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ORIGINAL ARTICLE

A previously unrecognized promoter of LMO2 forms part of a transcriptional regulatory circuit mediating LMO2 expression in a subset of T-acute lymphoblastic leukaemia patients

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The T-cell oncogene *Lim-only 2* (*LMO2*) critically influences both normal and malignant haematopoiesis. *LMO2* is not normally expressed in T cells, yet ectopic expression is seen in the majority of T-acute lymphoblastic leukaemia (T-ALL) patients with specific translocations involving *LMO2* in only a subset of these patients. Ectopic *lmo2* expression in thymocytes of transgenic mice causes T-ALL, and retroviral vector integration into the *LMO2* locus was implicated in the development of clonal T-cell disease in patients undergoing gene therapy. Using array-based chromatin immunoprecipitation, we now demonstrate that in contrast to B-acute lymphoblastic leukaemia, human T-ALL samples largely use promoter elements with little influence from distal enhancers. Active *LMO2* promoter elements in T-ALL included a previously unrecognized third promoter, which we demonstrate to be active in cell lines, primary T-ALL patients and transgenic mice. The ETS factors *ERG* and *FLI1* previously implicated in *lmo2*-dependent mouse models of T-ALL bind to the novel *LMO2* promoter in human T-ALL samples, while in return *LMO2* binds to blood stem/progenitor enhancers in the *FLI1* and *ERG* gene loci. Moreover, *LMO2*, *ERG* and *FLI1* all regulate the +1 enhancer of *HHEX/PRH*, which was recently implicated as a key mediator of early progenitor expansion in *LMO2*-driven T-ALL. Our data therefore suggest that a self-sustaining triad of *LMO2/ERG/FLI1* stabilizes the expression of important mediators of the leukaemic phenotype such as *HHEX/PRH*.

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Introduction

Lim-only 2 (*LMO2*), also known as *TTG2* or *RBTN2*, was originally reported as a T-cell oncogene where it was recurrently rearranged by chromosomal translocation in T-cell malignancies (Boehm *et al.*, 1991; Royer-Pokora *et al.*, 1991). *LMO2* encodes a 156 amino-acid transcriptional co-factor containing two LIM domain zinc-fingers (Kadrmas and Beckerle, 2004) that do not bind to DNA directly, but rather participate in the formation of multipartite DNA-binding complexes with other transcription factors, such as *LDB1*, *SCL/TAL1*, *E2A* and *GATA1* or *GATA2* (Osada *et al.*, 1995; Wadman *et al.*, 1997; Valge-Archer *et al.*, 1998). Although translocations involving *LMO2* are present in <10% of T-acute lymphoblastic leukaemia (T-ALL) patients, *LMO2* is ectopically aberrantly expressed in more than 60% of T-ALL samples (Rabbitts *et al.*, 1997; Raimondi, 2007). Ectopic expression of *lmo2* in thymocytes of transgenic mice has confirmed a role in the aetiology of T-ALL and the generation of T-ALL is greatly accelerated if *SCL* and *LMO2* are simultaneously overexpressed (Larson *et al.*, 1995, 1996). *LMO2* therefore represents one of the major oncogenes involved in the development of T-ALL.

Within the haematopoietic system, *LMO2* is expressed at all levels of maturity with the exception of mature T-lymphoid cells. *LMO2* expression has been shown to be critical for primitive haematopoiesis (Warren *et al.*, 1994; Yamada *et al.*, 1998). Moreover, *lmo2* null embryonic stem cells fail to contribute to adult haematopoiesis in chimeric mice, indicating that *LMO2* is essential for the generation of blood stem cells.

The majority of T-ALL samples expressing *LMO1/2* and *SCL/LYL1* do not have rearrangements of these genes (Asnafi *et al.*, 2004; Ferrando *et al.*, 2004). However, mechanisms mediating overexpression in cytogenetically normal T-ALL remain unknown. *LMO2* overexpression is seen most consistently in the more immature T-ALL phenotypes (van Grotel *et al.*, 2008), which have previously been associated with poor treatment outcome (Crist and Pui, 1993; Garand and

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Bene, 1993). Importantly, ectopic LMO2 expression due to transcriptional activation following retroviral vector integration into the *LMO2* locus has also been implicated in the development of clonal T-cell proliferation and subsequent T-ALL in patients undergoing gene therapy for X-linked severe combined immunodeficiency (Hacein-Bey-Abina *et al.*, 2003b, 2008; Howe *et al.*, 2008), and this has recently been recapitulated using murine retroviral mutagenesis models (Dave *et al.*, 2009). Taken together, the evidence from T-ALL patients, the transgenic mouse models and the insertional mutagenesis observations suggest that aberrant LMO2 expression in an immature haematopoietic cell represents a 'first-hit' to cause an accumulation of early lymphoid precursors with subsequent progression to T-ALL. Further evidence for this model has come from a recent study wherein it was demonstrated that *CD2*-driven *lmo2* is sufficient to permit the establishment of the leukaemia-initiating cell within the thymus many months before the development of leukaemia (McCormack *et al.*, 2010).

The regulation of LMO2 expression therefore has implications for both haematopoiesis and leukaemogenesis and accordingly has been of interest for some time. Studies of the regulation of LMO2 expression in normal haematopoiesis have shown two alternative transcripts (Royer-Pokora *et al.*, 1995), originating from the proximal and distal promoters, respectively, and that the proximal promoter drives the majority of LMO2 expression in endothelial cells (Landry *et al.*, 2005). In addition, a *cis*-regulatory element has been identified in the first untranslated exon of *LMO2*, which contains a PAR consensus-binding site (Crale and Anderson, 2003). Recently, further delineation of *LMO2* regulatory elements active in normal haematopoiesis (Landry *et al.*, 2005, 2009) has been reported but, thus far, the molecular mechanisms responsible for *LMO2* expression in leukaemic cells remain elusive, save for the recent identification of a cryptic chromosomal deletion in 4% of paediatric T-ALL patients deleting a region immediately upstream of *LMO2*, which was hypothesized to remove a putative negative regulatory element (Hammond *et al.*, 2005). Therefore, identification of upstream regulators of LMO2 in leukaemic cells may permit the discovery of critical transcription factors that characterize T-ALL transcriptional programmes and may thus open up new avenues for the development of future therapies.

Our interest was therefore to utilize chromatin immunoprecipitation (ChIP) with antibodies to activating histone modification marks in primary human samples coupled with microarray technology to discover regulatory sequences active in T-ALL. We have identified that (i) a proposed novel third promoter and both known promoters of *LMO2* are active in T-ALL and (ii) in contrast to B-acute lymphoblastic leukaemia (B-ALL), that the overexpression in T-ALL was directed from promoters with little influence by enhancer elements. We have also established that the proposed novel promoter is functional in reporter assays in cell lines and in F0 transgenic mice and that

transcripts originating at this promoter are present in T-ALL cell lines, primary T-ALL patient material and normal haematopoietic cells. We also identified FLI1 and ERG as likely transcriptional regulators of LMO2 in T-ALL and that LMO2 acts on FLI1 and ERG in a parallel manner. In addition, we demonstrate that these three factors bind to a powerful enhancer element in *HHEX/PRH*, the first time this has been demonstrated in human malignancy. Together with the data supportive of FLI1, ERG and LMO2 co-regulation, we suggest that these four transcription factors collaborate to form a key regulatory subcircuit of the wider transcriptional networks driving the development of T-ALL.

Results

A range of chromatin mark profiles are seen across the LMO2 locus in normal endothelial and blood cells

To demonstrate the feasibility of generating ChIP-on-chip profiles across the *LMO2* locus in human normal endothelial and blood cells and to show the range of profiles that may be derived in normal cells, we performed ChIP-on-chip analysis using antibodies to the histone modification marks H3K4Me3 and H3K9Ac in placenta-derived endothelial cells and in peripheral blood-derived CD34⁺ and T-cells (Figure 1). As expected from our previous mouse studies (Landry *et al.*, 2005), the proximal promoter was the dominant feature in endothelial cells and the locus was silent in T cells with internal control provided by the inclusion of the promoter of the neighbouring gene *CAPRIN1*. Three peaks were noted by H3K4Me3 ChIP-on-chip in *LMO2* in peripheral blood-derived blood progenitors (CD34⁺ cells): a peak each at the previously described proximal and distal promoters, and a third peak between these. In addition, a 5' H3K9Ac peak was seen in the CD34⁺ cells, which corresponded to the -64 kb element previously seen in mouse studies (Landry *et al.*, 2009) and shown to induce expression in haematopoietic cells in fetal liver.

The distribution of active histone marks across the LMO2 locus is different in LMO2-expressing T-ALL and B-ALL

To investigate the underlying cause of LMO2 overexpression in T-ALL, we next performed ChIP-on-chip analysis in primary T-ALL and B-ALL samples previously banked at the Sydney Children's Hospital and passaged through NOD-severe combined immunodeficiency mice (Lock *et al.*, 2002). In contrast to the silent *LMO2* locus in normal T-cells (Figure 1), there are strong peaks of H3K4Me3 and H3K9Ac seen over the previously described proximal promoter of *LMO2* in the three highly expressing T-ALL samples T-ALL 8, 16 and 27 with smaller peaks for the lowly expressing samples T-ALL 29 and 30 (Figure 2, Supplementary Figure 1A). Samples T-ALL 8 and 16 also showed an acetylation peak over the previously described distal promoter. In addition, there is a third peak of K4Me3 (strong in T-ALL 27 and weaker in T-ALL 16, 29 and 30) at a

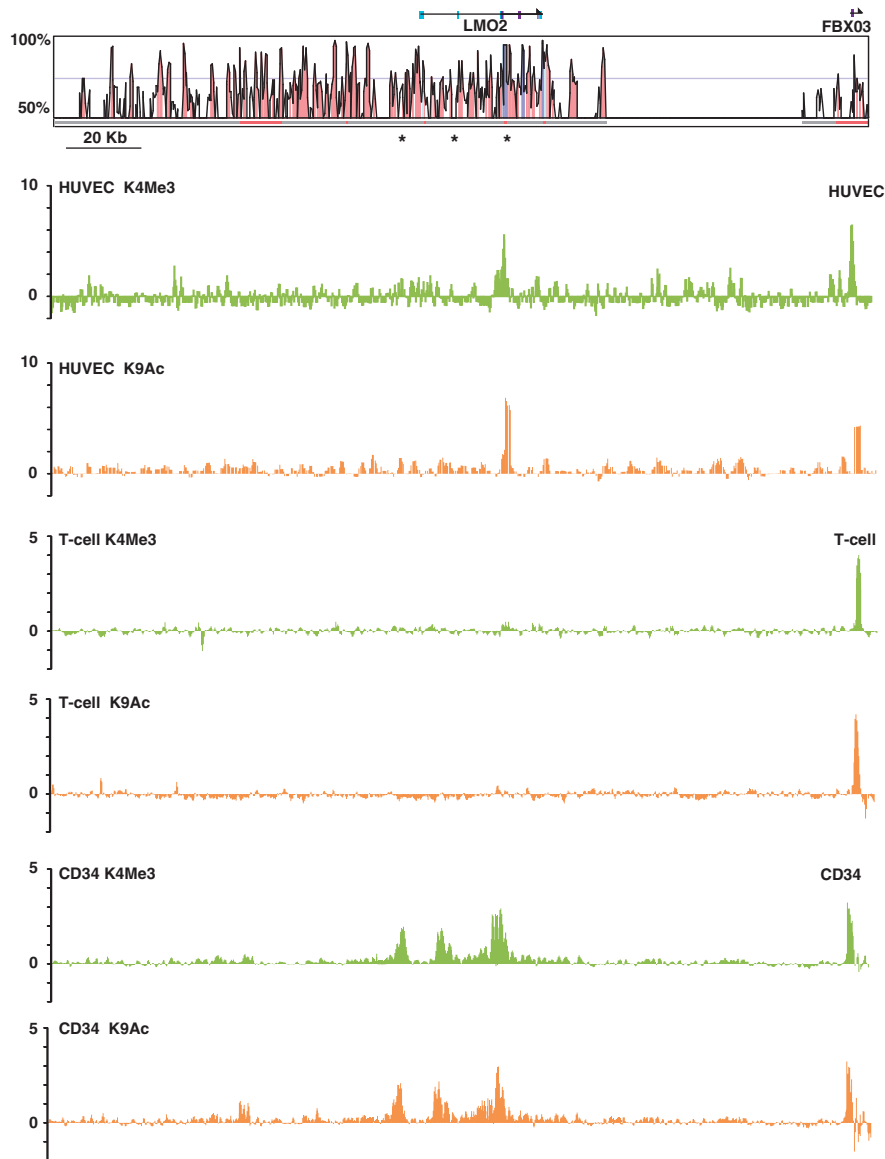


Figure 1 H3K4Me3 and H3K9Ac ChIP-on-chip demonstrates multiple peaks over the LMO2 locus in human primary cells, HUVECs, T cells and CD34⁺ cells. Shown at the top of the figure is a comparative genomics conservation plot generated using the Vista genome browser. The location of the exons of LMO2 and FBX03 are shown with non-coding exons in blue and coding exons in purple. Underneath are the results for ChIP-on-chip studies of placenta-derived endothelial as well as peripheral blood-derived T-cell and blood progenitor cells using antibodies to histone modifications H3K4Me3 (thought specific for promoters) and H3K9Ac (highlighting sequences with likely enhancer and/or promoter activity). The plot shows multiple peaks across the LMO2 locus. Endothelial cells demonstrate an H3K4Me3 peak over the LMO2 proximal promoter; the locus is silent in T cells and peaks are evident over both the LMO2 proximal and distal promoters and over a third peak between these over a presumed third promoter in peripheral blood-derived blood progenitors (CD34⁺ cells). Internal control is provided by inclusion of the promoter of the neighbouring gene, *FBX03*.

position just upstream of exon 2. These ChIP-on-chip findings were further corroborated by real-time PCR analysis of ChIP material (Supplementary Figure 1B).

On review of transcript databases, it is seen that there are transcripts beginning at this position in both human and mouse with the database of transcriptional start sites (<http://dbtss.hgc.jp/>) identifying transcripts starting at this position in fetal liver (Supplementary Figure 2). Moreover, multisequence alignment reveals near 100% sequence conservation across mammals (Supplementary Figures 3 and 4). Taken together, this evidence suggests

the presence of a previously unrecognized third promoter of LMO2, which is active in T-ALL. Comparison of the H3K4Me3 and H3K9Ac plots revealed that there is just one solely H3K9Ac positive region upstream of LMO2 (only seen in T-ALL 8) and one small region downstream of LMO2 (seen in T-ALLs 8, 16, 27 and 29), suggesting that LMO2 expression in T-ALL is largely driven by promoters with little additional enhancer activity.

We have previously shown that mouse *lmo2* expression depends on an array of distal regulatory elements

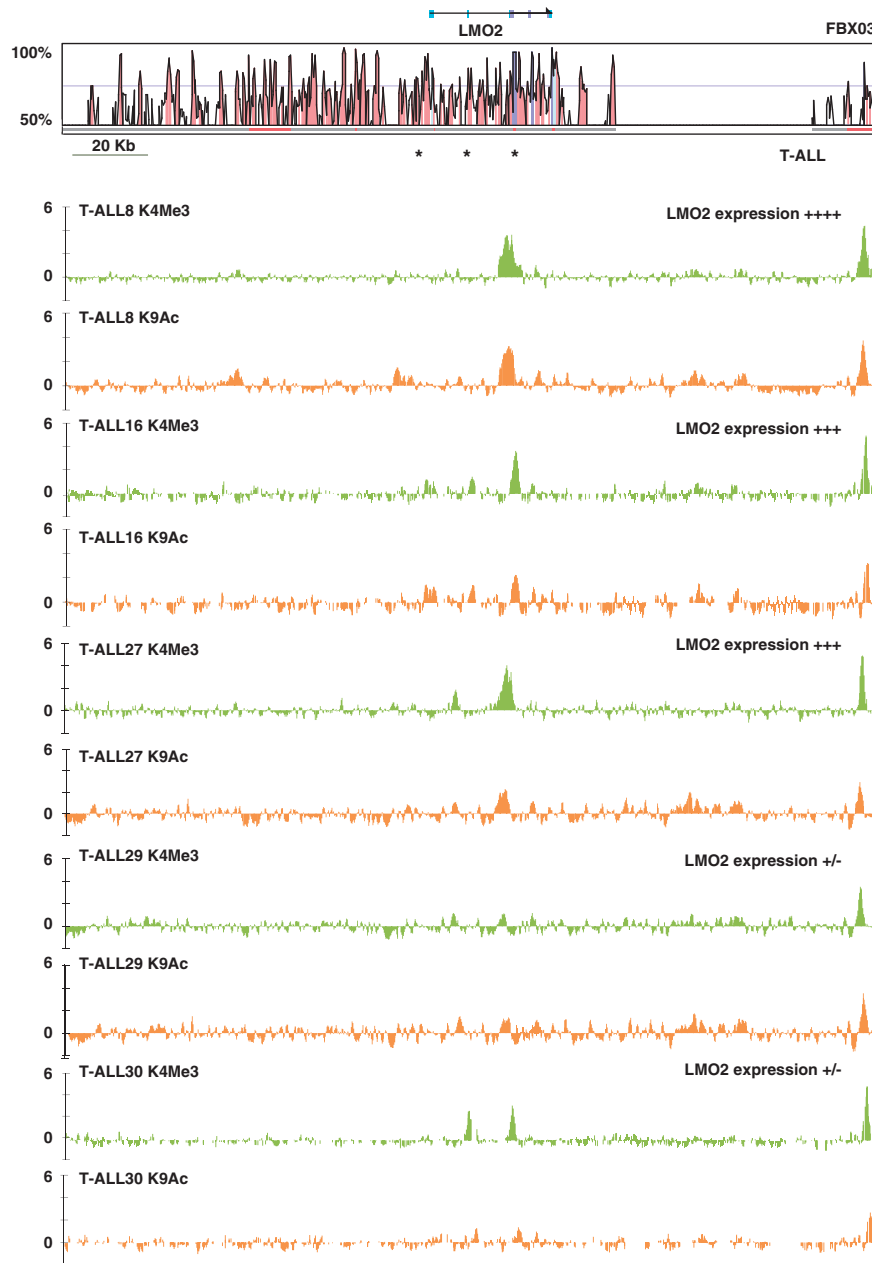


Figure 2 H3K4Me3 and H3K9Ac ChIP-on-chip demonstrates peaks largely restricted to promoters in the LMO2 locus in human T-ALL samples. Shown at the top of the figure is a comparative genomics conservation plot generated in Vista genome browser as in Figure 1. This is followed by ChIP-on-chip plots in xenograft T-ALLs, which demonstrate peaks over the two previously described promoters of LMO2 and over the newly described third promoter. There are no prominent H3K9Ac-only peaks evident, supportive of a promoter-predominant drive to transcription. LMO2 expression status is indicated.

spread over more than 100kb of genomic sequence (Landry *et al.*, 2009). To verify that our failure to pick up distal elements in the LMO2 H3K9Ac ChIP-on-chip analysis was due to the particular mode of LMO2 control in these ectopically expressing cells rather than fundamental differences between the control of human and mouse LMO2 expression, we also studied the human *LMO2* locus by ChIP-on-chip in B-ALL samples. In contrast to the T-ALL samples, we observed considerably more distal H3K9Ac peaks in the B-ALL samples upstream of LMO2 (Figure 3). Of note, the

smaller peaks in sample B-ALL 11 corresponded to peaks seen in the other two samples consistent with the notion that transcriptional control in B-ALL is more complex involving both promoter and enhancer elements. The main peaks seen in the H3K9Ac plots precisely correspond to regions previously identified to be important for murine *lmo2* expression (Landry *et al.*, 2009).

In summary, LMO2 expression in T-ALL appears to be driven predominantly by promoter activation in contrast with the promoter–enhancer cooperation seen

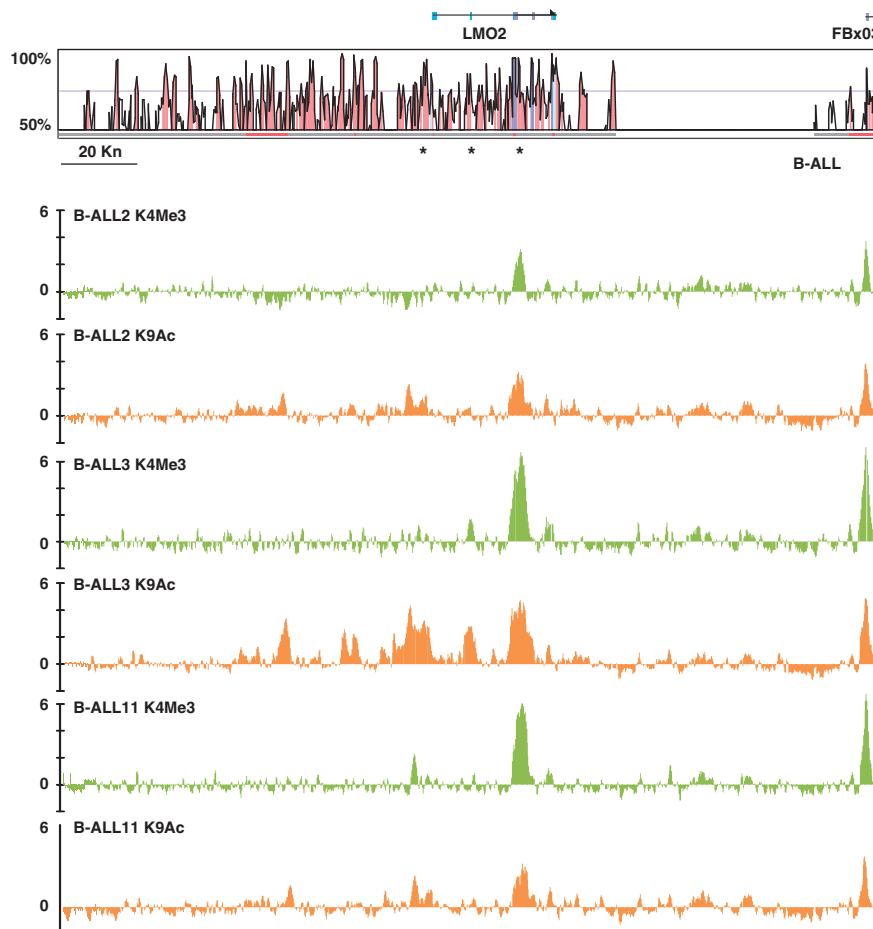


Figure 3 H3K4Me3 and H3K9Ac ChIP-on-chip demonstrates peaks over promoters and enhancer elements in the LMO2 locus in human B-ALL samples. Shown at the top of the figure is a comparative genomics conservation plot as in Figure 1. H3K4Me3 ChIP-on-chip plots in xenograft B-ALLs demonstrate peaks over the two previously described promoters of LMO2 and over the newly described third promoter. In contrast with the T-ALL samples, numerous H3K9Ac-only peaks are evident, supportive of promoter–enhancer cooperation to drive transcription.

in B-ALL. Additionally, these experiments have suggested the presence of a third previously undescribed promoter of LMO2. From now forward this region will be termed the putative intermediate promoter.

Additional evidence for the relevance of this promoter became evident when we analysed recently deposited data from ChIP sequencing human CD36-, CD19- and CD34-positive cells subjected to ChIP for the promoter mark H3K4Me3. Strong peaks were seen in the presumed novel promoter region in erythroid cells and CD34⁺ cells (Supplementary Figure 5). Furthermore, real-time PCR analysis of ChIP with an antibody to H3K4Me3 material from T-ALL cell lines and primary samples demonstrated the presence of the promoter specific mark on this new promoter not only in Lmo2-expressing cell lines but also in a subset of primary patient samples (Supplementary Figures 6A and B).

The putative intermediate promoter is active in T-ALL cell lines

Having identified a possible third promoter of LMO2, we next proceeded to validate this in experimental models. We first investigated the LMO2 expression status of a

panel of human T-ALL cell lines by real-time PCR. The 11p13 translocation status was ascertained using break-apart FISH (Supplementary Figure 7) and confirmed to be in agreement with published data and reference information in international cell banks. We were therefore able to identify three types of T-ALL cell lines with respect to LMO2: (i) non-LMO2-expressing, (ii) LMO2-expressing, not translocated and (iii) LMO2-expressing, translocated. In Figure 4a, we present data confirming that the human T-ALL cell lines Jurkat and All-Sil do not express LMO2. The cell lines Molt4, CCRFCEM, Peer and Loucy express LMO2 but do not show evidence of a translocation involving LMO2, whereas the cell lines KOPTK1 and Karpas45 express LMO2 and each have translocations involving LMO2 (Figure 4a, Supplementary Figures 6 and 7, Supplementary Table 1).

We next generated luciferase reporter constructs containing the two known promoters and the putative intermediate promoter (proximal promoter/LUC, intermediate promoter/LUC, distal promoter/LUC). Stable transfection assays demonstrated that activity of the three promoters in the LMO2-non-expressing cell lines or LMO2-expressing cell lines with a translocation involving

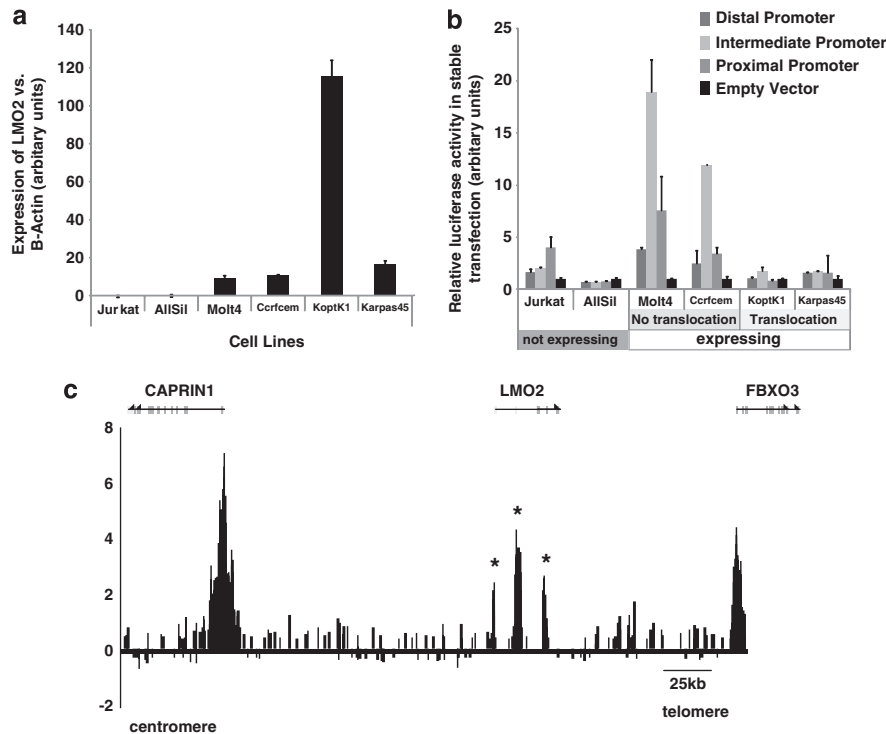


Figure 4 T-ALL cell line model systems. **(a)** LMO2 expression status in human T-ALL cell lines. Total LMO2 expression in T-ALL cell lines normalized for housekeeping gene expression is shown. No LMO2 expression in Jurkat and AllSil cells is observed; however, a moderate expression is seen in Molt4 and CCRFCEM cells, a moderate-high expression in Karpas45 and high expression in KoptK1 cells. **(b)** Luciferase reporter assays following stable transfection of promoter/LUC constructs. Luciferase reporter assays following stable transfection of LMO2 promoter constructs into T-ALL cell lines demonstrates low/no activity of the three promoter/LUC constructs in non-LMO2-expressing cell lines (Jurkat, AllSil) and in LMO2-expressing cell lines with an LMO2 translocation (Karpas45, KoptK1). By contrast, there is high relative luciferase activity of the promoters in non-translocated LMO2-expressing cell lines (Molt4, CCRFCEM), with the intermediate promoter being the most active of the three. Data shown represent four biological replicates each of four technical replicates. **(c)** H3K4Me3 ChIP-on-chip in Molt4, a LMO2-expressing non-translocated cell line, demonstrates three peaks over promoters in the LMO2 locus.

LMO2 was no higher than background in all instances with the exception of the proximal promoter, which showed modest activity in Jurkat cells. By contrast, all the promoters were significantly more active than empty vector (pGL2 basic) in LMO2-expressing cell lines without a translocation with the putative intermediate promoter displaying the highest activity (Figure 4b). Activity of the three promoters in LMO2-expressing non-translocated cell lines was confirmed by ChIP-on-chip using an antibody to the promoter-specific histone mark H3K4Me3 in Molt4 (Figure 4c). Taken together, these results allow us to draw the following conclusions: (i) there is a third promoter of LMO2; (ii) T-ALL cell lines represent suitable models in which to investigate aspects of its function further; (iii) the transfection data are consistent with the ability of promoters to respond to the T-ALL transcriptional environment in non-translocated, LMO2-expressing T-ALL.

Transcripts derived from the putative intermediate promoter are apparent in T-ALL cell lines and T-ALL primary samples

To quantify the contribution of transcripts starting at each of the three LMO2 promoters, PCR primers were designed to permit amplification of regions unique to the distal and intermediate promoters and a region shared

by all three transcripts (total LMO2), thereby permitting calculation of the contribution of transcripts sourced at the proximal promoter using a subtractive approach (Figure 5a). Transcripts beginning at each of the three promoters were quantified against a DNA template by real-time PCR and standardized for housekeeping gene expression (Figure 5b). A selection of expression patterns were seen in T-ALL cell lines. Lack of LMO2 expression was confirmed in All-Sill and Jurkat and high total LMO2 expression with low or no discernable transcripts from the intermediate or distal promoters in the 11q13-translocated cell lines Karpas45 and KOPT-K1. Transcripts starting at each promoter were evident in the untranslocated LMO2-expressing cell lines Molt4, CCRFCEM and Peer, whereas Loucy cells only showed activity for the distal and proximal promoters. Intermediate promoter transcripts are also seen as a variable proportion of all transcripts in primary T-ALL samples (Figure 5c) with the highest levels of intermediate promoter sourced transcripts in the sample with the most primitive immunophenotype, patient sample 1.

The LMO2 intermediate promoter directs reporter gene expression in F0 transgenic mice

To investigate the ability of the LMO2 intermediate promoter to direct expression *in vivo*, lacZ reporter

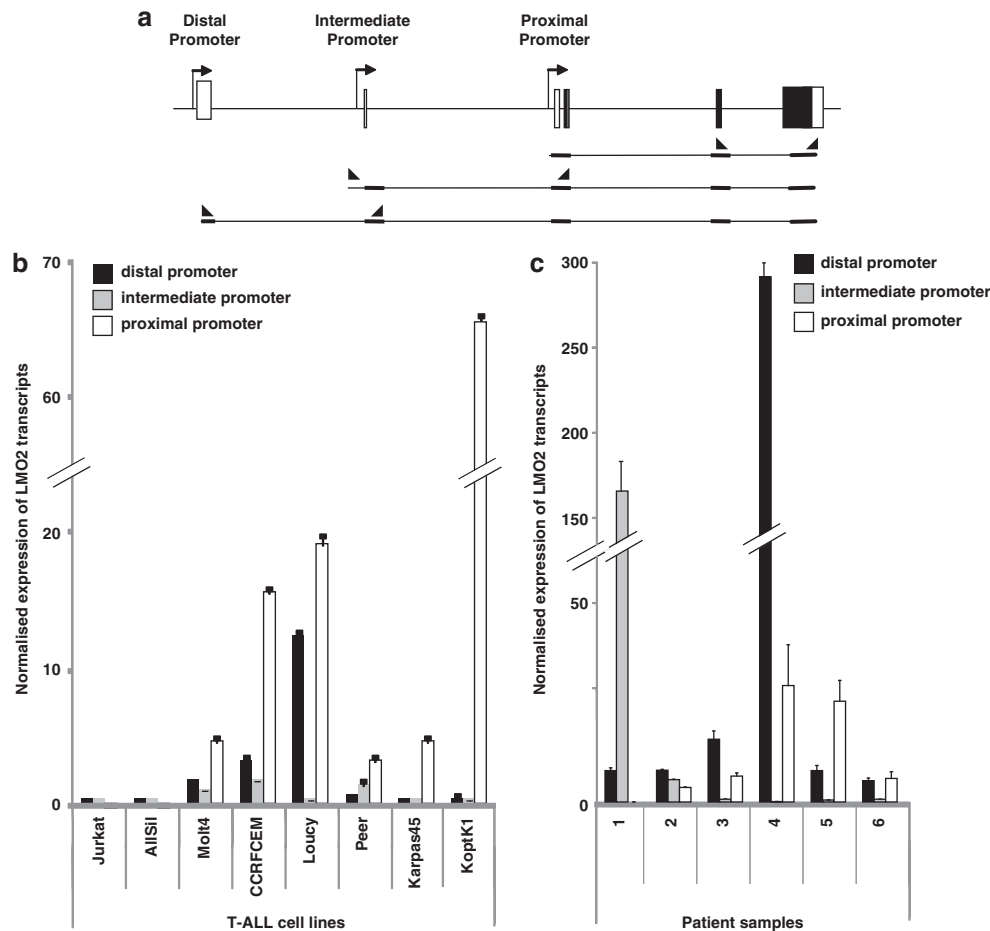


Figure 5 Analysis of promoter-specific expression. (a) Representation of expression primer pair positions. Shown is a sketch of the LMO2 locus with the three promoters designated by arrows, empty boxes to demonstrate non-coding exons and filled boxes to demonstrate coding exons. Beneath this, three lines represent the three transcripts starting at each promoter, with the exons as blocks on the line and real-time primer positions marked by filled triangles. Note that the 5' primer for intermediate promoter-sourced transcripts lies 5' of exon 2 in a region that is specific to transcripts starting at the intermediate promoter. (b) Expression of transcripts starting at each of the three LMO2 promoters against DNA template by quantitative real-time PCR (Q-RT-PCR) in T-ALL cell lines. Results are shown as standardized against β -actin expression. Minimal transcript levels are detected in the LMO2-non-expressing cell lines; transcripts starting at each promoter are seen in the LMO2-expressing non-translocated cell lines and the predominant amplicons detected in the translocated cell lines are total LMO2. (c) Expression of transcripts starting at each of the three LMO2 promoters against DNA template by Q-RT-PCR in T-ALL patient samples. Results are shown as standardized against β -actin expression. Variable levels of LMO2 expression are seen and of those that express highly, variable proportions of transcripts start at each promoter. Total LMO2 mRNA expression levels relative to β -actin for the eight T-ALL cell lines and six T-ALL patient samples from (b) and (c) are shown in Supplementary Figure 8.

constructs were generated. Following pronuclear microinjection, F0 transgenic mice were generated permitting the assessment of promoter activity by whole-mount staining of E11.5 embryos for *lacZ* expression. Similar to the distal and proximal promoter core regions (Landry *et al.*, 2005, 2009), the intermediate promoter on its own (IP/*lacZ*) does not direct any strong tissue-specific expression (see Figure 6a for a representative embryo). To demonstrate that the promoter can work in combination with enhancer elements, further constructs were generated coupling the intermediate promoter/*lacZ* to proposed enhancer elements identified in previous work (Landry *et al.*, 2005, 2009). In contrast with the minimal staining pattern seen with intermediate promoter/*lacZ* alone, when a proposed enhancer element immediately

adjacent to the intermediate promoter is included in the construct (IP/*lacZ*/-12), diffuse staining is seen throughout the embryo (Figure 6b). Similarly, combination of intermediate promoter/*lacZ* with a more distant enhancer element 1 kb downstream of the proximal promoter transcription start site (IP/*lacZ*/+1) creates a striking staining pattern with reporter gene expression directed to the tail tip, base of whiskers and apical ectodermal ridge (Figure 6c). This distinctive pattern mirrors the one that is seen when the same element is combined with the proximal promoter (Landry *et al.*, 2009). Taken together, therefore, comprehensive transgenic analysis has allowed us to validate the intermediate promoter using stringent *in vivo* assays as a bona fide promoter element within the LMO2 locus.

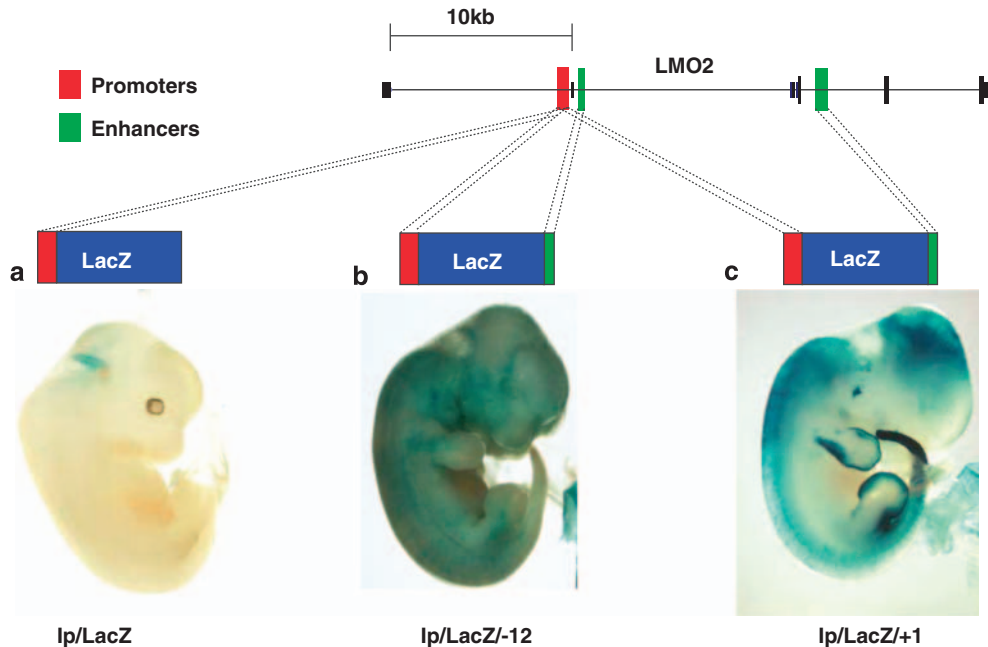


Figure 6 *In vivo* validation of promoter/LacZ and promoter–enhancer/LacZ F0 transgenic mice. F0 transgenic mice were generated by pronuclear microinjection with promoter and promoter–enhancer-coupled LacZ constructs. Specific staining patterns are seen in the promoter–enhancer/LacZ embryos. (a) Ip/LacZ: one of seven embryos stained; (b) Ip/LacZ/–12: three of eleven embryos stained; (c) Ip/LacZ/+1: two of six embryos stained.

The ETS transcription factors ERG and FLI1 bind to the LMO2 intermediate promoter and LMO2, ERG and FLI1 can form protein–protein complexes

The ETS family transcription factor FLI1 was recently identified as a candidate collaborating oncogene of LMO2 in retroviral mutagenesis models of T-ALL (Dave *et al.*, 2009). We therefore investigated potential interactions between LMO2 and FLI1 in human T-ALL. ERG was included in this analysis as it has been shown that FLI1 and ERG, which is closely related to FLI1, may co-regulate common target genes in haematopoietic stem cells and megakaryocytes (Kruse *et al.*, 2009) and high ERG expression has recently been shown to predict adverse outcome in T-ALL (Baldus *et al.*, 2006, 2007; Bohne *et al.*, 2009). ChIP using antibodies to ERG and FLI1 in Molt4 (LMO2-expressing non-translocated human T-ALL cell line) was therefore performed. Real-time PCR on FLI1 and ERG ChIP was then undertaken using primer sets designed for each of the LMO2 promoters and a negative control region 98 kb upstream of the transcription start site. Specific binding of ERG and FLI1 to the intermediate and proximal promoters was seen (Figure 7a) with binding at the distal promoter being only marginally above background.

Small groups of transcription factors have been previously associated with stabilizing cellular phenotypes, in particular when interconnected through reciprocal interactions as seen in the fully connected triads of OCT4/SOX2/Nanog (MacArthur *et al.*, 2009) and SCL/GATA2/FLI1 (Pimanda *et al.*, 2007) in embryonic and blood stem cells, respectively. Given the recent report of cooperation between LMO2 and FLI1

in retroviral insertional mutagenesis models of murine T-ALL and our identification of FLI1 and ERG as upstream regulators of LMO2 in human T-ALL, we wanted to explore whether reciprocal activation of FLI1 and ERG by LMO2 might also occur in human T-ALL. We have previously identified a +85 kb ERG enhancer (Wilson *et al.*, 2009) and +12 kb (Donaldson *et al.*, 2005) and –16 kb (Wilson *et al.*, 2009) FLI1 enhancers as being bound by the LMO2 partner protein SCL in myeloid progenitor cells. We therefore undertook the reverse experiment assessing the binding of LMO2 ChIP material to ERG and FLI1 elements. Specific binding of LMO2 was seen at the FLI1 promoter, +12 kb and –16 kb enhancers and the ERG proximal promoter and +85 kb enhancer element (Figure 7b). Of note, binding of LMO2 was also seen on the LMO2 IP and PP promoters consistent with LMO2 autoregulation in T-ALL.

Further biological validation of transcription factor binding to the intermediate promoter was provided by performing transcriptional assays. To this end, luciferase reporter constructs containing the intermediate promoter were co-transfected with Erg, Flil and LMO2 expression constructs alone or in combination and compared with a promoter-less construct (pGL2basic). Neither ERG nor LMO2 alone showed significant activation of the promoter, yet co-transfection of both resulted in a near twofold activation. FLI1 alone showed twofold activation and again this could be enhanced further by co-transfection with LMO2 (Figure 7c). These experiments therefore show that LMO2, ERG and FLI1 not only bind to the

intermediate promoter, but also have the capacity to enhance its activity. To investigate whether the synergistic transactivation was paralleled by protein–protein interactions, co-immunoprecipitation assays were performed using a haemagglutinin (HA)-tagged LMO2 expression vector, a myc-tagged FLI1 expression vector and an untagged ERG expression vector. These were transfected alone or in combination and proteins bound to LMO2 immunoprecipitated with an anti-HA antibody to confirm the association of LMO2 and ERG and of LMO2 and FLI1 proteins (Figure 7d). Taken together, these experiments not only show that LMO2, ERG and FLI1 proteins bind to the intermediate promoter, but also that this binding likely has a role in synergistic activation of this promoter.

LMO2/ERG/FLI1 function upstream of HHEX/PRH that is required for the growth of T-ALL cell lines

Following the recent identification of *hhex/prh* as a mediator of *lmo2*-induced thymocyte self-renewal during both the latency and leukaemic phases of *lmo2*-based mouse models of T-ALL (McCormack *et al.*, 2010), we also investigated whether the potentially self-sustaining triad of LMO2/ERG/FLI1 might directly regulate HHEX/PRH expression. We had previously identified an enhancer in the first intron of HHEX/PRH (+1 kb enhancer) (Donaldson *et al.*, 2005) and now show that indeed this element is strongly bound by LMO2, FLI1 and ERG in T-ALL cells (Figure 7e).

Further validation of this binding to the HHEX + 1 enhancer was confirmed by transactivation experiments using an *sv HHEX + 1* luciferase reporter construct co-transfected with ERG, FLI1 and LMO2 expression constructs alone or in combination. By comparison with the empty control vector, both the combination of LMO2 and ERG, the combination of LMO2 and FLI1 and the combination of LMO2, ERG and FLI1 has higher activity than the addition of LMO2, ERG or FLI1 alone (Figure 7f). Transactivation studies thus demonstrated that LMO2, FLI1 and ERG not only bind to the HHEX + 1 element but also have the capacity to enhance its activity.

The previous studies identifying HHEX/PRH as a mediator of the leukaemogenic phenotype of *Lmo2*-induced mouse models of T-ALL did not address whether there was a direct link between *Lmo2* and HHEX/PRH. Having established that there is indeed a direct link, we next asked whether HHEX/PRH was important for the growth of human T-ALL cells. Using vectors previously established to produce effective knockdown of HHEX/PRH (Noy *et al.*, 2010) we now show that following expression of short hairpin RNA against HHEX/PRH in the LMO2-expressing non-translocated cell line Molt4, cells were unable to grow, whereas cells transfected with a control vector could expand as expected (Figure 7g). Taken together, these results not only support the existence of a highly connected LMO2, FLI1, ERG triad in T-ALL but also

Figure 7 LMO2, ERG, FLI1 and HHEX/PRH form a subcircuit of T-ALL transcriptional programmes. (a) Real-time PCR in Molt4 FLI1 and ERG ChIP material. Real-time PCR demonstrates the binding to the three promoters of LMO2 in Molt4 chromatin subjected to immunoprecipitation with FLI1 and ERG antibodies with the highest amount of binding to the intermediate promoter. Bars represent mean enrichment relative to IgG and a negative control region, and error bars represent the s.d. of replicates. Data are representative of two replicates of experiments designed with duplicate data points. (b) Real-time PCR in Molt4 LMO2 ChIP material. Real-time PCR demonstrates binding to the LMO2 promoters, ERG and FLI1 promoters and enhancer element in Molt4 chromatin subjected to immunoprecipitation with LMO2 antibody. Results show binding of LMO2 to each of the LMO2 promoters, to the FLI1 enhancer elements and to the ERG promoters and enhancer element. Significantly, there is a moderate degree of binding to the LMO2 intermediate promoter, suggesting a role of LMO2 autoregulation via this element in those T-ALL samples lacking LMO2 translocations. Bars represent mean enrichment relative to IgG and a negative control region, and error bars represent s.d. of replicates. Data are representative of two replicates of experiments designed with duplicate data points. (c) Transactivation assays of the intermediate promoter with LMO2, ERG and FLI1 alone and in combination. Intermediate promoter luciferase construct was co-transfected with *Erg*, *Fli1* and LMO2 expression constructs alone or in combination and compared with a parallel experiment with control vector. Combination of LMO2/ERG and LMO2/*Fli1* has higher activity than the addition of single factors. Data shown represent intermediate promoter activity normalized for cell number and transfection efficiency and further normalized against the equivalent combination of expression vectors with the empty vector, pGL2basic. Four biological replicates each of three technical replicates were performed. (d) Co-immunoprecipitation of ERG and FLI1 with LMO2. Co-immunoprecipitation assays were performed using an HA-tagged LMO2 expression vector, a myc-tagged *Fli1* expression vector and an untagged ERG expression vector transfected alone or in combination. Control lanes were loaded with native whole-cell extract of HA-LMO2/ERG or HA-LMO2/myc-FLI1 transfected cells and demonstrate high levels of target protein production in these cells. No ERG or FLI1 protein is detected in the IP samples transfected with HA-LMO2 ERG or FLI1 alone. However, ERG or FLI1 protein is clearly detectable in cells transfected with HA-LMO2 in combination with ERG/FLI1, thus confirming the association of LMO2 and ERG and of LMO2 and *Fli1* proteins. Western blot images are cropped to remove IgG bands. (e) Real-time PCR in Molt4 ERG, FLI1 and LMO2 ChIP material. Real-time PCR demonstrates binding to the Hhex + 1 enhancer element in Molt4 chromatin subjected to immunoprecipitation with ERG, FLI1 and LMO2 antibody. Bars represent mean enrichment relative to IgG and a negative control region, and error bars represent s.d. of replicates. (f) Transactivation assays of the Hhex + 1 enhancer with LMO2, ERG, FLI1 alone and in combination. The minimal promoter *sv Hhex + 1* enhancer luciferase construct was co-transfected with *Erg*, *Fli1* and LMO2 expression constructs alone or in combination and compared with a parallel experiment with *sv luciferase*. Both the combination of LMO2/ERG and LMO2/*Fli1* has higher activity than the addition of just the single Ets factor and the combination of all three has the highest activity. Data shown represent activity on *sv Hhex + 1* normalized for cell number and transfection efficiency and further normalized against the equivalent combination of expression vectors with the empty vector, *sv-luciferase*. Four biological replicates each of three technical replicates were performed. (g) Knockdown of HHEX/PRH. The *Lmo2*-expressing T-ALL cell line Molt4 was transfected using vectors previously established to produce effective knockdown of Hhex. HHEX short hairpin RNA (shRNA) cells were unable to grow, while those transfected with a control vector expanded as expected following the initial loss of cells due to puromycin selection. At least six independent knockdown experiments were performed with each construct(s). (h) Representation of FLI1–ERG–LMO2–HHEX/PRH recursively wired circuit.

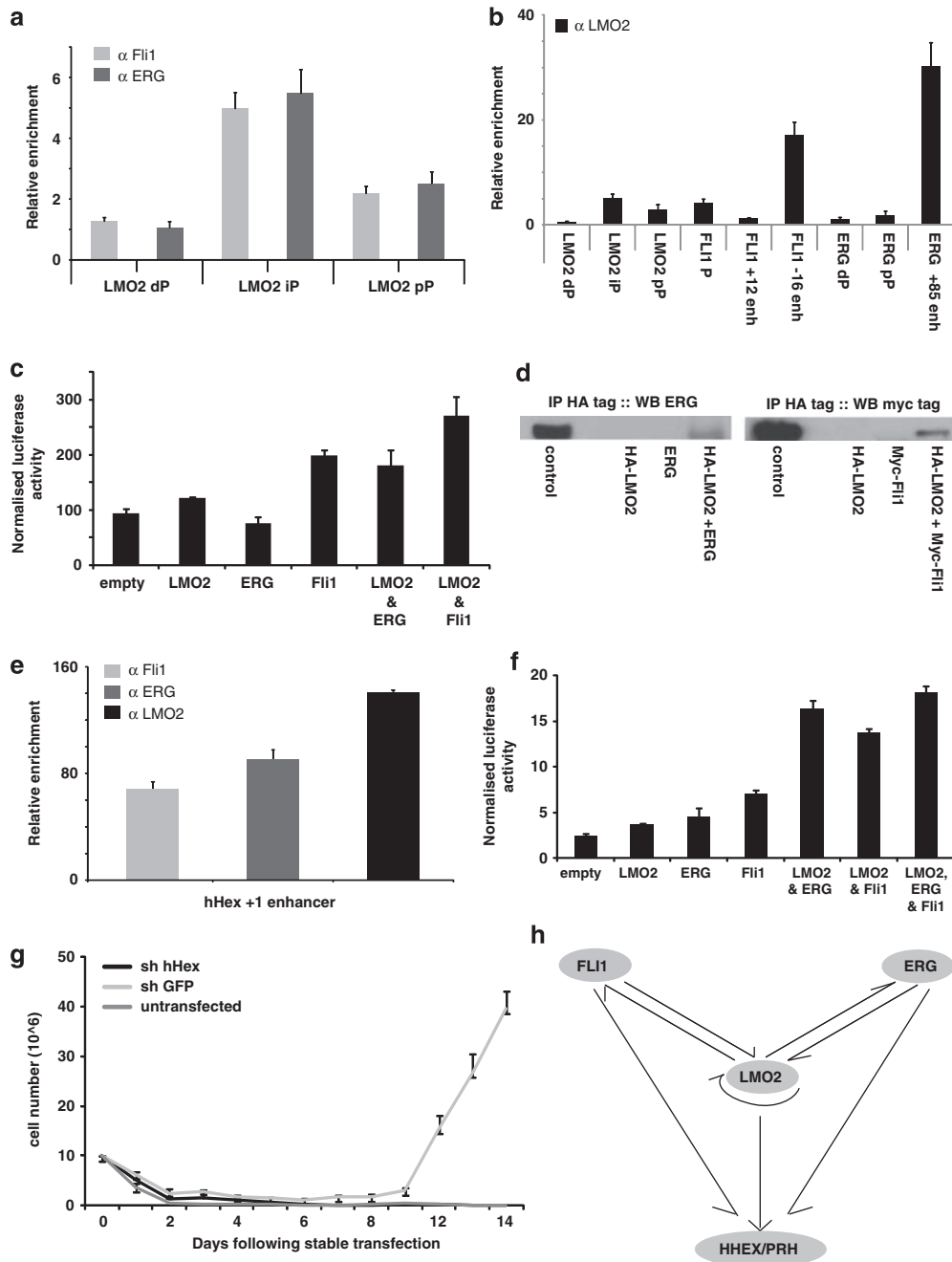
demonstrate that HHEX is a direct target of this triad with a likely role in human T-ALL (Figure 7h).

Discussion

Understanding the molecular mechanisms controlling the expression of LMO2 is likely to provide new insights into the pathogenesis of T-cell leukaemias and will also provide valuable information with regard to the transcriptional control of hematopoietic and endothelial development. Here, we have used ChIP-on-chip in patient T-ALL material to highlight the central nature

of promoter usage in TALL and also to the discovery of a previously unrecognized third promoter of LMO2. This novel promoter functions both *in vitro* and *in vivo* and participates in a recursively wired regulatory loop together with the ETS factors FLI1 and ERG. Moreover, all three members of this triad bind to an enhancer of the *HHEX/PRH* homeobox transcription factor gene thus linking a potentially self-sustaining regulatory circuit with expression of a recently identified candidate downstream mediator of LMO2-induced T-ALL.

It has long been noted that genes critical for blood and endothelial development contain functional ETS-binding sites, and using a combination of approaches,



expression of numerous ETS factors, including FLI1, SPI1/PU.1, ELF1 and ETS1, has been shown to control the expression of SCL/TAL1 (Gottgens *et al.*, 2004), LYL1 (Chan *et al.*, 2007), LMO2 (Landry *et al.*, 2005) and GATA2 (Pimanda *et al.*, 2007). What has been less well studied, however, is how dysregulation of this same constellation of genes might facilitate leukaemogenesis. Ectopic or aberrant expression of genes in leukaemia is an important and as yet relatively unexplored field. Indeed ectopic expression of stem cell-affiliated genes in the absence of translocation suggests that these cells may re-invoke some aspects of transcriptional control mechanisms normally used in more primitive cells.

Recent work from insertional mutagenesis models in mice (Dave *et al.*, 2009) has shown that the genes and signalling pathways deregulated in murine leukaemias with retroviral insertions at LMO2 are analogous to both those dysregulated in highly LMO2-expressing human leukaemias (Ferrando *et al.*, 2002; Yeoh *et al.*, 2002; Chiaretti *et al.*, 2004) and to the LMO2 retroviral insertion-mediated leukaemias induced in severe combined immunodeficiency-X1 patients (Hacein-Bey-Abina *et al.*, 2003a, b, 2008). Moreover, many of these genes are components of haematopoietic stem cell transcriptional regulatory networks.

Cell-fate mapping in *CD2-lmo2* transgenic mice (McCormack *et al.*, 2010) has shown that the leukaemia-initiating cell is established within the thymus many months before the development of leukaemia. In contrast with wild type, transgenic thymic cells were able to self-renew and to provide robust stable engraftment in primary, secondary, tertiary and quaternary recipients. Taken together, these data suggest that thymocyte self-renewal may have been reactivated by *lmo2*. Expression profiling of pre-leukaemic and wild-type thymocytes, and human T-ALL samples identified a gene-expression signature containing candidates with known roles in haematopoietic stem cell function, and a bone marrow reconstitution model showed that HHEX/PRH overexpression recapitulated the oncogenic potential of *lmo2*.

Dave *et al.* (2009) and McCormack *et al.* (2010) both suggest a possible role of the LMO2-ETS factor interaction and identify HHEX/PRH as a potential downstream collaborator in mice. However, although these studies have identified *lmo2*, the ETS factors and HHEX/PRH as collaborating oncogenes, they do not distinguish whether they function in a parallel, overlapping or hierarchical manner. We now present data showing that LMO2-ETS factors collaborate in T-ALL and that these directly interact with the *HHEX* +1 enhancer, the first time *HHEX/PRH* has been implicated in human T-ALL. A possible mechanism for these leukaemias may therefore be a failure to downregulate members of the LMO2-ERG-FLI1 triad identified in the current study. The molecular mechanisms responsible for downregulation of intermediate promoter activity are not known even though preliminary observation demonstrates that its activity is highly sensitive to the negative regulatory region upstream of the distal promoter (Göttgens B, unpublished observation).

Nevertheless, identification of upstream regulators responsible for ectopic expression of LMO2 represents an important milestone in furthering our understanding of aberrant transcriptional programmes in T-ALL.

Our approach to identification of critical elements is in no way T-ALL or LMO2 specific and may be considered generally applicable to other genes ectopically expressed in other malignancies. It has been previously shown possible to inhibit ETS factors in whole animal models without any harm coming to normal tissue (Huang *et al.*, 2006). As high LMO2 removal is sufficient to cause death of leukaemic cells even after acquisition of secondary genetic abnormalities (Appert *et al.*, 2009), identification of the molecular mechanisms involved in aberrant ectopic expression of LMO2 may open up new targeted therapeutic options directed towards the impediment of upstream regulators to block the maintenance of the leukaemia phenotype.

Materials and methods

Cell preparation and culture

Preparation of cell samples and patient information such as translocation status and immunophenotype is provided in Supplementary Materials.

Chromatin immunoprecipitation

ChIP was carried out as previously described (Wilson *et al.*, 2009) using 2×10^5 (primary samples) and 1×10^7 (cell lines) as per the condition using commercially available antibodies (Supplementary Information). The relative enrichment of immunoprecipitated DNA was estimated using real-time PCR as described in Supplementary Information. ChIP-chip assays were performed as described (Follows *et al.*, 2006). For details on transcription factor ChIP assays, see Supplementary Information.

RNA analysis

RNA was prepared from patient samples and cell lines with TRIZOL reagent (Invitrogen, Paisley, UK) and was DNase treated to eliminate residual genomic DNA using TURBO-DNase (Ambion, Huntingdon, UK). cDNA was prepared using random hexamers and TaqMan reverse transcriptase reagents kit (Applied Biosystems, Foster City, CA, USA). The procedure for quantitation of transcripts derived from each of the three promoters by quantitative real-time PCR is provided in Supplementary Information.

Quantitative PCRs (qPCR) were undertaken using Stratagene Brilliant Sybr Green QPCR Master Mix (Agilent Technologies, Stockport, UK). Standard curves for LMO2 and β -actin were created using dilutions of linearized plasmid templates.

Luciferase reporter assay and transgenic mouse analysis

The known promoters and candidate new promoter and enhancer regions were PCR amplified from human genomic DNA and subcloned into a luciferase reporter vector. Cells were transfected, subjected to antibiotic selection at an appropriate dose, lysate prepared and assayed as previously described (Landry *et al.*, 2009). The promoter and enhancer regions were PCR amplified from human genomic DNA and subcloned into a *lacZ* reporter vector. Transgenic mice were

generated as previously described (Landry *et al.*, 2009), and activity of the intermediate promoter and enhancers was assessed by whole-mount staining of E11.5 embryos for *LacZ* activity.

Co-immunoprecipitation

293T cells were transiently transfected with HA-tagged LMO2, untagged ERG and myc-tagged FLI1 expression constructs alone and in combination and co-immunoprecipitation assays performed following standard protocols using anti-HA antibody (Santa Cruz Biotechnology, Heidelberg, Germany, sc805G), Erg antibody (Santa Cruz, sc-354 ×) or c-myc antibody (Santa Cruz, sc-40).

Knockdown of HHEX/PRH

A total of 5×10^6 Molt4 cells were electroporated using 10 μ g green fluorescent protein short hairpin RNA (control) or 5 μ g PRH49 and 5 μ g PRH51 short hairpin RNA plasmids in combination previously as described (Noy *et al.*, 2010). Cells were selected with 1 μ g/ml puromycin and viable cells were

counted daily for 14 days. At least six independent knockdown experiments were performed with each construct(s).

Conflict of interest

The authors declare no conflict of interest.

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