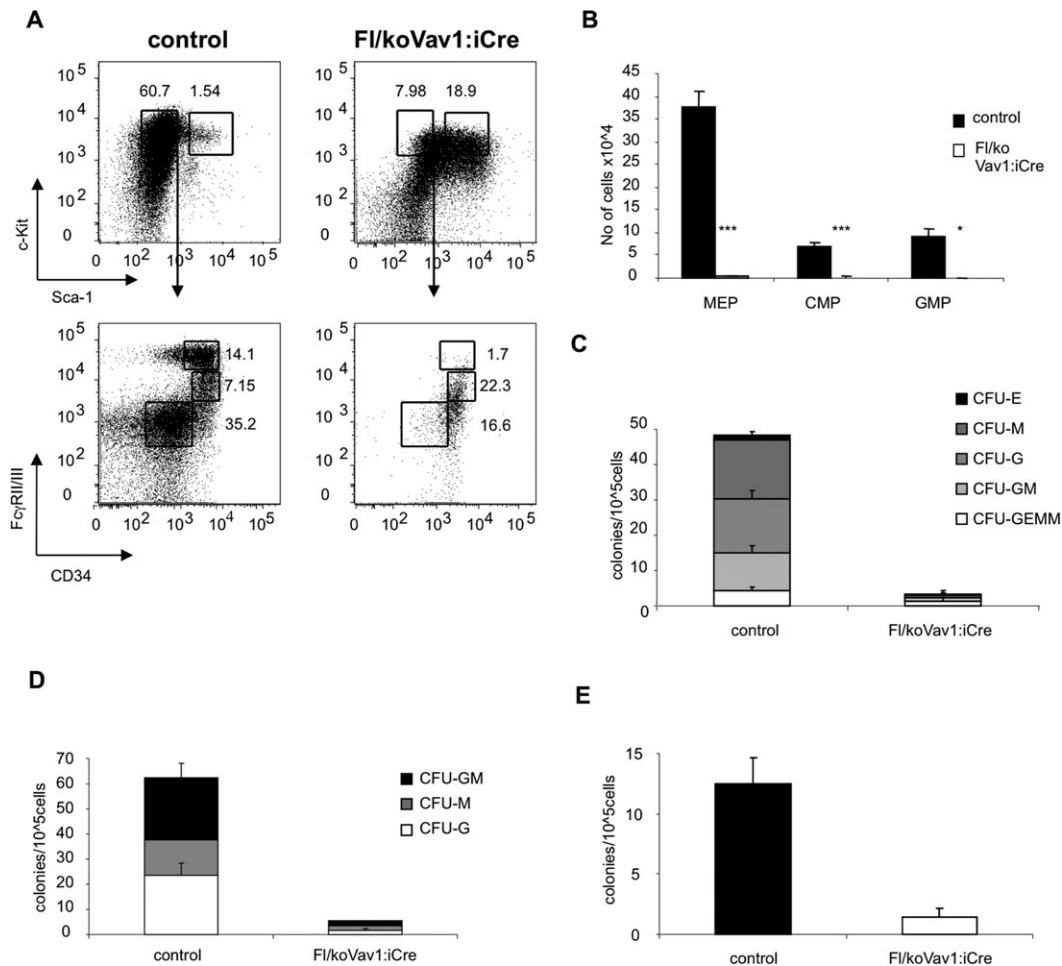
Fig. 2. Reduced numbers and self-renewal of HPCs in the absence of geminin. (A) Representative FACS analysis using antibodies against lineage, c-Kit, Sca1, FcγRII/III and CD34 as indicated. Values indicate the percentage of cells within the gate. (B) Quantification of the average absolute numbers of Lin$^-$ c-Kit^{high} Sca1$^-$ FcγRII/III^{lo} CD34$^+$ (CMP), Lin$^-$ c-Kit^{high} Sca1$^-$ FcγRII/III^{hi} CD34$^+$ (GMP) and Lin$^-$ c-Kit^{high} Sca1$^-$ FcγRII/III^{lo} CD34$^-$ (MEP) cells from E15.5 control (N=13) and Fl/koVav1:iCre (N=5) embryos. Values indicate average number of cells; error bars indicate s.e.m. *P<0.05, ***P<0.001 (two-tailed t-test). (C-E) Clonogenic in vitro colony-forming assays were performed using different cytokine cocktails promoting cell specification towards (C) CFU-GEMM: colony-forming unit (CFU) activity of granulocyte, erythroid, macrophage, megakaryocyte colonies, granulocyte-macrophage or mixed colonies; (D) CFU-GM: CFU activity of granulocyte-macrophage colonies; and (E) BFU-E: burst-forming unit activity of erythroid colonies. Values indicate number of colonies counted per 10^5 fetal liver cells seeded; error bars indicate s.e.m.

compared with controls [control, 2.63×10^3 (N=11); Fl/koVav1:iCre, 4.28×10^3 (N=9); P<0.01], confirming the observation that the absolute number of LT-HSCs is increased upon geminin ablation (Fig. 3A,D). The number of SLAM$^+$ LSK cells is not altered upon ablation of geminin in the adult hematopoietic system using Mx1-Cre (Shinnick et al., 2010).

We next investigated whether the increase in LT-HSCs was due to increased proliferation in this compartment. We performed Ki67 and 7-AAD staining on the LSK population from Fl/koVav1:iCre and control mice. Analysis of the cell cycle distribution of control and Fl/koVav1:iCre LSK HSCs revealed an increased percentage of geminin-deficient LSK HSCs in the S/G2/M phases [control, 21% (N=10); Fl/koVav1:iCre, 50% (N=5); P<0.05; Fig. 4]. In accordance with this, a reduced number of quiescent cells (Ki67$^-$) was observed among the geminin-deficient LSK population (Fig. 4). The percentage of cells with greater than 4N DNA content did not differ significantly between geminin-deficient and control fetal liver. Our data suggest that the observed accumulation of a population that is immunophenotypically equivalent to HSCs is due to an increase in the cycling LSK population in the absence of geminin.

Ablation of geminin alters transcription factor expression

Our in vivo and in vitro findings suggested that geminin is an essential regulator of hematopoiesis. To gain insight into the mechanism of geminin function, we compared the genome-wide transcriptome of geminin-deficient HSPCs (FACS-sorted Lin$^-$ fetal liver cells as described in the Materials and Methods) with that of their control counterparts. A total of 1929 genes were identified as differentially expressed in Fl/koVav1:iCre HSPCs compared with wild type, exhibiting a greater than 1.5-fold change (FC) and P<0.05 (Fig. 5A). The majority of the differentially expressed genes in HSPCs in the absence of geminin were upregulated (1389 genes, FC ranging between +1.5 and +11.9), while downregulated genes were considerably fewer (540 genes, FC between −1.5 and −8.02) (Fig. 5A). Comparison of our findings with previously established hematopoietic fingerprint genes (Chambers et al., 2007) showed that a significant number of genes associated with 'stemness' in hematopoietic cells were overexpressed in the Fl/koVav1:iCre HSPCs (supplementary material Table S1). Notably, expression of the erythroid-specific genes was downregulated in the absence of geminin, while genes associated with myeloid or lymphoid
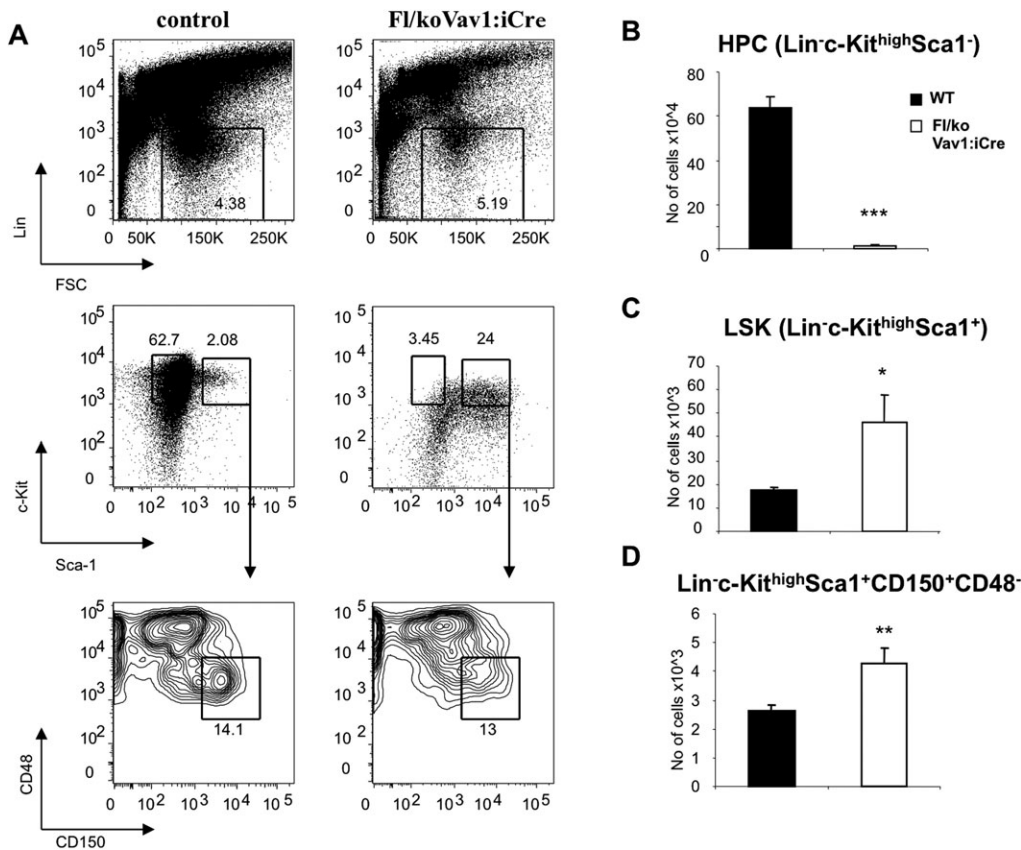
Fig. 3. Fetal liver of embryos lacking geminin is enriched for HSCs. FACS analysis of the hematopoietic stem and progenitor compartment in control and Fl/koVav1:iCre embryos. Fetal liver cells were stained with a cocktail of antibodies against lineage markers, Sca1, CD150, CD48 and c-Kit. (A) Representative FACS analysis of Lin⁻ c-Kit^high Sca1⁻ (HPC), Lin⁻ c-Kit^high Sca1⁺ (LSK) and Lin⁻ c-Kit^high Sca1⁺ CD150⁺ CD48⁻ cells from the fetal liver of E15.5 control or Fl/koVav1:iCre embryos. Numbers indicate percentage of population within gates. (B-D) Quantification of average absolute cell numbers for (B) HPCs, (C) LSK cells and (D) Lin⁻ c-Kit^high Sca1⁺ CD150⁺ CD48⁻ cells from FACS analysis of E15.5 control (N=14) and Fl/koVav1:iCre (N=10) embryos. Values indicate average number of cells; error bars indicate s.e.m. *P<0.05, **P<0.01, ***P<0.001 (two-tailed t-test).

lineages were under-represented in geminin-deficient HSPCs (supplementary material Table S1).

To examine whether the genes found to be differentially expressed upon geminin deletion were involved in specific biological processes or pathways, Gene Ontology (GO) or KEGG pathway analysis was performed using GSEA and DAVID, respectively. GO analysis showed significant enrichment for genes involved in the regulation of cell proliferation, hematopoiesis, transcriptional regulation and cellular homeostasis, among others (Table 1). Transcription factors associated with the maintenance of
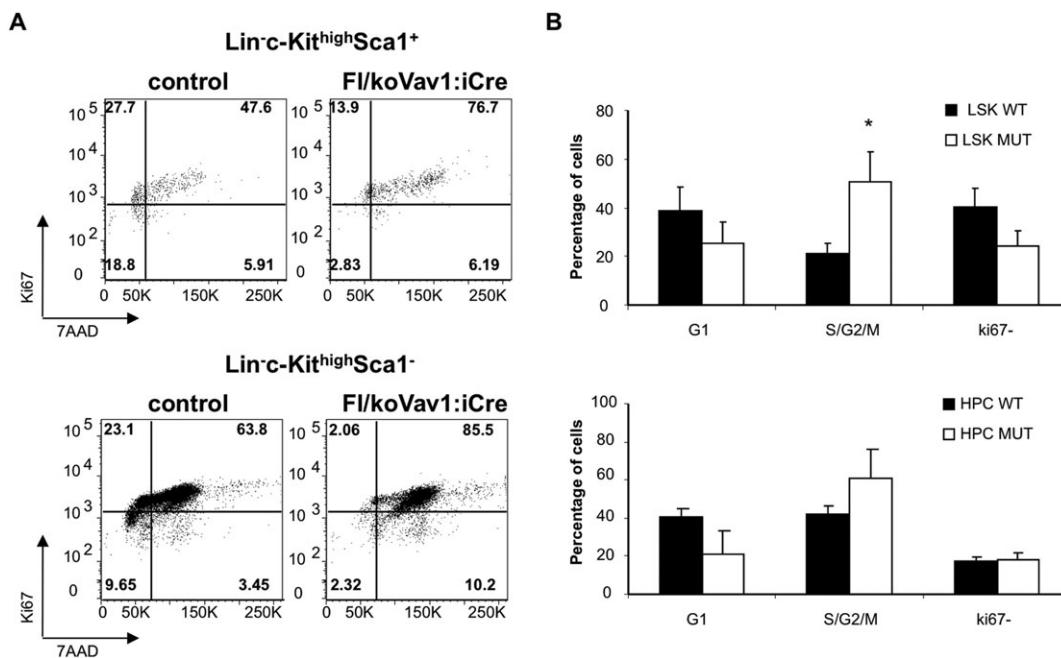

Fig. 4. Cell cycle defects of geminin-deficient hematopoietic stem and progenitor cells. (A) Cell cycle analysis of Lin⁻ c-Kit^high Sca1⁺ HSCs and Lin⁻ c-Kit^high Sca1⁻ HPCs from fetal livers of E15.5 control (N=10) and Fl/koVav1:iCre (N=5) embryos. Fetal liver cells were stained with a cocktail of antibodies against lineage, c-Kit and Sca1, fixed, permeabilized and stained with anti-Ki67 and 7-AAD. Representative FACS analyses of Ki67 and 7-AAD are shown. (B) The frequency of HSCs and HPCs in each phase of the cell cycle. Error bars indicate s.e.m. *P<0.05 (two-tailed t-test).
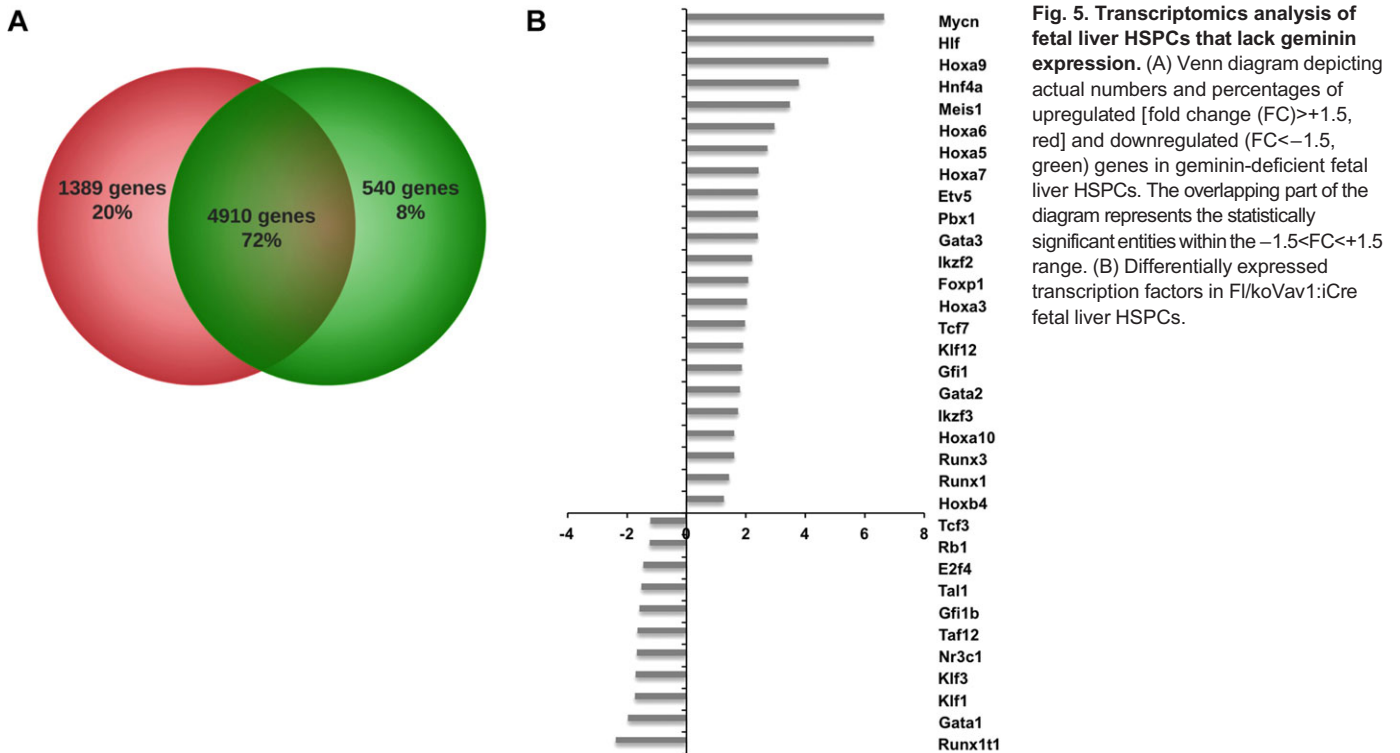
Fig. 5. Transcriptomics analysis of fetal liver HSPCs that lack geminin expression. (A) Venn diagram depicting actual numbers and percentages of upregulated [fold change (FC)>+1.5, red] and downregulated (FC<−1.5, green) genes in geminin-deficient fetal liver HSPCs. The overlapping part of the diagram represents the statistically significant entities within the −1.5<FC<+1.5 range. (B) Differentially expressed transcription factors in Fl/koVav1:iCre fetal liver HSPCs.

hematopoietic multipotency (Mycn, HoxA9, HoxA10, Gata2, Hlf, Meis1) were upregulated in geminin-deficient HSPCs (Fig. 5B; supplementary material Fig. S3, Table S1). The transcription factors that were differentially expressed in our analyses have been shown to be regulated by the concerted action of the Polycomb (PcG) and Trithorax (TrxG) groups of epigenetic regulators (supplementary material Table S2) (Cui et al., 2009; Schuettengruber et al., 2007). Moreover, a large fraction of the genes that were differentially expressed in geminin-deficient HSPCs have been characterized as Polycomb targets (supplementary material Table S3) or bivalent genes in embryonic stem cells (ESCs) (supplementary material Table S4).

Our results suggest that geminin regulates the transcription of several genes, among which are those encoding transcription factors involved in the maintenance of HSCs and the generation of committed progenitor cells. Moreover, geminin-dependent transcriptional control during hematopoiesis involves genes regulated by PcG and TrxG proteins, suggesting that geminin might be involved in modulating epigenetic marks.

Absence of geminin alters histone modifications and the recruitment of SUZ12 at *HOXA9* regulatory regions

Transcriptome analysis of geminin-deficient HSPCs revealed increased expression of a number of genes linked with the maintenance of stem and progenitor cells and a concomitant downregulation of genes that are expressed upon fate determination. It was previously shown that geminin and Rae28 co-immunoprecipitate in mouse embryonic extracts, while chromatin immunoprecipitation (ChIP) experiments showed that geminin binds to three Plzf binding sites located within the *Hoxd11* gene (Luo et al., 2004). Moreover, gain- and loss-of-function experiments in the chick neural tube demonstrate that geminin modulates transcription of *Hoxb9* (Luo et al., 2004). It has also been suggested that geminin is required for Polycomb binding and Polycomb-dependent histone modifications during early *Xenopus*

development (Lim et al., 2011). Transcriptomics analysis of HSPCs showed that several members of the homeobox family increase in expression upon geminin deletion. As transcriptional repression of homeobox genes is mediated by the PcG complex, we focused on *Hoxa9* regulation as an example to demonstrate that geminin affects epigenetic modifications and subsequently gene transcription through Polycomb-mediated repression. *Hoxa9* is expressed mainly in the early hematopoietic hierarchy and plays a key role in the maintenance of HSPCs (Lawrence et al., 2005; Thorsteinsdottir et al., 2002). Moreover, transcriptomics analysis showed that *Hoxa9* is among the top differentially expressed transcription factors in geminin-deficient HSPCs.

We performed knockdown experiments for geminin in human K562 erythroleukemia cells. These cells express high levels of geminin, whereas *HOXA9* mRNA is undetectable (Dorsam et al., 2004; Wilkinson et al., 2013). Upon siRNA treatment, geminin expression was significantly reduced and this resulted in a nearly sixfold increase of *HOXA9* mRNA expression (supplementary material Fig. S4). We performed ChIP experiments in K562 cells transfected with siRNAs against geminin (siGmnn) or luciferase (siLuc), a non-specific target used as a control, to assess histone modification changes upon geminin knockdown. Specifically, we examined the enrichment of active (H3K4me3, as well as RNA polymerase II recruitment) and repressive (H3K27me3 and H3K9me3) histone marks in the genomic region −500 bp to +500 bp relative to the transcription start site (TSS) of the *HOXA9* locus. The TATA box region of the beta-2 microglobulin (*B2M*), as well as the promoter region of *GAPDH*, provided controls for active marks (data not shown).

Consistent with the barely detectable expression of *HOXA9* in K562 cells, the active mark H3K4me3 was absent from the *HOXA9* regions examined, as was RNA polymerase II recruitment at these sites (Fig. 6A,B), whereas the H3K9me3 and the H3K27me3 repressive marks were significantly enriched (Fig. 6C,D). Upon geminin knockdown, RNA polymerase II recruitment was evident

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Table 1. GO analysis of biological processes affected upon geminin deletion from fetal HSPCs

Gene ontology	Biological process	Gene count	*P*-value
GO:0042127	Regulation of cell proliferation	189	2.03E–4
GO:0030097	Hematopoiesis	96	4.14E–4
GO:0002520	Immune system development	110	4.66E–4
GO:0048534	Hematopoietic or lymphoid organ development	104	9.02E–4
GO:0006357	Regulation of transcription from RNA polymerase II promoter	204	3.34E–3
GO:0001525	Angiogenesis	53	3.58E–3
GO:0019725	Cellular homeostasis	119	5.14E–3
GO:0045941	Positive regulation of transcription	159	6.06E–3
GO:0008285	Negative regulation of cell proliferation	81	6.84E–3
GO:0010628	Positive regulation of gene expression	162	7.90E–3
GO:0045449	Regulation of transcription	670	1.11E–2
GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	120	1.59E–2
GO:0006350	Transcription	533	2.43E–2
GO:0000082	G1/S transition of mitotic cell cycle	14	2.87E–2
GO:0009952	Anterior/posterior pattern formation	55	2.89E–2
GO:0000122	Negative regulation of transcription from RNA polymerase II promoter	79	3.09E–2

The top ranking processes as determined by DAVID are listed according to their *P*-value.

in the −500 bp region, with maximal occupancy at the TSS, thus signifying an actively transcribed gene (Fig. 6A). Enrichment of the active mark H3K4me3 was also increased fivefold in the −500 bp region and fourfold at the *HOXA9* TSS (Fig. 6B). Conversely, the repressive marks H3K9me3 and H3K27me3 were almost abolished (Fig. 6C,D).

To examine whether this might be the result of a direct interaction of geminin with the *HOXA9* regulatory regions we performed ChIP experiments using an anti-geminin polyclonal antiserum (Roukos et al., 2007), in order to detect geminin enrichment using a 500 bp tilling approach that spanned 4.0 kb in distal and proximal sites around the *HOXA9* TSS. Geminin binding was detected at a locus 4.0-3.5 kb upstream of the *HOXA9* TSS (Fig. 6E).

It was previously shown that *HOXA9* is regulated by Polycomb repressive complex 2 (PRC2) in hematopoietic cells (Nagel et al., 2010) and we therefore examined whether this regulation is altered in the absence of geminin. Using the same ChIP tilling approach described above, we examined enrichment for SUZ12, one of the three core components of PRC2, at the *HOXA9* gene locus. Chromatin was prepared from K562 cells that were stably transfected with shRNAs, either for a non-specific target (shControl) or for geminin (shGeminin). Significant enrichment of SUZ12 was observed by ChIP at the same distal site that geminin was previously detected, whereas upon geminin knockdown SUZ12 recruitment was significantly reduced (Fig. 6F). *SUZ12* mRNA expression was not altered by the ablation of geminin (data not shown).

Collectively, our results show that geminin binds to *HOXA9* regulatory regions and facilitates the recruitment of PcG components, and that geminin silencing leads to significant enrichment of the active histone mark H3K4me3 and RNA polymerase II recruitment, together with a sharp reduction of the repressive H3K9me3 and H3K27me3 marks. To further corroborate our findings, we examined the epigenetic marks in the promoter regions of the *HOXA10* and *GATA1* genes, the expression of which was increased and decreased, respectively, in HSPCs that lack geminin expression. We examined a region 1.0 kb downstream of the *HOXA10* TSS in K562 cells and found that, upon geminin knockdown, the repressive marks H3K27me3 and H3K9me3 were reduced, whereas the permissive mark H3K4me3 and RNA polymerase II loading were increased (supplementary material Fig. S5A). In the case of the lineage-specific transcription factor GATA1, a 1.5 kb region downstream of the *GATA1* TSS was examined by ChIP (supplementary material Fig. S5B). In K562 cells transfected with shGeminin, a decrease in RNA polymerase II occupancy and H3K4me3 modification was detected, whereas repressive marks were enriched (supplementary material Fig. S5B), in contrast to *HOXA9* and *HOXA10* and in agreement with the decrease in *GATA1* mRNA levels.

It has previously been shown that K562 cells can differentiate into erythrocytes and megakaryocytes (MKs) when cultured in the presence of hemin and phorbol ester (TPA), respectively (Lam et al., 2000; Hsu and Yung, 2003). Therefore, performing our *in vitro* differentiation experiments in this cell line makes the findings more coherent and directly comparable with those of the ChIP experiments. Silencing of geminin in K562 cells reduces the number of hemoglobin-expressing cells following hemin treatment from 80% in shControl to 55% when shGeminin was used (supplementary material Fig. S6). TPA treatment of K562 cells results in increased expression of the early MK marker CD41a (Itga2b) and the terminal MK differentiation marker CD61 (Itgb3) (supplementary material Fig. S7) (Mattia et al., 2002). Upon geminin knockdown, K562 cells treated with TPA exhibited reduced viability, while they showed increased numbers of CD41a^{+} cells and markedly reduced CD61^{+} cells, suggesting that MK terminal differentiation is defective in the absence of geminin (supplementary material Fig. S7). These results suggest that geminin downregulation reduces the ability of K562 cells to differentiate into erythrocytes and MKs, mimicking the differentiation defects observed in Fl/koVav1:iCre mice.

Our data thus suggest that geminin can directly modulate the expression of crucial transcription factors in hematopoiesis by modifying epigenetic marks in regulatory regions, thereby affecting cell fate choices.

DISCUSSION
During development, HSCs are required to coordinate the generation of different blood cell types and maintain a pool of constantly self-renewing cells. These processes are regulated by the concerted actions of transcription factors and epigenetic regulators, which ensure successful hematopoiesis and the maintenance of a progenitor pool that is sufficient to propagate mature cells throughout adult life (Cedar and Bergman, 2011; Iwasaki and Akashi, 2007).

Our results demonstrate that geminin is an essential regulator of definitive hematopoiesis, as its genetic ablation leads to severe defects in fetal liver hematopoiesis and premature embryonic lethality. Absence of geminin in fetal HSCs leads to reduced numbers of common progenitors of the lymphoid and myeloid lineages and, consequently, there is a dramatic decrease in terminally differentiated blood cell lineages. Moreover, our findings show that an immunophenotypically defined population that is equivalent to HSCs, characterized by Lin− c-Kit[high] Sca1+ CD150+ CD48−
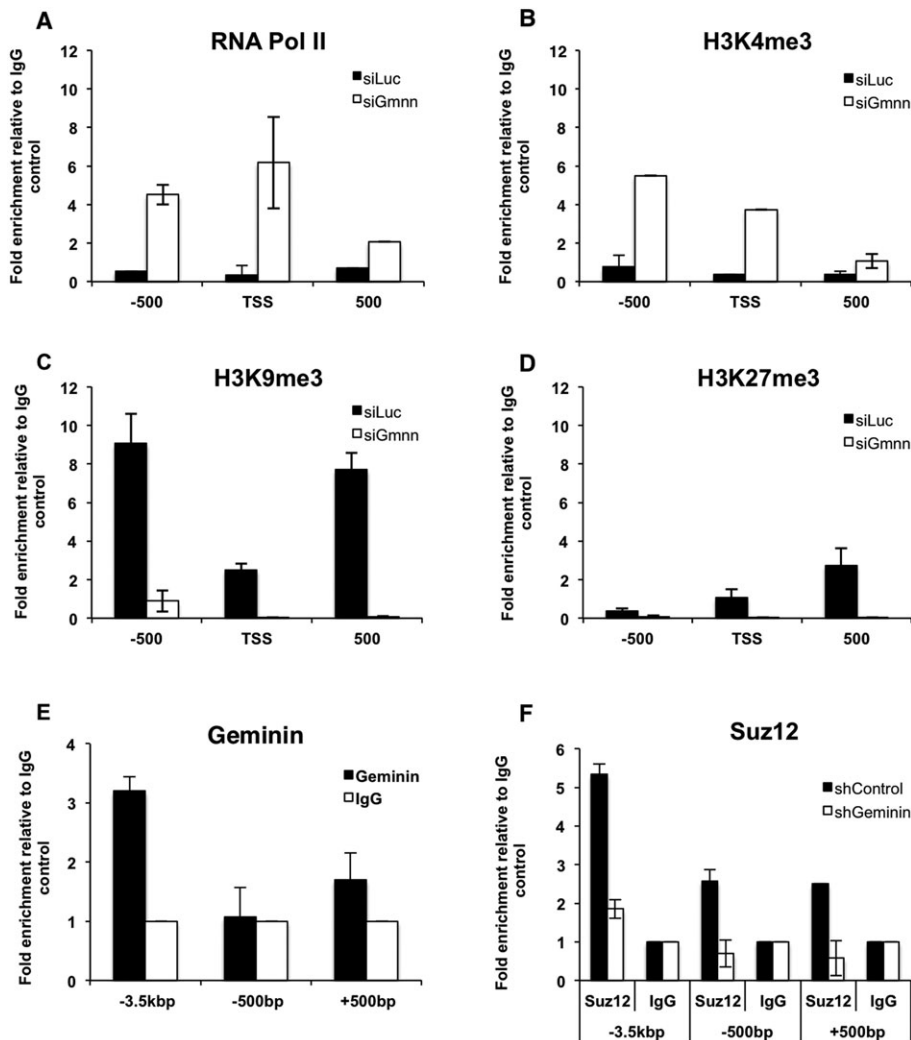
Fig. 6. Ablation of geminin modulates epigenetic marks and SUZ12 binding on the HOXA9 gene locus. ChIP in human K562 cells transfected with either siLuc or siGmnn, using antibodies recognizing (A) RNA polymerase II, (B) H3K4me3, (C) H3K9me3 and (D) H3K27me3. Chromatin isolated from K562 cells was incubated with a geminin-specific polyclonal antiserum and anti-Suz12 and a tilling approach encompassing −4.0 kb to +0.5 kb on the HOXA9 genomic locus was used. (E) Enrichment for geminin at distal loci 4.0 and 3.5 kb upstream of the HOXA9 TSS. (F) Immunoprecipitation using an anti-Suz12 antiserum and chromatin from K562 cells stably transfected with either an shControl vector or shGeminin showed that SUZ12 was enriched in the same distal elements as geminin, whereas geminin knockdown almost eliminated SUZ12 binding. Enrichment was determined as fold change over non-specific IgG anti-log values. Results shown are representative of three independent experiments performed in triplicate; error bars indicate s.e.m.

markers, is increased in the absence of geminin, similar to our previous observations for early cortical neuronal progenitors (Spella et al., 2011). The population within the LSK gate of the Fl/koVav1:iCre embryos presents an unusual phenotypic profile with lower levels of c-Kit expression. This profile is reminiscent of an LT-HSC-enriched, Kit intermediate population reported to proliferate extensively after transplantation in vivo (Grinenko et al., 2014), further reinforcing the observation of the accumulation of HSCs.

Our results demonstrate a distinct role for geminin in regulating fetal hematopoiesis when compared with previous work that investigated the role of geminin in the adult hematopoietic system (Shinnick et al., 2010). Shinnick et al. used the Mx1-Cre transgene to conditionally inactivate geminin in the adult hematopoietic system and showed that erythrocytes were reduced and white blood cells were only transiently affected (Shinnick et al., 2010). The numbers of SLAM^{+} LSK cells and of committed progenitor cells did not change significantly in the absence of geminin, whereas bone marrow MEPs presented skewed differentiation to MKs. Therefore, the authors proposed a regulatory role of geminin in erythrocyte/MK differentiation in adult hematopoiesis, without addressing the underlining molecular mechanism. Our results support a novel role for geminin in regulating the generation of HSCs and fate-restricted progenitors during fetal hematopoiesis. Differences in gene expression networks and function (Ciriza et al., 2012; Mayani, 2010), as well as hematopoiesis occurring in distinct tissue

compartments (i.e. fetal spleen, fetal liver bone marrow) (Kiel et al., 2005), may explain the phenotypic differences observed between adult and fetal hematopoiesis in the absence of geminin. Distinct roles in the genesis of primitive HSCs versus the maintenance of adult hematopoiesis have also been suggested for other transcriptional regulators (Chen et al., 2009; Ichikawa et al., 2008; Mikkola et al., 2003).

Previous studies have shown that overexpression of geminin at the midblastula stage in Drosophila and Xenopus embryos, as well as in cortical progenitor cells, promotes neuronal differentiation (Kroll et al., 1998; Quinn et al., 2001; Spella et al., 2011), while silencing of geminin impairs mouse ESCs from acquiring neural cell fate (Gonzalez et al., 2006; Yang et al., 2011; Yellajoshyula et al., 2011), emphasizing the role of geminin in regulating proliferation versus differentiation decisions in the nervous system. Our study extends previous observations and proposes that geminin controls self-renewal and differentiation decisions in fetal HSCs, implicating a more global function for geminin in the control of these decisions in different stem cell populations.

Towards a molecular understanding of the function of geminin in stem/progenitor cells, transcriptome analysis of Lin^{−} hematopoietic cells that lack geminin expression revealed increased expression of transcription factors associated with the self-renewal and maintenance of HSCs (such as Gata2, Hlf, Meis1 and Mpl). Ablation of geminin increases the expression of posterior HoxA

cluster genes (*Hoxa5*, *Hoxa6*, *Hoxa7*, *Hoxa9* and *Hoxa10*) and to a lesser extent of anterior Hox genes (*Hoxa1* and *Hoxa2*). Hox genes are known to play pivotal roles in the regulation of hematopoietic differentiation. Genes of the *HoxA* cluster, and specifically *Hoxa9* and *Hoxa10*, are mainly expressed during the early steps of hematopoiesis and are downregulated upon differentiation (Argiropoulos and Humphries, 2007; Lawrence et al., 1997; Magli et al., 1997; Singh et al., 2013; Thorsteinsdottir et al., 2002; Wheadon et al., 2011). In accordance with our findings, overexpression of *Hoxa9* and *Hoxa10* increases the pools of HSCs, suggesting a role for these genes in regulating self-renewal (Magnusson et al., 2007; Thorsteinsdottir et al., 2002). Interestingly, members of the *HoxA* cluster are also implicated in hematopoietic pathologies, including malignancies. For instance, *Hoxa5* and *Hoxb6* overexpression has been shown to inhibit erythropoiesis and induce myeloproliferative disorders (Fischbach et al., 2005; Luo et al., 2004; Magnusson et al., 2007; Thorsteinsdottir et al., 2002).

The observed changes in gene expression upon geminin deletion in mice indicate a direct role for geminin in repressing transcription factors associated with HSCs. They could also, however, reflect the increased proportion of HSCs in the HSPC population observed in Fl/koVav1:iCre mice. Our *in vitro* data make this latter scenario less likely, as upregulation of *Hoxa9* is recapitulated *in vitro* when geminin is depleted from K562 cells. *In vitro* experiments have, in addition, provided mechanistic insight into the pathway leading to transcriptional regulation by geminin. Hox genes are arranged in tandem arrays in the genome and their spatiotemporal expression patterns are regulated by antagonistic functions of PcG and TrxG, which define the expression domains of Hox genes in the genome as well as along the body axis (Hanson et al., 1999). It was previously shown that geminin is able to bind to *Hoxd11* regulatory sequences and directly interacts with PcG members, while it regulates the rostrocaudal axis of expression in Hox genes during chicken embryogenesis (Luo et al., 2004). We studied changes in the epigenetic status of the *HOXA9*, *HOXA10* and *GATA1* genes as examples so as to understand how geminin can modulate gene transcription. *Hoxa9* and *Hoxa10* presented attractive candidates owing to their well-documented role in the maintenance of the earliest stages of the hematopoietic hierarchy (Cellot et al., 2013; Ferrell et al., 2005; Sprussel et al., 2012; Wheadon et al., 2011; Magnusson et al., 2007) and to their regulation by PRC2 (Nagel et al., 2010). Furthermore, it has been shown that *Hoxa9* expression is regulated by the balanced introduction of suppressive (H3K27me3) and activating (H3K4me3) epigenetic modifications by the interacting Polycomb and Trithorax complexes, respectively (Erfurth et al., 2008; Guenther et al., 2005; Khan et al., 2013). Silencing of geminin in human K562 cells coincides with an increase in the permissive mark H3K4me3 and increased RNA polymerase II recruitment, whereas the repressive marks H3K9me3 and H3K27me3 were decreased in *HOXA9* and *HOXA10* regulatory regions. We have also shown that geminin is recruited to distal regulatory elements in the *HOXA9* gene and that this is needed for proper recruitment of PRC2 on the *HOXA9* locus. Our results indicate that geminin might modulate *HOXA9* gene expression by affecting PRC2 recruitment and, subsequently, the epigenetic organization of the *HOXA9* locus. By contrast, *GATA1* was negatively regulated upon geminin knockdown, and examination of its epigenetic organization showed that the permissive mark H3K4me3 and RNA polymerase II occupancy were decreased in favor of the repressive marks that were enriched in the locus examined. Consistently, a significant inhibition of K562 differentiation into erythrocytes and MKs (Fujiwara et al., 1996; Iwasaki et al., 2003) was observed following

geminin knockdown. These findings are in agreement with the phenotype of our *in vivo* model. They provide, for the first time, insight into the molecular network that is regulated by geminin, suggesting a pathway for the maturation of blood cells, which is indispensable for definitive hematopoiesis in the developing embryo.

The Polycomb and Trithorax complexes have been linked with epigenetic changes controlling cell fate decisions. Transcriptomics analysis showed that a significant number of the transcription factors with increased expression in the absence of geminin have been characterized as either targets of PcG proteins or exhibit a bivalent domain pattern in stem cells (Mikkelsen et al., 2007). Moreover, the enrichment of genes targeted by Polycomb proteins in ESCs (Boyer et al., 2006; Lee et al., 2006) in the pool of differentially expressed genes suggests that geminin might be involved in a more global epigenetic regulation of crucial transcription factors during hematopoiesis, as suggested for neural precursor cell differentiation (Yellajoshyula et al., 2012). A plausible hypothesis is that geminin participates in a specific composition of the PcG complex (Majewski et al., 2010) and could modify signals mediated by Polycomb and Trithorax for some of their targets genes (Schwartz et al., 2010), leading to aberrant or untimely expression in the absence of geminin.

In summary, we have identified a novel role for geminin in the regulation of fetal hematopoiesis. We provide evidence that geminin regulates the transition from the self-renewing HSC population to lineage-committed progenitor cells, and we propose that geminin modulates the expression of hematopoietic multipotency genes at the expense of lineage-specification transcriptional programs through the coordination and recruitment of epigenetic regulator complexes such as PRC2.

MATERIALS AND METHODS
Mice
Geminin*flox/flox* and geminin*+/−* Vav1:iCre mouse lines used for these studies were maintained on a C57BL/6 (CD45.2) background. B6.SJL-PtprcaPep3b/BoyJ (referred to as B6.CD45.1), *Rag2−/−* B6.JCD45.1 and C57BL/6 mice were obtained from the specific pathogen-free (SPF) facility of the National Institute for Medical Research, UK. All husbandry and animal experiments were conducted in accordance with the UK Home Office and the Achaia Animal Care and Use Committee, Prefecture of Western Greece, institutional guidelines of the Medical Research Council/ National Institute for Medical Research Ethical Committee and Patras University. Conditional inactivation of geminin was carried out using a mouse line in which exons 3 and 4 of the geminin gene are flanked by loxP sites (geminin*flox/flox*), permitting the conditional inactivation of geminin upon Cre expression. Mice and embryos were genotyped for the different geminin alleles by PCR using previously described primers (Karamitros et al., 2010b; Mantamadiotis et al., 1998). The *Vav1:iCre* transgene was detected by PCR as previously described (de Boer et al., 2003).

FACS analysis
Single-cell suspensions were prepared from E15.5 fetal livers and cells were stained with saturating concentrations of Pacific Blue, FITC, PE, PE-Cy7, PerCP, APC, APC-Cy7 or e-fluor780 conjugated antibodies in PBS containing 0.1% BSA and 0.05% NaN3 at 4°C for 30 min, then washed with PBS and subsequently analyzed using a BD Biosciences FACS CANTOII and FlowJo 8.7.1 software (Tree Star). For the analysis of hematopoietic stem and progenitor populations, 1×10^{6} fetal liver cells were stained with a biotinylated antibody cocktail against lineage markers CD3 (clone 145-2C11, eBioscience), CD8a (53-6.7, eBioscience), CD19 (clone MB19-1, eBioscience), B220 (RA3-6B2, eBioscience) Gr1 (clone GR-1, eBioscience), CD11b (M1/70, eBioscience), CD11c (N418, eBioscience) and Ter119 (TER-119, eBioscience) followed by streptavidin PerCP (BD Biosciences) or streptavidin APC (BD Biosciences) antibodies. In addition to the lineage markers for the analysis of LSK (Lin− c-Kit^high Sca1+) and LK

(Lin⁻ c-Kit^high Sca1⁻) populations, fetal liver cells were stained with antibodies against Pacific Blue-Sca1 (D7, BioLegend), PeCy7-CD150 (TC15-12F12.2, BioLegend), APC-CD48 (BCM1, eBioscience) and eFluor780-c-Kit (ACK2, eBioscience). Fetal liver cells were stained with PeCy7-Fcγ receptor II/III (93, eBioscience) and Alexa Fluor 647 anti-CD34 (RAM34, eBioscience) monoclonal antibodies to obtain GMP, CMP and MEP populations and with PE-IL7Ra (A7R34, eBioscience) and CD135 PECy7 (AF210, eBioscience) to obtain CLP cells. Cell sorting was performed on MoFlo machines (DakoCytomation) or FACS AriaII (Becton Dickinson).

Clonogenic assays

Fetal liver cells (1×10^5) were plated in duplicate in 35-mm cell culture dishes (Nalgene Nunc International). Methylcellulose-based medium (M3234, M3334 or M3434, StemCell Technologies) supplemented with 3 U/ml ng/ml recombinant human EPO, 10 ng/ml mouse IL3, 10 ng/ml recombinant human IL6, 50 ng/ml mouse SCF, 50 ng/ml mouse Flt3l, 20 ng/ml GM-SCF and 50 ng/ml mouse TPO (Peprotech) was used in the clonogenic assay. Colony formation of burst-forming unit-erythroid (BFU-E), colony-forming unit-granulocyte/macrophage (CFU-GM) and colony-forming unit-granulocyte/erythrocyte/monocyte/macrophage (CFU-GEMM) cells was analyzed after 10 days.

Cell cycle analysis

Fresh fetal liver cells were stained with antibodies against Sca1-PE (D7, eBioscience), eFluor780-c-Kit (ACK2, eBioscience) and a biotinylated cocktail of lineage antibodies (CD3, CD8, CD19, B220, GR1, CD11b, CD11c, Ter119) followed by streptavidin PerCP (BD Biosciences) or streptavidin APC (BD Biosciences). Cells were subsequently stained with anti-Ki67-FITC or an isotype control FITC conjugate along with 7-AAD according to the manufacturer's instructions (BD Biosciences).

Chromatin immunoprecipitation

K562 human erythroleukemia cells were grown in DMEM medium supplemented with 10% fetal bovine serum without antibiotics. Cells were reverse transfected with either control siRNA against luciferase (siLuc) or siRNA against human geminin (siGmnn) (300 μM, 0.5×10^6 cells) using RNAiMax (Invitrogen). The transfection was repeated after 24 h and geminin knockdown was assessed by western blot 72 h following the second transfection. Expression of *HOXA9* mRNA was assayed using quantitative real-time PCR (see methods in the supplementary material). Cells treated with siLuc or siGmnn were fixed in 1% formaldehyde and quenched with 0.125 M glycine prior to lysis and isolation of nuclei. Chromatin was sheared mechanically by sonication and quantified using a NanoDrop 2000 UV spectrophotometer (Thermo Scientific). ChIP assays were carried out using anti-RNA polymerase II [rabbit polyclonal; sc-9001 (H-224), Santa Cruz], anti-H3K4me3 [mouse monoclonal; Ab1012 (mAbcam1012), Abcam], anti-H3K27me3 [mouse monoclonal; Ab6002 (mAbcam6002), Abcam], anti-H3K9me3 (rabbit polyclonal; Ab8898, Abcam) and non-specific IgG antibodies conjugated to Protein G Dynabeads (Invitrogen) at 1 μg antibody per 50 μg chromatin. For geminin ChIP, 2.5 μg rabbit polyclonal antiserum (Roukos et al., 2007) was used per 40 μg chromatin. Monoclonal antibodies against Suz12 [mouse monoclonal; 05-1317 (clone 2AO9), Millipore] at 2 mg per 25 μg chromatin isolated from K562 cells stably expressing shRNAs (control or geminin; Origene) were used according to the manufacturer's instructions. Reverse cross-linked input and immunoprecipitated samples were cleaned by phenol-chloroform extraction, followed by precipitation with 3 M sodium acetate and isopropanol. Quantitative PCR was carried out using the primers listed in supplementary material Table S5. Immunoprecipitated DNA was quantified using the slope of primer-specific standard curves and fold enrichment was calculated relative to the non-specific IgG signals.

K562 differentiation assays

In vitro differentiation assays with K562 cells stably expressing shControl or shGeminin and treatment with hemin and TPA are described the methods in the supplementary material.

Microarray analysis

For whole-transcriptome analysis of E15.5 wild-type and Fl/koVav1:iCre mouse embryos, total RNA was isolated from FACS-sorted Lin⁻ fetal liver cells for probe synthesis and hybridization to Affymetrix Mouse Gene 1.0 ST chips. GO analysis was carried out using DAVID and gene set enrichment analysis (GSEA). Microarray data are deposited at GEO under accession GSE53056. For further details of RNA extraction and cDNA microarrays, see the methods in the supplementary material.

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