KLF4 acts as a tumor suppressor in human B-cells and patients' B-cell leukemias growing in mice

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Transcription factor Krüppel-like factor 4 (KLF4) regulates numerous basic biological processes and acts either as an oncogene or tumor suppressor. To characterize the functional role of KLF4 in B-cell transformation, we infected primary naïve B-cells with Epstein Barr virus (EBV), which is accompanied by rapid loss of KLF4 expression. EBV variants recombinantly expressing KLF4 were unable to transform B-cells into lymphoblastoid cells, indicating a tumor suppressor function. To study established B-cell tumors, we developed a novel technique allowing the inducible KLF4 expression in patient-derived xenograft (PDX) B-cell acute lymphoblastic leukemia (ALL) cells, for competitive *in vivo* trials. Wild type, but not mutant KLF4 reduced leukemia proliferation, with strongest effects in mice pre-treated with conventional chemotherapy and at minimal residual disease. Re-expression of KLF4 reduced stem cell potential and sensitized cells towards systemic chemotherapy *in vivo*. Azacitidine upregulated KLF4 levels and knockout of KLF4 in PDX B-ALL cells reduced Azacitidine-induced cell death, suggesting that KLF4, at least in part, mediates the anti-tumor effects of Azacitidine. Taken together, KLF4 acts as a tumor suppressor during EBV-induced lymphomagenesis and represents an interesting vulnerability in established B-ALL leukemias. As direct translational consequence, our data support applying Azacitidine in patients with B-ALL, to therapeutically target KLF4.

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Introduction

B-cell tumors appear as lymphomas or leukemias and require better treatment, especially upon disease relapse (1, 2). Here, we studied the functional role of Krüppel-like transcription factor 4 (KLF4) and asked whether KLF4 might represent a tumor suppressor and/or a putative therapeutic target for B-cell malignancies. KLF4 is implicated in stress-responsive regulation of cell cycle progression, apoptosis and differentiation as well as stemness and pluripotency, including generation of induced pluripotent stem cells (iPS) (3–9). KLF4 expression is deregulated in numerous solid tumors and cancers of various kinds (5, 8, 10–12). KLF4 acts either as an oncogene or a tumor suppressor, dependent on the cellular context, tumor type, sub-type and stage (8, 10, 13–15). KLF4 tumor suppressor functions led to the development of small molecules upregulating KLF4, which are currently in early clinical testing in solid tumors (16) and myeloid malignancies (17) (NCT02267863).

In hematologic malignancies the role of KLF4 is less well defined (6). KLF4 downregulation, e.g., by promoter hypermethylation has been described associated with a tumor suppressor function in T-cell acute lymphoblastic leukemia (T-ALL) (12, 18) and acute myeloid leukemia (AML) (11, 19, 20). In B-cell malignancies, KLF4 is either up- or downregulated (21). An oncogenic function of KLF4 was suggested in Burkitt´s lymphoma, chronic lymphocytic leukemia and multiple myeloma, where high KLF4 levels were associated with decreased response to therapy (22–24). In contrast, downregulated KLF4 was associated with adverse prognosis in most other subtypes of B-cell malignancies (24–30). Deletion of KLF4 in B-cells was not sufficient to drive leukemogenesis in mice (31), but KLF4 expression prevented transformation of murine pre-B-cells by the BCR-ABL oncogene (32), suggesting a tumor suppressor function that requires cooperation with other genetic events. Data on KLF4 function for B-cell lymphomagenesis remain entirely elusive. Overall, the functional role of KLF4 in B-cell malignancies remains insufficiently defined and functional data on established human B-ALL are entirely missing.

Here, we studied the function of KLF4 in two complementary model systems of B-cell malignancies to decipher its role in oncogenesis and tumor dependency, respectively. We found a clear tumor suppressor function of KLF4 during EBV-mediated B-cell transformation into lymphoblastoid cells. In established tumors from patients with precursor B-cell ALL (B-ALL), re-upregulating KLF4 reduced tumor growth and stem cell potential and increased treatment response in patient-derived xenograft (PDX) models *in vivo*. Knockout experiments showed that the anti-tumor effect of Azacitidine at least in part depended on KLF4. Our data indicate that KLF4 represents an interesting vulnerability in B-ALL and strengthen the use of KLF4 regulating drugs in clinical trials of B-ALL, e.g., the hypomethylating agent Azacitidine (NCT02828358).

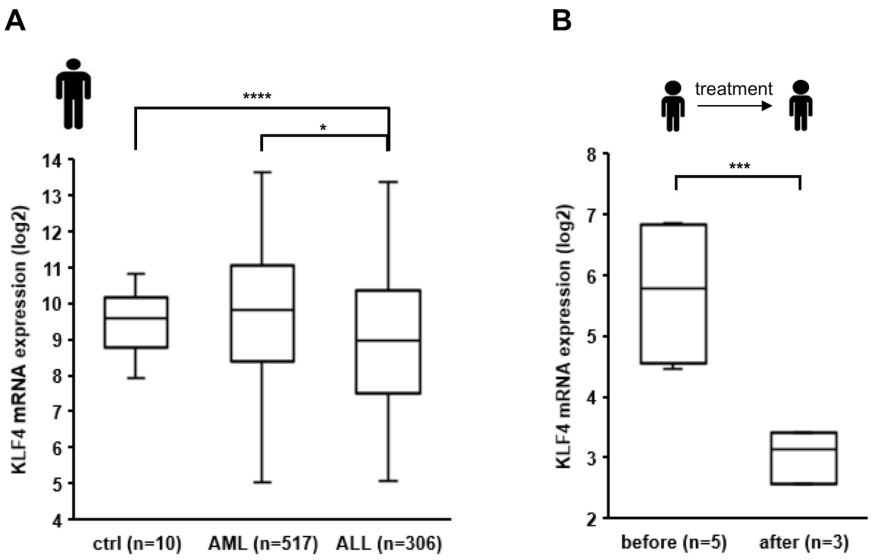

Fig. 1. *KLF4* mRNA is downregulated in primary patient leukemia cells.
A Expression of KLF4 mRNA in healthy bone marrow (ctrl, n=10), AML (n=517) and ALL (n=306) patient samples. Datasets are publicly available (GSE66006, GSE78132, GSE37642). * p<0.05 (p=0.048237398), **** p<0.0001 (p=1.67157x10-9) by two-tailed unpaired t test. **B** Expression of KLF4 mRNA in primary samples from pediatric ALL patients at diagnosis (n=5, before) and after the first block of treatment according to the BFM-2009 protocol (n=3, after, day 33). RNA-seq data are publicly available (GSE83142). *** p<0.005 (p=0.001427748) by two-tailed unpaired t test.

Results

We aimed at characterizing the role of KLF4 for B-cell transformation as well as survival of established B-cell tumor cells, using genetically engineered *in vitro* and *in vivo* model systems.

KLF4 is downregulated in patients' B-ALL cells, especially after treatment. Regarding KLF4 expression levels, publicly available datasets from primary samples from 306 adult ALL patients were analysed (33, 34). KLF4 expression levels were lowest in ALL, lower than in controls and AML (Figure 1A), demonstrating that adult ALL displays similarly reduced KLF4 levels than previously reported for pediatric ALL (12, 27, 30).

We had previously obtained transcriptome data from pediatric B-ALL patients before and after the first block of chemotherapy which allowed comparing therapy-naïve cells to cells at minimal residual disease (MRD) (35). *KLF4* mRNA levels were strongly reduced or nearly absent in primary ALL samples at MRD, suggesting that cells with low *KLF4* expression preferentially survived chemotherapy in patients (Figure 1B). Together, the expression data show that *KLF4* mRNA is downregulated in B-ALL, especially in cells surviving chemotherapy, suggesting a functional role for *KLF4* in B-ALL worth further pursuing.

KLF4 acts as tumor suppressor during EBV-induced transformation of B-cells. To decipher the role of KLF4 during B-cell oncogenesis, we studied infection and transformation of human B-cells by the Epstein Barr virus (EBV). EBV induces various human B-cell malignancies including lymphoma such as Burkitt Lymphoma and Hodgkin Disease, but also solid tumors (36, 37). As an *in vitro* surrogate model, EBV infection of primary naïve B-cells immortalizes and transforms them into lymphoblastoid cell lines (LCL) (Figure 2A) (38–40). In previous studies, we detected strong and persistent downregulation of *KLF4* mRNA levels during EBV-induced B-cell transformation (Figure S2A) (41). To study the functional relevance of KLF4 for EBV-induced LCL formation, we ectopically expressed KLF4 in this model. EBV mutants were generated expressing either wild type KLF4 (wtKLF4) or a variant (mutKLF4) as a control. It lacks the two C-terminal zinc fingers representing the DNA binding domain, disabling KLF4 to function as transcription factor (Figure S2BC). While mutKLF4 expression showed no major effects, persistent wtKLF4 levels completely abrogated EBV-induced LCL formation, disabled EBV-induced B-cell proliferation and induced apoptosis in the infected cells (Figure 2BC)). Cell cycle analysis indicated that KLF4 expression prevented S-phase entry of EBV-infected B-cells, required for LCL generation and expansion (Figure 2D). Thus, KLF4 acts as a tumor suppressor in the model of EBV-induced lymphomagenesis, depending on its C-terminal domain required for its function as transcription factor. To the best of our knowledge, this is the first evidence indicating that KLF4 acts as a tumor suppressor during human B-cell transformation and B-cell lymphomagenesis. Together with published data describing a tumor suppressor role of KLF4 for BCR-ABL-mediated transformation of murine pre-B-cells by BCR-ABL (32), recombinant KLF4 prevented oncogenesis in both models of B-cell malignancies studied so far, murine leukemia and human lymphoma.

An *in vivo* approach for inducible transgene expression in PDX cells. We next aimed at characterizing the role

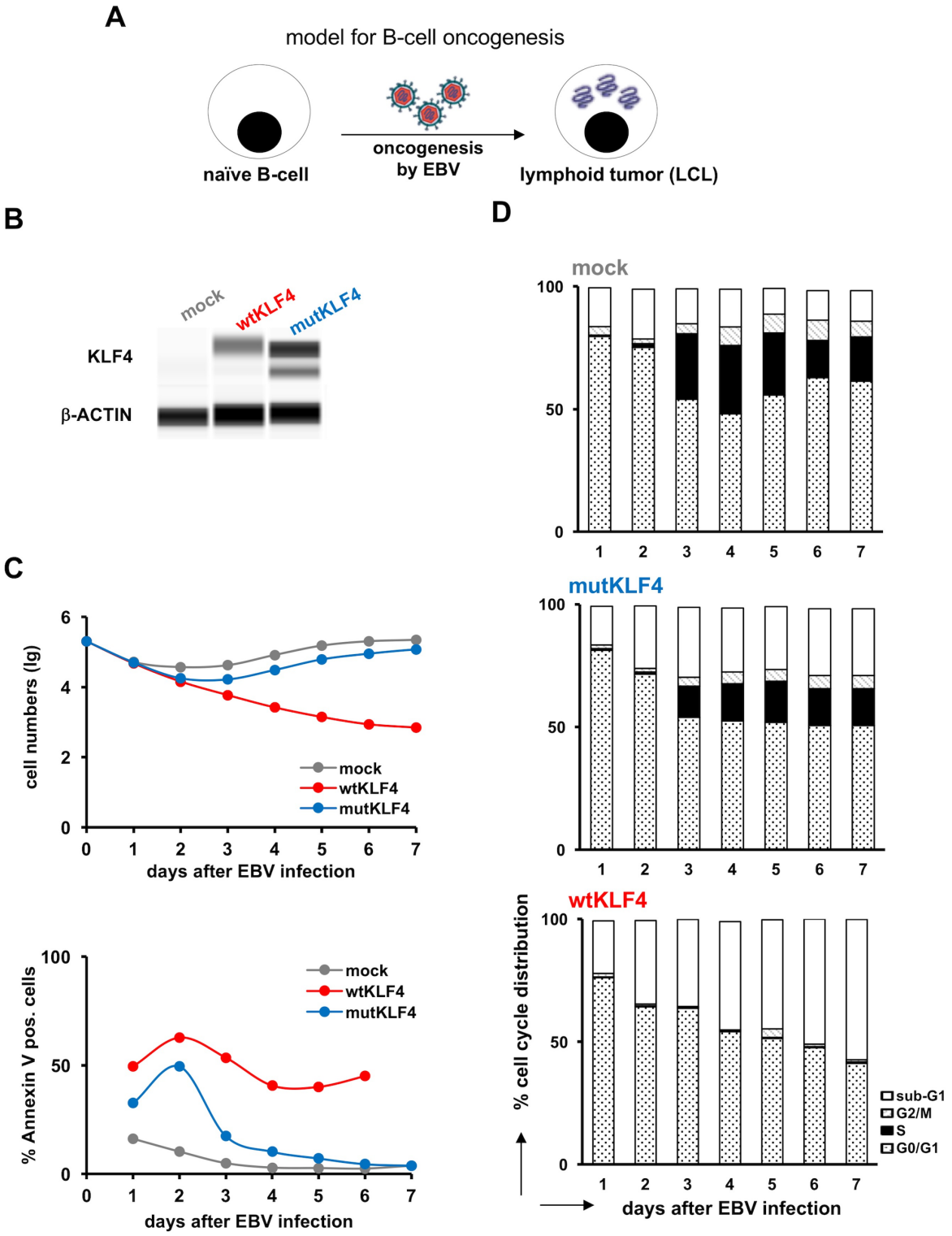

Fig. 2. KLF4 prevents EBV-induced transformation of naïve human B-cells. **A** Experimental design: Primary naïve B-cells were infected with EBV to generate lymphoblastoid cell lines (LCL); a MOI of 0.1 was used of either wildtype EBV (mock), or EBV strains with the wildtype KLF4 coding sequence (wtKLF4) or a mutated KLF4 sequence devoid of the DNA-binding domain (mutKLF4) co-expressed via a T2A element with the viral EBNA2 gene. **B** Western blot of KLF4 2 days after infection with the indicated EBV particles. β-actin was used as loading control. **C** $2x10^5$ B-cells infected with the indicated EBV viruses were plated and analyzed at the indicated time points. Upper panel: Numbers of viable, infected cells were analyzed by flow cytometry as detailed in the method section. Lower panel: Apoptosis of infected cells was assessed by quantification of Annexin V positive cells. **D** Cell cycle analysis; Cells were incubated with 5-Bromo-2'-deoxyuridine (BrdU) for 1 hour prior to harvest and analyzed by flow cytometry after permeabilization and staining with a BrdU specific antibody. The percentage of cells in the different phases of the cell cycle are indicated. One representative experiment out of two experiments with B-lymphocytes from two individual donors is shown.

of downregulated KLF4 for established B-cell tumours, in order to understand whether targeting KLF4 might represent a putative future therapeutic target. As a clinic-close model, we decided working with patients' ALL cells, to

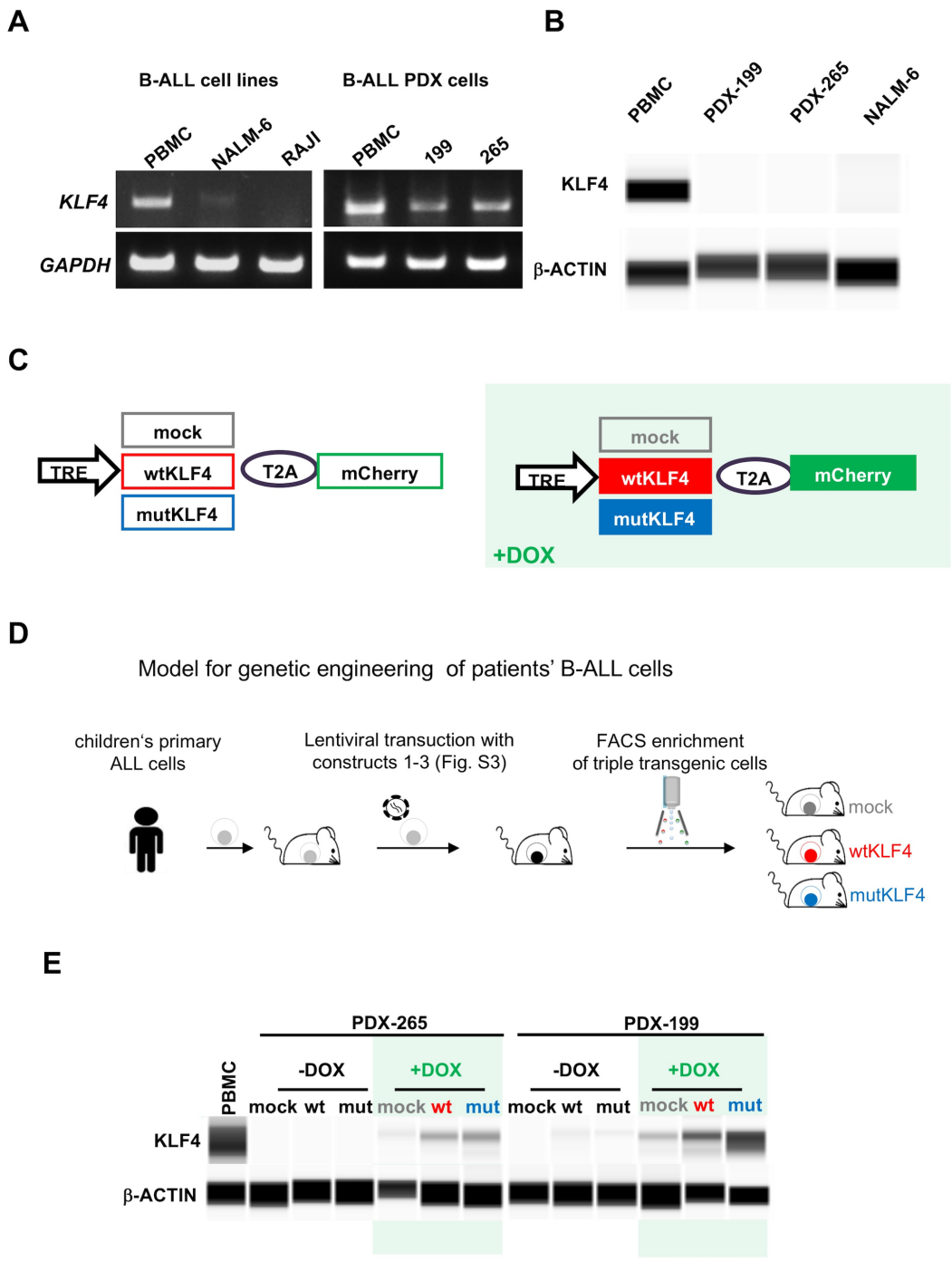

Fig. 3. A tet-on inducible system to re-express KLF4 in PDX ALL cells.AB KLF4 is downregulated in B-ALL and Burkitt Lymphoma cell lines and B-ALL PDX cells on mRNA level as determined by RT-PCR using GAPDH as loading control (**A**) and on protein level in Western blot using β-actin as loading control (**B**), as compared to PBMCs. **C** KLF4 expression vectors. The TRE promoter drives expression of KLF4, either as wildtype (wt)KLF4 or as a mutated (mut)KLF4 sequence devoid of two C-terminal zinc finger motifs comprising the DNA-binding domain (see Figure S2B), each linked by a T2A peptide to the fluorochrome mCherry as molecular marker; mock (empty vector only encoding the mCherry fluorochrome) was used as a control. Addition of Doxycycline (light green background throughout all Figures) leads to expression of mock (grey), wtKLF4 (red) or mutKLF4 (blue; colors identical throughout all Figures) and mCherry (green). **D** Experimental design: Primary B-ALL cells from patients were transplanted into immunocompromised mice to generate PDX models; PDX cells were transduced with three lentiviral constructs to express rtTA3 together with luciferase, tetR and either mock, wtKLF4 or mutKLF4 (vectors are detailed in Figure S3A). Following passaging through mice for amplification, transgenic PDX cells were enriched by flow cytometry based on additionally and constantly expressed fluorochromes (mTaqBFP for the rtTA3-luciferase construct, iRFP720 or T-Sapphire for the tetR construct and Venus under control of the constant PGK promoter for the KLF4 construct). Triple-transgenic cells were used for all further *in vivo* experiments. **E** PDX ALL-265 and PDX ALL-199 infected as indicated in D were cultured *in vitro* with or without addition of DOX for 48h and KLF4 protein expression was analyzed by Western blot using peripheral blood mononuclear cells (PBMC) from healthy donors and β-actin as controls. Representative immunoblots from triplicates are shown.

overcome limitations of cell lines (42). We studied primary B-ALL cells from two children with B-cell precursor ALL (data for ALL-265 in printed Figures and data for ALL-199 in suppl. Figures; clinical data for both patients

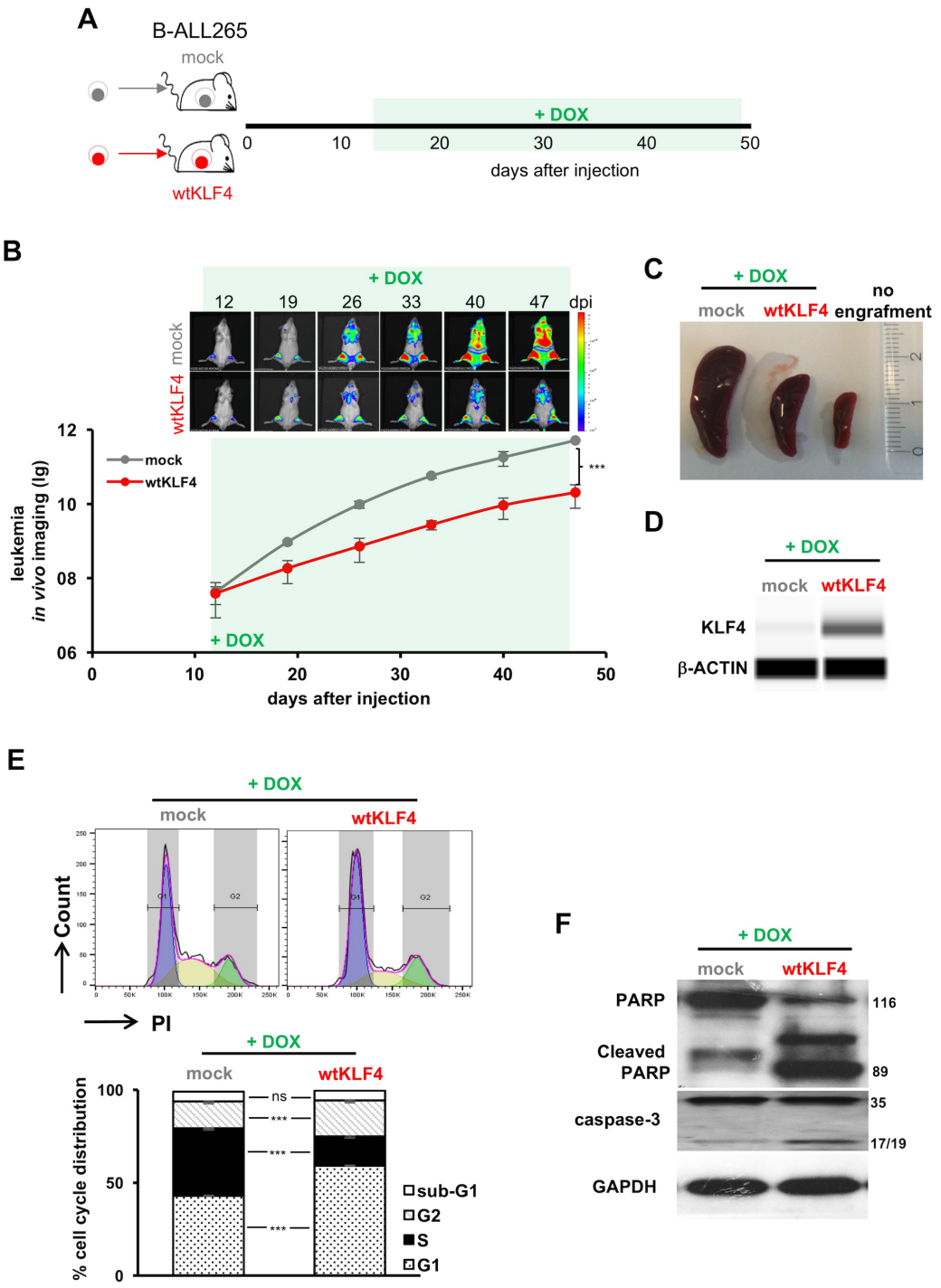

Fig. 4. Re-expressing KLF4 inhibits tumor growth through cell cycle arrest and apoptosis in B-ALL PDX cells *in vivo*. **A** Experimental design: 60,000 triple-transgenic, mock or wtKLF4 PDX ALL-265 cells were injected into 6 NSG mice each. After homing was completed and tumors were established, DOX (1mg/ml) was added to the drinking water on day 12 to induce KLF4 expression and leukemia growth monitored by bioluminescence *in vivo* imaging. On day 47 after cell injection, mice were sacrificed, spleens harvested and the successfully DOX-induced, mCherry positive mock or wtKLF4 expressing population enriched by flow cytometry for further analysis. **B** Bioluminescence *in vivo* imaging shown as representative images (upper panel) and after quantification of all 6 mice, depicted as mean ± SEM. *** $p<0.005$ ($p=0.0037$) by two-tailed unpaired t test. **C** Representative spleens of mice, using a healthy mouse without leukemic engraftment for comparison. **D** KLF4 protein level of mCherry positive splenic cells was analyzed by Western blot; β-actin served as loading control. Representative immunoblots from one out of three mice are shown. **E** Cell cycle analysis; 106 mCherry positive cells were fixed in 70% ethanol, stained with Propidium Iodide (PI) and cell cycle distribution was measured by flow cytometry. Upper panel: Representative histograms of mock (n=3) or wtKLF4-expressing (n=3) cells; lower panel: Quantification as mean ± SD is shown. *** $p<0.005$ by two-tailed unpaired t test; ns = not significant. **F** PARP and caspase-3 cleavage in mock- (n=3) or wtKLF4-transduced (n=3) PDX ALL-265 cells as determined by Western blot; GAPDH was used as loading control.

are published in Ebinger et al (35)). To consider the complex *in vivo* environment on tumor phenotypes, cells were transplanted into immunocompromised mice generating orthotopic patient-derived xenograft (PDX) models. Similarly to primary pediatric (12, 27, 30) and adult (Figure 1) primary B-ALL samples, both PDX B-ALL models revealed severely

diminished KLF4 mRNA and protein levels compared to normal control cells (Figure 3AB). KLF4 has been demonstrated to regulate tumor cell homing, growth, apoptosis and therapy response in different tumor entities (5, 7–9, 12, 18, 43). To be able to analyse the role of KLF4 in each of these individual processes in B-ALL, we aimed for inducible KLF4 expression in PDX B-ALL cells which would enable studying KLF4 at distinct disease stages (Figure 3C and S3A). We adapted a tetracycline-response system (44) and feeding mice with Doxycycline (DOX) induced expression of KLF4 in PDX B-ALL cells *in vivo*, at any time point or any given disease stage (indicated by green background in all Figures). PDX cells were transduced with three consecutive lentiviral constructs to express (i) the tet activator (rtTA3) together with a luciferase enabling bioluminescence *in vivo* imaging; (ii) the tet repressor (tetR) reducing leakiness together with one of two different fluorochromes used as molecular markers for competitive *in vivo* assays and; (iii) either a mock, wtKLF4 or mutKLF4 sequence under control of the tet-responsive element (TRE), linked to the fluorochrome mCherry via a T2A peptide to visualize successful DOX-induced gene expression (Figure 3CD and S3A). mCherry expression also served as control to re-assure similar expression levels of all recombinant proteins. Further constitutively expressed fluorochromes were required for flow cytometry-based enrichment of successfully transduced cells. Upon DOX-induction, the B-ALL cells started expressing wtKLF4 or mutKLF4 proteins (Figure 3E and S3BC), which turned back to baseline upon DOX withdrawal, proving reversibility of the system (Figure S3C). Of major importance, KLF4 was expressed below physiological levels of normal cells, avoiding unspecific effects by undesired overexpression (Figure 3E). Thus and for the first time to the best of our knowledge, we established an inducible system of transgene expression in PDX acute leukemias *in vivo* which might be of major usefulness for future studies on, e.g., individualized leukemia vulnerabilities.

Re-expressing wtKLF4 reduces growth of B-ALL PDX cells *in vivo*. The inducible system enabled studying whether turning off KLF4 was essential and required for survival and proliferation of established B-ALL malignancies. PDX ALL cells containing constructs for either mock or wtKLF4 were transplanted into groups of mice; after homing and early engraftment were completed, mice were fed with DOX which induced transgene expression during the exponential growth phase of PDX ALL tumors (Figure 4A and Figure S4A). *in vivo* imaging revealed that tumor growth of wtKLF4-expressing B-ALL PDX cells *in vivo* was diminished compared to controls (Figure 4B and S4B), resulting in reduced splenomegaly as compared to spleens from mock-bearing mice (Figure 4CD, S4CD). KLF4 significantly reduced the proportion of cells in S-phase (Figure 4E and S4E) and induced cleavage of PARP and Caspase-3 (Figure 4F and S4F), indicating that KLF4 impaired tumor growth by inducing cell cycle arrest and apoptosis; similar results were obtained in ALL cell lines (Figure S5).

The effect of KLF4 on tumor growth was further evaluated using pairwise competitive *in vivo* assays. Two different cell populations, a control population and the population of interest, were mixed and injected into the same animal; each population was followed up separately using recombinant fluorochromes. To control this approach, mice were injected either with the control mixture containing at a 1:1 ratio of mock and mutKLF4 expressing cells (indicated in purple); otherwise with the wtKLF4 mixture containing at a 1:1 ratio of mock and wtKLF4 expressing PDX B-ALL cells (indicated in orange) (Figure 5A). Leukemic load was monitored by *in vivo* imaging, revealing slightly reduced growth of the wtKLF4 mixture compared to the control mixture (Figure 5B, left panels). As expected, the phenotype was less pronounced compared to non-competitive experiments due to the fact that 50% of cells in the wtKLF4 mixture were control cells. When analysing the control mixture for the proportion of each subpopulation remained constant over time (Figure 5B, upper right panel). In contrast in the wtKLF4 mixture, the wtKLF4 sub-fraction significantly diminished and was nearly lost within 30 days of DOX treatment (Figure 5B, lower right panel). Thus, the competitive *in vivo* assay (Figure 5AB) reproduced the growth-inhibitory effect of re-expressed wtKLF4 seen in the non-competitive assay (Figure 4A-F). Competitive *in vivo* assays enable the identical *in vivo* environment for both populations leading to very minor inter-mouse variabilities and combine high sensitivity and high reliability with reduced numbers of animals; due to these advantages, all further experiments were performed as pairwise competitive *in vivo* experiments. In addition to reduced proliferation and increased apoptosis, re-transplantation experiments revealed reduced homing capacity of B-ALL PDX cells re-expressing KLF4 (see Figure S6 and suppl. information). As a sum of reduced homing and reduced proliferation, re-expressing KLF4 reduced stem cell potential of B-ALL PDX cells. Taken together, moderate re-expression of KLF4 to physiologic levels reduced the overall fitness of PDX B-ALL cells *in vivo*. KLF4 functions as a cell cycle inhibitor and pro-apoptotic transcription factor in PDX B-ALL which depend on the essential feature that KLF4 level is downregulated. KLF4 represents a therapeutic vulnerability in B-ALL and targeting KLF4 by upregulating its expression levels represents a putative therapeutic approach for B-ALL.

Treatment-surviving cells are especially sensitive towards re-expressing KLF4. In children with ALL, primary cells surviving chemotherapy and at minimal residual disease (MRD) displayed extremely low KLF4 levels, lower than cells at diagnosis (Figure 1B). We next asked whether tumor growth of MRD cells might reveal an especially strong dependency on KLF4 downregulation. To mimic the MRD situation in mice, we carefully titrated a combination of the routine drugs Vincristine and Cyclophosphamide at clinically relevant doses to effectively reduce tumor burden by several orders of magnitude over 5 weeks; at MRD, defined as below 1% leukemia cells in bone marrow, chemotherapy was discontinued and DOX was administered to induce KLF4 expression during the tumor re-growth phase (Figure 6A and S7A). In this experiment, KLF4 was re-expressed solely during the phase of tumor re-growth and not during chemother-

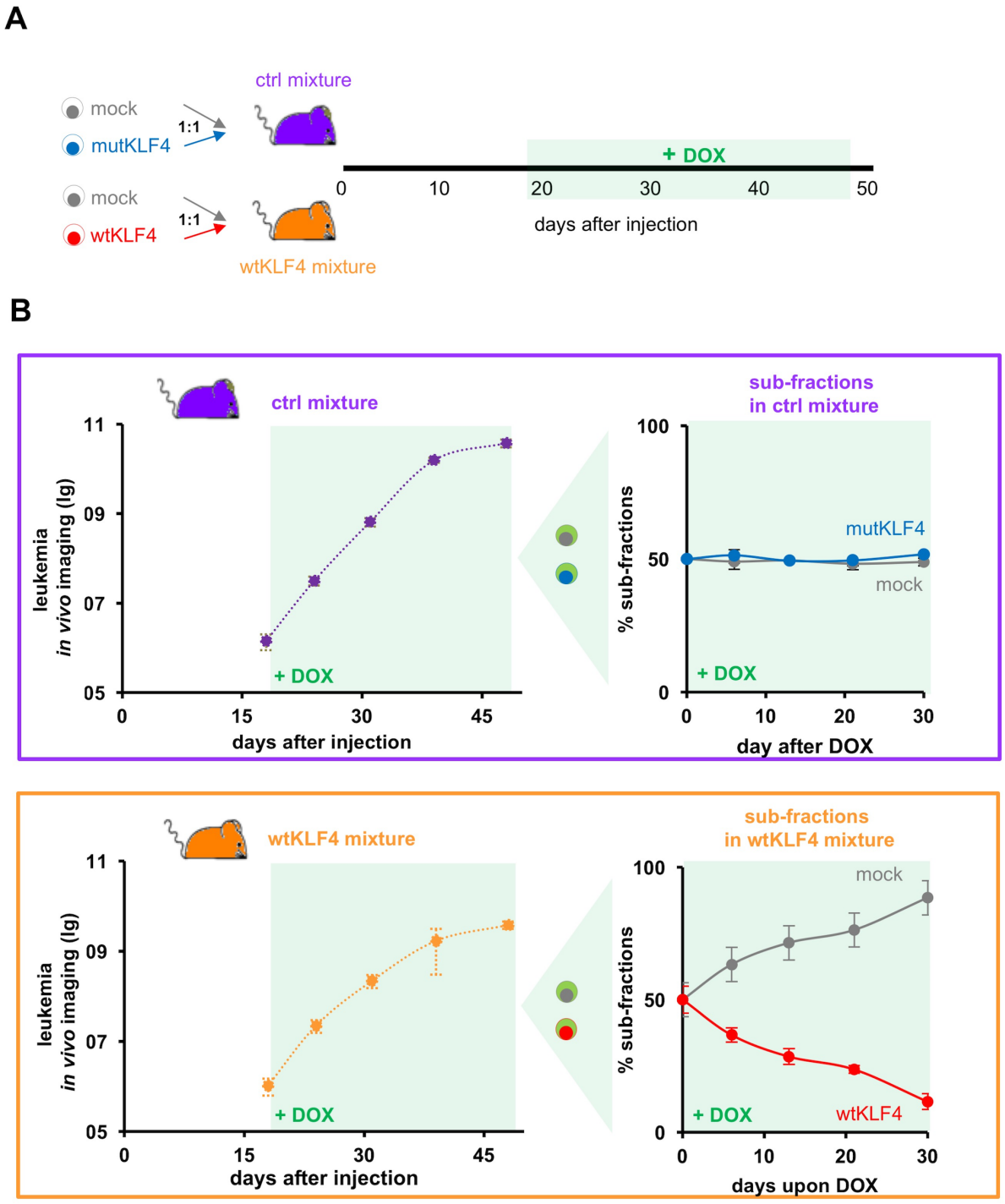

Fig. 5. KLF4 re-expressing cells are outcompeted during competitive *in vivo* assays.A Experimental procedure: NSG mice were injected with 30,000 cells each, consisting of two different 1:1 mixtures; the control mixture (framed in purple; n=10) consisted of a 1:1 ratio of mock and mutKLF4 expressing cells; the wtKLF4 mixture (framed in orange; n=10) consisted of a 1:1 mixture of mock and wtKLF4 expressing cells. After homing was completed and tumors were established, DOX was added to the drinking water on day 18 and leukemia growth monitored by bioluminescence *in vivo* imaging. 2 mice of each group were sacrificed every week and the proportions of the two sub-fractions in bone marrow analyzed by flow cytometry. **B** Left panel: Leukemia growth as determined by *in vivo* imaging. Right panel: sub-fraction analysis by flow cytometry gating on the subpopulation-specific fluorochrome markers T-Sapphire for mock subpopulation and iRFP720 for either mutKLF4 and wtKLF4 subpopulation (see Figure S3A for constructs). Quantification depicted as mean ± SEM.

apy. *In vivo* imaging showed identical response to treatment in mice carrying the control or wtKLF4 mixture, when transgenes were not induced. In contrast, upon DOX administration, tumor re-growth was clearly diminished in mice carrying the wtKLF4 mixture (Figure 6B and S7B, left panels). Sub-fraction analysis revealed that expression of wtKLF4, but not mutKLF4 lead to a significant growth disadvantage of MRD cells, depleting wtKLF4 cells to undetectable levels within two weeks (Figure 6B and S7B, right panels). While wtKLF4 expressing, therapy-naïve cells were dimin-

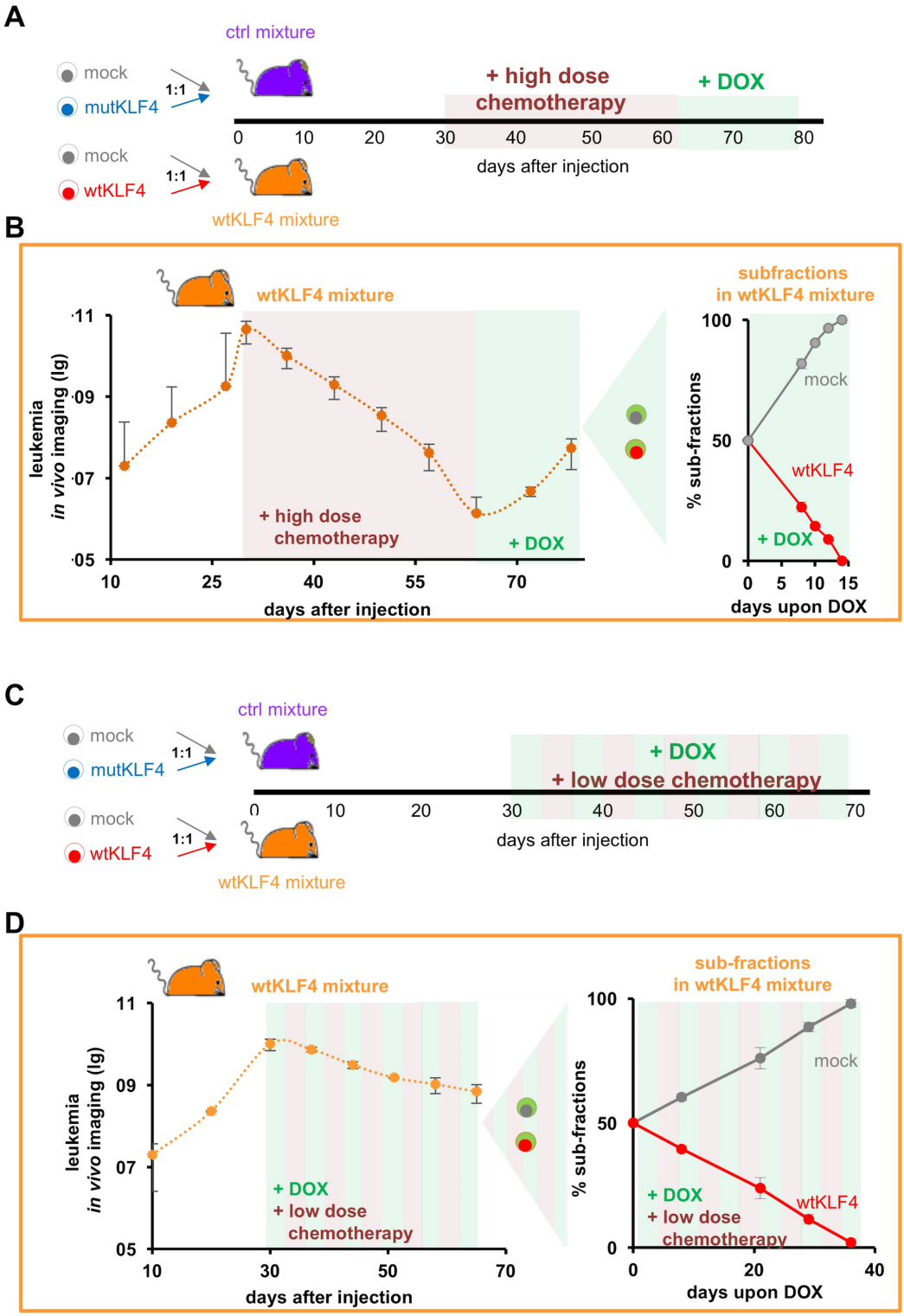

Fig. 6. Treatment-surviving cells are especially sensitive towards re-expressing KLF4.A Experiments were set up as in Figure 5, except that transgenes were expressed during tumor re-growth after treatment, while homing, growth and treatment were performed in the absence of transgene expression. At high tumor burden (day 31), mice were treated intravenously with high-dose combination chemotherapy (0.25 mg/kg vincristine + 100 mg/kg cyclophosphamide once per week, given on Mondays and Thursdays, respectively) to reduce tumor burden to MRD; at MRD (day 64), chemotherapy was stopped and DOX was added to the drinking water to induce transgene expression. **B** Tumor re-growth after treatment was monitored and analyzed as in Figure 5. **C** Experiments were set up as in Figure 5, except that transgene expression was initiated at high tumor burden (day 30) and maintained during low dose combination chemotherapy (0.2 mg/kg Vincristine + 35 mg/kg Cyclophosphamide; days 31-65). **D** Tumor growth was monitored and analyzed as in Figure 5.
See Figure S7 for results of the control mixture.

ished within 4 weeks (Figure 5B), pre-treated cells completely vanished within 2 weeks (Figure 6B); these results

indicate that KLF4 inhibited growth of MRD cells more drastically than growth of therapy-naïve PDX ALL cells (Figure S8), suggesting a stronger dependency of therapy surviving cells on downregulated KLF4 compared to untreated cells. The data suggest that chemotherapy selects for B-ALL cells with increased sensitivity towards re-expression of KLF4 and treatment upregulating KLF4 might be especially effective in the situation of MRD.

Re-expressing KLF4 sensitizes B-ALL PDX cells towards chemotherapy *in vivo*. As *in vivo* growth of both PDX ALL samples depended on KLF4 downregulation, especially if cells were pre-treated with chemotherapy, targeting KLF4 might be beneficial to improve treatment of B-ALL. We first studied whether re-expressing KLF4 influences the response of patients' B-ALL cells towards conventional chemotherapy of routine ALL treatment *in vivo*. PDX ALL control and wtKLF4 mixtures were injected into mice and allowed to grow to substantial tumor burden, before DOX was administered together with low-dose chemotherapy. Chemotherapy doses were chosen such that they induced minor effects alone, e.g., stop of tumor progression, but avoiding major tumor reduction; low dose chemotherapy facilitated identifying putative additional effects elicited by KLF4. (Figure 6C). In this experiment and in contrast to the previous experimental setup, KLF4 was re-expressed during chemotherapy treatment. Chemotherapy slightly reduced tumor load in mice with wtKLF mixture, but not control mixture (Figure 6D and S7D, left panels). When subfractions were analysed from each mixture, wtKLF4, but not mutKLF4 expressing cells were significantly decreased by chemotherapy; wtKLF4 cells were outcompeted within less than 40 days (Figure 6D and S7D, right panels). Similarly, KLF4 re-expression sensitized NALM-6 cells towards chemotherapy *in vitro* (Figure S9). These data demonstrate that re-expressing KLF4 sensitized PDX B-ALL cells towards chemotherapy *in vivo*. Upregulating KLF4 may synergize with standard therapeutic regimens to eliminate B-ALL cells and patients might benefit from targeting KLF4 in addition to conventional chemotherapy.

Azacitidine-induced cell death partially depends on KLF4. Transcription factors represent complex targets for treatment by drugs or compounds and activating KLF4 by compounds might prove challenging. The small molecule APTO-253 was introduced as KLF4 inducing drug (16) and APTO-253 indeed upregulated KLF4 in our hands, and sensitized cells towards Vincristine treatment *in vitro* (Figure S10AB). These data indicate that KLF4 can in general be re-upregulated by drugs, suggesting an epigenetic mechanism responsible for KLF4 downregulation in B-ALL. For straightforward clinical translation, we asked whether existing approved drugs might be able to re-upregulate KLF4 in B-ALL cells. The demethylating agent 5-Azacitidine (Aza) is known to reverse promoter hypermethylation, but its decisive mode of action remains at least in part elusive (45). Aza was able to upregulate KLF4 expression in other tumor entities (12, 25, 29, 46) and was tested for its putative therapeutic merit in tumors with proven tumor suppressor function of KLF4. Aza is increasingly used in clinical trials on patients with hematopoietic malignancies and during MRD, e.g., in acute myeloid leukemia in order to prevent or retard relapse (47). Here we found that Aza strongly upregulated KLF4 levels in B-ALL PDX cells and cell lines and similar to APTO-253 (Figure 7A, S11A and S10). We next asked whether KLF4 might be functionally involved in Aza-induced cell death and might mediate the effect of Aza in B-ALL. To prevent KLF4 upregulation on a molecular level, we generated PDX ALL cells with knockout (KO) of KLF4. PDX ALL cells were lentivirally transduced to recombinantly express Cas9, a technically challenging task due to the large size of the Cas9 gene and the limited transduction rates in PDX ALL cells (Figure 7B and S11B). By co-transducing a second vector encoding the single guide RNA, PDX B-ALL models with stable KO of KLF4 could readily be established (Figure 7C). Stimulation of PDX B-ALL cells with Aza at clinically relevant concentrations reduced cell viability in control cells; in contrast, PDX ALL and NALM6 cells with KLF4 KO displayed certain resistance against Aza-induced cell death and were partially rescued from Aza-mediated effects (Figure 7D and S11C). These data suggest that KLF4 participates in mediating the effect of Aza in B-ALL cells and that re-upregulating KLF4 represents an important mechanism how Aza induces cell death in B-ALL cells. The data presented here help to unravel the mode of action of Aza by introducing KLF4 as an important target of Aza; Aza requires re-upregulating KLF4 to exert its anti-leukemic function in B-ALL. Taken together, our data show that KLF4 represents a novel therapeutic target for B-ALL and that Aza, an established drug, can re-upregulate KLF4. Targeting KLF4 in B-ALL patients by introducing Aza into standard polychemotherapy protocols might reduce tumor burden and increase sensitivity towards conventional chemotherapy, for the benefit of patients.

Discussion

We demonstrate that KLF4 acts as a tumor suppressor in certain B-cell malignancies, namely i) during EBV-induced transformation of primary B lymphocytes; ii) in B-ALL patient-derived cells growing in mice, especially in the situation of MRD and; iii) during response of PDX ALL cells to therapy. We demonstrate that the well-described roles of KLF4 as a quiescence factor and apoptosis-inducer are maintained in the malignant B-cells studied (8). Of major translational importance, we show that Aza upregulates KLF4 in B-ALL and upregulated KLF4 mediates, at least in part, the anti-leukemia effect of Aza. KLF4 has been implicated in B-cell development (48), and ectopic expression of KLF4 has been found to block B-cell proliferation (31, 49) and transformation by the BCR-ABL oncogene (32), supporting a tumor suppressor role for KLF4 in B-cells. However, deletion of KLF4 in B-cells was not sufficient to drive leukemogenesis in mice (31), indicating that KLF4 cooperates with other genetic events. In line with this, multiple oncogenic signalling pathways including Notch1 (50, 51) or CDX2 (11) have been

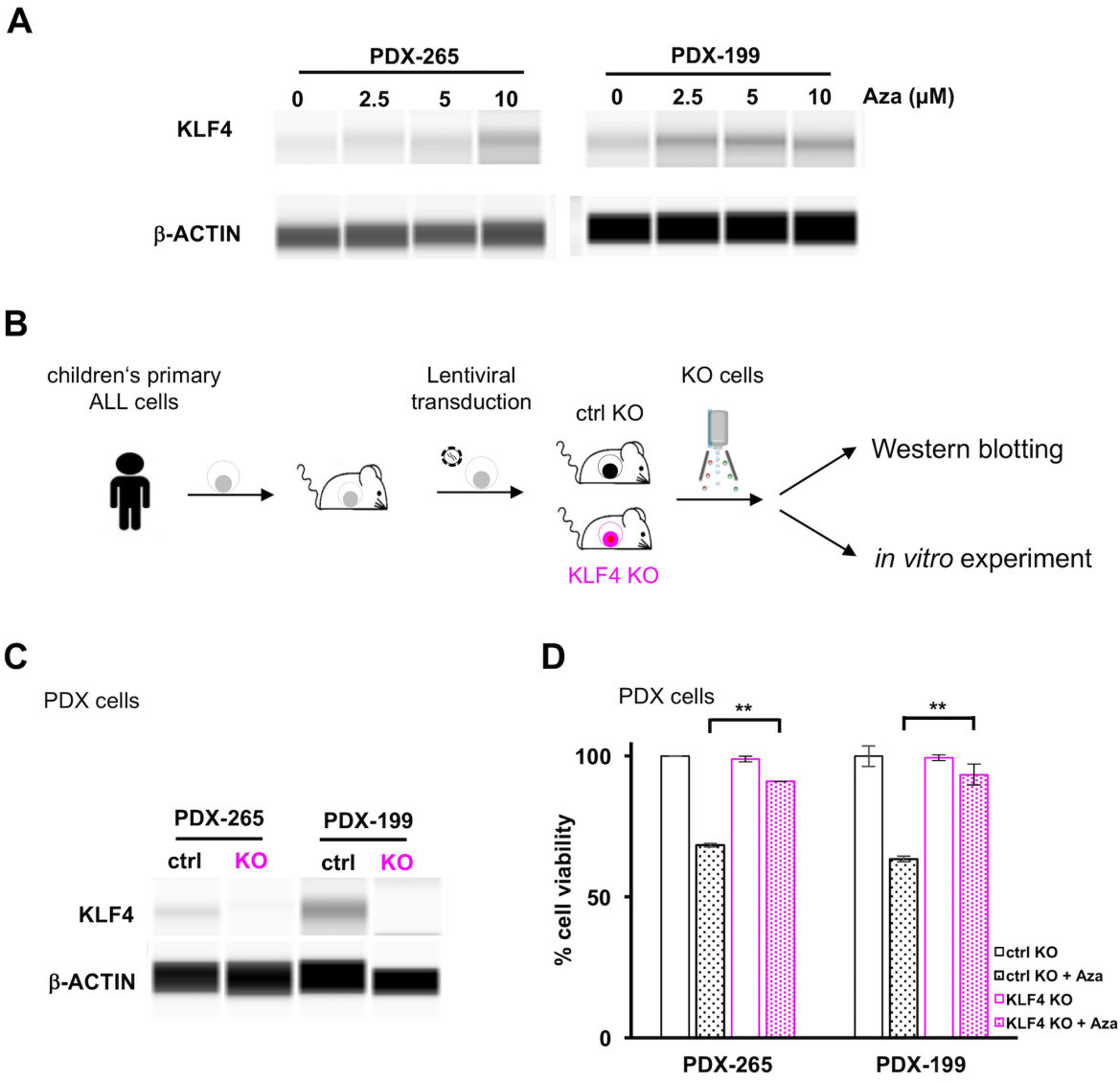

Fig. 7. Azacitidine-induced cell death depends on KLF4.A PDX-265 and PDX-199 cells were treated with different concentrations of Azacitidine (Aza) for 48h. KLF4 protein expression analyzed by Western blot, β-actin served as loading control. One representative immunoblot out of 2 independent experiments is shown. **B** Experimental procedure: B-ALL PDX cells were lentivirally transduced with Cas9, sgRNA and reporter expression vectors (Figure S10B) and injected into NSG mice to generate ctrl KO (black) and KLF4 KO (pink) PDX cells. Mice were sacrificed at full blown leukemia and marker-positive populations enriched by flow cytometry, gating on the recombinant markers (mTaqBFP for Cas9, mCherry for the sgRNAs and GFP for the reporter construct, see constructs in Figure S10B) and subjected to Western blot and *in vitro* culture. **C** KLF4 protein level of ctrl KO or KLF4 KO PDX-265 and PDX-199 cells were analyzed by Western blot. Due to low expression of KLF4 in the ctrl cells, images are displayed with strongly increased contrast; β-actin served as loading control. One representative immunoblot out of 2 experiments is shown. **D** PDX B-ALL ctrl KO cells and KLF4 KO cells were treated with 2.5 μM Aza *in vitro* for 48h and cell viability was measured by flow cytometry. Viability was normalized to non-treated cells. Mean ± SEM of duplicates is shown. ** $p<0.01$ by two-tailed unpaired t test.

shown to downregulate KLF4 expression to support tumor progression. Here, we show that KLF4 downregulation following EBV infection is necessary for EBV-induced transformation, suggesting that KLF4 downregulation occurs early during transformation and downstream of oncogenic events during B-cell lymphomagenesis. KLF4 is a stress-responsive transcription factor, and as part of the adaptive response to stress, KLF4 can protect cells from DNA-damaging insult such as γ-irradiation or chemotherapy (9, 24). Yet, KLF4 has also been described as a mediator of apoptosis, e.g. in chronic myeloid leukemia (43) or AML cells (7). Importantly, we did not detect any evidence of an antiapoptotic activity of KLF4 in our B-ALL model systems; rather we demonstrate that the proapoptotic effects of KLF4 are maintained under therapy, as KLF4-expressing cells are more susceptible to chemotherapy treatment *in vitro* and combination chemotherapy *in vivo*. Despite a proven clinical benefit of Aza treatment in hematological malignancies such as myelodysplastic syndrome and AML (45), only case reports exist on the clinical activity of Aza in ALL (52–54). Our data provide a mechanistic rationale for ongoing efforts (NCT02828358) to implement Aza in clinical regimens for treatment of B-ALL. Aza has previously been shown to upregulate KLF4 in other tumor entities (12, 29, 55–57) and we demonstrated that this ability is main-

tained in B-ALL. As molecular mechanism of Aza-mediated antitumor activity remain incompletely defined (45), our data suggests KLF4 upregulation as one important mechanism of action of Aza required to eliminate B-ALL cells. The data presented here suggests that targeting KLF4 may represent an attractive therapeutic approach in B-ALL. As Aza is able to upregulate KLF4 in B-ALL and KLF4 participates in mediating the anti-leukemia effect of Aza, our data strengthen the idea to apply Aza in patients with B-ALL.

Materials and Methods

Genetic engineering in EBV. In the maxi-EBV plasmid, wtKLF4 and mutKLF4 cDNAs were fused to the 3' open reading frame of the viral EBNA2 by a T2A element, mediating co-expression of both genes from the same promoter. While the wtKLF4 construct contained the entire open reading frame, the mutKLF4 construct lacked the two N-terminal zinc finger domains (58).

Genetic engineering of PDX B-ALL cells for inducible transgene expression. Primary patients' B-ALL cells were transplanted into severely immunocompromised mice to generate patient-derived xenograft (PDX) models. PDX B-ALL were lentivirally transduced and transgenic cells enriched using flow cytometry gating on recombinant fluorochromes as described (59). For inducible transgene expression, PDX B-ALL cells were lentivirally transduced with three consecutive constructs containing the tet activator, the tet repressor and KLF4 expression cassettes under control of the TRE promoter (44).

***in vivo* experiments.** Leukemia growth and treatment effects were monitored using bioluminescence *in vivo* imaging as described (59). Competitive experiments were performed by mixing two derivate cell populations, each expressing a different transgene and distinct fluorochrome markers, and injecting both into the same animal. Human PDX cells were isolated and enriched from murine bone marrow or spleen as described in Ebinger et al.(35) and distribution of each subpopulation measured by flow cytometry using the different recombinant fluorochrome markers.

Gene Expression analysis. *Array based expression analysis:* The data sets of T- and B-ALL (n=306) and AML (n=517) patients and healthy controls (n=10) were used for gene expression analysis (GSE66006, GSE78132, GSE37642) (33, 34, 60). Details of the diagnostic workup, sample preparation, hybridization, image acquisition and analysis have been described previously (33, 34). In brief, for probes to probe set annotation we used custom chip definition files (61). The HG-U133 A, B chips and HG-U133 Plus 2.0 chips were normalized separately by the robust multichip average method as described (62) and only probes present on all chips were included in the analysis (n=17,389). The batch effect resulting from the use of different chip designs was corrected by applying an empirical Bayesian method as described elsewhere (63). Gene Set Enrichment Analysis (GSEA) using default settings (version 3.0) was used for the association of defined gene sets (Hallmarks of cancer) with different subgroups (64, 65).
RNA sequencing and data analysis of primary ALL patient samples at diagnosis and 33 days following therapy was described previously (35).

Ethical Statements. For the two primary B-ALL patient samples, written informed consent was obtained from all patients or from parents/caregivers in cases when patients were minors. The study was performed in accordance with the ethical standards of the responsible committee on human experimentation (written approval by Ethikkommission des Klinikums der Ludwig-Maximilians-Universität Munich, number 068-08 and 222-10) and with the Helsinki Declaration of 1975, as revised in 2000. Animal trials were performed in accordance with the current ethical standards of the official committee on animal experimentation (written approval by Regierung von Oberbayern, tierversuche@reg-ob.bayern.de; July 2010, number 55.2-1-54-2531-95-10; July 2010, 55.2-1-54-2531.6-10-10; January 2016, ROB-55.2Vet-2532.Vet02-15-193; May 2016, ROB-55.2Vet-2532.Vet02-16-7 and August 2016, ROB-55.2Vet-2532.Vet03-16-56).

See supplemental Methods for details. Details are provided for constructs for KLF4 inducible expression and CRISPR/Cas9-mediated knockout, cell isolation and culture conditions, EBV infection, *in vitro* culture, *in vivo* growth of PDX-ALL, monitoring of tumor burden and drug treatments, homing assay, *in vitro* culture of PDX cells and drug treatment, cell cycle analysis, gene expression analysis, Western blotting and statistical analysis.

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