HIF-1α is over-expressed in leukemic cells from *TP53*-disrupted patients and is a promising therapeutic target in chronic lymphocytic leukemia

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*VG and CV contributed equally to this study.

Supplemental Information

Article title: HIF-1α is overexpressed in leukemic cells from *TP53*-disrupted patients and is a promising therapeutic target in CLL

Running Head: HIF-1α in *TP53*-disrupted CLL Cells

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Supplemental Methods

Cells preparation and culture

PBMC were separated using Ficoll-Hypaque (Sigma-Aldrich) and stained with anti-CD19 PerCP Vio700 and anti-CD5-APC antibodies (Miltenyi Biotec, Bologna, Italy). When the CD19+/CD5+ cells were < 90%, CLL cells were purified by negative selection and cultured in RPMI-1640 medium with 10% of fetal bovine serum (FBS) and penicillin/streptomycin. B lymphocytes from healthy donors were purified by positive selection using anti-CD19 micro beads (Miltenyi Biotec). Del(17p) was assessed by *fluorescence in situ hybridization* and the presence of *TP53* gene mutations was evaluated by Sanger sequencing. Immunoglobulin heavy chain variable region gene (IGHV) mutational status was assessed as already described[1]. Cell lines used in the experiments (i.e. Séraphine, Granta-519 and M2-10B4) were maintained in RPMI-1640 or DMEM medium with 10% fetal bovine serum, glutamine and antibiotics at 37 °C, 5% CO_2. A humidified hypoxia incubator chamber was used for hypoxic cultures.

Antibodies used for flow cytometry

Two- and three-color flow cytometry was performed with FACSCalibur and CELLQuest software (Becton Dickinson, Mountain View, CA) and with BD Accuri C6 flow cytometer (BD Bioscences). Data were analyzed with FlowJo software (Tree Star, Inc, Ashland, OR). Antibodies used for flow cytometry were: anti-CD19-PE (BD Biosciences, San José, CA), anti-CD19-PerCP Vio700 (Miltenyi Biotec), anti-CD5-APC (Miltenyi Biotec).

Western blot analysis

Cytosolic and nuclear protein extracts were obtained using the Nuclear Extract Kit (Active Motif, La Hulpe) following the manufacturer's instructions. Lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). The following antibodies were used: anti-HIF-1α (BD Biosciences, San José, CA), anti-ELK3

(Sigma Aldrich); anti-pVHL (EuroClone Spa, Milano); anti p(Thr202/Tyr204, Thr185/Tyr187)-ERK1–2 (Millipore, Bedford, MA); anti-ERK1-2 (Millipore); anti-RAS (Millipore); anti-RHOA (Santa Cruz Biotechnology Inc., Santa Cruz, CA) antibody anti-p(Ser 473)AKT (Millipore); anti-AKT (Millipore); anti-ACTIN (Sigma Aldrich), anti-GAPDH, anti-TUBULIN and anti-TATA-box binding protein (TBP) (Santa Cruz Biotechnology Inc.) used as control of equal protein loading for cytosolic and nuclear fractions, followed by the secondary peroxidase-conjugated antibodies (Bio-Rad, Hercules, CA). To exclude nuclei-cytosol contamination, we verified that GAPDH and TBP were always undetected in nuclear and cytosolic fractions, respectively (not shown). Blot images were acquired with a ChemiDocTM Touch Imaging System device (Bio-Rad Laboratories). Densitometric analysis was performed with the ImageJ software (NIH, Bethesda, MD).

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted and reverse transcribed as described[2]. The qRT-PCR was performed with the iTaqTM Universal SYBR® Green Supermix (Bio-Rad). The primer sequences were designed with the qPrimerDepot software (http://primerdepot.nci.nih.gov/). The primer sequences were: *HIF-1A*: 5'-GGCTGCATCTCGAGACTTT-3': 5'-GAAGACATCGCGGGGAC; *ENO1*: 5'-GCTCCGGGACAATGATAAGA-3', 5'- TCCATCCATCTCGATCATCA-3', *GAPDH*: 5'-GAAGGTGAAGGTCGGAGT-3', 5'-CATGGTGGAAT CATATTGGAA-3'; *VEGF*: 5'-ATCTTCAAGCCATCCTGTGTGC-3', 5'-GCTCACCGCCTCGGCTTGT-3'. The comparative CT method was used to calculate *HIF-1α*, *GLUT1*, *ENO1* and *VEGF* expression relative to the *GAPDH* product, used as a housekeeping gene, with the Bio-Rad Software Gene Expression Quantitation.

RAS and RHOA activity

The isoprenylated membrane-associated RAS or RHOA proteins and the non-

isoprenylated cytosolic forms were detected as previously described[2]. Total RAS and RHOA proteins were analyzed by WB; Briefly, the GTP-bound fraction, taken as an index of active G-proteins was measured, using a pull-down assay (with the RAF-1-GST fusion protein, agarose beads-conjugates, Millipore, Bedford, MA) and an ELISA assay (with the G-LISA™ RHOA Activation Assay Biochem Kit, Cytoskeleton Inc, Denver, CO), respectively.

Kinase inhibitors titration and kinase activity

For kinase inhibitors titration experiments we exposed CLL cells (10^6/mL) for 48 hours to PD98059, LY249002 and Y27632 at indicated increasing concentrations. ERK1-2, AKT and RHOA kinases activity was measured by spectrophotometric methods, using the CycLex RHO Kinase Assay Kit (CycLex, Nagano), the MAP Kinase/Erk Assay Kit (Millipore, Bedford, MA) and the AKT Kinase Activity Assay Kit (Abcam, Cambridge), as per manufacturer's instructions.

Cell viability assay

Cell viability was evaluated by flow cytometry using with Annexin-V/Propidium Iodide (Ann-V/PI) staining with the MEBCYTO-Apoptosis Kit (MBL Medical and Biological Laboratories, Naka-ku Nagoya). Normalized cell viability was arbitrarily defined as the ratio between the percentage of AnnV-/PI- CLL cells cultured in the presence of F-ara-A and the percentage of AnnV-/PI- CLL cells that were left untreated. CLL cells characterized by a normalized cell viability ≥0.5 were defined as *fludarabine-resistant*, otherwise, CLL cells were considered *fludarabine-sensitive*.

Table 1. Summary of genetic patient characteristics and treatment status

Unique Patient Number (UPN)	*TP53* subset	del(17)p, clone size in %	*TP53*mut, allele frequency in %	IGHV mutational status	Previous Treatment
UPN01	TP53dis	neg	mut, 84	M	y
UPN02	TP53dis	42	mut, 83	UM	y
UPN03	TP53dis	85	mut, 83	UM	n
UPN04	TP53dis	80	mut, 79	UM	n
UPN05	TP53dis	neg	mut, 75	UM	y
UPN06	TP53dis	79	mut, 86	M	n
UPN07	TP53dis	neg	mut, nd	UM	y
UPN08	TP53dis	nd	mut, nd	M	nd
UPN09	TP53dis	87	mut, 81	UM	n
UPN10	TP53dis	neg	mut, 88	M	y
UPN11	TP53dis	69	mut, 61	UM	y
UPN12	TP53dis	neg	mut, 36	M	n
UPN13	TP53dis	neg	mut, 50	M	n
UPN14	TP53dis	24	mut, 37	UM	y
UPN15	TP53dis	monosomy 17	mut, 50	UM	y
UPN16	TP53dis	96	mut, 67	nd	n
UPN17	TP53dis	neg	mut, 50	UM	y
UPN18	TP53dis	53	mut, 31	UM	n
UPN19	TP53dis	20	mut, 92	UM	y
UPN20	TP53dis	88	nd	UM	n
UPN21	TP53dis	21	mut, 25	UM	n
UPN22	TP53dis	46	mut, 51	UM	y
UPN23	TP53dis	70	nd	nd	y
UPN24	TP53dis	neg	mut, 34	M	y
UPN25	TP53dis	12	mut, 12	UM	y
UPN26	TP53dis	neg	mut, nd	M	y
UPN27	TP53dis	82	mut, nd	M	n
UPN28	TP53dis	80	mut, 63	UM	y
UPN29	TP53dis	68	mut, 75	UM	y
UPN30	TP53dis	nd	mut, 73	nd	n
UPN31	TP53dis	69	mut, 64	UM	n
UPN32	TP53dis	neg	mut, nd	UM	y

UPN33	TP53dis	43	mut, 25	M	nd
UPN34	TP53dis	neg	mut, nd	UM	n
UPN35	TP53dis	91	wt	M	n
UPN36	TP53dis	59	wt	nd	n
UPN37	TP53dis	50	wt	UM	y
UPN38	TP53dis	50	wt	UM	y
UPN39	TP53dis	46	wt	M	y
UPN40	TP53dis	45	wt	M	n
UPN41	TP53wt	neg	wt	M	y
UPN42	TP53wt	neg	wt	M	n
UPN43	TP53wt	neg	wt	UM	n
UPN44	TP53wt	neg	wt	UM	n
UPN45	TP53wt	neg	wt	M	y
UPN46	TP53wt	neg	wt	M	y
UPN47	TP53wt	neg	wt	M	y
UPN48	TP53wt	neg	wt	UM	n
UPN49	TP53wt	neg	wt	UM	n
UPN50	TP53wt	neg	wt	M	n
UPN51	TP53wt	neg	wt	UM	n
UPN52	TP53wt	neg	wt	UM	y
UPN53	TP53wt	neg	wt	UM	n
UPN54	TP53wt	neg	wt	UM	n
UPN55	TP53wt	neg	wt	UM	n
UPN56	TP53wt	neg	wt	M	n
UPN57	TP53wt	neg	wt	UM	n
UPN58	TP53wt	neg	wt	UM	n
UPN59	TP53wt	neg	wt	M	n
UPN60	TP53wt	neg	wt	UM	y
UPN61	TP53wt	neg	wt	M	n
UPN62	TP53wt	neg	wt	M	n
UPN63	TP53wt	neg	wt	M	y
UPN64	TP53wt	neg	wt	UM	n
UPN65	TP53wt	neg	wt	UM	n
UPN66	TP53wt	neg	wt	UM	n
UPN67	TP53wt	neg	wt	M	n
UPN68	TP53wt	neg	wt	M	n
UPN69	TP53wt	neg	wt	UM	n
UPN70	TP53wt	neg	wt	nd	n

UPN71	*TP53*wt	neg	wt	M	y
UPN72	*TP53*wt	neg	wt	UM	n
UPN73	*TP53*wt	neg	wt	UM	n
UPN74	*TP53*wt	neg	wt	M	n
UPN75	*TP53*wt	neg	wt	M	n
UPN76	*TP53*wt	neg	wt	UM	n
UPN77	*TP53*wt	neg	wt	UM	n
UPN78	*TP53*wt	neg	wt	M	n
UPN79	*TP53*wt	neg	wt	M	n
UPN80	*TP53*wt	neg	wt	UM	n
UPN81	*TP53*wt	neg	wt	M	y
UPN82	*TP53*wt	neg	wt	M	n
UPN83	*TP53*wt	neg	wt	M	n
UPN84	*TP53*wt	neg	wt	M	n
UPN85	*TP53*wt	neg	wt	nd	n
UPN86	*TP53*wt	neg	wt	UM	n
UPN87	*TP53*wt	neg	wt	UM	n
UPN88	*TP53*wt	neg	wt	M	n
UPN89	*TP53*wt	neg	wt	M	n
UPN90	*TP53*wt	neg	wt	UM	n
UPN91	*TP53*wt	neg	wt	nd	n
UPN92	*TP53*wt	neg	wt	UM	n
UPN93	*TP53*wt	neg	wt	UM	n
UPN94	*TP53*wt	neg	wt	nd	n
UPN95	*TP53*wt	neg	wt	UM	n
UPN96	*TP53*wt	neg	wt	M	n
UPN97	*TP53*wt	neg	wt	UM	n
UPN98	*TP53*wt	neg	wt	UM	y
UPN99	*TP53*wt	neg	wt	UM	n
UPN100	*TP53*wt	neg	wt	UM	y
UPN101	*TP53*wt	neg	wt	nd	n
UPN102	*TP53*wt	neg	wt	M	n

Abbreviations

Del(17)p	chromosome 17p13 deletion
neg	negative, <10% del(17p)
*TP53*mut	*TP53* gene mutation
mut	presence of *TP53* gene mutation
wt	wild type, absence of *TP53* gene mutation
nd	not determined
IGHV	Immunoglobulin heavy chian variable region genes

M   IGHV mutated

UM   IGHV unmutated

Supplemental Figures

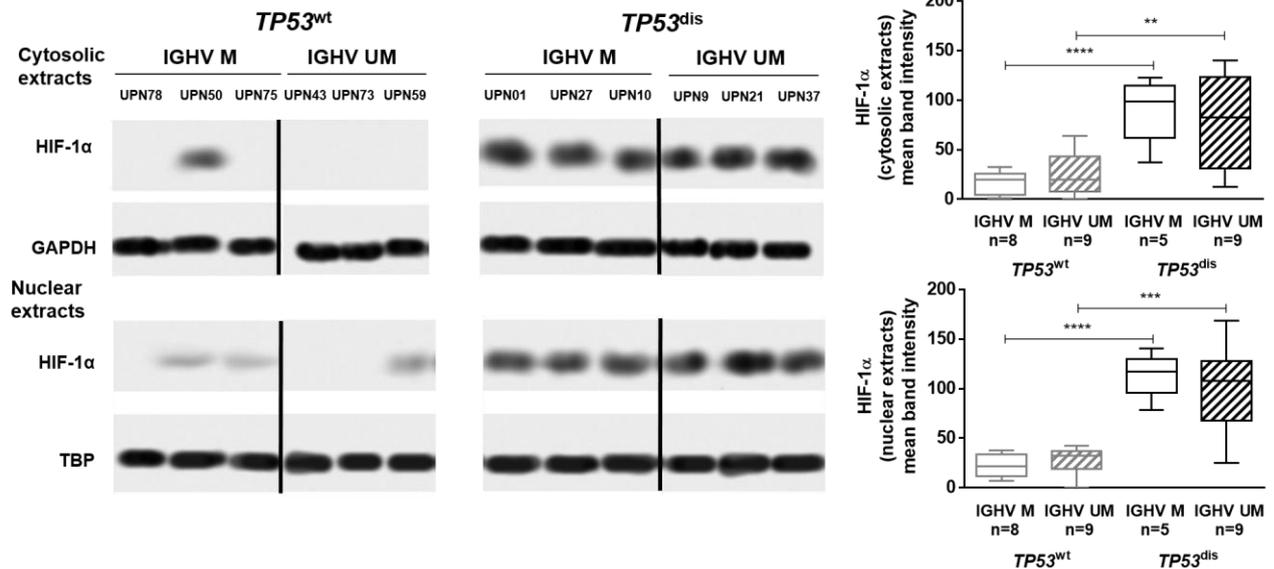## Supplemental Figure 1

Figure S1. The association between HIF-1α expression and the *TP53* status is not influenced by the IGHV mutational status. The expression of HIF-1α was measured in *TP53*wt and *TP53*dis CLL cells, accounting for the IGHV mutational status. *TP53*wt CLL included 8 IGHV M and 9 IGHV UM samples, and *TP53*dis CLL included 5 IGHV M and 8 IGHV UM samples. A representative blot is shown, together with Unique Patients Number (UPN) and cumulative band intensity data obtained from the analysis of the four subsets (i.e. *TP53*wt/IGHV M, *TP53*wt/IGHV UM, *TP53*dis/IGHV M and *TP53*dis/IGHV UM CLL patients). Box plots represent median values and 25%-75% percentiles, whiskers represent minimum and maximum values of band intensity for each group. Vertical lines have been inserted to indicate repositioned gel lanes. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$.

## Supplemental Figure 2
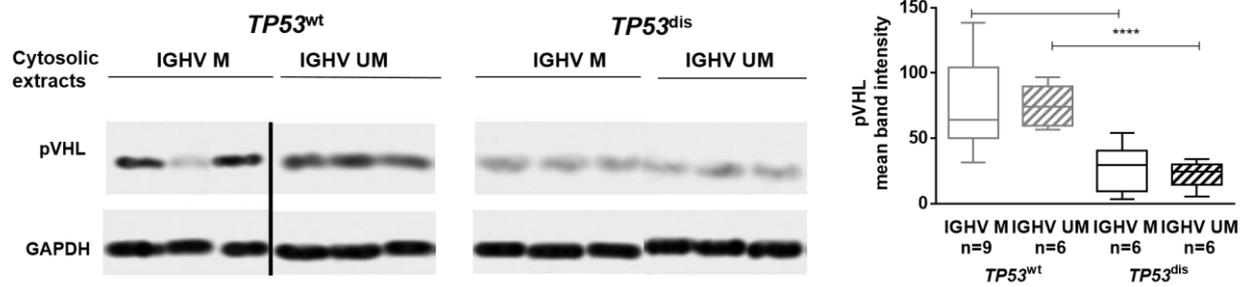
Figure S2. The expression of pVHL is not influenced by IGHV mutational status.

The expression of pVHL was measured in *TP53*[wt] and *TP53*[dis] CLL cells, accounting for the IGHV mutational status. *TP53*[wt] CLL included 9 IGHV M and 6 IGHV UM samples, and *TP53*[dis] CLL included 6 IGHV M and 6 IGHV UM samples. A representative blot is shown, together with UPN and cumulative band intensity data obtained from the analysis of the four subsets. Box plots represent median value and 25%-75% percentiles, whiskers represent minimum and maximum values of band intensity for each group. Vertical lines have been inserted to indicate repositioned gel lanes. **** $p < 0.0001$, ** $p < 0.01$.

Supplemental Figure 3
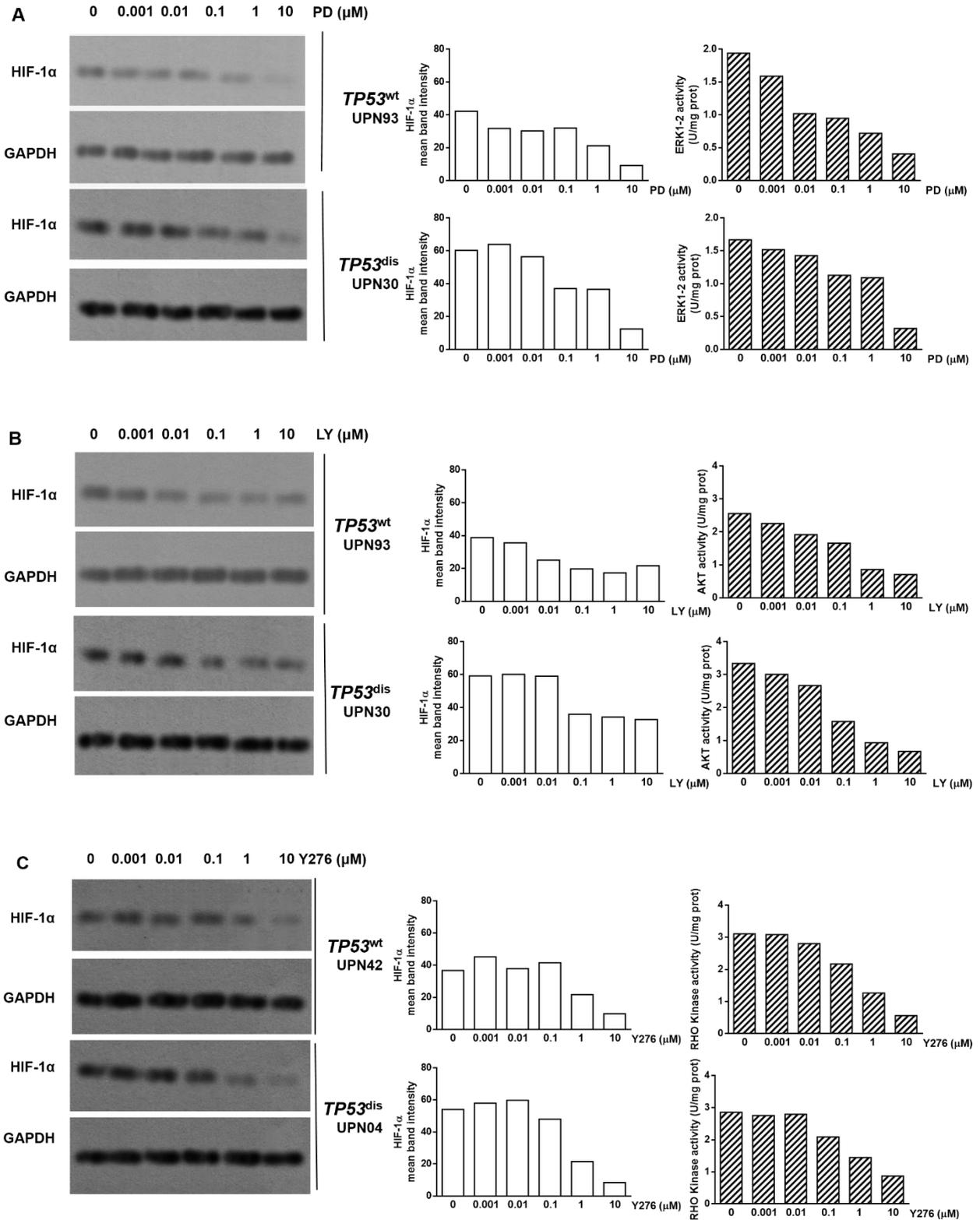
Figure S3. Increasing concentrations of ERK1-2, PI3K or RHOA kinase inhibitors determine a progressive reduction of HIF-1α levels. Primary CLL cells were cultured for 48 hours in the presence of increasing concentration of PD98059 (PD, 0.01 μM, 0.1 μM, 1 μM or 10 μM) **(A)**, LY294002 (LY, 0.01 μM, 0.1 μM, 1 μM or 10 μM) **(B)** or Y27632 (Y276 0.01 μM, 0.1 μM, 1 μM or 10 μM) **(C)**. WB analyses of HIF-1α protein expression for 1 *TP53*wt and 1 *TP53*dis representative CLL patient, together with UPN and the corresponding cumulative band intensity data, are shown. ERK1-2, AKT and RHOA kinase activities were evaluated by an immunomediated assay and are shown by bar graphs on the right.
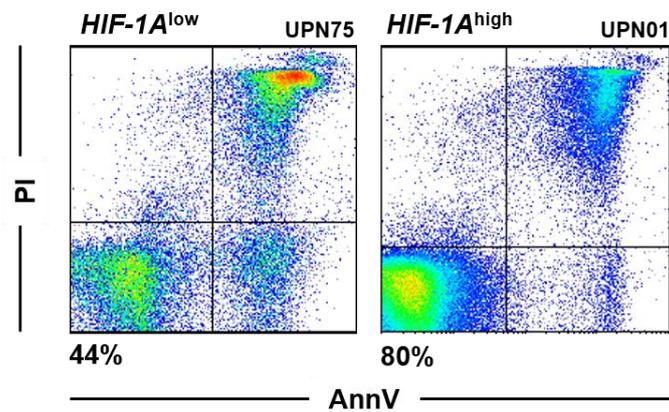
Supplemental Figure 4

Figure S4. Viability of *HIF-1A*high and *HIF-1A*low CLL cells. Representative flow cytometry analysis (relative UPN indicated) of AnnV/PI expression on *HIF-1A*high and *HIF-1A*low CLL cells after 48-hour culture in medium. CLL cells isolated from *HIF-1A*high samples had a significantly higher viability than CLL cells isolated *HIF-1A*low samples.
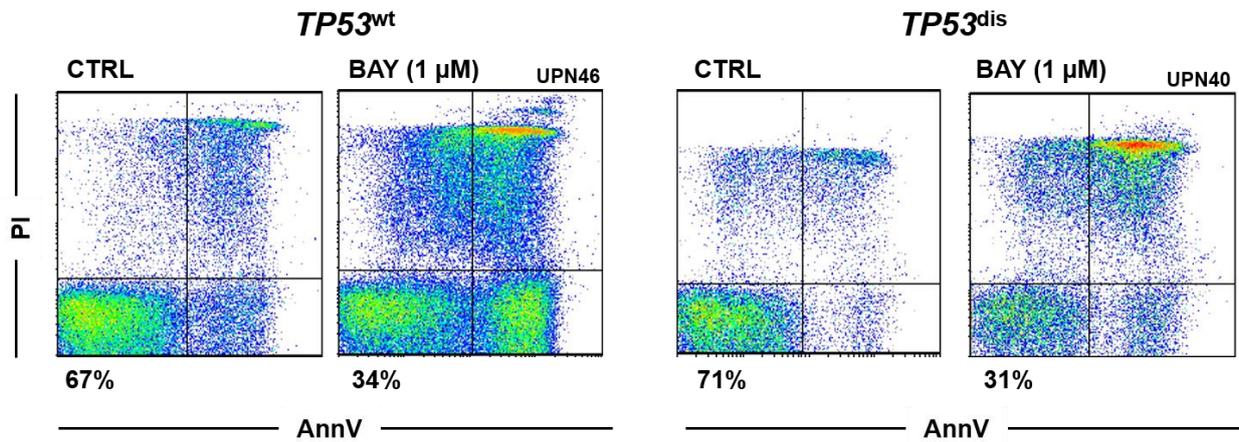
Supplemental Figure 5

Figure S5. Viability of *TP53*^{wt} and *TP53*^{dis} CLL cells exposed to BAY87-2243. Representative flow cytometry analysis (relative UPN indicated) of AnnV/PI expression on *TP53*^{wt} and *TP53*^{dis} CLL cells exposed to 1 µM BAY87-2243 (BAY) or left untreated for 48 hours. BAY87-2243 determined a direct cytotoxic effect toward leukemic cells isolated from both patient subsets.
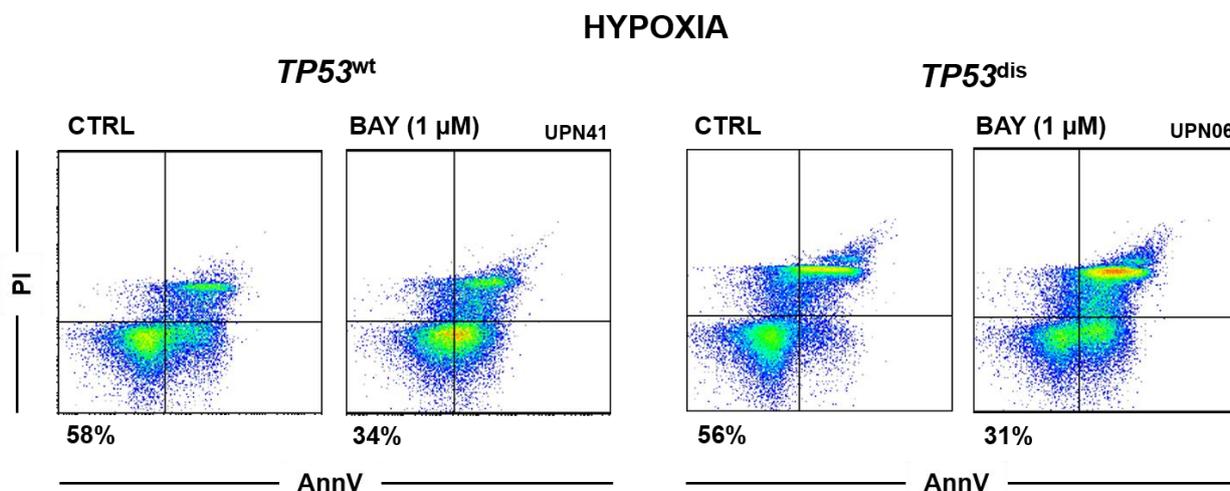
Supplemental Figure 6

Figure S6. Viability of *TP53*wt and *TP53*dis CLL cells exposed to BAY87-2243 under hypoxia. Representative flow cytometry analysis (relative UPN indicated) of AnnV/PI expression on *TP53*wt and *TP53*dis CLL cells exposed to 1 μM BAY87-2243 (BAY) or left untreated for 48 hours, under hypoxic conditions. BAY87-2243 exerted a cytotoxic effect also when *TP53*dis and *TP53*wt CLL cells were cultured in conditions of hypoxia.
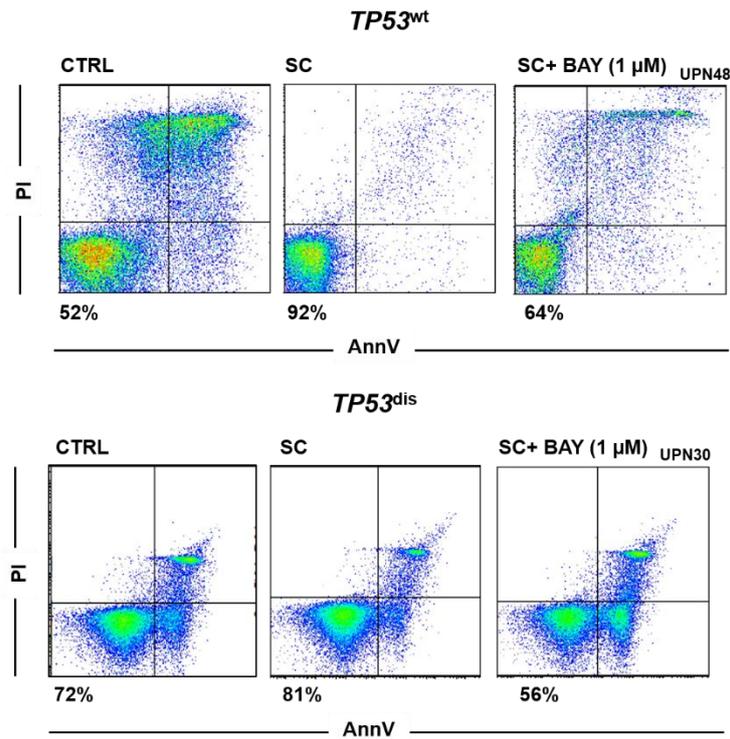
Supplemental Figure 7

Figure S7. Viability of *TP53*^{wt} and *TP53*^{dis} CLL cells exposed to BAY87-2243 in presence of SC. Representative flow cytometry analysis (relative UPN indicated) of AnnV/PI expression on *TP53*^{wt} and *TP53*^{dis} CLL cells exposed to 1 μM BAY87-2243 (BAY) or left untreated for 48 hours, in the presence or in the absence of the murine SC line M2-10B4. BAY87-2243 exerted a cytotoxic effect also when *TP53*^{dis} and *TP53*^{wt} CLL cells were co-cultured with SC.
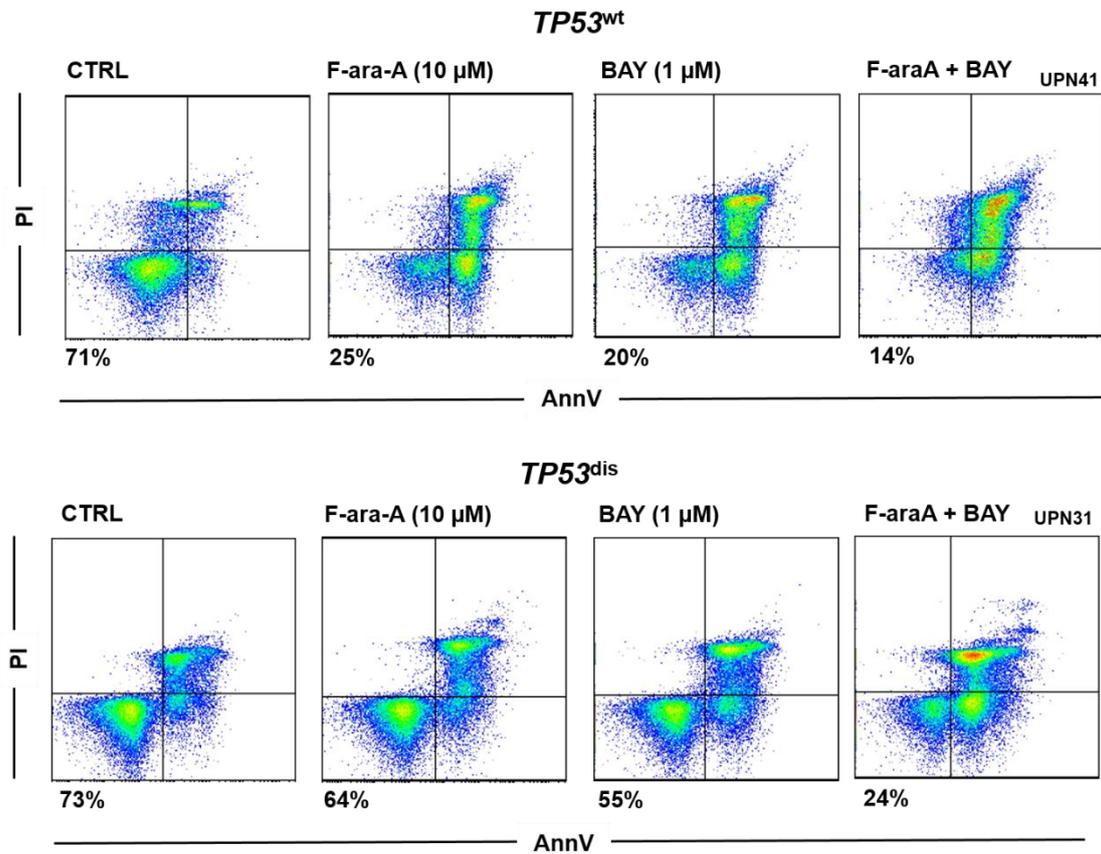
Supplemental Figure 8

Figure S8. Viability of *TP53*wt and *TP53*dis CLL cells exposed to BAY87-2243 + fludarabine. Representative flow cytometry analysis (relative UPN indicated) of AnnV/PI expression on *TP53*dis and *TP53*wt CLL cells exposed for 48 hours to 1 µM BAY87-2243 (BAY) and/or 10 µM F-ara-A. The combination BAY87-2243 + F-ara-A determined a significant decrease in the viability of *TP53*wt and *TP53*dis CLL cells, compared to each compound used as single agent and to untreated controls.

Supplemental Figure 9

F-ara-A (µM)	TP53^{wt}				TP53^{dis}			
BAY (µM)	0.01	0.1	1	10	0.01	0.1	1	10
0.01	1.58	6.9	**0.28**	1.1	57.5	1.44	**0.36**	**0.56**
0.1	**0.44**	**0.58**	**0.29**	1.21	**0.67**	**0.08**	**0.1**	**0.24**
0.5	**0.67**	1.74	**0.24**	**0.92**	**0.76**	2.8	**0.48**	**0.35**
1	1.53	**0.73**	**0.38**	**0.62**	6.66	**0.65**	**0.18**	**0.17**

Combination Index

Figure S9. CI of BAY87-2243 + fludarabine combinations. Figure showing combination indexes (CI) relative to 48-hour treatment with BAY87-2243 (BAY) and F-ara-A, used at different concentrations in TP53^{wt} and TP53^{dis} CLL cells. CI<1, highlighted in bold, indicate synergistic combinations.
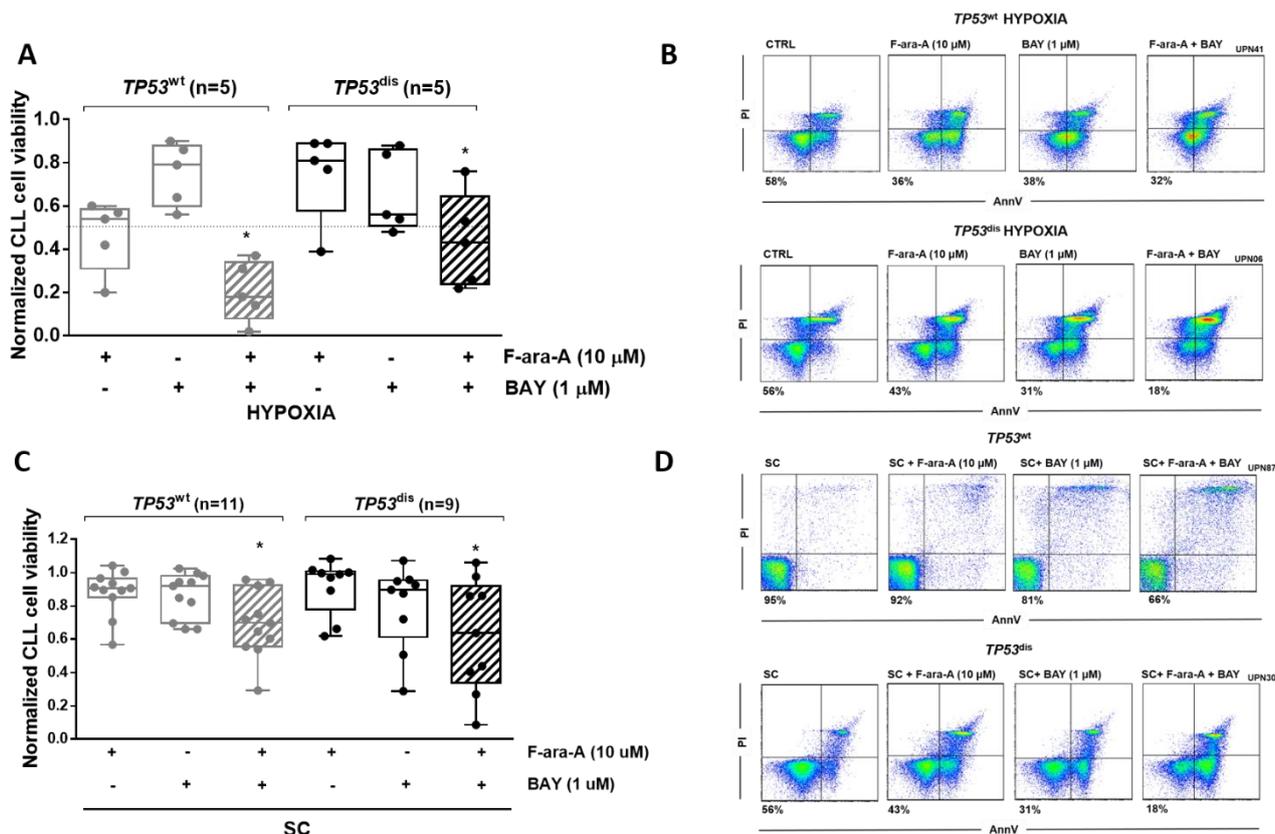
Supplemental Figure 10

Figure S10. BAY87-2243 + fludarabine combination exerts a significant cytotoxic effect on _TP53_[dis] and _TP53_[wt] CLL cultured under hypoxia or in the presence of SC. Normalized cell viability of _TP53_[dis] and _TP53_[wt] CLL cells exposed for 48 hours to 1 µM BAY87-2243 (BAY) and/or 10 µM F-ara-A, under normoxia and hypoxia, or in co-culture with SC. The combination BAY87-2243 + F-ara-A (striped pattern) determined a significant decrease in the viability of _TP53_[wt] and _TP53_[dis] CLL cells, compared to each compound used as single agent, in condition of hypoxia **(A, B)** or in the presence of SC **(C, D).** In panels A and C, box plots represent median values and 25%-75% percentiles, whiskers represent minimum and maximum values for each group, together with all the points. In panels B and D, representative flow cytometry analysis (relative UPN indicated) of

AnnV/PI expression on *TP53*dis and *TP53*wt CLL cells exposed for 48 hours to 1 μM

BAY87-2243 and/or 10 μM F-ara-A in the presence of SC.
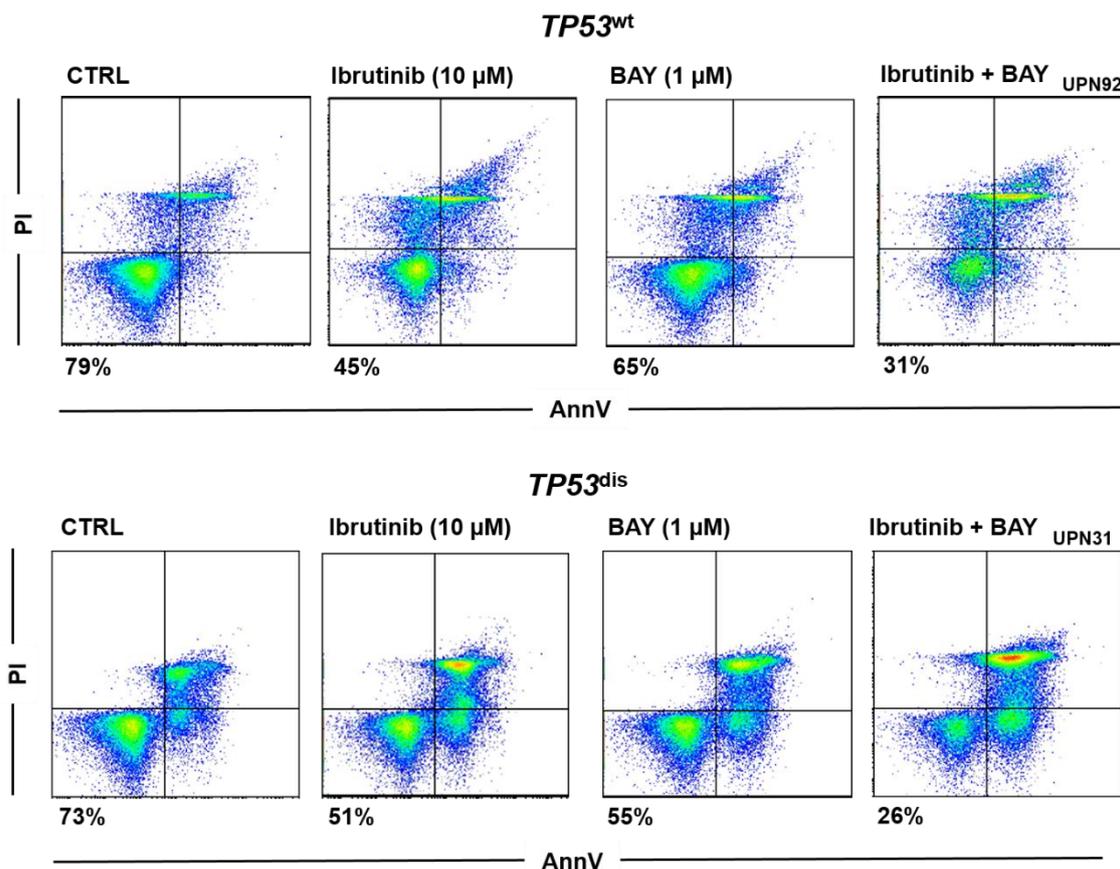
Supplemental Figure 11

Figure S11. Viability of *TP53*^{wt} and *TP53*^{dis} CLL cells exposed to BAY87-2243 + ibrutinib. Representative flow cytometry analysis (relative UPN indicated) of AnnV/PI expression on *TP53*^{dis} and *TP53*^{wt} CLL cells exposed for 48 hours to 1 µM BAY87-2243 (BAY) and/or 10 µM ibrutinib. The combination BAY87-3342 + ibrutinib determined a significant decrease in the viability of *TP53*^{wt} and *TP53*^{dis} CLL cells, compared to each compound used as single agent and to untreated controls.

Supplemental Figure 12

Ibrutinib (µM)	TP53wt				TP53dis			
BAY (µM)	0.01	0.1	1	10	0.01	0.1	1	10
0.01	**0.03**	**0.07**	**0.18**	**0.08**	**0.07**	**0.08**	**0.34**	**0.55**
0.1	**0.04**	**0.07**	**0.03**	**0.01**	**0.16**	**0.04**	**0.22**	**0.28**
0.5	**0.27**	**0.1**	**0.06**	**0.03**	**0.21**	**0.27**	1.17	**0.48**
1	**0.3**	**0.2**	**0.33**	**0.02**	**0.33**	**0.1**	**0.21**	**0.25**

Combination Index

Figure S12. CI of BAY87-2243 + ibrutinib combinations. Figure showing combination indexes (CI) relative to the 48-hour treatment with BAY87-2243 (BAY) and ibrutinib, used at different concentrations in *TP53*wt and *TP53*dis CLL cells. CI<1, highlighted in bold, indicate synergistic combinations.

Supplemental references

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2. Rigoni M, Riganti C, Vitale C, et al. Simvastatin and downstream inhibitors circumvent constitutive and stromal cell-induced resistance to doxorubicin in IGHV unmutated CLL cells. Oncotarget 2015;6(30):29833–29846.