RESCUE OF HIPPO CO-ACTIVATOR YAP1 TRIGGERS DNA DAMAGE-INDUCED APOPTOSIS IN HEMATOLOGICAL CANCERS

Francesca Cottini, Teru Hideshima, Chunxiao Xu, Martin Sattler, Martina Dori, Luca Agnelli, Elisa ten Hacken, Maria Teresa Bertilaccio, Elena Antonini, Antonino Neri, Maurilio Ponzoni, Magda Marcatti, Paul G. Richardson, Ruben Carrasco, Alec C. Kimmelman, Kwok-Kin Wong, Federico Caligaris-Cappio, Giovanni Blandino, W. Michael Kuehl, Kenneth C. Anderson and Giovanni Tonon

Supplemental Experimental Procedures

Experimental Procedures

Reagents. Bortezomib and doxorubicin were purchased from Selleck Chemicals LLC (Houston, TX, United States) and Sigma–Aldrich (St. Louis, MO, Unites States), respectively; ATM inhibitor (Ku55933) and JNK inhibitor (SP600125) were obtained from Calbiochem/Merck Millipore (Darmstadt, Germany). Imatinib, an ABL1 inhibitor, was purchased from Novartis (Basel, Switzerland).

Cell lines and culture. The MM cell lines UTMC–2, EJM, U266, H929, RPMI–8226, KMS–11, KMS–12PE, KMS–18, KMS–20, KMS–34, OCI–My5, JJN–3, and Karpas–620 were available in the labs, kindly provided by other researchers or purchased from American Type Culture Collection (ATCC). MM Dex–sensitive (MM.1S) or resistant (MM.1R) human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL). IL–6–dependent INA–6 cell line was provided by Dr. Renate Burger (University of Kiel, Germany). UTMC–2 and EJM human MM cell lines were established in our laboratory. All AML, ALL, Waldenström's macroglobulinemia (BCWM.1 and MWCL.1), and lymphoma cell lines were provided by collaborators or purchased from ATCC.

All MM, leukemia and lymphoma cell lines were cultured in RPMI–1640 media containing 10% fetal bovine serum (FBS, GIBCO, Life technologies, Carlsbad, CA, United States), 2 $\mu M\ L^{-1}$ glutamine, 100 U mL^{-1} penicillin, 100 $\mu g\ mL^{-1}$ streptomycin (GIBCO, GIBCO, Life technologies, Carlsbad, CA, United States), with 2.5 ng mL^{-1} of IL–6 only in INA–6 cells. 293T, HeLa, and HCT-116 cell lines were purchased from ATCC and were cultured in DMEM containing 10% fetal bovine serum (FBS, GIBCO, Life technologies, Carlsbad, CA, United States), 2 $\mu M\ L^{-1}$ glutamine, 100 U mL^{-1} penicillin, and 100 $\mu g\ mL^{-1}$ streptomycin (P/S, GIBCO, Life technologies, Carlsbad, CA, United States).

Primary cells. Blood samples collected from healthy volunteers were processed by Ficoll–Paque (GE Healthcare, Boston, MA, United States) gradient to obtain peripheral blood mononuclear cells (PBMCs). MM cells from individuals affected by MM were obtained from bone marrow samples after informed consent was obtained in accordance with the Declaration of Helsinki and approval by the Institutional Review Board of the Dana–Farber Cancer Institute or by the Ethical Committee at the San Raffaele Hospital. Mononuclear cells were separated using Ficoll–Paque density sedimentation, and plasma cells were purified (>95% $CD138^+$) by positive selection with anti–CD138 magnetic activated cell separation micro beads (Miltenyi Biotec, Cambridge, MA, United States).

DNA extraction and genomic deletion evaluation by PCR. Genomic DNA was extracted using DNA extraction kit (Qiagen, Hilden, Germany). To identify and confirm YAP1 deletion in MM cell lines the following primers were used:

Primer name	Sequence
YAP1 exon1–F	CTTCTCCACCTCGGCCC
YAP1 exon1–R	TCCAGGTCGGTCTCCGAGTC
YAP1 exon4–F	CATCGAATATCCCAAATTGC
YAP1 intron4/5–R	CAAAAGTGGAAGGCTGGTT
YAP1 exon7–F	CAGCCCTGATGTTAGCTTTTC
YAP1 exon7–R	AAATTTCCGGTGCATGTGTC
β-Actin–F	GTGGGGCGCCCCAGGCACCA
β-Actin–R	CTCCTTAATGTCACGCACGATTTC

Starting with 100 ng of genomic DNA, the following protocol was used: 94 ˚C for 30 s as initiation step; 94 ˚C for 15 s as denaturating phase; 58 ˚C or 59 ˚C for 15 s, according to primer set used, as annealing step; 72 ˚C for 1 min as elongation step. These steps were repeated for 25–30 cycles depending on the primer set and then 72 ˚C for 5 min as final elongation. PCR products were visualized on 1.0% TAE agarose gel after running for 1 h.

RNA extraction and reverse transcription polymerase chain reaction. RNA was extracted using Trizol (Invitrogen, Life technologies, Carlsbad, CA, United States) and quantified by a Nanodrop spectrophotometer (Labtech). Specifically, 5,000,000 cells were pelleted, washed with cold PBS, and

resuspended in 1 mL trizol. They were then incubated with 1–Bromo–3–chloropropane (Sigma), washed first with isopropyl alcohol and then with 75% ethanol, and resuspended in nuclease free–water (Invitrogen, Life technologies, Carlsbad, CA, United States). After quantification, 2000 ng of RNA were used to synthesize cDNA via the Superscript II First strand synthesis Kit (Invitrogen, Life technologies, Carlsbad, CA, United States), according to the manufacturer's instructions. To evaluate the expression levels of YAP1, p73, p21, PUMA, BAX, STK4, STK3 and GAPDH reverse transcription polymerase chain reactions (RT-PCR) were performed using SYBR GREEN PCR Master Mix (Applied Biosystems, CA, United States), after optimization of the primer conditions. cDNAs were diluted 1:100 or 1:1000 and amplified in a 20 μL reaction. Primers were used at 200 nmol or 400 nmol concentration. Thermal cycling conditions were: 10 min at 95 ˚C, 40 cycles at 95 ˚C for 15 s, followed by 1 min at 60 ˚C. Real–time quantitative PCR was performed on ABI Prism 7300 Sequence Detection System (Applied Biosystems, CA, United States). Data were analyzed using the delta delta Ct method. GAPDH was used for normalization.

Primer name	Sequence
YAP1–F	CAATAGCTCAGATCCTTTCCT
YAP1–R	TAGTATCACCTGTATCCATCTC
TA–p73–F	GCACCACGTTTGAGCACCTCT
TA–p73–R	GCAGATTGAACTGGGCCATGA
p21–F	CCTCATCCCGTGTTCTCCTTT
p21–R	GTACCACCCAGCGGACAAGT
PUMA–F	CCTGGAGGGTCCTGTACAATCT
PUMA–R	GCACCTAATTGGGCTCCATCT
BAX–F	CCAGCTCTGAGCAGATCATGAAG
BAX–R	GCAATCATCCTCTGCAGCTCCAT
STK4–F	AGTGCCAAAGGAGTGTCAATAC
STK4–R	GGATTCCTGGCGTTTCAGTTTC
STK3–F	CGATGTTGGAATCCGACTTGG
STK3–R	GTCTTTGTACTTGTGGTGAGGTT

GAPDH–F	GAAGGTGAAGGTCGGAGTCA
GAPDH–R	GGGGTCATTGATGGCAACAATA

Western blotting. MM cells were harvested and lysed using lysis buffer (50 mMTris–HCl (pH 7.4), 150 mM NaCl, 1% NP–40, 5 mM EDTA, 5 mM NaF, 2 mM Na_3VO_4, 1mM PMSF, 5 μg mL^{-1} leupeptine, and 5 μg mL^{-1} aprotinin). Nuclear extracts were prepared using Nuclear Extraction Kit (Affymetrix, Santa Clara, CA, United States). Cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with different antibodies: ATM, phospho–ATM (Ser1981), ATR, phospho–ATR (Ser428), CHK2, phospho–CHK2 (Thr68), CHK1, phospho–CHK1 (Ser296), p21, p53, YAP1, STK4, cleaved caspase 3, cleaved PARP, Histone H3, phospho–JNK (Thr183/Tyr185), HA–Tag, Myc–Tag, p63, GAPDH, and α-tubulin (from Cell Signaling, Beverly, MA, United States); STK4, phospho–YAP1 (Tyr357), Nuclear Matrix Protein p84 (from Abcam, Cambridge, MA, United States), p73 (from BD, San Diego, CA, United States), γ-H2A.X (Ser139) (from Millipore/Merck, Darmstadt, Germany); as well as ABL1, BAX, Puma, Noxa, and nucleolin (from Santa Cruz Biotechnology, Dallas, TX, United States). All antibodies were diluted 1:1000, except for GAPDH antibody (1:2000 dilution).

Immunofluorescence staining. 15,000 cells from MM cell lines, PBMCs or cells obtained from subjects affected by MM were cytospun for 5 min at 350–500 rpm, fixed in 4% paraformaldehyde (PFA) for 15 min, washed three times with PBS, and incubated with 0.1 M glycin for 10 min to quench PFA autofluorescence. After washing again, cells were permeabilized and stained for 90 min with a solution of 0.1% Triton X–100 and PBS + BSA 1%, containing primary antibodies at a ratio of 1:100. Cells were washed and incubated for 45 min with appropriate secondary–fluorescent antibodies. Alexa Fluor 488 anti–rabbit and Alexa Fluor 488 and Alexa Fluor 568 anti–mouse antibodies were purchased from Invitrogen (Life technologies, Carlsbad, CA, United States). After washes, the nuclear content was stained with DAPI reagent (Invitrogen, Life technologies, Carlsbad, CA, United States) for 5 min and washed. The entire procedure was performed at room temperature. The slides were then mounted with ProLong Gold Antifade Reagent (Invitrogen, Life technologies, Carlsbad, CA, United States), and images were taken using a Zeiss microscope (Carl Zeiss,Jena, Germany) equipped with Hamamatsu ORCA-ER camera (Hamamatsu Photonics, Hamamatsu, Japan) and analyzed with ImageJ software. Anti–γ-H2A.X was obtained from Millipore/Merck, (Darmstadt, Germany), Anti–ABL1 from Santa Cruz Biotechnology (Dallas,TX,United States) and Anti–cleaved caspase 3 (C3), and Anti–YAP1 from Cell signaling (Beverly, MA, United States).

Co-Immunoprecipitation for YAP1/p73 binding. KMS–20 MM cells were transiently transfected with YAP1–EGFP vector and treated with Dmso or 10 μM imatinib for 48 h. Cells were then lysed and diluted in NP–40 lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5mM EDTA, and 1% NP–40) plus protease and phosphatase inhibitors. The diluted cell lysates were incubated with 20 μl Protein A/G PLUS Agarose beads (Santa Cruz Biotechnology, Dallas, TX, United States) at 4 ^{o}C for 30 min on the rocker (pre-cleaning phase), and then incubated with 1:100 p73 antibody (Santa Cruz Biotechnology, Dallas, TX, United States) at 4 ^{o}C overnight on the rocker. Beads were then added, and samples were left on the rocker for additional 4 h. Lysates were then centrifuged at 800 g for 5 min; supernatant (unbound fraction) was isolated; beads (bound fraction) were washed three times, boiled in 4X Laemmli SDS sample buffer (Boston BioProducts, Ashland, MA, United States), and separated using 7.5% SDS-PAGE. YAP1 levels were detected using YAP1 antibody (Cell Signaling, Beverly, MA, United States).

γ-H2A.X foci quantification. MM cell lines and MM cells were stained and images acquired as described above. For each sample, at least three different images were taken, representative of different fields of the slide. Each image contained a field of at least 30 cells. Cells were considered positive if they have more than 6 foci. Mean and standard deviation were calculated among the triplicates.

Immunohistochemistry. For immunohistochemistry on clinical samples, polyclonal antibody (dilution 1:200) from Santa Cruz Biotechnology (Santa Cruz, CA, United States) was applied on 4 micron-thick sections, obtained from formalin–fixed, paraffin–embedded human specimens of MM, after antigen retrieval Tris–EDTA at pH 9, at 97 ˚C, for 30 min. The reaction was developed with Ultravision detection system HRP–Polymer (Thermo Scientific) and developed with DAB (Thermo Scientific, Tewksbury, MA, United States). Sections were counterstained with hematoxylin.

Stable gene knockdown using lentiviral vectors. Lentiviral short hairpin RNAs (shRNAs) were used to knockdown YAP1 and STK4 expression in MM cells. Scrambled control pLKO.1 shRNA vectors and specific YAP1 and STK4 shRNA constructs were kindly provided by Dr. William Hahn (Dana-Farber Cancer Institute, Boston, MA, United States). pTRIPZ vectors were purchased from Thermo Scientific Bio (Tewksbury, MA, United States).

Primer name	Sequence
YAP1 shRNA pLKO.1 clone #1	CCCAGTTAAATGTTCACCAAT
YAP1 shRNA pLKO.1 clone #3	CAGGTGATACTATCAACCAAA

STK4 shRNA pLKO.1 clone #1	AGTTGAGTGATAGCTGGGAAA
STK4 shRNA pLKO.1 clone #3	GCCCTCATGTAGTCAAATATT
STK4 shRNA pLKO.1 clone #4	GCCAAGCGGAATACAGTGATA
STK4 shRNA pLKO.1 clone #5	CTAAGAAGAGACGGCAACAAA
STK4 shRNA pTRIPZ V2THS_56763 (or 63)	TATTTGACTACATGAGGGC
STK4 shRNA pTRIPZ V2THS_56690 (or 90)	TAAACATAAACTCTCAAAG
STK4 shRNA pTRIPZ V2THS_56692 (or 92)	AATAGCAACAATCTGGCCG

The efficacy of each shRNA in silencing gene expression was tested in HCT–116 and HeLa cell lines using Mirus Bio Trans–IT LT1 transfection reagent (Mirus Bio LLC, Madison, WI, United States). To obtain stable silenced clones with YAP1/STK4 shRNA or pLKO.1 control plasmid, the following protocol was used. 293T cells were plated (300,000 cells on 6–cm plates) in DMEM/10% FBS/0.1% P/S. After 24 h when cells were 60–70% confluent, 1000 ng of vector of interest together with 100 ng p–VSV–G and 900 ng delta 8.94 (both packaging vectors purchased from Addgene, Cambridge, MA, United States) were co–transfected with MIRUS BIO TransIT-LT1 diluted in OPTI–MEM (GIBCO, Life technologies, Carlsbad, CA, United States).

After 12 h, 293T media was changed with DMEM/30% FBS/10% P/S to promote viral production. 24 and 48 h post transfection, supernatant containing lentiviral particles was harvested, filtered with 0.45 μM diameter filter, and used to infect 2,500,000 MM cells. MM cells were spinoculated at 750 g for 30 min with 8 μg mL^{-1} polibrene, incubated with viral supernatant for 6 h, and left in culturing media. After the second cycle of infection, cells were put under selection with a suitable concentration of puromycin (2 μg mL^{-1}). mRNA and protein expression were evaluated 48 and 72 h after selection. Functional studies were performed as described below.

Inducible vector cloning. STK4 shRNA pLKO.1 clone #4 sequence (see above) was sub–cloned into pLKO–TET–On–puro backbone. Briefly, pLKO.1–TET–On vector was first digested with EcoRI and AgeI restriction enzymes to release a 1.9 kb stuffer. The oligos (see below for sequence) were designed to contain the specific shRNA sequence flanked by sequences that are compatible with the sticky ends of EcoRI and AgeI, resuspended in distilled water, and then incubated for 5 min at 95 ˚C to promote oligo annealing. After ligation the ligation mix was transformed into competent DH5 alpha cells, following manufacturer's protocol. Colonies were screened by restriction enzyme digestion and then positive plasmids were sequenced, to confirm the present of the correct insert.

Oligo sequence	Sequence
STK4 shRNA #4–F	CCGGGCCAAGCGGAATACAGTGATACTCGAGTATCACT GTATTCCGCTTGGCTTTTTG
STK4 shRNA #4–R	AATTCAAAAAGCCAAGCGGAATACAGTGATACTCGAGT ATCACTGTATTCCGCTTGGC

Stable gene knockdown using lentiviral vectors. Lentiviral infection was performed as described above. For pTRIPZ and p–LKO.1–TET–On vectors, shRNA expression was induced adding 2 μg mL^{-1} doxycycline to the culturing media. The efficacy of the induction was confirmed by examining the cells microscopically for the presence of TurboRFP and by western blot analysis after 72 h of induction. Functional studies were performed as described below.

Transient transfection of MM cell lines. p–ENTR–YAP1–EGFP plasmid was kindly provided by Dr. Marius Sudol and subcloned into pLENTI4–V5DEST vector using GATEWAY strategy. p–LENTI4–LACZ vector was used as control. pJ3H–STK4 and pJ3M–STK4 K59R were purchased from Addgene (Cambridge, MA, United States), while dWW-YAP1-EGFP was kindly available in G.B. lab. KMS–18, KMS–20, MM.1S, RPMI–8226, UTMC–2, Jurkat, and OCI/AML3 cells were transiently transfected with p–LENTI4 YAP1, using 'Cell Line Nucleofector Kit V (Amaxa Biosystems, Köln, Germany), according to the manufacturer's instructions. AMAXA program: MM.1S →T–030; RPMI–8226 →C–015; KMS–18, KMS–20, UTMC–2, Jurkat and OCI/AML3 → X–001. Specifically, 3,000,000 cells were resuspended in 100 μL V solution and 2500 ng of plasmid. Following transfection, MM cells were subjected to mRNA analysis, Western blotting, as well as apoptosis, cell counting, and MTT assays.

***In vivo* studies.** Fox Chase Scid mice were purchased from Charles River Laboratories International Inc. (Wilmington, MA, United States) and housed in a BL2 lab at the Harvard Institutes of Medicine. All care of experimental animals were in accordance with Harvard Medical School/Dana–Farber Cancer Institute institutional animal care and use committee (IACUC) guidelines. The MM.1S scrambled, pLKO.1–TET shRNA #4 and pTRIPZ 63 cells were washed with PBS, and resuspended in serum–free medium (20,000,000 cells ml^{-1}) mixed with an equal amount of Matrigel (BD Biosciences, San Diego, CA, United States). Mice were injected with 200 μl/spot cell/matrigel suspension, each mouse with three injections: interscapular (MM.1S scrambled), left (pLKO.1–TET shRNA#4) and right flank (pTRIPZ 63). Induction of viral expression was obtained by addition of doxycycline to drinking water. Tumor volume was monitored starting from 4 weeks after implantation. At that time, more than 50% MM.1S-scrambled tumors reached a volume between 50–100 mm^3. Tumors were monitored twice weekly

and volumes were calculated with the formula: (mm^3) = width x width x length x 0.5. The significance was calculated by a Student t–test, using the GraphPad Prism software.

Viability and cellular growth assays. Viability of MM cells was evaluated by 3–[4,5–dimethylthiazol–2–yl]–2,5–diphenyltetrazolium bromide–MTT (Sigma–Aldrich, St. Louis, MO, Unites States) colorimetric survival assay. MM cells (20,000–50,000) were plated in 100 μL medium. At the various time points (24–96 h), 10 μL 5mg mL^{-1} MTT were added to cells. After 4 h incubation at 37 ˚C, medium was discarded and 100 μL MTT stop solution (Isopropanol with 1 N Hcl) was used to dissolve MTT metabolic products. Absorbance was read at 570 nm and background was subtracted at 630 nm, using the spectrophotometer SPECTRAMAX M2 and Softmax Pro v5 software. Cellular growth was estimated by cell counting in triplicates, excluding dead cells stained by trypan blue.

Apoptosis assays. Dead cells were detected by propidium iodide (PI) staining. Apoptosis was quantified using Annexin V–FITC/PI staining or Annexin V–PE/7–AAD staining on GFP–positive cells (BD Biosciences, San Diego,CA United States). In particular, cells were washed twice with room–temperature PBS, resuspended in 100 μL of Annexin binding buffer, and stained with specific antibodies for 20 min. After adding other 400 μL of Annexin binding buffer, samples were acquired using FACS Canto II machine from Becton Dickinson, BD (Franklin Lakes, NJ, United States) and analyzed with FCS EXPRESS 4 Flow Research Edition software. The percentage of cells undergoing apoptosis was defined as the sum of early apoptotic (Annexin V^+, PI^-) and late apoptotic (Annexin V^+, PI^+) cells.

Statistical analysis. Statistical significance was determined by Student t test. For YAP1 expression analysis, when more than two groups were compared, ANOVA one–way, followed by Tukey's Multiple Comparison test was performed. Kaplan–Meier survival curves for YAP1 were obtained with www.canevolve.org based on the GSE2658 dataset and GSE12417, probe set 224895_at. Individuals with MM were subdivided based on median YAP1 expression levels in tumor cells. To relate STK4 with YAP1 levels, the GSE2658 dataset was used, probe sets 211085_s_at for STK4 and 224895_at for YAP1. Subjects were subdivided in quartiles based on STK4 expression levels, and the higher and lower quartiles selected and compared with Student t–test for STK4 and YAP1 expression, respectively.

Supplementary Information Titles

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Corresponding Authors:	Kenneth C. Anderson, Giovanni Tonon

Supplementary Item & Number	Title or Caption
Supplementary Figure 1	MM cells do not undergo apoptosis despite pervasive ongoing DNA damage
Supplementary Figure 2	ABL1 localization in the nucleus of MM cells, but not of HeLa cells
Supplementary Figure 3	ABL1 nuclear localization is driven by ATM and JNK phosphorylation in MM cells
Supplementary Figure 4	Peripheral blood mononuclear cells (PBMCs) response to induced DNA damage
Supplementary Figure 5	YAP1 is consistently down-regulated in MM cells
Supplementary Figure 6	Effects on cell number and apoptosis after modulation of YAP1 expression in MM cells
Supplementary Figure 7	Role of ABL1 and engagement of p73 in YAP1–mediated apoptosis in MM cells
Supplementary Figure 8	YAP1 and STK4 regulation in MM cells
Supplementary Figure 9	Effects of STK4 silencing in MM.1S and H929 cells
Supplementary Figure 10	YAP1 is required for STK4–mediated growth inhibitory effects in MM cells
Supplementary Figure 11	STK4 kinase activity is required for YAP1 inactivation
Supplementary Figure 12	ABL1 cellular localization and YAP1 expression levels in hematological cancer cell lines
Supplementary Figure 13	Effect on apoptosis following YAP1 re–expression in Jurkat and OCI/AML3 cells

Supplementary Figure 14	YAP1 cellular localization in Jurkat cells, after STK4 silencing
Supplementary Figure 15	Effects of STK4–specific shRNAs on STK3 mRNA levels
Supplementary Methods	