METHODS

Generation of primary mouse lymphomas and leukaemias, and use of primary human B-cell lymphoma, B-CLL and AML samples. All animal protocols used in this study were approved by the governmental review board (Landesamt Berlin), and conform to the respective regulatory standards. Lymphomas with defined genetic defects were generated by intercrossing Eμ-*Myc* transgenic mice to mice carrying loss-of-function alleles at the *Suv39h1* locus[30,31] or to mice harbouring a 4-OHT-inducible *p53-ER^{TAM}* knock-in allele, encoding a p53–oestrogen receptor fusion protein that is inactive in the absence of 4-OHT[32], all in a C57BL/6 background. Eμ-*Myc* transgenic lymphomas that formed in Eμ-*Myc;p53-ER$^{Tam/+}$* mice with an allelic loss of the remaining *p53* wild-type allele were designated *p53-ER^{Tam}* lymphomas. *Suv39h1*$^{-}$ lymphomas reflect Eμ-*Myc* lymphomas that arose in *Suv39h1*$^{-/-}$ females or, owing to the X-linkage of the *Suv39h1* locus, in *Suv39h1*$^{Y/-}$ males[33]. Genotyping of the offspring by allele-specific genomic PCR, monitoring of lymphoma onset and isolation of viable lymphoma cells were carried out as described[8,34]. *Kras^{G12D}*;shp53-GFP-induced T-cell acute lymphoblastic leukaemias (T-ALL) with tetracycline (that is, doxycycline)-dependent shp53 expression ('DOX-on') were generated and isolated following a previously published protocol with minor modifications[23,35]. The *Nras^{G12D}*/MLL-AF9-driven mouse model of acute myeloid leukaemia (AML), co-expressing a reverse tetracycline transactivator ('Tet-on competent'), was generated as previously described[24]. Six- to eight-week-old C57BL/6 ('wild type') female mice were used as recipients for *in vivo* lymphoma or leukaemia propagation. No randomization or blinding was used to allocate experimental groups.

The use of tumour biopsies (that is, bone marrow aspirates, lymph-node biopsies or peripheral blood samples obtained for the initial diagnosis or follow-up analyses of patients with B-cell leukaemia (B-CLL), diffuse large B-cell lymphoma (DLBCL) or acute myeloid leukaemia (AML)) as anonymous samples after informed patient consent was approved by the local ethics commission of the Charité – Universitätsmedizin Berlin (reference EA4/085/07 and EA4/061/11).

Cell culture, plasmids and retroviral gene transfer. Isolated mouse lymphoma cells and primary human AML samples (tumour-cell-purified by Ficoll density-gradient centrifugation and red cell lysis) were short-term cultured in standard medium on irradiated NIH3T3 fibroblast feeders[36]. Primary human B-cell malignancies were cultivated in a 'CD40 system'[37], that is, in the same medium further supplemented with 100 IU ml^{-1} of recombinant human interleukin-4 (Peprotech) on irradiated NIH3T3 cells stably expressing the human CD40 ligand. Human cancer cell lines were obtained from DSMZ (Leibniz-Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), ATCC or Biomol: RCK8 (DSMZ; ACC-561), Eheb (DSMZ; ACC-67), K562 (DSMZ; ACC-10), Mec1 (DSMZ; ACC-497), Molm13 (DSMZ; ACC-554), SW480 (DSMZ; ACC-313), LS174T (DSMZ; ACC-759), DLD-1 (DSMZ; ACC-278), Caco-2 (DSMZ; ACC-169), SKMel28 (ATCC; HTB-72), MeWo (ATCC; HTB-65), WM266.4 (Biomol; WM266-4-01). Omm2.3 cells were provided by Martina J. Jager. The cells were cultivated according to the supplier's recommendations and regularly tested for mycoplasma contamination. The cell lines bought within last four years were not additionally authenticated (RCK8, Eheb, Mec-1). All other cell lines were authenticated by DSMZ using a single-nucleotide polymorphism-based multiplex approach in October 2017. Single-nucleotide polymorphism profiles matched known profiles or were unique (Omm2.3). Retroviral supernatants, generated by transient transfection of Phoenix-Eco packaging cells with murine stem-cell retrovirus (MSCV)-based constructs, were used to stably infect Eμ-*Myc* transgenic lymphomas, *Kras^{G12D}*;shp53–GFP T-ALL cells, *Nras*^{G12D}/MLL-AF9 AML cells or human cancer cell lines (engineered to express the ecotropic virus receptor as described[38]). Freshly isolated cells were first infected with an MSCV retrovirus encoding murine or human *Bcl2* and a blasticidin antibiotic resistance gene. *Bcl2*-overexpressing Eμ-*Myc;Suv39h1*$^{-}$ lymphoma were subsequently infected with *Suv39h1-ER^{T2}* cDNA, encoding murine full-length *Suv39h1,* fused in frame with the coding sequence of an 4-OHT-inducible oestrogen receptor mutant (ER^{T2}; see ref. 39), subcloned into MSCV-IRES–GFP or MSCV-IRES-DsRed vectors. GFP- or DsRed-positive cells were purified in a fluorescence-activated cell sorter (FACS Aria II, BD Biosciences). TOPflash and FOPflash reporter constructs (reflecting the wild-type or mutant TCF-binding promoter region followed by a firefly luciferase-encoding cDNA) were subcloned from the original pGL3 vector into a self-inactivating MSCV$_{SIN}$-DsRed plasmid, stably transferred into mouse lymphoma cells or human cell lines (expressing the ecotropic virus receptor), and flow-sorted for DsRed-positive cells. NF-κB inactivation was achieved by stable overexpression of an IκBαΔN construct (NF-κB super-repressor (NF-κB-SR)) in control;*Bcl2* cells as reported previously[40]. Wnt pathway activation was achieved by transducing control;*Bcl2* lymphomas with a stabilized murine β-catenin (encompassing an N-terminal 90-amino acid deletion, $^{\Delta N}$β-catenin)-encoding MSCV-IRES–GFP retrovirus. To stably knock down *β-catenin* expression, a previously published shRNA sequence[41] was subcloned into the pSuperRetro plasmid to infect *Suv39h1*$^{-}$*;Bcl2;Suv39h1*-ER^{T2} cells. An MSCV$_{SIN}$-based construct containing a miR30-shRNA against murine *p53* under a tetracycline-dependent promoter[42] was used to transfect *Nras^{G12D}*/MLL-AF9;*Bcl2* cells. Stable *TP53* knockdown in human cell lines RCK8, Molm-13 and LT174T was achieved by lentiviral transduction with a previously published shRNA against *p53* (ref. 43) in the pLKO.1-puro vector (Addgene plasmid 19119).

***In vitro* and *in vivo* treatments.** For the induction of cellular senescence *in vitro*, Adriamycin (ADR; Sigma), a topoisomerase II inhibitor widely used in the clinic to treat lymphomas and other malignancies, was added once at a concentration of 0.05 μg ml^{-1} in all experiments, with the following exceptions: Eheb, Mec1, Molm13 and RCK8 cell lines, treated with 0.01 μg ml^{-1} ADR, and the K562 cell line, treated with 0.025 μg ml^{-1} ADR. For conditional activation of ER^{Tam}- or ER^{T2}-fused constructs, the cells were additionally exposed over five days to 1 μM of 4-OHT (Sigma) or the equivalent volume of the ethanol-based solvent. Cellular senescence was assessed after five days of treatment. Pharmacological inhibition of the Wnt pathway or kinases involved in modulating Wnt signalling was performed by adding small molecule inhibitors to cells for the final 48 h of the senescence-inducing ADR ± 4-OHT treatment: Wnt inhibitors ICG-001 (10 μM; Enzo Life Sciences) and salinomycin (1 μM; Sigma), MAPK inhibitor PD325901 (10 nM; Selleckchem), MEK inhibitor PD98059 (25 μM; Selleckchem), PI3K inhibitor LY294002 (10 μM, Sigma-Aldrich), Akt inhibitor MK-2206 (200 nM, Selleckchem) or GSK3β inhibitor CHIR99021 (1 μM; Sigma-Aldrich). For Wnt-modulating treatments upon senescence-release, passage-2 never senescent and previously senescent cells were used (that is, ADR ± 4-OHT-pretreated Eμ-*Myc; Suv39h1*$^{-}$*;Bcl2;Suv39h1*-ER^{T2} cells, further propagated in 4-OHT/ADR-free medium for 14 days). Matched pairs of previously senescent and never senescent cells were exposed to Wnt inhibitors as described above, or to recombinant mouse Wnt3a (10 ng ml^{-1}, R&D Systems), recombinant mouse R-Spondin 2 (Rspo2; 20 ng ml^{-1}, R&D Systems), a combination of the two ligands (at the same concentration as for single treatments) or to the GSK3β inhibitor CHIR99021 (1 μM, Sigma-Aldrich) for 48 h regarding the gene expression analysis or for seven days (in methylcellulose medium) regarding colony formation assessment. The doxycycline (DOX)-dependent activation of an shRNA against *p53* in mouse *Kras^{G12D}*;shp53–GFP T-ALL or *Nras^{G12D}*/MLL-AF9 AML samples was achieved by supplementing the culture medium with 1 μg ml^{-1} of doxycycline (Sigma).

For *in vivo* experiments, 1 × 10^{6} Eμ-*Myc;Suv39h1*$^{-}$*;Bcl2;Suv39h1*-ER^{T2} lymphoma or 5 × 10^{6} *Kras^{G12D}*;shp53–GFP T-ALL leukaemia cells (or 1 × 10^{6} Lineage^{-} (Lin^{-}) cells as a positive control), if not otherwise indicated, were transplanted by tail-vein injection into immunocompetent recipient mice. In case of *Kras^{G12D}*;shp53–GFP T-ALL leukaemia samples, recipient mice were irradiated with 6 Gy, 24 h before transplantation. DOX was supplied with the drinking water (20 mg ml^{-1}; exchanged twice a week) and in food pellets (200 mg kg^{-1} of regular chow). Leukaemia manifestation was diagnosed by flow cytometry-based detection of GFP-positive cells in the peripheral blood at the time mice presented with general signs of pre-terminal sickness (greater than 20% weight loss or other symptoms of severe sickness). If no signs of sickness were noted, the experiments were ended by 70% tumour burden in peripheral blood. Lymphoma formation was diagnosed when palpable lymph-node enlargements had formed. A tumour size of 16 mm (corresponding to approximately 4 lymph nodes of 4 mm in diameter) was approved by Landesamt Berlin as an experiment end-point criterion and was not exceeded in any of the performed experiments. ICG-001 and salinomycin were applied intraperitoneally daily (both at a dose of 10 mg kg^{-1} body weight), starting from palpable lymphoma formation until a pre-terminal disease stage was reached. Time-to-death was defined as the latency between transplantation and a pre-terminal disease stage. Upon CO_2 euthanasia, single-cell suspensions were isolated from enlarged organs as described previously[8,36].

Analysis of growth parameters, viability, stem-cell and senescence markers. Cell-cycle analysis by 5-bromo-2′-deoxyuridine/propidium iodide (BrdU/PI)-based flow cytometric measurement was performed as described previously[33]. Cytospin preparations of suspension cultures for subsequent SA-β-gal analyses or immunostainings were carried out as described previously[6,44,45]. Carboxyfluorescein succinimidyl ester (CFSE) labelling was performed on day 3 after starting ADR ± 4-OHT treatment, using the CellTrace Far Red Cell Proliferation Kit for flow cytometry (Molecular Probes, C34564) according to the manufacturer's recommendations. CFSE^{high} cells were sorted on treatment day 5 on an S3e Cell Sorter (Bio-Rad). For β-catenin co-staining, CFSE-labelled cells were fixed in 4% paraformaldehyde, permeabilized by Saponin in 1% bovine serum albumin (LifeTechnologies, 10635), stained with Alexa Fluor 488 mouse anti-β-catenin antibody according to the manufacturer's recommendations (BD Pharmingen, 562505), and acquired on an ImageStreamX Mark II Imaging Flow Cytometer (Amnis, MerckMillipore). EdU labelling was performed on treatment day 5 using the Click-iT EdU Pacific Blue Flow Cytometry Assay Kit according to the manufacturer's recommendations (Molecular Probes, C10418). For the fluorescent SA-β-gal labelling, cells