Exploiting MYC-induced PARPness to target genomic instability in multiple myeloma

Daniele Caracciolo,[1] Francesca Scionti,[1] Giada Juli,[1] Emanuela Altomare,[1] Gaetanina Golino,[1] Katia Todoerti,[2,3] Katia Grillone,[1] Caterina Riillo,[1] Mariamena Arbitrio,[4] Michelangelo Iannone,[4] Eugenio Morelli,[5] Nicola Amodio,[1] Maria Teresa Di Martino,[1] Marco Rossi,[1] Antonino Neri,[2,3] Pierosandro Tagliaferri[1] and Pierfrancesco Tassone[1,6]

[1]Department of Experimental and Clinical Medicine, Magna Græcia University, Campus Salvatore Venuta, Catanzaro, Italy; [2]Department of Oncology and Hemato-oncology, University of Milan, Milan, Italy; [3]Hematology, Fondazione Cà Granda IRCCS Policlinico, Milan, Italy; [4]IRIB-CNR, Catanzaro, Italy; [5]Jerome Lipper Multiple Myeloma Center, Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA and [6]Sbarro Institute for Cancer Research and Molecular Medicine, Center for Biotechnology, College of Science and Technology, Temple University, Philadelphia, PA, USA

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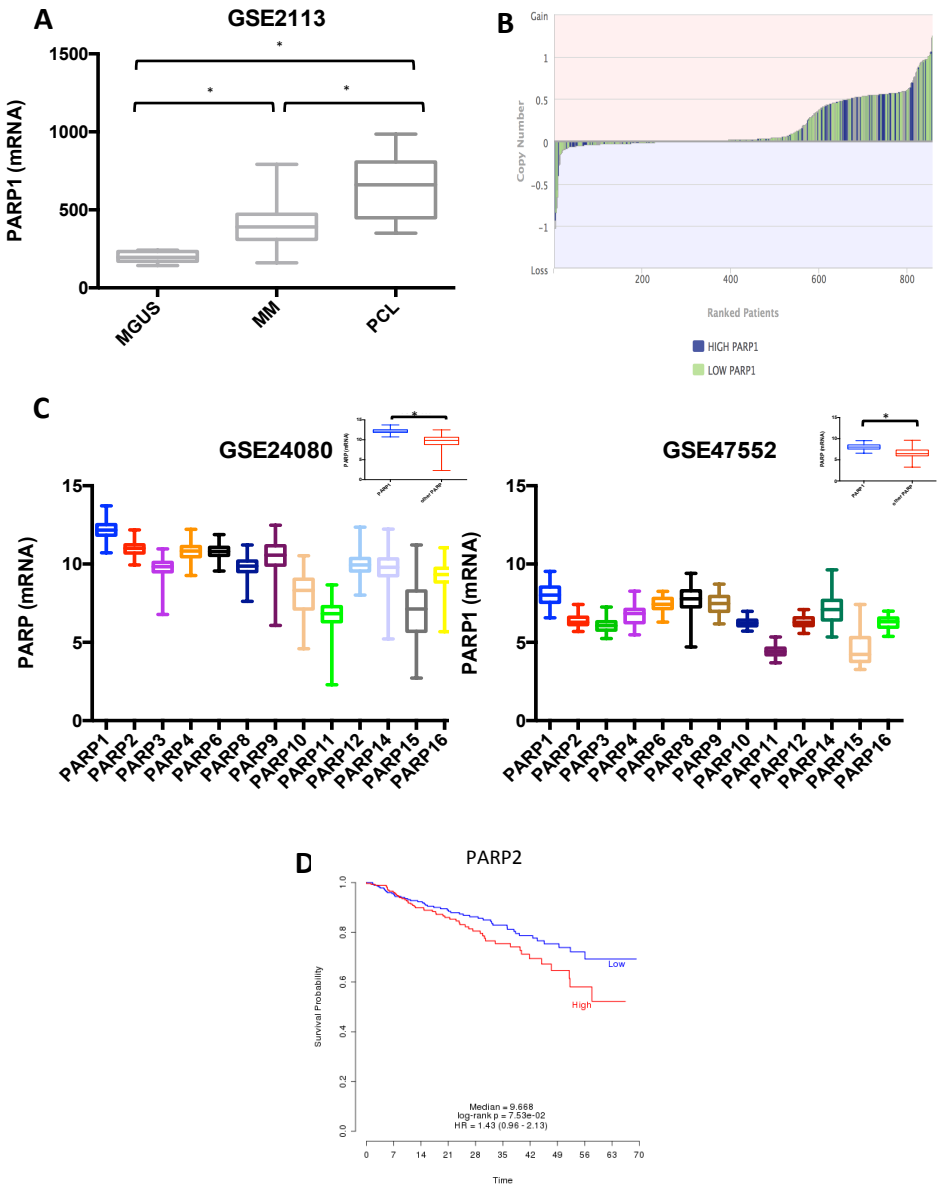
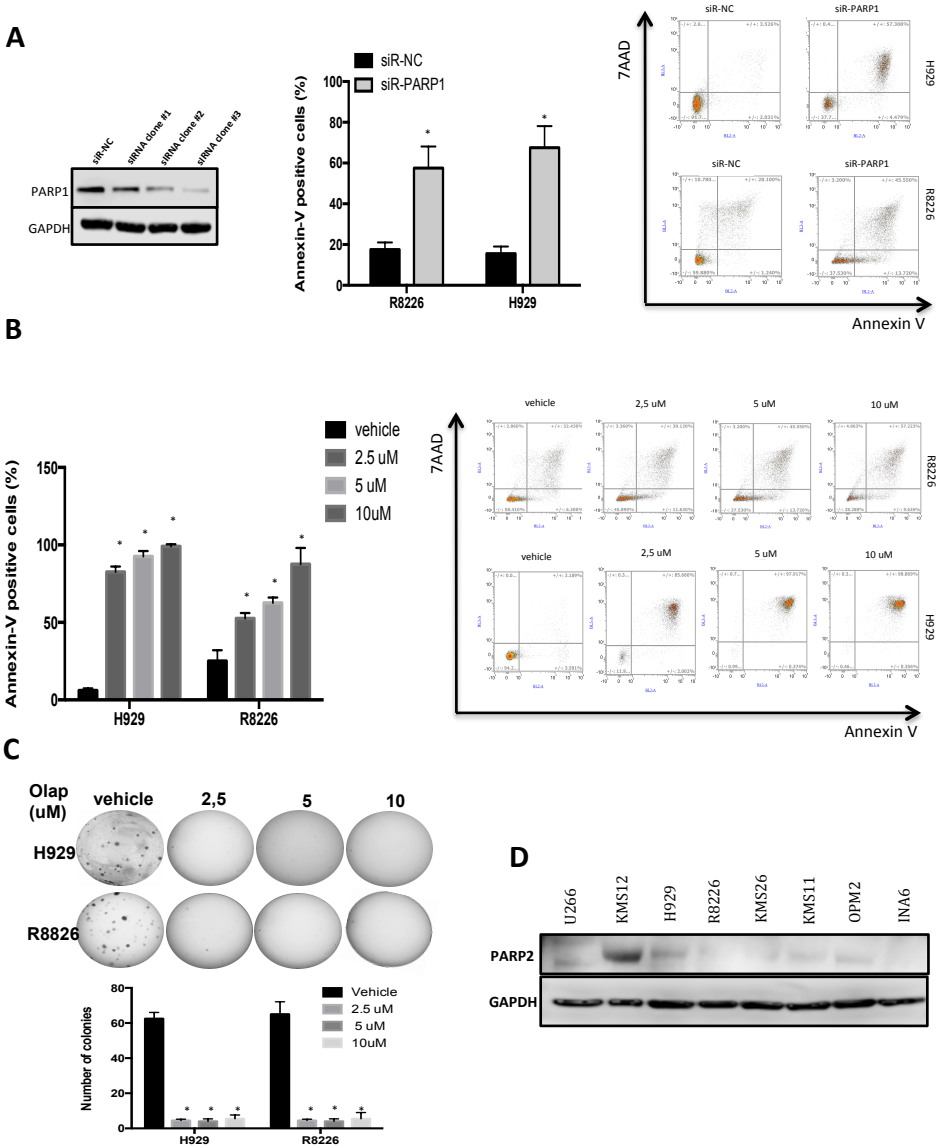
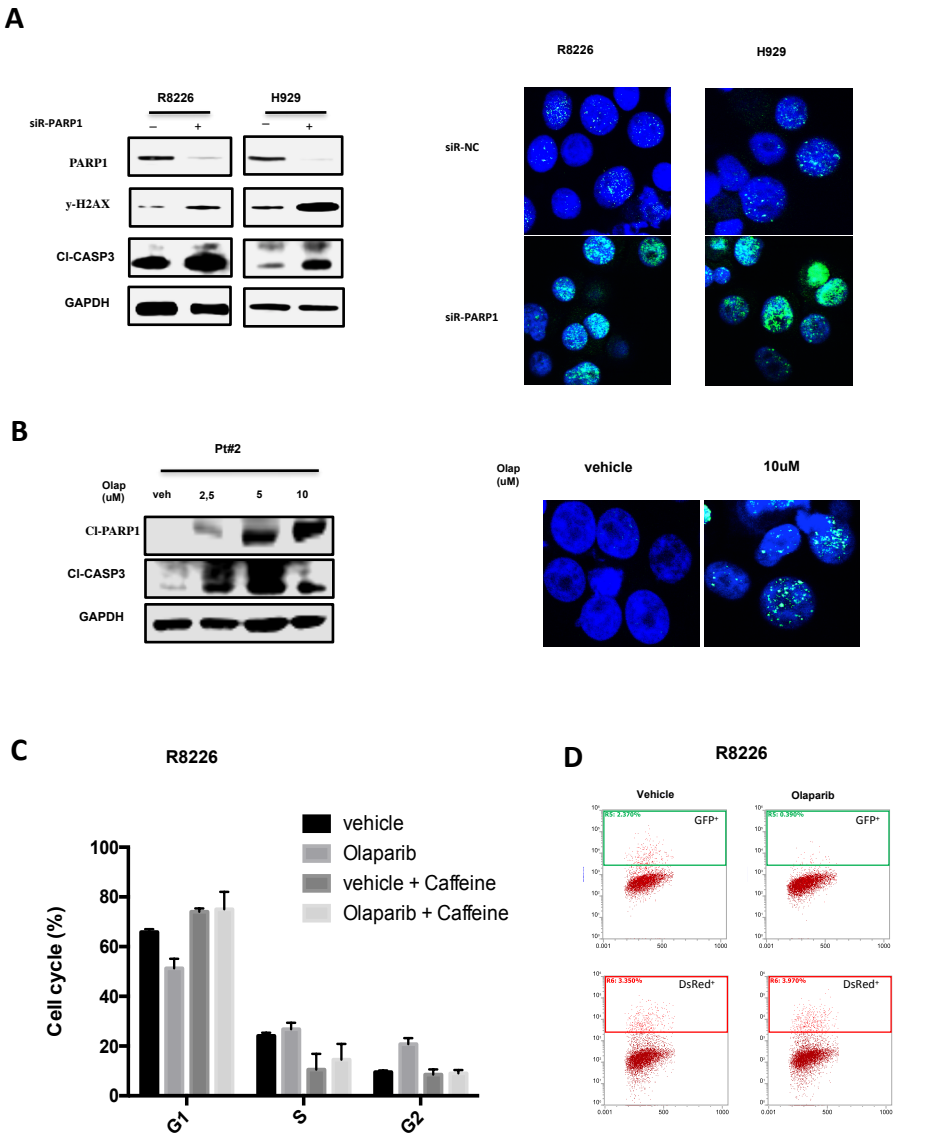
Correspondence: *PIERFRANCESCO TASSONE* - tassone@unicz.it



Supplementary Fig.1: (A) Analysis of GSE2113 dataset. PARP1 mRNA levels in MGUS, MM and PCL patients. *, P<0.001 **(B)** Copy number variation analysis of PARP1 gene in MM patients from MMRF researcher gateway portal (https://research.themmrf.org).
(C) Data obtained from GSE24080 and GSE47552 datasets showing PARP family members mRNA expression. **(D)** Data obtained interrogating GSE24080 dataset. Prognostic relevance of PARP2 on Overall Survival (OS)



Supplementary Fig.2: (A) *Left:* H929 cells were transfected with scramble control or 3 different PARP1-siRNAs. Immunoblot for PARP1 was performed 48h after transfection. *Right*: Annexin V-positive H929 and R8226 cells 48h transfected with siRNA-NC or PARP1-sIRNA clone #3. **(B)** Indicated MM cells were treated with increasing dose of Olaparib. Annexin V-positive cells 4 days from treatment are shown. **(C)** Colony formation of MM cells treated with vehicle or increasing dose of Olaparib. Light microscopy is shown. Numbers of colonies after 2 weeks (average of 3 independent experiments; *P<0.01) **(D)** Immunoblot analysis of PARP2 levels in MM cell lines



Supplementary Fig.3. (A) *Left*: Immunoblot analysis of PARP1, Cl-Caspase 3 and y-H2AX. GAPDH was used as a loading control. Analysis was performed 48h after transfection. *Right:* γ-H2AX foci evaluation by immunofluorescence 48h after cell transfection. DAPI (blue) was used for nuclear staining. **(B)** Primary MM cells (Pt#2) were treated with increasing dose of Olaparib. *Left*: Immunoblot analysis of PARP1 and Cl-Caspase 3. GAPDH was used as a loading control. Analysis was performed 24h after treatment. *Right:* γ-H2AX foci evaluation by immunofluorescence 24h after treatment. DAPI (blue) was used for nuclear staining. **(C)** R8226 cells were treated with Olaparib in presence or absence of Caffeine (4mM). Cell cycle analysis was performed 24h after treatment. **(D)** FACS traces of Alt-NHEJ repair assay performed on R8226 cells 48h after Olaparib (2,5 uM) treatment. Data are representative of at least three independent experiments

A

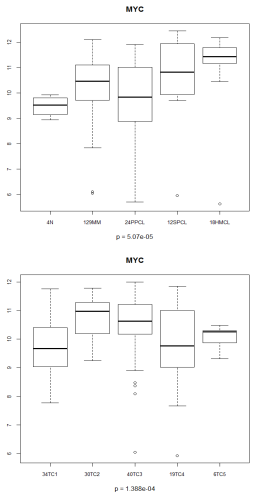
MM TC2

NAME	SIZE	NES	NOM p-val	FDR q-val
HALLMARK_MYC_TARGETS_V1	193	1,7856873	0,014373717	0,03468112
HALLMARK_DNA_REPAIR	142	1,6550231	0,017716536	0,058839813
HALLMARK_PROTEIN_SECRETION	95	1,6840036	0,01446281	0,06626201
HALLMARK_E2F_TARGETS	197	1,6056912	0,013861386	0,07338895
HALLMARK_MYC_TARGETS_V2	58	1,5217887	0,0945674	0,09294832
HALLMARK_G2M_CHECKPOINT	198	1,5295784	0,05162524	0,10125281
HALLMARK_OXIDATIVE_PHOSPHORYLATION	199	1,5424293	0,077731095	0,107795745
HALLMARK_UNFOLDED_PROTEIN_RESPONSE	110	1,4343021	0,08151093	0,15497208
HALLMARK_GLYCOLYSIS	197	1,4071164	0,025440313	0,16444612
HALLMARK_FATTY_ACID_METABOLISM	156	1,3859935	0,0977131	0,1699437
HALLMARK_MITOTIC_SPINDLE	198	1,3431541	0,1375969	0,2012267
HALLMARK_KRAS_SIGNALING_DN	191	-1,5732522	0,00203666	0,24661924

PCL

NAME	SIZE	NES	NOM p-val	FDR q-val
HALLMARK_PROTEIN_SECRETION	95	1,7689918	0,006012024	0,01914201
HALLMARK_MYC_TARGETS_V1	193	1,667538	0,023904383	0,05121669
HALLMARK_MTORC1_SIGNALING	195	1,556562	0,031746034	0,08666427
HALLMARK_OXIDATIVE_PHOSPHORYLATION	199	1,5660286	0,07456979	0,10556252
HALLMARK_MYC_TARGETS_V2	58	1,3731388	0,17254902	0,12093651
HALLMARK_MITOTIC_SPINDLE	198	1,4240882	0,04752066	0,12270054
HALLMARK_REACTIVE_OXIGEN_SPECIES_PATHWAY	47	1,377877	0,08262712	0,1270538
HALLMARK_DNA_REPAIR	142	1,4478067	0,09001957	0,13369577
HALLMARK_E2F_TARGETS	197	1,427879	0,09306931	0,13458158
HALLMARK_G2M_CHECKPOINT	198	1,3809469	0,14395393	0,13568851
HALLMARK_PEROXISOME	102	1,487821	0,010204081	0,13685687
HALLMARK_FATTY_ACID_METABOLISM	156	1,4614549	0,057026476	0,14129122
HALLMARK_ADIPOGENESIS	**195**	**1,3854795**	**0,06378601**	**0,14467503**
HALLMARK_GLYCOLYSIS	197	1,2754492	0,1663286	0,20073012
HALLMARK_KRAS_SIGNALING_DN	191	-1,6577327	0	0,13666743

B



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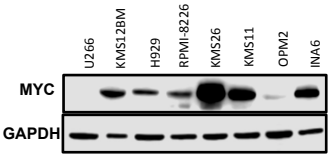

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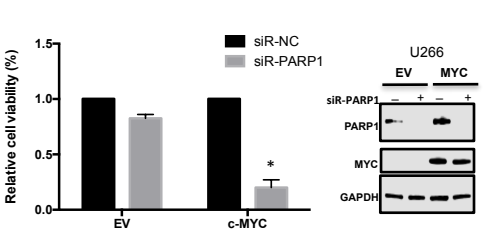

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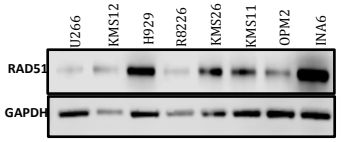
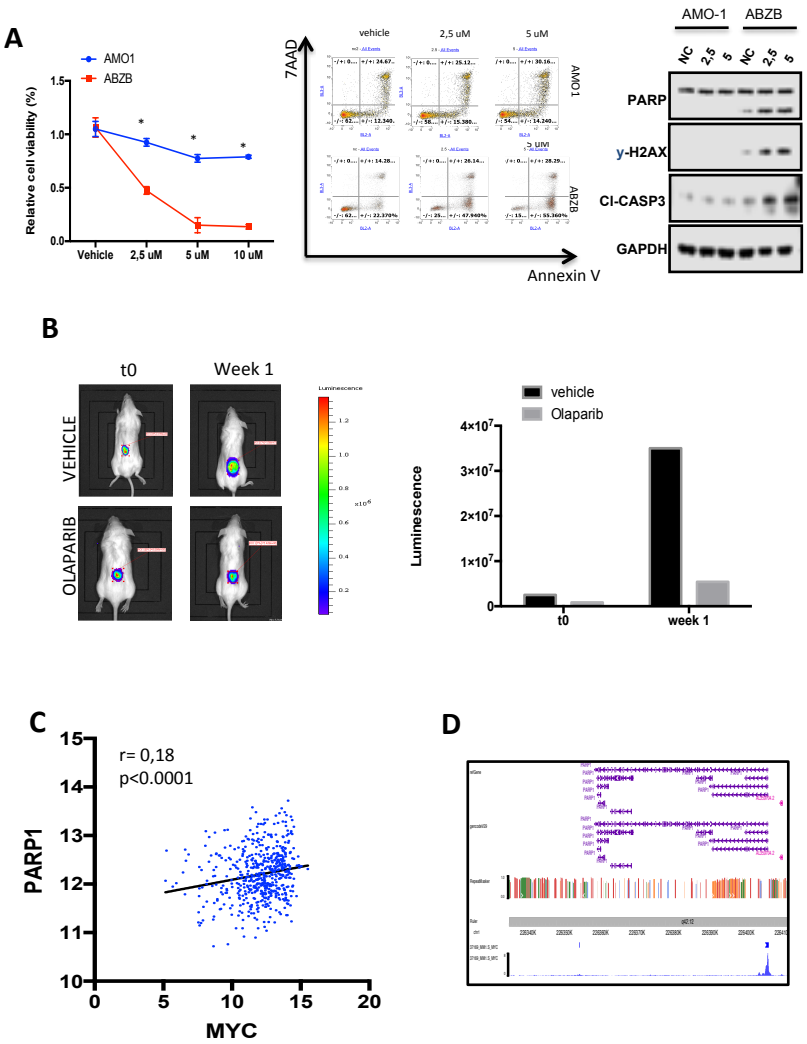


	1q WT	1q gain	TOT	Two-tailed P value equals 1.0000
PARPI-NEG	9	10	19	
PARPI-POS	4	6	10	
TOT	13	16	29	
	13q WT	del(13q)	TOT	The two-tailed P value equals 1.0000
PARPI-NEG	11	3	14	
PARPI-POS	13	3	16	
TOT	24	6	30	
	NHD	HD	TOT	The two-tailed P value equals 1.0000
PARPI-NEG	3	10	13	
PARPI-POS	4	11	14	
TOT	7	21	27	
	DIS3 WT	DIS3 mut	TOT	The two-tailed P value equals 0.2262
PARPI-NEG	13	0	13	
PARPI-POS	12	3	15	
TOT	25	3	28	
	BRAF WT	BRAF mut	TOT	The two-tailed P value equals 0.5956
PARPI-NEG	11	2	13	
PARPI-POS	13	2	15	
TOT	24	4	28	
	NRAS WT	NRAS mut	TOT	The two-tailed P value equals 1.0000
PARPI-NEG	8	5	13	
PARPI-POS	9	6	15	
TOT	17	11	28	
	KRAS WT	KRAS mut	TOT	The two-tailed P value equals 1.0000
PARPI-NEG	8	5	13	
PARPI-POS	9	6	15	
TOT	17	11	28	

Supplementary Fig.4: (A) List of significant gene sets (nominal p-value <0.05) from GSEA analysis, using Hallmark collection in MM-TC2 and PCL PARPi-positive versus PARPi-negative comparison. Normalized Enrichment Score (NES), Nominal p-value and FDR q-value are reported for each significant gene set. **(B)** Box plots of *MYC* gene expression profiles (log scale) in PC dyscrasias' dataset and e) in 129 MM samples stratified according to five TC classes. P-value by Kruskal-Wallis test is reported. **(C)** Immunoblot analysis of MYC protein levels in MM cell lines. GAPDH, used as a loading control, is the same shown in Fig-2A. **(D)** U266 EV or MYC$^+$ were transfected with scramble control or PARP1-siRNA. *Right panel*: Immunoblot analysis of PARP1. GAPDH was used as a loading control. Analysis was performed 48h after transfection. *Left panel*: CTG assay was performed on 4 days after transfection. **(E)** *Left panel:* Immunoblot analysis of RAD51 levels in MM cell lines. *Right panel:* Fisher's exact test on TC2-MM cases stratified according to main molecular alterations evaluated by FISH (cytogenetic abnormalities) and NGS (somatic mutations)



Supplementary Fig.5 (A) *Left panel*: Cell viability of AMO1 and ABZB, treated with vehicle or Olaparib for 7 days. *Middle panel*: Annexin V positive AMO1 and ABZB cells, treated with vehicle or Olaparib for 4 days. . *Right panel:* Immunoblot of PARP1, Cl-Caspase 3 and y-H2AX. GAPDH was used as a loading control. Analysis was performed 24h after treatment. **(B)** Representative IVIS images and BLI-based measurement of tumor volumes (5 animals for each group) of NOD SCID mice s.c. xenografted with luciferase gene-marked ABZB, were performed before (t0) and 1 week after treatment. **(C)** Graphs of correlations between endogenous mRNA expression levels of PARP1 and MYC in MM patients from GSE24080 dataset. **(D)** Graphical results of bio-informatic screening (cistrome.org) showing c-MYC binding consensus sequences to PARP1 promoter.
Data are representative of at least three independent experiments. *P<0.05.

Supplementary Material and Methods

Multiple myeloma cell lines, primary cells, and reagents

Peripheral blood mononuclear cells (PBMCs) and primary cells from multiple myeloma patient bone marrow aspirates, following informed consent and University Magna Graecia (Catanzaro, Italy) IRB approval, were isolated using Ficoll–Hypaque density gradient sedimentation as reported previously (1). Multiple myeloma patients' cells were separated from bone marrow samples by antibody-mediated selection using anti-CD138 magnetic-activated cell separation microbeads (Miltenyi Biotec). Purity of immunoselected cells was assessed by flow-cytometry analysis using a phycoerythrin-conjugated CD138 monoclonal antibody by standard procedures. CD138+ cells from MM patients pt#1, pt#2 and pt#3 were cultured in RPMI-1640 medium (Gibco®, Life Technologies) supplemented with 20% fetal bovine serum (Lonza Group Ltd.) and 1% penicillin/streptomycin (Gibco®, Life Technologies). Multiple myeloma cell lines (HMCLs) AMO1, NCI-H929, U266, KMS12-BM were purchased from DSMZ (Braunschweig, Germany). CAPAN1, RPMI-8226 and OPM2 were purchased from ATCC (Manassas, VA, USA). KMS-11 were kindly provided by Dr. K.C. Anderson (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA). KMS-26 were kindly provided by Dr. Giovanni Tonon (University of San Raffaele Scientific Institute, Milan, Italy). P493-6 were kindly provided by Dr.Dirk Eick (Max Planck Institute of Biochemistry, Helmholtz-Zentrum München, Germany and cultured in RPMI-1640 medium (Gibco® Life Technologies) supplemented with 10% fetal bovine serum (Lonza Group Ltd.) and 1% penicillin/streptomycin (Gibco®, Life Technologies). AMO1 bortezomib-resistant (ABZB) were kindly provided by Dr. Christoph Driessen (Eberhand Karls University, Tübingen, Germany). Multiple myeloma cell lines were cultured in RPMI1640 (Gibco, Life Technologies) supplemented with 10% FBS (Lonza Group). INA-6 cell line (kindly provided by Dr. Renate Burger, University of Erlangen- Nuernberg, Germany) was cultured in the presence of recombinant human IL6 (2,5 ng/mL, R&D Systems, Minneapolis, MN); this cell line was not further authenticated but confirmed for the described IL6 dependence. HS-5 human stromal cell line (purchased from ATCC, CRL-11882TM) was cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin . Co-culture experiments were performed in 6 well plate at a density of 2,5 × 10 5 cells/ ml in 1:1 HS-5 /MM cells ratio. All these cell lines were immediately frozen and used from the original stock within 6 months.

Transduction of cells

To generate cells stably expressing luciferase transgene, ABZB cells were transduced with pLenti-III-PGK-Luc (ABM Inc., Richmond, BC, Canada) vector, the manufacturer's instructions. To generate cells stably expressing c-MYC, U266 were transduced with Precision LentiORF human MYC (GE Dharmacon, Lafayette, Colorado, USA).

Gene expression profiling

PARPi signature was evaluated in a PC dyscrasias dataset, including samples from 129 MM, 24 primary PCL (PPCL) at onset and 12 secondary PCL (SPCL) cases, together with 18 HMCL and 4 healthy donors (N) samples, that were profiled on Affymetrix Gene 1.0 ST array (GSE66293)(2). MM samples were stratified according to TC classification (3) in 34 TC1, 30 TC2, 40 TC3, 19 TC4 and 6 TC5, respectively.

Global gene expression profiles were extracted for almost the entire PARPi gene list (27/28, 96% up-regulated and 111/119, 93% down-regulated genes). Hierarchical agglomerative clustering (Pearson's correlation and average linkage methods) was applied to identify sub-groups of samples with similar gene expression patterns.

A Gene Set Enrichment Analysis (GSEA) (4) was applied on global gene expression profiles of 16 MM-TC2 samples showing PARPi-positive versus 14 MM-TC2 cases carrying PARPi-negative expression patterns, grouped on the base of clustering analysis. Similarly, 13 PARPi-positive versus 23 PARPi-negative PCL samples were compared by GSEA analysis. Phenotype permutations and default analysis's conditions were applied on Hallmark gene set collection and significant gene sets were selected based on FDR q-value < 25%.

Differential expression analysis of MYC gene expression levels across PC dyscrasias and MM TC classes was performed by means of Kruskal-Wallis test, by using *stat* package in R software. Dunn's test was used for multiple pairwise comparisons and the Benjamini and Hochberg correction was applied to adjust significance of multiple tests (adjusted $p < 0.05$).

In vitro transfection of MM cells

Stealth™ PARP1 siRNA (clone IDs: HSS100243, HSS100244, HSS100245) and Silencer TM select MYC (clone IDs: s9129) were purchased from Invitrogen™ (Thermo Scientific). All the oligos were used at 100 nmol/L final concentration. MM cells were transfected using Neon Transfection System (Invitrogen™) (2 pulse at 1.050 V, 30 milliseconds). The same conditions were applied for transfection of MM cells with 2,5 μg of expression vectors carrying the ORFs of PARP1 NM_001618.3 (EX-Z8307-M68), with empty vector (EV) used as control (EX-NEG-M68) (GeneCopeia, Rockville, MD, USA).

DSB repair assay

In vivo DSB repair assays were performed as previously described (5, 6). Briefly, EJ2-GFP plasmid (#44025, Addgene) was linearized with I-SceI (Thermo Scientific) digestion and transfected into $1x10^6$ cells at a ratio of 1ug per well. In parallel, cells were transfected using with 0.1 μg of DsRed-N1 plasmid (kindly provided by Dr.Michele Cea, Dana-Farber Cancer Institute, Boston, MA) as the internal control. 48h after Olaparib treatment, the numbers of GFP+ and DsRed+ cells were determined by flow cytometry (Attune NxT, Thermo Fisher Scientific). For each experiment, FACS analyzed a minimum of 20,000 cells. The ratio between GFP+ and DsRed+ cells was used as a measure Alt-NHEJ repair efficiency.

ChIP

Cells (1.5 x107) were crosslinked in 1% formaldehyde, lysed and sheared by sonication for

10 cycles (each of 30 seconds) on a cold block with 90 seconds time intervals of cooling using the Bioruptor Plus (Diagenode). Chromatin was divided into equal amounts of immunoprecipitation with the MYC antibody (ab56), or rabbit IgG as negative control (Santa Cruz Biotechnology). Chromatin extracts were incubated on a rotator with 20 ml of ChIP Grade Protein A/G Plus Agarose for 3 h at 4°C. Bound agarose beads were harvested by centrifugation (12.000 rpm, 15 seconds) and washed; the precipitated protein-DNA complexes were eluted from washed beads and incubated twice at 65°C for 1.5 h with NaCl and Proteinase K to revert cross-links.

Purified DNA was subjected to qPCR using GoTaq qPCR Master Mix (Promega). Primer sequences for qPCR were:

PARP1(forward) 5′-GGTCTCAAACTCCTGCTACAA-3′

PARP1(reverse) 5'-AGGACACACTTAAGAGTTTGGG-3'

Ch22(forward) 5'- GGATGACAGGCATGAGGAATTA-3'

Ch22(reverse) 5'- TGCTGCTTACTTGGGATATGAG-3'

Promoter activity assay

ABZB cells were co-transfected with siRNA control or MYC–targeting siRNA and negative control (NEG-PG04) or PARP1 (HPRM43771-PG04) promoter constructs (GeneCopeia, Rockville, MD, USA). Measurement of promoter activity was performed with Secrete-Pair ™ Dual Luminescence Assay Kit (GeneCopeia, Rockville, MD, USA) according to manufacturers's instructions

SNP-array data analysis

DNA was extracted from the AMO1 and ABZB cells using the Perfect Pure DNA Blood kit (5 Prime) and analyzed using the Affymetrix Cytoscan HD array (Affymetrix, Inc., Santa Clara, CA) according to manufacturers's instructions, to estimate genomic instability and ongoing DNA rearrangements. This array consists of 2.67 million markers for copy number variation (CNV) analysis, including 750,000 SNP and 1.9 million non- polymorphic probes, with an average spacing for RefSeq genes of 880 bp. Analysis of intensity data (CEL file) was performed with Chromosome Analysis Suite v 3.1 (ChAS 3.1) software using the Affymetrix HapMap Reference Model File for comparison. We used 25 probes and > 25 kb and >50 kb as a minimum cutoff for deletions and gains respectively. Map position was based on GRCh37/hg19 assembly.

Histology and immunohistochemistry

Retrieved tumors from animals were fixed in 4% buffered formaldehyde and 24 hours later washed, dehydrated, and embedded in paraffin. For light microscopy analysis by an optical microscope Nikon i55 (Nikon Corporation, Tokyo, Japan), we performed staining with H&E on 4-mm tumor sections mounted on poly-lysine slides. For IHC staining, 2-mm–thick tumor slices were de-paraffinized and pretreated with the Epitope Retrieval Solution 2 (EDTA buffer, pH 8.8) at 98 C for 20 minutes. After washing steps, peroxidase blocking was carried out for 10 minutes using the Bond Polymer. All procedures were performed using the Bond Max Automated Immunohistochemistry. Tissues were washed and incubated with the primary antibody directed against Ki-67 (Dako, clone MIB-1; 1:150). Subsequently, tissues were incubated with

polymer for 10 minutes and developed with DAB–Chromogen for 10 minutes. Slides were counterstained with Hematoxylin.

List of Antibodies

Antibodies	**Sources**	**Catalog #**	**Applications**
Cleaved-Caspase 3 (Asp175)	Cell Signaling Technology	9661	WB (1:1000)
GAPDH	Santa Cruz	25778	WB (1:1000)
PARP	Cell Signaling Technology	9532	WB (1:1000)
Phospho-CHK1 (Ser345)	Cell Signaling Technology	2348	WB (1:1000)
phospho-CHK2 (Thr 68)	Cell Signaling Technology	2197	WB (1:1000)
phospho-ATM (Ser 1981)	Cell Signaling Technology	5883	WB (1:1000)
phospho-ATR (Ser 428)	Cell Signaling Technology	2853	WB (1:1000)
phospho-Histone H2A.X (Ser139)	Cell Signaling Technology	9718	WB (1:1000) IF (1:200) IHC (1:480)

c-Myc	Cell Signaling Technology	5605	WB (1:1000)
PARP2	Santa Cruz	393343	WB (1:1000)
RAD51	Cell Signaling Technology	8875	WB (1:1000)
Goat anti-rabbit IgG-HRP	Santa Cruz	2054	WB (1:3000)
Goat anti-mouse IgG-HRP	Santa Cruz	2055	WB (1:3000)

WB, western blot. ChIP, chromatin immunoprecipitaion. IF, immunofluorescence. IHC,

immunohistochemistry

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