Transcriptional Regulation of Hhex in Hematopoiesis and Hematopoietic Stem Cell Ontogeny

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Abstract

Hematopoietic stem cells (HSCs) emerge during development via an endothelial-to-hematopoietic transition from hemogenic endothelium of the dorsal aorta (DA). Using in situ hybridization and analysis of a knock-in RedStar reporter, we show that the transcriptional regulator Hhex is expressed in endothelium of the dorsal aorta (DA) and in clusters of putative HSCs as they are specified during murine development. We exploited this observation, using the Hhex locus to define *cis* regulatory elements, enhancers and interacting transcription factors that are both necessary and sufficient to support gene expression in the emerging HSC. We identify an evolutionarily conserved non-coding region (ECR) in the Hhex locus with the capacity to bind the hematopoietic-affiliated transcriptional regulators Gata2, SCL, Fli1, Pu.1 and Ets1/2. This region is sufficient to drive the expression of a transgenic GFP reporter in the DA endothelium and intra-aortic hematopoietic clusters. GFP-positive AGM cells co-expressed HSC-associated markers c-Kit, CD34, VE-Cadherin, and CD45, and were capable of multipotential differentiation and long term engraftment when transplanted into myelo-ablated recipients. The Hhex ECR was also sufficient to drive expression at additional blood sites including the yolk sac blood islands, fetal liver, vitelline and umbilical arteries and the adult bone marrow, suggesting a common mechanism for Hhex regulation throughout ontogenesis of the blood system. To explore the physiological requirement for the Hhex ECR region during hematoendothelial development, we deleted the ECR element from the endogenous locus in the context of a targeted Hhex-RedStar reporter allele. Results indicate a specific requirement for the ECR in blood-associated Hhex expression during development and further demonstrate a requirement for this region in the adult HSC compartment. Taken together, our results identified the ECR region as an enhancer both necessary and sufficient for gene expression in HSC development and homeostasis. The Hhex ECR thus appears to be a core node for the convergence of the transcription factor network that governs the emergence of HSCs.

Introduction

Stem cells are essential for homeostasis of adult organs and also have roles in development, disease, and regeneration. They have the remarkable potential to develop into a specific array of differentiated cell types and maintain this property through successive cell divisions. Hematopoietic stem cells (HSCs) are amongst the best characterized adult stem cells (Bryder et al., 2006). However, despite years of work, a detailed understanding of the molecular regulation of HSC ontogeny is still lacking. Such an insight would guide the in vitro generation of HSCs from pluripotent stem cells (PSCs), affording novel sources of HSCs for transplantation. In addition, it may inform efforts to regulate HSC behaviour in the context of stem cell expansion and provide mechanistic insights into HSC dysregulation in malignancy.

During mouse embryonic development, HSCs first emerge in clusters of hematopoietic cells which appear to bud from the floor of the dorsal aorta, from a specialized subset of endothelium, the hemogenic endothelium. Recently, live imaging visualized this process in in vitro and in vivo models of hematopoiesis, capturing the formation of blood cells from endothelium in real time (Bertrand et al., 2010; Boisset et al., 2010; Eilken et al., 2009; Kissa and Herbomel, 2010; Lancrin et al., 2009). This process appears to occur without obvious cell division (Eilken et al., 2009) through a so-called endothelium-to-hematopoiesis transition (Kissa and Herbomel, 2010). Prior to the generation of HSC, different types of uni-, bi-, and multi-lineage progenitor cells are generated in the yolk sac, placenta, vitelline and umbilical arteries and heart (Dzierzak and Speck, 2008; Medvinsky et al., 2011; Nakano et al., 2013). These include, among others, the primitive erythrocytes that are formed in the E7.5 blood islands of the yolk sac and the erythro-myeloid progenitors (EMPs) that emerge from the hemogenic endothelium of the yolk sac one day later (Palis et al., 1999; Silver and Palis, 1997). EMPs populate the fetal liver and are critical to the survival of the embryo (Chen et al., 2011). Definitive HSCs are present in the fetal liver from E11 onwards (Sanchez et al., 1996) and move to the fetal bone marrow (BM) shortly before birth, where they maintain the lifelong production of all blood lineages.

The homeobox transcription factor Hhex is expressed in a range of multipotent hematopoietic progenitor cells and cell lines, as well as in endothelial cells of the embryonic vasculature. Its expression is transient and is downregulated during differentiation (Bedford et al., 1993; Crompton et al., 1992; Manfioletti et al., 1995; Thomas et al., 1998), suggesting a role for this gene in the early stages of hematopoietic cell differentiation (Kubo et al., 2005). Indeed, analysis of microarray data from the Immunological Genome Project revealed Hhex expression in multiple hematopoietic lineages, with highest levels in hematopoietic stem and progenitor cells, and developing B cells (Goodings et al., 2015). Moreover, Hhex appears to be required for maturation and proliferation of definitive hematopoietic progenitors from ES-derived hemangioblasts, with Hhex-null embryoid bodies showing a decreased ability to form definitive hematopoietic colonies in vitro (Guo et al., 2003; Kubo et al., 2005) at least in part because Hhex null hematopoietic progenitors accumulate in the G2 phase of the cell cycle (Paz et al., 2010). Conditional deletion experiments reveal a requirement for Hhex in adult hematopoietic stem and progenitor cell differentiation with defects particularly apparent in the lymphoid compartment of Hhex deficient animals (Goodings et al., 2015; Schoenwaelder and Jackson, 2015). Both studies revealed that loss of Hhex increased HSC proliferation while the functional ability of HSCs to contribute to multi-lineage engraftment in transplant was compromised, with one study showing failure of myeloid and lymphoid engraftment (Schoenwaelder and Jackson, 2015) and the other an impact on lymphoid engraftment only (Goodings et al., 2015).

Hhex is also recognized as a major regulator of many other aspects of early embryonic development, as an essential regulator of both anterior-posterior axis formation and endoderm development (Zamparini, 2006). While a transcriptional role for Hhex in hematopoiesis has not been demonstrated, its expression pattern suggests that its induction may be a node in the gene regulatory network governing the generation of hematopoietic cells. Using a transgenic approach, Rodriguez et al. showed that the dynamic patterns of Hhex expression during early murine development were regulated by a number of distinct *cis*-acting elements, and suggested that a blood island enhancer was present within an 8 kb region of the Hhex locus. This region also drove reporter gene expression in the AGM (Rodriguez et al., 2001). Indeed, using a

computational approach, Donaldson et al. identified a region within Hhex intron 1 that had similarities to the *Scl* enhancer (Donaldson et al., 2005). This element could be used to drive reporter gene expression in fetal liver cells and could bind hematoendothelial transcription factors in vitro. However, the extent to which this or other regions in the Hhex gene are required for expression in authentic HSCs during homeostasis or during their specification in ontogeny remains unknown.

Given the evidence that Hhex is a critical regulator of hematopoietic cell development, maturation and proliferation, we sought to dissect the mechanisms that regulate dynamic Hhex expression in embryonic and hematopoietic differentiation. We found that Hhex was expressed in the endothelium and functional HSCs, and describe an evolutionarily conserved regulatory element that binds HSC-associated transcription factors Fli1, Pu.1, Scl, Ets1/2, Gata1, and Gata2 and was both necessary and sufficient for hematopoietic expression of Hhex. This element thus provides both an experimental tool for forced gene expression in these compartments and a route into further understanding and dissection of their regulatory networks.

METHODS

Embryo and Adult tissue Staining

Embryos were obtained from timed matings as previously described (Rodriguez et al., 2001). Embryos were embedded for either wax or cryo-sectioning. In situ hybridization on wax sections was carried out as previously described (Brickman et al., 2000). Hhex probe was generated from the Hhex ENE1 plasmid, linearized and then transcribed with T3 polymerase. For immunostaining on sections, embryos were fixed in 4% PFA, washed with PBS, incubated in 15% sucrose, embedded in OCT compound and snap-frozen on dry-ice/ethanol. Sections were stained with the nuclear dye, TO-PRO-3 (Molecular Probes) and incubated with CD31 (clone MEC 13.3 BD Pharmingen), CD34 (clone RAM34, BD Pharmingen) and anti-rat Cy3 (Jackson laboratories) primary antibodies. Images were taken on SPE inverted Leica confocal microscope.

For analysis of AGM tissues, embryos were harvested at 10.5-11 dpc. The AGMs were dissected, incubated for 45 min at 37C in 0.125% collagenase (Type I; Sigma) in PBS/10%FCS, and made into a single cell suspension. Cells were washed in PBS/10%FCS and spun twice at 1000rpm. The pellet was resuspended in PBS/10%FCS. For analysis of 14 dpc foetal livers, tissues were harvested passed through a 100 μm cell strainer (BD Pharmingen). For analysis of bone marrow, femur, tibia and hip bones were dissected from adult mice (9-16 weeks old). Bone marrow was flushed from bones using a 25-gauge needle and IMDM media.

Hhex genomic sequences for mouse, rat and human were obtained from NCBI and chicken, zebrafish and fugu were obtained from Ensembl, and the frog sequence was obtained by probing a BAC library. Genomic sequences were aligned to the mouse sequence using the Vista alignment tool (www-gsd.lbl.gov/vista/).

Transgenic model generation

The Hhex-GFP plasmid generated by Rodriguez et al. (2001) was digested to derived different transgenic constructs containing the eGFP gene cloned in frame into the exon 1 of the Hhex gene, and which spans 4.2 kb upstream and 3.8 kb downstream of the endogenous promoter. Mouse transient transgenic embryos and transgenic founder lines were generated via pronuclear injection into (CBAxC57BL/6)/F2 zygotes following standard procedures. Transient transgenic embryos and F0 founder males were genotyped by PCR on genomic DNA. Transgene expression in embryos was assessed by fluorescent microscopy. Transgenic lines were established and maintained on a mixed (CBAxC57BL/6) background.

The HBD targeting construct was generated using an approach similar to that previously described by Morrison et al. (2008). A schematic of the targeting vector and locus is shown in Supplementary Figure S5, (A). The cloning strategy is available upon request. Correctly targeted clones were expanded and characterized by Southern blot (Supplementary Figure S5, (B)). The selection cassette was excised and ESC clones HBD-7.5 and HBD-18.1 were selected for injection into blastocysts to generate the HBD mouse line. Mice were maintained on a mixed 129Ola × C57Bl/6 background.

Flow cytometry analysis of HSCs and hematopoietic cell immunophenotype

For analysis of AGM-HSC populations, cells were stained with either (i) cKit APC (2B8, DB Pharmingen) and CD34-PE (RAM34, BD Pharmingen), (ii) VE-Cadherin (clone 11D4.1, BD Pharmingen) followed by anti-rat Tricolour (PeCy5) antibody and CD45-PE (30-F11, eBioscience) (Invitrogen) or (iii) CD45-PE, VE-Cad:PeCy5, cKit-APC, CD34 staining the rat antimouse CD34 biotin (BD Pharmingen) followed by incubation with the secondary antibody anti-streptavidin PeCy7 (BD Pharmingen). Fetal liver cells were stained for lineage markers using purified rat anti-mouse antibodies the Lin- (Ter119, Gr1, CD4, CD8, B220) followed by anti-rat Tricolour (Pe-Cy5) secondary antibody. For cKit and Sca-1 staining, cKit-APC and anti-Sca-1-PE were used. . Lastly CD34 biotinylated antibody (BD Pharmingen) combined with the streptavidin PeCy7 was used for CD34 staining. For immunophenotyping of bone marrow, cells were stained for lineage analysis with purified anti mouse Ter119 (erythroid), Gr1 and Mac-1 (granulo-monocytic), B220 (B cells), CD3, CD8 and CD4 (T cell) followed by anti-rat Tricolour (PeCy5) antibody and ckit APC, Sca-1 PE and CD34 FITC. + Kit + Sca. For LT/ST HSC compartment analysis, cells were stained as before for KLS, and CD34. Cells were analysed for HSC and progenitor compartments by flow cytometry using the Cyan ADP flow cytometry platform (Beckman Coulter, High Wycombe, UK).

Adoptive transfer/transplantation experiments

Prior to adoptive transfer, recipient mice aged ~8-12 weeks were subjected to 9Gys of irradiation. AGM tissue was prepared as described above. GFP+ cells were isolated using a MoFlo cell sorter. To calculate embryo equivalents (e.e.), the total numbers of AGM cells were counted, divided by the number of AGM tissues analysed:

% of GFP population/% of total population x number of cells per AGM = number of cells per e.e.

Irradiated recipients were intravenously injected with 0.9-1.6 e.e. of GFP+ cells and engraftment from the bone marrow of each recipient was analysed by flow cytometry for GFP+ contribution to myeloid (Gr-1, Mac-1), erythroid (TER119) and lymphoid (B220, CD3) lineages at 2-5 months post-transplant.

Colony forming assay (CFU-C)

GFP+ and GFP- cell populations were isolated by flow cytometry from AGM single cell suspensions and plated into M3434 (Stem cell technologies) according to the manufacturer's instructions.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as previously described (May et al., 2013). Briefly, 10^8 FDCPmix cells were cross-linked with either 1% or 0.4% formaldehyde nuclei collected, lysed and then sonicated to yield chromatin fragments of 150-500bp, before immunoprecipitation with 10µg of the following antibodies; Gata1 (N6, sc265x), Gata2 (H-116, sc9008x), Pu.1 (D19, sc5949x), Fli-1 (C19, sc356x), Ets1/2 (C275, sc112x), Elf-1 (C20, sc631x), cMyb (M19, sc516x) and Aml1 (N20, sc 8563x) (Santa Cruz). SCL ChIP was performed using 20µl of anti-SCL serum (kind gift from Dr. Catherine Porcher). Control ChIPs used 10µg of normal rabbit IgG (sc2027, Santa Cruz).

ChIP qPCR analysis and normalization

ChIP enrichment was detected by qPCR. Probes of interest mapped to ECR1 (probes ecr -1a and ecr-1b) and ECR2 (ecr-2 probe) (see Figure S6, Table 4). Probes to the Gapdh gene and exon 4 of the Hex gene were used as controls. Each probe mix was made of a mix of 1µl of each forward and reverse primer (200µM), 1µl of FAM-TAMRA probe (100µM) and 37µl of HPLC water. Samples were run in duplicate wells under the programme; 50ºC for 2 minutes, 95ºC for 10 minutes, 95ºC for 15 seconds and 60ºC for 1 minute, for 40 cycles. For each probe, a standard curve was generated using input DNA and Ct values converted to equivalent ng DNA. All specific probe results were normalised to the Hex exon4 control probe; these relative enrichments were then further normalised to the IgG control sample. Statistical significance was determined using the paired students t test, with each probe enrichment compared to the Hex exon 4 probe enrichment.

RESULTS

Hhex expression marks the earliest stages of Hematopoietic Development

During embryonic development, the first HSCs emerge from the ventral aspect of the dorsal aorta around day 10.5 in mouse development. We sectioned day E10.5 mouse embryos that had been stained for Hhex RNA and found expression of Hhex in the lining of the dorsal aorta as well as in clusters of emerging presumptive HSC (Figure 1A). Consistent with this, embryos in which a red fluorescent protein (RedStar, RS) was knocked into the Hhex locus (Morrison et al., 2008) show Hhex expression in endothelial precursors of the head vasculature (visible as fine thread-like structures in Figure 1C) and intersomitic vessels, in addition to the heart, thyroid and AGM (Figure 1B and 1C). Sectioning of these embryos revealed expression in the dorsal aortic endothelium and blood clusters in a similar manner to that observed for endogenous Hhex RNA (Figure 1D). Importantly, although insertion of the Redstar reporter leads to haploinsufficiency of Hhex expression, there are no obvious phenotypic effects and so all cellular compartments develop normally and can be assessed for Hhex-RedStar expression. To identify the elements dictating Hhex expression in hematoendothelial precursors, we inserted a green fluorescent protein (GFP) reporter into an 8 Kb fragment that contains previously identified distinct enhancers known to control Hhex expression during early development (Rodriguez et al., 2001). Transgenic embryos generated with this construct (Figure 1E) recapitulated Hhex expression with expression in thyroid, heart, AGM and intersomitic vessels (Figure 1F). Sections through the dorsal aorta of embryos carrying this construct (Figure 1G) demonstrate that the transgene contains elements sufficient to regulate Hhex expression in the endothelium and budding hematopoietic clusters of the dorsal aorta.

A Conserved Regulatory Element Drives Hhex Expression in Early Hematopoietic Development

To further delineate the *cis* regulation of Hhex in the dorsal aortic region, we generated a deletion series guided by both evolutionary conservation (Figure 2I, Supplementary Figure S1) and transcription factor binding profiles, as determined by chromatin immunoprecipitation (ChIP) analysis of a murine multi-potential bone marrow-derived cell line, FDCP mix (Figure S2). FDCP

mix are karyotypically normal and non-leukemogenic, self-renew in IL-3 and differentiate in response to physiological cues, thus providing a tractable cell model to explore multipotent, adult hematopoietic progenitor biology. The expression patterns observed in all the Hhex-GFP embryos tested are summarized in Supplementary Figure S3, with the exception of Hhex construct D which did not express in blood-affiliated structures. We found that all constructs retaining the conserved sequence in the first intron of Hhex gave expression in emerging hematopoietic cells (Figure 2II). This is most clearly evidenced in construct Hhex*B* (Figure 2IIB) where an intronic fragment of approximately 1.2 kb is sufficient to drive expression. Notably, this 1.2 kb contains two evolutionarily conserved sub-regions (dubbed ECR1a/b and ECR2, Figure 2I), which bound a variety of hematopoietic-affiliated transcription factors (Scl, Fli1, Pu.1, Elf1, C-Myb, Gata2, and ETS1) in ChIP experiments using FDCP mix cells (Figure S2). The importance of this region was further emphasised when it was specifically deleted from the 8 kb parent fragment (construct HexD). Analysis of reporter gene expression in HhexD transient transgenic embryos demonstrated that, within the context of this promoter fragment, the ECR1/2 region was required for expression of Hhex within emerging hematopoietic endothelial cells. Removal of ECR2 alone in HhexC transient transgenics appeared to limit expression to hematopoietic cells in the dorsal aorta (Figure 2IIC), but this result was not reproduced in stable lines of transgenic mice where both hematopoietic and endothelial expression was observed (data not shown). Taken together, these results – particularly the analysis of HhexB and HhexD fragments - suggest that the ECR1/2 region may be both necessary and sufficient for Hhex expression in hematoendothelial cells at the developmental stage investigated in these transient transgenic embryos.

To explore the temporal regulation of early hematopoietic transcription driven by this intronic element, we generated a series of transgenic lines carrying the HhexB fragment. As a control, we also generated lines with the HhexD fragment, in which the conserved regulatory region in the first intron has been deleted but all other Hhex regulatory regions are retained. Examples of the analysis of both copy number and integrity of integrated material are shown in Supplementary

Figure 4, but, for simplicity, the expression patterns resulting from typical HhexB and HhexD lines are presented in Figure 3. Transgenic HhexB embryos at day E7.5 show GFP expression in the embryonic yolk sac and blood islands as well as in the endothelium (Figure 3A) consistent with the expression seen at the same developmental stage in Hhex RedStar knock-in mouse control embryos (Figure 3B). Analysis of the HhexB line at day E10.5 (Figure 3C) and E11.5 (Figure 3F) shows expression consistent with that seen in transient transgenic analyses. Thus GFP expression was observed in the endothelium of the intersomitic vessels, the heart and the AGM. Cryosectioning revealed transgene expression in the dorsal aorta (3D and 3E). AGM dissection at E11.5 reveals GFP expression in the dorsal aorta (Figure 3G, 3g) and umbilical cord (Figure 3I, 3i). Finally, sections of foetal liver at E14.5 (Figure 3H) show GFP-positive cells whose morphology and location is suggestive of hematopoietic identity (Donaldson et al., 2005). Thus, all hematopoietic expression observed during the early formation and development of the murine blood system – as revealed by the Hhex RedStar knock in mice as a point of reference –can be recapitulated by the ECR1/2-containing *cis* regulatory cassette present in HexB. In contrast, the HhexD line exhibited normal expression in the anterior definitive endoderm (ADE) and later endodermal derivatives, but lacked all blood-affiliated expression (Figure 3A'-I').

Function of HHex Expressing Progenitor Cells

We next tested the functional potential of HSCs marked by GFP expression driven by the ECR1/2 element in HhexB. AGMs were dissected at 10.5dpc and cells sorted on the basis of GFP expression were assessed for colony forming ability in vitro (CFU-C) in the presence of growth factors supporting multi-myeloid differentiation (Figure 4A-C). CFU-C potential was almost entirely restricted to the Hhex-GFP marked population (Figure 4C); lineage analysis reveals the multi-myeloid lineage potential of these cells, including the capacity to produce mixed lineage colonies (Figure 4D).We next tested the lymphomyeloid potential of the Hhex-expressing cells using a two-stage bipotential assay (Figure 4E-H). Analysis of the GFP-positive and GFP-negative fractions of VEcadherin/CD45 double-positive AGM cells revealed enhanced lymphomyeloid potential in the Hhex expressing cells. In contrast, the GFP-negative fraction of

AGM had a low frequency of CFU activity in both assay systems and generated predominantly unilineage colonies, consistent with reduction or loss of Hhex expression as progenitors progressively lose their multilineage potential and commit to a unique cell compartment. Finally, the primitive nature of Hhex-expressing AGM cells sorted from HhexB transgenic mice was assessed in adoptive transfer experiments. Critically, long-term reconstitution activity was restricted to the Hhex expressing cells, with close to 100% of all hematopoietic tissue being derived from the engrafted cells (Figure 4I). No engraftment was detected in either peripheral blood or bone marrow in 5 out of 5 recipients injected with GFP-negative AGM cells; as GFP-negative cells cannot be detected by expression of the fluorescent reporter, the level of the reporter construct in the genomic DNA of recipient mice was assessed by PCR, relative to actin as a control gene (data not shown). Taken together, these results indicate that the ECR1/2 region contained in HhexB is sufficient to mark emerging multipotent hematopoietic cells capable of reconstituting hematopoiesis. Consistent with these findings, analysis of the bone marrow of adult HhexB mice revealed ECR1/2-driven GFP expression in the long term reconstituting fraction identified by Lineage (Lin)- ckit+ Sca-1+ CD34- cell surface immunophenotype (data not shown, see also Figure 6).

The Conserved Regulatory Region is required for Embryonic and Adult Hematopoietic Expression from the Endogenous Hhex locus.

To unequivocally determine the requirement for this region in driving *Hhex* expression in the emerging hematoendothelial and adult stem cell compartments, we deleted the ECR1/2 regions from the endogenous Hhex locus in the context of the Hhex RedStar (HRS) allele. Figure 5A shows the targeting strategy and resulting recombined locus, termed HBD (for Hex Blood Deletion). Since expression of Hhex itself has already been compromised by insertion of the reporter, deletion of ECR1/2 should have no further effect on Hhex levels and thus no functional impact. Fluorescence imaging of 11 dpc embryos shows that HBD is not expressed in the AGM, in contrast to the intact HRS allele (Figure 5B). However, the two alleles are equally expressed in

hepatic endoderm of the developing liver (Figure 5B), indicating a specific requirement for ECR1/2 in blood-associated Hhex expression during development. Consistent with this, immunophenotypic analysis of hematoendothelial compartments delineated by staining with VEcadherin and CD45 shows a requirement for the ECR1/2 region to drive reporter expression in VECad-CD45+ blood progenitors. Interestingly, deletion of this region results in reduced dsRed expression in VECad+ CD45- endothelial cells and in newly formed hematopoietic stem and progenitor cells as they emerge from the AGM endothelium (VEcad+ CD45+; Figure 5D). Detection of residual dsRed protein in these newly formed stem and progenitor cells most likely represents perdurance of the dsRed protein from the endothelial stage. In support of this interpretation, we also note that while there is some dsRed expression from the HBD allele in the HSC compartment, the mean fluorescence intensity in these cells is 10-fold lower than HRS. Overall, deletion of ECR1/2 results in decreased dsRed expression in the AGM in both the CD45+ and CD45- populations. Finally, we assessed the requirement for the ECR1/2 region in the adult bone marrow HSC compartment (Figure 6). Redstar expression is clearly observed in the Lin-cKit+Sca-1+ CD34- fraction in HRS mice, which includes long term reconstituting HSCs; this expression is completely lost upon deletion of the ECR1/2 region in the HBD allele.

DISCUSSION

In this study, we have explored the transcriptional regulation of the locus encoding the homeobox transcription factor Hhex. Hhex is important for cellular identity in a variety of tissues including the prospective foregut, liver, thyroid and the developing blood system. We describe an evolutionarily conserved enhancer whose activity is confined to the hematoendothelial lineages. This element is sufficient to drive reporter gene expression in HSCs and hematopoietic progenitor cells as they emerge in embryonic development, as well as in their hematoendothelial precursors. Furthermore, it can direct reporter gene expression in the fetal liver and during adult hematopoiesis, including within the long term reconstituting HSC compartment. Using

homologous recombination, we also demonstrated that the expression of the endogenous Hhex locus in the blood system is critically dependent on this element. Taken together, these results define this intron 1 element of the Hhex locus as a key lineage-specific cassette coordinating hematoendothelial expression.

This element shares characteristics with enhancers described in loci of other key transcriptional hematopoietic regulators such as Runx1 and Scl. In the case of Runx1, an enhancer element located 23 kb downstream of the transcription start site (the +23 element) binds the Gata/Ets/Scl core complex and is able to drive reporter gene expression in all definitive HSCs in the mouse embryo (Nottingham et al., 2007). However, an absolute requirement for this element in the context of the endogenous Runx1 locus has not yet been demonstrated. Multiple hematopoietic enhancers have been described in the Scl locus driving expression in primitive and definitive hematopoiesis; however, there appears to be substantial redundancy and so far no single element has been demonstrated as uniquely responsible for regulating Scl expression in developing or adult HSC (Spensberger et al., 2012). Thus the element we have defined within the Hhex locus represents a tractable tool with which to dissect the transcriptional programming of HSCs.

A striking feature of this element is its capacity to regulate expression throughout all developmental stages of hematopoiesis, starting from the earliest hematoendothelial precursors of HSCs through to the long-term reconstituting HSC compartment in adults. In FDCPmix cells, which represent the adult, multipotential murine bone marrow-derived compartment (Spooncer et al., 1984), this element binds a pool of transcription factors including Gata2, Fli1, Elf1, Pu.1 and

SCL. The ECR1 component of this element corresponds to the Prh+1 enhancer identified in the Hhex locus by (Donaldson et al., 2005), who identified hematopoietic enhancer elements based on the presence of a medley of transcription factor binding sites similar to the +19 SCL element. While the ECR1 element was sufficient to drive reporter expression in the HhexC transient transgenic embryos, expression was not as robust as with the intact enhancer.

We found that the regulation of gene expression by Hhex regulatory elements in blood and endothelium could not be separated from each other, indicating that the Hhex enhancer and similar elements represent common blood-endothelial enhancers, which are likely to first be active in the HSC precursor. We have demonstrated that the ECR not only binds an array of hematopoietic-specific transcription factors, but also is functional in an assortment of cell types throughout hematopoietic development., We speculate that the capacity of the Hhex enhancer to regulate Hhex expression throughout the blood system and at different developmental stages is achieved through binding of a different complement of transcription factors and co-factors depending on the particular cell context. Such regulatory mechanisms have been demonstrated in the Pu.1 URE element (Hoogenkamp et al., 2007). More generally, as the Hhex enhancer appears absolutely required for hematopoietic expression at all stages, we suggest that it represents an integral sensor for the network (Hannah et al., 2011; Gillian May et al., 2013) that specifies and maintains the stem cell lineage in the blood system.

Ethics Statement

All animal work was carried in accordance with UK and European legislation and in particular according to the regulations described in the “The Animals (Scientific Procedures) Act of 1986 (UK).” All work in this manuscript was authorized by appropriate Project Licenses issued by the UK Home Office.

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Figure Captions

Figure 1. Analysis of endogenous and transgene driven Hhex expression during blood development.

(A) Hhex mRNA is expressed in the AGM at 10.5 dpc, in dorsal aorta endothelium (yellow arrow), cells in the underlying mesenchyme (red arrow) and putative ventrally emerging haematopoietic cell clusters (white arrow). (B) Schematic representation of the Hhex RedStar construct by Morrison et al. (2008) in which dsRed reporter gene (RFP) is inserted into exon 1 of Hhex. (C) Hhex expression reported by dsRed in whole embryos at 10.5 dpc indicates expression in endothelial precursors in head vasculature (white arrowhead, indicating the fine, thread-like structures), inter-somitic vessels (grey arrow), and heart (blue arrow), thyroid (white arrow) and AGM (blue arrowhead). (C’) Close up of the AGM region from image C. (D) Cross sections reveal reporter expression in dorsal aorta endothelium (yellow arrow) and clusters (white arrow). (E) Schematic representation of an 8kb Hhex-GFP transgene used in Rodriguez et al., (2001). (F) Hhex-GFP wholemount embryos recapitulate endogenous Hhex expression, as shown by GFP expression in thyroid (white arrow) heart (blue arrow), AGM (blue arrowhead) and inter-somitic vessels (grey arrow). (F’) Close up of AGM region from image F, blue arrowhead pointing at GFP expression in the AGM. (G) The transgene contains elements sufficient to regulate Hhex in dorsal

aorta endothelium (yellow arrow) and budding hematopoietic clusters (white arrow), as shown by embryonic cross sections (nuclei stained with TO-PRO-3 (blue).

Figure 2. Transient Hhex-GFP transgenics.

[I] Identification of putative regulatory elements in the Hhex locus. Comparative analysis of 15 kb region of mouse Hhex locus with the homologous human sequence. Evolutionarily conserved non-coding elements (pink bars) exist within intron 1 (ECR1 and ECR2) and intron 2 (not shown). Blue peaks indicate highly conserved coding DNA sequence.

Further dissection of important regulatory elements was carried by digestion of the Hhex-GFP construct to create other transgene fragments that spanned upstream and downstream regions, as shown schematically. Exons are represented in grey; each transgene is driven by the endogenous Hex promoter. The genomic co-ordinates of the regions of Hhex included in each construct are shown in Figure S1.

Above: Summary of transcription factor binding at ECR1 and ECR2, in vivo. ChIP experiments carried out in undifferentiated FDCP-mix cells reveal that transcription factors key to the regulation of blood development are bound to ECR1, and include an array of ETS proteins, SCL and Gata2, whilst only ETS proteins were shown to bind to ECR2. The reviewed results were generated from the combined findings of ChIP assays carried out at both 0.4% and 1% formaldehyde cross-linking conditions.

[II] (A-F, left). Wholemount transient transgenic Hhex embryos at 10.5 dpc, carrying GFP reporter constructs Hhex A-F. Only HhexB and HhexE, which contain ECRs 1 and 2, produced detectable GFP expression in AGM (yellow arrow), as well as expression in heart (red arrow), thyroid (white arrow) and inter-somitic vessels (blue arrow). HhexD embryos, with ECR 1 and 2 deleted, showed no obvious expression in whole mount analysis. HhexA showed expression in the anterior endothelium (white arrowhead), HhexC in yolk sac only (blue arrowhead) and HhexF in anterior endothelium (white arrowhead) and intersomitic vessels (blue arrow). Cross-sections of transgenic embryos (A'-F') provide additional detail of expression of each construct within the

AGM region. Cross-sections were stained with TOPRO3 (red in B' and blue in all other sections). Cross-sections of HhexA embryos (A') showed no expression in the dorsal aorta (DA) but cross-sectioning of transient HhexB (B') and HhexE (E') embryos revealed expression at the first sites of HSC ontogeny: dorsal aorta (DA), vitelline artery and umbilical artery (UA). (B'") Coexpression of GFP (green) and endothelial marker CD34 (red) in the endothelium lining the dorsal aorta and intra-aortic cell clusters (white arrow) but not in the underlying mesenchyme cells (white arrowhead) in the AGM region. (C'') Coexpression of GFP (green) and CD34 (red) in endothelial cells but not in budding haematopoietic cells (white arrows). In cross-sections of HhexD embryos (D'), expression of GFP was not detected in the DA, but in a region of the notochord. (E') GFP expression in emerging clusters and endothelium of HhexE embryos. (F') In HhexF embryos, expression of GFP was revealed in branchial arch (not shown) and DA as well as endothelium.

Figure 3. Expression of Hhex in transgenic embryos under control of regulatory elements HhexB and HhexD.

(A-I) Transgenic HhexB embryos. (A'-I') Transgenic HhexD embryos (A) Fluorescent microscopy image of wholemount transgenic HhexB embryo at 7.5 dpc shows GFP expression in embryonic yolk sac blood islands (red arrow) and in endothelium (yellow arrow) consistent with endogenous Hhex expression shown in (B). (B) Fluorescent microscopy image of HRS transgenic embryo at 7.5 dpc. (C) Expression of Hhex in the HhexB transgenic line at 10.5 dpc and at 11.5 dpc. Cross section of HhexB (D and E) reveals expression of transgene in the dorsal aorta (DA). (F), corresponds to GFP expression in the endothelium of the inter-somitic vessels (blue arrow), the heart (red arrow) and the AGM (yellow arrow). Dissection of AGM region (G) and florescence image (g) at 11.5 dpc shows GFP expression in dorsal aorta (red arrow), and GFP expression is shown in the umbilical cord (I, i). (H) Cross section of foetal livers of HhexB embryos at 14.5 dpc showing GFP positive cells which may be indicative of HSC (white arrows).

(A') Wholemount analysis of HhexD transgenic embryo showing absence of GFP expression in the blood islands at 7.5 dpc but presence of GFP in the region of the Anterior Definitive Endoderm (ADE) (white arrow). (B') Expression is seen later in the foregut (blue arrow) and

endodermal yolk sac (white arrow). (C') HhexD embryos show GFP expression at 9.5 dpc embryo in the otic vesicle (white arrow head) and also in the head mesenchyme (red arrow). (D' and E') Cross section of HhexD at 10.5-11 dpc embryos, expression was observed in the notochord (white arrowhead in D') and urogenital ridges, Ur (white arrow in E' and red arrow in g'). (F') Transgenic HhexD embryos lack GFP expression in the vitelline and umbilical arteries (dashed white arrows in F' and H' respectively), and in the dorsal aorta of the AGM (G', and dashed white arrow in g'). Additionally there was no co-expression of GFP and CD31 (red CD31, green GFP) as shown in D' and I' (pharynx, Px).
(Nt, neural tube); (hd, head);

Figure 4. CFU-C, transplant analysis and bipotential assay in HhexB line.

(A) Outline of the CFU-C assay method. (B) Representative FACs plot of the GFP^+ and GFP^- cells sorted from the AGM of 11 dpc embryos. (C) Graph showing CFU-C activity in total AGM cells and GFP^+ and GFP^- fractions. Comparison between the GFP^+ and GFP^- fractions reveals that there is a 16 fold enrichment of haematopoietic CFU-C activity in the GFP^+ compartment compared with the GFP^- fraction (data not shown). (D) Graph showing the different types of colonies produced from differentiated single cells from the AGM and $GFP^{+/-}$ compartments. From the total AGM cell population multipotential progenitors and all myeloid lineages are represented, where as in the GFP^+ fraction there is enrichment for multipotential progenitor activity and a loss in uni-potent erythroid activity. In contrast the GFP^- compartment exhibits no multipotential ability but does contain cells capable of lineage-restricted differentiation, and in both there is a loss of MegE/Meg activity. (E) Representative FACS plot of $GFP^{+/-}$ $CD45^+$ $VeCad^+$ populations sorted from 11 dpc AGM tissues. (F) Cytospin of progenitors after myeloid differentiation, demonstrating multi-myeloid outputs, blue arrows indicate monocytic lineage, red arrow indicates megakaryocytic lineage and black arrow indicates granulocytic lineages. (G) Table showing colony forming efficiency of single cells within the sorted populations, indicates that the GFP^+ fraction of the double-positive (DP) $CD45^+VeCad^+$ population is more clonogenic than the GFP^- fraction. (H) Graph showing the differentiation potential of all clones yielded from DP GFP

populations demonstrates that 50% of the clones derived from the DP GFP$^+$ compartment are bipotential precursors whilst the remainder are either lymphoid or myeloid restricted progenitors, or unable to undergo differentiation; these may be indicative of quiescent HSCs. Only 10% of the clones derived from the DP GFP$^-$ compartment show bipotency, whereas the majority has a myeloid restricted potential. Once again a small proportion of clones were demonstrated as being unable to undergo lineage differentiation. (I) Graph showing the level of reconstitution in the three engrafted recipients transplanted with GFP$^+$ cells. Recipient 1 and 2 were assayed for LT-HSC activity with BM being harvest at 5 months post-transplant whilst recipient 3 was analysed for ST-HSC activity at 2 months post-transplant. Graph also indicates the percentage contribution of GFP$^+$ cells to myeloid (Gr1, Mac1$^+$), lymphoid (CD3, B220$^+$) and erythroid (Ter119+) compartments in the engrafted recipient mice.

Figure 5. Hhex Blood Deletion.

(A) Targeting strategy to delete the blood element from HhexRedStar construct (HRS), and resulting recombined locus with deleted ERC1 and ERC2 regions termed HBD (for Hex Blood Deletion). (B) Fluorescence microscopy of HRS and HBD wholemount transgenic embryos at 11 dpc, livers and AGM regions. The AGM region of the HBD embryo doesn't show RFP expression in contrast to the HRS embryo. (C) Fluorescence microscopy of wild type, HRS and HBD sectioned livers from adult transgenic mice. Both HRS and HBD sectioned livers showing similar expression of RFP. (D) Phenotypic analysis of hemato-endothelial compartments. HBD cells showed reduced expression in endothelial cells (VECad$^+$, CD45$^-$) emergent stem/progenitors (VECad$^+$, CD45$^+$) and blood progenitors (VECad$^-$, CD45$^+$).

Figure 6. Analysis of Hhex Expression in the Adult HSC Compartment.

Lineage-negative bone marrow cells from HRS (left panels) and HBD (right panels) mice were analysed by flow cytometry for markers of HSC together with RS fluorescence reporter Hhex gene expression. RS expression is observed in the HSC enriched (Lineage-ckit$^+$Sca-1$^+$) KLS compartment of HRS mice, but not HBD mice. Analysis of the CD34$^-$ subset of KLS cells that

contain long-term HSCs shows RS expression indicating Hhex expression in HRS mice, but no HBD mice.

Figure S1

Schematic representation of the digestion of the Hhex GFP construct to create other transgene fragments that spanned upstream and downstream regions, and map within the 8 kb transgene. Exons are represented in blue and each transgene is driven by the endogenous Hhex promoter. (B) Table showing locus coordinates of regions covered by each transgene construct, and transgene size.

Figure S2. **ChIP analysis of FDCP-mix cells, after crosslinking with 1% (A) or 4% (B) formaldehyde.**

Fold-enrichments of ECR1a, ECR1b, ECR2 and Gapdh (negative control) after chromatin immunoprecipitation with the following antibodies: Fli1, Elf1, PU.1, GATA2, SCL, cMyb, Ets ½ and GATA1. All enrichments were normalised to the Hex Exon4 control probe and also to any non-specific enrichments observed with rIgG. (n=5 for all ChIPs in (A) apart from SCL (n=4) and cMyb (n=6) (n=3 for all experiments under 0.4% formaldehyde protein-DNA crosslinking conditions). Enrichments shown with SEM. Statistical significance were determined using the paired students t test.

Figure S3. Efficiency of transgenic generation and summary of tissue expression at 10.5 dpc.

Upper : Table summarizing efficiency of transgenic generation for the Hhex-GFP transgenic lines, scoring the total number of embryos analysed for each Hhex construct, the number of transgenic-positive embryos (by PCR), the number of transgenic GFP-expressing (GFP^+) embryos dissected at each developmental stage listed (dpc; days post-coitum), and the frequency of GFP-expressing embryos expressed as a percentage of the total number of transgenics obtained for

each construct. An embryo was scored as GFP-expressing if GFP was detected in any of the tissues analysed (listed in the table below).

Lower : Table showing tissue distribution of GFP expression in the transgenic embryos analysed at 10.5 dpc. For each Hhex construct and each tissue, the number of embryos expressing GFP is shown; the figure in brackets expresses this as a percentage of the number of embryos analysed. Expression for construct D is not included in the table, as HhexD was not expressed in any blood affiliated structures. IVS (intra-somitic vessels).

Figure S4.

(A) Schematic diagram of HhexB transgene with GFP reporter inserted into first exon, showing HindIII and BamHI restriction sites. (B) Southern blot of Hhex_B2 transgenic DNA, digested with Hind III and probed for GFP reporter insert. Lane1, 1Kb plus ladder; lanes 2-5, wt DNA spiked with x100, x50, x10, and x1 copy number equivalents; lane 6, wt DNA; and lanes 7-12, transgenic DNA. (C) Southern blot of Hhex_B2 transgenic DNA, digested with BamHI and probed for GFP reporter; lane 1, 1Kb plus ladder; lane 2, 3, 4, and 5, wt DNA spiked with x50, x10, x5 and x2 respectively; lane 6, wt x1 DNA; lane 7-10 & 12-13, transgenic DNA; lane 11, transgenic negative DNA. (D) Southern blot of Hhex_B5 transgenic DNA digested with Hind III. Lane1, 1Kb plus ladder; lanes 2-5, transgenic DNA; lane 6 wt DNA; 7- 9, wt DNA + x1, x10, x50 copy number equivalent. (E) Southern blot of Hex_B5 transgenic DNA digested with BamHI and probed for GFP. Lane1, 1Kb plus ladder, lanes 2-6, wt DNA spiked with copy number equivalents x50, x5, x10, x2 and x1 respectively. Lanes 7-9 & 11, transgenic DNA and lane 10, transgenic negative DNA.

Figure S5. **HBD targeting strategy**

(A) Schematic representation of the gene targeting strategy used for the generation of the Hhex Blood Deletion reporter line. Exon 1 was replaced by a polylinker and the blood element was deleted from the HhexRedStar construct by a series of PCR. CMVHygroTK cassette and dsRedT3 followed by an IRES sequence were inserted into the transfer vector. The selection

cassette was later removed. The HBD line expresses dsRedT3 in blood precursors. (B) Southern blot analysis of targeted cell lines. Genomic DNA digested with EcoRV was hybridised with the probe to reveal WT (11.3kb) or targeted (7.6kb) bands A7 and B6. (C) ES cells from two HBD clones (A7 and B6) were used to generate HBD transgenic mice line. Mice were genotyped using Hex primers (293bp band) and dsRed primers (219bp band) Line1, 1Kb ladder, HBD positive are 125, 127, 131, 133, and 135; GH are controls for dsRed and Hhex primers, positive and negative.

Figure S6. qPCR primers and probes for ChIP

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