RUNX1 regulates the *CD34* gene in haematopoietic stem cells by mediating interactions with a distal regulatory element

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The transcription factor RUNX1 is essential to establish the haematopoietic gene expression programme; however, the mechanism of how it activates transcription of haematopoietic stem cell (HSC) genes is still elusive. Here, we obtained novel insights into RUNX1 function by studying regulation of the human *CD34* gene, which is expressed in HSCs. Using transgenic mice carrying human *CD34* PAC constructs, we identified a novel downstream regulatory element (DRE), which is bound by RUNX1 and is necessary for human *CD34* expression in long-term (LT)-HSCs. Conditional deletion of *Runx1* in mice harbouring human *CD34* promoter-DRE constructs abrogates human *CD34* expression. We demonstrate by chromosome conformation capture assays in LT-HSCs that the DRE physically interacts with the human *CD34* promoter. Targeted mutagenesis of RUNX binding sites leads to perturbation of this interaction and decreased human *CD34* expression in

LT-HSCs. Overall, our *in vivo* data provide novel evidence about the role of RUNX1 in mediating interactions between distal and proximal elements of the HSC gene *CD34*. *The EMBO Journal* (2011) 30, 4059–4070. doi:10.1038/emboj.2011.285; Published online 26 August 2011
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Introduction

Understanding how transcription factors and *cis*-regulatory elements set up long-range interactions to orchestrate gene expression is a key issue in genome biology. Since the initial observation reporting chromatin looping at the β -globin gene locus (Carter *et al*, 2002; Tolhuis *et al*, 2002), similar interactions have been shown to occur between promoters and 5' and/or 3'UTRs of many genes, and even *trans*-interactions between loci on different chromosomes have been reported (Chen *et al*, 1998; Brown *et al*, 2002; Ling *et al*, 2006; Marenduzzo *et al*, 2007; Vernimmen *et al*, 2007; Barnett *et al*, 2008; Chavanas *et al*, 2008; Liu *et al*, 2009; Theo Sijtsse Palstra, 2009; Boney-Montoya *et al*, 2010). A model in which intervening inactive chromatin lying between distant elements is looped out, and forms an active chromatin hub, has been proposed (Patrinos *et al*, 2004; Theo Sijtsse Palstra, 2009). Structural proteins, transcription factors, or components of the preinitiation complex have been implicated as candidate molecules mediating chromatin looping (Kim *et al*, 2007, 2009; Marenduzzo *et al*, 2007; Williams *et al*, 2007; Liu *et al*, 2009; Theo Sijtsse Palstra, 2009; Deshane *et al*, 2010). However, while roles for individual factors in long-range interactions have been identified, little is known about the role of individual factor binding sites on the stability of these interactions and how the lack of such binding sites would impact on gene expression. In addition, it is still unknown how these mechanisms operate in stem cells.

The best-characterized adult stem cells are haematopoietic stem cells (HSCs), which are capable of life-long self-renewal and generation of the full spectrum of mature blood cells. It was recently revealed that a specific combination of transcription factor binding sites (GATA, ETS and SCL/TAL1) was able to mediate HSC-specific expression (Gottgens *et al*, 2002; Pimanda *et al*, 2007). One additional factor that is essential for the formation of HSCs and the establishment of a blood-cell-specific gene expression programme in the embryo is RUNX1 (Okuda *et al*, 1996; Okada *et al*, 1998; Cai *et al*, 2000; Ichikawa *et al*, 2004a, b, 2008; Gowney *et al*, 2005; Putz *et al*, 2006; Chen *et al*, 2009; Hoogenkamp *et al*, 2009; Jacob and Osato, 2009). However, while the importance of RUNX1 in the establishment of the expression of haematopoietic genes

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is well characterized, little is known about the mechanistic details of its role in activating the actual transcription of these genes, particularly at the HSC level. To gain further insights into the role of specific transcription factors regulating HSC-specific gene expression, we studied the regulation of the human *CD34* (*hCD34*) gene locus. The *hCD34* antigen is specifically expressed on human HSCs and early progenitors (Krause *et al*, 1996), and has been successfully used as a marker to identify, enrich and purify HSCs in clinical transplantation settings (Dunbar *et al*, 1995; Galy *et al*, 1995; Stella *et al*, 1995; Link *et al*, 1996; Michallet *et al*, 2000). Identification of the *hCD34* promoter revealed the presence of functional binding sites for c-myc, ets-2, MZF-1, Sp1, Sp3 and NFY (Melotti and Calabretta, 1994; Morris *et al*, 1995; Perrotti *et al*, 1995; Radomska *et al*, 1999). However, neither the promoter alone or in combination with a later-identified 3' enhancer is sufficient to drive *hCD34* expression in cell lines and/or transgenic mice (Radomska *et al*, 1998, 2002). In contrast, PAC clones carrying the entire *hCD34* gene on a *PvuI* fragment with 18.3 kb of 5'- and 25.6 kb of 3'-flanking regions contain the complete set of critical control elements necessary to direct *hCD34* expression in functional HSCs (Okuno *et al*, 2002a,b). We have previously shown that murine LT-HSCs are highly enriched in the murine *CD34*^{low} *hCD34*⁺ fraction of the LSK population (lineage⁻; Sca1⁺; c-kit⁺ cells), suggesting that the human and murine *CD34* genes are differently regulated in LT-HSCs (Okuno *et al*, 2002b).

To identify the critical regulatory elements required for *hCD34* expression in LT-HSCs, we generated different transgenic mouse lines carrying various combinations of *hCD34* genomic elements. The work described here identifies a novel regulatory element located at +19 kb, the downstream regulatory element (DRE), which is necessary for *hCD34* expression in LT-HSCs. The DRE contains four binding sites for RUNX together with other binding sites for factors known to be active in stem cells. Experiments with conditional *Runx1* knockout mice demonstrate that the presence of RUNX1 is essential for the activity of this element. By chromosome conformation capture (3C) analysis performed in LT-HSCs, we demonstrate that the DRE physically interacts with the *hCD34* promoter through the RUNX binding sites.

Our data are the first to demonstrate a role of a specific transcription factor in establishing chromatin looping in primary stem cells; they show that specific transcription factor binding sites are required for interactions between distal and proximal regulatory elements *in vivo*, and expand the role of RUNX1 in facilitating looping of distant regulatory elements in HSCs.

Results

A genomic region located between +17.4 and +19.6 kb of the *hCD34* gene is necessary for its expression in LT-HSCs

In order to identify which elements mediate expression of *hCD34* in LT-HSCs, we generated different PAC constructs containing deletions of the 3'-flanking region in the context of the original ~70 kb *PvuI* fragment containing all necessary *cis*-elements, as depicted in Figure 1A. Multiple founder lines for each transgenic construct were obtained, and

hCD34 antigen expression was quantified on LT-HSCs (*CD48*⁻ *SLAMF7*⁺ LSK cells; Kiel *et al*, 2005) using flow cytometry analysis of bone marrow cells from transgenic mice. This showed that all the LT-HSCs from mice carrying either 25.6 kb (construct A) or 19.6 kb (construct B) of the 3'-flanking region expressed the *hCD34* antigen on the cell surface (Figure 1B). Mice carrying construct A displayed 99.2 ± 0.5% (s.d.) of *hCD34*⁺ LT-HSCs, with a mean fluorescence intensity (MFI) of 8617 ± 125 (s.d.); mice transgenic for construct B displayed 99.1 ± 0.8% (s.d.) of *hCD34*⁺ LT-HSCs, with an MFI of 8611 ± 95 (s.d.). These values were obtained with all three founder lines transgenic for construct A (copy number ranged 3–5 per genome) and in the eight founder lines carrying construct B (copy number = 2–6). An additional deletion of 2.2 kb achieved in mice carrying 17.4 kb of 3'-flanking region (construct C) was sufficient to completely abolish *hCD34* expression *in vivo* in *SLAMF7*⁺ LSKs, in the three lines transgenic for construct C (copy number = 2–7; Figure 1A and B). These studies demonstrated that critical *cis*-regulatory element(s) are located between +17.4 and +19.6 kb. By conducting a computational sequence analysis of the 2.2-kb region, we identified multiple putative 'stem cell-related' transcription factor binding sites, such as two potential *E-Box*/*GATA* paired motifs, known to bind *SCL*/*LMO2*/*GATA2* (Wadman *et al*, 1997), and four potential RUNX binding sites (Figure 1C). These sites were located in a 0.8-kb region, spanning from +18.8 to +19.6 kb, which we named the DRE (Figure 1C).

The DRE is necessary and sufficient for *hCD34* gene expression in *SLAMF7*⁺ LSKs

In order to assess the functional role of the DRE in LT-HSCs, we generated construct D (Figure 2A), which contains a deletion of the DRE in the context of the 25.6-kb 3'-flanking sequence (construct A). All transgenic lines carrying this construct failed to express *hCD34* in *SLAMF7*⁺ LSKs *in vivo* (Figure 2B), demonstrating that the DRE is necessary for expression of *hCD34* in LT-HSCs. Three independent founder lines were obtained for construct D, with copy numbers per genome ranging between 3 and 7. To test whether the DRE is sufficient for *hCD34* expression *in vivo*, we placed it as the sole regulatory element 3' of the *hCD34* gene (construct E; Figure 2C). Majority of *SLAMF7*⁺ LSKs from mice carrying construct E exhibited high levels of *hCD34* expression (98.8 ± 0.4% (s.d.)), with an MFI of 8603 ± 68 (s.d.; Figure 2D). These values, obtained in nine independent transgenic lines (copy number = 3–7), were not statistically different from the percentages observed in LT-HSCs from mice carrying construct A (*P* = 0.15). Therefore, the DRE is required for expression in LT-HSC.

We next investigated whether expression of *hCD34* in phenotypic *SLAMF7*⁺ LSKs correlated with long-term (LT) haematopoietic reconstitution capacity. Bone marrow transplantation assays demonstrated the LT-reconstitution potential of FACS-purified *hCD34*⁺ *SLAMF7*⁺ LSKs from *hCD34*⁺ transgenic mice carrying either 25.6 kb (construct A), 19.6 kb (construct B) or the sole DRE as 3' element (construct E), indicating that sequences containing the DRE are sufficient to drive *hCD34* expression in functional LT-HSCs (Supplementary Table S1). Lethally irradiated mice that did not receive any transplanted bone marrow cells succumbed

Figure 1 A genomic region located between +17.4 and +19.6 kb of the human *CD34* gene is necessary for its expression in SLAM⁺ LSKs. (A) Diagram of human *CD34* genomic fragments used in transgenic mice. All fragments (A–C) were derived from PAC54A19 and digested as indicated. Thick vertical lines represent the eight *hCD34* coding exons. (B) Flow cytometry was performed on bone marrow cells from transgenic mice carrying constructs A–C. Cells were stained with anti-mouse c-kit and sca-1 antibodies to identify the LSK (lineage[−]; Sca1⁺; and c-kit⁺ population), as indicated in the left panels; anti-murine CD48 and CD150/SLAM antibodies were used to identify the CD150 single positive population (SLAM⁺ cells) contained within the LSK compartment, as indicated in the middle panels; and anti-hCD34 antibody was used to quantify the amount of hCD34⁺ cells contained within the SLAM⁺ population (right panel). The percentage of the gated populations is noted. All plots display a representative example from each of the three different transgenic lines. T-test (two-tailed; type 3) showed that the data, obtained in three independent transgenic lines from construct A ($n=3$ per founder, a part from one founder in which we did not observe germ line transgene transmission), were not statistically different ($P=0.55$) from the percentages observed in eight independent transgenic mice for construct B ($n=3$ per founder, a part from 1 founder that did not display germ line transmission) and were instead statistically different ($P=2.2$, E=22) from three independent transgenic lines from construct C ($n=3$ per founder). (C) *In silico* sequence analysis was carried out to identify regions of interest contained in the 2.2-kb genomic region spanning from +17.4 and +19.6 kb. The most 3' 0.8 kb, identified here as the downstream regulatory element (DRE), contains four RUNX sites and two E-Box/GATA motifs.

few weeks after irradiation, as expected. Therefore, the DRE is capable of mediating expression of hCD34 in functional LT-HSC.

RUNX1 binds to the DRE

RUNX proteins are transcription factors whose importance has been well established in haematopoiesis. In particular,

Figure 2 The DRE is necessary and sufficient for human *CD34* expression in SLAM⁺ LSKs. **(A)** Construct A (described in Figure 1) was modified to specifically delete the DRE (construct D). **(B)** Bone marrow cells from mice carrying construct D have been analysed by flow cytometry, performed as described in Figure 1. Deletion of the DRE is sufficient to abolish *hCD34* expression in SLAM⁺ LSKs (right panel). A representative FACS plot from one of the three founder lines is shown. *T*-test (two-tailed; type 3) indicated that the data obtained in three independent transgenic lines from construct D ($n = 3$ per founder) were statistically different ($P = 2.8$, $E = 21$) from the percentages observed in nine independent transgenic mice for construct A (Figure 1). **(C)** Construct A was engineered to contain the DRE as the sole sequences 3' of the human *CD34* gene (construct E). **(D)** Flow cytometry of bone marrow cells from mice carrying construct E. The DRE is sufficient as a sole 3' element to drive *hCD34* expression in SLAM⁺ LSKs. A representative FACS plot from one of the nine founder lines is shown. *T*-test (two-tailed; type 3) showed that the percentages observed in nine independent mice transgenic for construct E ($n = 3$ per founder, a part from two founders in which we did not observe germ line transgene transmission) were not statistically different from the data obtained in mice carrying construct A ($P = 0.15$). See also Supplementary Table S1.

RUNX1 is required for HSC generation (Cai *et al*, 2000; Chen *et al*, 2009), and inactivation of RUNX1 has been indicated to affect the HSC pool (Ichikawa *et al*, 2008; Jacob and Osato, 2009). Furthermore, RUNX1 is expressed in LT-HSCs (Supplementary Figure S1), leading us to hypothesize that RUNX1 could be a mediator of *hCD34* expression in those cells. Therefore, we tested the ability of RUNX1 to bind to the predicted consensus sites identified within the DRE (Figure 1C). Quantitative ChIP assay performed on human primary umbilical cord blood cells ($>95\%$ CD34⁺) showed that RUNX1 binds to a genomic DNA region containing RUNX consensus sites #1–3 and site #4, whereas we did not detect RUNX1 binding to the corresponding sequences in the

hCD34[−] cell line HL60 (Figure 3A). In addition, we detected histone H3 acetylated at lysines 9 and 14 of histone H3 (aH3-Ac) at sites #1–3 and #4 in cord blood cells, (Figure 3B). As histone acetylation is regarded as one of the epigenetic marks accompanying active chromatin (Roth and Sweatt, 2009), these data provide functional evidence that these sites are involved in an open chromatin complex in cord blood cells.

RUNX1 regulates *hCD34* expression in LT-HSCs through the DRE

To specifically address whether RUNX1 is involved *in vivo* in regulating *hCD34* expression in LT-HSCs, we conditionally

Figure 3 RUNX1 binds to the RUNX sites contained within the DRE. (A) Quantitative ChIP analysis was performed on primary human cord blood (CB) cells (>95% hCD34⁺) and HL60 cells (hCD34⁻). Primers and probes encompassing RUNX sites 1–3, and RUNX site 4, were used to amplify input genomic DNA, and DNA precipitated by antibodies against either normal IgG or Runx1 or histone 3 acetylated at lysines 9 and 14 (aH3-Ac). The Myf5 promoter was used as a negative control sequence. Values are normalized to input genomic DNA. The panel shows the values of fold enrichment obtained with anti-Runx1 antibodies (black columns for cord blood ■ and dotted columns for HL60 ▨) compared with IgG (white columns for cord blood □ and diagonally barred columns for HL60 ▤), used as a negative control. Values obtained for the fold enrichment in cord blood cells are statistically different, as shown (bars indicate standard deviations). (B) The panel shows the values of fold enrichment obtained with an antibody against aH3-Ac (black columns for cord blood ■ and dotted columns for HL60 ▨); IgG (white columns for cord blood □ and diagonally barred columns for HL60 ▤) served as control for the assay specificity. Values obtained for the fold enrichment in cord blood cells are statistically different, as indicated. All the data are representative of three independent experiments performed in duplicate. See also Supplementary Figure S1.

inactivated it in adult haematopoietic cells. *hCD34* transgenic mice carrying construct E (Figure 2C) were bred to conditional *Runx1* knockout mice (*Runx1*^{F/F}; Gowney *et al*, 2005) and *Mx1-Cre* mice (Kuhn *et al*, 1995) to obtain interferon-inducible *Runx1* gene excision (Figure 4A). One month after administration of seven doses of polyinosinic-polycytidylic acid (PIPC), FACS analysis was conducted on PIPC-treated *Mx1-Cre*⁺/*hCD34*⁺/*Runx1*^{F/F} mice (*hCD34*⁺ *Runx1* KO mice) and control littermates (either *Mx1-Cre*⁻/*hCD34*⁺/*Runx1*^{F/F} mice or *Mx1-Cre*⁺/*hCD34*⁺/*Runx1*^{wt/wt} mice, both referred to as *hCD34*⁺ *Runx1* WT mice). Flow cytometry showed almost complete abolishment of *hCD34* expression in SLAM⁺ LSKs of *hCD34*⁺ *Runx1* KO mice as compared with PIPC-treated *hCD34*⁺ *Runx1* WT mice (Figure 4B). Only 1.8 ± 0.7% (s.d.) of *Runx1* KO LT-HSCs were positive for *hCD34* (MFI 286 ± 25 (s.d.)) compared with 98.3 ± 0.5% (s.d.) of LT-HSCs from *hCD34*⁺ *Runx1* WT mice (MFI 8569 ± 112 (s.d.)). Percentages observed in either *Mx1-Cre*⁻/*hCD34*⁺/*Runx1*^{F/F} mice or *Mx1-Cre*⁺/*hCD34*⁺/*Runx1*^{wt/wt} mice were not statistically different (*P* = 0.4). Absence of RUNX1 expression in KO mice was verified by Q-RT-PCR (Supplementary Figure S2). As disruption of RUNX1 resulted in loss of *hCD34* expression, we conclude that RUNX1 is a critical transcription factor acting through the DRE to regulate *hCD34* gene expression in LT-HSCs.

The DRE physically interacts with the *hCD34* promoter

The results described above point to a crucial role of RUNX1 with respect to driving *hCD34* transcription in LT-HSCs. It has been previously shown that high-level transcription requires the interaction of enhancers with promoter elements. Expression of transcription factors such as EKLF is required to mediate these interactions (Chen *et al*, 1998; Brown *et al*, 2002; Pilon *et al*, 2006; Rathke *et al*, 2007; Chan *et al*, 2008; Gavrillov and Razin, 2008). Moreover, recent experiments in T cells indicate that RUNX is required to mediate the close proximity of *Cd4* and *Cd8* genes within the intranuclear space (Collins *et al*, 2011). However, this study did not address the question of chromatin looping. To test directly whether RUNX1 is involved in *hCD34* promoter–DRE communication, we performed 3C assays (Figure 5A) and assessed chromatin looping in two human haematopoietic cell lines, KG1a (CD34⁺) and HL60 (CD34⁻). In parallel, we assessed *hCD34* and *Runx1* expression status (Supplementary Figure S3a–c). The frequency of interaction was enhanced 6.18 ± 0.02 (s.d.) times in *hCD34*⁺ cells versus *hCD34*⁻ cells (Figure 5B). By performing 3C assays on FACS-purified SLAM⁺ LSKs (*hCD34*⁺) and *lin*⁺ cells (*hCD34*⁻) from mice carrying construct A (Figure 1), whose high purity was verified by resorting analysis (Supplementary Figure S3d), we demonstrated that the frequency of the DRE–promoter interaction correlates with the levels of *hCD34* expression (Figure 5C). In particular, *hCD34*⁺ SLAM⁺ LSKs showed a 10-fold increase in the DRE–promoter interaction as compared with *hCD34*⁻ *lin*⁺ cells (Figure 5C). In primary umbilical cord blood cells, the frequency of the DRE–promoter interaction follows a similar pattern, being 8.7-fold higher in FACS-purified *hCD34*⁺ cells compared with *hCD34*⁻ cells (Figure 5D and Supplementary Figure S3e). Overall, our *in vivo* data indicate that the DRE physically interacts with the *hCD34* promoter in LT-HSCs and in primary *hCD34*⁺ cord blood cells, and that the DRE is a critical element required for *hCD34* gene expression in these cells.

RUNX binding sites within the DRE are critical for *hCD34* expression in SLAM⁺ LSKs

To determine *in vivo* whether the activity of the DRE in LT-HSCs is mediated through the RUNX binding sites, we specifically mutated these sites (construct F; Figure 6A). Abrogation of RUNX1 binding to the mutated sites has been verified by electrophoretic mobility shift assays on 293T cells transiently overexpressing FLAG-tagged RUNX1 (Supplementary Figure S4a and b). Mice carrying construct F showed a decreased percentage of *hCD34* expression in SLAM⁺ LSKs and decreased MFI (Figure 6B), pointing to the importance of the RUNX1 binding sites for *hCD34* expression in LT-HSCs. In particular, 38.1 ± 4.2% (s.d.) of LT-HSCs expressed *hCD34* (versus 99.2 ± 0.5% of LT-HSCs from mice carrying construct A), with an MFI of 2342 ± 189 (versus 8617 ± 125 in LT-HSCs from mice carrying construct A). Six independent lines were generated (copy number = 2–6).

In contrast, when we deleted the neighbouring *E-Box*/*GATA* motifs (construct G; Supplementary Figure S4c), none of the transgenic lines was affected in its ability to express *hCD34* in LT-HSCs (Supplementary Figure S4d). Values obtained on the 11 transgenic lines (copy number = 2–9) showed that 98.9 ± 0.4% of LT-HSCs express *hCD34* with an MFI of

Figure 4 RUNX1 regulates human *CD34* expression in SLAM⁺ LSKs. **(A)** Schematic of the breeding strategy used to obtain *hCD34*⁺ mice (*hCD34*^{18.3kb5'/DRE3'}) with conditional deletion of *Runx1*. *hCD34* transgenic mice carrying construct E were bred to conditional *Runx1* knockout (KO) mice (*Runx1*^{F/F}) and *Mx1-Cre* mice, to obtain interferon-inducible *Runx1* gene excision. Mice have been treated with seven injections of PIPIC and analysed 1 month later. **(B)** Flow cytometry analysis on bone marrow cells from PIPIC-treated *hCD34*⁺ *Runx1* WT or *hCD34*⁺ *Runx1* KO mice. Representative FACS plots obtained from one WT and one KO mouse are shown. T-test (two-tailed; type 3) showed that the percentages observed in *hCD34*⁺ *Runx1* WT (*n* = 6) were statistically different (*P* = 6.7, *E* = 19) from the data obtained in *hCD34*⁺ *Runx1* KO mice (*n* = 6). See also Supplementary Figure S2.

8615 ± 130, indicating that these binding sites are not required for *hCD34* expression.

To address the effect of mutations in RUNX binding sites at the chromatin level, we FACS purified the LSK-HSCs into *hCD34*^{low} and *hCD34*^{int} populations from mice carrying construct F, according to the gating strategy utilized throughout this study to define *hCD34*⁺ and *hCD34*⁺ cells (see Figures 1B, 2B, D and 4B), and performed 3C assays (Figure 6C). A 5.2-fold decrease in the frequency of the DRE–promoter interaction was observed in *hCD34*^{low} HSCs versus *hCD34*^{int} HSCs. These data show that the levels of *hCD34* expression parallel the frequency of the promoter–DRE interaction (Figure 6C), which is affected when RUNX binding sites are mutated (Figure 6A). Levels of *hCD34* expression in *hCD34*^{low} and *hCD34*^{int} HSCs from mice carrying construct F have been normalized to the expression observed in LSK-HSCs from mice transgenic for construct A (Figure 6C), in which the RUNX binding sites are intact. In particular, *hCD34*^{low} LSK-HSCs from mice carrying construct F express 8%, and *hCD34*^{int} LSK-HSCs express 25% of the levels measured in LSK-HSCs from mice carrying construct A (Figure 6C).

Overall, these *in vivo* data show that to achieve *hCD34* expression, an optimal promoter–DRE interaction is required to occur through intact RUNX binding sites.

Discussion

To define genomic regulatory elements required to achieve gene expression in LT-HSCs, we focused on the regulation of the human *CD34* gene, which encodes a surface marker for

HSCs (Krause *et al*, 1996). Previously, we identified an element just downstream from exon 8 of the *CD34* gene, which exhibited enhancing activity in *CD34*⁺ cell lines in *in vitro* assays (Burn *et al*, 1992). However, subsequent studies showed that this 3' enhancer in combination with the *CD34* gene promoter lacked activity when stably integrated into the chromatin (Radomska *et al*, 1998), and furthermore, additional long-range element(s) were found to be critical for tissue-specific human *CD34* gene expression *in vivo* (Radomska *et al*, 1998; Okuno *et al*, 2002a). To map these elements precisely, we generated a series of transgenic mice carrying the *hCD34* genomic locus with different combinations of flanking sequences. The transgenic mouse experiments described here identified a 3' distal *cis*-regulatory element (DRE) that is necessary to drive *hCD34* expression in LT-HSCs and that depends on RUNX1 for its activity. When comparing the human DRE sequence with corresponding sequences from other vertebrates, such as rhesus and mouse, of the four RUNX1 binding sites present on the human sequence, only site 2 is conserved between these species, whereas the remaining sites are only present in humans and rhesus, and not in mouse (Supplementary Figure S6). Interestingly, the entire set of four RUNX1 binding sites is only present in those species in which *CD34* is expressed on LT-HSCs (Supplementary Figure S6), hinting to the possibility that differences in regulatory elements might account for the differential expression of *CD34* on human versus murine HSCs. Despite intensive investigation, the function of the *CD34* molecule on HSCs is still obscure, and the possibility that *CD34*⁺ cells are capable of LT reconstitution of the haematopoietic system has been

Figure 5 The DRE interacts with the human *CD34* promoter in SLAM⁺ LSKs. **(A)** Diagram showing the genomic position of the *hCD34* promoter (Pr); the eight coding exons (thick vertical lines); the DRE; and location of the *HindIII* (H3) restriction enzyme sites (vertical arrows) used to perform the 3C assay. The block arrows represent the interaction of the Pr fragment (7.4 kb) with the DRE fragment (4.9 kb) and with six other fragments located in the 3'-flanking region and upstream of the DRE (block arrows 1–6). The distance of each H3 fragment from the DRE is indicated in kb (vertical numbers). **(B)** Relative crosslinking frequency of the Pr with the DRE and six other 3' fragments located upstream of the DRE. Crosslinking frequency indicates how frequently distal genomic elements interact. Interaction of the 3' fragments and the DRE with the Pr are rare in HL60 cells (hCD34⁻), whereas they are increased in frequency in KG1a cells (hCD34⁺), with the DRE showing a 6.2-fold enrichment in the relative crosslinking frequency over the hCD34⁻ HL60 cell line ($n=4$ per cell line; two independent 3C experiments). **(C)** On the left panel, the relative crosslinking frequency of the Pr and DRE in hCD34⁺ SLAM⁺ LSKs ($n=4$; black column; ■) and hCD34⁻ lin⁺ cells ($n=4$; white column; □) from mice carrying construct A, as normalized to the levels in hCD34⁺ SLAM⁺ LSKs, is indicated. Interaction of the DRE with the Pr is rare in hCD34⁻ cells, whereas a 10-fold enrichment is observed in hCD34⁺ LT-HSCs. Only the relative crosslinking frequency between the Pr and the DRE has been analysed, given the small number of HSCs per mouse. On the right panel, Q-RT-PCR data verifying the expression levels of *hCD34* in the SLAM⁺ LSKs and the lin⁺ cells utilized for the 3C assay are shown. Sorted SLAM⁺ LSKs have been pooled from a total of 25 mice transgenic for construct A. **(D)** On the left panel, the relative crosslinking frequency of the Pr and DRE in hCD34⁺ cord blood cells ($n=4$; black column; ■) and hCD34⁻ cord blood cells ($n=4$; white column; □), as normalized to the levels in hCD34⁺ cord blood cells, is indicated. Interaction of the DRE with the Pr is weaker in hCD34⁻ cells, whereas an 8.7-fold enrichment is observed in hCD34⁺ cells. On the right panel, Q-RT-PCR data verifying the expression levels of *hCD34* in the cord blood populations utilized for the 3C assay are shown. See also Supplementary Figure S3.

Figure 6 Intact RUNX sites within the DRE are necessary for human *CD34* expression in SLAM⁺ LSKs and to maintain the promoter-DRE interaction. **(A)** Construct A (described in Figure 1) was modified to contain mutations in the *Runx* sites located in the DRE (construct F). The expanded section shows relative location of the mutated *Runx* sites in the DRE, and within the framed box the specific mutations are indicated relative to the wild-type sequence (Supplementary Figure S5). **(B)** Flow cytometry of bone marrow cells from mice carrying construct F. A representative example from one of the six founder lines is shown (three mice per each founder were analysed, a part from two founders in which we did not detect germ line transgene transmission). Mutation in the *Runx* sites strongly decreases *hCD34* expression in SLAM⁺ LSKs in comparison with SLAM⁺ LSKs from mice carrying construct A, in terms of percentage (from $99.2 \pm 0.5\%$ (s.d.) to $38.1 \pm 4.2\%$ (s.d.), respectively; $P = 3.44$, $E = 17$) and mean fluorescence intensity (from 8617 ± 125 (s.d.) to 2342 ± 189 (s.d.), respectively; $P = 2.1$, $E = 24$). **(C)** LSK-HSCs from mice carrying construct F have been subdivided into a *hCD34*^{low} and a *hCD34*^{int} population, in accordance to the *hCD34* gating strategy adopted throughout this study, to perform 3C assays. In the upper panel, post-sorting analysis indicates the purity of the sorted population. The lower left panel shows Q-RT-PCR data, indicating the levels of *hCD34* expression in the *hCD34*^{low} (black column; ■) and the *hCD34*^{int} population (white column; □), normalized to the levels of *hCD34* expression detected in LSK-HSCs from mice carrying construct A. The lower right panel indicates the relative crosslinking frequency of the promoter and DRE in the same *hCD34*^{low} (black column; ■) and *hCD34*^{int} (white column; □) FACS-purified cells, as normalized to crosslinking frequency of LSK-HSCs from mice transgenic for construct A. Interaction of the DRE with the Pr fragment is less frequent in *hCD34*^{low} cells ($n = 4$) than in *hCD34*^{int} LSKs ($n = 4$), showing a 5.2-fold enrichment in the relative crosslinking frequency in cells with higher *hCD34* expression. Sorted populations have been pooled from 18 mice transgenic for construct F. See also Supplementary Figure S4.

reported in the mouse (Zanjani *et al*, 1998, 1999, 2003). In addition, the activation status of the cells appears to influence the expression of the CD34 molecule (Sato *et al*, 1999), implying that identifying the molecular mechanisms regulating *CD34* expression may contribute to understand how HSC gene regulation is achieved and modulated during homeostasis, activation and stress-induced status.

Our data suggest that RUNX1 is crucially required for *hCD34* promoter-DRE interactions, and that other transcription factors in the DRE cannot fully compensate for its absence. The specific requirement of RUNX1 in the context of the *hCD34* DRE is also underlined by lack of any significant effect on *hCD34* gene expression when neighbouring GATA and SCL/TAL1 binding sites were deleted in the DRE,

although this combination of elements has been shown to be sufficient to mediate HSC-specific expression in the mouse (Gottgens *et al*, 2002; Pimanda *et al*, 2007).

It was recently shown that RUNX proteins regulate the intranuclear positioning of the *Cd4* and *Cd8* loci (Collins *et al*, 2011). An interesting hypothesis is therefore that RUNX1 may be required to bring together promoter-distal regulatory elements at nuclear 'transcription factories' (Jackson *et al*, 1993; Osborne *et al*, 2004), where genes are dynamically recruited during activation and abatement of their transcription (Schoenfelder *et al*, 2010). Moreover, RUNX1 proteins localize in defined subnuclear domains (Berezney *et al*, 1996; Stein *et al*, 1999; Li *et al*, 2005), and a unique intranuclear trafficking sequence that targets RUNX1 to these sites has been identified (Zeng *et al*, 1997, 1998; Zaidi *et al*, 2002). Interestingly, molecular alterations that cause misrouting of RUNX1 in the nucleus result in abnormal synergism with other transcription factors (Li *et al*, 2005), aberrant gene expression and development of disease (Jackson, 1997; Stein *et al*, 2000a, b), suggesting that the correct subnuclear targeting of RUNX1 is an integral component of multifactor interaction to control cell-specific gene expression.

We previously reported that the *PU.1* promoter directly interacts with a critical upstream regulatory element (URE) (Ebralidze *et al*, 2008). Similarly to what we observed with the *hCD34* DRE, we detected decreased interaction between the *PU.1* promoter and the URE in primary haematopoietic stem/progenitor cells when RUNX binding sites in the *PU.1* URE were mutated (PZ, AKE, Annalisa Di Ruscio, DGT, unpublished observations). These results suggest a more general role of RUNX proteins in mediating interactions of distal regulatory elements at its target genes.

Overall, our work contributes to elucidate the mechanism leading to gene regulation in the rare population of HSCs by identifying *trans*-acting regulatory proteins that establish the three-dimensional molecular organization of long-range regulatory elements. Our data open a new field of investigation aimed at identifying specific genetic mutations that lead to abnormal gene expression, therefore contributing to HSC defects and specific disease onset.

Materials and methods

Detailed protocols in the Supplementary data.

Mice

hCD34 transgenic mice contained *hCD34* PAC constructs derived from clone 54A19 (Radomska *et al*, 1998; Okuno *et al*, 2002b). To obtain the transgenic constructs, a combination of both homologous recombination and enzymatic restriction was used (Supplementary Figure S5) (Datsenko and Wanner, 2000). Transgenic mice carrying construct E were bred with Runx1^{F/F} (Growney *et al*, 2005) and Mx-Cre1 mice (Kuhn *et al*, 1995), and treated with PIPIC to achieve excision of the *Runx1* locus. Seven doses of 0.8 mg/mouse were given every other day. All animal experiments were performed by using procedures described in the protocol approved by our Institutional Animal Care and Use Committee.

Genotyping protocols

DNA was extracted from snip-tails, phenol/chloroform purified, and analysed by Southern blot and PCR, as described in Supplementary data.

Bone marrow transplantation

Fifty *hCD34*⁺ LT-HSCs (lineage⁻; *Scal*⁺; *c-kit*⁺; *CD150*⁺; and *CD48*⁻ cells) isolated from bone marrow of transgenic mice

(FVB/N, Ly5.1⁺) were tail vein injected into congenic FVB/N mice (Ly5.2⁺), with 5 × 10⁵ supporting bone marrow cells from Ly5.1/Ly5.2 heterozygous mice. Recipient mice were irradiated (10.1 Gray), and reconstitution assessed 5 months after transplantation by FACS analysis, as described in the Supplementary data.

RNA isolation and quantitative real-time PCR analysis

RNA was extracted using the RNeasy Mini Kit (Qiagen). Quantitative real-time PCR analysis was performed on a Corbett Rotor Gene 6000. Primers and probes were synthesized as follows and gene expression was compared with 18S expression (Eukaryotic 18S rRNA endogenous control, Applied Biosystems): *hCD34* sense: 5'-AACTACAACACCTAGTACCCTTGGAA-3', antisense: 5'-GAATTGTCATGTCGTTTCTGTGATG-3' and probe: 5'-FAM-CCCTGTGTCTCAACATGG-CAATGAGGCC-TAMRA-3'. *Runx1* sense: 5'-CGAGATTCAACGACCTCAGGT-3', antisense: 5'-AAGACGGT-GATGGTCAGAGTGA-3' and probe: 5'-FAM-TCGGGCGGAGCGGTAGAGGC-BHQ1-3'.

Flow cytometric analysis and cell sorting

Bone marrow cells were stained (see Supplementary data) and sorted on MOFlo (MOFlo-MLS, Cytomation) or FACS Aria. Data were analysed with FlowJo software (Treestar, Inc.).

Western blot

A quantity of 60 µg of nuclear extracts was diluted with 1 × PBS to 20 ml total volume, mixed with equal volume of 2 × Laemmli Sample Buffer and boiled at 100°C for 10 min. Samples were loaded on 7.5% SDS-PAGE gels and proteins transferred to nitrocellulose membranes. Following blocking in 5% milk/TBST (TBST: 25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl and 0.1% Tween 20), membranes were stained overnight at 4°C with anti-FLAG M2 monoclonal antibody (Sigma, no. F3165) diluted 1:10 000 in 5% BSA/TBST/0.1% sodium azide. FLAG-tagged proteins were detected following the staining with horseradish peroxidase-conjugated anti-mouse secondary antibody (1:3000 dilution; Santa Cruz, no. sc-2055) at room temperature for 1 h. Signals were detected by enhanced chemiluminescence and quantified by ImageQuant software (Molecular Dynamics).

ChIP assay

Cells (10⁶ for each antibody) were used to crosslink chromatin using the protocol from Millipore (Milton Keynes, UK), with minor modifications. HL60 cells were grown in RPMI supplemented with 10% FBS. Umbilical cord blood cells have been kept in culture (QBSF-60 Stem Cell Medium (Quality Biologicals Inc), supplemented with 20% FBS, 100 ng/ml SCF, 100 ng/ml Flt3 ligand, 50 ng/ml TPO and 50 ng/ml IL6) for eight passages. Briefly, cells were incubated in 1% formaldehyde for 10 min at room temperature. Glycine (0.125 M) was added to stop crosslinking. Crosslinked chromatin was sonicated for 150 s at 30% amplitude (Branson Digital Sonifer, Danbury, CT). Polyclonal antibodies raised against H3 acetylated at lysines 9 and 14 (aH3-Ac) from Millipore were used, whereas the RUNX1 antibody was purchased from Abcam and the IgG control from Sigma. DNA was purified by phenol-chloroform extraction, and specific regions were amplified by Q-PCR. Primers and probes used were the following: for the DRE *Runx* sites 1–3 region, sense 5'-CTCTCAGGTCACGCAGACAC-3'; antisense 5'-TAGGTTACCCACAGGCTTC-3'; probe 5'-FAM-TGTCCGTGTGGGAGGCAGGA-BHQ1-3'; for the DRE *Runx* site 4 region, sense 5'-CTCTGCCTTTGAGGAGCAAG-3'; antisense 5'-TACACCTTCCCTGACCATC-3'; probe 5'-FAM-ATTTGGAGCAGGCTGGGGC-BHQ1-3'; for the *Myf5* promoter region, sense 5'-CGAAACTGGGCTTCTTCTG-3'; antisense 5'-GAGCACCTTCTCTTTGTGC-3'; and probe 5'-FAM-TGCAGGTCTTTGGCCTGCTCA-BHQ1-3'.

Chromosome conformation capture

3C assay was used to detect genomic loci interactions (Dekker *et al*, 2002). Genomic DNA was uniformly digested, with an efficiency >90%, calculated following a published formula (Hagege *et al*, 2007). Oligonucleotides and probes for Q-RT-PCR to test for loci interactions and restriction enzyme efficiency were as described in the Supplementary data.

Statistical analysis

All values are presented either as mean ± s.d. or as representative images of at least three independent experiments. In the case of animal studies, three mice of each different founder line were

analysed. All comparisons were made using the unpaired Student's *t*-test for samples with unequal variance. Differences were considered statistically significant at $P < 0.05$ (indicated by asterisks).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: EL and DGT designed the study; EL, SL, HSR, GA, DSB and YO performed and planned research; EL, HSR, CJH, GA, MAJ, PZ, DEZ, AKE, CB, BG and DGT analysed data; CJH, MAJ, DG, JZ, NKW, SK and GH performed research; and EL and DGT wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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