Transcriptional regulation of the proto-oncogene Zfp521 by SPI1 (PU.1) and HOXC13

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Abstract
The mouse zinc-finger gene Zfp521 (also known as ecotropic viral insertion site 3; Evi3; and ZNF521 in humans) has been identified as a B-cell proto-oncogene, causing leukemia in mice following retroviral insertions in its promoter region that drive Zfp521 over-expression. Furthermore, ZNF521 is expressed in human hematopoietic cells, and translocations between ZNF521 and PAX5 are associated with pediatric acute lymphoblastic leukemia. However, the regulatory factors that control Zfp521 expression directly have not been characterized. Here we demonstrate that the transcription factors SPI1 (PU.1) and HOXC13 synergistically regulate Zfp521 expression, and identify the regions of the Zfp521 promoter required for this transcriptional activity. We also show that SPI1 and HOXC13 activate Zfp521 in a dose-dependent manner. Our data support a role for this regulatory mechanism in vivo, as transgenic mice over-expressing Hoxc13 in the fetal liver show a strong correlation between Hoxc13 expression levels and Zfp521 expression. Overall these experiments provide insights into the regulation of Zfp521 expression in a nononcogenic context. The identification of transcription factors capable of activating Zfp521 provides a foundation for further investigation of the regulatory mechanisms involved in ZFP521-driven cell differentiation processes and diseases linked to Zfp521 mis-expression.

KEYWORDS
B-cell leukemia, B-cell differentiation, AKXD mice, ZNF521, Evi3

1 | INTRODUCTION
Alterations in gene expression during lymphocyte differentiation can lead to malignancies including leukemia and lymphoma. Retroviral insertions that cause up-regulation of Zinc finger protein 521 (Zfp521) or its parologue, Zinc finger protein 423 (Zfp423), are associated with B-cell leukemia in mice (Hentges et al., 2005; Warming et al., 2003, 2004). Links between ZNF521 (the human orthologue of Zfp521) and human leukemia also exist, as a translocation that generates a chimaeric fusion protein of ZNF521 and PAX5 has been found in paediatric acute lymphoblastic leukemia (Mullighan et al., 2007). Retroviral insertions at the Zfp521 locus also promote the formation of B-cell acute lymphoblastic leukemia (B-ALL) in mice expressing the chimaeric oncogenic fusion protein E2A-hepatic leukemia factor (E2A-HLF), and ZNF521 overexpression is found in patients with translocations generating E2A-HLF fusion proteins (Yamasaki et al., 2010). Recent investigations have established a role for Zfp521 in B-cell differentiation, mediated through an interaction with the B-cell transcription factor EBF (Hentges et al., 2005; Hiratsuka et al., 2015; Mega et al., 2011). Additional functions for ZFP521 and its parologue ZFP423 have been identified, demonstrating that these multiple zinc-finger proteins participate in cell proliferation and differentiation events critical for the formation of a diverse set of cell types. An important role for ZFP521 in cell differentiation events has been documented for neural cells.
Therefore, we sought to identify factors that directly regulate important factors in determining cell fate, information regarding the activities. Despite the emerging evidence that ZFP521 and ZFP423 are 2011; Mega et al., 2011; Spina et al., 2013), suggesting multiple mecha-
extargets of murine leukemia retroviral insertions (Bijl et al., 2005).

differentiation. For example, over expression of human HOXB3 in mouse bone marrow results in a decrease in the total num-
ber of B220+ B-cells (Sauvageau et al., 1997). Conversely, deletion of HOXB3 in the bone marrow of adult mice also inhibits B-cell differentia-
tion, with knock out animals showing a reduced number of pro-B cells (Ko et al., 2007). Deletion of the homeobox gene Hoxa9 causes a reduction in the number of lymphocytes present in the spleen of adult mice, due to defects in the specification of committed B-lymphocyte progenitor cells in the bone marrow (Lawrence et al., 1997). In addition to these roles in B-cell differentiation, Hox genes are also associated with leukemia. For example, genes in the Hoxa cluster are frequent targets of murine leukemia retroviral insertions (Bijl et al., 2005).

HOX gene over-expression is also a common feature of human lymph-

Hox genes reveal roles for these factors in B-cell development and differentiation. The expression profile of Zfp521, and its over-expression in mouse B-cell leukemia, suggests that appropriate regulation of Zfp521 is important for B-cell function. We therefore sought to identify transcription factors required for expression of the Zfp521 gene, finding that SPI1 and HOXC13 synergistically regulate Zfp521 expression in a dose-
dependent manner. Furthermore, transgenic mice over-expressing Hoxc13 also have increased Zfp521 expression in the fetal liver, the site of B-cell differentiation during development. Further studies are needed to examine whether SPI1 and HOXC13 regulate Zfp521 in additional cell types, and whether alterations in the activity of these regulatory factors contributes to B-cell leukemogenesis.

2 | RESULTS

2.1 | Evolutionary analysis of Zfp521 and Zfp423

The close protein sequence similarity between ZFP521 and ZFP423 suggests that the genes encoding these proteins arose due to a gene duplication event during evolution. We identified orthologues of ZFP521 and ZFP423 based on protein sequences from genomes of both invertebrate and vertebrate organisms (Supporting Information Table 1), and assembled a phylogenetic tree. We found that the verte-
brates analysed have both ZFP521- and ZFP423-related protein sequences (Figure 1a,b), while organisms such as insects have only one single protein sequence that is equally related to ZFP521 and ZFP423. This finding suggests that vertebrates have retained both genes following a duplication event. A reason for paralogous genes to be retained after duplication events is that the paralogues have specialized such that the two paralogues no longer have conserved functions or conserved expression patterns. Given that both Zfp521 and Zfp423 cause leukemia in mice when over-expressed (Hentges et al., 2005; Warming et al., 2003; Warming et al., 2004), they may both retain similar func-
tions in lymphocytes. However, their expression patterns in B-cells have diverged (Warming et al., 2003; Warming et al., 2004), providing an explanation for the retention of both paralogues in vertebrate genomes. Due to the noted expression of Zfp521 in B-cells during dif-
ferentiation (Hiratsuka et al., 2015; Warming et al., 2003), and links between Zfp521 over-expression and B-cell leukemia (Hentges et al., 2005; Warming et al., 2003), we sought to identify transcriptional regulators of Zfp521.

2.2 | Zfp521 promoter analysis

In order to identify transcriptional regulators of ZNF521, an analysis of the promoter region was performed. We identified a 1Kb region upstream of the human ZNF521 gene transcription start site as anno-
tated in the UCSC genome browser (Figure 2a), and the corresponding
region 1Kb upstream of the annotated mouse Zfp521 transcriptional start site from the UCSC genome browser (Figure 2b). DNaseI hypersensitivity sites are present near the ZNF521 transcriptional start site, which were experimentally identified from GM12878 and K562 lymphocyte cells (Ho & Crabtree, 2010). The Zfp521 promoter region shows hypersensitivity to DNaseI cleavage in CD19+ B-cells isolated from an 8-week old mouse (Sabo et al., 2006), suggesting the Zfp521 promoter region has transcriptional activity in B-cells. Both the mouse and human promoter regions lack a consensus TATA box, but instead have a GC-rich region near the transcriptional start site (Figure 2a,b). There is a region of high conservation amongst 100 vertebrates in the ZNF521 promoter region overlapping with a K562 DNase I hypersensitivity hot spot (Figure 2a), although conservation of the mouse Zfp521 promoter region amongst placental mammals does not show a similar region of high conservation (Figure 2b). Sequence alignments between the mouse and human ZNF521 promoters revealed the presence of two conserved binding sites for the transcription factor SPI1, a known B-cell transcription factor (Figure 2c, gray boxes). We defined the proximal site as SPI1a and the distal site as SPI1b. Both SPI1 sites overlap with regions of DNase I hypersensitivity, suggesting these promoter regions are bound by regulatory proteins in lymphocytes and leukemia cells. A similar analysis of the ZNF423 gene promoter did not reveal any SPI1 binding sequences (data not shown). In addition to the predicted SPI1 binding sites in the ZNF521 promoter, we also found a predicted conserved binding site for HOXC13 distal to the predicted SPI1 binding sites (Figure 2c, underlined). HOXC13 has been demonstrated to bind the ETS domain of SPI1, and is coexpressed with SPI1 in erythroid leukemia cells (Yamada et al., 2008). No other conserved binding sites for transcription factors known to be involved in B-cell differentiation were identified. Therefore, we sought to test the hypothesis that SPI1 and HOXC13 may regulate ZNF521/Zfp521 expression, either individually or synergistically.

2.3 | SPI1 and HOXC13 regulate Zfp521 expression

To determine if SPI1 and HOXC13 could act as Zfp521 transcriptional regulators, we generated luciferase reporter constructs containing varying regions of the Zfp521 promoter (Figure 2d). The 1Kb construct contains the HOXC13 and both SPI1 predicted binding sites, while the 0.5Kb promoter only contains the predicted SPI1 binding sites. The 0.2Kb promoter contains only the most proximal SPI1 binding site. We then tested the ability of SPI1 or HOXC13 to activate these various reporters in HEK293 cells. We found that when transfected individually, both SPI1 and HOXC13 proteins modestly activated the Zfp521 luciferase reporter constructs. Upon cotransfection of SPI1 and HOXC13 we noted a greater than additive activation of the Zfp521 reporter, confirming that SPI1 and HOXC13 activate Zfp521 in a synergistic manner (Figure 2e). The greatest activation was detected from the 1Kb Zfp521 promoter reporter construct containing all HOXC13 and SPI1 predicted binding sites (Figure 2e). Similar results were found when the reporter assays were performed in BCL1 B-lymphoblast cells (data not shown).

2.4 | SPI1 and HOXC13 regulation of Zfp521 is dose-dependent

We detected the greatest activation of Zfp521 using the 1 Kb reporter construct, so we further examined Zfp521 regulation using this construct for transfections in HEK293 cells. To confirm that the activation depended on SPI1 and HOXC13, we generated truncated versions of
the SPI1 and HOXC13 proteins, lacking their respective DNA binding regions (Figure 3a). We confirmed that truncated mutant forms of SPI1 and HOXC13 proteins showed no Zfp521 reporter activation above background levels, even when cotransfected with a wild-type version of the partner protein (Figure 3b).

Following the observation that SPI1 and HOXC13 synergistically activate Zfp521 expression, we examined the effects of varying the dosage of SPI1 and HOXC13 on Zfp521 reporter activation. We performed cotransfection assays with decreasing amounts of either SPI1 or HOXC13 plasmid, while maintaining a
constant concentration of the other construct. We found that the interaction between SPI1 and HOXC13 is dose-dependent (Figure 3c).

2.5 | SPI1 and HOXC13 bind the Zfp521 promoter

A physical interaction between SPI1 and HOXC13 has been reported (Yamada et al., 2009). As our epitope-tagged constructs varied slightly from the ones previously reported, we verified that our full-length FLAG-tagged HOXC13 protein did indeed bind to full-length SPI1 (Figure 4). Following the confirmation that the SPI1 and HOXC13 proteins encoded by our epitope-tagged constructs shared a physical interaction, we next examined whether they could also bind the Zfp521 promoter directly. We performed chromatin immunoprecipitation assays on HEK293 cells transfected with either FLAG-SPI1 or FLAG-HOXC13 and the Zfp521 1Kb promoter construct. Following immunoprecipitation with an anti-FLAG antibody, we detected an enrichment of the Zfp521 promoter sequence from extracts individually transfected with either FLAG-SPI1 or FLAG-HOXC13 (Figure 3d). However, immunoprecipitation with a nonspecific antibody (IgG) did not allow amplification of the Zfp521 promoter. Likewise, transfection of the empty FLAG vector control did not produce enrichment of the Zfp521 promoter following immunoprecipitation. As a control, we demonstrated that we could detect the Zfp521 promoter in all input samples. We also detected a GFP vector control sequence in our input samples, but not in any reactions subjected to immunoprecipitation. The enrichment of the Zfp521 promoter region following immunoprecipitation of either SPI1 or HOXC13 indicates that SPI1 and HOXC13 proteins bind the Zfp521 promoter (Figure 3d) in a specific manner.

To provide further support for the hypothesis that SPI1 and HOXC13 bind the Zfp521 promoter, we assayed binding in vitro via EMSAs. We confirmed that the full-length SPI1 and HOXC13 proteins were indeed capable of binding to their predicted recognition sequences in the Zfp521 promoters, as indicated by reduced migration of the promoter DNA (Figure 5a–c). SPI1 and HOXC13 truncation mutants did not alter DNA migration. The addition of specific antibodies (anti-SPI1 or anti-FLAG) abolished the band shift observed in the EMSA, suggesting that the binding of the antibody interfered with the DNA-
binding activity of the SPI1 and FLAG-HOXC13 proteins. The addition of a nonspecific antibody (IgG) did not disrupt the migration of the promoter DNA sequence. Additionally, incubating the nonspecific antibody with the SPI1 and HOXC13 truncation mutant proteins did not produce a shift in DNA migration, demonstrating that the binding seen in reactions with the nonspecific antibody is due to SPI1 and HOXC13 proteins rather than the antibody.

We also wished to examine whether SPI1 and HOXC13 were colocalized on the Zfp521 promoter, in support of our finding that these proteins synergistically activate Zfp521 expression. We therefore sought to determine if the presence of anti-FLAG antibody (detecting FLAG-HOXC13 transfected protein) in a binding reaction including both SPI1 and HOXC13 full-length proteins would disrupt band shifting of either of the SPI1 binding sites of the Zfp521 promoter. A reciprocal reaction was performed using the SPI1 antibody and the putative HOXC13 binding site of the Zfp521 promoter. In all cases we found that the presence of the partner antibody disrupted the band shift (Figure 5d) in a manner similar to that seen for the specific antibodies (Figure 5a–c). From these results we conclude that the SPI1 and HOXC13 proteins are closely associated at their predicted binding site sequences in the Zfp521 promoter, supporting the model for synergistic regulation of Zfp521 expression.

2.6 | Knockdown of SPI1 and HOXC13 reduces ZNF521 expression

To confirm that SPI1 and HOXC13 are required for activation of Zfp521, we performed a knockdown experiment. shRNA plasmids containing sequences targeting SPI1 and HOXC13 were transfected either individually or jointly into THP-1 cells. A plasmid with a scrambled shRNA sequence was used as a control. Expression levels of SPI1, HOXC13, and ZNF521 were measured in each transfection condition by qPCR. We found that ZNF521 expression levels were significantly reduced upon cotransfection of the SPI1 and HOXC13 shRNA constructs (Figure 6a) as compared to the scrambled shRNA control. Transfection of either the SPI1 or HOXC13 shRNA constructs individually reduced ZNF521 expression, but the reduction was not as great as the cotransfection condition. SPI1 expression and HOXC13 expression were each reduced following the transfection of their specific shRNA construct, but not by transfection with the control shRNA plasmid (Figure 6a).

2.7 | SPI1 and HOXC13 expression rescues the effects of Zfp521 knockdown

Using the mouse BCL1 B-lymphoblast cell line, we have demonstrated that knockdown of Zfp521 reduces cell viability, increases apoptosis, and alters expression of Pro-B-cell genes (Al Dallal et al., 2016). To provide further evidence for a role for SPI1 and HOXC13 in Zfp521 regulation, we also assessed whether addition of these factors could restore cell viability following knockdown of Zfp521 in a BCL1 cells. We therefore cotransfected SPI1 and HOXC13 into BCL1 cells 3 days after an initial transfection with the Zfp521 shRNA or experimental control plasmids. On day 7 after the initial transfection, the ratio of viable cell number from cells receiving the rescue plasmids (denoted 7+) compared to control cells receiving no rescue plasmid (denoted 7−) was calculated. We found that cotransfection of SPI1 and HOXC13 into cells with Zfp521 knockdown led to a significant increase in viable cell number, as compared to cells with control transfections (Figure 6b). The percentage of dead cells, as indicated by trypan blue staining, was reduced in Zfp521 knockdown cells subsequently cotransfected with SPI1 and HOXC13 as compared to cells with a mock transfection (Figure 6c). Additionally, levels of activated CASPASE 3/7, indicative of apoptosis, were significantly reduced in Zfp521 knockdown cells cotransfected with SPI1 and HOXC13, as compared to cells with mock transfection (Figure 6d). A rescue transfection performed with an irrelevant empty vector (pCDNA3.1, denoted day 7−C) did not show a reduction in activated caspase 3/7 activity (Figure 6d). Because trypan blue stains both necrotic and apoptotic cells, we attribute the presence of trypan blue positive cells in the SPI1 and HOXC13 cotransfection condition (Figure 6c) to necrosis, since caspase 3/7 staining reveals minimal apoptosis in this sample (Figure 6d).

2.8 | Hoxc13, Spi1, and Zfp521 are coexpressed in immune tissues

During embryonic development B-cells begin to differentiate in the fetal liver (Yokota et al., 2006). If SPI1 and HOXC13 cooperatively regulate Zfp521 expression during B-cell differentiation, we hypothesized that they should both be expressed in the fetal liver. We found that both genes are expressed in mouse fetal liver from two separate animals at E16.5. Expression is also detected in adult mouse bone marrow, a well-documented location for B-cells at all stages of differentiation (Figure 7a,b). To better define Spi1 and Hoxc13 expression during B-cell differentiation, we FACS sorted B-cells from mouse bone
**FIGURE 5** In vitro assessment of SPI1 and HOXC13 binding to the Zfp521 promoter predicted binding sites. DNA sequences added to assay are shown above gels, and protein extracts added are shown below gels for each lane. 

**a:** EMSA reactions demonstrate that full-length SPI1 binds to the predicted SPI1a binding site of the Zfp521 promoter, because the migration of the DNA probe is reduced when protein extract is added prior to electrophoresis (arrow). Addition of an anti-SPI1 antibody abrogates the shift seen in DNA migration, while addition of a nonspecific antibody (IgG) does not affect the shift in DNA migration. The truncation mutant form of SPI1 (aa1-169) is not capable of binding to the Zfp521 promoter sequence. Adding specific or nonspecific antibody with the truncation mutant does not affect migration of the Zfp521 promoter SPI1a DNA sequence. A mutated version of the Zfp521 promoter SPI1a site is not bound by wild type SPI1 protein.

**b:** EMSA reactions demonstrate that full-length SPI1 binds to the predicted SPI1b binding site of the Zfp521 promoter (arrow). Addition of an anti-SPI1 antibody abrogates the shift seen in DNA migration, while addition of a nonspecific antibody (IgG) does not affect the shift in DNA migration. The truncation mutant form of SPI1 (aa1-169) is not capable of binding to the Zfp521 promoter sequence. Adding specific or nonspecific antibody with the truncation mutant does not affect migration of the Zfp521 promoter SPI1b DNA sequence. A mutated version of the Zfp521 promoter SPI1b site is not bound by wild type SPI1 protein.

**c:** EMSA reactions demonstrate that full-length FLAG-HOXC13 binds to the predicted HOXC13 binding site of the Zfp521 promoter (arrow). Addition of an anti-FLAG antibody abrogates the shift seen in DNA migration, while addition of a nonspecific antibody (IgG) does not affect the shift in DNA migration. The truncation mutant form of HOXC13 (HOXC13delta) is not capable of binding to the Zfp521 promoter sequence. Adding specific or nonspecific antibody with the truncation mutant does not affect migration of the Zfp521 promoter HOXC13 DNA sequence. A mutated version of the Zfp521 promoter HOXC13 site is not bound by wild type FLAG-HOXC13 protein. The use of the anti-FLAG antibody (recognising FLAG-HOXC13) eliminates the shift of the Zfp521 promoter predicted SPI1a and SPI1b binding sites (arrow), similar to the results seen for SPI1 protein and anti-SPI1 antibody on its own predicted promoter site (Figure 5a-b). The use of the SPI1 specific antibody eliminates the shift of the Zfp521 promoter predicted HOXC13 binding site, similar to the results seen for HOXC13 protein and anti-FLAG antibody on its own predicted promoter site (Figure 5c).

As shown in Figure 2c, the SPI1 binding site further away from the Zfp521 transcriptional start site was called SPI1a, and the SPI1 site closer to the Zfp521 transcription start site termed SPI1b.
marrow and spleen into different subpopulations. Zfp521 expression in these sub-populations has been confirmed (Hiratsuka et al., 2015). Spi1 expression was not detected in bone-marrow derived pro-B-cells, but was present in bone marrow derived pre-B-cells, spleen derived immature B-cells, and spleen derived mature B-cells (Figure 7c). We found that Hoxc13 was expressed in all subpopulations examined (Figure 7d). These data support the hypothesis that Spi1 and Hoxc13 are developmental regulators of Zfp521 expression.

### 2.9 | SPI1 and HOXC13 cooperatively up-regulate Zfp521 expression in vivo

We cotransfected Spi1 and HOXC13 expression vectors into the mouse B-lymphoblast BCL1 cell line, and tracked endogenous Zfp521 expression levels through qPCR. We found that endogenous Zfp521 expression increased with cotransfection of wild-type SPI1 and HOXC13 (Figure 7e). However, Zfp521 expression levels were not as greatly increased following either SPI1 or HOXC13 single transfections. This result indicates that SPI1 and HOXC13 do synergistically regulate Zfp521 expression in B-cells. An analysis of the same BCL1 cell cDNA samples by RT-PCR confirms the expression of Spi1 and Hoxc13 from transfection plasmids in these samples (Figure 7f). Similar results were obtained for transfections performed in Ba/F3 cells (Supporting Information Figure 1).

To extend our analysis, we also examined fetal liver expression of Zfp521 in embryos of Tg(Hoxc13)61B1Awg mice, a transgenic strain over-expressing Hoxc13 (Tkatchenko et al., 2001). The relative expression levels of both genes were compared within the same mouse
embryo. Transgenic embryos with high levels of Hoxc13 expression showed a significant increase in Zfp521 expression in the liver at E16.5 as compared to nontransgenic littermates (Figure 7g), Notably, not all Tg(Hoxc13)61B1Awg embryos demonstrated increased Hoxc13 expression in the liver, perhaps due to epigenetic alterations of transgene expression, but the expression levels of Hoxc13 and Zfp521 were strongly correlated in all embryos ($R^2 = 0.91$). As the fetal liver is a critical developmental location of B-cell differentiation (Yokota et al., 2006), we conclude that the HOXC13-dependent mechanism of Zfp521 regulation is relevant in vivo.
The large, multi-zinc finger proteins ZFP521 and ZFP423 were initially characterized as proto-oncogenes, both causing B-cell leukemia in mouse models (Hentges et al., 2005; Warming et al., 2003; Warming et al., 2004). Subsequently the critical roles that these factors play in cellular differentiation have been identified, and ongoing investigations will likely reveal additional details about the mechanisms by which they regulate gene expression. Both proteins contain zinc-fingers of the Krüppel type, which are predicted to mediate protein-protein interactions as well as possess DNA-binding ability. Whilst protein binding partners for ZFP521 have been identified, such as EBF (Mega et al., 2011), the DNA sequence to which ZFP521 binds is as yet unknown. Likewise, the DNA sequence bound by ZFP423 has not been identified. Although both proteins have been shown to regulate cellular differentiation processes, the overlap between their functions at a molecular level, such as whether they will both bind the same DNA sequence, is unclear. Our phylogenetic data combined with prior reports of different expression patterns for these two genes suggests that their expression patterns have diversified following a gene duplication event, which may explain why both genes were retained in vertebrate genomes.

As Zfp521 expression is detected early in the B-cell differentiation process (Hiratsuka et al., 2015; Warming et al., 2003), relevant transcription factors that initiate Zfp521 expression should be expressed at early stages of B-cell differentiation. In fact, SPI1 expression is found in committed lymphoid progenitor cells (DeKoter et al., 2002), making SPI1 a good candidate for the regulation of Zfp521 during B-cell differentiation. Spi1, Hoxc13, and Zfp521 are all expressed in the fetal liver, where B-cell differentiation originates during development (Yokota et al., 2006). Importantly, we find that if we cotransfect SPI1 and HOXC13 into mouse BCL1 cells, a B-lymphoblast cell line, the endogenous expression levels of Zfp521 in the cells are up-regulated (Figure 4e). Hoxc13 over-expressing mice display up-regulation of Zfp521 in the fetal liver, providing further evidence that SPI1 and HOXC13 coregulation of Zfp521 expression is relevant during developmental specification of B-lymphocytes.

Zfp521 up-regulation resulting from retroviral insertions at the 5’ end of the Zfp521 locus causes B-cell transformation in mice (Hentges et al., 2005; Warming et al., 2003). The tumors present in these mice express a variety of cellular markers known to be downstream of the B-cell transcription factor EBF, which are indicative of activated B-cell receptor (BCR) signalling (Hentges et al., 2005). Additionally, retroviral insertion up-regulation of Zfp521 has been identified as an important cooperative event which increased the incidence of B-ALL in mice expressing an EzA-HLF transgene as a model for human t(17;19) acute lymphoblastic leukemia (Yamasaki et al., 2010). The finding that SPI1 and HOXC13 coregulate Zfp521 expression highlights the possibility that over-expression of either of these factors could lead to B-cell leukemia via activation of Zfp521, resulting in enhanced BCR signalling in a manner similar to that displayed in AKXD27 mice (Hentges et al., 2005). During the process of bone differentiation Cyclin D1 has been identified as a ZFP521 target gene, promoting proliferation of growth plate chondrocytes (Correa et al., 2010). Tumors from mice with retroviral insertions within the Zfp521 gene have increased Cyclin D1 expression (Al Dallal et al., 2016), suggesting that over-expression of Zfp521 leads to an excessive proliferation of immature B-lymphocytes via Cyclin D1 up-regulation, culminating in B-cell leukemia. In humans ZNF521 over-expression is found in acute myeloid leukemia but not in B-cell leukemia (Mesuraca et al., 2015), although B-cell leukemia initiating cells do show increased expression of ZNF521 (Aoki et al., 2015). It has thus been proposed that up-regulation of ZNF521 in rare leukemia initiating cells may facilitate tumor progression, even though ZNF521 over-expression is not detected in the majority of leukemic B-cells from patients (Mesuraca et al., 2015).

The role of SPI1 in leukemia is complex. Over-expression of SPI1 is known to cause erythroid leukemia by inhibiting apoptosis and blocking the terminal differentiation of erythrocytes (Yamada et al., 2008). STAT3 activation of SPI1 is a key step in the disease progression of Friend erythroid leukemia (Hegde et al., 2009), reinforcing the finding that SPI1 over-expression alters erythroid cell differentiation. However, acute myeloid leukemia can result from loss-of-function mutations in SPI1 or reduced Spi1 expression (Sive et al., 2016; Will et al., 2015), while induced expression of SPI1 in myeloid leukemia cells restores their differentiation and reduces their proliferation rates (Tkatchenko et al., 2001). Likewise, expression of SPI1 is severely reduced in human patients with chronic myeloid leukemia due to aberrant promoter methylation in tumor cells (Yang et al., 2012). Deletion of SPI1 and the related ETS-transcription factor SPI-B in the B-cell lineage results in fully-penetrant pre-B-cell acute lymphoblastic leukemia (B-ALL), suggesting that SPI1 and SPI-B are tumor suppressors in the B-cell lineage (Sokalski et al., 2011). Likewise, mice lacking both SPI1 and IRF8 develop B-ALL (Pang et al., 2016). These results suggest that over-expression of SPI1 may play a part in erythroid leukemia, while reduced SPI1 expression contributes to myeloid and B-cell leukemia, although the many diverse functions of SPI1 at multiple time points during hematopoiesis complicate the analysis of SPI1 in specific lineages.

HOXC13 is a key downstream target of the polycomb group family gene B-cell specific Moloney murine leukemia virus integration site 1 (BMI-1). BMI-1 dysregulation leads to lymphoma and nonsmall cell lung carcinoma in humans (Jacobs et al., 1999; Vonlanthen et al., 2001). Additionally, in human cervical adenocarcinoma cells BMI-1 knockdown promotes the up-regulation of HOXC13 expression, contributing to a block in cell proliferation (Chen et al., 2011). Therefore, BMI-1 induced HOXC13 repression may cause abnormal cell proliferation contributing to adenocarcinoma. HOXC13 has additional links to leukemia, because fusions of NUP98 and HOXC13 cause human myeloid leukemia (Cheng & Reed, 2007). Also, an antagonistic role for SPI1 and HOXC13 in erythroid cell differentiation has been proposed based on data from erythro leukemia cell lines (Yamada et al., 2008). Cross-talk between SPI1 and the MEIS/HOX gene regulation pathway has been noted in mixed lineage leukemia (Zhou et al., 2014). Yet to our knowledge there are no prior reports of HOXC13 involvement in lymphoid leukemia. However, based on the results of this study, the potential role of
HOXC13 in lymphoid leukemia and B-cell gene expression via regulation of Zfp521 should be explored. Future experiments are required to determine the contribution of perturbations in Hoxc13 and Spi1 expression to dysregulation of Zfp521, and the potential for alternations in the expression of these transcription factors to promote lymphoid leukemia.

4 | MATERIALS AND METHODS

4.1 | Phylogenetic analysis

Protein sequences for orthologues of both ZFP521 and ZFP423 from multiple species were obtained from the online database of National Centre for Biotechnology Information (Supporting Information Table 1). Multiple sequence alignment was performed using MULTIPLE Sequence Comparison by Log-Expectation (MUSCLE) (Edgar, 2004). Phylogenetic trees were generated by MUSCLE, and branch length presented as either cladogram (Figure 1a) or real (Figure 1b).

4.2 | Bioinformatic promoter analysis

The 1Kb regions upstream of the annotated mouse and human Zfp521/ZNF521 and Zfp423/ZNF423 genes were obtained from the UCSC genome browser using the mm9 assembly for mouse and the hg38 assembly for human. Annotated tracks for DNAse I hypersensitivity (Ho & Crabtree, 2010) and the percentage of GC bases in the promoter regions were obtained from the UCSC genome browser on the hg38 and mm9 assemblies. DNase I hypersensitivity data was generated by the University of Washington ENCODE group (Sabo et al., 2006). The 1Kb mouse and human promoter regions were searched for transcription factor binding sites using TESS (Schug, 2008). Conserved sites were identified through manual inspection of annotated promoters.

4.3 | Cell culture

The BA/F3 cell line was maintained in RMPI with 2% mouse interleukin-3, 10% FBS in 5% CO2 at 37°C. BCL1 mouse lymphoblast cells (ATCC® TIB-197) were cultured in RPMI 1640 medium supplemented with 2mM L-glutamine (Lonza), 0.05mM 2-mercaptoethanol (Sigma-Aldrich), 15% Foetal Bovine Serum (FBS), 5% penicillin, 5% streptomycin at 37°C with 5% CO2. THP-1 cells were cultured in RMPI with 0.05 mM β-Mercaptoethanol and 10% FBS. The HEK293 cell line was maintained in DMEM with 10% FBS in 5% CO2 at 37°C.

4.4 | Constructs

IMAGE clone 3600260 was used as the wild-type Spi1 construct and as the template for site-directed mutagenesis. IMAGE clone 6171228 was used as the wild-type HOXC13 expression vector. Primer sequences for Zfp521 promoter amplification listed in Supporting Information Table 2. Promoter regions were cloned up stream of the luciferase gene in the pGL3 Basic plasmid. Nomenclature of the reporter plasmids is based on the approximate size of promoter regions. The human HOXC13 cDNA sequence was cloned in-frame into pFLAG-CMV2 (Sigma) to create the FLAG-tagged expression construct. The HOXC13 DNA-binding mutant construct (HOXC13-delta) was previously described (Potter et al., 2006).

4.5 | Site-directed mutagenesis

Site-directed mutagenesis was performed using Pfu Turbo DNA polymerase (Stratagene). Cycling conditions: 95.0°C for 30 seconds; followed by 12 cycles of: 95.0°C for 30 seconds, 55.0°C for 1.00 minute, 72.0°C for 6.00 min, and final hold at 37.0°C. PCR products were subjected to DpnI (NEB) digestion at 37.0°C for 4 h. Digested DNA was transformed and sequenced to confirm the presence of the point mutation creating a stop codon at SPI1 amino acid 170. Primer sequences are listed in Supporting Information Table 2.

4.6 | Transfection assays

0.5µg plasmid DNA was transfected into 5 × 10⁵ HEK 293 cells with Fugene 6 (Roche), and cells were cultured for 48 h. 2 µg plasmid was transfected into 2 × 10⁶ BA/F3 cells with Amaxa nucleofector reagent (Lonza). Cells were grown for 48 h to collect total RNA or protein for analysis. 1 µg plasmid DNA was transfected into 1 × 10⁶ BCL1 cells with FuGENE HD (Roche) in OptiMEM Media (Sigma). Cells were grown for 48 h for qPCR assays, and 3 or 7 days for knockdown rescue assays.

4.7 | Western blot

Cell lysate was prepared in RIPA buffer. 50 µg total protein was subjected to 12% SDS-PAGE and transferred to PVDF membrane. The membrane was incubated with mouse anti-FLAG antibody (anti-PU.1 D19, Santa Cruz). Protein was detected by ECL kit (GE Health care).

4.8 | Luciferase assay

0.1µg pSPORT6-Spi1 and 0.1 µg pSPORT6-HOXC13 were cotransfected into 1.25 × 10⁶ HEK293 cells with 0.05 µg pGL3-basic vector containing 0.2Kb, 0.5Kb or 1Kb Zfp521 promoter regions and 0.001µg pRLCMV using Amaxa nucleofector reagent (Lonza). Luciferase activity was determined by dual-luciferase Reporter Assay System (Promega). Ratios of firefly luciferase activity to renilla luciferase activity were calculated for each sample. Reactions were performed in triplicate, and all results represent combined analysis of three separate experiments. Expression levels are shown relative to transfection of luciferase reporter without cotransfection of protein expression constructs. Statistical significance was determined by t-test comparison to reporter control.

4.9 | Protein coimmunoprecipitation

HEK 293 cells cotransfected with 2 µg of Spi1 (wild type or mutant constructs) and Flag-HOXC13 were harvested 48 h following transfection and lysed in PBS with 1% Triton X100 and 0.01% Igepal CA-630.
The lysate was incubated with 2 μg anti-Flag (Sigma) or anti-SPI1 antibody (anti-PU.1 D19, Santa Cruz) and 20 μl protein G Dynabeads (Invitrogen) overnight, then washed with PBS with 1% Triton X-100 and 0.01% Igepal CA-630 and subjected to Western blot.

4.10 Chromatin immunoprecipitation

HEK 293 cells were transfected with pFlagCMV-Spi1 or pFlagCMV-HOXC13. Cells were also transfected with the pGL3-1kb Zfp521 promoter or pGL3-GFP plasmid control vector. The day before cells were harvested, 5 μl protein G Dynabeads were pre-incubated with 0.1mg/ml salmon sperm DNA and 1mg/ml BSA. 24 hours after transfection, the cells were fixed by 4% formaldehyde for 5 minutes, and the reaction was stopped by the addition of 0.125M glycine. Cells were washed twice with PBS and lysed with lysis buffer (0.1M NaCl, 1mM EDTA, 1% Triton-X 100, 50 mM Tris-HCl pH7.5). After the lysate was pre-cleared with pre-blocked Dynabeads, 1 μg anti-Flag antibody was added to the lysate, and incubated at 4°C overnight. The lysate was incubated with 5 μl Dynabeads for 1 hour, and then washed with low salt buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.1, 150 mM NaCl), high salt buffer (1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH8.1, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% (Igepal CA-630, 1 mM EDTA, 10 mM Tris-HCl pH8.1) and TE buffer. After washing, beads were incubated in elution buffer (1%SDS, 0.1M NaHCO3) twice, each 10 minutes. Elution buffer was collected and adjusted to a final concentration of 0.2 M NaCl, and then incubated at 65°C overnight. The eluate was RNase digested for 1 hour, incubated in proteinase K for 2 hours and purified by Qiaquick PCR purification kit (Qiagen). DNA was amplified with Faststart Taq DNA polymerase (Roche) with GC RICH solution with Zfp521 promoter primers or GFP plasmid primers listed in Supporting Information Table 2. PCR conditions were 95°C 4 minutes, and then followed by 95°C 30 s, 60°C 30 s and 72°C 1 min for 35 cycles, and 72°C 10 minutes.

4.11 Electrophoretic mobility shift assay (EMSA)

Following transfection with pCMVSPORT6-Spi1 and pCMVZFP-HOXC13, BCL1 adherent cells were trypsinized into 2 ml Trypsin (Gibco), pelleted by centrifugation, and the cell pellet re-suspended into cold high salt buffer (RIPA). Cells were lysed on ice for 15 minutes and then centrifuged for 30 minutes at 4°C. The supernatant was collected for use in binding reactions. Prior to use in EMSA, DNA oligonucleotides were denatured at 95°C for 5 minutes, and left to cool to room temperature. DNA probe sequences are listed in Supporting Information Table 2. Extracts were incubated with double stranded DNA fragments containing predicted SPI1 and HOXC13 binding sites of the Zfp521 promoter and EMSA binding buffer (10mM Tris-HCl pH 7.4, 150mM KCL, 0.1mM EDTA and 0.1mM DTT) at room temperature for 20 minutes, according to manufacturer's instructions (Life Technologies SYBR Green EMSA kit cat#E-33075). For reactions containing antibodies, anti-SPI1 (anti-PU.1 D-19, Santa Cruz), anti-FLAG (M2, Sigma), or anti-IgG (Santa Cruz) antibody was added to the reaction mix prior to room temperature incubation.

The products were resolved by electrophoresis on 6% nondenaturating polyacrylamide gel at 150 V in 1.0 X TBE buffer for 45 minutes. DNA migration was determined by staining the gel with SYBR Green (1:10,000; Molecular Probes, Eugene, OR) for 20 min at room temperature, and rinsing twice with deionized H2O. The gel was visualized with a Dark Reader Transilluminator.

4.12 SPI1 and HOXC13 knockdown assay

0.25ug shRNA targeting SPI1 (Origene TL316738) and/or shRNA targeting HOXC13 (Origene TL304059) were transfected into 2X10⁶ THP-1 cell with Amaxa nucleofector (Lonza VCA-1003); a scrambled noneffective shRNA sequence was used as a negative control. After 3 days, RNA was isolated from transfected cells and cDNA was prepared. The expression level of ZNF521, SPI1 and HOXC13 was determined by qPCR as described below for mouse cell line and tissue samples. The qPCR primers are listed in Supporting Information Table 2.

4.13 Zfp521 shRNA knockdown rescue assays

Knockdown of Zfp521 was performed by transfection of a cocktail of 4 vectors with shRNAs targeting Zfp521 into BCL1 cells as previously described (Al Dallal et al., 2016). The rescue assay with SPI1 and HOXC13 was performed by cotransfecting 0.5 μg each of Spi1 and HOXC13 expression constructs into knockdown or control cells 3 days after Zfp521 shRNA transfection. As a control, a rescue transfection was performed with pcDNA3.1 empty vector on day 3 post-shRNA transfection. Cell viability, trypan blue staining, and Caspase 3/7 activity analysis were performed as previously described (Al Dallal et al., 2016).

4.14 Flow cytometry

Bone marrow was extracted from the femurs of two 129S5 mice by flushing the bones with DMEM, pooling the cells, passing the cells through a 21 gauge needle and filtering the cells using a 0.7μm filter (BD). Splenocytes were isolated by pressing the spleen from 2 mice against a mesh, then suspending the cells in DMEM. Erythrocytes were lyzed by incubating the cells with red blood cell lysis buffer (Roche) for 5 minutes at room temperature. Cells were washed with FACS buffer (PBS, 1% FCS, 0.05% sodium azide), pelleted and resuspended with 1ug of FC receptor block and incubated for 20 minutes at 4°C. The cells were washed in FACS buffer, and resuspended in 0.313ug/ml anti-B220 (RA3-6B2) APC (ebioscience), 2.5ug/ml anti-IgM (ll/41) PE (ebioscience), 0.625ug/ml anti-IgD (11-26c) FITC (ebioscience), 2.5 μg/ml anti-C-kit (2B8) PE Cy5 (ebioscience) and 1 μl/ml Fixable viability dye eFluor 450 (ebioscience) and incubated for 40 min at 4°C in the dark. OneComp eBeads (ebioscience) were used for single stained controls. Cells were washed in FACS buffer, resuspended in DMEM with 25 mM HEPES at a concentration of 1 x 10⁶ cells/ml and filtered through a 50-μm filter (BD Biosciences). The cells were sorted into immature B cells (B220⁺ IgM⁺ IgD⁻), mature B cells (B220⁺ IgM⁺ IgD⁺), pro B cells (B220⁻ IgM⁺ IgD⁻ C-kit⁻) and pre B cells (B220⁻
IgM IgD C-kit) using a FACS Aria (BD Bioscience) and collected into a 5 ml polypropylene tube containing DMEM with 10% FBS.

4.15 Hoxc13 and Spi1 RT-PCR
RNA was prepared from mouse E16.5 fetal liver, adult bone marrow, E16.5 hind-limb, and adult spleen tissue samples, and FACS isolated B-cell fractions, with TRI-Reagent (Sigma), treated with DNase, and reverse-transcribed with Bioline RT. PCR primers are listed in Supporting Information Table 2. Cycling conditions were: 95°C 4 min, and then followed by 95°C 45 s, 58°C (Hoxc13) or 60°C (Spi1) 45 s and 72°C 1 min for 35 cycles, and 72°C 10 min.

4.16 Zfp521, Spi1, and Hoxc13 quantitative RT-PCR
BCL1 or BA/F3 cells were transfected with pFlagCMV-HOXC13 and pCMVSport6-Spi1 (Amaza nucleofector), or empty expression vector BCL1 or BA/F3 cells were transfected with pFlagCMV-HOXC13 and 4.16 | Zfp521 and Spi1 (Amaxa nucleofector), or empty expression vector 3. BCL1 or BA/F3 cells were transfected with pFlagCMV-HOXC13 and 4.16 | Zfp521 and Spi1 (Amaxa nucleofector), or empty expression vector BCL1 or BA/F3 cells were transfected with pFlagCMV-HOXC13 and 4.16 | Zfp521 and Spi1 (Amaxa nucleofector), or empty expression vector BCL1 or BA/F3 cells were transfected with pFlagCMV-HOXC13 and 4.16 | Zfp521 and Spi1 (Amaxa nucleofector), or empty expression vector

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