**Supplementary Methods**

**Analysis of aberrantly spliced MDM4 mRNA**

Semiquantitative RT-PCR reactions to investigate the presence of MDM4 transcripts with skipped exon 6 were performed as described (1). cDNA from individual BCR-ABL+ colonies was used as template.

The following primers were used:

Mdm4 skipped exon

Fw: 5’-TGTGGTGGAGATCTTTTGGG-3’

Rv: 5’-TCAGTTCTTTTTCTGGGATTGG-3’

**Chromatome**

**Sample preparation and phosphopeptide enrichment**

Phospho-chromatome was performed in accordance to the previously described protocol (2). The purified chromatin pellet was dissolved in Benzonase digestion buffer (10 mM HEPES pH 7.5, 2.5 mM CaCl2, 2.5 mM MgCl2), homogenized (Covaris S220) and subjected to Benzonase digestion at 4° for 35 min. SDS lysis (100 mM HEPES, 4%SDS) was performed for 10 min at room temperature and 5 min at 99°. The supernatant was collected by centrifugation at 16.000 x g at 20°C for 20min and transferred to fresh eppendorf tubes. Protein concentration was determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). FASP was performed using a 30 kDa molecular weight cutoff filter (VIVACON 500; Sartorius Stedim Biotech GmbH, 37070 Goettingen, Germany) essentially according to the procedure described by Wisniewski et al. (3). In brief, proteins were reduced by adding DTT at a final concentration of 83.3 mM followed by incubation at 99°C for 5 min. After cooling to room temperature, samples were mixed with 200 μL of freshly prepared 8 M urea in 100 mM Tris-HCl (pH 8.5) (UA-solution) in the filter unit and centrifuged at 14.000 × g for 15 min at 20 °C to remove SDS. Any residual SDS was washed out by a second washing step with 200 μL of UA. The proteins were alkylated with 100 μL of 50 mM iodoacetamide in the dark for 30 min at RT. Afterward, three washing steps with 100 μL of UA solution were performed, followed by three washing steps with 100µL of 50 mM TEAB buffer (Sigma-Aldrich). Proteins were digested with trypsin at a ratio of 1:35 overnight at 37 °C. Peptides were recovered using 40 μL of 50 mM TEAB buffer followed by 50 μL of 0.5 M NaCl (Sigma-Aldrich). Peptides were desalted using C18 solid phase extraction spin columns (The Nest Group, Southborough, MA). After desalting, peptides were labeled with TMT 6plex™ reagents according to the manufacturer (Pierce, Rockford, IL). After quenching of the labeling reaction, labeled peptides were pooled, organic solvent removed in vacuum concentrator and labelled peptides loaded onto a SPE column. Peptides were eluted with 300µL 80% acetonitrile containing 0.1% trifluoroacetic to achieve a final peptide concentration of ~1µg/µl. Eluate was then used for phosphopeptide enrichment applying a modified method of immobilized metal affinity chromatography (IMAC) published by Ficarro et al. (4). Briefly, two times 100 µL of Ni-NTA superflow slurry (QIAGEN Inc., Valencia, USA) were washed with LCMS-grade water and Ni2+ stripped off the beads by incubation with 100 mM of EDTA, pH 8 solution for 1 hr at room temperature. Stripped NTA resin was recharged with Fe3+-ions by incubation with a fresh solution of Fe(III)Cl3 and 75 µL of charged resin used for the enrichment of a total of ~300 µg TMT-labelled peptide. The unbound fraction was transferred to a fresh glass vial and used for offline fractionation for the analysis of the whole chromatome proteome. After washing the slurry with 0.1% TFA, phosphopeptides were eluted with a freshly prepared ammonia solution containing 3mM EDTA, pH 8 and all used for offline fractionation for the analysis of the phophoproteome.

**Offline Fractionation via RP-HPLC at high pH**

Tryptic peptides were re-buffered in 20 mM ammonium formiate buffer shortly before separation by reversed phase liquid chromatography at pH 10. The unbound fraction of the phosphopeptide enrichment was separated into 96 time-based fractions on a Phenomenex column (150 × 2.0 mm Gemini-NX 3 µm C18 110Å, Phenomenex, Torrance, CA, USA) using an Agilent 1200 series HPLC system fitted with a binary pump delivering solvent at 100 µL/min. Acidified fractions were consolidated into 40 fractions via a concatenated strategy described by Wang et al (5). The bound fraction containing the phosphopeptides was separated into 20 fractions on a Dionex column (500 µm × 50 mm PepSwift RP monolithic, Dionex Corporation, Sunnyvale, CA, USA) using an Agilent 1200 series nanopump delivering solvent at 4 µL/min. Peptides were separated by applying a gradient of 5 - 60% acetonitrile containing 20 mM ammonium formiate, pH 10 as described by (Gilar et al., 2005). Prepared samples were kept at -80°C until the analysis.

**2D-RP/RP Liquid Chromatography Mass Spectrometry**

After solvent removal in a vacuum concentrator, samples were reconstituted in 5% formic acid for LC-MS/MS analysis. Mass spectrometry was performed on an Orbitrap Fusion Lumos mass spectrometer (ThermoFisher Scientific, San Jose, CA) coupled to a Dionex Ultimate 3000RSLC nano system (ThermoFisher Scientific, San Jose, CA) via nanoflex source interface. Tryptic peptides were loaded onto a trap column (Pepmap 100, 5μm, 5 × 0.3 mm, ThermoFisher Scientific, San Jose, CA) at a flow rate of 10 μL/min using 2% ACN and 0.1% TFA as loading buffer. After loading, the trap column was switched in-line with a 30 cm, 75 µm inner diameter analytical column (packed in-house with ReproSil-Pur 120 C18-AQ, 3 μm, Dr. Maisch, Ammerbuch-Entringen, Germany). Mobile-phase A consisted of 0.4% formic acid in water and mobile-phase B of 0.4% formic acid in a mix of 90% acetonitrile and 10% water. The flow rate was set to 230 nL/min and a 90 min gradient used (6 to 30% solvent B within 81 min, 30 to 65% solvent B within 8 min and, 65 to 100% solvent B within 1 min, 100% solvent B for 6 min before equilibrating at 6% solvent B for 18 min). Analysis was performed in a data-dependent acquisition mode with full MS scans acquired from 375 to 1650 m/z in the orbitrap at a resolution of 120,000 (at 200Th). Automatic gain control (AGC) was set to a target of 2 × 105 and a maximum injection time of 50 ms. Precursor ions for MS2 analysis were selected using a TopN dependant scan approach with a cycle time of 3 seconds. For the analysis of the phosphoproteome an additional inclusion list containing 136 m/z values of preferentially selected phosphopeptides was enabled. MS2 spectra were acquired in the orbitrap (FT) using a quadrupole isolation window of 1 Da and higher energy collision induced dissociation (HCD) at a normalized collision energy (NCE) of 38%. AGC target was set to 5 × 104. For the global proteome a maximum injection time of 54 ms was set and an MS2 scans acquired at a resolution of 7,500 (at 200 Th). For the global phosphoproteome a maximum injection time of 150 ms was set and MS2 scans acquired at a resolution of 15,000 (at 200 Th). Dynamic exclusion for selected ions was 60 s. A single lock mass at m/z 445.120024 was employed (6). Xcalibur version 4.0.0 and Tune 2.1 were used to operate the instrument.

**Data Analysis**

Acquired raw data files were processed using the Proteome Discoverer 2.2.0. platform, utilising the Sequest HT database search engine and Percolator validation software node (V3.04) to remove false positives with a false discovery rate (FDR) of 1% on peptide and protein level under strict conditions. Searches were performed with full tryptic digestion against the mouse SwissProt database v2017.12 (25293 sequences and appended known contaminants) with up to two miscleavage sites. Oxidation (+15.9949 Da) of methionine was set as variable modification, whilst carbamidomethylation (+57.0214 Da) of cysteine residues and TMT 6-plex labelling of peptide N-termini and lysine residues were set as fixed modifications. For phosphopeptides phosphorylation (+79.9663 Da) of serine, threonine and tyrosine was additionally included as as variable modification. Data was searched with mass tolerances of ±10 ppm and 0.025 Da on the precursor and fragment ions (HCD), respectively. Results were filtered to include peptide spectrum matches (PSMs) with Sequest HT cross-correlation factor (Xcorr) scores of ≥1 and high peptide confidence. The ptmRS algorithm was additionally used to validate phospopeptides with a set score cutoff of 90. PSMs with precursor isolation interference values of ≥ 50% and average TMT-reporter ion signal-to-noise values (S/N) ≤ 10 were excluded from quantitation. Isotopic impurity correction and TMT channel-normalization based on total peptide amount were applied. For statistical analysis and p-value calculation, the integrated ANOVA hypothesis test was used. TMT ratios with p-values below 0.01 were considered as significant.

**Kinase Assay**

The kinase-reactions were performed in Kinase Reaction buffer (40mM Tris pH 7.5, 20mM MgCl2, 0.1 mg/ml BSA, 50μM DTT). The following purified recombinant proteins (100ng per 25 µl kinase reaction) were used as substrates: EBF-1 (CloudClone), NFYA (OriGene) and ZBTB7A (OriGene). CDK6 activity was detected in the presence or absence of ATP (250µM), Palbociclib (500 nM) and recombinant substrate proteins using the CDK6/CyclinD3 Kinase Enzyme System and the ADP-Glo™ Kinase Assay Kit following manufacturer’s instruction.

**Microarray Analysis**

The quality of the RNA was evaluated using the Laboratory-Chip technique (Agilent Bioanalyzer). Samples were amplified and fluorescent-labeled by *in vitro* transcription using the Two-Color Microarray-Based Gene Expression Analysis (Agilent) kit, following manufacturer’s instructions. For hybridization, the Mouse Gene Expression G3 60K (Agilent) containing ∼56000 60-mer probes was used. Images were acquired and quantified by means of confocal scanner and software (Agilent G2505C and Feature Extraction). The expression levels were processed in FlexArray 1.6.3 using standard methods of normalization, significance analysis and multiple testing correction. Data were Loess-normalized and a linear model was applied to obtain a list of genes that was differentially expressed between the three genotypes. These values were corrected for multiple testing using the Benjamini-Hochberg algorithm. Genes showing an adjusted p-value below 0.05 were considered as differentially expressed. Gene ontology analysis was performed according to the PANTHER classification system (7). Heatmaps were generated using the R package gplots version 3.0.1. The microarray data reported in this article has been deposited in the Gene Expression Omnibus (GEO) database (Accession ID: GSE87420).

**Motif enrichment analysis**

The peaks of the ChIP-Seq analysis were annotated with Homers annotate.pl script using default parameters. Only peaks within promoter regions (promoter-TSS tag) were considered for further analysis. A list of proximal downstream genes of the promoter peaks was overlaid with differentially expressed genes found in colonies, cell lines or both. The promoter peaks of these intersected genes were used for a motif enrichment analysis using Homers findMotifsGenome.pl with the default size 200 base pairs. The motif analysis was performed 3 times and motifs that i) were not marked as potentially false positives within Homers *de novo* results and ii) appeared in at least 2 replicates, were considered enriched.

**Analysis of publically available leukemia datasets – genome wide expression analysis**

Microarray data (Affymetrix GeneChip® Human Genome U133 Plus 2.0) data of healthy bone marrow control samples, MDS, c-ALL/Pre-B-ALL with t(9;22) and without t(9;22) translocation from the MILE study were downloaded from the NCBI GEO series GSE13159 (8,9). Additionally, the acute lymphoblastic leukemia dataset from the Phase I TARGET study (Affymetrix GeneChip® Human Genome U133 Plus 2.0 data, https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study\_id=phs000218.v16.p6) was obtained via the TARGET data matrix (https://ocg.cancer.gov/programs/target/data-matrix). CEL files were normalized using the R packages affy version 1.50.0 and frma version 1.24.0. For the correlation of CDK6 levels with the incidence of p53 mutations in Thymoma and Glioblastoma, data was downloaded from the (TCGA) database (10,11). CDK6 expression was calculated as means of expression for probes 224847\_at,  224848\_at,  224851\_at, 235287\_at, 243000\_at. Expression levels for these probes were normally distributed. Probes 207143\_at and 231198\_at which also hybridize to CDK6 were excluded based on low correlations to the gene expression of other CDK6 probes. As gene expression of many probes was not normally distributed we calculated pairwise Spearman correlation coefficients between mean CDK6 expression and all other probes on the array. To establish significance we permuted mean CDK6 gene expression across patients 500 times and recalculated pairwise Spearman correlation coefficients for each of the permuted datasets. With 54676 probes on the array this gave a total of 2733800 correlation coefficients. We then considered correlation coefficients as significant when they exceeded levels coefficients observed in the permuted data. The probability of getting a stronger correlation is 1/500 =0.002 already corrected for multiple testing based on the genome wide permutations. (All analysis were performed with R 3.2.3. https://www.R-project.org). To generate a heat-map depicting commonly CDK6- associated genes we chose the following procedure: each column represents an individual patient and each row one CDK6 regulated transcript. This matrix was plotted using the R function heatmap.3.R (https://github.com/obigriffith/biostar-tutorials). In addition, probes were grouped into positive (blue) and negative (red) correlating probes based on their Spearman’s Rank correlation coefficient as indicated for each probe. For the Gene Set Enrichment Analysis (GSEA; http://software.broadinstitute.org/gsea/index.jsp) gene sets were downloaded from the MSigDB database. A gene list ranked by the correlation coefficients was analyzed with the standard parameters. The median of correlation coefficients was used in cases when multiple probe sets were present.

For the analysis of an association between the CDK6 level and the p53 mutational status in Thymoma, AML, ALL and Glioblastoma RNA-Seq and Microarray data, the Wilcoxon Rank-sum test with continuity correction was used.

**ChIP: anti-CDK6**

Negative Control

Fw: 5’-CCCTCTTCTCATTCGTTTTCCA-3’

Rv: 5’-CCAGGAAAGAATTTGAGAAAAATCA-3’

*Vegf-A*

Fw: 5’-GGCAGGGACGTATGAGGATA-3’

Rv: 5’-GCATGCATGTGTGTGTGTGT-3’

*Prmt5*

Fw: 5’-CGGCCCAATAACGATACTTCA-3’

Rv: 5’-GCTCAGCAATCTCCCAGAAT-3’

*Mdm4*

Fw: 5’-GAGGAGTTGGTTCAGTTGCT-3’

Rv: 5’-TCTGTCTTGTCACTGTGTGATG-3’

ChIP: anti-NFYA Re-ChIP: anti-CDK6

*Prmt5*

Fw: 5’-GAGCGTCTTTACAGCTCCAG-3’

Rv: 5’-CACGTCACTGTGAGAATTGC-3’

Sp1

Fw: 5’-AGCTCTTCCTATCCCTACCG-3’

Rv: 5’-AAACTCAGGAAGTGGTGGTG-3’

*CyclinG1*

Fw: 5’-ACTTCCCGTTGTCCAGACTT-3’

Rv: 5’-GGAAATGCTCCAATGAGAGA-3’

*Cdk1*

Fw: 5’-CTCTGATTGGCTCCTTTGAA-3’

Rv: 5’-CAGCAACTCCAGGAGCTTAGA-3’

ChIP: anti-CDK6

ChIP: anti-p53

Primers were designed based on the overlapping peaks between the p53 and CDK6 ChIP-Seq data.

*Prmt5*

Fw: 5’-CACTGTGAGAATTGCGGATTG-3’

Rv: 5’-AGTCGCCTTAACAACCAGAG-3’

*Mdm4*

Fw 5’-AGCCTTTCTAGCTCTCTAGTCC-3’

Rv: 5’-CACTTCCGGCCTCTCAAAC-3’

*Ppm1D*

Fw: 5’-CAGAAGTCTCACGTTAGGGATTT-3’

Rv: 5’-TCCTTGCCCGGTTTGTTT-3’

**shRNA Sequences**

For knockdown of SP1, pRFP-C-RS shRNA plasmids were used (TF502115, Origene). shRNAs targeting CDK6 and NFYA were cloned into the pLENC (pMSCV-miRE-PGK-NeoR-IRES-mCherry) vector.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| shRNA ID | Gene name | Entrez ID | 22nt-shRNA guide | 97mer shRNA PCR oligo |
| Cdk6.1663 | Cdk6 | 12571 | TTTATTGTTTACTGCTAGCAAA | TGCTGTTGACAGTGAGCGCTTGCTAGCAGTAAACAATAAATAGTGAAGCCACAGATGTATTTATTGTTTACTGCTAGCAAATGCCTACTGCCTCGGA |
| Cdk6.897 | Cdk6 | 12571 | TTTAGCTGGATTAAACGTCAGG | TGCTGTTGACAGTGAGCGACTGACGTTTAATCCAGCTAAATAGTGAAGCCACAGATGTATTTAGCTGGATTAAACGTCAGGTGCCTACTGCCTCGGA |
| Nfya.2652 | Nfya | 18044 | TTTTGATGTATCAACATTGGTC | TGCTGTTGACAGTGAGCGAACCAATGTTGATACATCAAAATAGTGAAGCCACAGATGTATTTTGATGTATCAACATTGGTCTGCCTACTGCCTCGGA |
| Nfya.2705 | Nfya | 18044 | TAGTCTTTGGATTGACTGCGTT | TGCTGTTGACAGTGAGCGCACGCAGTCAATCCAAAGACTATAGTGAAGCCACAGATGTATAGTCTTTGGATTGACTGCGTTTGCCTACTGCCTCGGA |
| Nfya.3311 | Nfya | 18044 | TACTCTGTGTCTTTGAAGCCAT | TGCTGTTGACAGTGAGCGCTGGCTTCAAAGACACAGAGTATAGTGAAGCCACAGATGTATACTCTGTGTCTTTGAAGCCATTGCCTACTGCCTCGGA |

**Supplementary Figure legends**

**Figure S1: Tumor development of *Cdk6*+/+ and *Cdk6*-/- BCR-ABL+ cells**

A) Cloning of *Cdk6*+/+ and *Cdk6*-/- bone marrow cells transduced with BCR-ABL in growth factor free methylcellulose at day 10. Colony numbers are indicated (CFU: Colony Forming Units).

B) Documentation of the gating strategy used for quantification of the individual pre-pro-B cell markers expressed on BCR-ABL+ cell lines. Depicted are representative FACS blots for *Cdk6*+/+ and *Cdk6*-/- cell lines (n=3 different biological replicates).

C) Apoptotic cell deathanalysis of *Cdk6*+/+ and *Cdk6*-/- bone marrow cells that were transduced with BCR-ABL. The plots show representative Annexin V/7-AAD stainings during the immortalization process. The numbers represent the mean±SD (n=3 different biological replicates).

D) Mean fluorescence intensity (MFI) of GFP+ BCR-ABL+ *Cdk6*+/+ or *Cdk6*-/- cell lines.

**Figure S2: *Cdk6*-/- cells fail to induce apoptosis after BCR-ABL-transformation**

A) Documentation of the gating strategy used for quantification of the individual pre-pro-B cell markers expressed on primary bone marrow cells.

B) *Cdk6*+/+ and *Cdk6*-/- bone marrow cells were isolated and exposed to -irradiation with a single dose of 5 Gy. Apoptosis induction in pre-pro-B cell was analyzed 24 hours thereafter by combined surface marker and Annexin V/7-AAD stainings. Numbers represent the mean±SD (n=3 different biological replicates per genotype, one representative example is depicted).

C) *Cdk6*+/+ and *Cdk6*-/- thymocytes were isolated and exposed to -irradiation with a single dose of 2.5 Gy. Apoptosis induction was analyzed eight hours thereafter by Annexin V/7-AAD stainings. Numbers represent the mean±SD (n=3 different biological replicates per genotype, one representative example is depicted).

D) Cultures of *Cdk6*+/+ and *Cdk6*-/- pre-pro-B cells. Annexin V/7-AAD staining upon neocarzinostatin (NCS) treatment is shown. Numbers represent the mean±SD (n=2 different biological replicates per genotype, one representative example is depicted).

E) qPCR analysis of p21, PUMA and NOXA in e*x vivo* -irradiated pre-pro-B cells isolated from *Cdk6*+/+ and *Cdk6*-/- mice at 4 hours post-treatment. Expression levels are shown relative to housekeeping gene rplp0 (n=3 different biological replicates per genotype).

F) qPCR analysis of the p53-target genes p21, PUMA and NOXA in e*x vivo* -irradiated thymocytes isolated from *Cdk6*+/+ and *Cdk6*-/- mice at 4 hours post-treatment. Expression levels are shown relative to housekeeping gene rplp0 (n=3 different biological replicates per genotype).

G) qPCR analysis of the p53-target genes p21, PUMA and NOXA in BCR-ABL+ *Cdk6*+/+ and *Cdk6*-/- cell lines upon treatment with NCS (50ng/ml) for 4 hours. Expression levels are shown relative to housekeeping gene rplp0 (n=3 different biological replicates per genotype).

H) qPCR analysis of the p53 target genes p21, PUMA and NOXA in BCR-ABL+ *Cdk6*+/+ and *Cdk6*-/- cell lines upon treatment with DMSO or etoposide (1 µM) for 4 hours. Expression levels are shown relative to housekeeping gene rplp0 (n=3 different biological replicates per genotype).

I) BCR-ABL+ *Cdk6*+/+ (left) and *Cdk6*-/- (right) cells were -irradiated with 2.5 Gy. Annexin V/7-AAD staining was performed 8 hours (upper panel) and 24 hours (lower panel) post-irradiation. The numbers represent the mean±SD (n=3 cell lines per genotype, one representative example is depicted).

J) Annexin V/7-AAD staining of BCR-ABL+ *Cdk6*+/+ (left) and *Cdk6*-/- (right) cells upon treatment with NCS (50ng/ml) for 48 hours. Numbers represent the mean±SD (n=3 cell lines per genotype, one representative example is depicted).

**Figure S3: Altered response of BCR-ABL+ *Cdk6*+/+ and *Cdk6*-/- cells to DNA damage and nutlin-3**

A) Cell cycle analysis (PI staining) of neocarzinostatin-treated BCR-ABL+ cells (50ng/ml, 4 and 24 hours, n=3 cell lines per genotype, one representative example is depicted in the histogram; UT: Untreated).

B) Annexin V/7-AAD staining of BCR-ABL+ *Cdk6*+/+ and *Cdk6*-/- cells upon treatment with etoposide for 24 hours (concentration: 1 µM). The numbers represent the mean±SD (n=3 cell lines per genotype, one representative example is depicted).

C) Cell-cycle analysis (PI staining) of etoposide-treated BCR-ABL+ cells (1 µM for 24 hours; n=3 cell lines per genotype, one representative example one representative example is depicted in the histogram).

D) Western blot showing p53 and p21 expression levels in BCR-ABL+ *Cdk6*+/+ and *Cdk6*-/- cell lines upon treatment with nutlin-3 (30 µM) for 4 hours. HSC-70 was used as loading control.

E) Cell-cycle analysis (PI staining) of BCR-ABL+ cells treated with nutlin-3 (30 µM, 24h) (n=3 cell lines per genotype, one representative example one representative example is depicted in the histogram).

F) Annexin V/7-AAD staining of BCR-ABL+ *Cdk6*+/+ and *Cdk6*-/- cells treated with nutlin-3 (30 µM, 24h). Numbers represent the mean±SD (n=3 cell lines per genotype, one representative example is depicted).

G) qPCR analysis of the p53 target p21 in BCR-ABL+ cell lines upon nutlin-3 treatment (30 µM, 24h, n=3 different biological replicates).

H) Wild type CDK6 or the kinase-dead CDK6 mutant CDK6K43M was expressed in BCR-ABL+ *Cdk6*-/- cell lines. Western blot analysis for p53 and CDK6 is depicted.

I) Annexin V/7-AAD staining of reconstituted BCR-ABL+ *Cdk6*-/- cells upon treatment with NCS (50ng/ml) for 48 hours. Numbers represent the mean±SD (n=3 cell lines per genotype, one representative example is depicted).

J) qPCR analysis of the p53 target p21 in reconstituted BCR-ABL+ cell lines upon treatment with neocarzinostatin (50ng/ml) for 4 hours (n=3).

**Figure S4: Altered response to chemotherapy in *Cdk6-/-* cells**

A) Drug screening of BCR-ABL+ *Cdk6*+/+and *Cdk6*-/-cell lines with 272 FDA approved drugs. Relative viability is indicated as % DMSO control, p-values are color-coded (n=3 cell lines per genotype).

B) 8-point dose-response curve with selected compounds from the drug screen presented in Figure 1H and S4A.

C) Annexin V/7-AAD staining of BCR-ABL+ *Cdk6*+/+ and *Cdk6*-/- cells upon treatment with mitomycin C (MMC) for 48 hours (concentration: 1.5 µM). Numbers represent the mean±SD (n=3 cell lines per genotype, one representative example is depicted).

D) Cell-cycle profile of MMC-treated BCR-ABL+ cells using DMSO as solvent-control (n=3 cell lines per genotype, one representative example is depicted in the histogram).

E) Annexin V/7-AAD staining of *Cdk6*+/+ and *Cdk6*-/- cells upon treatment with etoposide for 24 hours (concentration: 1 µM). The numbers represent the mean±SD (n=3 cell lines per genotype, one representative example is depicted).

F) Cell-cycle analysis (PI staining) of the etoposide-treated cells (1 µM for 24 hours; n=3 cell lines per genotype, one representative example is depicted in the histogram).

G) Annexin V/7-AAD staining of *Cdk6*+/+ and *Cdk6*-/- cells upon treatment with fluorouracil (5-FU) for 24 hours (concentration: 80 µM). The numbers represent the mean±SD (n=3 cell lines per genotype, one representative example is depicted).

H) Cell-cycle analysis (PI staining) of the 5-FU-treated cells (80µM, 24 hours, n=3 cell lines per genotype, one representative example is depicted in the histogram).

**Figure S5: p53-deficiency abrogates the necessity for CDK6 during immortalization**

A)Corresponding human mutation located in the DNA-binding domain of p53 found in murine BCR-ABL+ cell lines listed in Figure 2A.

B) Annexin V/7-AAD staining of *Cdk6*-/- cells treated with etoposide (1 µM), PRIMA-met (1 µM and 30 µM) or the combination of both drugs for 24 hours. Numbers represent the mean±SD (n=3 cell lines per genotype, one representative example is depicted).

**Figure S6: CDK6 induces a transcriptional program to antagonize p53 during immortalization**

A) Density plot of the microarray probe set intensities before and after Loess normalization.

B) Volcano plot of adjusted p-values and fold change of genes differentially regulated between *Cdk6*+/+, *Cdk6*-/-and *Cdk6K43M*colonies obtained from the microarray analysis of colonies.

C) Western blot of initially transformed cells purified by density gradient centrifugation from *Cdk6*+/+ and *Cdk6*-/-bone marrow cultures at day 10, 16 and 19 after BCR-ABL transduction. Stable BCR-ABL+ cell lines were used as controls.

D) Validation of the microarray by qPCR using RNA isolated from single colonies isolated at day 10 after BCR-ABL transduction.

E) RT-PCR analysis showing the ratio between Exon 6-including (MDM4) or lacking (MDM4-S) mRNA. cDNA obtained from *Cdk6*+/+, *Cdk6*K43M and *Cdk6*-/- colonies was used as template.

F) CDK6 expression reconstitutes PRMT5 and MDM4 expression. qPCR analysis in *Cdk6*-/- colonies concomitantly transduced with BCR-ABL and CDK6 or a control vector.

**Figure S7: CDK6 regulates a complex transcriptional program in colonies and cell lines**

A) Pie chart showing the functional classification of gene ontology terms identified in the gene sets that are differentially expressed between *Cdk6*+/+ and *Cdk6*-/- colonies or cell lines.

B) Representative example of ChIP-Seq peaks in the promoter region of the p53 regulators PPM1D.

C) ChIP in BCR-ABL+ cell lines verifies the presence of CDK6 on the PRMT5 and MDM4 promoter. VEGF-A was used as positive control. The fold-enrichment over a negative control region is shown. The PCR products are shown in the right panel (NC: Negative control, PC: Positive control).

D) Venn diagram showing the number of overlapping peaks between CDK6- and HA-CDK6-ChIP-Seq datasets performed in BCR-ABL+ cell lines.

E) Pie chart showing the functional classification of gene ontology terms identified in the gene sets that are bound by CDK6 and show expression changes in BCR-ABL+ colonies or cell lines.

**Figure S8: CDK6 phosphorylates transcriptional regulators and shares common binding sites with p53**

A) Initially transformed *Cdk6*-/- cells (day 10 after BCR-ABL transduction) show reduced levels of chromatin-bound p53 antagonists PRMT5, MDM4 and PPM1D.

B) Quantification of ADP formation in a luminescent kinase assay using recombinant ZBTB7A or EBF1 and increasing amounts of active CDK6 protein. Photometrically acquired relative luminescence units (RFU) are shown. Luminescent kinase assays were performed in the presence or absence of substrate, ATP or CDK4/6 inhibitor Palbociclib (500 nM).

C) Protein levels of NFYA and CDK6 after immunoprecipitation (IP) with antibodies against NFYA. In and SN indicate the input lysate or supernatant after IP.

D) CDK6 chromatin immunoprecipitation (CDK6 ChIP) followed by qPCR analysis of target gene promoters. Fold enrichment over the established *Cd19* negative region is shown

E) Annexin V/7-AAD staining of initially transformed *Cdk6*+/+;*p53*+/+ and *Cdk6*+/+;*p53*-/- bone marrow cells where CDK6, NFY and SP1 were transiently knocked-down using shRNAs (48 h). A non-targeting shRNA was used as control (n=3 biological replicates).

F) Motif analysis of p53 ChIP-Seq peaks from -irradiated B-cells cells that overlap with CDK6 ChIP-Seq peaks.

**Figure S9: CDK6 and p53 do not physically interact in immunoprecipitation assays**

A) Protein levels of p53 and CDK6 after immunoprecipitation (IP) with antibodies against CDK6 or HA-CDK6. In and SN indicate the input lysate or supernatant after IP. BCR-ABL+ *Cdk6*+/+ cells were treated with nutlin-3 (4 h, 30 µM) to induce p53 protein accumulation.

B) Protein levels of p53 and CDK6 after immunoprecipitation (IP) with antibodies against p53. In and SN indicate the input lysate or supernatant after IP. BCR-ABL+ *Cdk6*+/+ cells were treated with nutlin-3 (4 h, 30 µM) to induce p53 protein accumulation.

C) qPCR analysis of the p53 target genes PUMA and NOXA in individual colonies isolated from methylcellulose on day 10 after BCR-ABL transduction.

**Figure S10: Transcription factor programs correlate with CDK6 expression in human leukemia**

A) Gene set enrichment analysis of the E2F transcription factor program in publically available leukemia datasets.

B) Gene set enrichment analysis of the NFY transcription factor program in publically available leukemia datasets (MDS - Myelodysplastic Syndrom, BCR-ABL+ and BCR-ABL- ALL - MILE study, BCR-ABL- - Target study).

**Figure S11: The CDK6-regulated expression signature in human leukemia**

A) Circular heatmap showing color-coded correlation coefficients of CDK6-coexpressed transcripts in the indicated disease entities. The circles depict the genomic location and the color indicates the correlation coefficients of transcripts that are significantly correlated to CDK6 expression levels. Each disease entity and healthy bone marrow samples are depicted as individual circle. The interconnecting lines highlight transcripts that are consistently correlated to CDK6 expression levels in all disease entities. Red colored-lines show consistently correlated transcripts that have a ChIP-Seq promoter peak.

B) Venn diagram showing the number of transcripts correlated with CDK6 levels in publically available leukemia datasets. The numbers in the intersecting areas show the overlap between the different datasets.

C) Venn diagram showing the overlap between transcripts correlated in all leukemia datasets and healthy bone marrow control samples.

D) Venn diagram showing the overlap between transcripts correlated in at least one leukemia dataset and healthy bone marrow control samples.

E) Box plot showing the distribution of CDK6 expression levels in patients harboring wild type or mutant p53 (thymoma: W = 157, p-value = 0.0813, glioblastoma: W = 13025, p-value = 0.0208).

F) Box plot showing the distribution of CDK6 expression levels in acute leukemia patients (AML and ALL) with or without monosomy 7 or 7q deletion (-7/del(7q)) (W = 2191, p-value = 0.0315).

**Figure S12: *Cdk6*-/- cells show increased levels of p19ARF mRNA**

qPCR analysis of p19ARF mRNA levels in colonies isolated at day 10 after BCR-ABL transduction and in BCR-ABL+ cell lines.

**References in Figure S5A:** (12–20)

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