Supplementary Methods

Cell lines

Leukemia cell lines were purchased from DSMZ (Braunschweig, Germany). Cell lines were cultured in RPMI+Glutamax (Gibco BRL, Life Technologies, Bleiswijk, The Netherlands) or DMEM+Glutamax (HEK293T) containing 100 IU/ml penicillin, 100 µg/ml streptomycin (pen-strep), 0.125 µg/ml fungizone (Gibco) and 10% Fetal Calf Serum (Integro, Zaandam, The Netherlands), or 20% Fetal Calf Serum for SUP-B15, TOM-1, MHH-CALL-2, MHH-CALL-3, and MHH-CALL-4. Cells were cultured at 37ºC in a humidified incubator with 5% CO_2. RNA was isolated with an RNeasy minikit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol.

Primary samples

Patients and/or parents/guardians granted written informed consent to use excess of diagnostic material for research purpose, as approved by institutional ethical review boards. Bone marrow (BM) or peripheral blood (PB) samples from children with newly diagnosed ALL and from children without hematological disorder (normal bone marrow; nBM) were obtained and processed as previously described.[1] Mononuclear cells were isolated and ALL samples were enriched to contain >90% leukemic blasts. T-ALL was identified by flow cytometry of cell surface markers. Patients with precursor B-ALL were divided into the main genetic subtypes hyperdiploid (>50 chromosomes), MLL/11q23-rearranged, ETV6-RUNX1/t(12;21)(p13;q22), TCF3/19p13-rearranged, BCR-ABL1/t(9;22)(q34;q11), or 'B-other' (negative for afore-mentioned features) as described before.[2] The data of patients enrolled in two consecutive German Co-operative Study Group for Childhood ALL (COALL) trials 06-97 and 07-03 were combined for survival and cumulative risk of relapse analyses, and this cohort is hereafter referred to as COALL-97/03.

Gene expression analysis

RNA was extracted from 859 ALL patients and 8 nBM samples using TRIzol reagent (Invitrogen, Life Technologies, Bleiswijk, The Netherlands) according to the manufacturer's protocol. The Affymetrix (Santa Clara, CA, USA) One-Cycle cDNA Synthesis kit and the GeneChip IVT Labeling kit were used to synthesize cRNA. Gene expression was analyzed with Affymetrix U133 plus 2.0 GeneChips as described before.[2] Data was normalized using variance stabilizing normalization and robust multichip average.[3] Batch effects were corrected for by the empirical Bayes method described by Johnson et al.[4] These procedures were performed with R v2.13.0 and the following R Bioconductor packages: affy v1.30.0, vsn v3.20.0 and Biobase v2.12.1. The R code for the ComBat procedure was downloaded from the author's website (http://jlab.byu.edu/ComBat). To compare mRNA expression levels of PLK1 between different subtypes of ALL and normal mononuclear bone marrow cells, we used the gene expression data of one probeset for PLK1 (Affy probe ID 202240_at).

Reverse Phase Protein Arrays

Reverse Phase Protein Arrays (RPPA) were performed in collaboration with E. Petricoin (George Mason University, Manassas, VA, USA) as previously described.[5–7] In short, 172 pediatric ALL samples (118 BM and 54 PB; see Supplementary Table S1 for patient characteristics) and ten nBM samples were lysed in Tissue Protein Extraction Reagent (TPER; Pierce Biotechnology, Rockford, IL, USA), supplemented with 300 mM NaCl, 1 mM orthovanadate and protease inhibitors, and spotted twice in triplicate on glass-backed nitrocellulose-coated array slides (FAST slides; Whatman Plc, Kent, UK) by the Aushon Biosystems 2470 arrayer (Aushon Biosystems, Billerica, MA, USA). Using a DAKOcytomation autostainer, the slides were stained with an antibody against PLK1 (Cell Signaling Technology, Danvers, MA, USA; #4535), Thr210-phosphorylated PLK1 (BD Pharmingen, Breda, The Netherlands; #558400), Aurora A (AbD Serotec, Kidlington, UK; MCA2249) or Aurora B (Cell Signaling Technology; #3094), followed by incubation with a biotinylated secondary antibody. Slides were scanned using a NovaRay CCD fluorescent scanner (Alpha Innotech, San Leandro, CA, USA) and analyzed with MicroVigene v2.8.1.0. software (VigeneTech, Carlisle, MA, USA). Finally, protein levels were calculated relative to the total amount of protein per sample, determined by staining one in every fifteen slides with Sypro Ruby Protein Blot Stain (Invitrogen, Life Technologies, Grand Island, NY, USA).

Lentiviral infection
HEK293T cells were transfected as described before[8] with lentiviral helper vectors psPAX2 (Addgene plasmid 12260) and pMD2.G (Addgene plasmid 12259), provided by Prof. D. Trono (Geneva, Switzerland), and a pLKO.1 Mission® vector (Sigma-Aldrich, Zwijndrecht, Netherlands) containing a puromycin selection marker and a short hairpin RNA (shRNA) either against PLK1 (TRCN0000121222) or against eGFP (SHC005), which was used as a non-targeting control (NTC).
Virus-containing supernatant was concentrated by ultracentrifugation, and viral titers in pg/ml were determined with a HIV-1 p24 Antigen ELISA kit (ZeptoMetrix, Buffalo, NY, USA) according to the manufacturer's instructions, and expressed as Transforming Units per ml (TU/ml) (with 1 pg = 10 TU). Cell lines were spin-infected with a multiplicity of infection (MOI) of 2.5 and in the presence of 5 µg/ml polybrene (Sigma-Aldrich). Infected cells were then cultured for 24 hours before selection on puromycin (Sigma-Aldrich) in concentrations killing >90% of non-infected cells within 24 to 48 hours. A trypan blue exclusion assay was used to determine cell viability.

Flow cytometric analysis of caspase-3 cleavage
Cells were fixed with 2% formaldehyde and 37% acetone and subsequently incubated with Cleaved Caspase-3 (Asp175) antibody (Cell Signaling Technology, #9661) in 0.5% BSA for 30 min. at room temperature. Samples were incubated with a FITC-conjugated secondary anti-rabbit antibody (DAKO, Glostrup, Denmark; #F0054) for 30 min. in the dark at room temperature. Measurements were performed on a FACS Calibur (BD Biosciences, Breda, The Netherlands) and data was analyzed with FlowJo v7.6.5.

Cell cycle analysis
Cell cycle distribution was analyzed by flow cytometry of propidium iodide-stained nuclei as described previously.[8] Measurements were performed on a FACS Calibur and data was analyzed with FlowJo v7.6.5.

Protein electrophoresis and Western Blot
Protein was isolated from lysed cell pellets and Western Blot was performed as previously described.[8] Primary antibodies were from Cell Signaling Technology (PLK1 (208G4), #4513; cleaved Poly (ADP-ribose) polymerase (PARP), #9541; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), #2118) and Abcam (Cambridge, UK) (β-actin, ab6276). Proteins were detected with either a horseradish peroxidase (HRP)-tagged secondary antibody (Cell Signaling Technology) and scanned with a SynGene Chemigenius (SynGene, Cambridge, UK), or detected with a fluorescently labeled secondary IRDye antibody (LI-COR Biosciences, Lincoln, NE, USA) and scanned with an Odyssey Infrared Imaging System (LI-COR Biosciences).

MTS drug sensitivity assay
NMS-P937 was provided by Nerviano Medical Sciences (Nerviano, Italy) as part of the Kids Cancer Kinome consortium and kindly distributed by Dr. J.J. Molenaar, AMC, Amsterdam, The Netherlands. Sensitivity of 15 leukemic cell lines and 15 BM samples of newly diagnosed ALL patients to the PLK1-selective inhibitor NMS-P937 in DMSO was determined with an MTS assay as described previously.[8] Primary cells were tested in the presence of 0.12µM NMS-P937 and 0.0012% DMSO. Cell viability did not further decrease in the presence of up to 0.033% DMSO. Cell survival of treated cells was therefore calculated as a percentage of untreated control cells. GI_{50} values represent the drug dose required to inhibit cell growth by 50% within 72 hours of exposure. Cell line experiments were performed three times.

Sequencing
DNA from 38 ALL patient samples and four cell lines was isolated using TRIzol reagent (Invitrogen, Life Technologies, Bleiswijk, Netherlands) according to the manufacturer's protocol. Samples were screened by 454 sequencing of twelve PCR products covering exons 1–7 of PLK1, encompassing the kinase domain(s) predicted by the COSMIC (http://www.sanger.ac.uk/genetics/CGP/cosmic/) and Ensembl (http://www.ensembl.org) databases (Supplementary Table S2). The average number of sequence reads achieved per PCR product was 161, and the sequencing success rate for PCR products (defined as >30 reads) based on all samples was 92% (Supplementary Table S3). Mutations were confirmed by Sanger sequencing.

Statistical analyses

The following statistical analyses were performed in IBM SPSS Statistics v20.0.0.1. Statistical significance of differential expression was determined with the non-parametric Mann-Whitney U test. Significance of correlation between expression levels and drug sensitivity was determined with a Spearman's rank correlation (r_s) test.

Survival analyses were performed on the COALL-97/03 patient cohort only (n=123) (see Supplementary Table S4 for patient characteristics). Univariate and multivariate analyses of potential prognostic factors were performed with Cox's proportional hazards model. Event-free survival (EFS), where an event was defined as relapse, non-response to induction therapy at day 56, secondary malignancy or death due to leukemia or toxicity of treatment, was stratified for treatment arms based on patient risk classification. Risk classification took place prior to and during treatment and consisted of high risk (HR) and low risk (LR) categories based on initial white blood cell count, age at diagnosis, immunophenotype, chromosomal rearrangements, response to induction therapy, and *in vitro* drug sensitivity.

The statistical environment R v2.14.0 was used to analyze the cumulative incidence of relapse (CIR) for the COALL-97/03 cohort with death as a competing risk event according to the method of Fine and Gray. Patients were censored at the end of follow-up or at the time of death.

$P<0.05$ was considered statistically significant.

References

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Supplementary Tables

Supplementary Table S1. Characteristics of 172 patients included in reverse phase protein array (RPPA) analysis. Cut-offs for age (10 years) and white blood cell (wbc) count (50 wbc/nl) are based on cut-offs commonly used for risk classification.

	N patients (total N=172)
Gender	
[male]	99/172 (57.6%)
Age	
[<10 yrs]	123/172 (71.5%)
Wbc count	
[<50 wbc/nl]	94/172 (54.7%)
Genetic subtype	
Precursor B-ALL	141/172 (82.0%)
Hyperdiploid	29/141 (20.6%)
MLL-rearranged	9/141 (6.4%)
ETV6-RUNX1	29/141 (20.6%)
TCF3-rearranged	19/141 (13.5%)
BCR-ABL1	14/141 (9.9%)
B-other	41/141 (29.1%)
T-ALL	31/172 (18.0%)

Supplementary Table S2. PCR primer locations and sequences for 454 sequencing.

PCR product ID	Ensembl exon annotation	Forward primer 5'-3'	Reverse primer 5'-3'	Start nt	Stop nt	Genome position HG19
PLK1_exon1_1	ENSE00001107240	CTGGGTCCGGGTTTAAAGG	CTGCGTGGGTCCACTAGG	20	331	chr16:23690051-23690399
PLK1_exon1_2	ENSE00001107240	CTTCGGGAGCATGAGTGC	TTGAGCAGCAGAGACTTAGGC	212	454	chr16:23690244-23690525
PLK1_exon1_3	ENSE00001107240	CACCGGCGAAAGAGATCC	GCAGGCAGTTCCAGTTCC	325	628	chr16:23690357-23690696
PLK1_exon2_1	ENSE00001107217	CTGGTAACCCTCTCCCTTCC	CCTCCAGATCTTCATTCAGG	46	240	chr16:23691330-23691564
PLK1_exon2_2	ENSE00001107217	CTGCACAAGAGGAGGAAAGC	CTTGTGGGTTGTCTCCTTCC	133	341	chr16:23691417-23691665
PLK1_exon3	ENSE00001107239	TGGTTCTGGATGGTCAAACC	CGAAAGGTCCACAGAAAAGG	28	316	chr16:23692142-23692470
PLK1_exon4	ENSE00001107237	GCCTGACCTTTGTTCTGACC	GCTGGGCTCCATAAACAGC	21	231	chr16:23693295-23693544
PLK1_exon5_1	ENSE00001107229	GGAGAACTTGGCATTGAACC	CTGGGAGCAATCGAAAACC	21	313	chr16:23695032-23695363
PLK1_exon5_2	ENSE00001107229	CTCCCTCATCCAGAAGATGC	GGGACTAAGCTGCACACACC	200	436	chr16:23695211-23695487
PLK1_exon6_1	ENSE00001107234	TGATTCAGTTTCCCCAAAGC	ACCTTGCCTGACC	21	262	chr16:23698673-23698945
PLK1_exon6_2	ENSE00001107234	GGTTCGAGAGACAGGTGAGG	GGAAACCAATGGTCCTATGC	187	379	chr16:23698839-23699071
PLK1_exon7	ENSE00001107238	GGAGCAGAGGGGAAGAGG	TACCTGGGAGCTCAAAAAGG	60	250	chr16:23699930-23700158

Supplementary Table S3. Sequence reads for 38 primary ALL samples and 4 cell lines. The number of sequence reads and percentage of successful reads are given for each PLK1 PCR product defined in Supplementary Table S2, for primary samples with IDs 076–113 and cell lines U0C-B6, SUP-B15, HPB-ALL and SEM.

	Sample ID																							
PCR ID	**076**	**077**	**078**	**079**	**080**	**081**	**082**	**083**	**084**	**085**	**086**	**087**	**088**	**089**	**090**	**091**	**092**	**093**	**094**	**095**	**096**	**097**	**098**	**099**
PLK1_exon1_1	102	169	162	25	71	26	141	59	0	76	63	565	232	95	93	96	228	56	37	83	49	158	48	104
PLK1_exon1_2	167	250	265	103	287	9	160	78	61	134	94	396	195	144	229	75	158	62	79	123	107	180	31	132
PLK1_exon1_3	110	136	147	86	60	37	112	37	37	31	41	336	207	83	50	99	210	61	20	59	75	164	107	67
PLK1_exon2_1	189	292	275	164	292	124	221	76	22	85	169	833	613	254	132	157	347	184	56	180	159	401	264	155
PLK1_exon2_2	173	284	316	154	359	98	368	62	87	123	151	470	532	368	251	209	322	109	85	306	175	356	161	170
PLK1_exon3	141	183	150	41	173	81	125	49	16	38	78	388	181	115	117	66	261	45	32	66	121	123	117	86
PLK1_exon4	163	310	226	132	800	98	233	110	122	163	289	750	474	411	249	90	646	61	70	427	173	270	137	112
PLK1_exon5_1	0	192	177	95	339	90	133	51	38	20	182	318	588	162	218	138	183	134	60	190	187	197	15	155
PLK1_exon5_2	116	236	247	16	161	78	146	41	35	84	116	208	280	94	61	85	198	53	55	184	115	219	183	106
PLK1_exon6_1	79	143	159	15	104	81	87	17	22	58	22	159	129	62	13	52	91	0	0	76	43	76	74	32
PLK1_exon6_2	234	317	357	37	251	96	258	63	0	173	174	404	368	190	139	105	306	78	33	57	175	278	210	127
PLK1_exon7	246	360	321	32	240	97	233	53	56	57	62	381	316	49	129	105	240	47	34	35	134	274	204	147
No. primer reads >30	**11**	**12**	**12**	**9**	**12**	**10**	**12**	**11**	**7**	**11**	**11**	**12**	**12**	**12**	**11**	**12**	**12**	**11**	**10**	**12**	**12**	**12**	**11**	**12**
% primers successful	**91.7**	**100**	**100**	**75**	**100**	**83.3**	**100**	**91.7**	**58.3**	**91.7**	**91.7**	**100**	**100**	**100**	**91.7**	**100**	**100**	**91.7**	**83.3**	**100**	**100**	**100**	**91.7**	**100**

Supplementary Table S3. Continued.

	Sample ID																			
PCR ID	**100**	**101**	**102**	**103**	**104**	**105**	**106**	**107**	**108**	**109**	**110**	**111**	**112**	**113**	**U0C-B6**	**SUP-B15**	**HPB-ALL**	**SEM**	**No. primer reads >30**	**% primers successful**
PLK1_exon1_1	63	61	130	47	31	47	71	35	41	63	38	90	77	107	64	15	140	26	**37**	**88.1**
PLK1_exon1_2	84	0	49	39	4	13	34	16	13	36	0	47	82	188	282	19	297	76	**34**	**81.0**
PLK1_exon1_3	235	168	138	193	73	132	217	81	155	161	110	220	72	145	148	126	205	65	**41**	**97.6**
PLK1_exon2_1	311	331	237	391	57	189	254	86	268	391	57	153	127	240	339	38	262	101	**41**	**97.6**
PLK1_exon2_2	367	222	277	296	188	230	209	136	196	292	114	568	79	286	339	31	235	311	**42**	**100.0**
PLK1_exon3	259	213	187	206	81	169	0	74	170	243	154	367	61	156	79	131	248	73	**40**	**95.2**
PLK1_exon4	318	222	175	205	117	201	207	139	247	279	181	312	110	224	268	112	198	269	**42**	**100.0**
PLK1_exon5_1	249	158	142	134	31	122	131	44	94	150	36	441	95	196	294	39	288	311	**39**	**92.9**
PLK1_exon5_2	1828	0	176	135	225	131	133	165	111	142	244	117	103	112	204	60	22	139	**39**	**92.9**
PLK1_exon6_1	170	110	87	130	78	89	101	78	86	167	104	75	47	65	91	12	60	20	**33**	**78.6**
PLK1_exon6_2	358	228	266	305	50	195	325	186	220	385	190	161	199	276	77	37	242	26	**40**	**95.2**
PLK1_exon7	329	278	178	308	167	132	257	57	220	351	82	144	190	282	0	0	163	37	**40**	**95.2**
No. primer reads >30	**12**	**10**	**12**	**12**	**11**	**11**	**11**	**11**	**11**	**12**	**11**	**12**	**12**	**12**	**11**	**8**	**11**	**9**		
% primers successful	**100**	**83.3**	**100**	**100**	**91.7**	**91.7**	**91.7**	**91.7**	**91.7**	**100**	**91.7**	**100**	**100**	**100**	**91.7**	**66.7**	**91.7**	**75**		

Supplementary Table S4. Characteristics of ALL patients (N=123) included in survival analyses. The cut-offs for low and high PLK1 and Thr210 phospho-PLK1 (pPLK1) classification are based on the highest tertiles when ranking the complete RPPA cohort (172 patients) from low to high PLK1 protein expression or T210 phosphorylation. Percentages are relative to the number of patients in the column head. Cut-offs for age (10 years) and white blood cell (wbc) count (50 wbc/nl) are based on cut-offs commonly used for risk classification. Risk categories include high-risk (HR) and low-risk (LR) and are based on initial wbc count, age at diagnosis, immunophenotype, chromosomal rearrangements, response to induction therapy, and *in vitro* drug sensitivity.

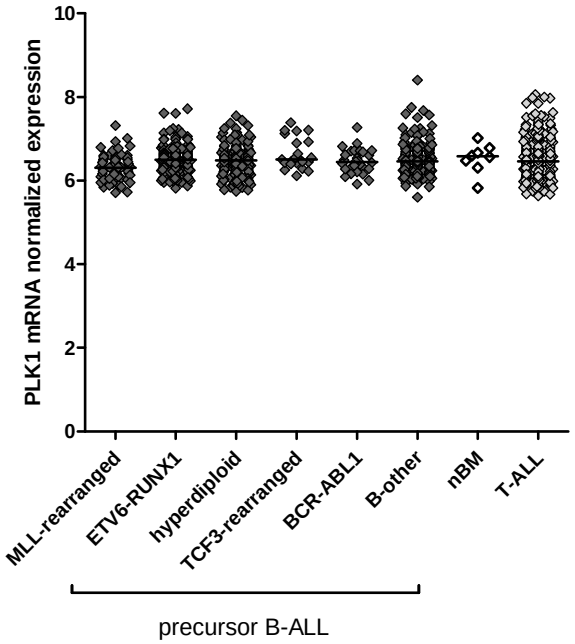
	PLK1		pPLK1	
	low (N=83)	**high (N=40)**	**low (N=86)**	**high (N=37)**
Gender				
[male]	46 (55.4%)	23 (57.5%)	48 (55.8%)	21 (56.8%)
Age				
[<10 yrs]	57 (68.7%)	26 (65.0%)	59 (68.6%)	24 (64.9%)
Wbc count				
[<50 wbc/nl]	46 (55.4%)	24 (60.0%)	53 (61.6%)	17 (45.9%)
Genetic subtype				
Hyperdiploid	9 (10.8%)	5 (12.5%)	11 (12.8%)	3 (8.1%)
MLL-rearranged	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
ETV6-RUNX1	22 (26.5%)	2 (5.0%)	15 (17.4%)	9 (24.3%)
TCF3-rearranged	3 (3.6%)	12 (30.0%)	12 (14.0%)	3 (8.1%)
BCR-ABL1	6 (7.2%)	2 (5.0%)	5 (5.8%)	3 (8.1%)
B-other	29 (34.9%)	9 (22.5%)	31 (36.0%)	7 (18.9%)
T-ALL	14 (16.9%)	10 (25.0%)	12 (14.0%)	12 (32.4%)
Risk classification				
[HR]	71 (85.5%)	31 (77.5%)	71 (82.6%)	31 (83.8%)
Events (N)	23 (27.7%)	12 (30.0%)	20 (23.3%)	15 (40.5%)
Relapses (N)	14 (16.9%)	9 (22.5%)	13 (15.1%)	10 (27.0%)

Supplementary Table S5. Multivariate Cox proportional hazards model for age, wbc count and PLK1 protein expression or Thr210 phosphorylation. Cut-offs for age (10 yrs) and white blood cell (wbc) count (50 wbc/nl) are based on cut-offs commonly used for risk classification. The cut-offs for low and high PLK1 and Thr210 phospho-PLK1 (pPLK1) classification are based on the highest tertiles when ranking the complete RPPA cohort (172 patients) from low to high PLK1 protein expression or Thr210 phosphorylation. HR, hazard ratio; CI95%, 95% confidence interval.

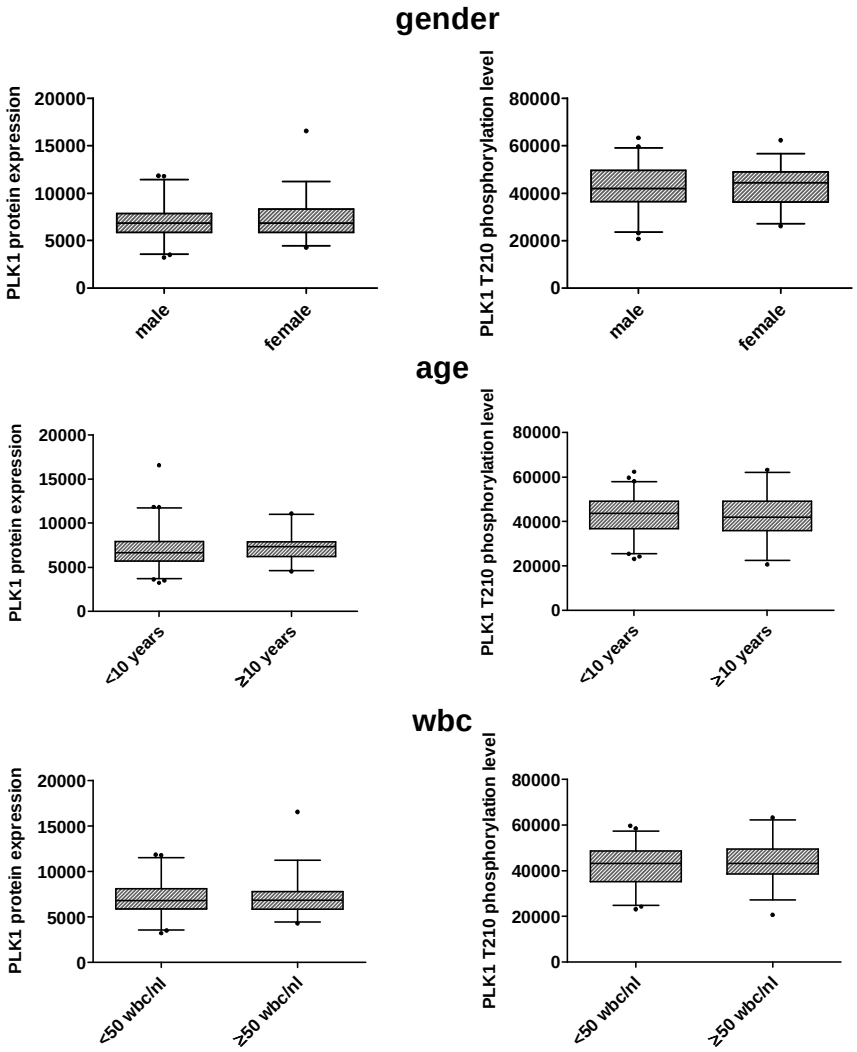
Model for PLK1			
Covariate	**p-value**	**HR**	**HR CI95%**
PLK1 level			
high vs low	0.728	1.13	0.56–2.29
Age (yrs)			
>10 vs <10	0.161	1.64	0.82–3.26
Wbc (wbc/nl)			
>50 vs <50	0.084	1.86	0.92–3.74

Model for pPLK1			
Covariate	**p-value**	**HR**	**HR CI95%**
pPLK1 Thr210 level			
high vs low	0.065	1.89	0.96–3.71
Age (yrs)			
>10 vs <10	0.160	1.63	0.83–3.20
Wbc (wbc/nl)			
>50 vs <50	0.131	1.71	0.85–3.45

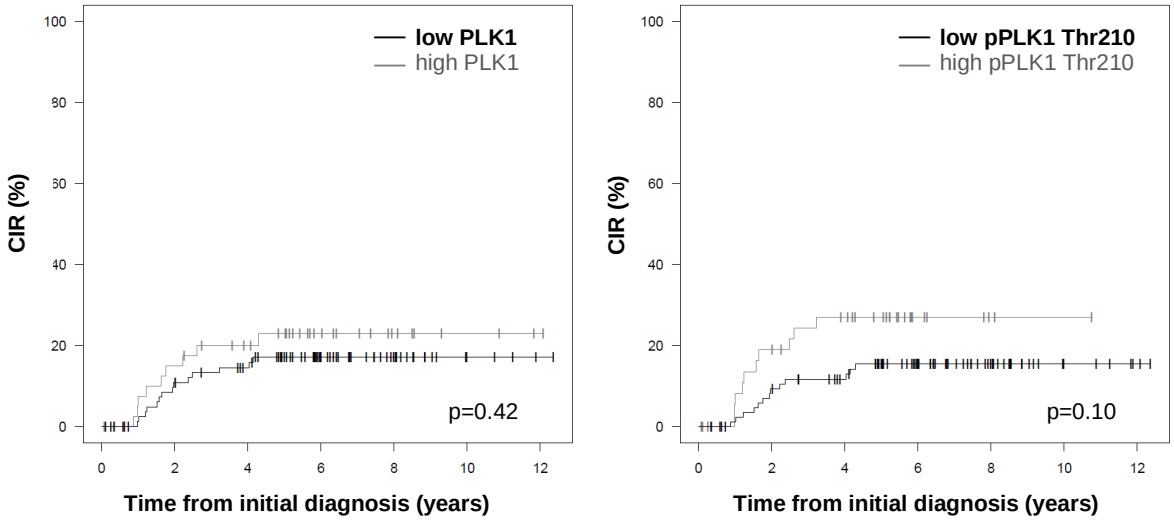
Supplementary Figures



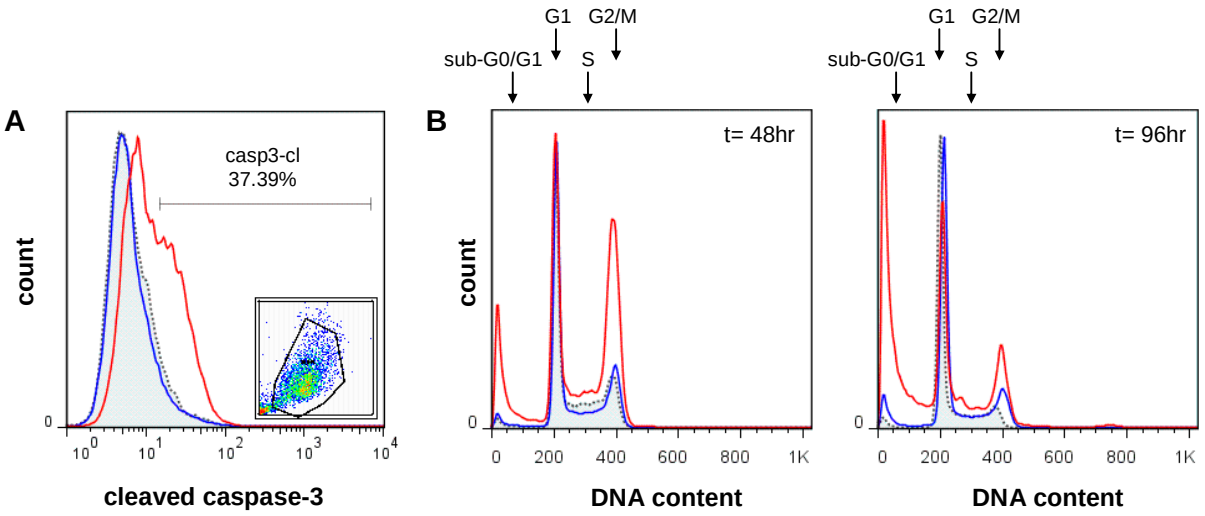
Supplementary Figure S1. PLK1 mRNA expression in pediatric ALL and normal bone marrow. Gene expression of PLK1 in genetic subtypes of ALL (N=859) and normal bone marrow cases (nBM; N=8) was analyzed by gene expression array (probeset 202240_at). Horizontal lines indicate median expression levels.



Supplementary Figure S2. PLK1 protein and T210 phosphorylation levels are not linked to gender, age and white blood cell count. PLK1 protein (left) and T210 phosphorylation levels (right) of 172 primary ALL patient samples were determined by RPPA and are expressed in normalized fluorescence units. Levels were compared for male (N=99) vs female (N=73), below (N=123) vs above (N=49) 10 years of age, and a white blood cell (wbc) count at diagnosis below (N=94) vs above (N=78) 50 wbc/nl. Patients' age and wbc count at diagnosis were categorized according to the cut-offs widely used for risk categorization. Whiskers present the 95% confidence intervals.



Supplementary Figure S3. Cumulative incidence of relapse for pediatric ALL cases with high and low PLK1 expression and phosphorylation. Patients treated according to the German COALL-97/03 protocol were divided into groups with high (n=40) and low (n=83) PLK1 protein expression, and high (n=37) and low (n=86) Thr210 phosphorylation. The cut-offs for the categories high and low were determined by the highest tertile of 172 patients when ranking from low to high PLK1 protein expression and phosphorylation level, respectively. Graphs show the cumulative incidence of relapse (CIR) with death as a competing risk event for PLK1 expression (left) and PLK1 Thr210 phosphorylation (right). Cases were censored (vertical ticks) at the end of follow-up or time of death.



Supplementary Figure S4. Effect of PLK1 knockdown on caspase-3 cleavage and cell cycle distribution. Jurkat cells were transduced with a short hairpin against PLK1 (shPLK1) or a non-targeting control (NTC). Cells were analyzed by flow cytometry. A) Detection of caspase-3 cleavage (casp3-cl) at t=96 hours in shPLK1-treated (red), NTC-treated (blue) and non-transduced (dotted grey) cells. Transduced cells were not selected on antibiotics to prevent false-positive signal from apoptosis induced in a subpopulation of non-transduced cells. Data was gated on live cells (see insert). B) Cell cycle analysis by propidium iodide-staining of nuclei 48 and 96 hours after PLK1 knockdown in shPLK1-treated (red), NTC-treated (blue) and non-transduced (dotted grey) cells. Arrows indicate where cells reside in the G1, S and G2/M phases of the cell cycle, and an apoptotic sub-G0/G1 fraction.