In vivo inducible reverse genetics in patients’ tumors to identify individual therapeutic targets

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High-throughput sequencing describes multiple alterations in individual tumors, but their functional relevance is often unclear. Clinic-close, individualized molecular model systems are required for functional validation and to identify therapeutic targets of high significance for each patient. Here, we establish a Cre-ER\textsuperscript{T2}-loxP (causes recombination, estrogen receptor mutant T2, locus of X-over P1) based inducible RNAi- (ribonucleic acid interference) mediated gene silencing system in patient-derived xenograft (PDX) models of acute leukemias in vivo. Mimicking anti-cancer therapy in patients, gene inhibition is initiated in mice harboring orthotopic tumors. In fluorochrome guided, competitive in vivo trials, silencing of the apoptosis regulator \textit{MCL1} (myeloid cell leukemia sequence 1) correlates to pharmacological \textit{MCL1} inhibition in patients’ tumors, demonstrating the ability of the method to detect therapeutic vulnerabilities. The technique identifies a major tumor-maintaining potency of the \textit{MLL-AF4} (mixed lineage leukemia, ALL1-fused gene from chromosome 4) fusion, restricted to samples carrying the translocation. \textit{DUX4} (double homeobox 4) plays an essential role in patients’ leukemias carrying the recently described \textit{DUX4-IGH} (immunoglobulin heavy chain) translocation, while the downstream mediator \textit{DDIT4L} (DNA-damage-inducible transcript 4 like) is identified as therapeutic vulnerability. By individualizing functional genomics in established tumors in vivo, our technique decisively complements the value chain of precision oncology. Being broadly applicable to tumors of all kinds, it will considerably reinforce personalizing anti-cancer treatment in the future.
Translating comprehensive cancer sequencing results into targeted therapies has been limited by shortcomings of model systems and techniques for preclinical target validation\(^3\). The methodological gap contributes to the fact that only below 10% of drugs, successful in preclinical studies, pass early clinical evaluation and receive approval\(^4\).

Functional genomic tools including RNA interference (RNAi) proved of utmost importance to annotate the numerous alterations detected by multi-omics profiling and significantly deepened our understanding of the merit of individual genes as drug targets\(^5\). As limitation, functional studies have largely been restricted to cancer cell lines, which often fall short in predicting the role of alterations in individual human tumors\(^2\). To approximate the situation of the patient, the predictive power of primary tumor cell cultures\(^6\) and organoids\(^9\) is currently under intense investigation\(^10\).

For mirroring the clinical situation even closer, patient-derived xenograft (PDX) mouse models have been demonstrated to faithfully recapitulate the complexity of tumors in humans. PDX models are available for the vast majority of human cancers, and their preclinical value for biomarker identification and drug testing is well established\(^11-15\). It is increasingly recognized that the drug development process might profit from studying PDX models with molecular techniques, routinely used in cell line models and genetically engineered mouse models (GEMM)\(^16,17\). Still, RNAi techniques were only rarely applied for in vivo mechanistic studies in PDX, mainly due to technical challenges such as low transduction efficiencies and the need for continuous in vivo growth and associated high demand on resources\(^16\). As an advantage over constitutive systems, inducible gene silencing prevents overestimating in vivo gene function by avoiding influences from, e.g., transplantation and engraftment, and allows mimicking the treatment situation in patients with established tumors. The use of Cre-ERT2-loxP combines the properties of high ligand sensitivity while maintaining tight control of shRNA expression in the un-induced state, thus minimizing leakiness, an advantage over tet-regulated systems\(^16,18-20\).

Here, we report a Cre-ER\(^T2\) inducible RNAi in PDX models in vivo, using acute leukemia (AL) as prototype disease where ex vivo investigation on primary cells is challenging, but orthotopic PDX models are promising\(^21,22\). In proof of principle studies, we demonstrated that MCL1 silencing in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) PDX models correlates to response to pharmacological MCL1 inhibition. We confirmed a tumor-maintaining potency of the MLL-AF4 fusion protein in PDX models in vivo and used the technique to identify DDIT4L as therapeutic targets in PDX ALL carrying the recently described DUX4-IGH translocation.

Results

Development of a Cre-ER\(^T2\) inducible shRNA knockdown approach in vivo. To test the suitability of the inducible knockdown system across a broad range of leukemia subtypes, primary tumor cells from 5 patients with AL (3 pediatric ALL, 1 adult ALL, 1 adult AML; clinical patient data in Table S1) were transplanted into NOD scid gamma (NSG) mice (Fig. 1a). Resulting PDX cells were genetically engineered first with a construct encoding a Tamoxifen (TAM)-inducible variant of Cre recombinase, Cre-ER\(^T2\), together with a red fluorochrome for enriching transgenic cells and Gaius luciferase (Luc) for bioluminescence in vivo imaging\(^23\) (Fig. 1a). Transduction efficiencies were typically well below 30% (Table S2), putatively indicating a single viral integration per genome according to literature\(^24\), leading to homogenous expression levels of Cre-ER\(^T2\) (Fig. S1a), minimal toxicity and neglectable leakiness in all samples, thus overcoming one of the challenges of TRE-based inducible expression systems\(^19\).

In a second step, PDX cells were transduced with the small hairpin (sh) RNA expression vectors (Figs. 1a and S1b). The miR30-based knockdown cassette was directly coupled to a fluorochrome and both were cloned in antisense orientation, flanked by two pairs of loxP sites. In the absence of TAM, neither the inducible fluorochrome nor the shRNA were expressed. TAM administration induced a two-step Cre-ER\(^T2\)-mediated recombination process which flipped the fluorochrome-shRNA insert into sense orientation, initiating its expression (Fig. S1b-c\(^25,26\)). A set of 4 recombinant fluorochromes was used to monitor shRNA transduction and recombination and to enable competitive in vivo assays (Fig. S1c). Transduction efficiency was tracked by iRFP the control vector encoding an shRNA targeting Renilla luciferase (shCTRL), or by mTagBFP in the vector encoding a gene of interest (GOI)-specific shRNA (shGOI) (Table S2). Upon TAM administration, Cre-ER\(^T2\)-mediated recombination deleted the constitutively expressed fluorochromes iRFP and mTagBFP and induced expression of the second set of fluorochromes\(^27\) (Figs. 1a and S1b-c\(^\)). T-Sapphire and eGFP were chosen as inducible fluorochromes due to their high similarities in sequence and expression kinetics\(^28\) and replaced iRFP and mTagBFP expression upon TAM treatment. The two knockdown vectors enabled pairwise competitive in vivo experiments in the same animal to increase reliability and sensitivity, while saving resources.

Mice were transplanted with a 1:1 mixture of PDX cells from the same patient expressing either of the two RNAi vectors, shCTRL or shGOI (Fig. 1a). For exemplary purposes and to describe distinct aspects of the method, the apoptosis regulator MCL1 was chosen as GOI (Figs. 1 and S1). As quality control, expression of constitutive markers revealed equal engraftment of both populations at the time of TAM administration (Fig. 1b).

To induce gene silencing, TAM was administered to mice with pre-established leukemias when homing and initial engraftment to the murine bone marrow was achieved and PDX cells were in the exponential growth phase, mimicking treatment of patients with pre-existing tumors. Systemic TAM administration induced expression of the inducible fluorochromes T-Sapphire or EGFP, in similar amounts for both constructs, starting as early as 24 h, with highest expression levels obtained at 72 h after TAM (Fig. 1b). The functional consequences of control and GOI knockdown were monitored by quantifying each population according to their fluorochromes, using flow cytometry (Fig. 1b-c\(^\)). TAM was dosed to obtain substantial Cre-ER\(^T2\) induced recombination in the absence of toxicity and with recombination efficiencies independent of tumor load (Fig. S1d).

Several quality controls were performed to exclude unspecific toxicities; the distribution of both populations remained stable over time after TAM treatment, if both populations expressed shCTRL (shCTRL/shCTRL mixture in Fig. 1c, upper lane) in all PDX samples analyzed (Fig. S1d). Similarly, the distribution of the shCTRL/shGOI mixture remained unchanged, if mice received the carrier solution alone (Fig. S1f-g\(^\)). These results are in line with our previous studies\(^29\), where we found that transduction and enrichment of PDX cells was not associated with clonal selection, and that PDX samples largely maintained their sample-specific mutational pattern.

In contrast and upon treatment with TAM, the population expressing a shRNA targeting an essential GOI (shMCL1) decreased over time and was overgrown by control cells (Fig. 1c, lower lane and Fig. S1h). Loss of cells with GOI knockdown in vivo proved a functional importance of the GOI on the molecular level, mimicking elimination of tumor cells in patients upon treatment with a targeted drug.
Inducible silencing of MCL1 correlates response to small molecule MCL1 inhibitors in vivo. To test whether inducible knockdown of the GOI correlates to targeted inhibitors, we first analyzed the response of PDX samples to shRNA-mediated inhibition of MCL1. We selected MCL1 as proof of principle target gene from literature as certain, but not all leukemias seem responsive to MCL1 inhibition30,31. The anti-apoptotic gene MCL1 was chosen as it is dysregulated in numerous tumor entities32 and MCL1 inhibitors are currently investigated in clinical trials yielding mixed results33 (NCT03218683). Predicting treatment response for selecting patients who will profit from MCL1 directed therapy remains a major challenge and functional in vivo assays might provide helpful insights34.

We studied PDX models from three different patients with acute leukemia (AML-388, ALL-199, ALL-265). In the AML-388 PDX model, we found a clear decrease of cells with MCL1 knockdown compared to control cells in vivo, accompanied by efficient knockdown on protein level (Fig.2a–b), validating MCL1 as important vulnerability. Importantly, these effects were independent of tumor load at the time of TAM administration, supporting the use of the inducible knockdown system at any disease stage (Fig. S2a). In contrast, knockdown of MCL1 in two...
ALL samples showed minor to no effects on growth, proving patient-individual sensitivities (Figs. 2c and S2b). Silencing MCL1 in AML-388 induced rapid cell death, which was already detectable within the first 2 h after TAM administration (Fig. S2c–e). Gene set enrichment analysis from RNA sequencing data comparing shCTRL and shMCL1 PDX cells indicated that MCL1 knockdown was associated with activation of the apoptosis pathway, verified using Annexin-V staining (Fig. S2d–e). To visualize selective loss of individual GFP-positive cells upon MCL1 silencing, re-transplantation experiments into wildtype zebrafish (danio rerio) were performed, which confirmed significant and rapid depletion of PDX cells upon MCL1 knockdown between 48 and 72 h after TAM in an independent in vivo model (Fig. S2f).

Taken together, using the inducible knockdown approach, MCL1 could be identified as a therapeutic vulnerability in one of 3 PDX samples, for which functional relevance could not be predicted by expression levels of anti-apoptotic BCL-2 family members, highlighting the need for functional assays (Fig. S2g).

As silencing of MCL1 induced cell death in PDX AML-388, but not in ALL-199 nor ALL-265, we next asked whether this prediction by expression levels of anti-apoptotic BCL-2 family members correlated to response of PDX samples to the pharmacological inhibition of MCL1. We studied the small molecule antagonist S63845 (Fig. 2d), which has previously been shown to be effective in AML cell lines and PDX samples, and is currently under clinical investigation as single agent (NCT02979366) or in combination regimens (NCT03672695). Treatment of mice bearing AML-388 significantly diminished tumor burden as monitored by in vivo bioluminescence imaging (Fig. 2e), reduced splenomegaly (Figs. 2f and S2h) and number of PDX cells (Fig. S2i) re-isolated from the murine spleens or bone marrow. In contrast, the MCL1 inhibitor had no effect on ALL-199, recapitulating effects observed in the inducible knockdown system. Thus, the inducible knockdown system correlated to response of PDX samples to the pharmacological inhibition, confirming the use of this technique as surrogate to study sample-specific vulnerabilities on a molecular level in a highly clinically relevant setting.

Because MCL1 has been shown to confer resistance to several anticancer drugs, we examined in a next step whether knockdown of MCL1 strengthens the response of AML PDX models towards drug treatment in vivo. Groups of mice were treated either with the BCL-2 inhibitor ABT-199 (Venetoclax) (Fig. 2g), or the conventional chemotherapeutic drug Cytarabine (Figs 2j–k) at doses that do not significantly reduce tumor burden in mice. Both treatments further decreased the MCL1 knockdown population in a synergistic way, indicating that sensitivity towards ABT-199 or Cytarabine might be increased by MCL1 directed treatment in patients (Fig. 2g, Fig. 2j–k). Thus, using MCL1 as exemplary target, we provide evidence that our approach enables distinguishing between subgroups of tumors in order to select patients, which might profit from therapies targeting a certain GOI, and to evaluate treatment combinations.

Specific targeting of the fusion oncogene MLL-AF4. To further validate the specificity of our approach, we next studied a bona fide positive control with high likelihood of harboring an essential function in established PDX tumors in vivo. The translocation t(4;11) and corresponding expression of the MLL-AF4 fusion (KMT2A-AFF1) is present in 80% of infant B-precursor ALL patients, and is associated with poor prognosis. Several studies elucidated its role in ALL cell lines and mouse models, but up to date no molecular investigations on its function have been carried out in patient cells or established tumors growing in vivo. We designed a shRNA targeting a mRNA breakpoint shared by several patients, which significantly reduced expression of the fusion transcript (Fig. 3a). Because the shRNA sequence targeted neither of the individual wildtype genes, MLL or AF4 (Figs. 3a and S3a–b), no major adverse effects on normal tissue are expected when applied in vivo, e.g., by systemic gene therapeutic approaches. Inducible knockdown of MLL-AF4 significantly reduced ALL cells in the t(4;11)-positive PDX model tested, but not in a translocation-negative sample, proving a tumor maintaining role of MLL-AF4 in established patient tumors in vivo (Figs. 3b and S3a). Variations between the different animals were neglectable reflecting the high reliability of our approach (Fig. 3b). Reduced tumor growth of the shMLL-AF4 mixture was visible using in vivo imaging, even though 50% of injected tumor cells expressed shCTRL (Fig. 3c). Gene expression analysis demonstrated that shCTRL cells expressed a set...
of genes characteristic for samples with the MLL-AF4 translocation, which was no longer present upon shMLL-AF4 knockdown, where an expression signature similar to non-MLL rearranged samples prevailed (Fig. S3c–d).

These results prove the selectivity and operability of our technique and showed that MLL-AF4 harbors an essential function in established patient-derived leukemias growing in vivo. We provide strong molecular evidence in a clinically relevant model that the translocation transcript represents an attractive therapeutic target for future therapies.

DDIT4L is a therapeutic vulnerability in DUX4-IGH rearranged acute lymphoplastic leukemia. In a last step, we examined a less well studied tumor alteration, the recently discovered rearrangement t(4;14) which occurs in 7% of ALL patients and results in the DUX4-IGH gene fusion. Because cells with t(4;14) display high levels of otherwise absent DUX4, we asked whether DUX4 represents a vulnerability in this subgroup of ALL in vivo. Using our technique, we demonstrated an essential function for DUX4 in t(4;14) rearranged ALL-811 (Fig. 3d). Expression of the DUX4-IGH translocation was reported to be associated with a defined gene expression...
signature, previously referred to as the "ERG subtype"42–46. We performed gene expression analysis of shDUX4 and shCTRL ALL-811 cells (Fig. 3e) and performed gene set enrichment analysis (GSEA) with two published datasets45,46. We found genes over-expressed in DUX4 knockdown NALM6 cells45 also enriched in our shDUX4 PDX sample (Figs. 3f and 5s3e Set 1). Accordingly, genes downregulated in DUX4 knockdown NALM-6 cells (Fig. 3s3e Set 2) and genes highly expressed in the cluster of patients characterized by DUX4 translocation and ERG deletion45 (Fig. 3s3f, Set 3) were enriched in the shCTRL sample (Fig. 5s3g). These data confirm the presence of the typical DUX4 signature in shCTRL PDX cells and demonstrate reversal of this signature upon DUX4 knockdown in a PDX model in vivo (Figs. 3e–f and 5s3e–g). Our technique could thus identify DUX4 as attractive therapeutic target to treat the recently detected subgroup of DUX4-IGH rearranged ALL.

To further confirm the relevance of the detected genes for tumor maintenance of DUX4-rearranged samples we tested the role of one gene that was downregulated upon DUX4 silencing in PDX ALL-811 and in NALM-6 cells (Fig. 3g), the DNA-damage-inducible transcript 4-like (DDIT4L; also known as Redd2 or Rtp801L), which has been shown to regulate mitOR signaling and autophagy in mammalian cells. DDIT4L expression is induced in the presence of different types of pathological stress, suggesting a possible involvement of DDIT4L in stress response47–49. Interestingly, we found DDIT4L highly expressed in DUX4-expressing ALL (Fig. 5s3h). Inducible knockdown of DDIT4L significantly diminished leukemic growth within 2 weeks of in vivo tumor growth (Fig. 3h–i), suggesting that downregulation of DDIT4L might have mediated, at least in part, the growth inhibitory effects observed in the shDUX4 population. Taken together, we identify DDIT4L as a therapeutic vulnerability in the DUX4-IGH subtype of B-ALL.

Discussion
We have established a method which combines an in vivo approach with patient-derived tumor cells and pre-established tumors for inducible knockdown and allows validating vulnerabilities on an individual patient level. We established the technique, as preclinical molecular approaches are lacking which faithfully mimics the situation of a pre-existing tumor, avoiding in vivo predictions of drug sensitivity. Our molecular approach closely mimics the clinical situation of a pre-existing tumor, avoiding in vivo predictions of drug sensitivity. We envision a major potential of our method on a proof-of-concept level, where deeper knowledge on tumor dependencies will improve clinical decision making to individualize treatment. Due to its major potential to tailor drug development, improve patient care and increase the success rate of clinical trials, our technique will foster personalized oncology in the future.

Methods
Ethical statement. Written informed consent was obtained from all patients and from parents/carers in the cases where 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 München, Ethikkommission@med.uni-muenchen.de, April 15/2008, number 068-08; September 24/2010, number 222-10; January 18/2019, number 2008).
All animal trials were performed in compliance with the ARRIVE guidelines (https://arriveguidelines.org) and 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, January 15/2016, Az. ROB-55.2Vet-2532.Vet_02-16-7; Az. ROB-55.2Vet-2532.Vet_02-15-193; ROB-55.2Vet-2532.Vet_03-16-56).

Animal model. Six to 16 weeks old male and female NOD.Cg-Prkdcscid IL2rgtm1Wjl/SzJ (NSG) mice (The Jackson Laboratory, Bar Harbour, ME, USA) were included. Mice were kept under specified pathogen-free (SPF) conditions with a 12/12 h light cycle, temperature of 20–24 °C and 45–65% humidity according to Annex A of the European Convention 2007/526 EC. The maximum stocking density of the cages corresponds to Annex III of the 2010/63 EU. The cages were constantly filled with structural enrichment and the animals had unlimited access to food and water. During the experiment, mice were kept in individually ventilated cages (IVCs). Hygiene monitoring was carried out at least quarterly in accordance with the current FELASA recommendation.

Donor mice used for PDX cell amplification were sacrificed at advanced leukemic disease (more than 60% leukemic cells within peripheral blood) or when first clinical signs of illness appeared (rough fur, hunchback, reduced motility, paralysis). Experimental mice were sacrificed at specified time points.

Generating transgenic patient derived xenograft (PDX) models. Establishing serially passaged AML and ALL PDX models in NSG mice, re-isolating PDX cells from mice, PDX cell culture, lentiviral transduction, enrichment of transgenic cells and in vivo imaging were described previously29,52,53.

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Generation of AML and ALL-PDX models. Fresh primary AML or ALL cells were isolated by Ficoll gradient centrifugation from peripheral blood or bone marrow aspirates that had been obtained from leftovers of clinical routine sampling before onset of therapy and injected into 6–12 weeks old NSG mice via the tail vein. Engraftment was monitored by 2-weekly flow cytometry measurement of human cells in peripheral blood starting at week 4. Mice were sacrificed at first clinical signs of disease, as measured by quantification of human cells in peripheral blood. From engrafted mice
first generation), PDX AML or ALL cells were resuspended out of femurs, tibiae and spleen by mincing the tissues and filtration through a cell strainer, followed by Ficoll gradient centrifugation in case of spleenic cells. PDX AML cells were identified by staining for human CD45, CD33, CD3 and CD19 (CD38 for PDX ALL) and human CD14 and CD11b in case of splenic cells. Without further enrichment or manipulation, 1×10⁶ total BM cells were reinjected into recipient NSG mice for reexpansion (secondary transplantation).

Lentiviral transduction and cell enrichment. Lentiviral transduction was performed as previously described. Briefly, PDX cells freshly isolated from mouse spleen or BM were re-suspended in RPMI-Medium (Life Technologies) supplemented with 20% fetal calf serum (Biochrom AG, Berlin, Germany), 5% d-Gluta- 
mine, 1% Gentamicin, 1% Penicillin/Streptomycin, 0.66% mixture of rh-insulin/ 
human transferrin/sodium selenite (Life Technologies), 1 mM sodium pyruvate, 
and 50 µM 1-thioglycerol (Sigma-Aldrich, Hannover, Germany). 1×10⁶ cells in 1 
ml medium were transferred to a cell culture plate and were transduced overnight 
with lentiviral constructs in the presence of 8 µg/ml polybrene (Sigma-Aldrich). To 
save one round of passaging through mice, PDX cells freshly transduced with 
lentiviruses were kept in culture for 4 days to allow marker expression and 
enrichment of transgenic cells using a FACSaria III (BD Bioscience) and the 
FACSDiva software 8.0.2 (BD Bioscience). Sorted cells were then re-injected 
to next generation recipient mice.

Bioluminescence in vivo imaging. In vivo bioluminescence imaging (BLI) BLI was performed as previously described. The IVIS Lumina II Imaging System was used (Caliper Life Sciences, Manz, Germany). Mice were anesthetized using isoflurane, placed into the imaging chamber in a supine position and fixed at the lower limbs and by the inhalation tube. Coelenterazine (Synchem OHG, Felsberg/Altenburg, 
Germany) was dissolved in acidified methanol (HPLC grade) at concentration 
10 mg/ml and diluted shortly before injection in sterile HBG buffer (HEPES-buf- 
fered Glucose containing 20 mM HEPES at pH 7.1, 5% glucose w/v). Immediately 
after intravenous tail vein injection of 100 µg of native Coelenterazine, mice were 
 imaged for 15 s using a field of view of 12.5 cm with binning 8, U/stop 1 and open 
filter setting. To monitor tumor growth, mice were imaged once weekly; after 
thrapy, mice were imaged every other day.

Quantification of BLI pictures. Quantification of BLI signal was performed as previously described. The Living Image software 4.4 (Caliper Life Sciences, Manz, Germany) was used for data acquisition and quantification of light emission using a scale with a minimum of 1.8×10⁶ photons per second per cm² per solid angle of one steradian (sr). Different regions of interest (ROI) were defined and 
signals were considered positive, when light emission exceeded background in each 
ROI. Background was measured in mice harboring Gluc negative leukemias. A 
ROI covering the entire animal was used (background 4×10⁶ photons per second). As 
an exception to determine early engraftment, a small ROI covering the femurs 
was used (background 6×10⁶ photons per second), as light emission became visible 
there first. Overt leukemia was considered above 10⁶ photons per second using the 
ROI covering the entire animal.

Cloning. For constitutive expression of the CreER²T recombinase, the coding sequence of the enzyme was PCR amplified from the CreER²TFlrNeoFr cassette (gift from MSS) using a 5’ primer carrying NsiI and a 3’ primer carrying PsaI-NasiI and ligated into the NsiI digested pCDH-SFFV-GLC-T2A-mCherry vector downstream of the T2A peptide (Fig. S2a) (pCDH-vector, System Bioscience). For inducible knockdown of target genes, the lentiviral FLIP vector system was optimized to link shRNA expression to 
fluorescein expression. We used the lentiviral pCDH backbone, digested the vector 
with SspI and Sall and introduced the following elements as a pre-synthesized stretch of 
DNA (GenScript®, Picataway, NJ, USA): SspI - SFFV -lox2272 - mTagBFP (T-Sapphire) 
lox2272 - lox5171 - mir-30 cassette-eGFP (T-Sapphire) -lox2272 - lox5171 – Sall (Fig. S2b). The 
shRNA sequences targeting the different genes (MCL1, DUX4, DDIT4L; see Table S2) 
were designed using theSplashRNA algorithm, with the exception of MALL-AF4 where 
sequences were designed to be distinguishable from the patient-specific transcript 
breakpoint (Table S2). As control, a shRNA targeting the Renilla luciferase was used in all 
experiments (shCTRL). The shRNA sequences were introduced into the miR30 cassette 
of the KD vector as part of pre-synthesized and annealed, complementary single strand 
DNA oligos (110 bps, Table S2; Integrated DNA Technologies, USA), having XhoI 
and BssHII as 5’ and 3’ restriction sites, respectively. For knockdown of MALL-AF4, 
the miR-KE knockdown cassette was used and concatenated to enhance the knockdown 
efficiency.

Flow cytometric analysis of competitive in vivo experiments. Freshly isolated PDX cells were analyzed using LSRII (BD Bioscience) to determine fluorescentemoces. Forward/Side scatter analysis was used to gate on living cells, followed by 
gating on mCherry (CreER²T positive PDX cells). At the beginning, the two cell 
populations of the mixture were distinguished by expression of either eGFP or 
mTagBFP. Upon CreER²T recombination, cells expressing shCTRL started expressing 
T-Sapphire (instead of eGFP), while cells expressing shGOI expressed eGFP (instead of 
mTagBFP) (Fig. S1b); the color switch was monitored in two separate histograms for 
either T-Sapphire or eGFP (Fig. 1b). The final analysis combined the two histograms, 
compared all cells expressing either of the two shRNAs, either T-Sapphire/shCTRL or 
eGFP/shGOI (Fig. 1b and c).
To determine the sensitivity of different PDX samples to inhibition of selected GOI, the percentage of cells with knockdown of the GOI (eGFP-expressing cells) were compared between starting conditions (3 days after TAM) to later time points, using at least \( n = 3 \) data points per time point and condition. A significant depletion in the amount of eGFP/shGOI positive cells over time characterized PDX samples sensitive to the knockdown of the GOI. For target genes inducing rapid cell death upon knockdown, data after TAM administration can be used for comparison. To separate shCTRL and shGOI populations for further investigations, cells were sorted using FACSaria III (BD Bioscience).

**Statistical analysis.** Statistical significance of pairwise competitive in vivo experiments was analyzed by comparing the percentage of eGFP-positive cells out of all recombined cells (sum of T-Sapphire positive plus eGFP positive cells) between the shCTRL/shGOI mix at 72 h after TAM administration with the shCTRL/shGOI mix at the end of each experiment. Statistical analyses were performed using GraphPad Prism 8. Student’s t-test was used, if not differently stated in the legends. A p-value of \( \leq 0.05 \) was considered significant.

**In vivo drug treatment.** For in vivo treatment with ABT-199 (Venetoclax, SelleckChem, USA) or Cytarabine (Cell Pharma GmbH, Bad Vilbel, Germany), mice were injected with a 1:1 mixture of shCTRL/shMCL1 AML-388 PDX cells (3×10^5 cells/mouse) and TAM was administered one week thereafter to all animals. 72 h after TAM, three mice were sacrificed to determine recombination efficiency. The remaining animals were divided into three groups and treated either with solvent (\( n = 3 \)) or ABT-199 (100 mg/kg in Carboxymethyl cellulose (1% w/v)/DMSO (2% v/v) by oral gavage for 5 consecutive days and 2 weeks; \( n = 3 \)) or Cytarabine (100 mg/kg in PBS by intraperitoneal injection) for two consecutive days and 1 week; \( n = 3 \). At the end of the experiment, mice were sacrificed. BM processed and PDX cells analyzed by flow cytometry for subpopulations’ distribution.

Synergistic effect was calculated using the fractional product method27. Measured survival rates were 0.39 upon MCL1 KD and 1.0 upon Venetoclax. Expected apoptosis induction of independent application of MCL1 knockdown and Venetoclax was calculated as \([1 \text{ minus (survival after simulation with MCL1 knockdown) times (survival after simulation with VCR)](\text{times})\] which resulted to be 0.61; measured apoptosis by the combination of MCL1 and Venetoclax was 0.94 and thus much higher than the expected apoptosis of 0.61, proving that the combination acted in a synergistic way.

For in vivo treatment with S63845 (Roche, Mannheim, Germany), using a Femtojet microinjector (Eppendorf, Hamburg, Germany), with 200 to 500 AML-388 PDX cells per embryo of the 75 µM) (Sigma-Aldrich, P7629) wild type zebrafish embryos/larvae were studied exclusively within the first 5 days after fertilization, harvested, handled compliant to local animal welfare regulations and maintained according to standard protocols (www.ZFIN.org) which does not require a special permit according to German Laboratory Animal Protection Law.

**Flow cytometric analysis of BH3 proteins’ level and Annexin V staining.** To determine intracellular expression levels of BH3 proteins, cells were fixed in 2% paraformaldehyde, permeabilized using perm/wash buffer (BD Bioscience, Franklin Lakes, NJ, USA) and subsequently stained with fluorescently labeled antibodies against BCL-2 (clone Bcl-2/100, BD Bioscience), BCL-X, (clone 54H11, Cell Signalling, Cambridge, UK), MCL-1 (Clone D2W9E, Cell signalling) or respective isotype controls (Cat.: 55637, BD Bioscience; clone DALE, Cell Signaling). Dead cells were excluded by Fixable Viability Dye staining. If not otherwise stated, reagents and antibodies were purchased from eBioscience. Flow cytometric analysis was performed on a BD FACS Canto II (BD Bioscience) and data were analyzed using Flowjo software (TreeStar Inc., Ashland, OR, USA).

For Annexin V staining was performed on PDX AML-388, ALL-199 and ALL-265 cells isolated from the mouse BM 72 h after TAM treatment or thawed and treated in vitro, using PE/Cy7 Annexin V (BioLegend, 640949) according to the manufacturer’s instruction and analyzed by flow cytometry (LSRII, BD Bioscience).

**Targeted genome sequencing.** The MLL-ALF4 breakpoint was sequenced at the certified laboratory for Leukemia Diagnostics, Department of Medicine III, University Hospital, LMU Munich, Munich, Germany.

**Real-time quantitative PCR.** Total RNA from flow cytometry enriched populations was enriched using RNeasy Mini Kit (Qagen, Venlo, Netherlands) and reverse transcription using MMLV reverse transcriptase (Promega, Madison, WI). qPCR was performed using SYBR green master mix following the supplier’s recommendations. Statistical analysis was performed using the R 3.6.1 software package (R Core Team, 2019). In case of multiple testing, p-values were adjusted using the Benjamini-Hochberg procedure (FDR-cutoff <0.05). Gene expression profiling was performed on a LightCycler 480 (Roche, Mannheim, Germany) using the corresponding LightCycler 480 Probes Master and the pre-designed Probes of the Universal ProbeLibrary (Roche, Mannheim, Germany). The primer and probes for all gene sets were as follows. The forward primer is in bold and the reverse primer is in italics.

**Gene expression profiling.** Gene expression analysis was performed by applying a bulk subtracted SCRP-seq protocol on sorted subpopulations from PDX samples as described previously25. Briefly, for library preparation 2,000 cells of each individual sample were sorted and lysed in RLT Plus (Qiagen) lysis buffer with 2-mercaptoethanol (Sigma–Aldrich) and stored at –80 °C until processing. A modified SCRP-seq protocol (6, 7) was used for library preparation. Briefly, proteins in the lysate were digested by Protease K (Ambion), RNA was cleaned up using SPRI beads (GE, 22% PEG). In order to remove isolated DNA samples were treated with DNase I for 15 min at RT. DNA was generated using MACE bulk adjusted SCRB-seq protocol (6, 7) was used for library preparation. Brie...
Protein immunassay. To quantify protein of low PDX cell numbers, the Simple Western capillary protein immunassay (ProteinSimple) was performed using the following antibodies: MCL1 (D3CA5, Cell Signaling Technologies). Antibodies used were MCL1 (D3CA5, Cell Signaling Technologies), β-actin (NB600-501S, Novus Biologicals). Western blot analysis of PDX ALL-265 was performed as previously described68, using the following antibodies: MCL1 (S-19, Santa Cruz Biotechnology) and GAPDH (6C5, Merck Millipore).

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Author contributions
M.C. designed and performed experiments, analyzed data and wrote the manuscript. K.V. designed, performed experiments and analyzed data with contributions from J.V. (establishing the technique), M.B. (DUX4-IGB), D.S. (data analysis and writing the manuscript), Y.G. (PCR, MCL-1 inhibitor treatment), W.H.L. (cloning), B.V. (establishment of treatment regimens), J.F.S. (quality control experiments); J.W.B. performed and T.H. and V.J. analyzed DUX4-SCRB-seq data; A.W. and R.M. analyzed MLF-44 RNAseq data; A.A. and V.B. performed zebrafish experiments; V.D. and P.I. quantified BH3 protein expression; B.F. and K.R. designed fluorochrome use; C.B., L.B., L.L. and D.M.S. provided PDX models; C.M., M.S.S. and M.B. developed cloning strategies; I.J. supervised the study, and contributed to experimental design, data analysis and writing the manuscript.

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