

Appendix

X-linked inhibitor of apoptosis protein represents a promising therapeutic target for relapsed/refractory ALL

Running Title: Targeting XIAP in r/r ALL

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Appendix Table S1: Combination indices (CI) values of LBW242 at concentrations applied in combination with vincristine, doxorubicin, L-Asparaginase and dexamethasone in LOUCY cells.

LBW (μ M)	VCR (ng/ml)			Doxo (μ M)			L-Asp (IE/ml)			Dexa (μ M)
	CI (EC ₆₀)	CI (EC ₅₀)	CI (EC ₂₅)	CI (EC ₅₀)	CI (EC ₂₅)	CI (EC ₁₀)	CI (EC ₆₀)	CI (EC ₅₀)	CI (EC ₂₅)	CI (EC ₂₀)
5	0,76	0,81	0,81	n.d.	n.d.	2,67	0,34	0,96	0,97	0,45
10	0,73	0,78	0,84	n.d.	n.d.	4,72	0,39	0,97	1,04	0,55
20	0,74	0,78	0,85	n.d.	n.d.	8,51	0,47	0,91	0,98	0,53

Combination indices (CI) with synergism (CI<1), additivity (CI=1) or antagonism (CI>1) were calculated as described in material and methods using the Chou-Talalay method. n.d.: not determined.

Appendix Table S2: Combination indices (CI) values of LBW242 at concentrations applied in combination with vincristine, doxorubicin, L-Asparaginase and prednisone in NALM-6 cells.

LBW (μ M)	VCR (ng/ml)			Doxo (μ M)			L-Asp (IE/ml)			Predni (μ M)
	CI (EC ₆₀)	CI (EC ₅₀)	CI (EC ₂₅)	CI (EC ₅₀)	CI (EC ₂₅)	CI (EC ₁₀)	CI (EC ₆₀)	CI (EC ₅₀)	CI (EC ₂₅)	CI (EC ₁₀)
5	0,71	0,57	0,71	0,99	0,73	n.d.	0,45	0,23	0,87	0,62
10	0,77	0,63	0,74	0,97	0,72	n.d.	0,25	0,31	0,82	0,65
20	0,74	0,66	0,78	0,92	0,29	n.d.	0,34	0,51	1,01	0,95

Combination indices (CI) with synergism (CI<1), additivity (CI=1) or antagonism (CI>1) were calculated as described in material and methods using the Chou-Talalay method. n.d.: not determined.

Appendix Table S3: Exact p-values of the data displayed in the main figures and expanded views .

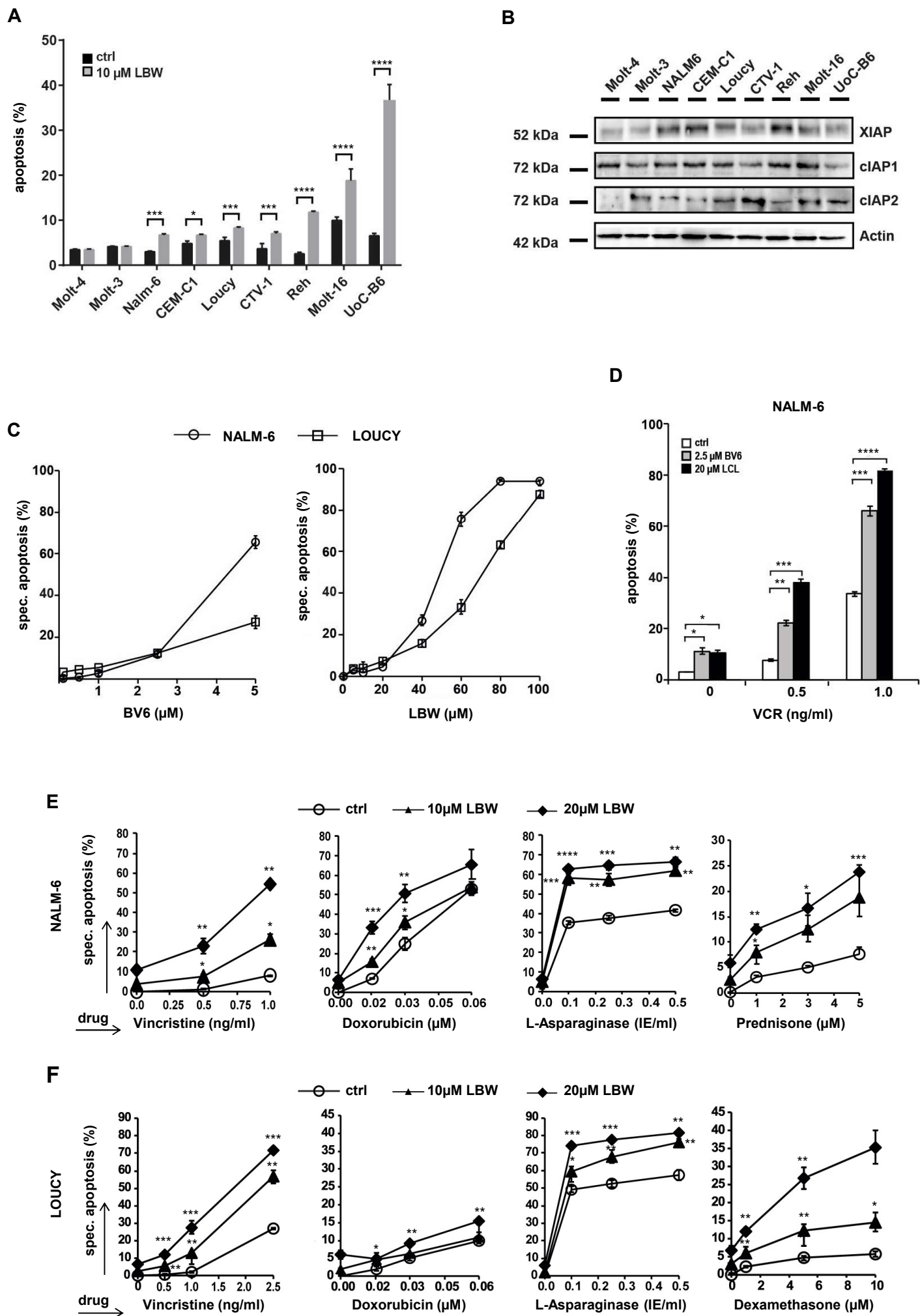
Figure 1A	Student's t-test							
CTRL vs treated	0	0,5ng/ml VCR	1ng/ml VCR					
2,5 µM BV6	0,084963905	0,000582774	0,001029077					
20 µM LCL	0,281727762	5,35344E-06	0,000140644					
20 µM LBW	0,000308131	1,19497E-05	2,96861E-05					
Figure 1C	Student's t-test							
CTRL vs treated	0	1ng/ml VCR	2,5ng/ml VCR	0.25IE/ml L-Asp	10 µM Dexa			
20 µM LBW	0,0130	0,0009	0,0003	0,0082	0,0026			
Figure 1D	Student's t-test							
CTRL vs treated	0	1ng/ml VCR	2,5ng/ml VCR	0.25IE/ml L-Asp	10 µM Dexa			
20 µM LBW	0,000369316	4,1109E-05	1,13E-05	0,000733103	0,000353214			
Figure 1E	Student's t-test							
CTRL vs treated	0	1ng/ml VCR	2,5ng/ml VCR	0.25IE/ml L-Asp	10 µM Dexa			
20 µM LBW	0,0012	0,0000	0,0001	0,0001	0,0034			
Figure 2A	Student's t-test							
CTRL vs treated	0	1ng/ml VCR	20µM LBW	VCR1+20LBW	10ng/mlTNF	20LBW+10TNF		
100 µM Nec	0,0580	0,0097	0,0698	0,1011	0,1878	0,0170		
Figure 2B	Student's t-test							
CTRL vs KD	0	1ng/ml VCR	20µM LBW	VCR1+20LBW	10ng/mlTNF	20LBW+10TNF		
negsiRNA/RIP1KsiRNA	0,3913	0,9423	0,1745	0,0964	0,2336	0,0004		
Figure 2D	Student's t-test							
CTRL vs KD	0	2,5ng/ml VCR	20µM LBW	VCR2,5+20LBW				
negsiRNA/NEMOsRNA	0,04811472	0,367441258	0,059994962	0,681266411				
Figure 3A	Games-Howell							
ctrl vs. AML	0.00964							
ctrl vs. ALL	1,96E-05							
AML vs. ALL	4,24E-09							
Figure 4H	Student's t-test							
shCTRL vs shXIAP.1	0,00873							
Figure EV1A	Student's t-test							
CTRL vs treated	0	1 ng/ml VCR	2,5ng/ml VCR	20ng/ml TNF	20µM LBW	1 VCR+20LBW	2,5VCR+20LBW	20TNF+20LBW
10µg/ml ADM	0,292812641	0,777831278	0,870322417	0,870407399	0,717239546	0,491204268	0,414450383	5,63691E-05
Figure EV1D	Student's t-test							
CTRL vs treated	0	20ng/ml TNF	20µM LBW	20TNF+20LBW				
10µg/ml ADM	0,969276919	0,526891977	0,93525466	0,000294946				
Figure EV1F	Student's t-test							
CTRL vs treated	0	2,5ng/ml VCR	20µM LBW	2,5 VCR+20LBW				
30µM Nec	0,85439	0,00536	0,23620	0,13289				
Figure EV1G	Student's t-test							
CTRL vs treated	0	20ng/ml TNF	20µM LBW	20TNF+20LBW				
30µM Nec	0,052883274	0,101156039	0,292082893	0,017500033				

Appendix Table S3: Exact p-values of the data displayed in the appendix figures.

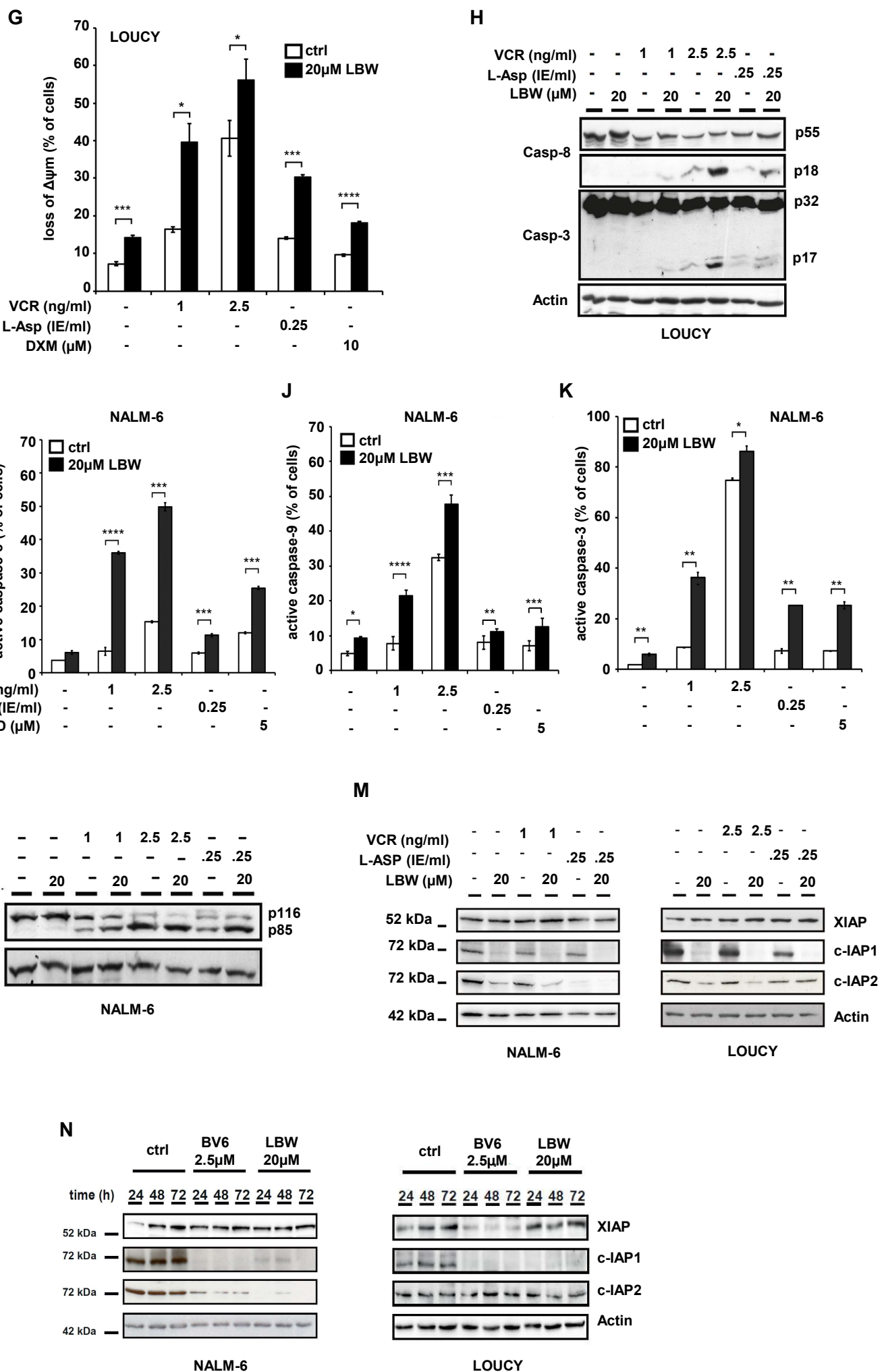
Figure S1A	Student's t-test							
CTRL vs treated	10µM LBW							
Molt-4	1							
Molt-3	1							
Nalm-6	0,00363241							
CEM-C1	0,0494052							
Loucy	0,00351995							
CTV-1	0,00480327							
Reh	2,49647E-06							
Molt-16	0,000612923							
UoC-B6	0,000115696							
Figure S1D	Student's t-test							
CTRL vs treated	0	0,5ng/ml VCR	1ng/ml VCR					
2,5 µM BV6	0,011012234	0,000442628	3,52E-05					
20 µM LCL	0,011156842	0,001224023	0,000581156					
Figure S1E	Student's t-test							
CTRL vs treated	0	0,5ng/ml VCR	1ng/ml VCR					
10 µM LBW	0,000349609	0,004646706	0,007630418					
20 µM LBW	0,008804797	0,015317905	0,015304673					
CTRL vs treated	0	0,016	0,03125	0,0625	µM Doxo			
10 µM LBW	0,033481909	0,000143641	0,027568743	0,805117584				
20 µM LBW	0,010891842	0,009356303	0,004489487	0,134202301				
CTRL vs treated	0	0,1	0,25	0,5	IE/ml Asp			
10 µM LBW	0,033481909	0,000378345	0,008107675	0,003487407				
20 µM LBW	0,010891842	3,51491E-05	0,000845131	0,002932937				
CTRL vs treated	0	0,016	0,03125	0,0625	µM Predni			
10 µM LBW	0,020100403	0,025778705	0,05801541	0,242626027				
20 µM LBW	0,033109433	0,002592965	0,026476042	0,000228633				
Figure S1F	Student's t-test							
CTRL vs treated	0	0,5ng/ml VCR	1ng/ml VCR	2,5 ng/ml VCR				
10 µM LBW	0,003512038	0,001635095	0,000752515	0,0045211				
20 µM LBW	0,00593424	0,000753875	0,0083219	0,000485581				
CTRL vs treated	0	0,016	0,03125	0,0625	µM Doxo			
10 µM LBW	0,009080952	0,010852514	0,281395048	0,460737129				
20 µM LBW	0,018791506	0,125144237	0,001543763	0,001103613				
CTRL vs treated	0	0,1	0,25	0,5	IE/ml Asp			
10 µM LBW	0,009080952	0,018587429	0,009592439	0,002883264				
20 µM LBW	0,018791506	0,00022726	0,000737238	0,003574289				
CTRL vs treated	0	1	5	10	µM Dexa			
10 µM LBW	0,003512038	0,006437393	0,003387514	0,051182878				
20 µM LBW	0,00593424	0,001626988	0,006609678	0,01059855				

Appendix Table S3: Exact p-values of the data displayed in the appendix figures

Figure S1G	Student's t-test							
CTRL vs treated	0	1 ng/ml VCR	2,5 ng/ml VCR	0.25 L-Asp (IE/ml)	10 Dexa (µM)			
20µM LBW	0,000163436	0,020126905	0,041600748	0,000229908	9,77228E-05			
Figure S1I	Student's t-test							
CTRL vs treated	0	1 VCR	2,5 VCR	0.25 L-Asp	5 µM Predni			
20µM LBW	0,0802	0,0000	0,0004	0,0003	0,0005			
Figure S1J	Student's t-test							
CTRL vs treated	0	1 VCR	2,5 VCR	0.25 L-Asp	5 µM Predni			
20µM LBW	0,0340	0,0000	0,0003	0,0050	0,0006			
Figure S1K	Student's t-test							
CTRL vs treated	0	1 VCR	2,5 VCR	0.25 L-Asp	5 µM Predni			
20µM LBW	0,0085	0,0042	0,0184	0,0024	0,0024			
Figure S2D	Student's t-test							
CTRL vs treated	0	0.5ng/ml VCR	1ng/ml VCR	10µM Dexa				
10 µM A4	0,0192	0,0000	0,0007	0,0218				
CTRL vs treated	0	0.5ng/ml VCR	1ng/ml VCR	10µM Dexa				
10 µM A4	0,0130	0,0006	0,0072	0,0004				
Figure S3I	Student's t-test							
shCTRL vs shXIAP.1	0,009							
shXIAP.1 vs shXIAP/OE	0,0017							
shCTRL vs shXIAP/OE	0,0025							



Appendix Figure S1



Appendix Figure S1: SM sensitize r/r ALL to cytotoxic drugs

A Apoptosis induction by SM (LBW242, 10 μ M) in the indicated ALL cell lines determined 48h after treatment by flow cytometry. Mean \pm SEM of 3 independent experiments with * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ unpaired Student's t-test is shown.

B Abundance of IAP in different ALL cell lines. Protein levels of XIAP and c-IAP1/2 were analyzed by Western blot. Actin served as loading control. One representative immunoblot out of 3 independent experiments is shown.

C Limited sensitivity towards SM alone. NALM-6 and LOUCY cells were treated with the indicated SM for 48h. Apoptosis was measured following staining with Annexin-V/propidium iodide. Mean \pm SEM of 3 independent experiments is shown.

D Synergistic cell death of SM in combination with cytotoxic drugs. Apoptosis induction by different SMs in combination with vincristine (VCR) in NALM-6 cells. Flow cytometry analysis (Annexin-V/propidium iodide) after 48h of treatment is depicted. Mean \pm SEM of 3 independent experiments with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by unpaired Student's t-test is shown.

E-F NALM-6 (**E**) and Loucy (**F**) cells were treated for 48h with the indicated concentrations of LBW242, VCR, doxorubicin, L-asparaginase (L-ASP), prednisone or dexamethasone. Apoptosis was determined by flow cytometry after Annexin V and PI staining. Background apoptosis of untreated control cells was subtracted. Mean \pm SEM of 3 independent experiments with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by unpaired Student's t-test is shown.

G Mitochondrial depolarization was analyzed using flow cytometry by detection of the loss of mitochondrial membrane potential ($\Delta\psi_m$) in LOUCY ALL cells. Mean \pm SEM of 3 independent experiments with * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ by unpaired Student's t-test is shown.

H Caspase-8 and Caspase-3 activation in LOUCY cells was determined using Western blotting of the active (p18) caspase-8 subunit and the active p17 caspase-3 subunit 48h after treatment with the indicated drugs. One representative immunoblot out of 3 independent experiments is shown.

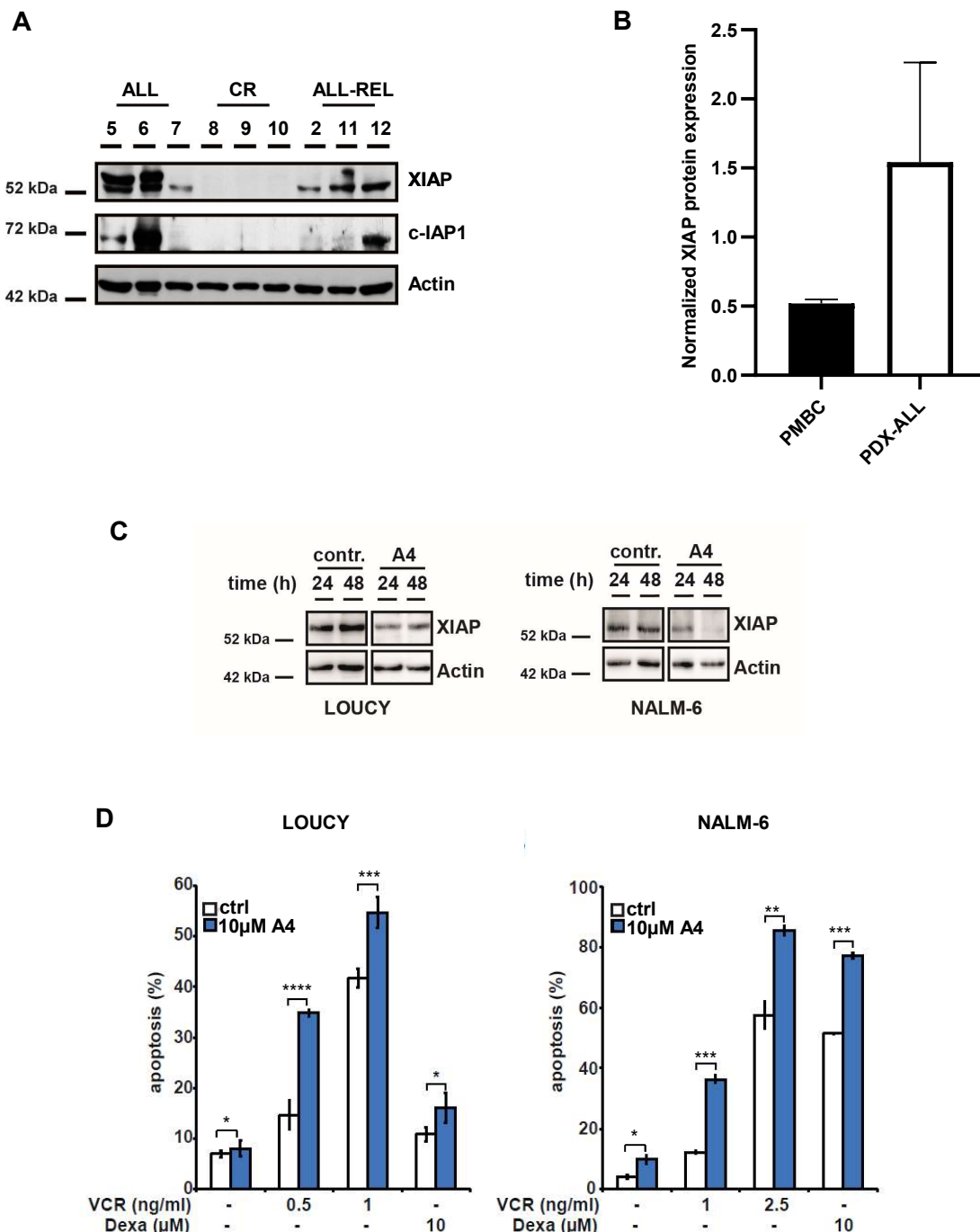
I-K Caspase activation. Activation of (**I**) Caspase 8, (**J**) Caspase-9, (**K**) Caspase-3 was analyzed by flow cytometry 48h after treatment of NALM-6 cells with the indicated drugs as described in Figure 1. Mean \pm SEM of 3 independent experiments with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ by unpaired Student's t-test is shown.

L Cleavage of PARP was measured by Western Blot in NALM-6 cells 48h after the indicated treatment. Actin was used as loading control. One representative immunoblot out of 3 independent experiments is shown.

M SM and cytotoxic drugs degrade cIAP-1/2, but not XIAP. XIAP, cIAP-1 and cIAP-2 protein levels were determined after treatment of NALM-6 and LOUCY cells with VCR, L-ASP and LBW242 (LBW) for 24h by Western blot. Actin served as loading control. One representative immunoblot out of 3 independent experiments is shown.

N Protein abundance of XIAP, cIAP-1 and cIAP-2 after treatment of NALM-6 and LOUCY cells with SM at the indicated concentrations was detected by Western blot analysis. Actin served as loading control. One representative immunoblot out of 3 independent experiments is shown.

Source data for Appendix and Expanded View are available online.



Appendix Figure S2: XIAP expression in acute leukemias and pharmacological inhibition

A Western blot analysis of XIAP and cIAP-1 expression levels in primary tumor cells from nine individual ALL patients (see Table EV1 for patient characteristics) at diagnosis (ALL), complete remission (CR) and relapse (ALL-REL).

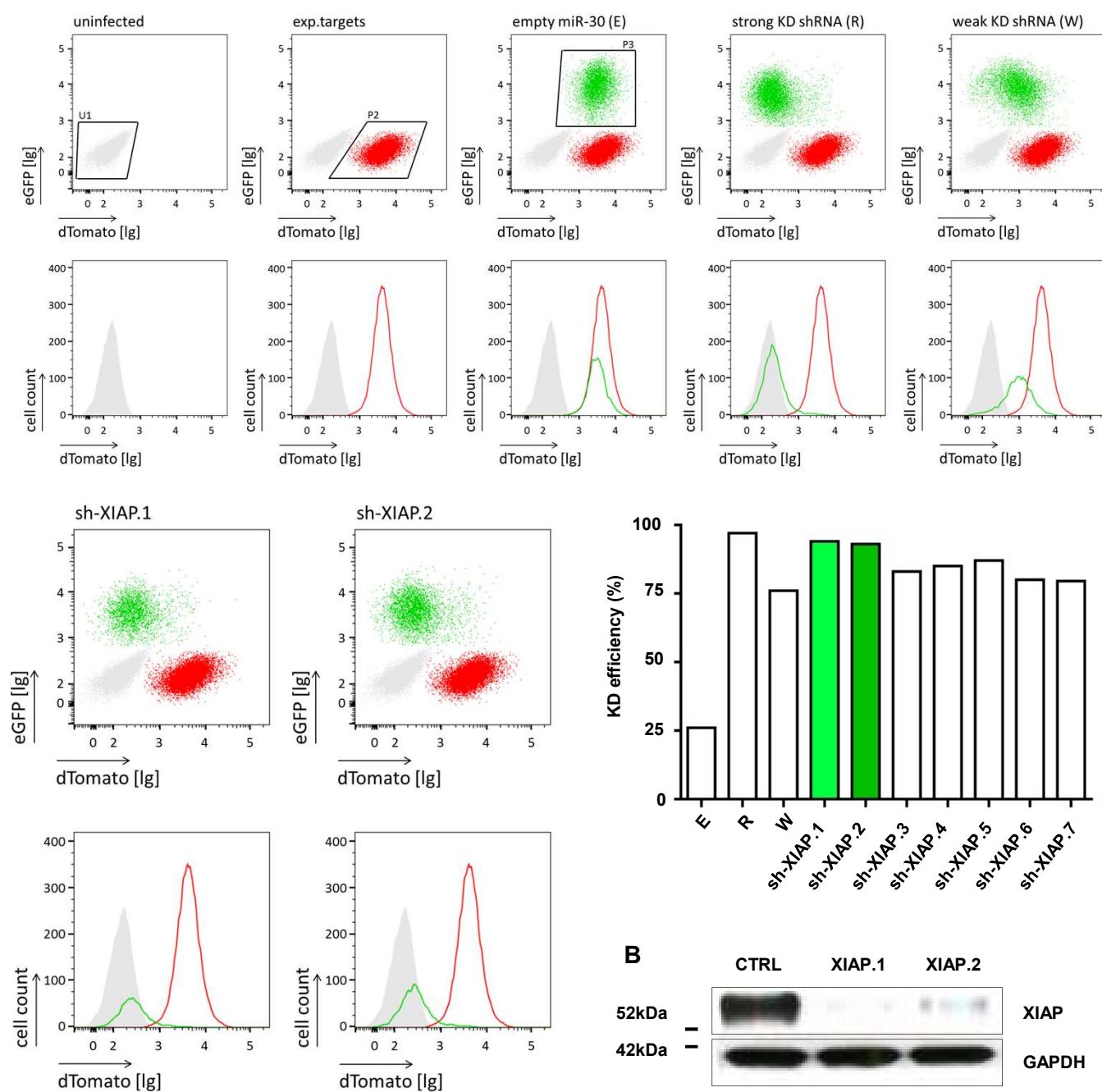
B XIAP protein expression in all ALL PDX samples (n=10) analysed in Figure 3B compared to PBMCs (n=2).

C The ARTS mimetic A4 decreases XIAP protein levels. LOUCY and NALM-6 cells were treated with 10μM A4 for the indicated time and XIAP protein levels were analyzed by western blot. Actin served as loading control. One representative immunoblot out of 3 independent experiments is shown.

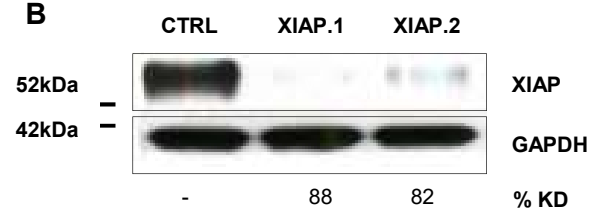
D A4 synergistically induces apoptosis in combination with cytotoxic drugs. LOUCY and NALM-6 cells were treated with the indicated drugs or A4 alone or with a combination of both drugs for 48h. Apoptosis was quantified in AnnexinV / PI stained cells by flow cytometry. Mean \pm SEM of 3 independent experiments with * p < 0.05, ** p < 0.01, *** p < 0.001, ****p < 0.0001 by unpaired Students t-test with Welch's correction is shown.

Source data for Appendix and Expanded View are available online.

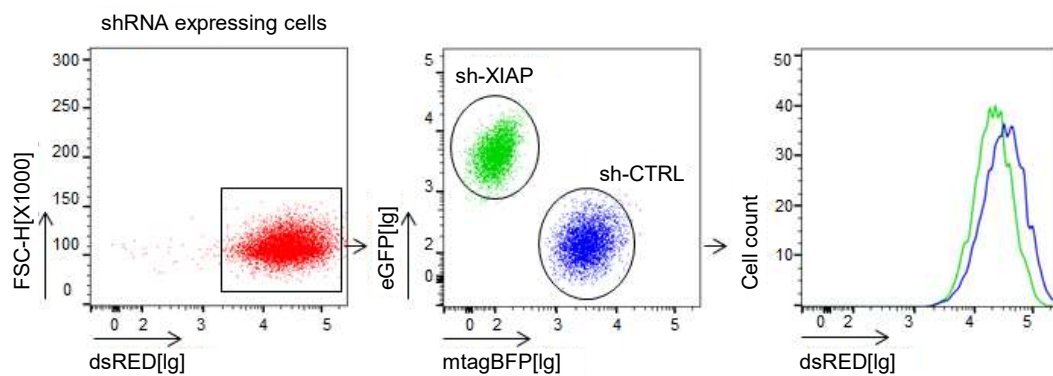
A NALM-6

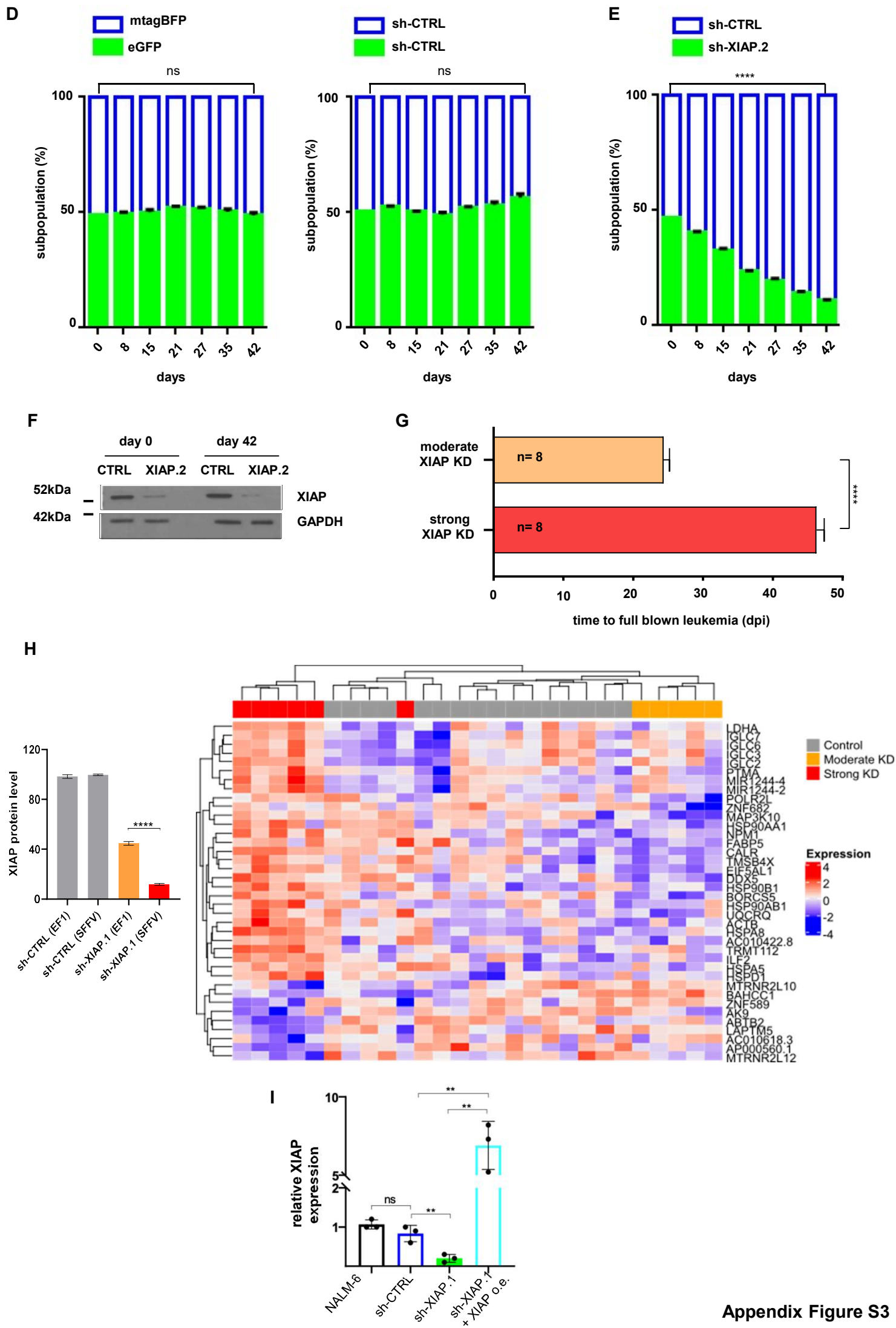


B



C





Appendix Figure S3

Appendix Figure 3: Silencing XIAP sensitizes NALM-6 cells towards VCR *in vivo*.

A-B Selection of efficient shRNA sequences targeting XIAP: **A** The shRNA reporter system (Fellmann *et al*, 2013) enables to determine the efficiency of several shRNA sequences. Multiple genomic shRNA target sequences were cloned into the 3'UTR of a dTomato marker gene and constitutively expressed in NALM-6 cells (red). dTomato-positive NALM-6 cells were subsequently transduced with a second lentiviral vector expressing a specific mir30-shRNA sequence and eGFP as marker for transduction. Reduced expression of dTomato intensity in the GFP/dTomato double-positive population takes place only if the shRNA sequence/eGFP recognizes the specific target sequence located in the 3'UTR of dTomato. **Note that the grey and red populations are repeated in all panels as references to compare the shift of the dTomato/eGFP double positive population.** Representative flow cytometry plots of NALM-6 (untransduced, transduced with experimental targets, empty mir30, strong or weak shRNA) are shown. Quantification of KD efficiency is summarized and plotted as % of KD efficiency using shRenilla as CTRL. shXIAP.1 and shXIAP.2 sequences have been selected for further studies. E = empty control; R and W are positive controls for strong and weak KD, respectively. **B** KD efficiency of selected XIAP KD sequences. sh-XIAP.1 and sh-XIAP.2 were selected for further studies. Western blot analysis in NALM-6 cells confirm efficient XIAP KD on protein level. GAPDH was used as loading control. One representative immunoblot out of 3 independent experiments is shown. **C** Gating strategy. To study the subpopulations' distribution of sh-CTRL/mTagBFP versus sh-XIAP/eGFP ALL cells, ds-RED cells were analyzed for expression of mTagBFP and eGFP. Back-gating allowed to compare dsRED expression between both subpopulations. **D** Expression of transgenes and control shRNA sequences does not affect proliferation of NALM-6 *in vitro*. NALM-6 cells expressing either mTagBFP or eGFP alone (left panel) or in combination with sh-CTRL (right panel) were mixed at a 1:1 ratio and monitored by flow cytometry for up to 6 weeks. Relative proportion of each subpopulation is depicted. Mean \pm SEM of 3 independent experiments is depicted; ns (not significant) by unpaired Students t-test. **E** Strong KD of XIAP impairs NALM-6 *in vitro*. Relative proportion of NALM-6 cells expressing sh-CTRL/mTagBFP versus sh-XIAP.2/eGFP *in vitro* was measured by flow cytometry at the indicated time points. Mean \pm SEM of 3 independent experiments, **** $p < 0.0001$ by unpaired Students t-test. **F** XIAP KD remained stable over time. Representative immunoblot indicating XIAP KD NALM-6 cell lines at the beginning (day 0) and at the end (day 42) of the *in vitro* experiments depicted in E. GAPDH was used as a loading control. One representative immunoblot out of 3 independent experiments is shown. **G** Strong XIAP KD prolongs life span of leukemic mice. Summary of competitive *in vivo* assays performed with a 1:1 mixture of sh-CTRL/mTagBFP and sh-XIAP/eGFP KD NALM-6 cells with moderate (orange, n=8) or strong (red, n=8) XIAP KD. Mice of each group were sacrificed at first sign of leukemia. Shown is mean \pm SEM, **** $p < 0.0001$ by unpaired Students t-test. **H** Gene expression analysis. NALM-6 control cells and NALM-6 cells with moderate and strong XIAP KD were subjected to RNA SCRB-seq analysis. XIAP KD was confirmed by protein immunoassay (Simple Western). Quantification of XIAP protein expression of 3 independent experiments is depicted; mean \pm SEM, **** $p < 0.0001$ by unpaired t-test. Heatmap of 38 genes differentially expressed between any two groups is shown (adjusted p-value ≤ 0.25). All gene expressions have been scaled to a mean value of 0 and a variance of 1. **I** Quantification of XIAP protein expression in the different cell populations via Simple Western (Immunoassay). Data are expressed as mean \pm SD (n=3). ns, not significant, ** $p \leq 0.01$ by unpaired Student's t-test.

Source data for Appendix and Expanded View are available online.