Acute B-cell lymphoblastic leukemia (B-ALL) results from oligo-clonal evolution of B-cell progenitors endowed with initiating and propagating leukemia properties. The activation of both the Rac guanine nucleotide exchange factor (Rac GEF) Vav3 and Rac GTPases is required for leukemogenesis mediated by the oncogenic fusion protein BCR-ABL. Vav3 expression becomes predominantly nuclear upon expression of BCR-ABL signature. In the nucleus, Vav3 interacts with BCR-ABL, Rac, and the polycomb repression complex (PRC) proteins Bmi1, Ring1b and Ezh2. The GEF activity of Vav3 is required for the proliferation, Bmi1-dependent B-cell progenitor self-renewal, nuclear Rac activation, protein interaction with Bmi1, mono-ubiquitination of H2A(K119) (H2AK119Ub) and repression of PRC-1 (PRC1) downstream target loci, of leukemic B-cell progenitors. Vav3 deficiency results in derepression of negative regulators of cell proliferation and repression of oncogenic transcriptional factors. Mechanistically, we show that Vav3 prevents the Pihp2-sensitive and Akt (S473)-dependent phosphorylation of Bmi1 on the regulatory residue S314 that, in turn, promotes the transcriptional factor reprogramming of leukemic B-cell progenitors. These results highlight the importance of non-canonical nuclear Rho GTPase signaling in leukemogenesis.
the Philadelphia positive (Ph⁺) t(9;22) (q34;q11.2) translocation that generates the constitutively active BCR-ABL oncprotein is frequently found in older adult (60%) and pediatric/adolescent-young adult (AYA) (25%) B-ALL cases1,2. The p190 fusion protein, the predominant form found in 60–80% of pediatric/AYA Ph⁺ B-ALL, a leukemia that derived from the transformation of a B-cell progenitor3. Ph-like B-ALL possess other driver mutations that induce the same transcriptional signatures as in Ph⁺ B-ALL7. Ph⁺ and Ph-like B-ALL have a much poorer prognosis compared to other cytogenetic or molecular abnormalities1,2. Genetic abnormalities including Bmi1 upregulation8–10, homozygous deletion of INK4A/ARF11 and mutations in the lymphoid-lineage transcription factor loci expressing PAX5, IKZF1, and EBF112–14, collaborate with BCR-ABL in the progression to aggressive B-ALL. These mutations, however, are not present in all cases of children’s BCR-ABL⁺ B-ALL13,15 and, when present, they are usually monallelic, which suggests that loss-of-heterozygosity drives B-ALL transformation. Recurrent epigenetic alterations in genes shown to be transcriptional programs and the induction of self-renewing stem cell like phenotype via the repression of B-cell progenitors in a GEF-dependent manner16. Nuclear VAV3 predominantly controls proliferation of B-cell leukemogenesis17.

BMI1, a major component of the polycomb repression complex (PRC) type I, is upregulated in patients with advanced stages of BCR-ABL-induced chronic myelogenous leukemia (CML)8 and through its repressive activity on the Cdkn2a locus18 and other less well-characterized activities results in leukemic transformation19. The overexpression of BMI1 reprograms lymphoid leukemia progenitors into a self-renewing and transplantable stem cell like phenotype via the repression of a B-cell transcriptional programs and the induction of self-renewing genes10,20,21. Rho GTPases play essential roles in transformation, initiation, and progression of BCR-ABL driven leukemias. Deficiency of Rac proteins impairs myeloid leukemogenesis induced by p210-BCR-ABL expression22,23. Vav proteins (Vav1, Vav2, and Vav3) are guanine nucleotide exchange factors (GEF) for Rac GTPases24–29. We have previously shown that genetic deletion of Vav3, but not Vav1 and Vav2, delays BCR-ABL-induced lymphoblastic leukemia and increases the therapeutic vulnerability during TKI treatments30. Human and murine BCR-ABL⁺ B-cell progenitors show increased expression and activation of Vav330 and we have demonstrated that a first-in-class, Vav3 inhibitor significantly impairs human and murine B-cell progenitor lymphoblastic leukemogenesis in vitro and in vivo31. Vav3 deficiency impairs Rac GTPase activation, survival, and proliferation of BCR-ABL⁻ B-cell progenitors30. The survival of Vav3-deficient B-cell progenitors negatively correlates with the level of expression of pro-apoptotic proteins30. However, the underlying molecular mechanisms of Vav3 regulation of lymphoid leukemia B-cell progenitor proliferation remains unclear. In the present study, we demonstrate that the mechanism by which Vav3 controls leukemic cell proliferation is through a non-canonical nuclear function associated with the nucleation of PRC complex components and the control of PRC1 activity.

Results
Nuclear VAV3 predominantly controls proliferation of B-cell progenitors in a GEF-dependent manner. Previous results have shown that VAV3 activity plays important tumorigenic functions in a number of cancer models32–35. Our previous work has also shown that Vav3 plays roles in acute lymphoblastic leukemia, a function linked to the regulation of the survival of B-cell progenitors30. This work, however, could not identify the signaling mechanism involved in this process30. It was inferred that this leukemogenic process was associated with the engagement of the canonical GEF activity of Vav3 at the plasma membrane. To approach this issue, we first examined the expression and cellular distribution of this GEF in murine and human BCR-ABL⁺ B-ALL cell progenitors. Consistent with our earlier study30, we found that p190-BCR-ABL induces Vav3 expression with predominant nuclear localization in murine B-cell progenitors (Fig. 1A, B). Western blot analyses using different subcellular cell extracts revealed that Vav3 is preferentially located in the nucleus under these conditions (Fig. 1C). Nuclear Vav3 is probably in an active state, as inferred by the detection of substantial levels of phosphorylation of the key residue involved in the activation step of the GEF (Y174) (Fig. 1C). Similarly, human B-cell progenitors from Ph⁺ or Ph-like B-ALL patients, containing secondary pathogenic mutations (including loss of CDKN2A/B, mutant PAX5 and loss of IKZF1; see Supplemental Methods), show increased VAV3 expression with a predominant nuclear distribution (Fig. 1D, E).

As expected36, a significant fraction of BCR-ABL localizes in the nucleus of murine and human B-ALL B-cell progenitors (Supplementary Fig. 1A). To determine whether ABL and VAV3 interact, we performed a proximity ligation assay (PLA) that allows the in situ detection of the protein-protein interactions with subcellular location resolution37. Using this method we found that the nuclear fraction of VAV3 does reside in close proximity with BCR-ABL in both murine and human B-ALL cell progenitors (Fig. 1F, G). By contrast, no interaction is seen in B-cell progenitors from non-leukemic mice (Supplementary Fig. 1B). These data indicate that BCR-ABL and VAV3 are in close physical proximity in transformed B cell progenitors. The nuclear distribution of leukemic VAV3 prompted us to investigate its role in regulating genes involved in leukemic B-lymphoid progenitor proliferation using a Vav3⁻/⁻ cell model (Supplementary Fig. 1C). We found that the Vav3 deficiency significantly attenuates the proliferation of p190-BCR-ABL⁺ B-cell progenitors (Fig. 1H). Re-expression of either full length (FL) Vav3 or constitutively active (CA) Vav3 rescues the proliferation of Vav3⁻/⁻ leukemic progenitors (Fig. 1I, J and Supplementary Fig. 1D, E). The expression of a catalytically inactive version of Vav3 (N369A, referred to as Vav3 NA) does not show this rescue activity (Fig. 1I, J and Supplementary Fig. 1D, E), indicating that this biological activity is GEF- and GTPase-dependent. Likewise, we found that xenotransplants of shRNA-mediated knockdown of VAV330 in leukemic progenitors from a Ph⁺ B-ALL patient (CD34⁺/CD19⁻; Fig. 1K) reduces by ~60–75% the level of human leukemic progenitor chimera (hCD45⁺ hCD34⁺ hCD19⁺ EGFP⁺) present in the bone marrow of the transplanted immunodeficient recipients (Fig. 1L and Supplementary Fig. 1F). Taken together, these data indicate that VAV3 has a crucial role in the proliferation of Ph⁺ B-ALL progenitors.

Nuclear Vav3 interacts with Bmi1 and regulates PRC1 activity. To unravel the mechanism involved in the Vav3-mediated regulation of p190-BCR-ABL⁺ leukemogenesis, we first performed genome-wide expression analyses in Vav3-deficient and control transformed B cell progenitors (Fig. 2A). We identified the differential expression of 1110 genes (450 upregulated and 660 downregulated) in Vav3⁻/⁻ leukemic B-cell progenitors (<0.5 to >1.5-fold, p < 0.05; Supplementary Data 1). Gene ontology (GO) analyses revealed the differential expression of genes involved in
nuclear division, regulation of cell cycle, G1/S phase transition, chromosome organization, and covalent histone modifications (Fig. 2B and Supplementary data 2). We also found similarities of this transcriptome with those previously identified in cells bearing gain-of-function of the cyclin-dependent kinase inhibitors Cdkn2a and Cdkn2b (GSE125841)\(^3^8\) and of the B-cell differentiation master regulator Pax5 (GSE126375)\(^3^9\). All those genes are frequently altered in B-ALL\(^4^0,4^1\). Consistent with this, we found that the deficiency of Vav3 results in the upregulation of all those transcripts (Fig. 2C and Supplementary Fig. 2A) as well as increased expression of p16Ink4a and p15Ink4b (Fig. 2D). However, the expression of Ebf1 and Ikaros, two other crucial B-cell differentiation factors, remains unchanged in the absence of Vav3 (Supplementary Fig. 2A).

Transcriptional profiles of human BCR-ABL\(^+\) lymphoid and myeloid blast crisis progenitors are enriched for PRC1- and
**Fig. 1** VAV3 expression is upregulated and predominantly nuclear in murine and human B-ALL B-cell progenitors, and its guanine nucleotide exchange activity is essential for leukemic B-cell progenitor proliferation. **A.** Confocal immunofluorescence microscopic images (A) and quantification of mean fluorescence intensity (B) showing upregulation and nuclear distribution of Vav3 in p190-BCR-ABL+ murine B-cell progenitors (n = 13 per group). **C** Representative immunoblots for Vav3, pVav3-Y174, Gapdh and Parp in the cytoplasmic and nuclear fraction of p190-BCR-ABL+ B-cell progenitors. Vav3 is primarily distributed in the nuclear fraction. **D** Representative confocal immunofluorescence images of VAV3 expression in healthy donor and B-ALL patients derived B-cell progenitors (CD34+/CD19−). **E** Western blot and microscopic images are representative of a minimum of two independent experiments. Statistical significance was determined using the unpaired Student-t or Anova tests when more than two groups were compared. *p < 0.05; **p < 0.01; ***p < 0.001.

**Fig. 2** Nuclear Vav3 regulates Cdkn2a and Cdkn2b expression by modulating PRC1 activity. **A** Schema depicting the assays performed in Fig. 2. **B** Comparative transcriptome and gene-ontology [molecular and biological functions] of differentially expressed genes in WT and Vav3−/− leukemic B-cell progenitors showing the differential regulation of genes involved in nuclear division, cell cycle regulation, G1/S phase transition, and covalent modifications of histone. **C** Quantitative real time PCR (Q-RT-PCR) analyses of Cdkn2a and Cdkn2b in Vav3 deficient p190-BCR-ABL+ B-cell progenitors in comparison to their WT counterparts (n = 4–12 per group). **D** Representative immunoblots for p16/INK4a, p15/INK4b and Actin in the whole cell lysates of WT and Vav3−/− leukemic B-cell progenitors. **E** Representative immunoblots for H2AK119Ub, H3K27me3 and Parp in the nuclear fraction of WT and Vav3−/− leukemic B-cell progenitors. **F** Representative immunoblots for H2AK119Ub and Parp in the nuclear fraction of WT leukemic B-cell progenitors transduced with empty vector and Vav3−/− leukemic B-cell progenitors transduced with empty vector or Vav3 FL or Vav3N369A GEF mutant vectors. **G** Q-RT-PCR analyses of Cdkn2a and Cdkn2b in empty vector transduced WT and empty or Vav3 or Vav3N369A vector transduced Vav3−/− leukemic B-cell progenitors (n = 6–8 per group). Data are presented as mean ± SD of a 2 or 3 independent experiments. Statistical significance was determined using the unpaired Student-t or Anova tests when more than two groups were compared. *p < 0.05; **p < 0.01; ***p < 0.001.
depleted for PRC2-related gene sets, respectively.42 Our transcriptional data also suggest a role for Vav3 in the regulation of histone covalent modifications like those produced by the PRC complexes (Fig. 2B and Supplementary Data 2). This led us to analyze the status of PRC activity in Vav3−/− leukemic progenitors. We found that the loss of Vav3 decreases (60 ± 1%, p < 0.05) the global level of H2A(K119) mono-ubiquitination (H2A(K119Ub), a hallmark of PRC1 activity (Fig. 2E). However, the Vav3 deficiency does not affect the levels of PRC2 activity as assessed by the analysis of the global levels of H3K27 trimethylation (H3K27me3) (Fig. 2E). The re-expression of full-length Vav3 restores global H2A(K119Ub) levels and transcriptional repression of Cdkn2a/2b (Fig. 2F, G). Further underscoring the GEF-dependency of this process, we could not observe any rescue activity when using the catalytically inactive version of Vav3 (Fig. 2F, G).

The interaction between the enzyme Ezh2, a known member of the PRC2 complex, and Vav family proteins has been reported before.43 The dynamic interaction between PRC2 and PRC1 has also been described.44 Based on our data, we hypothesized that VAV3 and BMI1, a crucial component of the PRC1 complex protein responsible for H2A(K119) mono-ubiquitination,45 could be involved in this process. To test this idea, we performed BMI1 and Vav3 co-immunoprecipitations using both the cytoplasmic and nuclear extracts from Ba/F3 cells (Fig. 3A). Similar to primary murine B-cell progenitors, we observed that p190-BCR-ABL promotes the expression and nuclear localization of Vav3 in these cells (Fig. 3B and Supplementary Fig. 2B, C). As expected, the PRC proteins BMI1 and Ring1B are present in the nuclear fraction (Fig. 3B). We found that Vav3 immunoprecipitated with BMI1, Ring1B, and the PRC2 protein Ezh2 (Fig. 3C). Conversely, we found that Vav3 also co-immunoprecipitates with BMI1 in these cells (Fig. 3D). Although predominantly cytoplasmic, we also found that the Rac1 and Rac2 GTPases are co-immunoprecipitated with nuclear Vav3 in p190-BCR-ABL-expressing Ba/F3 cells (Fig. 3B, C).

Validating these observations, we also found that Vav3 and BMI1 are in close proximity within the nucleus (Fig. 3E, F). BMI1 and Vav3 also associate with primary murine progenitors, and interaction is boosted by the ectopic expression of p190-BCR-ABL (Fig. 3G, H). Compared to the full-length Vav3 protein, the interaction of the catalytically deficient Vav3(N369A) mutant proteins with BMI1 is reduced ~2-fold (Fig. 3I, J). A similar effect is seen in Rac2-deficient leukemic B-cell progenitors (Fig. 3K, L), further indicating that the Vav3–BMI1 interaction that takes place inside the nucleus is both GEF and GTPase-dependent.

**Vav3 collaborates with BMI1 in B-cell leukemogenesis.** To determine whether Vav3 and BMI1 interaction modulates the PRC1 activity and p190-BCR-ABL-induced B-ALL progression, we co-transduced WT and Vav3−/− low-density bone marrow cells (LDBM) with p190-BCR-ABL (plus EGFP) and BMI1 (plus EGFP) bicistronic integrating vectors, and then transplanted them into lethally irradiated congenic female mice to monitor leukemogenesis (Fig. 4A). As control, we used vectors expressing EGFP alone. We confirmed nuclear BMI1 overexpression in the BMI1-transduced leukemic progenitors (FSChi EGFP++EGFP +B220loCD19+IgM+CD43+) (Supplementary Fig. 3 and Fig. 4B). Consistent with our previous reports,10,30, the transplantation of p190-BCR-ABL-expressing WT or Vav3−/− leukemic LDBM cells leads in both cases to leukemogenesis as demonstrated by the detection of lymphadenopathies, hepatosplenomegaly, and extranodal tumors in head and neck (data not shown). However, this process is significantly delayed in mice recipient of Vav3-deficient LDBM cells (Fig. 4C). The overexpression of BMI1 in WT leukemic cells results in the development of a more aggressive leukemia that results in ~40% of the transplanted mice dying much faster than controls (Fig. 4C). However, such an acceleration in vivo leukemogenesis in not observed when BMI is ectopically expressed in Vav3-deficient cells (Fig. 4C). This differential behavior was further amplified when using serial transplantations of different cell doses of B-cell progenitors derived from WT and Vav3−/− primary leukemic mice (Fig. 4D). Whole genome sequencing and copy number variation analyses identified no large-scale mutations or copy number variation in regions containing genes associated with leukemogenesis in the serially propagated WT and Vav3−/− leukemias (Supplementary Data 3). However, we did find a heterozygous point mutation in Pax5 (P80R) in three out of the four analyzed secondary WT leukemias but not in the Vav3−/− leukemias. Interestingly, this difference in survival of secondary recipients induced by the Vav3 deficiency is not rescued by BMI1 overexpression (Fig. 4D), reinforcing the concept that Vav3 is required for BMI1-induced leukemic acceleration.

Further analyses indicated that Vav3−/− leukemic B-cell progenitors proliferate less than their control counterparts (Fig. 4E and Supplementary Fig. 4A). BMI1 overexpression results in increased proliferation of both WT and Vav3−/− leukemic B-cell progenitors (Fig. 4E and Supplementary Fig. 4A). However, the proliferation of BMI1-expressing Vav3−/− cells is significantly lower (~20% reduction) than the WT controls (Fig. 4E and Supplementary Fig. 4A). This suggests that BMI1 overexpression cannot overcome the proliferative deficiency exhibited by Vav3−/− cells. We also found that the Vav3 deficiency and the BMI1 overexpression impairs and increases the self-renewal ability of leukemic progenitors, respectively (Fig. 4F). The overexpression of BMI1 partially rescues the effect of Vav3 deficiency on the frequency of primary clonogenic leukemic progenitors but not the negative effects caused by the Vav3 deficiency in both leukemic progenitor proliferation and self-renewal (Fig. 4F). The biochemical level, BMI1 overexpression does not rescue the impaired H2AK119Ub of Vav3-deficient leukemic B-cell progenitors (Fig. 4G). These results indicate that the BMI1-induced self-renewal, in vivo tumor initiation/propagation and stimulation of PRC1 activity are all Vav3-dependent.

**BMI1-dependent nuclear Rac activation depends on Vav3.** To understand the role of Vav3 in nuclear Rac activation in the context of BMI1 overexpression, we performed Rac GTPase activation assays in the nuclear fraction of p190-BCR-ABL+ B-cell progenitors. BMI1 overexpression increases while Vav3 deficiency abrogates Rac activation (Fig. 4H). The BMI1-induced Rac activation in WT leukemic B-cell progenitors is associated with augmented expression and activation of Vav3 (Supplementary Fig. 4B). The genetic ablation of Rac2 GTPase phenocopies the Vav3 deficiency, abrogating the expansion effect of BMI1 overexpression on p190-BCR-ABL+ leukemic B-cell colony formation (Fig. 4I). To further analyze the role of BMI1 in p190-BCR-ABL+ B-cell progenitors in the context of Vav3 and Rac2 deficiencies, we depleted BMI1 by shRNA-mediated knockdown (Supplementary Fig. 5A). Serial clonogenic CFU-prob assays show that the repleting ability of B-cell progenitors transformed by p190-BCR-ABL is completely dependent on endogenous BMI1 expression (Supplementary Fig. 5B). These data reinforce the role of Vav3 and Rac2 in BMI1-mediated transformation and self-renewal of leukemic progenitors.

**Vav3 is required to maintain homeostatic PRC1.4 epigenetic programs.** To evaluate the effects of Vav3 on PRC1.4 activity at the genome-wide level, we performed cleavage under target and...
release using nuclease followed by next-generation sequencing (CUT&RUN-seq) experiments in WT and Vav3−/− leukemic B-cell progenitors. This technique cleaves DNA sequences that are associated with protein A-conjugated micrococcal nuclease, thus allowing the detection of both protein binding and histone mark enrichments in DNA regulatory regions throughout the whole genome. To this end, we performed CUT&RUN-seq for in WT and Vav3−/− leukemic B-cell progenitors using specific antibodies. An anti-Bmi1 antibody, validated to be specific for nuclear Bmi1 as assessed in Bmi1-deleted (CRISPR/Cas9) pre-B-leukemic Ba/F3 cells (Supplementary Fig. 6A, B) and antibodies previously validated for chromatin immunoprecipitation followed by next-generation sequencing for Ring1b and H2AK119Ub antibodies were used. Subsequently, the Bmi1, Ring1b, and H2AK119Ub differential binding in WT and Vav3−/− leukemic B-cell progenitors was evaluated as indicated in Methods (Supplementary Figs. 7 and 8). Correlations between replicates of each of the CUT&RUNseq datasets (Supplementary Fig. 7A)
confirmed good correlations for anti-Bmi1 binding (r ≥ 0.84) with lower correlations for Ring1b and H2AK119Ub marks. We found no overt differences in the distribution of antibody-binding peaks (Supplementary Fig. 7B), although we did detect statistically significant quantitative differences in the mean signal generated by antibody-binding peaks (Supplementary Figs. 7C-E and 8A-C) for Bmi1, Ring1b, and H2AK119Ub. Specifically, 349 loci are significantly depleted of Bmi1 binding in Vav3−/− p190-BCR-ABL+ B-cell progenitors (Supplementary Fig. 8A and Supplementary Data 4). The differential binding analyses of Ring1b and H2AK119Ub CUT&RUN dataset showed higher number of loci with reduced binding in Vav3−/− leukemic B-cell progenitors (5607 Ring1b bound and 2605 H2AK119Ub bound) (Supplementary Data 5 and 6). To identify the PRC1.4 specific targets, we performed intersection analysis of all three datasets and found 75 common loci (5607 Ring1b bound and 2605 H2AK119Ub bound) (Supplementary Figs. 7C and 8A and Supplementary Data 4). Analyses of loci with enhanced binding (Supplementary Figs. 7C and 8A and Supplementary Data 4) as well as those encoding cell cycle regulators (Supplementary Data 4) associated with decreased association of Bmi1, Ring1b and H2AK119Ub in Vav3−/− leukemic B-cell progenitors (Fig. 5A–C; Supplementary Fig. 9A, B; and Supplementary data 7). These data further supports the role of Vav3 in the regulation of PRC1-dependent processes such as Cdkn2a/2b expression and cell cycle progression described above (Figs. 1H, J and 2B–E, and Supplementary Fig. 10A). They also suggest that the regulation of the foregoing loci at the epigenetic level can represent a plausible mechanism to explain the loss of heterozygosity in leukemic B-cell leukemias which frequently contain heterozygous deletions of only one of Cdkn2a/b alleles.43,44 Conversely, we also found increased Bmi1 presence in 345 loci (Supplementary Figs. 7C and 8A and Supplementary Data 4) as well as a higher number of genes associated with increased Ring1b (535 loci) and H2AK119Ub binding (3506 loci) in cells lacking Vav3 expression (Supplementary Figs. 7D, E and 8B, C and Supplementary Data 5 and 6). Analyses of loci with enhanced Bmi1, Ring1b and H2AK119Ub binding in those cells identified 50 common loci (p = 4.5 × 10−48) with direct or RNA pol-II binding dependent transcription regulatory activities (Fig. 5D–F, Supplementary Figs. 9C, D and 10B, and Supplementary data 8). We confirmed reduced expression of the tumorigenic transcription factors Id1, Zfhx3, Meis2, and Blhle22 in Vav3-deficient cells (Supplementary Fig. 10C), indicating that the increased binding of PRC1.4 complex to these loci is also linked to the repression of these leukemogenic genes in Vav3−/− leukemic B-cell progenitors.

The Vav3/Phlp2/Akt axis modulates nuclear Bmi1 phosphorylation and PRC1 activity. It has been shown that the phosphorylation of Bmi1 on S174 by activated Akt leads to the disassembly of Bmi1 from PRC1 complexes and the ensuing derepression of Cdkn2a52. To understand how the Vav3 deficiency ablates the oncogenic effect of Bmi1, de-represses the genes associated with proliferation and differentiation, and results in the rewiring of Bmi1 binding to genes involved in self-renewal, we next decided to compare the expression and phosphorylation levels of Bmi1 in p190-BCR-ABL+ WT and Vav3−/− B-cell progenitors. Using these analyses, we found that the loss of Vav3 results in a 59 ± 4.9% (p < 0.01) increase in phospho-S174 Bmi1 protein levels in the nuclear fraction of p190-BCR-ABL+ Vav3−/− B-cell progenitors (Fig. 6A). Although total Bmi1 expression remains unchanged, Vav3 deficiency was associated with shuttling of Bmi1 from being bound to chromatin to the nuclear matrix (Fig. 6B). This increased phosphorylation level correlated with enhanced expression of Cdkn2a, Cdkn2b, and Pax5 transcripts in Rac2−/− leukemic B-cell progenitors (Supplementary Fig. 11A, B). Together, these data indicate that Vav3−/Rac2 activity is required to ensure nuclear expression and chromatin binding of Bmi1.

To further examine if the deficiency of VAV3 leads to increased BM1-1 phosphorylation and growth arrest of human B-cell precursor leukemias, we silenced VAV3 in four independent Ph+ human B-precursor leukemia cell lines (BV-173, NALM-1, TOM-1, and SUP-B15)53–56. These cells contain additional secondary mutations in addition to those targeting the BCR and ABL loci. Similar to primary cells, we found that VAV3 expression is predominantly nuclear in these B-ALL cell lines (Supplementary Fig. 12A). More importantly, we found that upon downregulation of VAV3, the residual expression of VAV3 inversely correlates with BM1I phosphorylation (Supplementary Fig. 12B). The silencing of VAV3 also leads to reductions in the frequency of CBF-blast formation and the ability to expand in liquid culture (Supplementary Fig. 12C, D). To verify that the Vav3−Rac2 axis is involved in the down-modulation of the dephosphorylated status of Bmi1, we transduced WT and Vav3−/− leukemic B-cell progenitors with either wild-type or a phosphorylation-deficient version (S314A) of Bmi1 using a lentiviral delivery method (Supplementary Fig. 13A). As anticipated, the expression of Bmi1(S314A) rescues the leukemic cell growth (Fig. 6C), H2AK119 ubiquitination (Supplementary Fig. 13A), and the repression of Cdkn2a of Vav3−/− p190 BCR-ABL+ B-cell progenitors (Fig. 6D).

To further understand this process, we analyzed the levels of activated Akt (phospho-S473) in the cytoplasmic and nuclear fractions of both WT and Vav3−/− leukemic B-cell progenitors. We observed that Akt preferentially localizes in the cytoplasmic fraction regardless of the genotypes of the cells analyzed (Fig. 6E). However, we found 6.0 ± 1.1 higher levels of activated Akt in the nuclear fraction of Vav3−/− leukemic B-cell progenitors when compared to the wild-type controls (p < 0.001; Fig. 6E). The allosteric Akt specific inhibitor MK2206 reduces Bmi1 phosphorylation found in Vav3-deficient cells (Fig. 6F), indicating that the enhanced expression of phospho-Bmi1 found in these cells depends on nuclear Akt activation. Akt activation is...
downregulated by the concurrent action of the protein phosphatase 2A (Pp2a) and the pleckstrin homology domain leucine-rich repeat protein phosphatase 2 (Phlpp2) that target the phosphorylated T308 and S473 residues of Akt, respectively\textsuperscript{57,58}. We observed reduced levels (40 ± 2\%, \textit{p} < 0.01) of Phlpp2 in the nuclear fraction of Vav3\textsuperscript{−/−} leukemic B-cell progenitors (Fig. 6E). This change is not observed at the transcript level (Supplementary Fig. 13B), indicating that this phosphatase is regulated at the posttranscriptional level. The expression of Pp2a is also reduced in the absence of Vav3 (41 ± 15\%, \textit{p} < 0.05, \textit{n} = 2). However, in this case, we could not observe any variation in the levels of phospho-Akt(T308) in Vav3\textsuperscript{−/−} leukemic progenitor nuclear fraction (Supplementary Fig. 13C). This suggest that Pp2a is not likely involved in this regulatory step. Further strengthening the
Fig. 4 Deficiency of Vav3 abrogates oncogenic effect of Bmi1 over-expression in leukemic B-cell progenitors. A Wild type or Vav3−/− LDBM cells co-transduced with p190-BCR-ABL retroviruses and empty or Bmi1 lentiviruses were transplanted into lethally irradiated C57Bl/10 mouse for the development of p190-BCR-ABL-induced B-cell acute lymphoblastic leukemia (B-ALL). B Representative Immunoblots for Bmi1, Parp and Gapdh in the cytoplasmic and nuclear extracts of B-cell progenitors derived from p190-BCR-ABL retrovirus and empty or Bmi1 lentivirus co-transduced and transplanted leukemic mice (n = 10 mice per group). C Kaplan-Meier overall survival analyses of primary recipient mice transplanted with WT or Vav3−/− LDBM cells (10^6 cells/mouse) co-transduced with p190-BCR-ABL retroviruses and empty or Bmi1 lentiviruses. Vav3 deficiency resulted in significant delay in chimeric mouse death. Bmi1 overexpression in WT, but not of Vav3−/−, leukemic cells resulted in significantly decreased latency to chimeric mouse death. D Kaplan-Meier survival analyses (90-days) of secondary recipient mice transplanted with 10^4 (dotted lines, black-WT empty; gray-Vav3−/− empty; light blue-Vav3−/− Bmi1), 3 × 10^4 (dashed lines, black-WT empty; gray-Vav3−/− empty; light blue-Vav3−/− Bmi1), and 10^5 (solid lines, black-WT empty; gray-Vav3−/− empty; light blue-Vav3−/− Bmi1) leukemic B-cell progenitors derived from primary leukemic mice. Vav3 deficiency significantly prolongs the survival. No significant difference in survival between Vav3−/−/+ empty and Vav3−/−/+ Bmi1 at any of the three cell doses tested was found (n = 10 mice per group). E Quantification of BrdU uptake of WT and Vav3−/− B-cell progenitors co-expressing p190-BCR-ABL and Bmi1. Vav3 deficiency partially impairs the Bmi1 overexpression effect (n = 3 per group). F Serial plating of CFU-proB showing abrogation of CFU generating ability of Vav3 deficient empty or Bmi1-transduced p190-BCR-ABL expressing B-cell progenitors. Representative immunoblots of H2AK19Ub, and β-actin in empty or Bmi1 over-expressed WT or Vav3−/− leukemic B-cell progenitors (n = 3 per group). H2AK119Ub

Fig. 5 Nuclear Vav3 modulates PRC1 mediated repression of regulators of proliferation and transcriptional factors. A Venn diagrams depicting intersection of genes where CUT&RUNseq signal for Bmi1, Ring1b, and H2AK119Ub was decreased in Vav3−/− leukemic B-cell progenitors (Diffbind: Log2 Fold Change>1, p < 0.05). B Gene-ontology analyses of molecular and biological function of the genes with reduced Bmi1, Ring1b, and H2AK119Ub binding. Genes associated with negative regulation of protein kinase activity, negative regulation of protein phosphorylation, and cell cycle G1/S transition show reduced PRC1 components binding. C Representative density map of 75 common genes with decreased binding of Bmi1, Ring1b, and H2AK119Ub in Vav3−/− leukemic B-cell progenitors. Tracks shown are for Bmi1 binding. Diffbind analyses were performed between two CUT&RUN replicates of WT and Vav3−/− leukemic B-cell progenitors, and one representative example density map is presented. D Venn diagrams showing intersection of genes where binding of Bmi1, Ring1b, and H2AK119Ub in Vav3−/− leukemic B-cell progenitors was increased (Diffbind: Log2 Fold Change>1). E Gene-ontology analyses of molecular and biological function of the genes with increased Bmi1, Ring1b, and H2AK119Ub binding. Genes associated with RNA polII transcription regulatory region-specific DNA binding, transcription factor regulator activity show increased PRC1 components binding. F Representative density map of 50 common genes with increased binding of Bmi1, Ring1b and H2AK119Ub in Vav3−/− leukemic B-cell progenitors. Tracks shown are for Bmi1 binding. Diffbind analyses was performed between two CUT&RUN replicates of WT and Vav3−/− leukemic B-cell progenitors, and one representative example density map is presented.

signaling connection between Vav3 and Phlp2, we found that these two proteins are in close nuclear proximity (Fig. 6G). Such a proximity is not found between Bmi1 and Phlp2, indicating that it is unlikely that Phlp2 itself regulates Bmi1 phosphorylation (Supplementary Fig. 13D). These data support the concept that Vav3 associates with Phlp2 to maintain low levels of Akt and Bmi1 phosphorylation in the nucleus of B-ALL progenitors.
Ectopic expression of Phlpp2 restores nuclear Bmi1 expression and leukemogenesis of Vav3-deficient leukemic B-cell progenitors. To understand the role of Phlpp2 in Vav3-dependent B cell leukemogenesis, we isolated Lineage− /− c-Kit+/Sca-1+/ (LSK) bone marrow progenitors from WT or Vav3−/− mice that, after transductions with p190-BCR-ABL-encoding retroviruses plus either control or Phlpp2-expressing lentiviruses, were transplanted into C57/Bl10 congenic recipients (Fig. 6H). Mice transplanted with BCR-ABL+/LSK cells transduced with Phlpp2-expressing and control lentiviral particles develop B-ALL with a median survival of 50 and 45 days, respectively (Fig. 6I). As expected, mice transplanted with Vav3−/− LSK co-transduced with p190-BCR-ABL and empty vector survive significantly longer (median survival 100 days) than the previous ones. However, the ectopic expression of Phlpp2 in those cells abrogates the effect of the Vav3 deficiency, leading to a significant shortening of the median time of death down to 70 days (Fig. 6I). The overexpression of Phlpp2 also restores the clonogenicity of Vav3−/− leukemic B-cell progenitors (Fig. 6J) as well as normal levels of Bmi1 phosphorylation, H2AK119 ubiquitination, and
**Discussion**

In the present study, we demonstrate that Vav3 becomes unexpectedly upregulated and activated in the nucleus of both human and murine lymphoblastic leukemia progenitors upon expression of BCR-ABL. Furthermore, we have shown that the exchange activity of Vav3 found in the nucleus is important for the proliferation and progression of p190-BCR-ABL+ leukemia using a PRC1- and to a lesser extent PRC2-dependent mechanism. Leukemic B-cell progenitors lacking Vav3 have impaired PRC1.4 activity associated with increased phosphorylation and inactivation of Bmi1. We have previously reported the anti-apoptotic activity of VAV3 and its targeting by a Phlpp2 phosphatase Phlpp2. Phlpp2 was found not to be in direct proximity with p190-BCR-ABL retroviruses and empty or Phlpp2 lentiviruses followed by transplantation into C57Bl/10 mice. Kaplan-Meier survival analyses of primary recipient mice transplanted with WT or Vav3−/− mice showed significant prolongs the survival. Phlpp2 overexpression in Vav3−/−/B-cell progenitors restores leukemogenesis. The anti-proliferative and anti-survival content of empty vector or Phlpp2 transduced WT and Vav3−/−/leukemic B-cell progenitors (n = 3 per group). In WT and Vav3−/−/leukemic B-cell progenitors transduced with empty or Phlpp2 lentiviruses.

Bmi1 deletion accelerates B-cell differentiation through upregulation of lymphoid specification genes and the transcriptional repressor activity of Bmi1 is required for normal and leukemic stem cell self-renewal. Bmi1 phosphorylation on residue S314 by Akt leads to its inactivation and its dissociation from the PRC1 complex resulting in de-repression of Cdkn2a locus. This effect is residue specific because the phosphorylation of Bmi1 on other Akt-dependent sites S251, S253, and S255 results in Bmi1 activation. We found increased Bmi1 phosphorylation on S314 in the absence of expression of Vav3 or Rac2, indicating that Vav3/Rac2 prevent Bmi1 phosphorylation. Increased phosphorylation of Bmi1 is associated with increased Akt activation in the nucleus and reduced level of phospho-Akt(S473) specific protein phosphatase Phlpp2. Phlpp2 was found not to be in direct proximity to Bmi1 suggesting that Phlpp2 is unlikely to be acting directly on Bmi1 phosphorylation. Overexpression of Phlpp2 or the mutant Bmi1 (S314A), but not wild-type Bmi1, results in the restoration of leukemogenesis and transcriptional repression. The mechanisms by which Vav3 controls the nuclear expression of the tumor suppressor Phlpp2 remain unclear but Phlpp2 stabilization by p27kip1, a cyclin-dependent kinase inhibitor upregulated by BCR-ABL in leukemic progenitors, has been highlighted and it is possible that Vav3 participates in the stabilization of Phlpp2.

Nuclear Rac plays a critical role in chromatin modifications and gene expression and downstream actin polymerization plays a major role in gene relocalization between heterochromatin and euchromatin that controls differentiation of embryonic stem cells. Rac and its downstream target Wave are required for transcriptional reprogramming and polymerized actin has strongly suggest that Vav3-dependent transcriptional repressive program is not universal, with a number of loci in which Vav3-dependent PRC1.4 repressive activity is probably replaced by alternative systems that activate PRC1.4 repressive activity in absence of Vav3.

Bmi1 is upregulated in patients with advanced stages of BCR-ABL-induced chronic myelogenous leukemia (CML) and through its upregulative activity on the Cdkn2a locus and other less well-characterized Cdkn2a independent activities results in leukemic transformation. Co-expression of Bmi1 and BCR-ABL in human cord blood CD34+ cells induces leukemia that can be propagated serially in immunodeficient mice with a bias towards lymphoid blast crisis. Our group has demonstrated that Bmi1 transforms and reprograms CML B-lymphoid progenitors into self-renewing, leukemia initiating cells. Bmi1 deletion accelerates B-cell differentiation through upregulation of lymphoid specification genes and the transcriptional repressor activity of Bmi1 is required for normal and leukemic stem cell self-renewal.
been shown to control polyclomb-mediated gene silencing\textsuperscript{70}. In leukemic progenitors, Rac2 suffices to phenocopy the effect of Vav3 deficiency while Rac1 is dispensable for BCR-ABL-induced proliferation\textsuperscript{71,72,73,74,75}. Along with our published data\textsuperscript{76}, this report supports a selective role for nuclear Vav3 and Rac2 in the regulation of PRC1.4 dependent transcription repression and reprogramming in B-cell leukemogenesis and suggests they control higher level nuclear structures of transcriptional repression with selectivity to act on specific loci. Anti-Bmi1 binding allowed us to establish sufficient signal/noise ratio to distinguish the most differentially bound loci in Vav3-deficient leukemic progenitors and identify 75 loci with reduced PRC1.4 binding/activity relevant to proliferation and differentiation of B-cell progenitors while other 50 loci, relevant in self-renewal, have increased PRC1.4 binding/activity. The mechanisms of this differential binding are unclear but it may arise on the selectivity of high-order chromatin structures for preferential binding and the use of alternative, non-canonical PRC1.4 complexes (reviewed in ref\textsuperscript{77}).

In summary, the results of the present study show that Vav3 predominantly resides in the nucleus of leukemic B-cell progenitors where it plays a pivotal role in Ph\textsuperscript{+} leukemogenesis by modulating PRC1.4 activity and is dependent on nuclear Phlp2-regulated Akt activity. This description of a nuclear GEF activity associated with an oncogenic polyclomb repression program, opens a whole area of research to better understand the role of nuclear Rho GTPases in leukemogenesis.

**Methods**

**Animals.** The generation of Vav3-deficient (Vav3\textsuperscript{-/-}) mice\textsuperscript{78} and Rac2-deficient (Rac2\textsuperscript{-/-}) mice\textsuperscript{79} have been described previously. All mutant mice were backcrossed >10 generations onto C57Bl/10 or C57Bl/6 mice, respectively. To avoid possible interference with androgen signaling, 6- to 8-week-old female wild-type (WT) C57Bl/10 and C57Bl/6 mice were obtained commercially (Jackson Laboratory, Bar Harbor, ME and Harlan Laboratories, Indianapolis, IN, respectively) and used as donors and/or recipients for transduction/transplantation models. All mouse strains were maintained at an Association for Assessment and Accreditation of Laboratory Animal Care accredited, specific-pathogen–free animal facility at Cincinnati Children’s Research Foundation, Cincinnati, under an Institutional Animal Care and Use Committee approved protocol. The transgenic mice used in the study were between 6 and 12 week of age at the time of experimentation.

**Human specimens.** Four B-ALL patient samples were obtained from The Pediatric Leukemia Avatar Program within the Cancer & Blood Diseases Institute (CBDI) at Cincinnati Children’s Hospital Medical center, under IRB approval and informed consent. All leukemic specimens contained more than 80% blasts and were collected at diagnosis or relapse. Genotyping characterization of these four leukemias had the following monoallelic mutations: the generation of Vav3-deleted (Vav3\textsuperscript{-/-}; Vav3\textsuperscript{-/-}) mice and the generation of Vav3-deleted (Vav3\textsuperscript{-/-}; Vav3\textsuperscript{-/-}) mice have been described previously. All mutant mice were backcrossed >10 generations onto C57Bl/10 or C57Bl/6 mice, respectively. To avoid possible interference with androgen signaling, 6- to 8-week-old female wild-type (WT) C57Bl/10 and C57Bl/6 mice were obtained commercially (Jackson Laboratory, Bar Harbor, ME and Harlan Laboratories, Indianapolis, IN, respectively) and used as donors and/or recipients for transduction/transplantation models. All mouse strains were maintained at an Association for Assessment and Accreditation of Laboratory Animal Care accredited, specific-pathogen–free animal facility at Cincinnati Children’s Research Foundation, Cincinnati, under an Institutional Animal Care and Use Committee approved protocol. The transgenic mice used in the study were between 6 and 12 week of age at the time of experimentation.

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leukemic BM cells or sorted p190-BCR-ABL expressing B-cell progenitors in M3134 methylcellulose (Stem Cell Technologies, Vancouver, BC), 2 mML-glutamine (Invitrogen, Waltham, MA), 1% antibiotics (penicillin-streptomycin; Invitrogen), 100 μM β-mercaptoethanol (Thermo Fisher Scientific, Waltham, MA), and 1% BSA (Sigma-Aldrich, St. Louis, MO). 

**Prostate and survival assays.** Survival and proliferation of B-cell progenitors were determined by in vivo BrdU uptake and annexin V binding assay, respectively. For in vivo proliferation analysis, mice were intraperitoneally injected 500 μg of BrdU 1 h after the last BM transfer and cells were harvested and counted for BrdU incorporation for the identification of B-cell progenitors (B220-APC-Cy7; CD45.2-PerCP-Cy5.5). The cells were fixed, permeabilized, and stained with APC-conjugated anti-Brdu antibody (α-Brdu-APC) according to the manufacturer’s protocol (BD Biosciences, San Jose, CA) and then FACS analyzed using a FACS Canto II analyzer (BD Biosciences). For FACS Canto II analysis, 4% BCR-ABL+ WT, Vav3−/− leukemic cells transduced with mock or Bmi1 or Bmi1(S314A) lentivector were cultured in 24-well plate, and cell growth was counted every 3rd or 4th day following initial seeding, and plotted. To evaluate the survival, freshly isolated BM cells from leukemic mice were stained for cell surface markers and the identification of B-cell progenitors (B220-APC-Cy7; CD45.2-PerCP-Cy5.5) and APC-conjugated annexin V Ab for 30 min at RT. Cells were washed to remove unbound antibodies, and FACS analyzed using a FACS Canto II analyzer (BD Biosciences).

**Western blot analyses and co-immunoprecipitation.** B-cell progenitors (FSCθ 84% B220+ IgM+ CD45+ CD19+) were sorted from BM cells of primary leukemic mice belonging to WT, Vav3−/−, Rac2−/− transduced with empty or Bmi1 or Phlp2b lentivector vectors. B-cell progenitors (hCD45+ hCD34+ hCD19+) from human B-ALL patient samples were FACS sorted. The EGFPP+ cells were sorted from Ntg shRNA or Vtg3 shRNAs lentiviral transduced B-ALL cell line Bm173 and NALM1. Freshly sorted murine progenitors, human B-cell progenitors, and VAV3 (or non-targeted, NTG) shRNA transduced B-ALL cell lines were processed for cytoplasmic and nuclear fractionation using NE-PER Nuclear and Cytoplasmic Extraction reagents as per manufacturer’s instruction (ThermoFisher Scientific). For nuclear fractionation, isolated nuclei were treated with chromatin isolation buffer (30 mM Tris-HCl, pH 7.9, 200 mM NaCl, 0.5 mM EDTA, 0.5% Triton-X 100) containing Protease inhibitor cocktail, lysed by pipette up and down, incubated on ice for 10 min, and then centrifuged for 5 min (8000 x g, 4 °C). The pellet contained the chromatin fraction and the supernatant contained the nuclear matrix which was depleted of chromatin. The cytoplasmic and nuclear fractions were dissolved in Laemmli buffer, boiled for denaturation, and electrophoresed through 4-15% SDS-PAGE gel followed by transfer to PVDF membrane. The membranes were blocked and treated with primary antibodies against Vav3/Va33 (rabbit polyclonal, from Dr. Bustelo’s laboratory, dilution 1:200 v/v) or -Bmi1 (anti-Bmi1 Ab O/N with constant shaking at 4 °C). The blot was then revealed using HRP conjugated secondary antibody, and the bands were visualized with ChemiDoc XRS+ Imaging System (Bio-Rad). All specific bands were quantitated by using ImageJ software, and normalized against Actin, Gapdh or Parp band intensity of the corresponding lane.

**Confocal immunofluorescence microscopy and proximity ligation assay.** Sorted murine B-cell progenitors derived from WT or Vav3−/− leukemic mice and human B-cell progenitors (patient samples were derived from a B-ALL patient (RetroNectin) containing t(11;19) were transduced with empty or Bmi1 (RetroNectin) co-transduced with mock or Bmi1 (RetroNectin) WT, Vav3−/− leukemic cells transduced with mock or Bmi1 or Bmi1(S314A) lentivector were cultured in 24-well plate, and cell growth was counted every 3rd or 4th day following initial seeding, and plotted. To evaluate the survival, freshly isolated BM cells from leukemic mice were stained for cell surface markers and the identification of B-cell progenitors (B220-APC-Cy7; CD45.2-PerCP-Cy5.5) and APC-conjugated annexin V Ab for 30 min at RT. Cells were washed to remove unbound antibodies, and FACS analyzed using a FACS Canto II analyzer (BD Biosciences).

**Rac activation assays.** WT and Vav3−/−, p190-BCR-ABL+ B-cell progenitors transduced with empty or Bmi1 lentivector were processed for the evaluation of activated Rac and GTPases in the nuclear fraction. Freshly isolated cells were subjected to cytoplasmic and nuclear fractionation. The cytoplasmic and nuclear fractions were dissolved in Laemmli buffer, boiled for denaturation, and electrophoresed through 4-15% SDS-PAGE gel followed by transfer to PVDF membrane. The PVDF membranes were subjected to western blot analyses as described above.

**Confluent immunofluorescence microscopy and proximity ligation assay.** Sorted murine B-cell progenitors derived from WT or Vav3−/− leukemic mice and human B-cell progenitors (patient samples were derived from a B-ALL patient (RetroNectin) containing t(11;19) were transduced with empty or Bmi1 (RetroNectin) co-transduced with mock or Bmi1 (RetroNectin) WT, Vav3−/− leukemic cells transduced with mock or Bmi1 or Bmi1(S314A) lentivector were cultured in 24-well plate, and cell growth was counted every 3rd or 4th day following initial seeding, and plotted. To evaluate the survival, freshly isolated BM cells from leukemic mice were stained for cell surface markers and the identification of B-cell progenitors (B220-APC-Cy7; CD45.2-PerCP-Cy5.5) and APC-conjugated annexin V Ab for 30 min at RT. Cells were washed to remove unbound antibodies, and FACS analyzed using a FACS Canto II analyzer (BD Biosciences).
Santa Cruz Biotechnology, Inc. Freshly passaged murine pre B-ALL Ba/F3 cells (2 x 10^7) were nucleofected with 2 μg of scramble or Bmi1 targeting gRNA containing 25%SPCR/Cas9 plasmid vector using SC Cell line 4D-Nucleofector X Kit and 100 ml nucleocuvette vessel (Lonza, Catalog V4XC-3012) and nucleofection program CM-147, as per manufacturer’s instruction. Following nucleofection, cell were immediately resuspended in fresh media and transferred to a 12-well non tissue culture plate, and 72 h post nucleofection the EGF-P (nucleofected) were used for western blot (anti-Bmi1 rabbit mAb) and confocal immunofluorescence microscopy (anti-Bmi1 Rabbit mAb at 21.7 nM concentration).

Transcriptome and bioinformatics analysis. Total RNA was extracted from sorted p190-BCR-ABL+ B-cell progenitors (FSC<ref>120</ref> EYFP<ref>120</ref>CD19<ref>120</ref> IgM<ref>120</ref>CD43<ref>120</ref>) derived from WT and Van3−/− leukemic primary mice using RNaseasy Mini Kit (QiAGEN; catalog no. 74104) following manufacturer’s instructions. cDNA was prepared using TaqMan reverse transcription reagent (Applied Biosystems, Life technologies, catalog no. 44080234). The mRNA expression levels of Cdkn2a, Cdkn2b, Pax5, Ebf1, Ikaros, and Phlp2p were measured using qRT-PCR (qRT-PCR) assay with TaqMan Universal PCR master mix and gene-specific TaqMan primers (Roche Applied Science, Life technologies). The expression level was normalized to the expression of internal control gene Gapdh.

Differential binding analysis and description for specific loci in Supplementary Figs. 10A-B included, in sequence, the integer score of each peak, the fold-change at peak summit and the statistical analysis (FDR) presented as -log10(p value at the peak summit). The correlation matrix of Bmi1, Ring1b, and H2AK119Ub CUT&RUN datasets for WT and Van3−/− was calculated based on the read coverages computed across the entire genome with the bin size of 5 Kb (Pearson’s r test). All calculations were made in deepTools package using multibamSummary and ploCorrelation command.<ref>38</ref>

Differentially Bmi1, Ring1b and H2AK119Ub bound sites between WT and Van3−/− leukemic B-cell progenitors (n = 2/group) were identified by using DifBind- Differential Binding Analysis of ChiP-Seq Peak Data - 1.0.0<ref>39</ref> pipeline attached to ScIdAP platform, using "Deseq2" analyses method. Only significant differential in values between sites with p < 0.05 and with a minimum EDF change (LogFold Change ≥ 2 and ≤ 1) were reported. Based on this, all differential peaks were divided into two groups: (1) LogFC ≥ 2, (2) LogFC ≤ 1. Each peak group was cleaned from duplicates based on the peak start and end coordinates and centered by peak center. Re-centered peaks were used for generating tag-density plots within 20 kb radius from peak center with "Homer". For gene TSS-centered tag density plots, each peak was assigned to the nearest gene within 20 kb radius from TSS. The resulting two groups of genes were deduplicated and intersected based on the gene names thus obtaining three groups of genes. Genes from every group were re-centered on the TSS and used for generating tag-density heatmap within 20 kb radius from gene TSS with Homer. The tag density maps were generated using https://software.broadinstitute.org/morpheus/. The representative genome browser map of specific loci was obtained from IGV browser in "SciDAP" platform. The Venn diagram of genes with differential Bmi1, Ring1b and H2AK119Ub binding between WT and Van3−/− leukemic B-cell progenitors were generated using online tool "Multiple List Comparator" (https://www.molbiotools.com/morecompare.php). The significance of differentially bound loci from Van3−/− leukemic B-cell progenitors in comparison to their WT counterparts were subjected to gene ontology analyses (molecular and biological functions and pathway analyses) using ToppGene Suite (https://toppgene.cchmc.org/enrichment.jsp) and confirmed using bioinformatics platform DAVID (Database for Annotation, Visualization and Integrated Discovery, v6.8<ref>40</ref>).

Whole exome sequencing. For whole exome sequencing, genomic DNA from p190-BCR-ABL-induced leukemic mice [WT (n = 4) and Van3−/− (n = 4)] were isolated following lysis of cells in DNA extraction buffer (5 mM EDTA, pH-8; 200 mM NaCl; 10 mM Tris, pH-8; 0.2% SDS, and 20 mg/ml proteinase K), precipitated with 100% isopropanol and washing with 70% ethanol. Following an initial Quality Control evaluation, 50 ng of genomic DNA for each sample was used for NGS library preparation and multiplex exome enrichment (both Twist Biosciences). Libraries were evaluated for quantity and quality and pooled together for sequencing on an Illumina Novaseq 6000 instrument. Each sample targeted 30M PE-100 read pairs.

Raw sequencing data was aligned to the mm10 genome with BWA-MEM version 0.7.17<ref>41</ref> using the non-default parameter “-Y”. Alignment files were sorted and duplicate reads identified with the bamsammpad program found in the bioconductor suite of tools (version 2.0.87). Variants were called with GATK4 v4.1.8.0. The HaplotypeCaller tool was first used to create gvcfs for each sample with parameters “-max-alternate-alleles 3 -ip 100” and the bed containing capture regions provided by the manufacturer. Finally, variants were genotyped with the GenotypeGVCFS tool. In order to achieve maximal sensitivity, SNPs were not filtered and the default calling thresholds were set using GATK. However, indels were filtered with bcftools v1.10.2 and the expression of “TYPE != "snp" & &QD < 20.0 || ReadPosRankSum < -20.0 || FS > 200.0 || SOR > 10.0”. Finally, gene annotations and variant consequences were annotated using the Ensembl REST web server. Copy number variation was analyzed using the CNVKit tool<ref>42</ref> and the UCSC Reference Genome Browser database (http://genome.ucsc.edu). CSTB/murine reference was used for alignment and data was filtered for clinically relevant loci.

Statistical analysis. Quantitative data is given as mean ± standard deviation (SD). Statistical significance was determined using the unpaired Student-t or Anova tests and differences in survival were examined using the log-rank P test. A value of p < 0.05 was considered to be statistically significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The authors declare that all data supporting the findings of this study are available, to the best of our effort, within this manuscript and supplementary information files. Source data are provided with this paper. DNA sequencing data can be accessed at BioProject PRJNA657836. RNA and CUT&RUN sequencing data can be accessed at GEO dataset GSE196378. Source data are provided with this paper.
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R.C.N., K.H.C., A.S., M.D., A.M.W., M.W., J.B., B.M., and M.C. performed experiments and generated crucial reagents. M.K., P.D., and A.B. performed bioinformatic analyses. R.C.N. and J.A.C. analyzed data and wrote the manuscript, R.C.N., K.H.C., M.D., and J.A.C. designed experiments. X.B.R. and N.N. providing insightful views and critiques and edited the manuscript. J.A.C. led the group and supervised their overall work.

Competing interests
A.B. is a co-founder of Datium, LLC. Datium developed SciDAP bioinformatics platform used for data analysis in this study. Other authors have no relevant conflicts of interest to declare.

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