**SUPPLEMENTARY INFORMATION**

# Blocking ETV6/RUNX1-induced MDM2 overexpression by Nutlin-3 reactivates p53 signaling in childhood leukemia

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## Supplementary Methods

### Cell culture

All cell lines were cultured as reported previously[1] and primary leukemic cells were resuspended in X-VIVO™ 10 Medium (Lonza, Visp, CH). Nutlin-3 was purchased from Calbiochem (San Diego, CA, USA), dissolved in DMSO and used at 2.5-10 µM concentrations. Daunorubicin, Vincristine (Pfizer, Inc., NY, USA) and L-Asparaginase (Medac GmbH, Wedel, Germany) were diluted in NaCl and used at indicated concentrations. HCT116 cells were transfected by Lipofectamine 2000 (Invitrogen) according to the manufacturer´s recommendations. E/R knock-down was performed as described previously [2] and RNA was isolated 2-3 weeks after lentiviral transduction and confirmation of chimeric protein depletion.

### Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

For qRT-PCR analysis of MDM2 transcripts, 2 µg of RNA was reverse transcribed using a mixture of oligo$(dT)_{15}$, random hexamer primers and M-MLV Reverse Transcriptase to generate cDNA. To amplify mouse MDM2 and GAPDH transcripts, the following primer/probe combinations were used: MDM2, (f) 5'-CTGTGTCTACCGAGGGTGCT-3'; (r) 5'-ATGTGCTGCTGCTTCTCGT-3'; (p) 5'-FAM-CGTTGGAGCGCAAAACGACA-TAMRA-3'; GAPDH, (f) 5'-TGTGTCCGTCGTGGATCTGA-3'; (r) 5'-CCTGCTTCACCACCTTCTTGAT-3'; (p) 5'-FAM-CCGCCTGGAGAAACCTGCCAAGTATG-TAMRA-3'. GAPDH was used as a standard reference for normalization. The quantitative PCRs were done in a total volume of 25 µl containing 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 100 nmol/l forward and reverse primers each and 50 nmol/l Taqman probe. The following cycling conditions were used: 95°C for 10', 95°C for 15'' and 58 - 63°C according to the different primer and probe combinations for 1'. The last two steps were repeated 50 times.

### Western blot analysis

After the addition of 2x loading buffer samples were incubated at 95°C for 5 min. Sixty µg of protein were size separated on 10 - 15 % SDS PAGE, blotted on nitrocellulose transfer membrane (Whatman, GE Healthcare, Chalfont St. Giles, UK), blocked with 1x blocking reagent (Roche) in TBS and incubated with the respective antibody at 4°C overnight. Secondary antibodies were HRP- or (Biorad, Hercules, USA) infrared dye-labeled (LI-COR

Biosciences, Lincoln, NE) and proteins were visualized with enhanced chemiluminscence detection system (Thermo Scientific, Waltham, MA, USA) or membranes were scanned with Odyssey Infrared Imaging System (LI-COR Biosciences, NE, USA) respectively. All western blots shown are representative of at least three independent experiments.

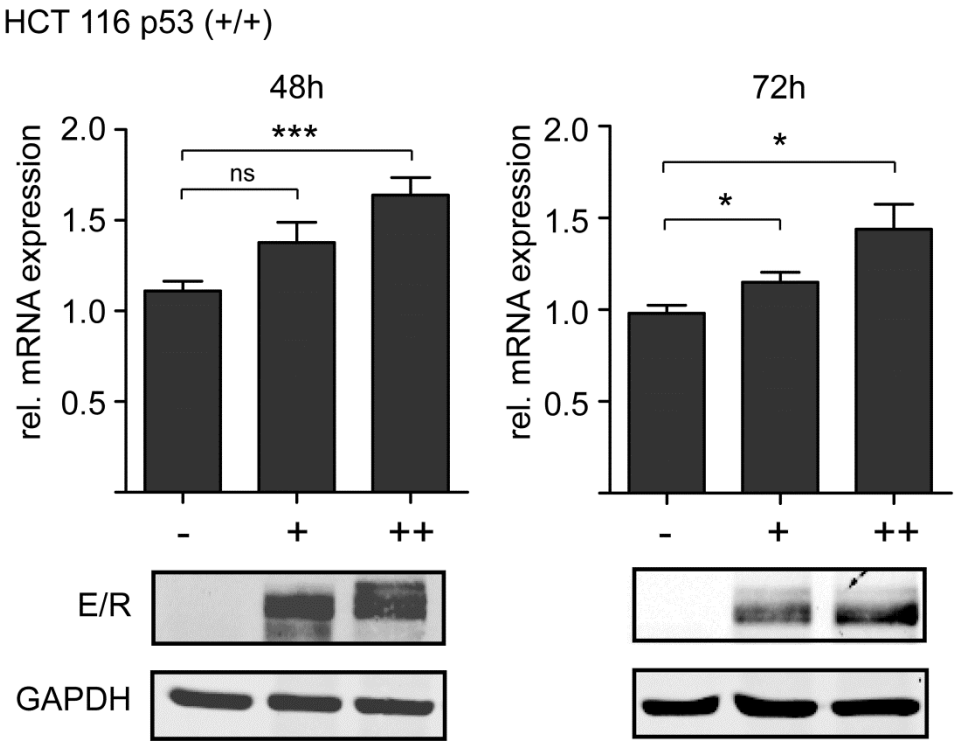
**ChIP buffers**

ChIP lysis buffer: 1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0 and protease inhibitors; ChIP dilution buffer: 0.01% SDS, 1% Triton-X 100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0, 167 mM NaCl and protease inhibitors; Low-salt buffer: 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl; High-salt buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl pH 8.1, 500 mM NaCl; LiCl buffer: 0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA and 10 mM Tris-HCl pH 8.1; TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA; Elution buffer: 1% SDS and 0.1 M $NaHCO_3$

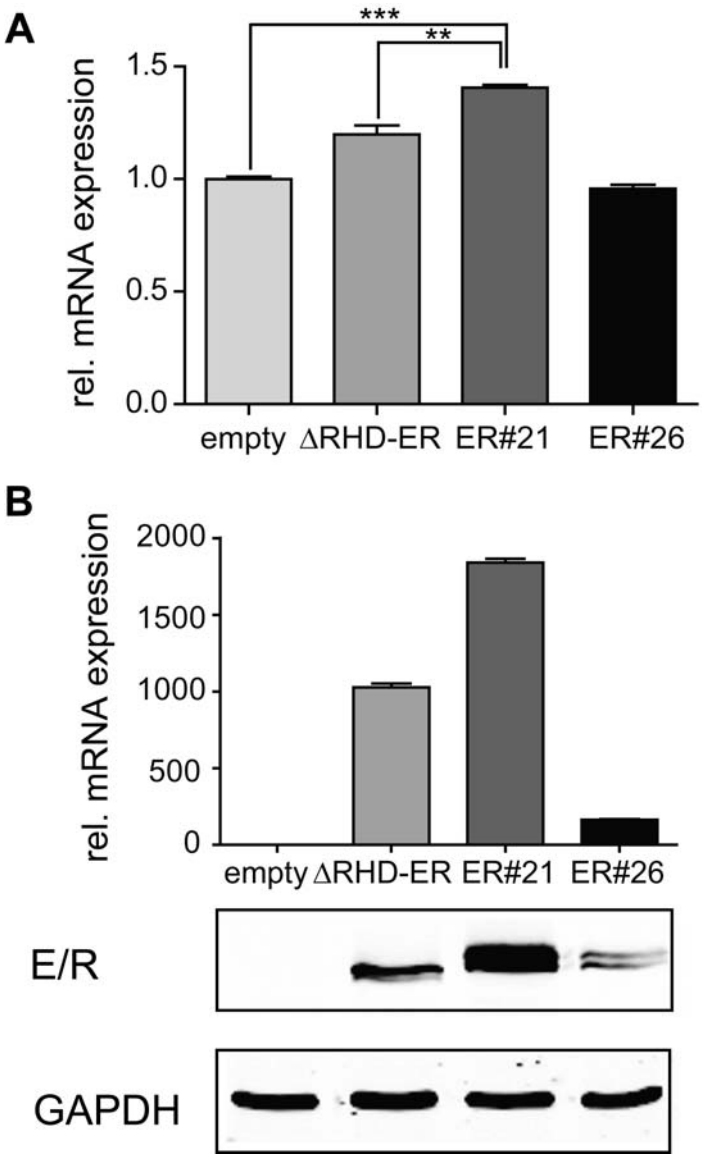
**Supplementary Table 1.** Top 50 upregulated genes by E/R

| | Gene symbol | Entrez Gene ID | E/R KD REH | E/R KD AT2 | E/R KD mean | P-value | adj. P-value | ER+ vs ER- | P-value ER+ vs ER- | adj. P-value ER+ vs ER- |
|---|---|---|---|---|---|---|---|---|---|---|
| **1** | BMP2 | 650 | -2.29 | -0.98 | -1.63 | 0.0006 | 0.0171 | 1.6556 | 0.00000 | 0.0000 |
| **2** | DCHS1 | 8642 | -1.24 | -1.97 | -1.60 | 0.0001 | 0.0059 | 1.0079 | 0.00000 | 0.0000 |
| **3** | SEMA6A | 57556 | -1.55 | -1.58 | -1.56 | 0.0000 | 0.0033 | 0.3505 | 0.00036 | 0.0041 |
| **4** | ANO1 | 55107 | -1.93 | -1.10 | -1.52 | 0.0004 | 0.0126 | 0.4811 | 0.00000 | 0.0000 |
| **5** | DRAM1 | 55332 | -1.16 | -1.16 | -1.16 | 0.0000 | 0.0019 | 0.3776 | 0.00000 | 0.0000 |
| **6** | MDM2 | 4193 | -1.02 | -1.21 | -1.11 | 0.0000 | 0.0019 | 0.5817 | 0.00000 | 0.0000 |
| **7** | TRIB1 | 10221 | -0.34 | -1.84 | -1.09 | 0.0001 | 0.0047 | 1.0848 | 0.00000 | 0.0001 |
| **8** | INPP5D | 3635 | -0.98 | -1.07 | -1.03 | 0.0009 | 0.0210 | 0.3949 | 0.00686 | 0.0411 |
| **9** | HIST2H2BE | 8349 | -1.61 | -0.21 | -0.91 | 0.0008 | 0.0194 | 0.8964 | 0.00087 | 0.0081 |
| **10** | FYB | 2533 | -0.68 | -1.15 | -0.91 | 0.0003 | 0.0117 | 0.6357 | 0.00000 | 0.0000 |
| **11** | SOX11 | 6664 | -1.14 | -0.54 | -0.84 | 0.0003 | 0.0107 | 2.2622 | 0.00000 | 0.0000 |
| **12** | PVRIG | 79037 | 0.43 | -2.08 | -0.82 | 0.0038 | 0.0466 | 0.5947 | 0.00116 | 0.0103 |
| **13** | ARHGEF4 | 50649 | -1.03 | -0.59 | -0.81 | 0.0002 | 0.0080 | 1.1283 | 0.00000 | 0.0000 |
| **14** | TLE4 | 7091 | -0.56 | -1.01 | -0.79 | 0.0021 | 0.0334 | 0.6286 | 0.00125 | 0.0108 |
| **15** | KHDRBS3 | 10656 | -0.39 | -1.11 | -0.75 | 0.0000 | 0.0040 | 0.9730 | 0.00001 | 0.0002 |
| **16** | PRKAR2B | 5577 | -0.59 | -0.89 | -0.74 | 0.0000 | 0.0043 | 0.4625 | 0.00103 | 0.0093 |
| **17** | B4GALT6 | 9331 | -0.73 | -0.71 | -0.72 | 0.0000 | 0.0043 | 0.2224 | 0.00017 | 0.0022 |
| **18** | PTPRK | 5796 | -0.70 | -0.69 | -0.70 | 0.0001 | 0.0049 | 0.5882 | 0.00379 | 0.0261 |
| **19** | PIK3C3 | 5289 | -0.46 | -0.93 | -0.69 | 0.0001 | 0.0068 | 0.8371 | 0.00000 | 0.0000 |
| **20** | NDFIP1 | 80762 | -1.07 | -0.25 | -0.66 | 0.0041 | 0.0482 | 0.4428 | 0.00049 | 0.0052 |
| **21** | ZNF268 | 10795 | -0.94 | -0.36 | -0.65 | 0.0006 | 0.0165 | 0.1535 | 0.00008 | 0.0012 |
| **22** | SCARB1 | 949 | -0.51 | -0.77 | -0.64 | 0.0015 | 0.0282 | 0.6792 | 0.00000 | 0.0000 |
| **23** | RAB1A | 5861 | -0.75 | -0.51 | -0.63 | 0.0002 | 0.0104 | 0.6657 | 0.00006 | 0.0009 |
| **24** | SLC35E3 | 55508 | -0.44 | -0.79 | -0.62 | 0.0002 | 0.0083 | 0.6119 | 0.00133 | 0.0114 |
| **25** | AKAP12 | 9590 | -0.56 | -0.56 | -0.56 | 0.0010 | 0.0222 | 0.9180 | 0.00762 | 0.0440 |
| **26** | BTBD3 | 22903 | -0.48 | -0.62 | -0.55 | 0.0002 | 0.0100 | 1.0385 | 0.00000 | 0.0000 |
| **27** | FAM134B | 54463 | -0.59 | -0.50 | -0.54 | 0.0027 | 0.0387 | 0.4522 | 0.00016 | 0.0021 |
| **28** | TNFRSF21 | 27242 | -0.33 | -0.75 | -0.54 | 0.0013 | 0.0257 | 2.4108 | 0.00000 | 0.0000 |
| **29** | GPR125 | 166647 | -0.76 | -0.30 | -0.53 | 0.0011 | 0.0238 | 0.5559 | 0.00000 | 0.0000 |
| **30** | RGL1 | 23179 | -0.48 | -0.55 | -0.52 | 0.0006 | 0.0160 | 0.7800 | 0.00007 | 0.0010 |
| **31** | TLE3 | 7090 | -0.14 | -0.90 | -0.52 | 0.0021 | 0.0336 | 1.6644 | 0.00000 | 0.0000 |
| **32** | TSPAN9 | 10867 | -0.69 | -0.35 | -0.52 | 0.0008 | 0.0196 | 0.4682 | 0.00002 | 0.0003 |
| **33** | TBC1D9 | 23158 | -0.53 | -0.50 | -0.52 | 0.0006 | 0.0160 | 1.2922 | 0.00000 | 0.0000 |
| **34** | GNAQ | 2776 | -0.25 | -0.78 | -0.52 | 0.0010 | 0.0227 | 0.6355 | 0.00001 | 0.0003 |
| **35** | MME | 4311 | -0.49 | -0.49 | -0.49 | 0.0029 | 0.0401 | 0.9211 | 0.00204 | 0.0160 |
| **36** | EIF4A2 | 1974 | -0.18 | -0.78 | -0.48 | 0.0004 | 0.0142 | 0.2684 | 0.00624 | 0.0382 |
| **37** | KIAA0232 | 9778 | -0.78 | -0.16 | -0.47 | 0.0037 | 0.0456 | 0.5108 | 0.00376 | 0.0259 |
| **38** | ERO1LB | 56605 | -0.43 | -0.49 | -0.46 | 0.0021 | 0.0334 | 0.2934 | 0.00708 | 0.0420 |
| **39** | GNG11 | 2791 | -0.40 | -0.52 | -0.46 | 0.0009 | 0.0206 | 2.9448 | 0.00000 | 0.0000 |
| **40** | GRAMD1B | 57476 | -0.54 | -0.36 | -0.45 | 0.0015 | 0.0276 | 0.3283 | 0.00007 | 0.0011 |
| **41** | OTUD4 | 54726 | -0.42 | -0.46 | -0.44 | 0.0008 | 0.0201 | 0.3520 | 0.00066 | 0.0066 |
| **42** | GNPTAB | 79158 | -0.35 | -0.51 | -0.43 | 0.0037 | 0.0456 | 0.8936 | 0.00000 | 0.0000 |
| **43** | DYRK2 | 8445 | -0.38 | -0.44 | -0.41 | 0.0040 | 0.0472 | 0.5562 | 0.00000 | 0.0000 |
| **44** | FAM171A1 | 221061 | -0.47 | -0.34 | -0.41 | 0.0032 | 0.0423 | 0.6799 | 0.00079 | 0.0076 |
| **45** | GAL3ST4 | 79690 | -0.42 | -0.34 | -0.38 | 0.0033 | 0.0428 | 0.2898 | 0.00009 | 0.0013 |
| **46** | C13orf18 | 80183 | -0.46 | -0.26 | -0.36 | 0.0037 | 0.0456 | 3.0346 | 0.00000 | 0.0000 |
| **47** | BRP44L | 51660 | 0.26 | 0.40 | 0.33 | 0.0037 | 0.0456 | -0.9280 | 0.00000 | 0.0000 |
| **48** | DENND5A | 23258 | 0.31 | 0.38 | 0.34 | 0.0036 | 0.0452 | -0.4684 | 0.00275 | 0.0202 |
| **49** | BLK | 640 | 0.11 | 0.57 | 0.34 | 0.0030 | 0.0412 | -1.0290 | 0.00000 | 0.0000 |
| **50** | PCK2 | 5106 | -0.37 | 1.10 | 0.37 | 0.0033 | 0.0428 | -0.1446 | 0.00352 | 0.0245 |

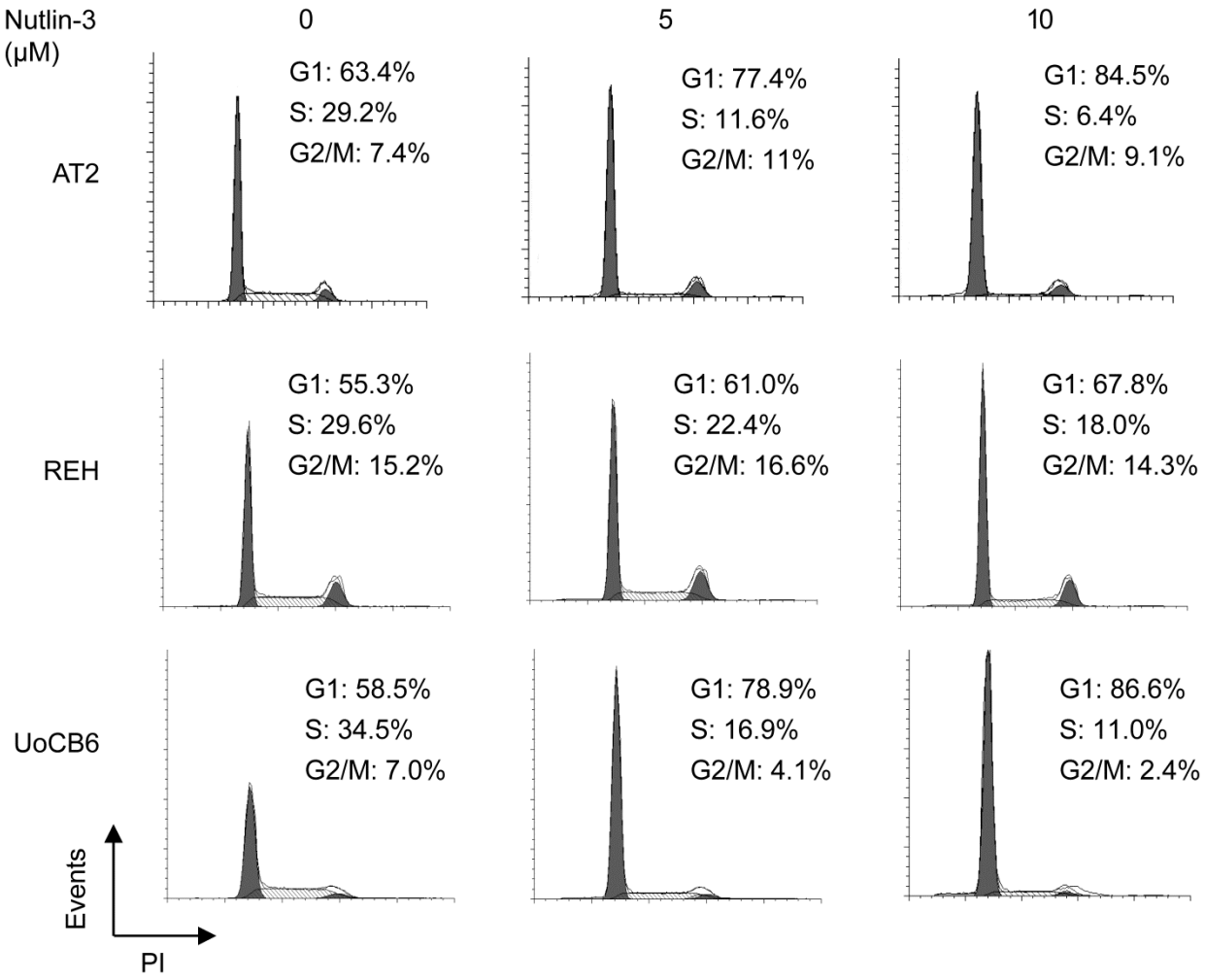
**Supplementary Figures**



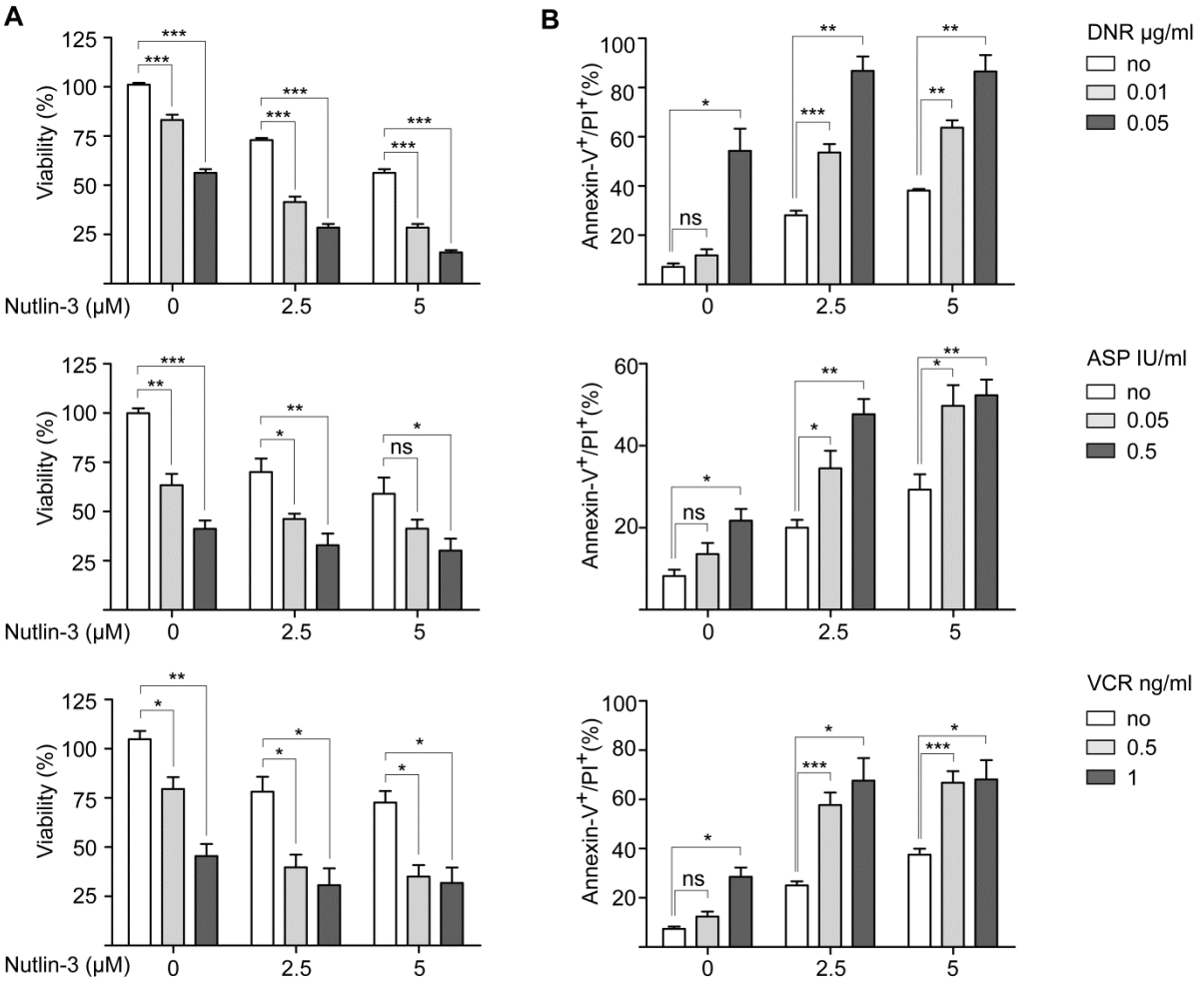

**Supplementary Figure 1.** MDM2 P2 transcripts upon ectopic expression of E/R in HCT116 $p53^{+/+}$ cells. Quantification of MDM2 transcripts 48h and 72h post transfection. MDM2 expression was normalized to GUS and is shown relative to the empty vector control. Depicted is pooled data of three independent experiments. Welch's t-test, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. E/R expression was confirmed by western blot analysis (bottom).



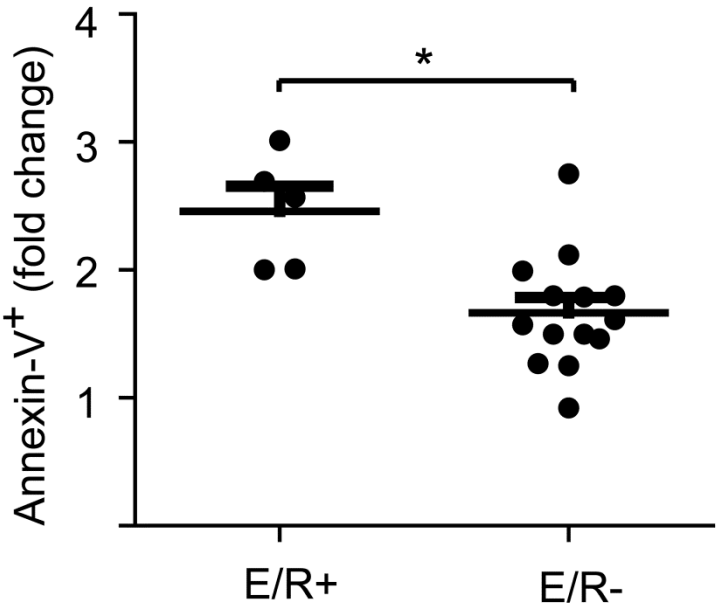

**Supplementary Figure 2.** Expression of MDM2 as a function of E/R. (A) Quantification of MDM2 mRNA levels in HEK 293T clones expressing different amounts of ETV6-RUNX1 (ER#21 and #26), ΔRHD-ER or the empty vector. MDM2 expression was normalized to GUS and is shown relative to the empty vector control. Welch's t-test, **P ≤ 0.005, ***P ≤ 0.0001. (B) Quantification of E/R transcript (top) and corresponding protein levels (bottom) in ETV6/RUNX1 (ER), ΔRHD-ER and empty vector (empty) expressing HEK 293T clones. HEK 293T ER#21 expresses the fusion transcripts at an approximately 10-fold higher level than clone#26. Welch's t-test, for all: ***P ≤ 0.001




**Supplementary Figure 3.** Reactivation of p53 by Nutlin-3 induces cell cycle arrest in E/R-expressing leukemic cell lines. Cell cycle distribution was assessed by propidium iodide staining using BD Cycletest$^{TM}$ Plus-DNA Reagent Kit. Representative DNA histograms showing cell cycle profiles of three E/R-expressing cell lines upon exposure to Nutlin-3. Data were generated after 24 h exposure to Nutlin-3 at 5 and 10 μM. Welch's t-test, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.




**Supplementary Figure 4.** Nutlin-3 enhances effects of chemotherapeutic drugs in E/R-expressing cell lines. UoCB6 cells were exposed to either daunorubicin (DNR), asparaginase (ASP) or vincristine (VCR) together with Nutlin-3. (A) Viability was determined by MTT assay and is indicated in % of carrier control. (B) Apoptotic fractions were measured by flow cytometry. The percentage of apoptotic cells (Annexin V+/PI+) is depicted. Quantification of at least three independent experiments is shown. Welch's t-test, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.




**Supplementary Figure 5.** Primary E/R-positive leukemic samples are more sensitive to Nutlin-3 compared to E/R-negative ones. Apoptosis levels (Annexin-V single positive cells) of E/R-positive (E/R+, n=5) and E/R-negative (E/R-, n=14) BCP-ALL samples after exposure to Nutlin-3 for 24 h are indicated as fold-changes of carrier control. Welch's t-test, *P ≤ 0.05.

1. Inthal A, Krapf G, Beck D, et al. Role of the erythropoietin receptor in ETV6/RUNX1-positive acute lymphoblastic leukemia. *Clin Cancer Res*. 2008;**14**(22):7196-7204.
2. Fuka G, Kantner HP, Grausenburger R, et al. Silencing of ETV6/RUNX1 abrogates PI3K/AKT/mTOR signaling and impairs reconstitution of leukemia in xenografts. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK*. 2012;**26**(5):927-933.