The PAX5-JAK2 translocation acts as dual-hit mutation that promotes aggressive B-cell leukemia via nuclear STAT5 activation

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Abstract

While PAX5 is an important tumor suppressor gene in B-cell acute lymphoblastic leukemia (B-ALL), it is also involved in oncogenic translocations coding for diverse PAX5 fusion proteins. PAX5-JAK2 encodes a protein consisting of the PAX5 DNA-binding region fused to the constitutively active JAK2 kinase domain. Here, we studied the oncogenic function of the PAX5-JAK2 fusion protein in a mouse model expressing it from the endogenous PAX5 locus, resulting in inactivation of one of the two PAX5 alleles. Pax5<sup>5J2</sup>-mice rapidly developed an aggressive B-ALL in the absence of another cooperating exogenous gene mutation. The DNA-binding function and kinase activity of PAX5-Jak2 as well as IL-7 signaling contributed to leukemia development. Interestingly, all Pax5<sup>5J2</sup>-mice lost the remaining wild-type PAX5 allele, allowing efficient DNA-binding of Pax5-Jak2. While we could not find evidence for a nuclear role of Pax5-Jak2 as an epigenetic regulator, high levels of active phosphorylated STAT5 and increased expression of STAT5 target genes were seen in Pax5<sup>5J2</sup>- B-ALL tumors, implying that nuclear Pax5-Jak2 phosphorylates STAT5. Together, these data reveal Pax5-Jak2 as an important nuclear driver of leukemogenesis by maintaining phosphorylated STAT5 levels in the nucleus.

Keywords: B-cell acute lymphoblastic leukemia; dual-hit mutation; mouse Pax5-Jak2 knock-in model; nuclear function of Pax5-Jak2; PAX5-JAK2 rearrangement

Introduction

Acute lymphoblastic leukemia is the most common pediatric cancer and, in the majority of cases, originates within the B cell lineage (Hunger & Mullighan, 2015). Genome-wide studies demonstrated that 60% of all B-cell precursor acute lymphoblastic leukemia (B-ALL) cases carry genetic alterations in genes coding for regulators of B cell development, with the most commonly affected transcription factor genes being PAX5, EBF1, and IKZF1 (Ikaros) (Kuiper et al, 2007; Mullighan et al, 2007, 2008). Mutations leading to aberrant activation of tyrosine kinase and cytokine receptor signaling were also identified in about 15% of all B-ALL cases, which are collectively referred to as Philadelphia chromosome-like (Ph-like) B-ALLs and have a poor prognosis (Roberts et al, 2012, 2014; Ofran & Izraeli, 2017). One Ph-like B-ALL subtype is characterized by the PAX5-JAK2 rearrangement (Nebral et al, 2009; Roberts et al, 2012), which we have studied here with regard to its molecular and oncogenic function in a mouse model.

The transcription factor Pax5 is an essential regulator of B cell commitment and development (Nutt et al, 1999; Horcher et al, 2001; Medvedovic et al, 2001). It acts as a transcriptional repressor to suppress B-lineage-inappropriate genes (Delogu et al, 2006) and functions as an activator to induce gene expression required for B cell development and function (Schepstel et al, 2007; Revilla-Domingo et al, 2012). Notably, mature B cells upon conditional loss of Pax5 cannot only be converted into functional T cells via dedifferentiation to uncommitted bone marrow progenitor cells, but also give rise to an aggressive progenitor cell leukemia (Cobaleda et al, 2007). Hence, Pax5 maintains B cell identity throughout B-lymphopoiesis and functions as a tumor suppressor in the B-lymphoid lineage.

PAX5 was identified as a haploinsufficient tumor suppressor gene in human B-ALL, as heterozygous PAX5 deletions, rearrangements, and loss-of-function mutations are present in one third of all cases (Kuiper et al, 2007; Mullighan et al, 2007). PAX5 translocations occur at a frequency of 2.5% in human B-ALLs and are currently known to involve 28 different partner genes, generating novel PAX5 fusion proteins (Gu et al, 2019). The different partner genes code for proteins of diverse functions such as transcription
factors (exemplified by ETV6), signal transducers (JAK2), chromatin regulators (BRD1), structural proteins (ELN), and proteins of unknown function (NOL4L) (Cazzaniga et al., 2001; Nebral et al., 2009; Coyaud et al., 2010). The PAX5 fusion proteins contain the N-terminal DNA-binding paired domain, but lack the potent C-terminal transactivation domain of PAX5 (Nebral et al., 2009; Coyaud et al., 2010) and were therefore thought to act as dominant-negative proteins to prevent genomic DNA binding of the full-length PAX5 protein expressed from the second allele (Kawamata et al., 2012; Fortschegeger et al., 2014). However, we could demonstrate in a mouse model that the PAX5-ETV6 fusion protein does not interfere with the expression of regulated PAX5 target genes and hence does not act as a dominant-negative protein (Smeenk et al., 2017). Instead, PAX5-ETV6 functions as a potent oncprotein to promote B-ALL development in combination with loss of the tumor suppressors Cdkn2a and Cdkn2b (Smeenk et al., 2017). Similarly, heterozygous loss of PAX5 is not sufficient for tumor formation, as it additionally requires constitutive activation (ca) of STAT5 for leukemia development in transgenic caStat5b Pax5+/− mice (Heltemes-Harris et al., 2011). Hence, heterozygous PAX5 alterations promote B-ALL development in cooperation with a second oncogenic “hit.”

The Janus kinase 2 (JAK2) belongs to a family of nonreceptor tyrosine kinases that mediate signal transduction downstream of many cytokine and growth hormone receptors, regulating hematopoiesis, immunity, growth, and development (Chen et al., 2012; Villarino et al., 2017). Upon signaling, JAK2 phosphorylates STAT transcription factors in the cytoplasm, which promotes their dimerization and translocation to the nucleus, where they control the expression of genes involved in cell survival, differentiation, and metabolism (Malin et al., 2010; Villarino et al., 2017; de Araujo et al., 2019). In addition to this canonical JAK-STAT signaling function, JAK2 was also shown to be present in the nucleus and to directly phosphorylate histone H3 on tyrosine 41 (H3Y41ph), which, in turn, prevents interaction of the heterochromatin protein 1a (HP1α) with H3, thus leading to the activation of oncogenes such as Lmo2 and Myc (Dawson et al., 2009; Rui et al., 2010). Hence, these studies uncovered a second role of JAK2 as an “epigenetic writer” that stimulates expression of leukemogenic genes. JAK2 has directly been implicated in the development of Ph-like B-ALL by activating mutations, which are predominantly located in its autoinhibitory pseudokinase domain (JH2) (Chen et al., 2012). JAK2 also participates in translocations with at least 22 different partner genes, which all contain the catalytically active JAK2 kinase domain (JH1) as a common denominator (Nebral et al., 2009; Chen et al., 2012; Roberts et al., 2012; Akkari et al., 2020).

The PAX5-JAK2 rearrangement codes for a fusion protein consisting of the DNA-binding paired domain of PAX5 fused to only the kinase domain (JH1) of JAK2 (Nebral et al., 2009) (Appendix Fig S1A). As shown by detailed characterization in transfected cell lines, PAX5-JAK2 is a monomeric nuclear protein that can bind PAX5 recognition sequences and possesses constitutive kinase activity. Consequently, PAX5-JAK2 activates STAT5 by phosphorylation, which likely induces a STAT5-dependent gene program (Schinnerl et al., 2015). Moreover, JAK2 inhibitors efficiently block the constitutive kinase activity of PAX5-JAK2 in transfected cells, suggesting that these inhibitors may be beneficial for the treatment of PAX5-JAK2 B-ALL (Roberts et al., 2014; Schinnerl et al., 2015). As the PAX5-JAK2 rearrangement inactivates one functional PAX5 allele, resulting in haploinsufficiency, and simultaneously leads to STAT5 activation, it may function as a dual-hit mutation to promote aggressive B-ALL. However, the function of PAX5-JAK2 in B cell development and leukemogenesis has not yet been investigated in an in vivo model system.

Here, we have generated a mouse model that expresses the PAX5-JAK2 protein under the control of the Pax5 locus. Pax5+/jak2/− mice exhibited normal B cell development up to 3 weeks of age, but thereafter rapidly developed an aggressive B-ALL tumor in the absence of another cooperating exogenous gene mutation. The DNA-binding function and kinase activity of Pax5-Jak2 both contributed to leukemia development, as evidenced by mutation of the paired domain of Pax5 or the catalytic center of Jak2. Unexpectedly, the wild-type Pax5 allele was lost in all Pax5+/jak2/− B-ALLs by acquired uniparental dysomy, which facilitated efficient binding of Pax5-Jak2 to its genomic recognition sequences, thus pointing to an important oncogenic function of the fusion protein in the nucleus. While we could not find evidence for an epigenetic role of Pax5-Jak2 in the nucleus, STAT5 was highly phosphorylated in the earliest pre-leukemic B220low B cells of 4-week-old mice. Consistent with this finding, activated STAT5 target genes were upregulated in Pax5+/jak2/− B-ALLs. Together, these data indicate that the constitutively active Pax5-Jak2 kinase maintains active STAT5 at high levels in the nucleus, thus leading to continuous expression of STAT5 target genes in Pax5+/jak2/− B-ALL cells.

Results

Pax5-jak2 expression from the Pax5 locus leads to development of an aggressive B-ALL

To study the role of PAX5-JAK2 (Appendix Fig S1A) in B-ALL development, we used ES cell targeting to generate a mouse model by inserting human cDNA sequences, starting with exon 4 and encoding the remaining PAX5-JAK2 protein, into the mouse Pax5 locus to recaputlate the corresponding human rearrangement as closely as possible (Fig 1A and Appendix Fig S1B and C). Additionally, we inserted an IRES-luciferase indicator gene downstream of the Jak2 sequence and turned the endogenous Pax5 exon 4 into a loxP-stop-loxP (LSL) cassette by the insertion of a stop codon together with six polyadenylation sequences to generate the Pax5LSL-Jak2-Luc allele, which expresses the DNA-binding paired domain of Pax5 instead of the full-length Pax5-Jak2 protein (Appendix Fig S1B). The Pax5LSL-Jak2 allele was subsequently created by Dre recombinase-mediated deletion of the IRES-luciferase gene (Appendix Fig S1B). To enable the expression of the Pax5-Jak2 fusion, we eliminated the LSL cassette by ubiquitous Cre expression from the Meox2 locus (Talquist & Soriano, 2000) in Meox2-Cre Pax5LSL-Jak2+/− or Meox2-Cre Pax5LSL-Jak2-Luc+/− mice, which will be thereafter referred to as Pax5jak2/− or Pax5jak2-Luc−/− mice, respectively. The LSL cassette was efficiently deleted in cells of Pax5jak2/− and Pax5jak2-Luc−/− mice, as shown by PCR analysis (Appendix Fig S1D). Immunoblot analysis of nuclear extracts with an anti-Pax5 antibody recognizing the N-terminal paired domain demonstrated that the Pax5-Jak2 wild-type Pax5 proteins were similarly expressed in pro-B cells of 3-week-old Pax5jak2/− mice (Fig 1B and Appendix Fig S1E and F). These results therefore identified the Pax5jak2/− mouse as a valid
Figure 1.

A. Pax5\(^{+}\) and Pax5\(^{Jak2-}\) genetic constructs with ATG, paired, and stop sites indicated. Pax5\(^{+}\) constructs include Pax5\(^{Jak2-}\) with Jak2 and TEV-Biotin for epitope tagging. B. Anti-Pax5 (N-term) Western blot showing Pax5\(^{Jak2-}\) and Pax5\(^{+}\) bands at 70 kDa and 55 kDa, respectively. C. Flow cytometry analysis of Pax5\(^{+}\) and Pax5\(^{Jak2-}\) mice showing CD19 expression in B220+CD19+IgM+IgD+ cells. D. Bar graph showing cell counts for Total B, Pro-B, Pre-B, Imm B, Small pre-B, and Large pre-B in Pax5\(^{+}\) and Pax5\(^{Jak2-}\) mice. E. Flow cytometry images of B220+CD19+IgM+IgD+ cells in bone marrow and spleen of Pax5\(^{Jak2-}\) mice at 4 weeks, 4 weeks, 4 weeks, and 6 weeks. F. Survival curve for Pax5\(^{+}\) (n = 15) and Pax5\(^{Jak2-}\) (n = 48) mice over 300 days. G. Histological sections of spleen showing Pax5\(^{Jak2-}\) tumor growth. H. Histological sections of lung and liver showing Pax5\(^{Jak2-}\) tumor growth. I. Flow cytometry images of Pax5\(^{+}\) and Pax5\(^{Jak2-}\) lymph node at 10 weeks. J. Flow cytometry images of Pax5\(^{Jak2-}\) bone marrow at 4 weeks.
A Schematic diagram of the Pax5-Jak2 allele. Human cDNA sequences, starting in exon 4 and encoding the remaining Pax5-Jak2 protein, were inserted in frame into exon 4 of the mouse Pax5 locus to generate the Pax5-Jak2 allele (Appendix Fig S1B). The C-terminal tag sequence (grey) contains an epitope for the VS antibody, two cleavage sites for the TEV protease, and a biotin acceptor sequence (Biot). A black oval denotes the B cell-specific enhancer (En) in intron 5 (Decker et al, 2009). pA, polyadenylation site. Notably, the human and mouse Pax5 protein sequences encoded from exon 1 to exon 5 contain only one amino acid substitution (human Ser13 vs. mouse Ile13 in exon 1) located upstream of the paired domain (Adams et al, 1992).

B Expression of the Pax5 protein in Pax5Jak2+/− pro-B cells. Nuclear extracts of short-term cultured pro-B cells from 3-week-old Pax5Jak2+/− and Pax5+/− mice were analyzed by immunoblot analysis with an anti-Pax5 antibody recognizing the N-terminal paired domain. The size of marker proteins is indicated in kilodaltons (kDa) to the left.

C Flow-cytometric analysis of bone marrow cells from the two hindlegs of 3-week-old Pax5+/− and Pax5Jak2+/− mice. The percentage of the cells in each gate or quadrant is indicated.

D Absolute cell numbers of the indicated cell types were determined by flow-cytometric analysis of the bone marrow from 3-week-old Pax5+/− and Pax5Jak2+/− mice. Average cell numbers are shown with SEM and were statistically analyzed by multiple t-tests (unpaired two-tailed with Holm–Šidák’s correction); n.s. not significant (P > 0.05). See Methods section for flow-cytometric definition of the different cell types. One of three independent experiments is shown.

E Flow-cytometric analysis of bone marrow cells and splenocytes from Pax5Jak2+/− mice at the age of 4 or 6 weeks. The newly emerging B220low B cells are highlighted by black boxes.

F Kaplan–Meier survival analysis of Pax5Jak2+/− (black) and control Pax5+/− (grey) mice. n, number of mice analyzed. A P value of < 0.0001 was determined for the survival curves by statistical analysis with the log-rank (Mantel–Cox) test.

G Size comparison of the spleen and lymph nodes from a control Pax5+/− mouse and a Pax5Jak2+/− tumor mouse.

H Eosin-hematoxylin-stained sections of the lung and liver of a moribund mouse Ile13 (Fig 1E). Infiltrating and blasting tumor cells are indicated by an arrow.

I Flow-cytometric analysis of lymph node cells from a control Pax5+/− mouse and a 10-week-old Pax5Jak2+/− tumor mouse.

J Flow-cytometric analysis of B220lowCD19+ B cells from the bone marrow of a 4-week-old Pax5Jak2+/− mouse.

Source data are available online for this figure.

model for studying the developmental and leukemogenic role of Pax5-Jak2 in B cells.

Flow-cytometric analysis of B cell development in 3-week-old mice revealed that total B, pro-B, large and small pre-B, as well as immature B cells were present at similar numbers in the bone marrow of Pax5Jak2+/− and control Pax5+/− mice (Fig 1C and D). These data therefore indicate that heterozygous expression of Pax5-Jak2 had no apparent effect on B cell development in young mice.

The first sign of aberrant B cell development appeared in the bone marrow of Pax5Jak2+/− mice at around 4 weeks of age with the emergence of B220low B cells in some of these mice (Fig 1E). At 6 weeks of age, most B lymphocytes in the bone marrow were B220low and proved to be tumorigenic, as their transplantation into wild-type C57BL/6 mice resulted in tumor development within 72 days (Appendix Fig S1G). With some delay, these tumorigenic B220low B cells also appeared in the spleen (Fig 1E), in agreement with their expansion in the bone marrow. None of these tumorigenic B220low B cells led to the rapid death of Pax5Jak2+/− mice with a median survival of 74 days, as shown by Kaplan–Meier survival analysis (Fig 1F). Moribund Pax5Jak2+/− mice exhibited enlarged lymph nodes and splenomegaly (Fig 1G) as well as infiltration of leukemic cells in other organs such as the lung and liver (Fig 1H). Flow-cytometric analyses revealed that the leukemic B cells from lymph nodes were blasting, as shown by their large size, and expressed surface markers characteristic of early B lymphopoiesis, such as CD93, IL-7Ra (CD127), and Flt3 (CD135) (Fig 1I). The cell surface phenotype of the Pax5Jak2+/− tumors was B220low/CD93+IL-7Rαlow/Flt3−/CD135−/IgM−/CD2−/CD21+/CD23−, while the expression of CD19 was variable, with some tumors being positive, negative, or mixed (CD19− to CD19+) (Fig 1I and Appendix Fig S1H). Moreover, the Pax5Jak2+/− tumor cells from lymph node gave rise to overt leukemia within 30 days after transplantation in wild-type C57BL/6 mice, thus highlighting their aggressive nature (Appendix Fig S1G). Notably, the B220low B cells in the bone marrow of 4-week-old Pax5Jak2+/− mice already expressed CD93, IL-7Ra, and Flt3 (Fig 1J) and thus had a similar cell surface phenotype as the B-ALL tumors in the lymph nodes of Pax5Jak2+/− mice (Fig 1I). In summary, these data demonstrate that Pax5-Jak2 expression from the Pax5 locus initially did not interfere with normal B cell development in young mice, but then rapidly led to the development of an aggressive B-ALL tumor.

The Pax5Jak2 allele does not provide any canonical Pax5 function

To gain insight into the role of Pax5-Jak2 in early B cell development, we performed RNA-sequencing (RNA-seq) with ex vivo sorted pro-B cells (CD19+ B220+ Kit+ CD2− IgM−) from the bone marrow of Pax5Jak2+/− and control Pax5+/− mice at the age of 3 weeks, before leukemic B220low cells were detected. Differentially expressed genes were identified by an expression difference of > 3-fold, an adjusted P value of < 0.05 and an expression value of > 5 TPM in at least one of the two cell types (Dataset EV1). Few gene expression changes were observed between Pax5Jak2+/− and Pax5+/− pro-B cells. Only 35 and 28 genes were up- or down-regulated, respectively, in Pax5Jak2+/− pro-B cells compared with control Pax5+/− pro-B cells (Fig 2A), consistent with the absence of a B cell developmental phenotype in Pax5Jak2+/− mice (Fig 1C and D). For comparative analyses, we identified 331 activated and 327 repressed Pax5 target genes with an expression difference of > 3-fold by performing RNA-seq analysis with ex vivo sorted Pax5−/− and Pax5+/− pro-B cells as well as Bio-ChIP-seq analysis with ex vivo sorted Pax5Bio/Bio RAG2−/− pro-B cells (Appendix Fig S2A and Dataset EV2; see Appendix Supplementary Methods). Comparison of the differentially expressed genes identified in Pax5Jak2+/− pro-B cells with these regulated Pax5 target genes revealed that 26 (74%) of the 35 upregulated genes corresponded to repressed Pax5 target genes, while 25 (89%) of the 28 downregulated genes qualified as activated Pax5 target genes (Fig 2B and Dataset EV1). Interestingly, a previous RNA-seq comparison of Pax5−/− and Pax5+/− pro-B cells identified a similarly low number of Pax5-regulated genes (Smeek et al, 2017). Together, these data suggest that the Pax5Jak2 allele behaves like a Pax5 null allele with regard to Pax5 function.
Figure 2.
To corroborate this finding, we generated Pax5<sup>Jak2/-</sup> and control Pax5<sup>Pro-/.</sup> mice, which were unable to generate CD19<sup>+</sup> B cells in the bone marrow (Appendix Fig S2B), suggesting that theJak2 kinase domain does not provide transcriptional activity to the Pax5-Jak2 fusion protein. Importantly, B cell development in both mouse strains was arrested at an uncommitted B220<sup>-</sup>CD19<sup>+</sup> progenitor cell stage, expressing Pax5-Jak2 or the Pax5 paired domain (Prd) from the Pax5 locus (Appendix Fig S2C and D). We conclude therefore that Pax5-Jak2 does not function as a transcriptional regulator, as the Jak2 kinase domain cannot substitute for the loss of the central and C-terminal Pax5 sequences encoding a potent transactivation domain (Dörfler & Busslinger, 1996).

**Pax5-dependent gene expression signature of Pax5<sup>Jak2/-</sup> B-ALL cells**

To investigate the oncogenic function of Pax5-Jak2 in the Pax5<sup>Jak2/-</sup> B-ALL model, we next performed RNA-seq analysis with tumors isolated from the lymph nodes of moribund Pax5<sup>Jak2/-</sup> mice. As reference tumors, we analyzed B-ALLs that developed in the lymph nodes of Pax5<sup>-/-</sup> Cdkn2ab<sup>-/-</sup> mice, a tumor model that lacks constitutively activated JAK-STAT signaling (Smeenk et al, 2017). The Pax5<sup>Jak2/-</sup> and control Pax5<sup>-/-</sup> Cdkn2ab<sup>-/-</sup> B-ALL tumors were assigned by principal component analysis (PCA) between pro-B and large pre-B cells in early B cell development (Appendix Fig S2E) and yet differed from each other in expression (Appendix Fig S2F). Moreover, the Pax5<sup>Jak2/-</sup> B-ALL cells were of oligoclonal origin, as they predominantly expressed a few V<sub>H</sub> genes of the immunoglobulin heavy-chain locus (Appendix Fig S2G). By defining differentially regulated genes by an expression difference of >3-fold and the aforementioned criteria, we identified 254 upregulated and 144 downregulated genes in Pax5<sup>Jak2/-</sup> B-ALLs relative to the control B-ALLs (Fig 2C and Dataset EV3). Unexpectedly, gene set enrichment analyses (GSEA) revealed that 76 repressed Pax5 target genes, identified in pro-B cells (Appendix Fig S2A and Dataset EV2), were significantly enriched as downregulated genes in Pax5<sup>Jak2/-</sup> B-ALL tumors (Fig 2F and G). Hence, 29% of the differentially expressed genes in Pax5<sup>Jak2/-</sup> B-ALL cells correspond to regulated Pax5 target genes. These observations strongly suggest that the function of wild-type Pax5 may also be compromised in Pax5<sup>Jak2/-</sup> B-ALL tumors. While the Ptprc (CD45) gene was similarly expressed in the Pax5<sup>Jak2/-</sup> and control B-ALL tumors (Appendix Fig S2H), the increased expression of the repressed Pax5 target gene Hmnrpl (Fig 2E) may explain the decreased B220 expression on Pax5<sup>Jak2/-</sup> B-ALL cells, as the RNA-binding protein hRNPL regulates the alternative splicing of exons 4–6 of the Ptprc (CD45) mRNA (Oberdoerffer et al, 2008) (Fig 2H and Appendix Fig S2I). Low expression of hRNPL, as observed upon Pax5-mediated repression in control B-ALL and B cells, leads to the inclusion of all three exons in the Ptprc mRNA giving rise to the CD45 isoform RABC (known as B220). In contrast, reads at exon 4 are barely detectable and reads at exon 6 are strongly reduced in the mRNA of Pax5<sup>Pro-/-</sup> B-ALL cells, which likely gives rise to the CD45 isoforms RBC and RB (see Appendix Fig S2O).

**Loss of wild-type Pax5 in B-ALL by uniparental disomy of the Pax5<sup>alleles**

To study the function of Pax5 may be impaired in Pax5<sup>Jak2/-</sup> B-ALL cells, we next compared the RNA-seq expression pattern at the Pax5 locus in Pax5<sup>-/-</sup> pro-B cells and Pax5<sup>Jak2/-</sup> B-ALL tumors. Whereas all 10 Pax5 exons were expressed in Pax5<sup>-/-</sup> pro-B cells, abundant expression was detected in the Pax5<sup>Jak2/-</sup> B-ALL cells only from exon 1 to exon 5, which code for the N-terminal paired domain present in the Pax5-Jak2 fusion protein (Fig 3A). Consistent with the absence of wild-type Pax5 mRNA, Pax5<sup>Jak2/-</sup> B-ALL cells failed to express full-length Pax5 protein, in contrast to the Pax5-Jak2 protein, as shown by immunoblot analysis with a Pax5 paired domain-specific antibody (Fig 3B). Absence of the wild-type Pax5 protein was confirmed by intracellular Pax5 staining of Pax5<sup>Jak2/-</sup>.
Figure 3.
B-ALL cells with a C-terminal Pax5-specific antibody that is unable to detect the Pax5-Jak2 protein (Fig 3C).

To investigate the developmental onset of the Pax5 expression loss, we took advantage of the Pax5<Δ19/> allele, which carries an IRES-hCd2 gene in the 3’ untranslated region of Pax5 and thus reports Pax5 expression by giving rise to the expression of human (h) Cd2 (Fuxa & Busslinger, 2007). To this end, we generated Pax5<Δ19/><hCd2/> mice and analyzed the bone marrow of these mice at the age of 4 weeks by flow cytometry, which revealed that hCd2 was expressed by B220<+/> cells but was already lost in all B220<low/> cells (Fig 3D). These data further confirmed that the downregulation of Pax5 expression is an ideal surrogate marker for monitoring the loss of wild-type Pax5 expression in Pax5<Δ19/><Jak2/> B-ALL cells.

Next, we examined whether the wild-type Pax5 allele was lost in leukemic Pax5<Δ19/><Jak2/> B cells. For this, we sorted B220<+/> and B220<low/> B cells from the bone marrow of 6-week-old Pax5<Δ19/><Jak2/> mice and analyzed genomic DNA of these cells by PCR with specific primers that amplified exon 4 of the wild-type Pax5 allele or unique sequences of the Pax5<Δ19/> allele, respectively (Fig 3E). While the Pax5<Δ19/> allele was identified in both B220<+/> and B220<low/> cells, the Pax5 exon 4 could only be amplified from the B220<+/> B cells in all Pax5<Δ19/><Jak2/> mice analyzed (Fig 3E). Moreover, copy number alteration in the Pax5-containing genomic region could not be observed by whole genome sequencing at a 10-fold coverage. As wild-type Pax5 sequences are present on both sides of the Jak2<Δ2> allele (Fig 1A), it is conceivable that the Jak2<Δ2> insertion may be copied by interchromosomal homologous recombination into the wild-type Pax5 allele by a process known as acquired uniparental disomy or copy-neutral loss of heterozygosity (Tuna et al., 2009). To investigate a possible copy-neutral gain of the Pax5<Δ19/> allele, we performed quantitative PCR analyses with primers that amplified the unique sequences at the Pax5 exon 5–Jak2 exon 19 junction of the Pax5<Δ19/> allele (Fig 3F). To allow for normalization of the PCR data, we amplified a control region in intron 2 of the Car8 gene, located 35 Mb upstream of Pax5, and then set the ratio obtained with B220<+/> B cells from Pax5<Δ19/><Jak2/><neo/> bone marrow to 1 copy number for the Pax5<Δ19/><neo/> allele (Fig 3F). This analysis revealed the gain of a second Pax5<Δ19/><Jak2/> allele in B220<low/> B cells and B-ALL cells of Pax5<Δ19/><Jak2/> mice (Fig 3F). Conversely, the loss of the wild-type Pax5 allele in B220<low/> B cells and B-ALL cells was confirmed by quantitative PCR analyses with primers amplifying a 150-bp sequence in Pax5 intron 3 that was absent on the Pax5<Δ19/><Jak2/> allele (Appendix Fig S3A). Together, these data revealed a strong selection pressure to lose the wild-type Pax5 allele and to gain a second Pax5<Δ19/><Jak2/> allele by acquired uniparental disomy in leukemic B220<low/> B cells of the Pax5<Δ19/><Jak2/> mouse model.

Pax5 loss is not essential for B-ALL formation, but accelerates tumor progression

To investigate whether the loss of Pax5 is a prerequisite for leukemia formation, we reasoned that ectopic transcription of Pax5 from a heterologous locus may maintain Pax5 expression in B cells of Pax5<Δ19/><Jak2/> mice. For this, we took advantage of the Ikzf1<neo/> allele, which contains a loxP-flanked neomycin (neo) resistance gene upstream of a Pax5 mini-gene in the Ikzf1 locus (Souabni et al., 2002). Cre-mediated deletion of the neo cassette results in Pax5 expression from the Ikzf1<Δax> allele (Souabni et al., 2002). As ectopic Pax5 expression in the T-lymphoid lineage leads to the development of an aggressive T cell lymphoma (Souabni et al., 2007), we used the Cd79a-Cre line (Hobeika et al., 2006) to convert the Ikzf1<neo/> to the Ikzf1<Δax> allele only at the onset of B cell development. Hence, we generated Cd79a-Cre Ikzf1<neo/><Pax5<Δ19/><Jak2/><neo/> and control Cd79a-Cre Pax5<Δ19/><Jak2/><neo/> mice, which we monitored for the development of B cell leukemia. Kaplan–Meier survival analysis revealed that the control Cd79a-Cre Pax5<Δ19/><Jak2/><neo/> mice had a median survival of 79 days (Fig 3G) and thus died as rapidly as Pax5<Δ19/><Jak2/><neo/> mice (Fig 1F). Cd79a-Cre Ikzf1<neo/><Pax5<Δ19/><Jak2/><neo/> mice also developed B-ALL, although with a longer latency and incomplete penetrance, as 27% of these mice were still alive after one year.
(Fig 3G). B-ALL tumors from the lymph nodes of Cdc79a-Cre Lkz2flox/+ Pax5SL-Jak2f/+ mice had a similar cell surface phenotype as Pax5flox/Jak2+ B-ALL tumors except for higher expression of B220 and CD19 (Fig 3H), although they still lost the wild-type Pax5 allele (Appendix Fig S3B). Consistent with normal B220 expression, intracellular Pax5 staining revealed that the B-ALL tumors of Cdc79a-Cre Lkz2flox/+ Pax5SL-Jak2f/+ mice expressed high Pax5 levels similar to the wild-type B cells (Fig 3H). In summary, we conclude that the loss of Pax5 expression was not strictly required for leukemia formation, although it clearly accelerated tumor development.

To determine whether the wild-type PAX5 allele is present or absent in human PAX5-JAK2+ B-ALLs, we interrogated the RNA-seq data of 8 human PAX5-JAK2+ B-ALL tumors to identify sequence reads spanning the unique exon junctions of the wild-type PAX5 gene (exon 5-exon 6) and PAX5-JAK2 rearrangement (PAX5 exon 5-JAK2 exon 19). Sequence reads could be detected at both unique exon junctions in all human PAX5-JAK2+ B-ALLs, in contrast to the absence of sequence reads at the Pax5 exon 5-exon 6 junction in the murine Pax5flox/+ tumors (Appendix Fig S3C, left). Interestingly, the Pax5-JAK2 transcripts were increased in 6 of the 8 B-ALLs, resulting in an average percentage of 68.4% for PAX5-JAK2 mRNA compared with 31.6% for full-length PAX5 mRNA (Appendix Fig S3C, right), whereas a similar analysis of PAX5-ETV6+ B-ALLs revealed a 1:1 ratio of both Pax5 transcripts (Smeenk et al., 2017). Analysis of the RNA-seq expression pattern at the human PAX5 locus corroborated that all 10 PAX5 exons were expressed in PAX5-JAK2+ B-ALLs (Appendix Fig S3D), contrary to the situation observed in murine Pax5flox/+ B-ALLs (Fig 3A). These data therefore demonstrate that the wild-type PAX5 allele is not lost in human PAX5-JAK2+ B-ALLs. The discrepancy between the human PAX5-JAK2 rearrangement and the murine Pax5flox/+ model is likely caused by the insertion of the Jak2 cDNA sequence in the mouse Pax5 locus, which provides an ideal substrate for acquired uniparental disomy due to the presence of Pax5 sequence homologies on both sides of the Jak2 cDNA insertion.

The Jak2 kinase activity is required for the development and maintenance of Pax5flox/Jak2+ B-ALL

We next investigated whether the kinase activity of Pax5-Jak2 is essential for leukemia development. As mutation of the full-length JAK2 protein at lysine (K) 882 to glutamic acid (E) in the ATP-binding loop was previously shown to abolish its kinase activity (Feng et al., 1997), we introduced the equivalent K272E mutation in the Pax5flox allele to generate a kinase-dead (KD) Pax5-Jak2 protein (Fig 4A and Appendix Fig S4A). The Pax5-Jak2-KD protein was expressed in pro-B cells of Pax5flox/Jak2+/- mice, albeit at a 4-fold lower level relative to the wild-type Pax5 protein, as shown by immunoblot analysis of nuclear pro-B cell extracts (Appendix Fig S4B). All B cell subsets were present at normal frequencies and did not downregulate B220 expression in the bone marrow and spleen of Pax5flox/Jak2-KD+/- mice at the age of 2 months (Fig 4B and C). Notably, no mice succumbed to leukemia during the observation period of 12 months (Appendix Fig S4C). While the 4-fold lower expression of the Pax5-Jak2-KD protein in pro-B cells is expected to significantly delay the tumor onset, the complete absence of B-ALL in 1-year-old Pax5flox/Jak2-KD+/- mice nevertheless indicates a critical role of the Pax5-Jak2 kinase activity in the initiation of leukemia development.

To determine whether the kinase activity is required for tumor maintenance, we investigated the in vivo sensitivity of Pax5flox/Jak2+/- B-ALLs to Jak2 inhibition. Previous studies showed that in vitro cultured cell lines ectopically expressing PAX5-JAK2 rapidly lose cell viability upon treatment with the JAK1/2 inhibitor ruxolitinib (Roberts et al., 2014; Schinnerl et al., 2015; Hurtz et al., 2020). We next studied the effect of ruxolitinib on the maintenance of the murine Pax5flox/Jak2+/- B-ALL tumors in vivo. As wild-type pro-B and pre-B cells abundantly express Jak1 but not Jak2 (ImmGen database), ruxolitinib may mediate its effect by inhibition of the endogenous Jak1, transgenic Pax5-Jak2, or both kinases in Pax5flox/Jak2+/- B-ALL tumors. To perform the ruxolitinib inhibition experiment, we took advantage of the Pax5flox/Jak2-Luc+/- mice (Appendix Fig S1B), which additionally expressed luciferase in B cells, thus facilitating tumor monitoring by in vivo bioluminescence measurement. The pro-B cells of Pax5flox/Jak2-Luc+/- mice expressed 2-fold lower levels of Pax5-Jak2 protein compared with Pax5flox/Jak2+/- pro-B cells (Appendix Fig S1E and F). Consequently, the Pax5flox/Jak2-Luc+/- mice develop B-ALL with a 3-fold longer latency of 214 days (Appendix Fig S4D and E) relative to the Pax5flox/Jak2+/- mice (74 days; Fig 1F). Tumor cells from lymph nodes of moribund Pax5flox/Jak2-Luc+/- mice (Ly5.2+) were harvested, sorted as B220low cells by flow cytometry, and
Figure 4.
10^5 cells were transferred into sublethally irradiated Ly5.1^+ CS7BL/6 recipient mice. Tumor cell engraftment was verified by in vivo bioluminescence measurement 14 days post-transfer, followed by twice-daily treatment with either ruxolitinib or vehicle (Appendix Fig S4F). Bioluminescence monitoring revealed that ruxolitinib treatment significantly slowed down leukemia progression in two independent experiments (Fig 4D and E, and Appendix Fig S4G). Consequently, ruxolitinib treatment prolonged the survival of the transplanted mice (with a median survival of 74 days) by 20 days compared with vehicle-treated mice (with a median survival of 54 days; Fig 4F). In summary, we conclude that the kinase activity of Pax5-Jak2 is important for both the development and maintenance of the Pax5^+Jak2^+/-- B-ALL tumors.

### Inefficient competition of Pax5-Jak2 for DNA binding in the presence of wild-type Pax5

The consistent loss of the wild-type Pax5 allele in Pax5^+Jak2^+/-- tumor cells raised the question of whether the full-length Pax5 protein may interfere with the function of Pax5-Jak2. Since the N-terminal DNA-binding paired domain is the only common region between Pax5-Jak2 and full-length Pax5 (Appendix Fig S1A), it is conceivable that both proteins may compete for DNA binding in Pax5^+Jak2^+/-- B cells. To test this hypothesis, we analyzed the genome-wide binding pattern of both Pax5 proteins. In order to distinguish the two proteins, we added a biotin acceptor sequence in frame to the C-terminus of the Pax5-Jak2 protein encoded by the Pax5^+Jak2^-allele (Fig 5A and Appendix Fig S1B), which allowed Pax5-Jak2 to be specifically biotinylated in vivo by the E. coli biotin ligase BirA expressed from the Rosa^26^ allele (de Boer et al., 2003; Driegen et al., 2005). We thus generated Pax5^+Jak2^+/-- Rosa26^BirA^+/-- mice and used in vitro cultured pro-B cells from young mice (expressing Pax5) or B-ALL tumors (lacking Pax5) to determine the genome-wide DNA-binding pattern of Pax5-Jak2 by streptavidin-mediated chromatin precipitation coupled with deep sequencing (Bio-ChIP-seq; Revilla-i-Domingo et al., 2012). For comparison, we also determined the DNA-binding profile of full-length Pax5 by Bio-ChIP-seq analysis of Pax5^+Bio/Bio^ pro-B cells, which carried a C-terminal biotin acceptor sequence together with an IRES-BirA gene insertion in the 3’ untranslated region of Pax5 (McManus et al., 2011).

Peak calling with a stringent P value of < 10^-10 identified 36,917 Pax5 peaks in Pax5^+Bio/Bio^ pro-B cells, 2,717 Pax5-Jak2 peaks in Pax5^+Jak2^+/-- Rosa26^BirA^+/-- pro-B cells, and 34,068 Pax5-Jak2 peaks in Pax5^+Jak2^+/-- Rosa26^BirA^+/-- B-ALL cells (Fig 5B). Analysis of the 300 top-ranked peaks with the de novo motif-discovery program MEME-ChIP (Machanick & Bailey, 2011) identified the Pax5 motif in both Pax5-Jak2 and Pax5 peaks (Fig 5C), consistent with the two proteins sharing the same DNA-binding domain. Four sites, which exhibited differential binding of Pax5 and Pax5-Jak2 in the Bio-ChIP-seq data (Appendix Fig S5A), were selected for validation by Bio-ChIP-qPCR analysis of independently prepared Pax5^+Bio/Bio^ pro-B cell, Pax5^+Jak2^+/-- Rosa26^BirA^+/-- pro-B cell, and Pax5^+Jak2^+/-- Rosa26^BirA^+/-- B-ALL samples (Appendix Fig S5B). The Bio-ChIP-seq and Bio-ChIP-qPCR methods both detected the same binding pattern at each selected site in the three different cell types (Fig 5D and Appendix Fig S5A). Notably, the Bio-ChIP-seq analysis identified a 13.6-fold lower number of Pax5-Jak2 peaks in Pax5^+Jak2^+/-- Rosa26^BirA^+/-- pro-B cells compared with the Pax5 peaks in Pax5^+Bio/Bio^ pro-B cells (Fig 5B), which unequivocally demonstrated that the Pax5-Jak2 protein was unable to efficiently compete for DNA binding in the presence of wild-type Pax5.

### Experimental procedures

**Figure 5.** The DNA-binding function of Pax5-Jak2 is important for leukemia formation.

A Schematic diagram of the Pax5-Jak2 protein containing a C-terminal biotin acceptor sequence that can be biotinylated in vivo by the E. coli biotin ligase BirA upon expression from the Rosa^26^ allele (Driegen et al., 2005).

B Genome-wide binding of Pax5-Jak2 in in vitro cultured pro-B cells from Pax5^+Jak2^+/-- Rosa26^BirA^+/-- mice at the age of 3 weeks (expressing Pax5) and ex vivo Pax5^+Jak2^+/-- Rosa26^mut^+/-- B-ALL tumors (lacking Pax5), as determined by Bio-Chip-seq analysis (Revilla-i-Domingo et al., 2012). The DNA-binding pattern of full-length Pax5 was determined by Bio-ChIP-seq analysis of ex vivo sorted Pax5^+Bio/Bio^ pro-B cells, which carried a C-terminal biotin acceptor sequence together with an IRES-BirA gene insertion in the 3’ untranslated region of Pax5 (McManus et al., 2011).

C Two independent Bio-Chip-seq experiments were performed for each cell type. Representative binding patterns of Pax5 and Pax5-Jak2 in the three B cell types are shown for a selected genomic region, with horizontal bars indicating Pax5 and Pax5-Jak2 peaks that were identified by MACS peak calling (left). The number of Pax5 (white) and Pax5-Jak2 (grey or black) peaks, which were defined by stringent MACS peak calling with a P value of < 10^-10 in the three B cell types, are shown to the right.

D Bio-Chip-qPCR analysis of Pax5 binding at four sites, which were selected for their differential Pax5 binding in the three cell types, as determined by Bio-Chip-seq analysis (Appendix Fig S5A).

E Statistical data are shown as mean percentages with SEM and were analyzed by multiple t-tests (unpaired and two-tailed with Holm-Sidak’s correction; *P < 0.05, **P < 0.01, ***P < 0.001). Each dot corresponds to one mouse. See Methods section for flow-cytometry definition of the different cell types.

F Kaplan-Meier survival analysis of Pax5^+mut^+/-- (black) and Pax5^+Jak2^+/-- (grey) mice. A P value of < 0.0001 was determined for the survival curves by statistical analysis with the log-rank (Mantel-Cox) test.

G Flow-cytometric analysis and intracellular Pax5 staining of lymph node tumor cells from two moribund Pax5^+mut^+/-- mice (black line), which differed by the absence (mouse #1) or presence (mouse #2) of Pax5 expression in the tumor cells. Pax5^+^ (WT) B cells (filled grey) were used as controls. The apparently higher Pax5 expression level in the blasting tumor cells of mouse # 2 compared with that of quiescent Pax5^+^ B cells may reflect a difference in cell size rather than in Pax5 expression. The percentage and number of Pax5^+^ and Pax5^+Jak2^+/-- tumors analyzed is shown to the right.
binding in the presence of the full-length Pax5 protein. Conversely, the loss of Pax5 in Pax5<sup>jak2<sup>+/+</sup></sup> Rosa26<sup>BirA</sup>+/+ B-ALL cells resulted in a similarly high number and extensive overlap of the Pax5-Jak2 peaks compared with the Pax5 peaks identified in Pax5<sup>Bio/Bio</sup> pro-B cells, although the binding density was still higher for full-length Pax5 compared with Pax5-Jak2 (Fig 5B and Appendix Fig S5C and
D). We therefore conclude that the consistent loss of Pax5 in Pax5<sup>–/–</sup>-Jak2<sup>–/–</sup> B-ALL tumors allows Pax5-Jak2 to bind to its target sites in the genome, which strongly argues for a nuclear function of Pax5-Jak2.

**The DNA-binding function of Pax5-jak2 contributes to leukemia formation**

We next investigated whether the DNA-binding activity of Pax5-Jak2 is essential for leukemia formation. To this end, we mutated the N-terminal region of the paired domain by introducing five amino acid changes (N29K, G30S, R31Q, P32L, V36I) by CRISPR/Cas9-mediated mutagenesis in the Pax5<sup>Prd–/–</sup> allele (Fig 5E and Appendix Fig S5E). The selected five amino acid residues are known to bind to the DNA backbone in the minor groove of the Pax5-binding sequence (Garvie et al, 2001) and are thus predicted to affect DNA-binding of the N-terminal paired domain region, consistent with the fact that the amino acids N29, R31, and P32 were shown to be mutated in human B-ALLs (Gu et al, 2019). As shown by immunoblot analysis, the Pax5<sup>Prd<sup>–/–</sup>-Jak2<sup>–/–</sup> protein was expressed at a lower level relative to the Pax5 protein in Pax5<sup>Prd<sup>+/+</sup>-Jak2<sup>+/+</sup> B cells (Appendix Fig S5F), which may be caused by an interference of the five mutant amino acid residues with efficient recognition of the Pax5<sup>Prd<sup>+/+</sup>-Jak2<sup>+/+</sup> protein by the anti-Pax5 paired domain antibody. We therefore analyzed the expression of the Pax5<sup>Prd<sup>–/–</sup>-Jak2<sup>–/–</sup> allele by RT-qPCR analysis of Pax5<sup>Prd<sup>–/–</sup>-Jak2<sup>–/–</sup></sup> pro-B cells, which indicated that the Pax5<sup>Prd<sup>–/–</sup>-Jak2<sup>–/–</sup></sup> mRNA was expressed at a similar level as Pax5<sup>–/–</sup>-Jak2<sup>–/–</sup> and Pax5<sup>–/–</sup>-Jak2<sup>–/–</sup>-Luc mRNA in Pax5<sup>Prd<sup>+/+</sup>-Jak2<sup>+/+</sup></sup> and Pax5<sup>Prd<sup>+/+</sup>-Jak2<sup>+/+</sup></sup>-pro-B cells (Appendix Fig S5G).

B cell development in the bone marrow and spleen of 6–7-week-old Pax5<sup>Prd<sup>–/–</sup>-Jak2<sup>–/–</sup></sup> mice was largely normal except for a modest decrease in pre-B and mature B cells (Fig 5F). Importantly, leukemic B220<sup>low</sup> cells could not be detected in the bone marrow of 6–7-week-old Pax5<sup>Prd<sup>–/–</sup>-Jak2<sup>–/–</sup></sup> mice (Fig 5G). However, the Pax5<sup>Prd<sup>+/+</sup>-Jak2<sup>+/+</sup></sup> mice still developed B-ALL with a median survival of 178 days (Fig 5H) and thus lived on average 100 days longer than Pax5<sup>Prd<sup>–/–</sup>-Jak2<sup>–/–</sup></sup> mice (Fig 1F). The Pax5<sup>Prd<sup>–/–</sup>-Jak2<sup>–/–</sup></sup> tumor cells also downregulated B220 and CD19 expression (Fig 5I) and had a similar cell surface phenotype as the Pax5<sup>Prd<sup>+/+</sup>-Jak2<sup>+/+</sup></sup> tumor cells (Fig 5I). Interestingly, 31% of the tumors did not lose wild-type Pax5 expression, as revealed by intracellular Pax5 staining (Fig 5I). However, stratification of the Pax5<sup>Prd<sup>–/–</sup>-Jak2<sup>–/–</sup></sup> tumors according to their Pax5 expression status did not reveal any survival advantage for mice with tumors that still expressed the wild-type Pax5 protein (Appendix Fig S5H). The delayed development of Pax5<sup>Prd<sup>–/–</sup>-Jak2<sup>–/–</sup></sup> B-ALLs could be caused by an altered DNA-binding potential of the mutant paired domain. To test this hypothesis, we used in vitro cultured Pax5<sup>Prd<sup>+/+</sup>-Jak2<sup>+/+</sup></sup> and Pax5<sup>Prd<sup>–/–</sup>-Jak2<sup>–/–</sup></sup> B-ALL cells, which lost the wild-type Pax5 allele, for ChIP-seq analysis with an antibody detecting the N-terminal paired domain of Pax5. Notably, the mutant Pax5-Jak2 protein still bound to 20,692 sites in the genome (Appendix Fig S5I). However, the mutant protein uniquely bound to 52% of these sites and furthermore failed to interact with 33% of all Pax5-Jak2-binding sites (Appendix Fig S5I), suggesting that the five N-terminal amino acid substitutions resulted in an altered DNA-binding specificity of the paired domain. In summary, these data indicate that the DNA-binding activity of Pax5-Jak2 contributes to leukemia development.

**Nuclear Pax5-Jak2 does neither phosphorylate H3Y41 nor induce active chromatin**

To gain further insight into the nuclear function of Pax5-Jak2, we next studied the subcellular localization of Pax5-Jak2 by nuclear-cytoplasmic cell fractionation and immunoblot analysis, which revealed that the Pax5-Jak2 protein was almost exclusively present in the nucleus of Pax5<sup>Prd<sup>+/+</sup>-Jak2<sup>+/+</sup> B-ALL cells (Appendix Fig S6A). This evidence further supported the concept of an essential nuclear function for Pax5-Jak2 that involves DNA binding in the absence of a classical transcriptional activity. We therefore investigated whether Pax5-Jak2 may indirectly control gene expression by phosphorylating histone H3 on tyrosine 41 (H3Y41ph), as it was previously proposed for nuclear JAK2 (Dawson et al, 2009). Due to the discontinued commercial availability of the published rabbit polyclonal anti-H3Y41ph antibody, we generated a new mouse monoclonal anti-H3Y41ph antibody (clone 8B2-C1; IgG1/IgG2a) that specifically detected an H3 peptide containing the phosphorylated Y41 (pY41) in ELISA assays and identified the pY41-peptide conjugated to the ubiquitin protein in immunoblot analysis (Fig 6A–C). We next extensively purified the anti-H3Y41ph antibody from 8B2-C1 hybridoma cell supernatants by negative and positive selection on peptide columns (Appendix Fig S6B). Immunoblot analysis of whole-cell extracts prepared from Pax5<sup>Prd<sup>+/+</sup>-</sup> or control Pax5<sup>Prd<sup>Env<sup>+/+</sup>-</sup></sup> Cdkn2ab<sup>+/–</sup>-tumor cells revealed that phosphorylated STAT5 could be readily detected in both tumor cell types (Fig 6D). However, the monoclonal anti-H3Y41ph antibody (8B2-C1) could not detect H3Y41 phosphorylation in the Pax5<sup>Prd<sup>+/+</sup>-</sup> tumor cells, although it readily identified the pY41-peptide conjugated to ubiquitin (Fig 6D). Moreover, the H3Y41ph modification could also not be detected in HEL, TMD8, and K1106 cells (Fig 6D), which were previously shown to contain abundant H3Y41ph levels upon detection with the published polyclonal anti-H3Y41ph antibody (Dawson et al, 2009; Rui et al, 2010, 2016). As a control, abundant H3 expression was observed in all tumor cell lines by analysis with an anti-H3 antibody (Fig 6D). Based on these data, we conclude that Pax5-Jak2 does not phosphorylate H3Y41 in Pax5<sup>Prd<sup>+/+</sup>-</sup> B-ALL cells.

Phosphorylation of H3Y41 was also shown to expel HP1α from nucleosomes, thus inducing active chromatin leading to gene activation (Dawson et al, 2009; Rui et al, 2010). We next investigated a possible role of Pax5-Jak2 in chromatin regulation. For this purpose, Pax5<sup>Prd<sup>+/+</sup>-</sup> B-ALL cells could, however, not be analyzed due to the absence of a reference cell type that would be blocked at the same developmental stage as the B-ALL cells. As lymphopoiesis was arrested at the same uncommitted progenitor stage in Pax5<sup>Prd<sup>+/+</sup>-</sup> and control Pax5<sup>Prd<sup>+/+</sup>-</sup> mice (Appendix Fig S2C), we performed ChIP-seq analysis with in vitro cultured Pax5<sup>Prd<sup>+/+</sup>-</sup> and Pax5<sup>Prd<sup>+/+</sup>-</sup> progenitor cells (Appendix Fig S6C) to investigate the genome-wide distribution of the active histone marks H3K4me3 and H3K27ac as well as the repressive histone modification H3K27me3. We initially focused our bioinformatic analysis on Pax5-Jak2-binding regions identified in Pax5<sup>Prd<sup>+/+</sup>-</sup> B-ALL cells (Figs 5B and 6E). Heat maps and density profiles did not reveal any obvious difference in the distribution of the active H3K4me3 and H3K27ac marks at the Pax5-Jak2-binding sites, while the repressive H3K27me3 modification was absent at these sites (Fig 6E and Appendix Fig S6C). We subsequently used a window-based approach (Lun & Smyth, 2016) to scan the entire genome for regions with a differential abundance of...
Figure 6. Pax5-jak2 does not function as an epigenetic regulator.

A Schematic diagram of histone H3 indicating the position of Y41. The pY41-peptide (amino acids 37–46, red) was used for immunizing mice and the subsequent generation of the hybridoma cell line 8B2-C1 (see Methods). The longer phosphorylated (pY41) and nonphosphorylated peptides (amino acids 29–53, black) were used for ELISA and immunoblot analysis.

B Specific detection of the pY41-peptide by the antibody produced by the hybridoma cell line 8B2-C1. The plate-bound pY41-peptide, in contrast to the corresponding nonphosphorylated peptide, is specifically detected by antibodies in the serially diluted supernatant of the hybridoma cells by ELISA assays, as measured by absorption at the wavelength of 450 nm. Mean values with SEM are shown for two independent experiments.

C Specificity of the anti-H3Y41ph antibody, as shown by immunoblot analysis. One phosphorylated (pY41) or nonphosphorylated peptide was coupled to ubiquitin (Ubi, 8.5 kDa), followed by separation of the protein conjugates on SDS-PAGE and immunoblot detection with the anti-H3Y41ph antibody present in the 8B2-C1 cell supernatant. Following treatment with calf intestinal alkaline phosphatase (CIP), the dephosphorylated Ubi-pY41 peptide conjugate could no longer be detected with the anti-H3Y41ph antibody.

D Immunoblot analysis of whole-cell extracts prepared from Pax5jak2+/− and control Pax5jak2+/+ B-ALL cells as well as from the human HEL, TM08 and K1106 cell lines. Phosphorylated (p) STAT5 was detected with an anti-STAT5 (pY694) antibody and the pY41-peptide with the purified anti-H3Y41ph antibody (Appendix Fig S6B). One to five pY41-peptides were coupled to ubiquitin, which was added in the range of 50 ng per well. The Gapdh and histone H3 proteins were analyzed as loading control. An unspecific protein is denoted by an asterisk. One representative of 5 immunoblot experiments is shown.

E No evidence for an epigenetic function of Pax5-jak2 in early B cell development. The genome-wide distribution of the histone marks H3K4me3, H3K27ac and H3K27me3 in in vitro cultured Pax5jak2+/+ and Pax5jak2+/− progenitor cells was determined by ChIP-seq analysis (Appendix Fig S6C). The average density of the three histone marks in these progenitor cells was determined for a region from −2.5 kb to +2.5 kb from the summit of the Pax5-jak2 peaks identified in Pax5jak2+/− B-ALL cells (Fig S5B). The results of two different ChIP-seq experiments per cell type are shown. The expression of the Pax5-jak2 protein and paired domain (Prd) polypeptide in Pax5jak2+/+ and Pax5jak2+/− progenitor cells is shown in Appendix Fig S2D.

Source data are available online for this figure.
the three individual histone marks, which also did not identify any
significant differences. We therefore conclude that Pax5-Jak2 does
not function as an epigenetic regulator.

IL-7 signaling promotes the development of Pax5<sup>Jak2/−</sup> B-ALL

While establishing Pax5<sup>Jak2/−</sup> tumor cells in culture, we noticed that
IL-7 strongly stimulated the proliferation of these cells (Appendix
Fig S7A), which is consistent with expression of the IL-7
receptor on these cells (Fig 1I) and with a recent report demonstrat-
ing that the IL-7 sensitivity of leukemic B cells is increased upon
Pax5 loss (Ramamoorthy et al, 2020). To investigate a possible IL-7
dependency in vivo, we again took advantage of the Pax5<sup>Jak2-Luc/−</sup>
tumor cell transplant system and injected freshly harvested tumor
cells into Il<sup>−/−</sup>, Il<sup>+/−</sup>, or Il<sup>+/+</sup> recipient mice (Appendix Fig S7B).
Weekly boltamivirone analysis revealed that tumor development
was delayed in Il<sup>+/−</sup> mice compared with control Il<sup>−/−</sup> and Il<sup>+/−</sup>
mice in two independent experiments (Fig 7A and B, and
Appendix Fig S7C). Moreover, the survival of tumor-bearing mice
was modestly improved in Il<sup>+/−</sup> recipient mice compared with the
control Il<sup>−/−</sup> and Il<sup>+/−</sup> recipient mice (Fig 7C). These results indi-
cate that IL-7 promotes the growth of Pax5<sup>Jak2/−</sup> tumor cells also
in vivo. It therefore appears that IL-7 signaling and the constitutively
active Pax5-Jak2 kinase cooperatively support the development of
Pax5<sup>Jak2/−</sup> B-ALL.

STATS-mediated gene regulation in Pax5<sup>Jak2/−</sup> B-ALL

As the constitutively active Jak2 kinase domain of the Pax5-Jak2
protein was shown to phosphorylate STAT5 when ectopically
expressed in a JAK2-deficient human cell line (Schinammer et al,
2015), we next investigated the status of STAT5 phosphorylation in
B cells of Pax5<sup>Jak2/−</sup> mice. Intracellular staining combined with flow-
cytometric analysis revealed a strong increase of phosphorylated
STAT5 (p-STAT5) in B-ALL cells compared with residual B220<sup>+</sup>
B cells in the lymph nodes of Pax5<sup>Jak2/−</sup> mice (Fig 7D). Moreover,
the p-STAT5 levels in Pax5<sup>Jak2/−</sup> B-ALL cells were reduced at 30 min
and lost at 1 h after ruxolitinib treatment (Appendix Fig S7D), while
they were reduced at 2 h and lost at 4 h after IL-7 withdrawal
(Appendix Fig S7E), suggesting that STAT5 phosphorylation in these
tumor cells depends on both the Pax5-Jak2 kinase and IL-7 signal-
ing. To investigate whether the Pax5-Jak2 kinase could also phos-
phorylate STAT5 in a B cell type lacking IL-7R signaling, we
analyzed p-STAT5 levels in follicular (FO) B cells (B220<sup>+</sup>CD19<sup>+</sup>CD21<sup>+</sup>CD23<sup>+</sup>
from the spleen of 3–5-week-old Pax5<sup>Jak2/−</sup> mice. A small but significant increase of p-STAT5 was
detected in Pax5<sup>Jak2/−</sup>/FO B cells compared with control Pax5<sup>+</sup>/FO
B cells (Fig 7E and Appendix Fig S7F). Importantly, this increase in
p-STAT5 was not observed in Pax5<sup>Jak2-KD/−</sup> FO B cells due to inact-
vation of the Jak2 kinase by the K272E mutation (Fig 7E). We next
analyzed B cells from the bone marrow of Pax5<sup>Jak2/−</sup> mice at the
time of 4–5 weeks. A small but significant increase of p-STAT5 levels
was again observed in Pax5<sup>Jak2/−</sup>/pro-B cells compared with Pax5<sup>+</sup>/pro-B cells,
whereas the p-STAT5 levels were strongly increased in the
leukemic B220<sup>low</sup> B cells (Fig 7F). We therefore conclude that
the strong increase of STAT5 phosphorylation occurs already in
early leukemogenesis concomitant with loss of the wild-type Pax5
allele and increased DNA-binding of Pax5-Jak2. These data therefore
provide strong support for a nuclear function of Pax5-Jak2 in main-
taining high levels of p-STAT5 in Pax5<sup>Jak2/−</sup> B-ALL cells.
Figure 7.
To evaluate the role of STAT5 in controlling gene expression in Pax5\(^{Jak2/2}\) B-ALL tumors, we first generated a reference dataset by identifying STAT5-bound and STAT5-regulated genes in pro-B cells. ChiP-seq analysis of short-term cultured pro-B cells with an anti-STAT5 antibody identified 2,377 STAT5 peaks by stringent MACS peak calling with a P value of \(<10^{-10}\), which resulted in 1,606 STAT5-bound genes and the identification of the consensus STAT5-binding motif (Appendix Fig S7G). RNA-seq analysis of \(ex\ vivo\) sorted pro-B cells from Vav-Bel2 Rag1-Cre \(Stat5^{fl/fl}\) and control Vav-Bel2 Rag1-Cre \(Stat5^{+/+}\) mice (Malin et al, 2010) identified 57 STAT5-activated and 53 STAT5-repressed genes with an expression difference of \(>2\)-fold, an adjusted P value of \(<0.05\) and an expression value of \(>5\) TPM in at least one of the two cell types (Appendix Fig S7H and Dataset EV4). Combining differential gene expression with STAT5 binding defined 19 activated and 4 repressed STAT5 target genes in pro-B cells (Appendix Fig S7H). We next investigated whether the STAT5-binding sites identified in pro-B cells were enriched at the 254 upregulated and 144 downregulated genes in Pax5\(^{Jak2/2}\) B-ALLs (Fig 2C). Indeed, STAT5 binding was significantly increased from 8.5% of all nonregulated genes to 14.9% of the 398 regulated genes in Pax5\(^{Jak2/2}\)/B-ALL tumors (Fig 7G), which was also confirmed by GSEA analysis (Appendix Fig S7I). Although we identified only 19 activated STAT5 target genes in pro-B cells, nine of these genes were shown by GSEA to be significantly enriched as upregulated genes in Pax5\(^{Jak2/2}\)/B-ALL cells compared with control Pax5\(^{+/+}\) -Cdkn2a\(^{−/−}\)/B-ALL cells (Fig 7H). Cxcr5, Syndig1l, and Sema4a are shown as representative genes that exhibited strong upregulation in Pax5\(^{Jak2/2}\)/B-ALL cells (Fig 7I) and displayed STAT5-dependent activation as well as STAT5 binding in pro-B cells (Fig 7J and K). In summary, we conclude that the nuclear Pax5-Jak2 kinase activity phosphorylates STAT5, which in turn activates a STAT5-dependent gene expression program in Pax5\(^{Jak2/2}\)/B-ALL cells.

**Discussion**

The Pax5-JAK2 \(^{−}\) B-ALLs belong to the Philadelphia chromosome-like (Ph-like) ALL subgroup, which is characterized by genetic alterations leading to constitutive activation of kinase signaling (Roberts et al, 2012, 2014). The Pax5-JAK2 protein, consisting of the DNA-binding paired domain of Pax5 fused to the kinase domain of Jak2 (Nebral et al, 2009), was shown to bind Pax5 recognition sequences and to function as a constitutively active kinase in established cell lines (Schinnerl et al, 2015). Here, we have generated a mouse model to investigate the oncogenic role of the Pax5-Jak2 protein in leukemia formation. The Pax5\(^{Jak2/2}\)/B-ALL mice rapidly developed an aggressive B-ALL in the bone marrow without the need of introducing another cooperating exogenous gene mutation. Both the DNA-binding function and kinase activity of Pax5-Jak2 were contributing to leukemogenesis. Unexpectedly, the cells of all Pax5\(^{Jak2/2}\)/B-ALL tumors analyzed lost the wild-type Pax5 allele, which allowed the Pax5-Jak2 protein to efficiently bind to genomic target sites in the nucleus. The strong selection for loss of heterozygosity identified Pax5-Jak2 as a nuclear oncprotein and important driver of leukemia development, which functions by maintaining high levels of phosphorylated STAT5 in the nucleus.

Although Pax5-Jak2 contains a DNA-binding domain, it does not function as a classical transcription factor, as the Jak2 kinase domain cannot substitute for the lack of the Pax5 transactivation domain. This is best evidenced by the lack of CD19 \(^{+}\) B cells due to a developmental block at an uncommitted lymphoid progenitor stage (Kit\(^{B220}\)/CD19\(^{−}\)) in Pax5\(^{Jak2/2}\) mice similar to Pax5\(^{−/−}\)/B-ALL mice (Nutt et al, 1999), demonstrating that the Pax5\(^{Jak2/2}\)-null allele behaves like a Pax5 null allele with regard to Pax5 function. Like other B-ALL types, the Pax5\(^{Jak2/2}\) tumors arise in committed Pax5-expressing B cells in the bone marrow. However, different to most other B-ALLs, loss of the wild-type Pax5 allele is a genetic alteration leading to accelerated leukemia development in Pax5\(^{Jak2/2}\)/B-ALL mice. Consequently, the leukemic Pax5\(^{Jak2/2}\) cells lack normal Pax5 function, which is evidenced by the downregulated expression of activated Pax5 target genes and the reactivation of repressed Pax5 target genes in Pax5\(^{Jak2/2}\)/B-ALL cells. Hence, tumor development is initiated in committed B cells, but then leads to dedifferentiation of the Pax5\(^{Jak2/2}\)/B-ALL cells due to the loss of Pax5 function similar to what is observed upon conditional Pax5 deletion in pro-B cells (Mikkola et al, 2002).

By genome-wide binding analysis, we previously demonstrated that a Pax5 protein consisting only of the DNA-binding paired domain (Prd) competes very inefficiently with full-length Pax5 protein for binding to genomic Pax5 target sites in Pax5\(^{Prd/Prd}\)/B-ALL cells (Smeenk et al, 2017). A possible explanation for this binding competition may be that full-length Pax5 can be incorporated into transcription factor complexes through interaction via its central and C-terminal protein sequences in contrast to the Pax5 paired domain polypeptide, which cannot be stabilized on genomic DNA by such protein interactions. Here, we have shown that the Pax5-Jak2 protein, containing only the paired domain of Pax5, is equally inefficient in competing with full-length Pax5 for binding to genomic Pax5 recognition sequences in Pax5\(^{Jak2/2}\)/B-ALL cells. Upon loss of full-length Pax5, the Pax5-Jak2 protein was, however, able to interact with most genomic Pax5-binding sites in Pax5\(^{Jak2/2}\)/B-ALL cells, strongly indicating that Pax5-Jak2 functions as DNA-binding oncoprotein in the nucleus. The potent selection pressure to lose the wild-type Pax5 allele in these Pax5\(^{Jak2/2}\)/B-ALL cells was dependent on both the Jak2 kinase activity and the DNA-binding function of Pax5-Jak2, as no tumors developed in Pax5\(^{Jak2/2-KD/−}\)/B-ALL cells, while one third of the Pax5\(^{Prd/+,Jak2/2}\)/B-ALL tumors still contained the wild-type Pax5 allele. Notably, the loss of heterozygosity in Pax5\(^{Jak2/2}\)/B-ALL tumors occurred by acquired uniparental disomy through copying the Jak2 cDNA insertion of the Pax5\(^{Jak2/2}\) allele by interchromosomal recombination into the wild-type Pax5 allele. The observed oligoclonal origin of the Pax5\(^{Jak2/2}\)/B-ALL tumors furthermore suggests that rare independent events of Pax5 loss by uniparental disomy were strongly selected for cell expansion to contribute to tumor development.

Loss of heterozygosity by uniparental disomy depends on homology regions on both sides of the mutant sequence to be inserted by homologous recombination into the wild-type allele (Tuna et al, 2009) and was previously reported to account for the homozygosity of the Jak2-V617F or Pax5-P80R mutation in myeloid or B cell leukemia, respectively (Vilainé et al, 2011; Li et al, 2014; Bastian et al, 2019; Gu et al, 2019). In this regard, the mouse Pax5\(^{Jak2/2}\)/B-ALL model differs from the human Pax5-JAK2\(^{−}\) B-ALLs, as the Pax5\(^{Jak2/2}\) allele was created by an in-frame insertion of the partner Jak2 cDNA sequences into the mouse Pax5 locus, whereas the human Pax5-JAK2 rearrangements are generated by fusion of the 5'
part of the PAX5 locus with the 3’ region of the JAK2 locus (Nebral et al., 2009). Consequently, only the Ptx5/Jak2 allele can undergo loss of heterozygosity by homologous recombination in marked contrast to the PAX5-JAK2 rearrangement. Consistent with this idea, the wild-type PAX5 allele was present and expressed in all 8 human PAX5-JAK2 B-ALLs. Notably however, the expression of Pax5 from the heterologous Ikaros locus delayed but did not prevent B-ALL development in Pax5/Jak2+/- mice, indicating that the Pax5-Jak2 protein could still induce tumor development in the presence of full-length Pax5 in the mouse, which is analogous to the situation observed with human PAX5-JAK2 B-ALLs. Hence, the mouse Pax5/Jak2+/- model appears to generate, through loss of the wild-type Pax5 allele, a more aggressive leukemia and thus does not recapitulate all aspects of the human PAX5-JAK2 B-ALL disease. However, the more aggressive mouse Pax5/Jak2+/- tumor model allowed us to identify the Pax5-Jak2 protein as an important oncogenic driver of leukemia development, which might have been more difficult to demonstrate in the presence of the competing full-length Pax5 protein. The Pax5/Jak2+/- mouse model also allowed us to demonstrate by genetic mutation that both the DNA-binding function and kinase activity of the Pax5-Jak2 protein contribute to B-ALL development. The Jak2 kinase activity is also required for tumor progression, as treatment of Pax5/Jak2+/- tumor-bearing mice with the Jak1/2 inhibitor ruxolitinib delayed tumor growth leading to prolonged survival. However, this treatment could not eradicate the tumor cells similar to the observed development of resistance to ruxolitinib in clinical applications (Meyer & Levine, 2014).

In addition to the canonical role of cytoplasmic JAK2 in JAK-STAT signaling (Chen et al., 2012), nuclear JAK2 was identified as an “epigenetic writer” that phosphorylates histone H3 on tyrosine 41 (H3Y41ph; Dawson et al., 2009). H3Y41 phosphorylation was shown to prevent heterochromatin formation by interfering with HP1α binding to H3, which leads to gene activation as exemplified by the oncogenes Lmo2 and Myc (Dawson et al., 2009; Rui et al., 2010). Consistent with a role in gene expression, high levels of H3Y41 phosphorylation correlate with high abundance of the active H3K4me3 mark at active promoters (Dawson et al., 2012; Rui et al., 2016). A critical reagent for obtaining these results was a rabbit polyclonal anti-H3Y41ph antibody that is no longer commercially available. Based on these published data, we hypothesized that the nuclear Pax5-Jak2 protein with its constitutive Jak2 kinase activity may also control gene expression by phosphorylating H3Y41. By generating a mouse monoclonal antibody that specifically detects pY41 in the H3 sequence context, we could, however, not detect Jak2-dependent phosphorylation of H3Y41 in Pax5/Jak2+/- B-ALL cells. Moreover, we also did not observe differences in the abundance of the active H3K4me3 or H3K27ac mark at genomic Pax5-Jak2-binding sites in experimental Pax5/Jak2+/- and control Pax5/Jak2-/- progenitor cells. Based on these data, we therefore conclude that Pax5-Jak2 is unlikely to control gene expression as an epigenetic regulator.

Here, we have demonstrated that Pax5-Jak2 phosphorylates STAT5 in Pax5/Jak2+/- B-ALL cells, consistent with previous data obtained with transfected human cell lines (Roberts et al., 2014; Schinnerl et al., 2015). While STAT5 phosphorylation was minimally induced by Pax5-Jak2 in Pax5/Jak2+/- pro-B cells, it was strongly increased in leukemic B220high B cells of 4-week-old Pax5/Jak2+/- mice. Hence, the strong increase of STAT5 phosphorylation occurred already in early leukemogenesis concomitant with loss of the wild-type Pax5 allele and increased DNA-binding of Pax5-Jak2, which provides compelling evidence for a nuclear function of Pax5-Jak2 in maintaining high levels of p-STAT5 in Pax5/Jak2+/- B-ALL cells. ChIP- and RNA-seq analyses furthermore implicated Pax5-Jak2 in the regulation of a STAT5-dependent gene expression program in Pax5/Jak2+/- B-ALL cells. In this context, it is important to note that STAT5 has been identified as an important oncogenic driver of leukemia development (Heltemes-Harris et al., 2011; Katerndahl et al., 2017; Wingelhofer et al., 2018; de Araujo et al., 2019).

The almost exclusive localization of Pax5-Jak2 in the nucleus raises the question of how nuclear Pax5-Jak2 can promote gene expression, if newly synthesized and unphosphorylated STAT5 needs to be phosphorylated by Jak kinases in the cytoplasm in order to relocate to the nucleus in response to cytokine signaling (Villarino et al., 2017) (Fig 8). Notably, the Pax5/Jak2+/- B-ALL cells still depend on IL-7 signaling even in the presence of the constitutively active Pax5-Jak2 protein, which may explain why the

Figure 8. Model explaining the role of PAX5-JAK2 in maintaining active STAT5 levels in the nucleus.

In wild-type pro-B cells, IL-7-mediated activation of the IL-7 receptor, consisting of the IL-7Rα and CD127 chains, leads to phosphorylation and activation of JAK1 and JAK3. These active kinases phosphorylate STATs, which results in the formation of parallel STAT5 dimers that are transported across the nuclear membrane by importins. In the nucleus, STAT5 binds to DNA and regulates genes involved in cell survival, proliferation, and metabolism (Villarino et al., 2017). Phosphatase-mediated inactivation of the DNA-bound STAT5 complexes disengages the dimer from the DNA, followed by formation of the anti-parallel STAT5 dimer and its export via nuclear exportins to the cytoplasm. In Pax5/Jak2+/- B-ALL cells, cytoplasmic STAT5 is similarly activated by IL-7R signaling and transported across the nuclear membrane. In the nucleus however, the constitutively active (ca) PAX5-JAK2 protein antagonizes the action of phosphatases by re-phosphorylating STAT5 and thus maintaining active STAT5 levels, leading to sustained expression of STAT5 target genes, which promotes leukemogenesis.
Pax5Jak2/^+ tumors are preferentially located in the IL-7-rich environment of lymph nodes (Link et al., 2007). Based on this finding, we propose the following model for the nuclear function of Pax5-Jak2 (Fig 8). In control pro-B cells, IL-7 signaling promotes Jak1,3-dependent STAT5 phosphorylation, which leads to parallel dimer formation and nuclear transfer of p-STAT5. In the nucleus, p-STAT5 promotes gene expression until it is dephosphorylated and relocated to the cytoplasm to initiate a new cycle of phosphorylation and nuclear transfer in response to IL-7 signaling. In Pax5Jak2/^+ B-ALL cells, IL-7 signaling is also responsible for the transfer of p-STAT5 from the cytoplasm to the nucleus. However, p-STAT5 can now be maintained at a higher level in the nucleus by the constitutively active Pax5-Jak2 kinase, which antagonizes DNA-binding function of Pax5-Jak2 likely contributes to the nuclear Jak2 activity by retaining the fusion protein in the nucleus either through specific recognition of Pax5-binding sites or through a more general DNA-binding mode. This paradigm explaining the nuclear function of Pax5-Jak2 may also be valid for all other 13 nuclear JAK2 fusion proteins identified in human B-ALL, as they also retain the catalytic kinase domain (JHI) of Jak2 (Roberts et al., 2014; Akkari et al., 2020).

Finally, B-ALL development requires at least two cooperating mutations, as exemplified by the constitutive activation (ca) of STAT5 combined with heterozygous loss of Pax5 in transgenic caStat5a Pax5^5+/− and caStat5b Pax5^5+/− mice (Heltemes-Harris et al., 2011; Smeenk et al., 2017). In contrast, the Pax5Jak2 allele behaves like a dual-hit mutation, as it gives rise to the expression of a constitutively active Jak2 kinase in the absence of Pax5 function. In summary, our detailed analysis of the Pax5-Jak2 rearrangement as a dual-hit mutation that promotes B-ALL development by maintaining high STAT5 activity in the nucleus.

Material and Methods

Detailed methods can be found in the Appendix Supplementary Methods available online.

Mice

The following mice were maintained on the C57BL/6 genetic background: Pax5^5−/− (Urbaneck et al., 1994), Pax5<sup>bc32/bc32</sup> (Fuxa & Busslinger, 2007), Pax5<sup>bio/bio</sup> (McManus et al., 2011), Pax5^5Dp/+ (Smeenk et al., 2017), Pax<sup>5E6/+</sup> (Smeenk et al., 2017), Cdkn2a<sup>−/−</sup> (Krimpenfort et al., 2007), Il7r<sup>−/−</sup> (von Freeden-Jeffry et al., 1995), Rosa26<sup>BirA/+</sup> (Driegen et al., 2005), Ikzf1<sup>pro/+</sup> (Sousa et al., 2002), Stat5a<sup>−/−</sup> (Cui et al., 2004), Cd79a<sup>Mb1</sup>Cre<sup>+</sup> (Hobeika et al., 2006), Meox2<sup>Cre</sup> (Tallquist & Soriano, 2000), Rag1<sup>Cre</sup> (McCormack et al., 2003), transgenic FLPe (Rodriguez et al., 2000), transgenic CAGGs-Dre (Anastassiadis et al., 2009), and transgenic Vav-Bcl2 (Ogilvy et al., 1999). The Meox2<sup>Cre</sup>, Rag1<sup>Cre</sup> and Cd79a<sup>Cre</sup> genotypes are referred to as Meox2-Cre, Rag1-Cre and Cd79a-Cre, respectively. All animal experiments were carried out according to valid project licenses, which were approved and regularly controlled by the Austrian Veterinary Authorities.

Generation of Pax5Jak2/−, Pax5Jak2-Luc/+ and Pax5^5Dp/KD/+ mice

The Pax5Jak2− and Pax5Jak2 alleles were generated by ES cell targeting using the targeting vector shown in Appendix Fig S1B and described in detail in Appendix Supplementary Methods. The Pax<sup>5LSL-Jak2-KD/+</sup> and Pax<sup>5LSL-Pro^5-Jak2/+</sup> mice were generated by CRISPR/Cas9-mediated genome editing in mouse Pax5<sup>LSL-Jak2/+</sup> zygotes that were injected with Cas9 mRNA, an sgRNA specific for the sequence to be mutated and a single-stranded DNA repair template of 200 nucleotides (Appendix Fig S4A and SSE). The Pax5Jak2-Luc/+ and Pax5Jak2/+ mice were generated by deletion of the LSL cassette with Meox2-Cre.

Antibodies

The following antibodies were used for flow-cytometric analysis: B220/CD45R (RA3-6B2), CD2 (RM2-5), CD11b/Mac1 (M1/70), CD19 (1D3), CD21/CD35 (7G6), CD23 (B34B), CD25/IL-2Rα (PC61), CD93/AA4.1 (A4.1), CD117/c-Kit (2B8), CD127/IL-7Rα (A7R34), CD135/Fli3 (A2F10), Gr1 (RB6-8C5), IgD (11.26c), Igx (187.1), Igm (II/41 or eB121-15F9), and TCRβ (H57-597).

The anti-Pax5 (C-terminal; D198F; Cell Signaling Technology) anti-p-STAT5 (47/Stat5 pY694; BD Biosciences) antibodies were used for intracellular staining. The following antibodies were used for immunoblot analysis: anti-Pax5 (directed against amino acids 17–145; Adams et al., 1992), anti-p-STAT5 (C115 pY694; Cell Signaling Technology), anti-Tbp (3TF1-3G3; Active Motif), anti-H3, HRP-coupled (D112; Cell Signaling Technology), and anti-Gapdh, HRP-coupled (14C10; Cell Signaling Technology). The following antibodies were used for ChIP analysis: anti-STAT5A and anti-STAT5B (PA-ST5A and PA-ST5B, R&D Systems), anti-STAT5B (PAST5A and PA-ST5B, R&D Systems), anti-STAT5A (C-terminal; D198F; Cell Signaling Technology), anti-p-STAT5 (47/Stat5 pY694; BD Biosciences) antibodies were used for immunoblot analysis: anti-Pax5 (directed against amino acids 17–145; Adams et al., 1992), anti-p-STAT5 (C115 pY694; Cell Signaling Technology), anti-Tbp (3TF1-3G3; Active Motif), anti-H3, HRP-coupled (D112; Cell Signaling Technology), and anti-Gapdh, HRP-coupled (14C10; Cell Signaling Technology). The following antibodies were used for ChIP analysis: anti-STAT5A and anti-STAT5B (PA-ST5A and PA-ST5B, R&D Systems), anti-H3K4me3 (paB003-050; Diagenode), anti-H3K27ac (ab4729, Abcam), and anti-H3K27me3 (C36B11; Cell Signaling Technology) antibody.

Generation of a monoclonal H3Y41ph-specific antibody

Fifty milligrams of the phosphorylated H3 peptide (amino acids 37–46 with phosphorylated (p) Y41; Fig 6A) in complete Freund’s adjuvant were subcutaneously injected into one mouse. After 3 immunizations, 30 mg of the phosphorylated H3 peptide (without adjuvant) were intravenously injected as final boost. After 4 days, spleenocytes were fused with the myeloma cell line X63-Ag8.653 by using polyethylene glycol. The cells were seeded into 96-well plates, and fused hybridoma cells were selected in HAT-containing growth medium. Hybridoma supernatants were screened for H3Y41ph-specific antibodies by ELISA against unmodified or pY41-modified H3 peptides (amino acids 29–53, Fig 6A).

Flow cytometric sorting and definition of mouse hematopoietic cell types

Cell types were defined as follows: Pax5-deficient progenitors (CD19^B220^Kit^Ly6D^), pro-B cells (CD19^B220^Kit^CD2^-IgM^-IgD^-), pre-B cells (CD19^B220^Kit^-CD2^-IgM^-IgD^-), large pre-B cells (CD19^B220^Kit^-IgM^-IgD^-FSChi), small pre-B cells (CD19^B220^Kit^-IgM^-IgD^-FSClo), immature B cells (CD19^B220^-IgM^-IgD^-FSChi), mature B cells (CD19^B220^-IgM^IgD^-FSClo), small B cells (CD19^B220^-IgM^-IgD^-FSClop), and CD19^B220^-IgM^-IgD^-FSClop, immature B cells (CD19^B220^-IgM^-IgD^-FSClop), and CD19^B220^-IgM^-IgD^-FSClop, immature B cells (CD19^B220^-IgM^-IgD^-FSClop).
IgM⁺IgD⁺), recirculating B cells (CD19⁺B20⁺IgD⁺), mature splenic B cells (CD19⁺B20⁻IgD⁺), follicular (FO) B cells (CD19⁺B20⁺CD21hi CD23⁻), marginal zone (MZ) B cells (CD19⁺B20⁺CD21lo CD23⁺), T cells (TCRβ⁺), myeloid cells (CD11b⁺Gr1⁺).

**Transplantation experiments and ruxolitinib treatment**

Tumor cells from lymph nodes of moribund Pax5⁺Jak2⁻/⁻ and Pax5⁺Jak2⁻/⁻ Rosa26⁺BirA⁺/⁺ mice as well as B20⁺ and B20⁻ B cells from the bone marrow of Pax5⁺Jak2⁻/⁻ mice were sorted by flow cytometry and transfected by intravenous injection (10⁵ cells per mouse) into sublethally irradiated C57BL/6 mice (4.5 Gy). Ruxolitinib (R-6688; LC Laboratories) was administered twice daily by oral gavage of 45 mg/kg in 0.5% methylcellulose (M0512; Sigma). For bioluminescence imaging, mice were intraperitoneally injected with D-luciferin (150 mg/kg, Goldbio) and imaged with an IVIS Spectrum Xenogen machine (Caliper Life Sciences).

**In vitro culture of pro-B cells and B-ALL cell lines**

Pro-B cells were cultured on OP9 feeder cells in IL7-containing IMDM as described (Nutt et al., 1997). B-ALL cell lines were isolated from lymph nodes of Pax5⁺Jak2⁻/⁻ Rosa26⁺BirA⁺/⁺ or Pax5⁺Etv6⁺/⁺ Cdkn2a⁻/⁻ tumor mice and were established as cell lines by culturing them on ST2 feeder cells in IL7-containing IMDM medium.

**ChiP analysis of Pax5-Jak2 and STAT5 binding**

*Ex vivo* B-ALL cells from Pax5⁺Jak2⁻/⁻ Rosa26⁺BirA⁺/⁺ tumor mice, *ex vivo* sorted Pax5⁺BirA⁺/⁺ pro-B cells, short-term cultured Pax5⁺Jak2⁻/⁻ Rosa26⁺BirA⁺/⁺ pro-B cells, and short-term cultured Pax5⁺Jak2⁻/⁻ and Pax5⁺Sta⁻/⁻ precursor cells were crosslinked at room temperature with 1% formaldehyde (Sigma) for 10 min. The sheared chromatin was immunoprecipitated with specific antibodies or by streptavidin-mediated pull-down of *in vivo* biotinylated Pax5 and Pax5-Jak2 proteins, as described (Ebert et al., 2011; Revilla-i-Domingo et al., 2012). The precipitated DNA at selected genomic sites was determined by qPCR analysis (Table EV1). For analysis of STAT5 binding, wild-type pro-B cells were cultured on OP9 cells in the presence of IL-7, before IL-7 was withdrawn for 4 h and the pro-B cells were restimulated with IL-7 (10 ng/ml) for 30 min prior to formaldehyde fixation, nuclei preparation, and ChiP analysis with a mixture of anti-STAT5A and anti-STAT5B antibodies. ChiP-precipitated DNA (0.5–5 ng) was used for library preparation and Illumina deep sequencing.

**RNA-sequencing**

RNA from *ex vivo* sorted pro-B cells and B-ALL cells was isolated with the RNeasy Plus Mini Kit (Qiagen). mRNA was obtained by two rounds of poly(A) selection and used for library preparation and Illumina deep sequencing, as described (Smeenk et al., 2017).

**Bioinformatic analysis of RNA- and ChiP-seq data**

Bioinformatic analysis of the RNA- and ChiP-seq data was performed as described in Smeenk et al (2017) and in Appendix Supplementary Methods.

**Statistical analysis**

Statistical analysis was performed with the GraphPad Prism 8 software. Two-tailed paired Student’s t-test analysis and multiple t-tests (unpaired and two-tailed with Holm-Sidak’s correction) were used to assess the statistical significance of one observed parameter between two experimental groups. For comparison of multiple groups and analysis of repeated measurements, the ANOVA analysis or mixed model effect analysis (REML) were used together with post-hoc multiple comparison tests (Sidak or Tukey, for two or more groups, respectively).

**Data availability**

The RNA-seq and ChiP-seq data generated for this study (Table EV2) are available at the Gene Expression Omnibus (GEO) repository under the accession number GSE174775 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE174775). The human RNA-seq data are available upon request.

**Expanded View** for this article is available online.

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**Author contributions**

SJ performed most experiments; ASF carried out p-STAT5 analyses; DK-P generated the Pax5⁺Jak2⁻/⁻ Rosa26⁺BirA⁺ allele by ES cell targeting; SGM performed the ChiP- and RNA-seq experiments to identify regulated STAT5 target genes; CCAM provided PAX5-JAK2⁻/⁻ B-ALL RNA-seq data; SS provided human PAX5-JAK2⁻/⁻ B-ALL samples and advice on these tumors; MF and MJ performed the bioinformatic analysis of the RNA-seq and ChiP-seq data, respectively; SJ and MB planned the project, designed the experiments, and wrote the manuscript.

**Disclosure and competing interests statement**

The authors declare that they have no conflict of interest.

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