Inducible transgene expression in PDX models *in vivo* identifies KLF4 as therapeutic target for B-ALL

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The process of oncogenesis leads to dysregulation of mRNA and protein levels of multiple molecules in tumor cells. A minority of them harbors an essential function and might represent attractive therapeutic targets. Unfortunately, clinic-close methods are lacking which validate alterations for their functional implications, at best in individual tumors. We developed a novel technique for inducible transgene expression in established patient-derived xenograft (PDX) models *in vivo*. Transcription factor Krüppel-like factor 4 (KLF4) acts either as an oncogene or tumor suppressor and its role in B-cell acute lymphoblastic leukemia (B-ALL) is insufficiently defined. In competitive preclinical *in vivo* trials, we found that re-expressing wild type, but not mutant control KLF4 reduced leukemia load in PDX models of B-ALL, with strongest effects after conventional chemotherapy, at minimal residual disease. Re-expressing KLF4 sensitized PDX models towards systemic chemotherapy *in vivo*. Of major translational relevance, Azacitidine upregulated KLF4 levels and knockout of KLF4 in PDX models reduced Azacitidine-induced cell death, suggesting that Azacitidine acts via KLF4. Taken together, our novel technique enabled identifying an essential role for KLF4 in established B-ALL leukemias and support applying Azacitidine in patients with B-ALL, for therapeutically targeting KLF4.

PDX models of acute leukemia | inducible transgene expression | KLF4 | Azacitidine

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Introduction

The process of oncogenesis induces a broad variety of changes in tumor cells leading to multiple alterations on the level of mRNA and protein expression. While non-genomic changes are described in depth by techniques like RNA sequencing or proteomics(1–3), the functional consequences of the numerous dysregulations remain poorly understood. Only a small minority of alterations might play an essential function for growth and maintenance of the tumor. But these essential alterations are of major clinical importance as they represent putative targets for treatment.(1–4) While techniques are missing to validate alterations for their functional consequence, we established a clinic-close method to identify alterations with life-sustaining function in individual tumors *in vivo*. So far, researchers mostly used established cancer cell lines to study the function of signaling molecules; as limitation, developing an established cell line from a primary tumor cell might induce severe changes, especially in acute lymphoblastic leukemias (ALL).(5–7) As additional technical challenge, primary ALL cells undergo rapid apoptosis *ex vivo* where they are reluctant to grow, disabling in depth studies. In contrast, patient-derived xenograft (PDX) models serve as qualified preclinical surrogates and faithfully recapitulate genetic and phenotypic tumor characteristics.(8, 9) Although resource intensive and complex in handling, orthotopic PDX models represent the most clinical-related approach up to date, for studying individual tumors.(6, 10) We studied B-cell precursor ALL (B-ALL) as B-ALL represents a highly frequent tumor disease in children and requires better treatment, especially upon disease relapse.(11) B-ALL represents a suitable model disease to established advanced PDX techniques. To identify signaling molecules with essential function, we aimed at establishing a method closely mimicking the clinical situation. In direct reflection of patients harboring pre-existing tumors, the preclinical surrogate should address tumors pre-existing in animals. Inducible *in vivo* expression systems were required and we established such a system in PDX models, while they grow in mice. We are not aware that inducible expression systems have been used in orthotopic PDX models before. We applied our novel method to an exemplary signaling molecule which is downregulated in pediatric B-ALL, namely KLF4.(12–15) KLF4 is implicated in stress-responsive regulation of cell cycle progression, apoptosis and differentiation as well as stemness and pluripotency.(16–22) KLF4 expression is downregulated in numerous cancers, but upregulated in others, and abnormal KLF4 expression elicits either oncogenic or tumor suppressor function, dependent on the cellular context, tumor type, sub-type and stage.(17, 20, 23–30) In the different B-cell malignancies, it remains unclear whether dysregulated KLF4 has (i) no effect, (ii) an oncogenic or (iii) a tumor suppressor function.(12–14, 31–37) In particular in patient B-ALL cells *in vivo*, it remains unclear whether or not downregulation of KLF4 evokes functional relevance. We developed a tetracycline-inducible expression system to re-express KLF4 in pre-existing PDX B-ALL models *in vivo* and found that

KLF4 reduced tumor growth and increased chemotherapy response. CRISPR/Cas9-mediated knockout of KLF4 in PDX cells showed that the anti-tumor effect of the hypomethylating agent Azacitidine depended, at least in part, on KLF4. Our data indicate that KLF4 represents an interesting therapeutic target in B-ALL and strengthen the use of KLF4 regulating drugs in clinical trials of B-ALL.

Results

We aimed at establishing a technique which enables validating the functional relevance of downregulated expression levels in individual tumors *in vivo*. We established PDX models of B-ALL as clinic-close surrogates, used inducible transgene expression as approach and discovered KLF4 as putative therapeutic target in B-ALL.

KLF4 acts as tumor suppressor during EBV-induced oncogenesis of B-cells We decided studying KLF4 in B-ALL as we suspected a role of KLF4 during transformation of normal human B-cells with 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.(38, 39) While primary naïve normal human B-cells rapidly die *in vitro*, EBV immortalizes them and transforms them into B-blasts (Figure 1A).(40–42) EBV transformation of normal B-cells is considered a surrogate model for B-cell oncogenesis. In a recent paper(43), we had detected strong and persistent downregulation of KLF4 mRNA levels during EBV-induced B-cell transformation (*Supplementary Figure 1A*). We now asked whether KLF4 downregulation might be of functional relevance during EBV-induced oncogenesis of B-cells.

To study the functional relevance of KLF4, we expressed KLF4 in primary normal human B-cells. We cloned two different variants of KLF4, namely wild type KLF4 (wtKLF4) or a variant form of KLF4 (mutKLF4) such that they are immediately expressed upon EBV infection of primary B-cells (*Supplementary Figure 1B*). mutKLF4 lacks the two C-terminal zinc fingers representing the DNA binding domain, which disables KLF4 to function as transcription factor, while other KLF4 functions such as via protein-protein interactions remain (*Supplementary Figure 1B*). Naïve human B-cell were infected with EBV to induce oncogenic transformation into B-blasts by viral oncogenes (Figure 1A, mock). To study KLF4, EBV was genetically modified as a vector shuttle to allow expression of wtKLF4 or mutKLF4 in normal B-cells, exactly during the time period of EBV-induced oncogenesis (Figure 1B and *Supplementary Figure 1C*). While mutKLF4 expression showed no major effects over mock EBV-infected B-cells, persistent wtKLF4 levels completely abrogated EBV-induced B-cell transformation, disabled EBV-induced B-cell proliferation and induced apoptosis in the infected cells (Figure 1C). Cell cycle analysis indicated that KLF4 expression prevented S-phase entry of EBV-infected B-cells, required for generation and expansion of B-blasts. Thus, KLF4 acts as a tumor suppressor in the model of *in vitro* EBV-induced oncogenesis of normal B-cells into B-blasts.

To the best of our knowledge, this is the first evidence demonstrating that KLF4 might act as a tumor suppressor during human B-cell transformation and B-cell oncogenesis, in the model of EBV-induced oncogenesis of normal B-cells into B-blasts. Together with published data describing a tumor suppressor role of KLF4 for BCR-ABL-mediated transformation of murine pre-B-cells by BCR-ABL(44), ectopic expression of 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 As KLF4 was important during EBV-induced B-cell oncogenesis, we wondered whether it might still be of relevance in established B-cell tumors. KLF4 is downregulated in pediatric tumors including B-ALL (12, 14, 27), especially after therapy(45) (*Supplementary Figure 2A*). We found indications for downregulated KLF4 also in adult ALL, especially in T-ALL and BCR-ABL-like ALL (*Supplementary Figure 2B*). Thus, we aimed at understanding whether deregulation of KLF4 might harbor an important function in established B-ALL tumors so that KLF4 might represent a therapeutic target.

To answer the question in individual tumors growing *in vivo*, we studied two PDX established from primary B-ALL cells from two children with relapsed B-cell precursor ALL (clinical data for both patients are published in Ebinger et al.(45)). Similarly to pediatric(12, 14, 27) and adult (*Supplementary Figure 2B*) primary B-ALL samples, both PDX B-ALL models revealed low KLF4 mRNA and protein levels (Figure 2AB). Patients always harbor pre-existing, established tumors. To mimic the clinical situation in a preclinical molecular model, we aimed at studying the role of KLF4 in mice with pre-existing tumors and set-up a novel method for inducible KLF4 expression in PDX B-ALL cells using a tetracycline-response system. Inducible transgene expression allows studying characteristics of PDX models independently from tumor cell transplantation and at different time points, e.g., before, during and after treatment and at minimal residual disease.

PDX cells were consecutively transduced with three lentiviral constructs (Figure 2CD and *Supplementary Figure 3A*) 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 (*Supplementary Figure 2B*) under control of the tet-responsive element (TRE)(46), linked to the fluorochrome mCherry via a T2A peptide (Figure 2D and *Supplementary Figure 3A*). We did not experience promoter silencing in PDX models over time, despite that TRE harbors a mini-CMV promoter and CMV promoters are rapidly silenced in leukemias. Doxycycline (DOX) treatment (indicated by green background in all Figures) induced expression of KLF4 alongside with mCherry, which served as control to re-assure similar expression levels of all recombinant

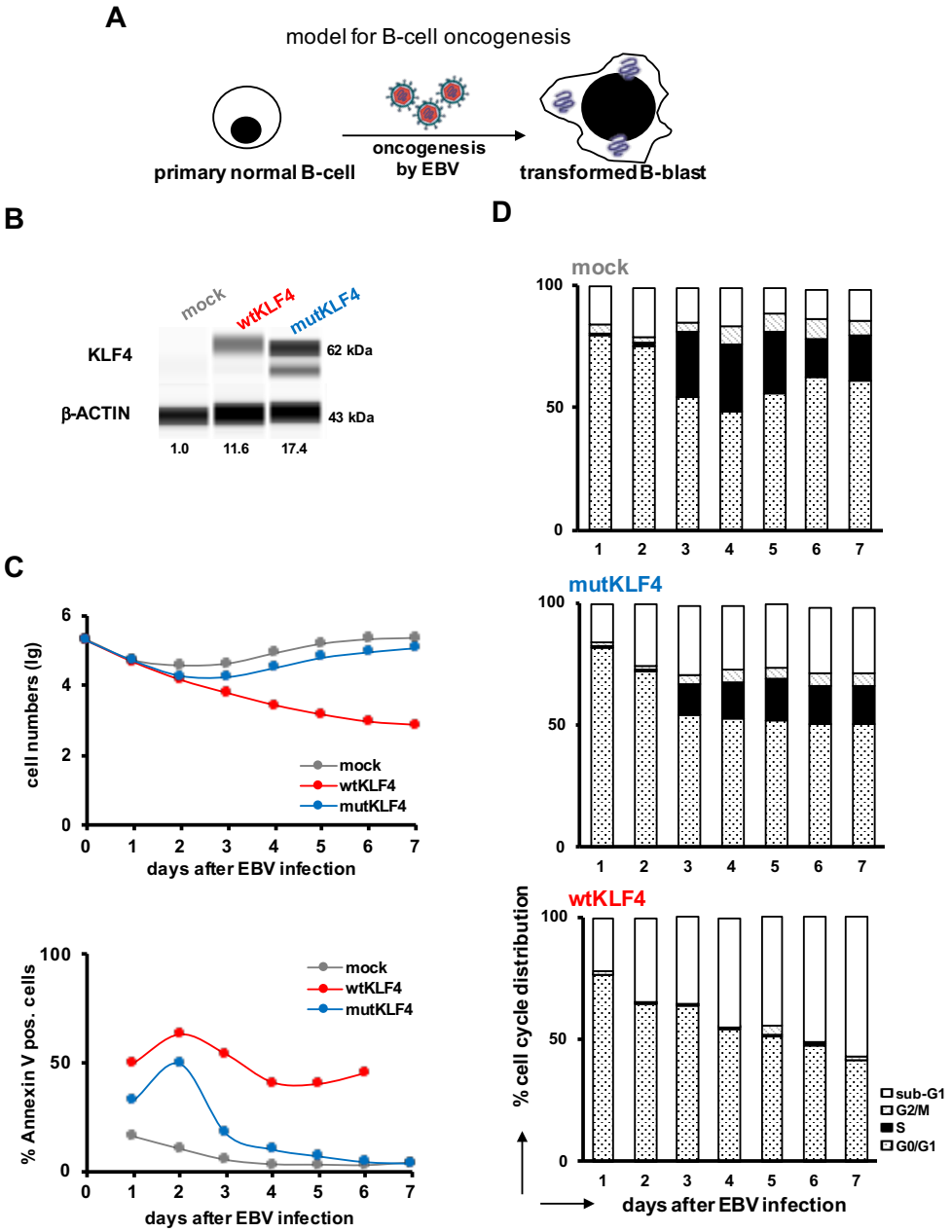

Fig. 1. KLF4 re-expression prevents EBV-mediated oncogenesis.
$2x10^5$ normal naïve primary B-cells were infected with EBV *in vitro* to induce their transformation. Either the wildtype EBV virus was used which contained all viral genes required for oncogenesis (mock); alternatively, EBV additionally expressed wild type KLF4 in normal B-cells (wtKLF4) or a mutant KLF4 derivative, lacking the DNA-binding domain (mutKLF4). After EBV infection, B-cells were harvested every day for a total of 7 days. **A** Experimental design **B** Capillary immunoassay of KLF4, two days after infection with the indicated EBV viruses. β-actin was used as loading control. **C** Cell numbers were quantified by flow cytometry (upper panel) and apoptosis measured using Annexin V staining (lower panel). **D** Cell cycle analysis; daily, an aliquot of cells was incubated with 5-Bromo-2'-deoxyuridine (BrdU) for 1 hour prior to harvest. The cells were 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 is shown using sorted naïve normal B-cells from two individual donors.

proteins. Accordingly, DOX treatment of transduced B-ALL cells induced expression of mCherry (*Supplementary Figure 3B*) and wtKLF4 or mutKLF4 protein (Figure 2E and *Supplementary Figure 3C*), which turned back to baseline upon DOX withdrawal, proving reversibility of the system (*Supplementary Figure 3C*). Of note, KLF4 expression was moderate and did not exceed expression observed in, e.g., PBMC, avoiding undesired overexpression (Figure 2E). Importantly,

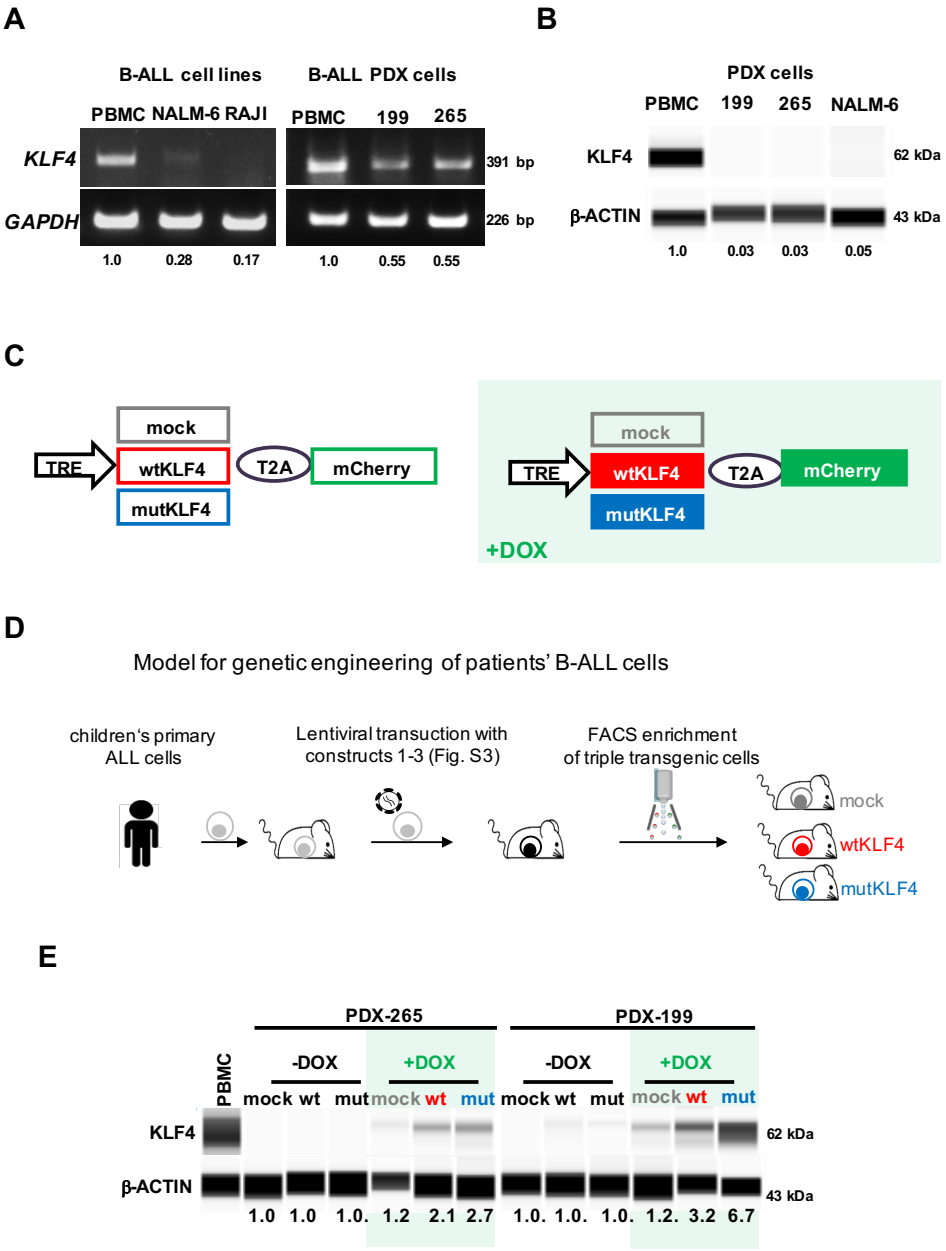

Fig. 2. Re-expression of KLF4 in PDX ALL cells using a tet-on inducible system. **A,B** KLF4 is downregulated in B-ALL cell lines, B-cell lymphoma cell lines and B-ALL PDX cells on mRNA level as determined by RT-PCR using GAPDH as loading control (**A**) and on protein level in capillary immunoassay using β-actin as loading control (**B**), as compared to PBMCs. **C** KLF4 expression vector. The TRE promoter drives expression of KLF4, either as wildtype (wt)KLF4 or a mutated (mut)KLF4 sequence devoid of two C-terminal zinc finger motifs comprising the DNA-binding domain (see *Supplementary Figure 1B*), 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 panel B and *Supplementary Figure 3A*). 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 capillary immunoassay using peripheral blood mononuclear cells (PBMC) from healthy donors and β-actin as controls. Representative analysis from triplicates are shown.

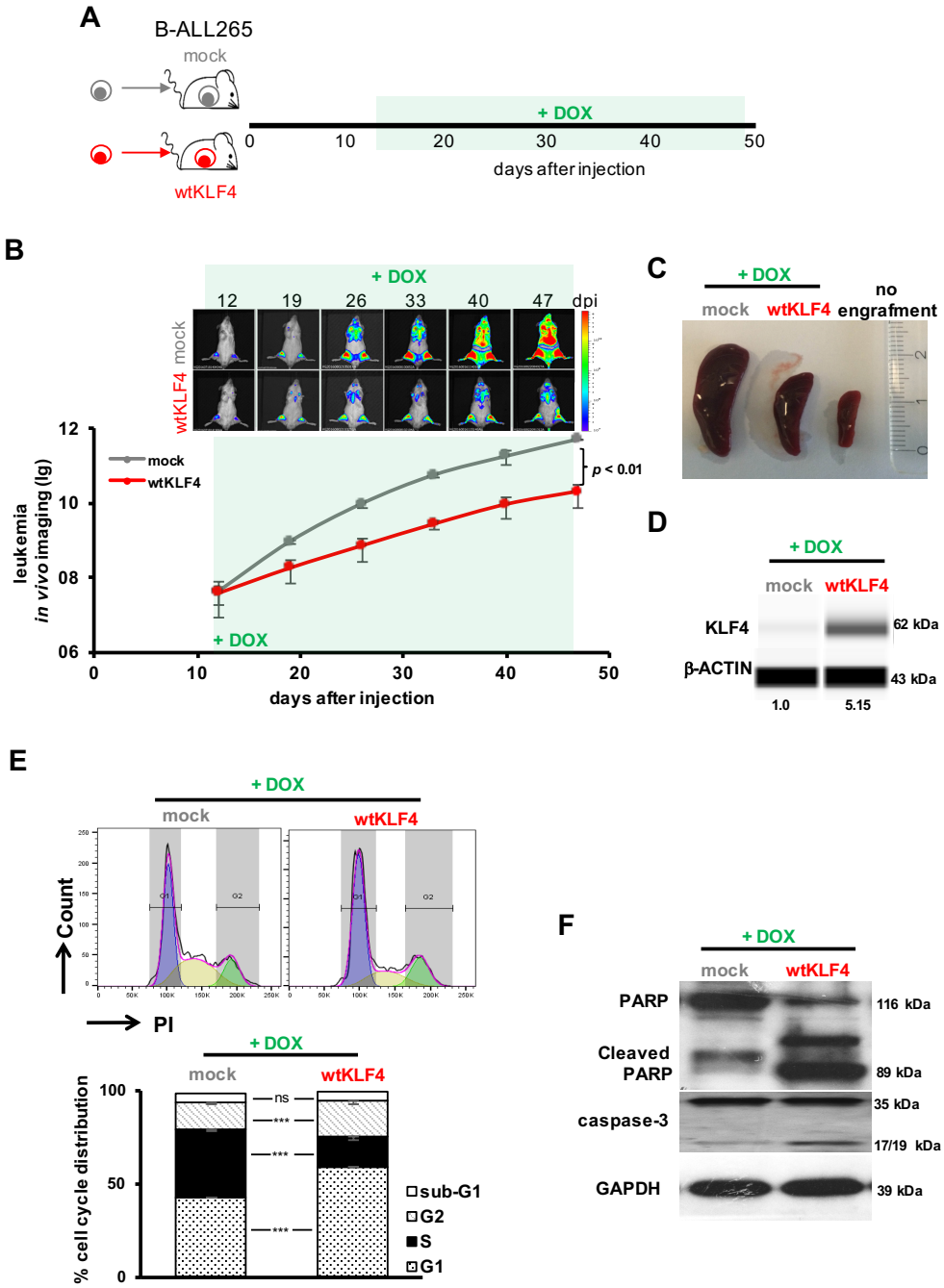

Fig. 3. Re-expressing KLF4 inhibits tumor growth through cell cycle arrest and apoptosis in B-ALL PDX cells *in vivo*. **A** Experimental design: 60,000 triple-transgenic, mock or wtKLF4 PDX ALL-265 cells were injected into 6 NSG mice each. After homing was completed and tumors were established, DOX (1mg/ml) was added to the drinking water on day 12 to induce KLF4 expression. On day 47 after cell injection, mice were sacrificed, spleens harvested and the 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<0.01 (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 capillary immunoassay; β-actin served as loading control. Representative analysis from one out of three mice are shown. **E** Cell cycle analysis; 10^6 mCherry positive cells were 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.

feeding mice with Doxycycline (DOX) induced expression of mCherry/KLF4 in PDX B-ALL cells *in vivo* (*Supplementary Figure 3B*), enabling the analysis of KLF4-mediated effects at any given time point in mice. Marker fluorochromes al-

lowed performing pairwise competitive *in vivo* experiments, having, e.g., PDX cells with wtKLF4 and mutKLF4 in the same animal.

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 and homing of B-ALL PDX cells *in vivo*. In a first step, we determined whether KLF4 downregulation was essential and required for survival and proliferation of established B-ALL tumors (data for ALL-265 in main Figures and for ALL-199 in supplemental Figures). PDX B-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 to induce transgene expression during the exponential growth phase of PDX B-ALL tumors (Figure 3A and *Supplementary Figure 4A*). *In vivo* imaging revealed that tumor growth of wtKLF4-expressing B-ALL PDX cells was diminished compared to controls (Figure 3B and *Supplementary Figure 4B*), resulting in reduced splenomegaly as compared to spleens from mock-bearing mice (Figure 3CD and *Supplementary Figure 4CD*). KLF4 significantly reduced the proportion of cells in S-phase (Figure 3E and *Supplementary Figure 4E*) and induced cleavage of PARP and Caspase-3 (Figure 3F and *Supplementary Figure 4F*), indicating that KLF4 impaired tumor growth by inducing cell cycle arrest and apoptosis; similar results were obtained in B-ALL cell lines (*Supplementary Figure 5)*.

The effect of KLF4 on tumor growth was further evaluated using pairwise competitive *in vivo* assays, wherein the control population (mock) and the population of interest (wtKLF4) were mixed at a ratio of 1:1 and injected into the same animal (indicated in orange, wtKLF4 mixture) (Figure 4A). To control this approach, a second group of mice was injected with the control mixture containing at a 1:1 ratio of mock and mutKLF4 expressing cells (indicated in purple) (Figure 4A). At the end of the experiment, each population was quantified using the respective recombinant fluorochromes. Leukemic load was monitored by *in vivo* imaging, revealing slightly reduced growth of the wtKLF4 mixture compared to the control mixture (Figure 4B, left panels). As expected, the phenotype was less pronounced compared to non-competitive experiments, because 50% of cells in the wtKLF4 mixture were control cells. When analyzing the control mixture, the proportion of each subpopulation remained constant over time (Figure 4B, upper right panel). In contrast in the wtKLF4 mixture, the wtKLF4 sub-fraction significantly diminished and was nearly lost within 30 days (Figure 4B, lower right panel). Thus, the competitive *in vivo* assay (Figure 4AB) reproduced the growth-inhibitory effect of wtKLF4 seen in the non-competitive assay (Figure 3). As major advantaes, competitive *in vivo* assays enable the identical *in vivo* environment for both populations, leading to very minor inter-mouse variabilities, high sensitivity and high reliability, while requiring reduced numbers of animals; due to these advantages, all further *in vivo* experiments were performed as pairwise competitive assays.

In a next step, homing capacity of KLF4-expressing PDX cells to the murine bone marrow was assessed in re-transplantation experiments. First generation mice were transplanted with cells either expressing mock, wtKLF4 or mutKLF4 and transgene expression was induced by DOX administration at advanced disease stage, shortly before cell isolation (*Supplementary Figure 6A*). Cells were re-transplanted as control and wtKLF4 mixture, respectively, and homing assessed 3 days later. While equal amounts of mock and mutKLF4-expressing cells were re-isolated from mice injected with the control mixture, wtKLF4-expressing cells were strongly diminished in the wtKLF4 mixture (*Supplementary Figure 6B*), indicating that wtKLF4 expression impairs homing of PDX B-ALL cells to the murine *in vivo* environment. Taken together, re-expression of KLF4 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 and abrogates homing to the murine bone marrow.

Treatment-surviving cells are especially sensitive towards re-expressing KLF4. In B-ALL, persistent minimal residual disease (MRD) is the most predictive factors for disease-free survival(11), emphasizing the need to target and eradicate MRD. As technical challenge, the number of primary MRD cells isolated from each patient is small by definition. As surrogate, elaborate *in vivo* models are required to functionally characterize the MRD cell subpopulation.(45) In children with B-ALL, primary cells surviving chemotherapy and at MRD displayed extremely low mRNA KLF4 levels, lower than in cells at diagnosis (*Supplementary Figure 1A*). Therefore, we hypothesized that MRD cells might depend on KLF4 downregulation in an especially strong way. To mimic the MRD situation in mice, we 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 less than 1% leukemia cells in bone marrow, chemotherapy was discontinued and DOX was administered to induce KLF4 expression during the tumor re-growth phase (Figure 4C). We took advantage of our inducible expression system and initiated KLF4 expression at MRD, while primary growth and treatment were performed in the absence of KLF4.

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 at MRD, tumor re-growth was clearly diminished in mice carrying the wtKLF4 mixture (Figure 4D, left panel). Sub-fraction analysis revealed that expression of wtKLF4, but not mutKLF4 led to a significant growth disadvantage of MRD cells, depleting wtKLF4 cells to undetectable levels within two weeks (Figure 4D, right panel). For comparison, therapy-naïve wtKLF4 expressing cells were diminished within 4 weeks (Figure 4B), indicating that KLF4 inhibited growth of MRD cells more drastically than growth of therapy-naïve PDX B-ALL cells (*Supplementary Figure7*).

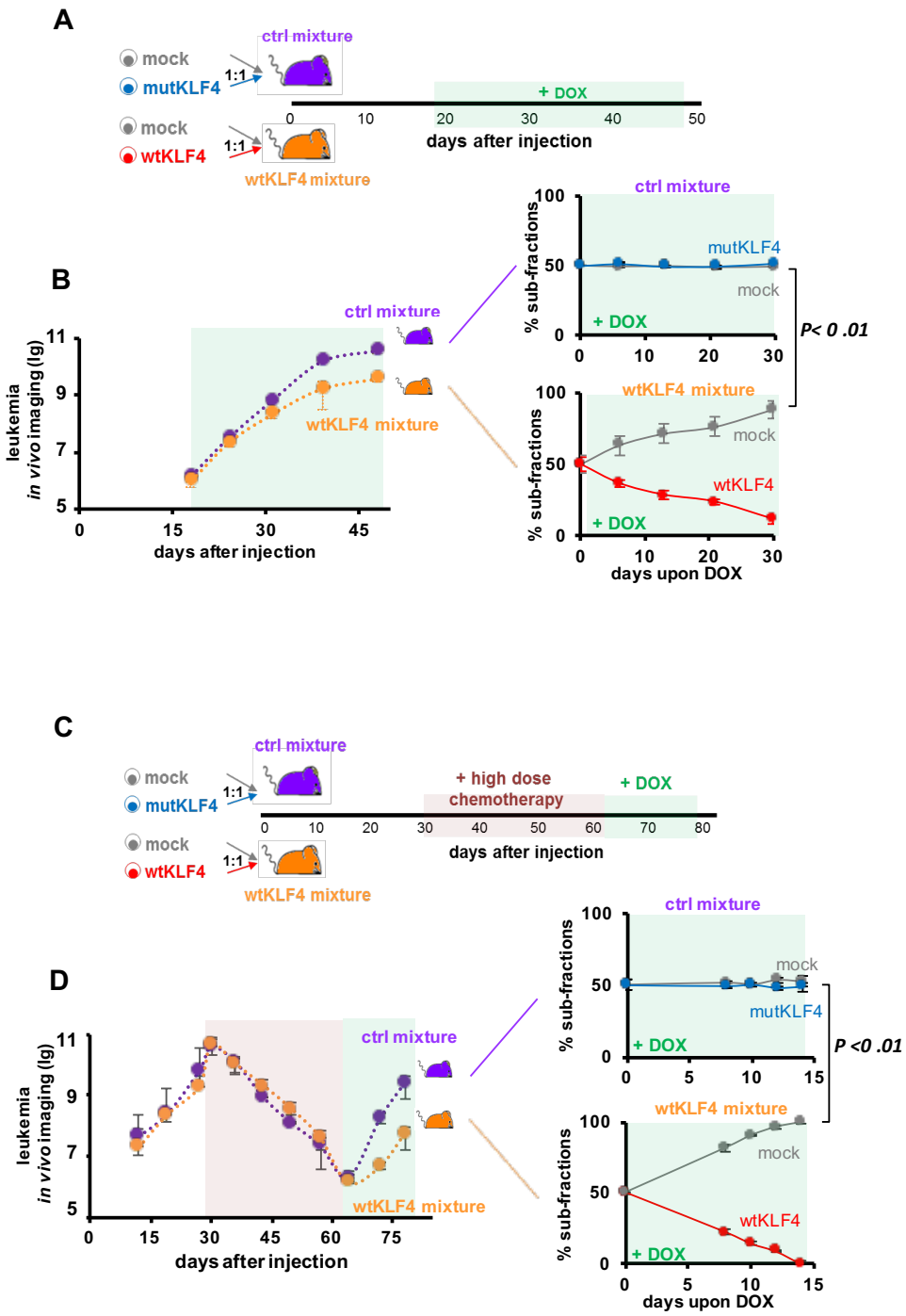

Fig. 4. KLF4 re-expressing cells are outcompeted in competitive *in vivo* assays, especially after treatment. **A** Experimental procedure: NSG mice were injected with 30,000 cells each, consisting of two different 1:1 mixtures; the control mixture (purple; n=10) consisted of a 1:1 ratio of mock and mutKLF4 expressing cells; the wtKLF4 mixture (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 in mice bearing the wtKLF4 or the control mixture. 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 *Supplementary Figure 3B* for constructs). Quantification depicted as mean ± SEM. **p< 0.01 two-tailed Student's t-test. **C** Experiments were set up as in **A**, except that transgenes were induced 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. **D** Tumor re-growth after treatment was monitored and analyzed as in **B**.

The data suggest that MRD cells are especially sensitive towards upregulating KLF4 and treatment upregulating KLF4 might be especially effective in consolidation regimens, to eliminate MRD cells.

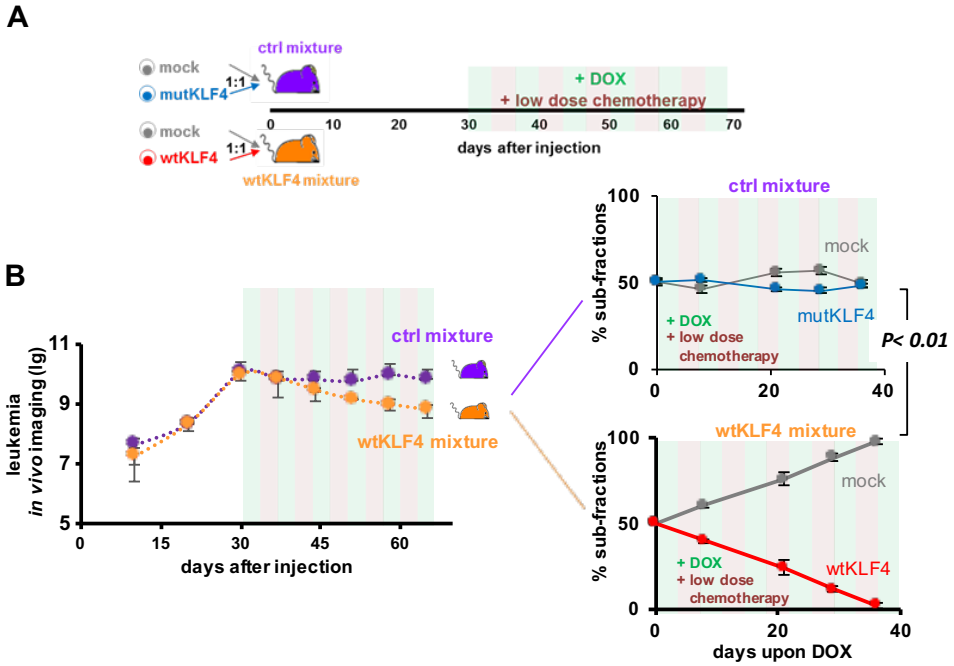

Fig. 5. KLF4 expression sensitizes PDX ALL cells to chemotherapy. **A** Experiments were set up as in Figure 3, except that transgene expression was initiated at high tumor burden (day 30) and maintained during low dose combination chemotherapy (0.2 mg/kg Vincristine + 35 mg/kg Cyclophosphamide; days 31-65). **B** Tumor growth was monitored and analyzed as in Figure 3.

Re-expressing KLF4 sensitizes B-ALL PDX cells towards chemotherapy *in vivo*. As *in vivo* growth of both PDX B-ALL samples depended on KLF4 downregulation, especially if cells were pre-treated with chemotherapy, we next asked whether targeting KLF4 might be beneficial to improve treatment of B-ALL, i.e. in the setting of induction therapy. 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 B-ALL control and wtKLF4 mixtures were injected into mice and DOX was administered together with low-dose chemotherapy at high tumor burden (Figure 5A). Chemotherapy doses were chosen such that they induced minor effects alone, e.g., stop of tumor progression, but avoiding major tumor reduction, to facilitate identifying putative additional effects elicited by KLF4.

Chemotherapy slightly reduced tumor load in mice injected with the wtKLF mixture, but not the control mixture (Figure 5B, left panel). When sub-fractions were analyzed from each mixture, wtKLF4, but not mutKLF4 expressing cells were significantly decreased by chemotherapy; wtKLF4 cells were outcompeted within less than 40 days (Figure 5B, right panel). Similarly, KLF4 re-expression sensitized NALM-6 cells towards chemotherapy *in vitro* (*Supplementary Figure 8*). 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. Having established the growth-suppressive and pro-apoptotic function of KLF4 in B-ALL PDX, we next asked whether KLF4 can be therapeutically targeted. We first tested the small molecule APTO-253, which was introduced as a KLF4 inducing drug(47), but was recently shown to also regulate MYC(48). APTO-253 indeed upregulated KLF4 in our hands and sensitized cells towards Vincristine treatment *in vitro* (*Supplementary Figure 9*). 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 thought that existing approved drugs might be able to re-upregulate KLF4 in B-cell tumors. The demethylating agent 5-Azacitidine (Aza) was shown to upregulate KLF4 expression in other tumor entities.(27, 31, 34, 49) Although Aza is known to reverse promoter hypermethylation, its decisive mode of action remains at least in part elusive.(50) 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.(51) Here we found that Aza strongly upregulated KLF4 levels in B-ALL PDX cells as well as in B-ALL cell lines (Figure 6A and *Supplementary Figure 10A*), and decreased cell viability *in vitro* at clinically relevant doses (*Supplementary Figure 10A*).
To test whether KLF4 is functionally involved in Aza-

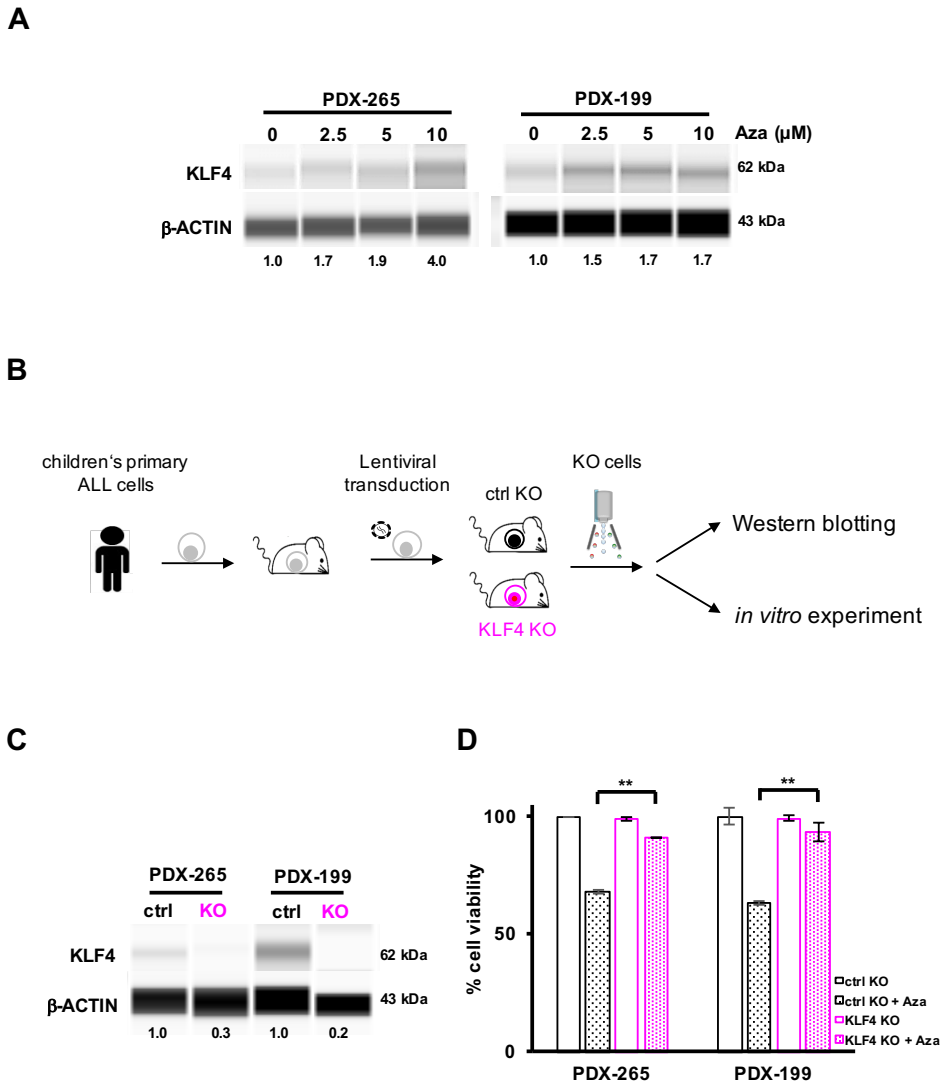

Fig. 6. Azacitidine-induced cell death depends on KLF4. **A** PDX-265 and PDX-199 cells were treated with different concentrations of Azacitidine (Aza) for 48h. KLF4 protein expression was analyzed by capillary immunoassay, β-actin served as loading control. One representative analysis of 2 independent experiments is shown. **B** Experimental procedure: B-ALL PDX cells were lentivirally transduced with Cas9, sgRNA and reporter expression vectors (*Supplementary Figure 10B*) 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 *Supplementary Figure 10B*) and subjected to capillary immunoassay and *in vitro* culture. **C** KLF4 protein level of ctrl KO or KLF4 KO PDX-265 and PDX-199 cells were analyzed by capillary immunoassay. 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 analysis 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.

induced cell death in B-cell tumors on a molecular level, we generated cells with knockout (KO) of KLF4, which is challenging in PDX. For B-ALL PDX, cells were first lentivirally transduced to recombinantly express Cas9 (Figure 6B and *Supplementary Figure 10B*). By co-transducing a second vector encoding the single guide RNA, along with a reporter

construct to enrich successfully gene edited cells, PDX B-ALL cells with stable KO of KLF4 could readily be established (Figure 6C). Stimulation of PDX B-ALL cells with Aza at clinically relevant concentrations reduced cell viability in control cells; in contrast, PDX B-ALL or NALM-6 cells with KLF4 KO displayed decreased sensitivity towards Aza-induced cell death and were rescued from Aza-mediated effects (Figure 6D and *Supplementary Figure 10C*). These data suggest that KLF4 partially mediates the effect of Aza in B-cell tumor cells and that upregulating KLF4 represents an important mechanism how Aza induces cell death.

Taken together, our data show that KLF4 represents a novel therapeutic target for B-ALL and that Aza, an established drug, can upregulate KLF4. Targeting KLF4 in B-ALL patients by introducing Aza into standard poly-chemotherapy protocols might reduce tumor burden and increase sensitivity towards conventional chemotherapy, for the benefit of patients with B-ALL.

Discussion

We describe a novel method which allows characterizing which alterations in tumor cells harbor functional relevance, in preclinical PDX models *in vivo*. Using the technique, we demonstrate that PDX B-ALL models depend on downregulation of KLF4 which plays an essential function *in vivo*; re-upregulating KLF4 using the inducible expression system or by treatment with Aza reduces the tumor. We work with genetically engineered PDX models that we propose calling GEPDX models, in accordance to genetically engineered mouse models (GEMM). Inducible GEMM were designed to allow determining the role of single molecules independently from, e.g., gestation. In analogy, our novel inducible GEPDX models allow determining the role of single molecules in pre-existing tumors, independently from, e.g., tumor transplantation into mice. Inducible GEPDX models might be established in a broad range of different tumor types beyond B-ALL. In fact, for all tumor entities allowing PDX models, GEPDX models and inducible versions thereof might be generated by adapting, e.g., promoters driving transgene expression.

Using these approaches, we demonstrate that KLF4 exerts a tumor suppressor function in certain B-cell malignancies, namely i) during EBV-induced transformation of primary B-cells; ii) in pre-existing B-ALL PDX models growing in mice, especially in the situation of MRD and; iii) during response of PDX B-ALL cells to therapy. We show that the well-described roles of KLF4 as a quiescence factor and apoptosis-inducer are maintained in the malignant B-cells studied.(17) 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. Aza has previously been shown to upregulate KLF4 in other tumor entities(27, 34, 52–54) and we prove that this ability is maintained in B-ALL. A proven clinical benefit of Aza treatment in hematological malignancies was first obtained in myelodysplastic syndrome and AML(50). In B-ALL, case reports exist on the clinical activity of Aza in ALL(55–58) and an ongoing clinical study applies Aza in patients with KMT2A rearranged B-ALL (NCT02828358). As molecular mechanism of Aza-mediated antitumor activity remain incompletely defined(50), our data suggests that KLF4 upregulation represents an important mechanism of Aza to eliminate B-ALL cells.

The data presented here highlight the power of molecular / functional testing to improve understanding of alterations in individual tumors. As we discovered that downregulating KLF4 plays an essential role for established PDX models of B-ALL to grow *in vivo*, KLF4 represents an attractive therapeutic target. Our data strengthen the idea of applying Aza in patients with B-ALL.

Materials and Methods

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).

Genetic engineering in EBV In the maxi-Epstein Barr virus (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.(59)

Genetic engineering of PDX B-ALL cells for inducible transgene expression. Primary patients' B-ALL cells were transplanted into immunocompromised mice to generate PDX models. PDX B-ALL were lentivirally transduced and transgenic cells enriched using flow cytometry gating on recombinant fluorochromes as described.(60) 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.(46)

***In vivo* experiments.** Leukemia growth and treatment effects were monitored using bioluminescence *in vivo* imaging as described.(60) 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.(45) and distribution of each subpopulation measured by flow cytometry using the different recombinant fluorochrome markers.

Methods detailed in the supplement. Details are provided for constructs for inducible expression of KLF4 and CRISPR/Cas9-mediated knockout, cell isolation and culture conditions, EBV infection, gene expression analysis, *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, capillary immunoassay and statistical analysis.

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