Cyclin C promotes development and progression of B-cell acute lymphoblastic leukemia by counteracting p53-mediated stress responses

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Abstract

Despite major therapeutic advances in the treatment of acute lymphoblastic leukemia (ALL), resistances and long-term toxicities still pose significant challenges. Cyclins and their associated cyclin-dependent kinases are one focus of cancer research when looking for targeted therapies. We discovered cyclin C to be a key factor for B-cell ALL (B-ALL) development and maintenance. While cyclin C is not essential for normal hematopoiesis, *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{+} B-ALL cells fail to elicit leukemia in mice. RNA sequencing experiments revealed a p53 pathway deregulation in *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{+} cells resulting in the inability of the leukemic cells to adequately respond to stress. A genome-wide CRISPR/Cas9 loss-of-function screen supplemented with additional knock-outs unveiled a dependency of human B-lymphoid cell lines on *CCNC*. High cyclin C levels in B-cell precursor (BCP) ALL patients were associated with poor event-free survival and increased risk of early disease recurrence after remission. Our findings highlight cyclin C as a potential therapeutic target for B-ALL, particularly to enhance cancer cell sensitivity to stress and chemotherapy.

Introduction

The Philadelphia (Ph) chromosome, a product of the reciprocal translocation t(9;22)(q34;q11) between chromosomes 9 and 22, encodes the BCR::ABL1 fusion oncoprotein.[1] The constitutively active BCR::ABL1 tyrosine kinase is a hallmark of chronic myeloid leukemia (CML) and drives a subset of acute lymphoblastic leukemia (ALL). The incidence of Ph positive (Ph^{+}) ALL correlates with age, from only 3% in pediatric ALL to around 25% in older adults.[2] Direct targeting of the BCR::ABL1 kinase with tyrosine kinase inhibitors (TKI) has been a breakthrough in targeted cancer therapy. Despite efforts to counteract TKI resistance and improve safety profiles, refractory BCR::ABL1^{+} leukemia, as well as toxicities and long-term side effects of TKI, present particular therapeutic challenges.[3-5]

The clinical relevance of cyclins and their associated cyclin-dependent kinases (CDK) has been a major focus of research for several years. Cyclin-CDK complexes do not

only drive the cell cycle, but are also important players in various other cellular processes including transcriptional and epigenetic regulation, metabolism or stem cell self-renewal.[6] In line with their importance in different pathways, cyclin-CDK complex dysregulation is implicated in many different types of cancer.[7]

Cyclin C belongs to the transcriptional cyclins, with its most notable role being the activating partner of the CDK8/19 serine / threonine kinases. Both CDK8 and cyclin C are essential for embryonic development, as transgenic mice lacking either protein are not viable while deletion in adult mice is generally well tolerated.[8-10] The cyclin C-CDK8 kinase complex can regulate transcription by phosphorylation of different substrates including histone H3,[11] the NOTCH intracellular domain,[8,12] STAT transcription factors,[13] and SMAD proteins.[14] In addition, cyclin C plays a transcriptional role as a member of the CDK8/19 kinase module (CKM) of the Mediator, a regulatory subunit that acts as modulator of the interaction between the large Mediator complex and RNA Polymerase II and consists of MED12/12L, MED13/13L, CDK8/19, and cyclin C.[15] Apart from its transcriptional function, some studies also report that cyclin C in complex with CDK1/2/3 has a role in cell-cycle regulation.[8,16] In addition, non-canonical functions of cyclin C in mitochondrial fragmentation have been elucidated which are separate from its binding to CDK.[17,18] The diverse pathways through which cyclin C operates might also account for its highly context-dependent role in cancer; for example, in T-ALL and osteosarcoma it functions as a tumor suppressor.[8,19] In thyroid tissue, cyclin C co-operates with PTEN to suppress cancer development.[20] Conversely, *CCNC* amplification is associated with poor survival in colon adenocarcinoma[21] and high *CCNC* levels correlate with poor relapse-free survival in breast cancer patients.[22,23] Cyclin C-specific roles in B-cell malignancies have not yet been investigated.

Understanding the mechanisms driving the progression of B-cell acute lymphoblastic leukemia (B-ALL) is a fundamental prerequisite for developing innovative pharmacological inhibitors and refining treatment strategies. In this study, we investigated the role of cyclin C in the development and maintenance of B-ALL and demonstrate its pivotal oncogenic role in leukemic cells both *in vitro* and *in vivo*.

Methods

Study approvals

All experiments were conducted with gender- and age-matched 6-12-week old mice in accordance with the Ethics and Animal Welfare Committee of the University of Veterinary Medicine, Vienna, Austria, and the national authority, according to the 2012 Animal Experiments Act (Tierversuchsgesetz) (licence numbers: BMWFW-68.205/0093-WF/V/3b/2015, 2022-0.404.452 and BMBWF-68.205/0174-V/3b/2018), and according to the guidelines of the FELASA and ARRIVE.

Leukemia monitoring

To monitor leukemia development over time, gender- and age-matched 8-12-week old NOD *scid* gamma (NSG) mice were injected with 2,500 BCR::ABL1^{p185+} cells via the tail vein. Mice were monitored daily and sacrificed upon manifestation of disease symptoms (hindleg paralysis, hunched posture, decreased mobility, weight loss). Alternatively, leukemic cell infiltration at a specific time point was assessed by sacrificing the whole cohort of transplanted mice 26 days post injection.

Patient data analysis

For comparison of *CCNC* levels in leukemia patients, the preparation of the RNA sequencing (RNA-Seq) libraries and the pre-processing of sequencing data was carried out as previously described at the MLL Munich Leukemia Laboratory.[24] Bone marrow (BM) mononuclear cells from healthy donors served as controls. Raw counts were normalized by applying the Trimmed mean of M-values method from the edgeR package,[25] producing $\log_2$ CPM values.

To determine the event-free survival (EFS) probability for patients with high *versus* low *CCNC*-expressing B-cell precursor (BCP) ALL, expression levels were analyzed in a cohort of 573 pediatric BCP-ALL patients described by van der Veer *et al.*[26] Expression of *CCNC* at first diagnosis was determined with Affymetrix U133 plus2.0 gene expression microarrays using probeset 201955_at after vsnrma normalization.[27] Expression data are published by Polak *et al.*[28] and have been deposited at GEO (accession number: GSE87070). Median expression among BCP-ALL samples was used as high/low cut-off for *CCNC* expression.

Results

Malignant B-lymphoblastic cells depend on cyclin C

To analyze the impact of deleting components of the cyclin C-CDK8/19 kinase complex, we queried the genome-wide CRISPR/Cas9-based loss-of-function screens performed for the Cancer Dependency Map (DepMap) Project and investigated the effects of *CCNC*, *CDK8* or *CDK19* knock-out across a panel of 1,095 human cancer cell lines (Figure 1A). Among these 1,095 cell lines, 258 cell lines exhibited a dependency on cyclin C, whereas only 50 displayed a dependency on CDK8 and none were reliant on CDK19. Notably, the loss of *CCNC* had a pronounced impact on distinct cell lines, particularly those associated with plasma cell myelomas and cell lines of lymphoid origin, specifically B-cell lymphoblastic leukemia / lymphoma cell lines (Figure 1B, *Online Supplementary Figure S1A*). The dependency of plasma cell myelomas on cyclin C was accompanied by a dependency on CDK8 while leukemic cells were not among the enriched lineages for CDK8 dependency (*Online Supplementary Figure S1B*). This prompted us to investigate the role of cyclin C in the development and progression of B-cell leukemia and

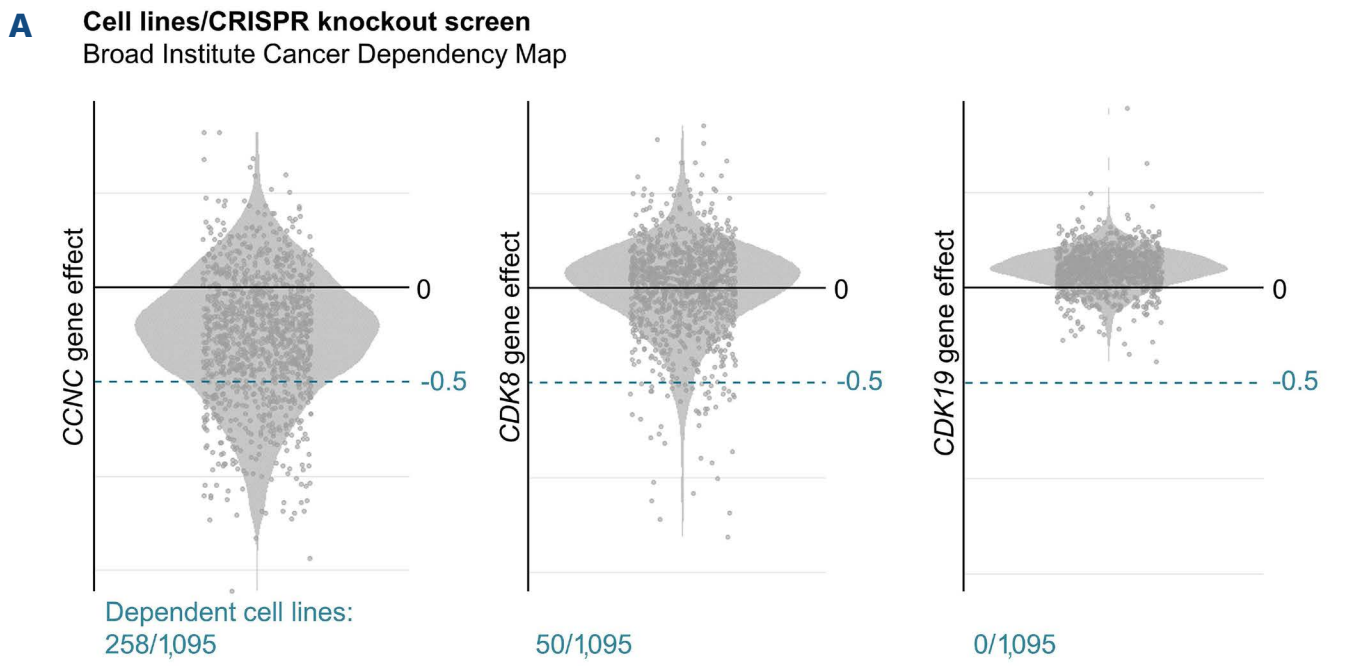

Figure 1. Malignant B-lymphoblastic cells depend on cyclin C. (A) CRISPR dependencies for *CCNC*, *CDK8* and *CDK19* in a panel of human cancer cell lines obtained from the Broad Institute Cancer Dependency Map (DepMap). Dots correspond to individual cell lines (N=1,095), y-axis represents CRISPR dependency score (DepMap Public 23Q2+Score, Chronos). Negative gene effect scores imply dependency of a cell line on a given gene as gene knock-out results in impaired cell line growth and/or death. Scores are normalized so that all non-essential genes have a median score of 0 and all common essential genes have a median score of -1. Cell lines with probability of dependency >0.5 are considered dependent. (B) Representation of the top 8 significantly enriched lineages (P<0.0005, *t* test) in the Depmap CRISPR dependency screen for *CCNC*. The number of cell lines included in each lineage subset is denoted in parentheses. Ranking was based on effect size.

to validate its potential as novel therapeutic vulnerability.

Cyclin C is not essential for normal hematopoiesis

We first studied the consequences of cyclin C deficiency for hematopoiesis and crossed conditional *Ccnc*$^{fl/fl}$ mice[8] with *VavCre*[29] mice. *Ccnc*$^{fl/fl}$ *VavCre*$^{-/-}$ (*Ccnc*$^{fl/fl}$) and *Ccnc*$^{fl/fl}$ *VavCre*$^{+/-}$ (*Ccnc*$^{fl/fl}$ *VavCre*) mice were born at the expected Mendelian ratio (*Online Supplementary Figure S2A*) and the ablation of cyclin C in BM and spleens of *Ccnc*$^{fl/fl}$ *VavCre* mice was confirmed via immunoblotting (Figure 2A). Total cell counts in BM and spleen remained unchanged by *Ccnc* deletion (*Online Supplementary Figure S2B*) and peripheral blood analysis indicated comparable numbers of white and red blood cells, as well as platelets (*Online Supplementary Figure S2C, D*). Flow cytometric analyses failed to detect any alterations in the numbers of Lin^{-}Sca-1^{+}c-kit^{+} (LSK) cells and progenitor cell subsets in *Ccnc*$^{fl/fl}$ *VavCre* animals (Figure 2B, C and *Online Supplementary Figure S2E, F*).

As the analysis of human cell lines indicated a role of cyclin C in malignancies of B-lymphoid origin, we conducted a detailed analysis of B-cell development in *Ccnc*$^{fl/fl}$ *VavCre* mice. We failed to detect any alterations in B-cell developmental stages starting from common lymphoid progenitors (CLP) in the BM (Figure 2D, *Online Supplementary Figure S2G*). In essence, cyclin C deficiency in the hematopoietic system is well tolerated.

Cyclin C plays a pivotal role in the oncogenic transformation and immortalization of BCR::ABL1^{p185+} B-cell acute lymphoblastic leukemia

To investigate the role of cyclin C in B-ALL development and maintenance, we focused on Ph^{+} leukemia which represents the most common chromosomal aberration in adult ALL patients,[30] and utilized well-established murine

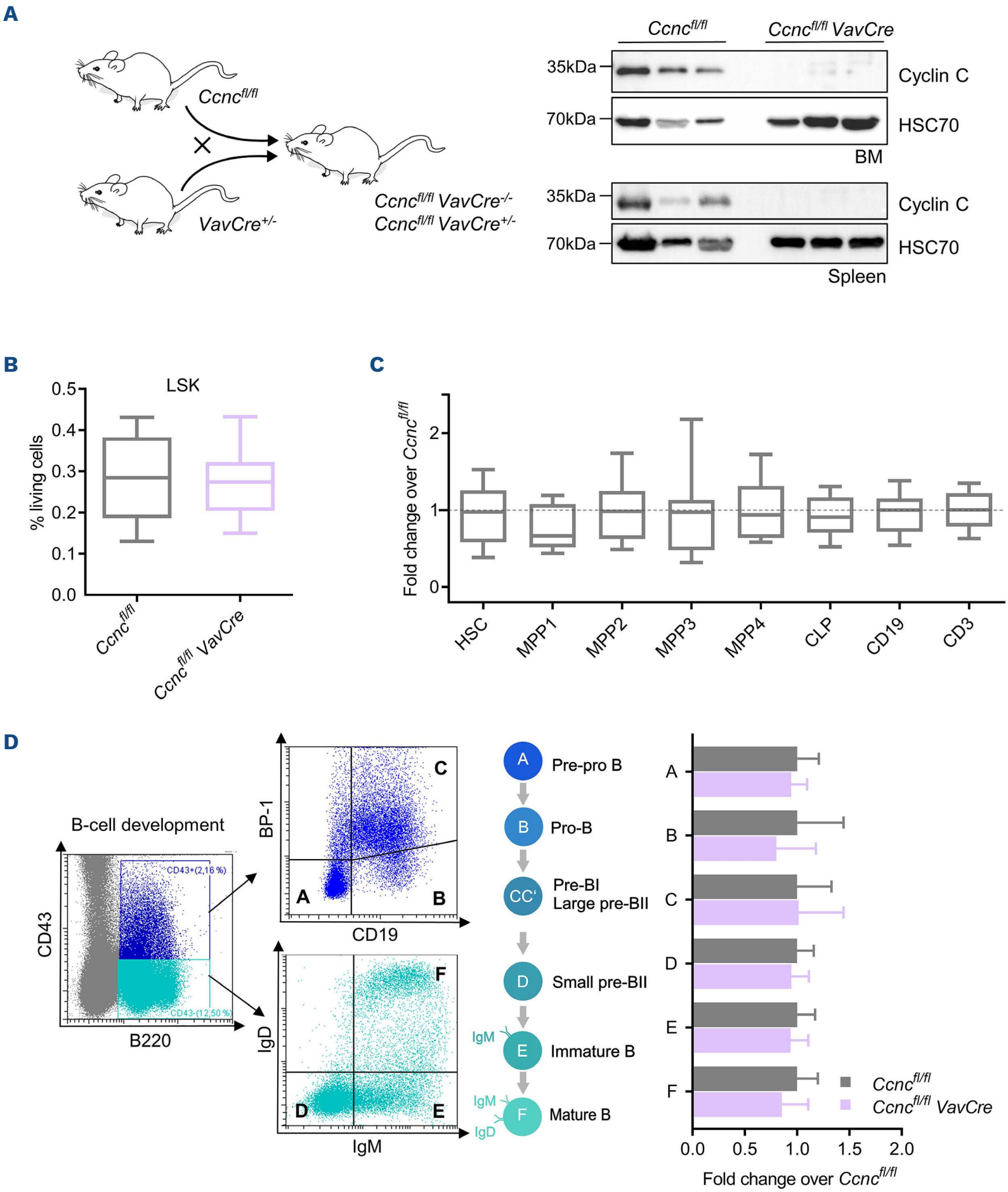

Figure 2. Cyclin C is not essential for normal hematopoiesis. (A) (Left) Breeding scheme for the generation of *Cncᶠˡ/ᶠˡ VavCre*$^{-/-}$ (*Cncᶠˡ/ᶠˡ*) and *Cncᶠˡ/ᶠˡ VavCre*$^{+/-}$ (*Cncᶠˡ/ᶠˡ VavCre*) mice. (Right) Cyclin C immunoblot analysis of bone marrow (BM) and spleen from *Cncᶠˡ/ᶠˡ* and *Cncᶠˡ/ᶠˡ VavCre* mice (N=3 per genotype). HSC70 served as loading control. (B) Lin–Sca-1^{+}c-kit^{+} (LSK) frequencies in BM of *Cncᶠˡ/ᶠˡ* (N=12) and *Cncᶠˡ/ᶠˡ VavCre* (N=11) mice, one representative out of 2 independent experiments is shown. (C) Relative fold change of BM subpopulations from *Cncᶠˡ/ᶠˡ VavCre* normalized to the mean value from *Cncᶠˡ/ᶠˡ* mice (N=9-12). (D) (Left) Representative flow cytometry plots depicting the gating scheme for early B-cell development populations according to expression of the markers CD43, B220, BP-1, CD19, IgM, IgD and (right) summary of frequencies in the BM of *Cncᶠˡ/ᶠˡ* (N=12) and *Cncᶠˡ/ᶠˡ VavCre* (N=10) mice normalized to mean values from *Cncᶠˡ/ᶠˡ* mice. Details on flow cytometric analyses are provided in the *Online Supplementary Methods*. Bar graphs represent mean ± Standard Deviation. Box and whiskers plot center values represent median, the box 25th to 75th percentiles, and whiskers minimum to maximum. (B-D) Levels of significance were calculated using Mann-Whitney U test. HSC: hematopoietic stem cells; MPP: multipotent progenitor population; CLP: common lymphoid progenitors.

models which reliably and specifically promote B-ALL development. A retroviral pMSCV-*Bcr-Abl1-p185*-IRES-eGFP construct was used to transform BM cells isolated from *Ccnc*$^{fl/fl}$ and *Ccnc*$^{fl/fl}$ *VavCre* mice. In contrast to CDK8 deficiency,[10] absence of cyclin C impaired the initial leukemic transformation, as evidenced by a significantly reduced number of *Ccnc*$^{\Delta/\Delta}$ colonies derived from BM of *Ccnc*$^{fl/fl}$ *VavCre* mice in growth factor-free methylcellulose (Figure 3A). Primary BM cultured in methylcellulose supplemented with interleukin-7 (IL-7) served as control and proved that cytokine-dependent colony growth of non-transformed cells is unaffected by cyclin C deletion (Figure 3B). Cancer progression is a multistep process which requires cancer cells to overcome intrinsic checkpoint mechanisms for tumorigenesis.[31] To test the potential for immortalization, individual clones were picked and the outgrowth of stable cell lines in fetal calf serum (FCS)-supplemented medium was monitored. Cyclin C ablation significantly decreased the ability to form monoclonal cell lines, indicative of its impact on immortalization (Figure 3C). To control the experimental setting, we also directly infected BM from *Ccnc*$^{fl/fl}$ and *Ccnc*$^{fl/fl}$ *VavCre* mice with pMSCV-*Bcr-Abl1-p185*-IRES-eGFP retrovirus prior to seeding in IL-7-supplemented medium. While this approach facilitates the simultaneous emergence of multiple transformed clones, ensuring the reliable establishment of immortalized, stable cell lines, we noted again a significant decrease in outgrowing cell lines in the absence of cyclin C. In contrast, all wild-type BM samples successfully underwent immortalization upon transformation with the *BCR::ABL1*^{p185} oncogene (*Online Supplementary Figure S3A*, B). Comparable results were obtained using the v-ABL^{p160} oncoprotein, a murine variant of BCR::ABL1^{p185} (*Online Supplementary Figure S3C, D*). Cyclin C deficiency increased the apoptotic cell fractions during cell-line establishment (in not yet stable cell lines), indicating elevated apoptosis might account for the reduced capability of *Ccnc*$^{\Delta/\Delta}$ cells to immortalize (Figure 3D).

Cyclin C regulates stress responses in BCR::ABL1^{p185+} leukemic cells

Deletion of cyclin C reduced the number of emerging stable cell lines (*Online Supplementary Figure S3A, B*). The cyclin C-deficient BCR::ABL1^{p185+} cell lines that managed to immortalize showed comparable oncogene levels, as indicated by the mean fluorescence intensity (MFI) of the co-expressed GFP (*Online Supplementary Figure S3E*). Absence of cyclin C in these cell lines had no impact on the expression of CDK8/19 (*Online Supplementary Figure S3F*). All *Ccnc*$^{fl/fl}$ and *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cell lines stained positive for the pan B-cell markers B220 and CD19. While all cell lines of both genotypes lacked the expression of maturation markers IgM and IgD, CD43 expression was reduced in *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells, suggesting a more differentiated developmental stage (*Online Supplementary Figure S3G-I*). All immortalized, stable cell lines proliferated in FCS-supplemented medium, but we observed a slight, statistically significant growth defect upon cyclin C deletion (Figure 3E). Following initial studies in yeast, later reports underscored the importance of cyclin C in mammalian stress response.[8,17,32] This prompted us to evaluate the impact of serum starvation on the stable *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cell lines. Nutrient deprivation for 24 hours led to an increase in the sub-G1 fraction that was more pronounced in cyclin C-deficient cells, indicating a role for cyclin C in stress response regulation of leukemic cells (Figure 3F).

Cyclin C deficiency impairs *in vivo* leukemia establishment

To test the disease-initiating potential of the cyclin C-deficient cell lines *in vivo*, we transplanted *Ccnc*$^{fl/fl}$ and *Ccnc*$^{\Delta/\Delta}$ leukemic cells into NSG mice (Figure 4A). The severe immunodeficiency of these animals allowed us to study the tumor-intrinsic properties of BCR::ABL1^{p185+} B-ALL *in vivo*. Wild-type BCR::ABL1^{p185+} cells initiated an aggressive leukemia and infiltrated BM, spleen and blood of the recipient mice within 26 days. In contrast, *Ccnc*$^{\Delta/\Delta}$ cells failed to elicit disease in the injected animals 26 days post transplantation despite being still detectable at low numbers (Figure 4B, C, *Online Supplementary Figure S4A-C*). This prompted us to monitor disease progression over time in a second independent experiment (Figure 4D). NSG mice injected with wild-type BCR::ABL1^{p185+} cells rapidly developed leukemia within 3-5 weeks, while *Ccnc*$^{\Delta/\Delta}$ cells (with one exception) did not elicit a disease over the period of 40 weeks. One animal from the *Ccnc*$^{\Delta/\Delta}$ cohort developed a B-ALL after 54 days (Figure 4E). The experiment was terminated after 281 days; at that time point no disease symptoms were detectable in the remaining *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+}-injected mice (Figure 4F, *Online Supplementary Figure S4D-F*).

Cyclin C represses p53 responses in transformed cells

To understand how cyclin C interferes with BCR::ABL1-induced transformation, we performed RNA-Seq using *in vitro* cultured *Ccnc*$^{fl/fl}$ and *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cell lines. In addition, the cells were injected into NSG mice to allow adaption to the *in vivo* microenvironment. *Ccnc*$^{fl/fl}$ or *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells were injected in high amounts (5x10^5 cells compared to 2,500 cells for disease initiation), which facilitated retrieval of sufficient numbers of *Ccnc*$^{\Delta/\Delta}$ cells from the BM ten days post transplantation despite their reduced infiltration compared to control cells (Figures 4, 5A, *Online Supplementary Figure S5A*).

In vitro, cyclin C deficiency affected the transcription of 446 genes: 146 upregulated and 300 downregulated. In contrast, differential gene expression analysis from the *ex vivo*-derived samples showed profound transcriptional changes, with 3,179 deregulated genes in *Ccnc*$^{\Delta/\Delta}$ cell lines compared to *ex vivo* controls; 1,465 genes were upregulated and 1,714 downregulated (Figure 5B, *Online Supplementary Figure*

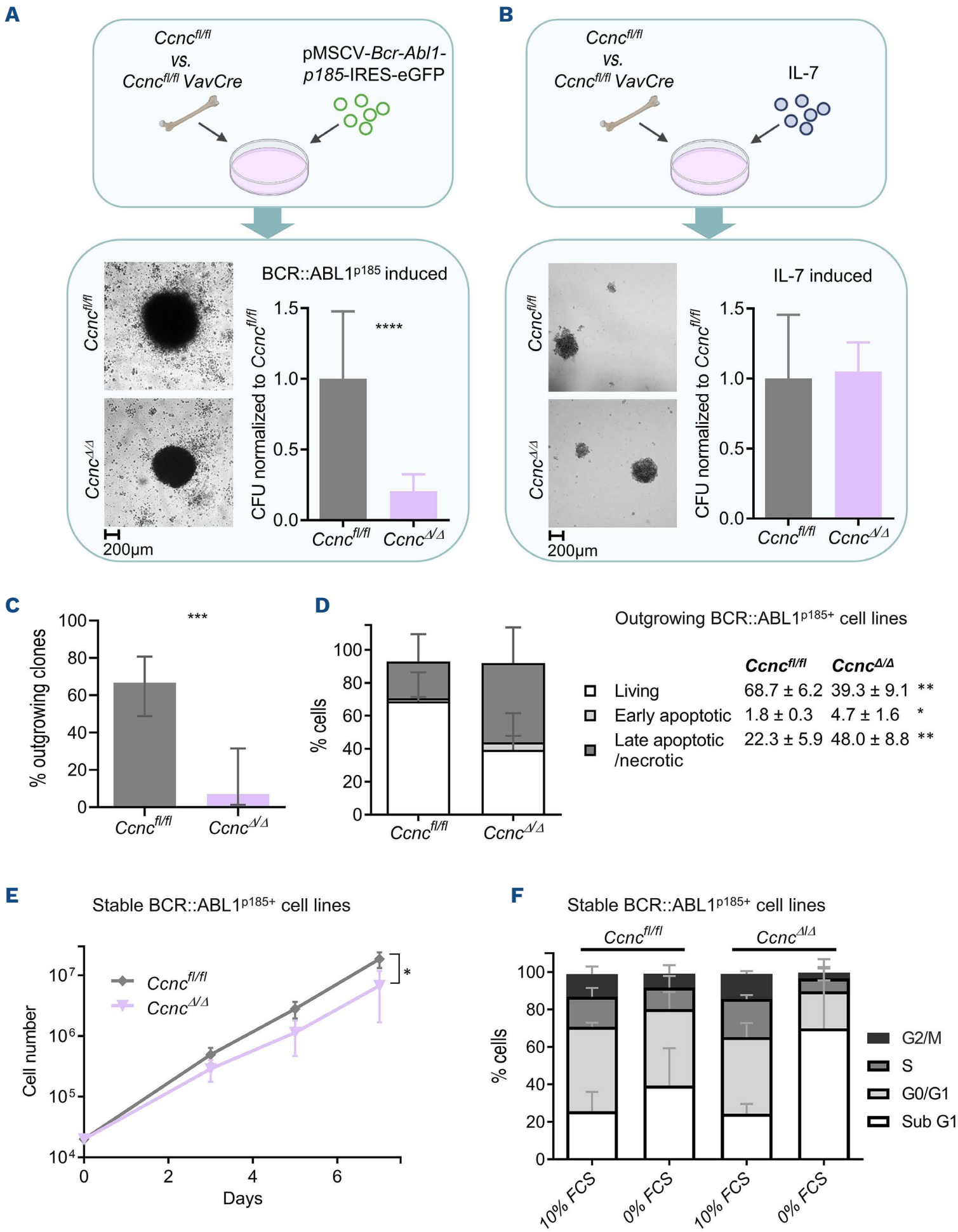

Figure 3. Cyclin C plays a pivotal role in the oncogenic transformation and immortalization of BCR::ABL1^{p185+} B-cell acute lymphoblastic leukemia. Bone marrow (BM) cells from *Cnnc*$^{fl/fl}$ and *Cnnc*$^{fl/fl}$ *VavCre* mice were isolated and either (A) infected with a retrovirus encoding BCR::ABL1^{p185} prior to plating in growth-factor free methylcellulose or (B) directly plated in methylcellulose containing interleukin-7 (IL-7). Pictures show individual colonies for each genotype. The number of colonies (colony forming units [CFU]) were counted and are depicted normalized to mean values from *Cnnc*$^{fl/fl}$ mice. (A) N=12 per genotype, pooled from 4 independent experiments, performed in technical duplicates. (B) N=3 per genotype, performed in technical duplicates. One representative result from 2 experimental set-ups with different concentrations of plated BM cells is shown. (C) Statistics on the percentage of immortalized monoclonal cell lines proliferating in FCS-supplemented medium after picking individual colonies from

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a BCR::ABL1^{p185}-induced colony formation assay (N=14-30 picked colonies / genotype). Error bars represent Confidence Intervals (CI) calculated using the Wilson Score interval (95% CI). (D-F) BM cells from *Ccnc*$^{fl/fl}$ and *Ccnc*$^{fl/fl}$ *VavCre* mice were isolated, transformed with a pMSCV-*Bcr-Abl1*-p185-IRES-eGFP-based retrovirus, and cultured in liquid medium to monitor outgrowth. (D) Bar graphs summarizing the result of Annexin/7-AAD stainings performed six weeks after transformation of BM of *Ccnc*$^{fl/fl}$ and *Ccnc*$^{fl/fl}$ *VavCre* mice with the *BCR::ABL1*^{p185} oncogene. Frequencies of living (Annexin$^-$ 7-AAD$^-$), early apoptotic (Annexin$^+$ 7-AAD$^-$), and late apoptotic/necrotic (Annexin$^+$ 7-AAD$^+$) fractions from N=6-8 biological replicates / genotype are shown. (E) Growth curves of stable *Ccnc*$^{fl/fl}$ and *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cell lines 12 weeks after initial transformation (N=4-5 cell lines/genotype, performed in technical duplicates). One representative result out of 3 independent experiments is depicted. (F) Flow cytometric analysis after PI cell cycle staining of *Ccnc*$^{fl/fl}$ and *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cell lines (N=3 stable, independently established cell lines / genotype) in standard medium supplemented with 10% FCS and 24 hours after FCS removal (0% FCS). Experiments were performed in technical duplicates 12-18 weeks after initial transformation. (A, B, D, E, F) Graphs represent mean ± Standard Deviation. Levels of significance were calculated using (A, B, D) Mann-Whitney U-test, (C) Fisher's exact test, or (E) unpaired *t* test on log-transformed counts from day 7 post seeding. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

S5B, C). Gene set enrichment analysis (GSEA) revealed 27 altered pathways in the *ex vivo* samples, of which 9 were also deregulated *in vitro*, such as interferon responses or Myc targets (*Online Supplementary Figure S5D*). Among the pathways which were exclusively upregulated in *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells *ex vivo* were the apoptosis and the p53 pathways (Figure 5C). Notably, enhanced expression of the p53 targets *Plk*, *Sfn* and *Gadd45a* contributed significantly to the enrichment of the p53 pathway *ex vivo* (*Online Supplementary Table S1*). GADD45a serves as an anti-oncogenic stress sensor in transformed cells and can inhibit the CDK1/cyclin B1 complex together with p21 and 14-3-3σ (encoded by *Sfn*).[33,34] The polo-like kinase PLK2 (SNK) likewise plays a role in stress signaling and has been described as a tumor suppressor in B-cell malignancies.[35,36] As the p53 gene set was not among the significantly enriched pathways in the *in vitro* samples, we subjected *in vitro* cultured cells to stress by reducing FCS or cell density. Inducing stress *in vitro* via serum starvation or sparse seeding inhibited the proliferation of *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells and enhanced the upregulation of several p53 pathway members including GADD45a, PLK2 and 14-3-3σ in cyclin C-deficient cells (Figure 5D, E, *Online Supplementary Figure S5E*). Upregulation of *Cdkn2b*, encoding the tumor suppressor p15^{INK4b}, which likewise contributed to the enrichment of the p53 pathway, could also be confirmed via RT-qPCR (*Online Supplementary Figure S5E*). Notably, the enhanced expression of these p53 pathway genes *in vitro* only reached significance after provoking a stress response in *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells. In addition, the p53 target *Cdkn1a* encoding p21 was significantly upregulated *in vitro* in the *Ccnc*$^{\Delta/\Delta}$ cell lines on mRNA as well as at the protein level (*Online Supplementary Figure S5F, G*), although no upregulation was observed in the *ex vivo* samples on mRNA level. The tumor suppressors p16^{INK4a} and p19^{ARF}, both encoded by the *Cdkn2a* gene, are also members of the p53 pathway and upregulated in *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells in accordance with the RNA-Seq data (*Online Supplementary Figure S5H*).

Disruption of functional p53 signaling restores the leukemogenicity of cyclin C-deficient BCR::ABL1^{p185+} cells

As reducing cell density or serum concentrations *in vitro* increased the overexpression of the p53 pathway genes *Plk2*, *Sfn* and *Gadd45a* in *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells, we employed CRISPR/Cas9 to test the effects of silencing the genes under the same stressors. Individually depleting either of these p53 targets partially rescued the impaired proliferation of *Ccnc*$^{\Delta/\Delta}$ cell lines, most pronouncedly under sparse seeding conditions (Figure 6A, *Online Supplementary Figure S6A*). Since our data suggest that cyclin C deletion amplifies overall p53 responses in BCR::ABL1^{p185+} cells, we hypothesized that cumulative effects of aberrant p53 signaling might account for the distinct disease-initiating potential observed in one *Ccnc*$^{\Delta/\Delta}$ cell line (Figure 4E, highlighted turquoise). Sanger sequencing analysis of *ex vivo*-derived spleen-infiltrating BCR::ABL1^{p185+} cells from the single mouse in the *Ccnc*$^{\Delta/\Delta}$ cohort which succumbed to leukemia revealed a *Tp53* mutation accompanied by accumulation of mutant p53 protein (Figure 6B), potentially counteracting the cyclin C deficiency and resulting in an aggressive disease phenotype. Analyses of spleens from mice injected with *Ccnc*$^{fl/fl}$ BCR::ABL1^{p185+} cells revealed either wild-type or mutant p53, proving that in the presence of cyclin C, BCR::ABL1^{p185+} cells are able to elicit leukemia irrespective of *Tp53* mutational status.

To determine if functional loss of p53 signaling indeed restores the leukemogenicity of *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells *in vivo*, we screened for *Ccnc*$^{\Delta/\Delta}$ cell lines carrying spontaneous *Tp53* mutations, injected them into NSG mice, and monitored disease progression. Unlike *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells with intact p53 (Figure 4E), BCR::ABL1^{p185+} cells lacking functional p53 rapidly caused a fatal B-ALL, despite loss of cyclin C (Figure 6C, *Online Supplementary Figure S6B*). In addition, inhibiting p53 by expressing dominant negative p53 (dn p53) in parental BCR::ABL1^{p185+} cells without spontaneous *Tp53* mutations (*Online Supplementary Figure S6C*) restored the disease-initiating potential of *Ccnc*$^{\Delta/\Delta}$ B-ALL cells (Figure 6D, *Online Supplementary Figure S6D*). These data indicate that enhanced p53 activity in cyclin C-deficient BCR::ABL1^{p185+} cells could mechanistically explain their inability to give rise to B-ALL *in vivo*.

Cyclin C as a potential new target for B-cell acute lymphoblastic leukemia

To mimic a therapeutic setting, and to target cyclin C in already transformed leukemic cells, we generated

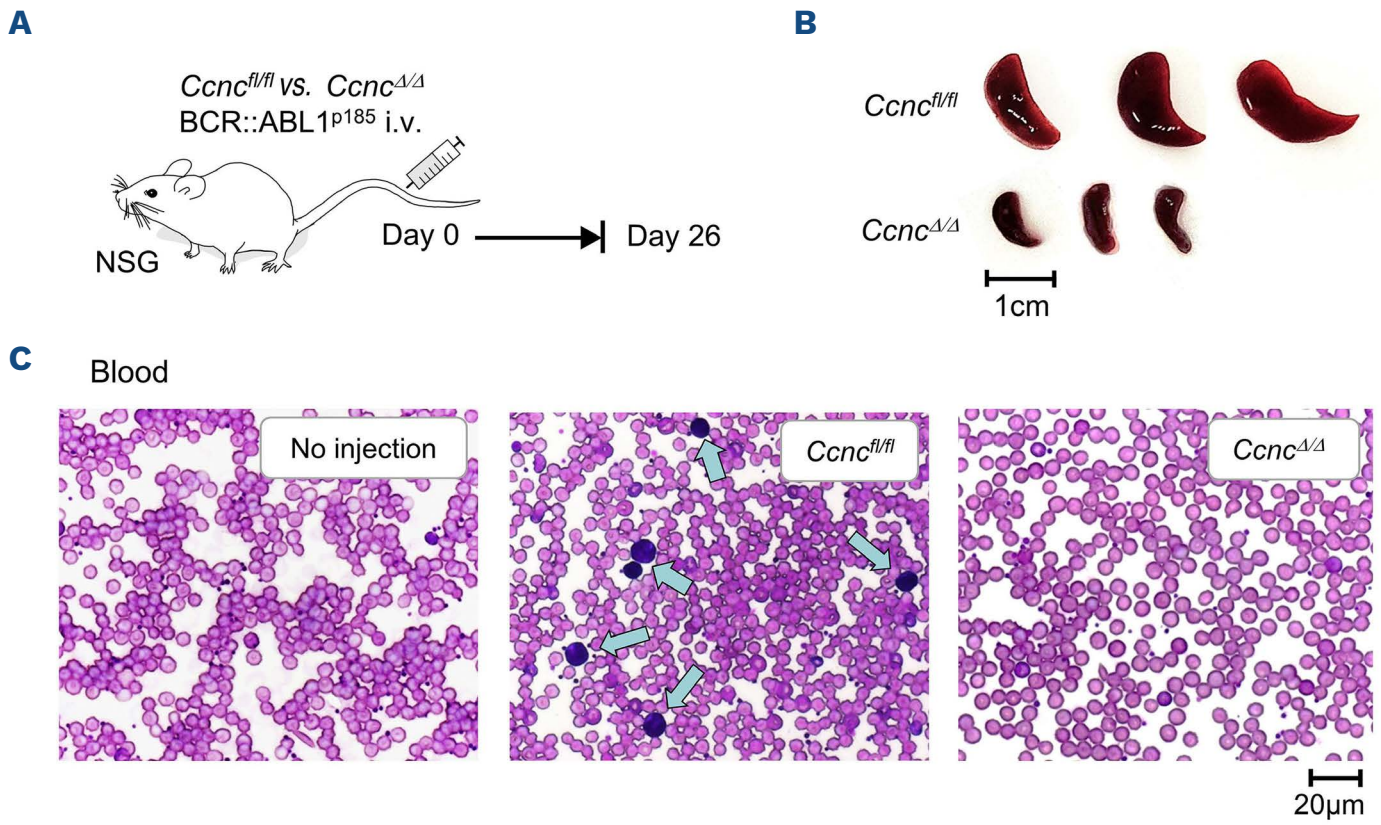
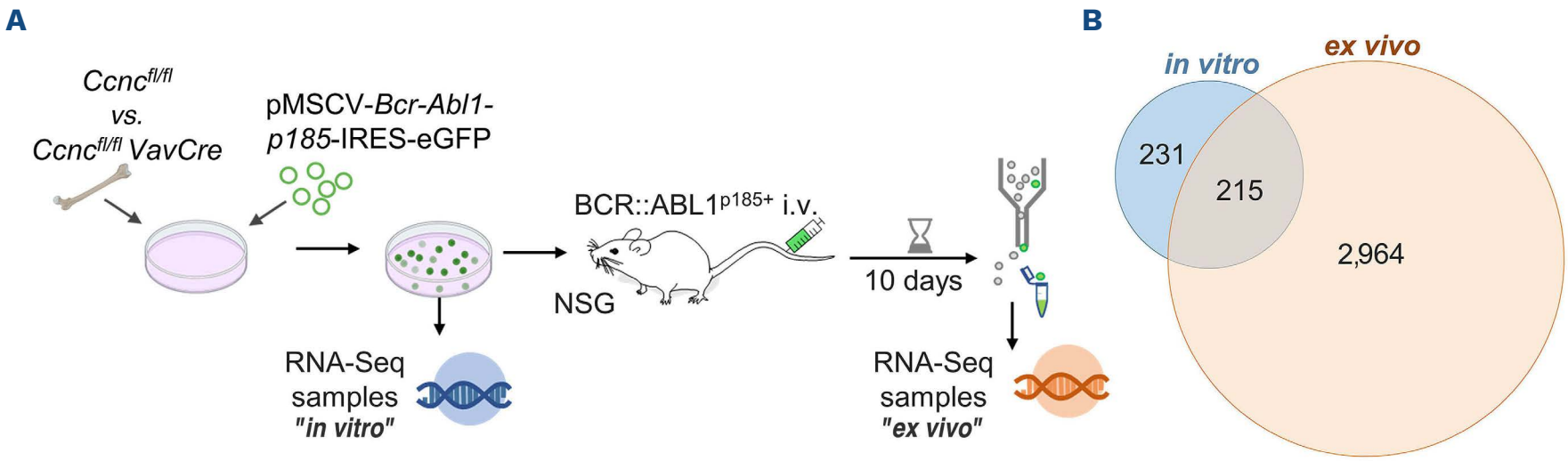
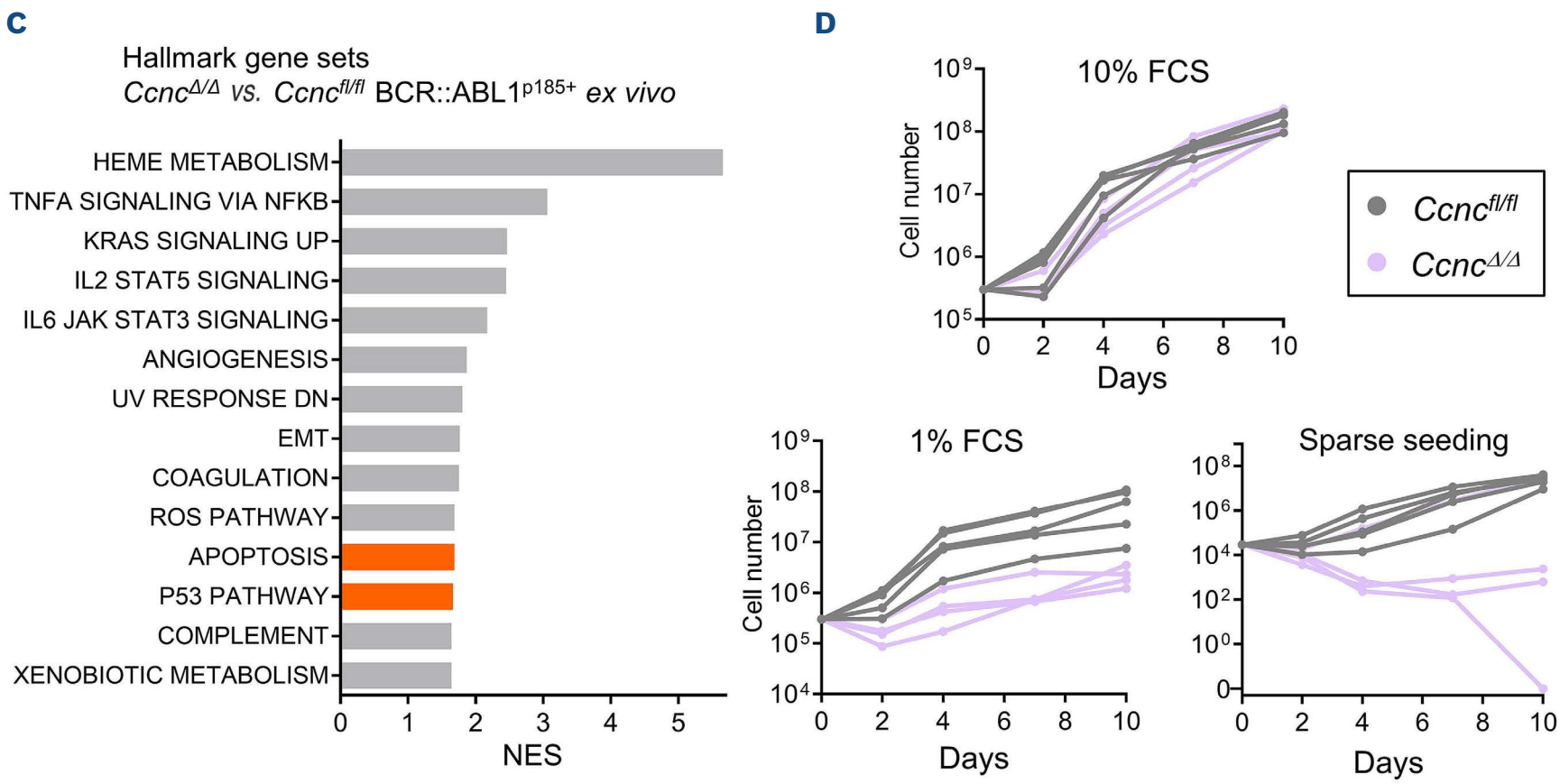


Figure 4. Cyclin C deficiency impairs *in vivo* leukemia establishment. (A) Scheme depicting experimental setup of data shown in (B) and (C). 2,500 *Ccnc*$^{fl/fl}$ or *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells were injected intravenously (i.v.) into NSG mice (N=9-10/genotype, 3 independent cell lines per genotype were injected). (B) Representative pictures of spleens on day 26 post injection. (C) Representative pictures of blood smears on day 26 post injection after Hemacolor Rapid staining. Blasts are indicated with turquoise arrows. (D) Scheme depicting experimental setup for data shown in (E) and (F). (E) Intravenous injection of *Ccnc*$^{fl/fl}$ and *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells into NSG mice (N=9-10/genotype, 3 independent cell lines per genotype were injected, data pooled from 2 independent experiments). Survival of recipient mice was monitored for up to a maximum of 224-281 days in case of absent disease symptoms. Median survival of mice receiving *Ccnc*$^{fl/fl}$ injections was 23 days; survival of the only diseased mouse in the *Ccnc*$^{\Delta/\Delta}$ cohort (highlighted turquoise) was 54 days. Black triangles indicate time points at which mice were eliminated without appearance of disease symptoms. Level of significance was calculated using log rank (Mantel-Cox) test. (F) Flow cytometric analysis of bone marrow (BM) infiltration in mice receiving *Ccnc*$^{fl/fl}$ and *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} injections as depicted in (D) and (E). Graph shows mean ± Standard Deviation. Level of significance was calculated using Mann-Whitney U test. ****P<0.0001.





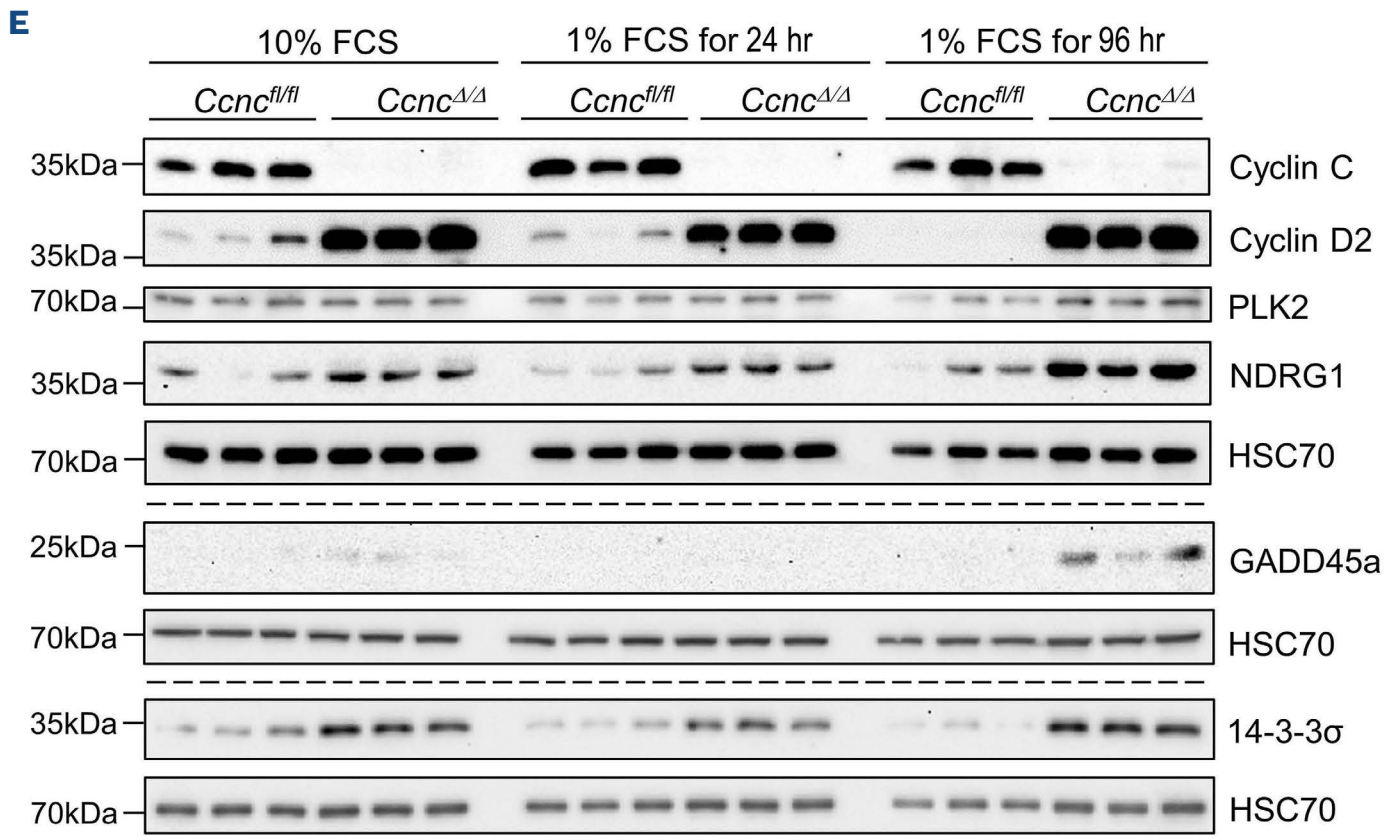


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Figure 5. Cyclin C represses p53 responses in transformed cells. (A) Scheme depicting experimental setup for RNA sequencing (RNA-Seq) analyzed in (B) and (C). Bone marrow (BM) from *Ccnc*$^{fl/fl}$ and *Ccnc*$^{fl/fl}$ *VavCre* mice was isolated and infected with retrovirus encoding BCR::ABL1^{p185} to generate stable cell lines ("*in vitro*" samples) which were intravenously (i.v.) injected into NSG mice and retrieved from bone marrow of recipient mice ten days later ("*ex vivo*" samples). (B) Venn Diagram showing the number of differentially expressed genes (Benjamini-Hochberg adjusted *P* value <0.1) in *Ccnc*$^{\Delta/\Delta}$ *versus Ccnc*$^{fl/fl}$ BCR::ABL1^{p185+} cells *in vitro* (blue) and *ex vivo* (orange). Intersecting area shows the overlap between the *in vitro* and *ex vivo* datasets. (C) Significantly upregulated hallmark gene sets (normalized enrichment score >1, false discovery rate <0.2, *P*<0.05) from Gene Set Enrichment Analysis (GSEA) of *ex vivo* derived *Ccnc*$^{\Delta/\Delta}$ *versus Ccnc*$^{fl/fl}$ BCR::ABL1^{p185+} cells which were not significantly enriched in cyclin C-deficient BCR::ABL1^{p185+} cell lines *in vitro*. (D) *In vitro* proliferation of *Ccnc*$^{fl/fl}$ and *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells in standard culture conditions: medium supplemented with 10% fetal calf serum (FCS) (top), after reducing FCS to 1% (bottom left) and in reduced cell density (bottom right) (N=4-5 cell lines / genotype). (E) Immunoblot analysis of stable *Ccnc*$^{fl/fl}$ and *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cell lines (N=3 per genotype) in standard culture medium (10% FCS) and after reducing FCS to 1% for 24 hours (hr) and 96 hr. Levels of cyclin C, cyclin D2, PLK2, NDRG1, GADD45a and 14-3-3σ (*Sfn*) were assessed on 3 separate immunoblots with the same lysates. HSC70 served as loading control. EMT: epithelial-mesenchymal transition; ROS: reactive oxygen species.

BCR::ABL1^{p185+} cell lines using BM from *Ccnc*$^{fl/fl}$ Mx1Cre mice. Here, an interferon-responsive *Mx1* promoter allows Cre-mediated activation and excision of *Ccnc* upon interferon treatment / induction (*Online Supplementary Figure S7A*). Deletion of cyclin C via treatment with interferon-beta (Ifn-β) inhibited leukemic cell proliferation *in vitro* (*Online Supplementary Figure S7B*).

We next conducted a thorough analysis of RNA-Seq data from leukemia patients, revealing elevated *CCNC* levels in different hematologic malignancies, including BCP-ALL (*Online Supplementary Figure S7C*). Further examination of different BCP-ALL subtypes showed that cyclin C is overexpressed in all studied subclasses (Figure 7A). Notably, there was no upregulation observed in myeloid leukemia patient samples, and cell lines of myeloid origin were not among the significantly enriched lineages for *CCNC* in the DepMap CRISPR / Cas9 knock-out screen (Figure 1B). To confirm this, we performed a colony formation assay using the *BCR::ABL1*^{p210} oncogene, detecting no significant differences in myeloid colony growth and affirming no dependency on cyclin C in CML (*Online Supplementary Figure S7D*). Conversely, 5 out of 6 Ph$^-$ human B-lymphoblastic cell lines with intact p53 analyzed in the DepMap screen exhibited a dependency on *CCNC* (Figure 7B).

Subsequently, we employed CRISPR / Cas9 to target *CCNC* in the Ph$^-$ human BCP-ALL cell line, NALM-6. This resulted in a notable reduction in outgrowing single cell clones with guide RNA #1 and #2 (Figure 7C, D). Despite an initial cyclin C reduction in the bulk cell culture, we were unable to generate cyclin C knock-out single cell clones after screening over 100 clones from 3 individual approaches. Moreover, continuous culture of the bulk cells following the knock-out approach resulted in the outgrowth of wild-type cells. This indicates that B-ALL cell lines are not compatible with cyclin C deficiency, and the few remaining clones that survive the initial knockout have a proliferation or survival disadvantage.

To further assess the impact of cyclin C levels on disease outcome in human patients, we compared the EFS of pediatric BCP-ALL patients with high *versus* low *CCNC* expression. Above-median *CCNC* levels were associated with a significant decrease in EFS probability (Figure 7E). High *CCNC* expression was particularly associated with early relapse / death in patients who had already reached remission after induction therapy (Figure 7F).

In summary, our data show that cyclin C is important for the transformation and maintenance of BCR::ABL1^{p185+} leukemia, and cyclin C deficiency precludes leukemia development *in vivo*. This is in line with our analysis of human data sets showing a reliance of B-cell malignancies on cyclin C and increased risk of disease recurrence in BCP-ALL patients with high levels of *CCNC*.

Discussion

Cyclin C is a multifaceted protein with functions in transcriptional regulation and mitochondrial fragmentation. Its impact on oncogenesis is intricate and context-dependent. Found on chromosome 6q21, a segment frequently deleted in cancer,[37] cyclin C may act as tumor suppressor or promoter. We here add a novel angle to the story by showing that in Ph$^+$ B-ALL, cyclin C acts as an oncogene by suppressing p53 responses.

Cyclin C deficient BCR::ABL1$^+$ cells lines can be established *in vitro*, albeit to a lesser extent, but are incapable of eliciting disease *in vivo*, which indicates a function of cyclin C in cellular adaptation and reaction to environmental stress. Similar to its oncogenic role, cyclin C involvement in regulating stress response seems highly fine-tuned to the type of cell and stressor, influencing signaling pathways for either cell death or survival. Absence of cyclin C inhibits stress-mediated mitochondrial fission and cell death responses in yeast and mouse embryonic fibroblast (MEF) cells.[17,38] On the other hand, studies in yeast have highlighted cyclin C degradation via the ubiquitin-proteasome system following unfavorable environmental cues prior to nuclear release, thereby eliciting survival signals.[39] In MEF cells, oxidative stress or mTOR inhibition cause distinct changes in CKM promoter occupancy to fine-tune the transcriptional stress reponse.[32] Adding another layer of complexity, cyclin C has been reported to play a role at

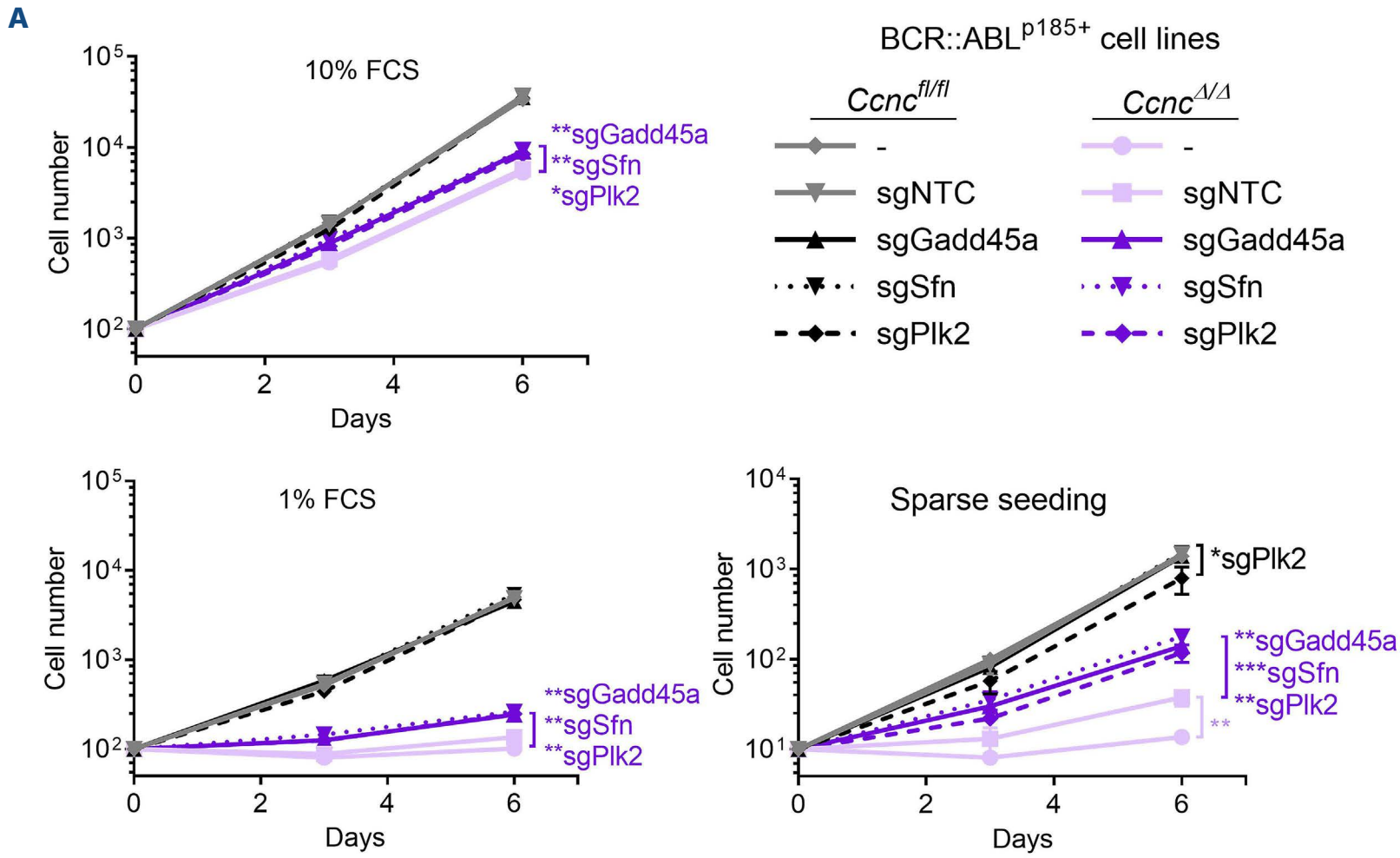

Figure 6. Disruption of functional p53 signaling restores the leukemogenicity of cyclin C-deficient BCR::ABL1^{p185+} cells. (A) *In vitro* proliferation of *Ccnc*$^{fl/fl}$ and *Ccnc*$^{Δ/Δ}$ BCR::ABL1^{p185+} cells six days after direct delivery of a ribonucleoprotein (RNP) complex consisting of Cas9 enzyme and guide RNA targeting *Gadd45a*, *Sfn* or *Plk2*. A non-targeting control (sgNTC) and mock treated cells (-)

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served as controls. Growth curves were performed in technical duplicates in standard culture conditions: medium supplemented with 10% fetal calf serum (FCS) (top), with lowered FCS reduced to 1% (bottom left) or reduced cell density (bottom right). Graphs show mean ± Standard Deviation. Levels of significance were determined by one-way ANOVA followed by Dunnett's test comparing log-transformed counts from day 6 post seeding with sgNTC as control group for *Ccnc*$^{fl/fl}$ and *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells, respectively. (B) Immunoblot analysis showing levels of cyclin C and p53 in spleen infiltrating BCR::ABL1^{p185+} cells of diseased mice from experiment shown in Figure 4E. HSC70 served as loading control. (C) Kaplan-Meier plot of NSG mice after intravenous injection of *Ccnc*$^{fl/fl}$ and *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells carrying spontaneous mutations in the DNA binding domain of p53 (p53^{mut}) (N=4 per genotype, median survival 16 *vs*. 18 days). (D) Stable *Ccnc*$^{fl/fl}$ and *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cell lines were infected with retrovirus encoding dominant negative p53 (dn p53), intravenously injected into NSG mice and survival was monitored: 2 independent cell lines per genotype were used, N=5 mice were injected with *Ccnc*$^{fl/fl}$ + dn p53 and N=5 received *Ccnc*$^{\Delta/\Delta}$ + dn p53 BCR::ABL1^{p185+} cells; median survival 20 *vs*. 22 days. Levels of significance were determined using (C and D) log rank (Mantel-Cox) test . *P<0.05, **P<0.01, ***P<0.001.

different stages of the cell cycle by phosphorylating Rb and regulating E2F-dependent transcription factor activity.[8,16,40] There are limited data available on the regulation of cyclin C expression itself, except that it is induced by various mitogenic signals such as vitamin D and IL-3.[41,42] We now show that cyclin C is vital for leukemic transformation and stress-adaptive responses in B-ALL. Cyclin C deficient BCR::ABL1^{+} cells display restricted transformation and immortalization. The outgrowth of fewer transformed clones suggests the presence of compensatory mechanisms in the surviving *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells, which could be explored in future studies. Nonetheless, these cyclin C-deficient cells continue to display impaired stress adaptation even in their already transformed state (e.g., serum starvation, low cell density, and upon transfer into immunocompromised mice). This concept is supported by the RNA-Seq results showing a significant upregulation of the p53 and apoptosis pathways in *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells *in vivo*. The importance of the p53 pathway in stress responses is well established. Upon sensing stress, p53 regulates the expression of genes involved in diverse cellular processes, including DNA repair, cell cycle arrest and apoptosis, and prevents the propagation of damaged or compromised cells that could contribute to tumorigenesis or other abnormalities.[43] GADD45a and PLK2, amongst our top hits in the p53 pathway, are known anti-oncogenic stress sensors.[33,35,36] *in vitro*, their increased expression in *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells was not as pronounced as *in vivo*, possibly masked by optimal growth conditions. However, after introducing additional stress through a cell density or nutrient reduction *in vitro*, cyclin C ablation again caused significantly enhanced expression of the p53 pathway genes. The upregulation of several p53 pathway genes was also validated at the protein level. Their significance was further demonstrated by knock-out experiments, showing that loss of individual p53 targets partially rescued the impaired proliferation of cyclin C-deficient leukemic cells under stress. The enhanced p53 activity observed in *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells under stress could thus at least partly explain why cyclin C deficiency is particularly harmful for leukemic cells during early transformation and in the absence of optimal growth conditions.

Disruption of the p53 pathway through a genomic *Tp53* mutation or via introduction of dn p53 restored the leukemic potential of *Ccnc*$^{\Delta/\Delta}$ BCR::ABL1^{p185+} cells. While p53 mutations are more common in many solid tumors, aberrations of *TP53* in B-ALL increase with age and upon relapse, and are associated with poor prognosis.[44,45]

Based on our findings, cyclin C emerges as a potential new therapeutic target for B-ALL. While transformation and mechanistic studies were only conducted in BCR::ABL1^{p185+} cell lines, we also provide supporting evidence that cyclin C plays an essential role for Ph^{-} B-ALL (Figures 1, 7), suggesting that our findings are broadly relevant to B-ALL. The essential role of cyclin C in B-ALL, but not in normal B-cell development, may stem from its overexpression and critical involvement in promoting oncogene-driven proliferation and survival in leukemic cells. This dependency creates synthetic lethal vulnerabilities which might be therapeutically exploited, offering a promising avenue for selectively targeting B-ALL cells. However, targeting molecules like cyclin C, given their intracellular location, lack of kinase activity, and complex protein interactions, has been challenging. In addition, caution must be exercised due to potential detrimental effects on the heart.[46] Proteolysis-targeting chimeras (PROTAC) are small molecule compounds that can selectively degrade previously undruggable molecules. PROTAC offer high specificity, and strategies to additionally increase their safety profile include selective, cell-type-specific PROTAC delivery using nanoparticle delivery systems or antibody-PROTAC conjugates which could enable B-cell-specific targeting.[47] Loss of cyclin C renders B-ALL cells more vulnerable to stress, suggesting it as an interesting target for combination therapies as it may allow sensitization of cancer cells, for example, to chemotherapy or radiotherapy. *In silico* analysis of pediatric BCP-ALL patients supports this notion: cyclin C expression is associated with decreased EFS, and high *CCNC* levels show a particularly strong correlation with early disease recurrence in systematically treated patients. This suggests that cyclin C could compromise the prolonged efficacy of systemic therapy in patients who have achieved remission, and that additional targeting of cyclin C might provide synergistic effects. Successful cyclin-degrading compounds have been previously developed and tested.[48,49] Developing degraders specifically targeting cyclin C and further studying its role in various tumor entities is, therefore, of high interest.

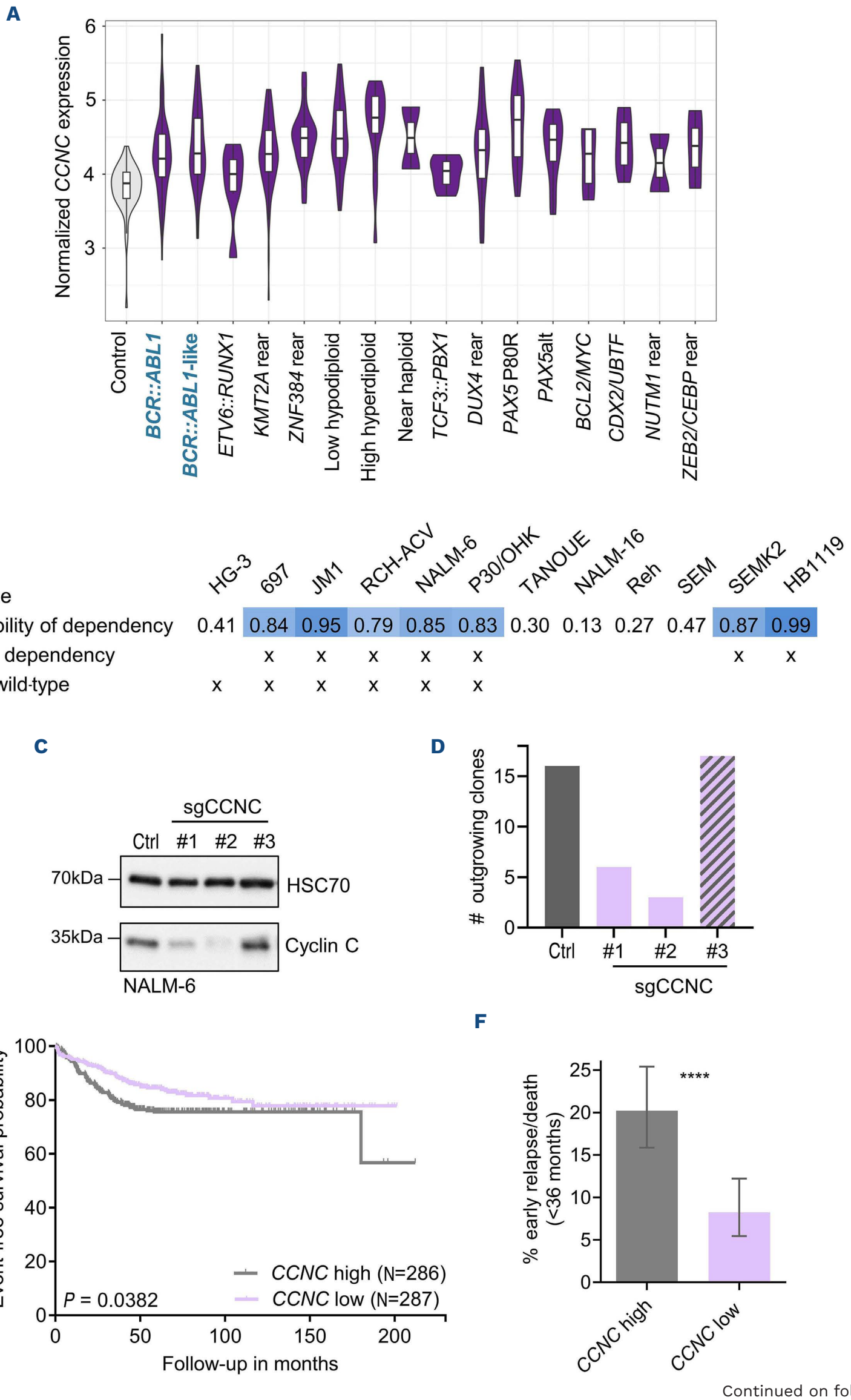

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Figure 7. Cyclin C as a potential new target for B-cell acute lymphoblastic leukemia treatment. (A) Gene expression profile of *CCNC* in control (bone marrow [BM] mononuclear cells, N=56) *versus* primary B-cell precursor acute lymphoblastic leukemia (BCP-ALL) samples (N=362), presented as violin plot. Sample size per subtype: *BCR::ABL1*: N=97; *BCR::ABL1*-like: N=51; *ETV6::RUNX1*: N=6; *KMT2A* rear: N=56; *ZNF384* rear: N=20; low hypodiploid: N=45; high hyperdiploid: N=17; near haploid: N=2; *TCF3::PBX1*: N=6; *DUX4* rear: N=22; *PAX5* P80R: N=14; *PAX5alt*: N=10: *BCL2/MYC*: N=4; *CDX2/UBTF*: N=7; *NUTM1* rear: N=2; *ZEB2/CEBP*: N=3. (B) Analysis of 12 human B-lymphoblastic leukemia / lymphoma cell lines showing p53 status and dependency probabilities for *CCNC* from a genome-wide CRISPR/Cas9 knock-out screen (DepMap Public 23Q2+Score, Chronos). Cell lines with dependency probabilities >0.5 are considered dependent. (C) Immunoblot showing cyclin C levels in bulk cell culture after CRISPR/Cas9 mediated targeting of cyclin C in NALM-6 cells. Three different guide RNA targeting cyclin C (sgCCNC) were used, guide RNA targeting *HPRT1* served as control (Ctrl). HSC70 was used as loading control. (D) Bulk cell lines depicted in (E) were single cell sorted using a BD FACSAria III cell sorter and outgrowth of single cell clones was monitored. One representative result is shown; a similar result was obtained using limiting dilution to generate monoclonal cell lines. (E) Probability of event-free survival (EFS) in pediatric BCP-ALL patients with high *versus* low *CCNC* expression. The high / low *CCNC* cut-off was based on median expression among BCP-ALL samples in cohort. Death in induction, death, relapse, non-response, and secondary malignancy were counted as events for EFS. (F) Proportion of pediatric BCP-ALL patients who reached remission after induction treatment, but for which an event (relapse / death) was reported prior to 36 months. Error bars represent 95% Confidence Interval calculated using the adjusted Wald method. Levels of significance were determined using (E) log rank (Mantel-Cox) test and (F) Fisher's exact test. ****P<0.0001.

Disclosures

PS has been a consultant at Novartis, Genovis, Guidepoint, The Planning Shop, ORIC Pharmaceuticals, Cedilla Therapeutics, Syros Pharmaceuticals, Blueprint Medicines, Curie Bio, Differentiated Therapeutics, Excientia, Ligature Therapeutics, Merck, Redesign Science, Sibylla Biotech, and Exo Therapeutics; his laboratory receives research funding from Novartis. GHo and WW are employed by MLL Munich Leukemia Laboratory.

Contributions

JT, VS and DG conceived the study. JT, JL, KK, MPM, AWS, PT, SS, SM, FB and DG performed the experiments. JT, VS and DG analyzed the data. FB and MPM helped with the experiments and data analysis. LLF and AV were involved in experimental design and scientific discussions. TK and JT analyzed sequencing data. GHo, WW, JMB and MLdB provided bioinformatic patient data analysis. GHe provided bioinformatic support and scientific input. FB, MPM and PS established methods. MPM and VS obtained the ethical permits for the experiments and oversaw ethical aspects. LLF, DG and VS provided reagents. DG and VS supervised the study. JT and DG wrote the manuscript. All authors revised the final version for publication.

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Data-sharing statement

The RNA-Seq data reported in this article have been deposited in the Array Express database (Accession number: E-MTAB-13728). All other relevant data that support the conclusions of the study are available from the authors on request. Please contact: dagmar.gotthardt@vetmeduni.ac.at

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