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Supplementary Information for

Ancient Drug Curcumin Impedes 26S Proteasome Activity by Direct Inhibition of Dual-Specificity Tyrosine-Regulated Kinase 2.

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Supplementary Information Text

SI methods

General methods, antibodies and reagents

All recombinant DNA procedures, electrophoresis, immunoblotting, affinity-purification and tissue culture were performed using standard protocols. Antibodies used in this study were: RPT3 (clone TBP7-27, BML- PW-8765), 20S α subunits (clone MCP231, BML-PW8195) from Enzo Life Sciences; anti-Flag M2 (F3165) from Sigma; DYRK2 (#8143), p21 (#2947), IκBα (#9242) from Cell Signaling; GFP (for YFP blot) (clone JL-8, 632381) from BD Clontech; and GAPDH (clone 6C5, CB1001, 1:5,000) from Millipore. All antibodies were diluted 1:1000 for western blot unless otherwise noted. Rabbit anti-pT25 RPT3 polyclonal antibody was generated in-house as described previously (37). Cycloheximide (CHX), dimethyl sulfoxide (DMSO) and curcumin were purchased from Sigma. Bortezomib and carfilzomib were from Selleck Chemicals. Bortezomib and carfilzomib were diluted/dissolved in DMSO. Calyculin A was from Cell Signaling.

Curcumin preparation and treatment

For all cell based and *in vitro* assays, curcumin was diluted in DMSO at a stock concentration of 10 mM in the dark and prepared fresh prior to each experiment and the excess solution was never stored. Thiol scavengers like DTT or β-mercaptoethanol was added to all reaction/lysis buffers. Curcumin treatment was always carried out in media containing 10% FBS unless otherwise mentioned to maintain maximum stability of curcumin (41) and final concentration was maintained between 0.1-10 μM to avoid aggregation. Treatment time varied from 0.5-24 hr in the dark depending on experiment. For xenograft mouse experiments, curcumin was mixed with 1% carboxymethycellulose (Millipore) in 0.1 M citrate buffer pH 6 and 300 mg/Kg body weight was administered via intraperitoneal injection every alternate day. Curcumin solution was prepared fresh every time in the dark.

IC_{50} determination

IC_{50} determination was carried out at The International Centre for Protein Kinase Profiling (http://www.kinase-screen.mrc.ac.uk/). Active DYRK1A, DYRK2, DYRK3, IKKβ and GSK3β were purified as reported previously (42). Curcumin IC_{50} measurements were carried out against the kinases with final concentrations between 0.1 nM to 10 μM *in vitro* (curcumin was added to the kinase reaction prior to ATP master mix). The values were expressed as a percentage of the DMSO control. DYRK isoforms (5-20 mU diluted in 50 mM Tris pH7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol) are assayed against Woodtide (KKISGRLSPIMTEQ) in a final volume of 25.5μl containing 50 mM Tris pH 7.5, 0.1 mM EGTA, 300 μM substrate peptide, 10 mM Magnesium acetate and 0.025-2 mM [^{33}P-γATP] (0.05mM for DYRK1A and 0.005mM for DYRK3) (50-1000 cpm/pmole) and incubated for 30 min at room temperature. GSK3β (5–20 mU diluted in 20 mM MOPS pH 7.5, 1 mM EDTA, 0.01% Brij35, 5% glycerol, 0.1% β-mercaptoethanol, 1 mg/ml BSA) is assayed against Phospho-GS2 peptide (YRRAAVPPSPSLSRHSSPHQS(PO4)EDEEE) in a final volume of 25.5 μl containing 8 mM MOPS pH 7.0, 0.2 mM EDTA, 20 μM Phospho GS2 peptide, 10 mM magnesium

acetate and 0.005 mM [^{33}P-γATP] (50-1000 cpm/pmole) and incubated for 30 min at room temperature. IKKβ (5-20 mU diluted in 50mM Tris (pH 7.5), 0.1mM EGTA, 1mg/ml BSA, 0.1%, β-Mercaptoethanol) is assayed against substrate peptide (LDDRHDSGLDSMKDEEY) in a final volume of 25.5µl containing 50mM Tris (pH 7.5), 0.1mM EGTA, 0.1%, β-Mercaptoethanol, 300µM substrate peptide, 10 mM magnesium acetate and 0.005 mM [^{33}P-γATP] (500-1000 cpm/pmole) and incubated for 30 mins at room temperature. Assays are stopped by addition of 5 µl of 0.5 M (3%) orthophosphoric acid and then harvested onto P81 Unifilter plates with a wash buffer of 50 mM orthophosphoric acid. IC_{50} curves were developed and IC_{50} values were calculated using GraphPad Prism software.

Protein kinase profiling

Kinase inhibitor specificity profiling assays were carried out at The International Centre for Protein Kinase Profiling (http://www.kinase-screen.mrc.ac.uk/). Curcumin kinase specificity was determined against a panel of 140 protein kinases as described previously (42, 43). Results are presented as a percentage of kinase activity in DMSO control reactions. Protein kinases were assayed *in vitro* with 1 µM final concentration of curcumin and the results are presented as an average of triplicate reactions ± SD or in the form of comparative histograms.

Cell lines

All cell lines were originally obtained from American Type Culture Collection (ATCC) unless otherwise stated. HEK293T, MDA-MB-231, Hs578T, MDA-MB-468, HaCaT were maintained in DMEM with 10% fetal bovine serum (FBS) and Penicillin/Streptomycin (Life Technologies). HCC1187, HCC1937, MM.1R, MM.1S, MM.1S.BR, RPMI8226, RPMI8226.BR, NCI-H929, U266, 5TGM1-GFP (kindly provided by Dr. Babatunde Oyajobi, UT San Antonio, TX, USA) and ANBL6 (kindly provided by Dr. Robert Orlowski, UT MD Anderson Cancer Center, Houston, TX, USA) were maintained in RPMI-1640 with 10% fetal bovine serum (FBS) and Penicillin/Streptomycin. MCF10A cells were cultured in DMEM/F-12 medium supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin and Penicillin/Streptomycin (Life Technologies). AHH1 cells were cultured in RPMI-1640 with 10% horse serum, 10 mM HEPES, and 1.0 mM sodium pyruvate (Life Technologies).

Cell transfection

Transient transfection of HEK293T and MDA-MB-231 cells was carried out using Lipofectamine 2000 (Life Technologies) as recommended by the manufacturer. Stable overexpression of DYRK2 and Rpn11-TBHA were carried out by Retroviral transduction followed by puromycin selection as reported previously (37).

Cell lysis

Post-treatment and/or transfection, cells were lysed in lysis buffer containing 50 mM Tris/HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 50 mM NaF, 10 mM sodium 2-glycerophosphate, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.27 M sucrose, 1 mM benzamidine (added before lysis), 1 mM PMSF (added before

lysis) and 0.1% β-mercaptoethanol (added before lysis). For proteasome purification or activity assays, cells were lysed in 50 mM Tris/HCl (pH 7.5), 0.5% Nonidet P-40, 1 mM ATP, 10 mM $MgCl_2$, 1 mM DTT and a phosphatase inhibitor cocktail (10 mM NaF, 20 mM β-glycerophosphate and 50 nM Calyculin A).

Genome editing

Crispr/Cas9 mediated genome editing of DYRK2 loci in MDA-MB-231 cells was carried out using guide RNA: CCTGGATCTGTCCGTGAGCG as described previously (37). DYRK2 KO MDA-MB-231 clones were ascertained by DNA sequencing. HaCaT cells with DYRK2 KO were generated as reported previously (37).

Protein expression and purification

$DYRK2^{74-479}$ with an N-terminal 6xHis tag was expressed in *E. coli* Rosetta (DE3). For protein expression, cultures were grown at 37 °C in LB medium to an OD_{600} of 0.6-0.8 before induced with 0.5 mM IPTG overnight at 18 °C. Cells were collected by centrifugation and frozen at −80 °C.

The cells were suspended in the lysis buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 10 mM imidazole, 5 mM β-mercaptoethanol, 1 mM PMSF) and disrupted by sonication. The insoluble debris was removed by centrifugation. The supernatant was applied to a Ni-NTA column (GE Healthcare). The column was washed extensively with the wash buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 50 mM imidazole and 5 mM β-mercaptoethanol) and eluted using the elution buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 250 mM imidazole, 5 mM β-mercaptoethanol). $DYRK2^{74-479}$ was then further purified by gel filtration chromatography using a Superdex 200 column (GE Healthcare), and eluted using the final buffer (25 mM HEPES, pH 7.5, 500 mM NaCl, 5 mM DTT). Purified $DYRK^{74-479}$ was concentrated to 8 mg ml^{-1} and flash-frozen with liquid nitrogen.

Crystallization, structure determination and alignment studies

$DYRK2^{74-479}$ was incubated with 100 μM curcumin on ice before crystallization. The DYRK2-curcumin crystals were grown at 18 °C by the sitting-drop vapor diffusion method, using a reservoir solution containing 12%-20% PEG 3350 and 0.2 M ammonium citrate tribasic, pH 7.0. Crystals grew to full size in several days and were transferred to 20% PEG 3350, 0.2 M ammonium citrate tribasic, pH 7.0, and 15% ethylene glycol before frozen in liquid nitrogen.

The diffraction data were collected at Shanghai Synchrotron Radiation Facility (SSRF) beamline BL17U. The diffraction data were indexed, integrated, and scaled using HKL2000 (HKL Research). The structure was determined by molecular replacement using the published DYRK2 structure (PDB ID: 3K2L) (44) as the search model using the Phaser program (45). Curcumin was placed using LigandFit program in Phenix (46). The structural model was then adjusted in Coot (47) and refined using Phenix. The quality of the structural model was checked using the MolProbity program in Phenix.

Structural alignments were performed in PyMOL (Schrödinger) using the *align* command. Sequence alignments were performed in Clustal Omega (48) and manually adjusted for clarity of interpretation in the final figure.

Proteasome activity assays

Proteasome activity assays were analysed either directly on cell lysates or on human 26S proteasome affinity-purified from MDA-MB-231 cells stably expressing Rpn11-TBHA as described previously (37). Cells were treated with or without indicated concentration of curcumin and/or carfilzomib prior to lysis. Peptidase activities of purified proteasome or proteasomes in whole cell or tumor lysates were assayed using fluorogenic peptide substrates (Enzo Life Sciences) (49) with phosphatase inhibitors present in the lysis and assay buffers. The measured activity was normalized either against total protein concentration of cell lysates or against western blot of proteasome subunits. Fluorescence signal in whole cells or cell lysates were quantified using a Tecan Infinite® M200 Pro multi-well plate reader. For casein degradation, 1 μg of self-quenching bodipy-casein (EnzChek® Protease Assay Kit, green fluorescence, Life Technology) and 1 μg of purified, 26S proteasome were mixed in the 26S assay buffer with 5 mM ATP. Reaction was carried out for 40 mins at 37°C. Fluorescence was measured in a Tecan Infinite® M200 Pro multi-well plate reader.

Cell proliferation, viability and invasion assays

Cell proliferation was carried out by cell counting as reported previously (37) in the presence or absence of 1 (for 5TGM1-GFP cells) or 3 μM (for MDA-MB-231 cells) curcumin. Media containing curcumin or DMSO control was replaced every day for 5 days to avoid excess degradation of curcumin in cell culture media. Cell viability assays were carried out with or without 24 hr treatment of carfilzomib or curcumin or both using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit. Synergistic effects of drugs on cytotoxicity were analysed using combination index (CI) by CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). CI<1 is considered synergism; CI=1 is considered additive while CI>1 is accepted to be antagonism. To analyse whether cytotoxicity induced by curcumin was apoptotic, we analysed Caspase3/7 activity in cell lysate using the CellEvent™ Caspase-3/7 Green Detection Reagent (Life Technologies) using manufacturer's instructions. The Matrigel invasion assay was performed using 8 μm pore size transwells coated with Matrigel (BD Biosciences) as described previously (43). The bottom chamber contained normal growth media (DMEM with 10% FBS with or without 5 μM curcumin) as a chemoattractant. MDA-MB-231 or Hs578T cells were seeded into the upper chamber (20,000 cells/insert) in DMEM with 1% BSA with or without 5 μM curcumin. After 24 hr of culture, cells that migrated through the matrix were quantified using Cyquant following manufacturer's instructions (Life Technologies).

Tumor study

Female NSG™ (Jackson Labs) mice were housed and maintained at the University of California-San Diego (UCSD) in full compliance with policies of the Institutional Animal Core and Use Committee (IACUC). MDA-MB-231 (parental or DYRK2 KO) cells were counted and suspended in PBS containing 50% Matrigel (BD Bioscience). 500,000 cells

(100 µl) were injected subcutaneously into the neck of each mouse, 10 mice for parental and 5 mice for DYRK2 KO cells. When tumor became palpable, mice bearing parental MDA-MB-231 cells were randomized into 2 groups with 5 mice per group. Curcumin 300 mg/Kg body weight was administered to one group of n=5 mice bearing parental 231 cells. Vehicle 1% carboxymethylcellulose in 0.1 M citrate buffer pH 6 was injected into the control group of n=5 mice bearing parental 231 cells. Tumor dimensions were measured twice per week using a digital caliper and tumor volume was calculated as (length × width^2)/2. Mice were euthanized 42 days after injection and tumors were excised and weighed. For immunohistochemistry, tumor tissue was fixed in 10% neutral buffered formalin over night at room temperature, rinsed in 70% ethanol and placed in histology cassettes for paraffin embedment. Hematoxylin and eosin (H&E) staining and anti-Ki-67 staining were performed by the UCSD Tissue Technology core. For immunoblotting and proteasome assays, tumors were lysed in proteasome assay buffer using Bullet Blender Navy Bead Lysis Kit (Next Advance Inc., NY, USA) using manufacturer's instructions.

Statistics and data presentation

Most experiments were repeated 3 times with multiple technical replicates to be eligible for the indicated statistical analyses, and representative image has been shown. All results are presented as mean ± SD unless otherwise mentioned. For animal studies, five mice per group is the standard sample size for tumor xenograft experiments, and no statistical power analysis was used to predetermine sample size. Mice bearing parental MDA-MB-231 derived tumors were randomized into two groups of n=5 prior to vehicle or drug treatment. The investigators were not blinded to allocation during experiments and outcome assessment. Data were analysed using Graphpad Prism statistical package.

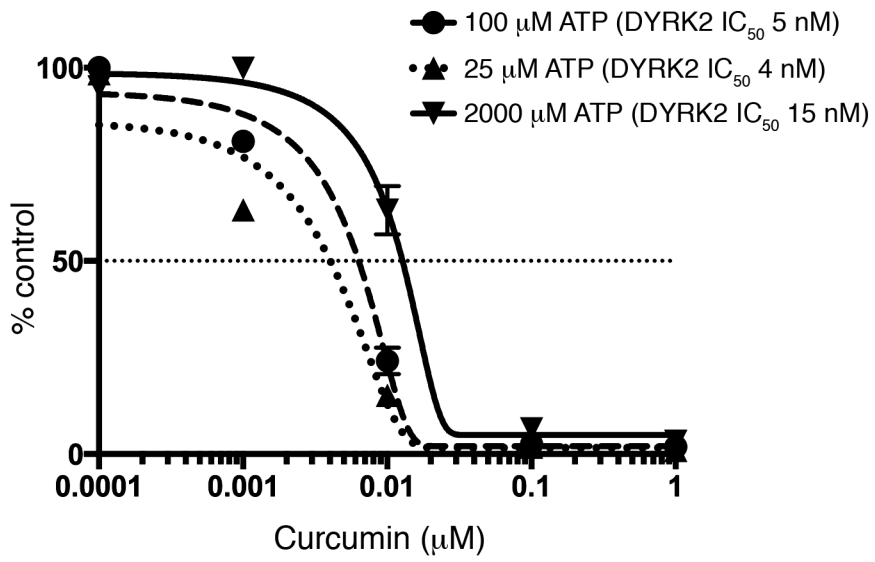

Fig. S1. Changes in ATP concentration moderately affected the IC_{50} value of Curcumin toward DYRK2. 5-20 mU DYRK2 was treated with indicated concentrations of curcumin or DMSO control and assayed using 300 μM Woodtide in the presence of 0.025 mM or 0.1 mM or 2 mM ATP [γ-^{32}P]ATP with the indicated concentrations of curcumin. The IC_{50} graph was plotted using GraphPad Prism software. The results are presented as the percentage of kinase activity relative to the DMSO-treated control. Results are means ±SD for triplicate reactions.

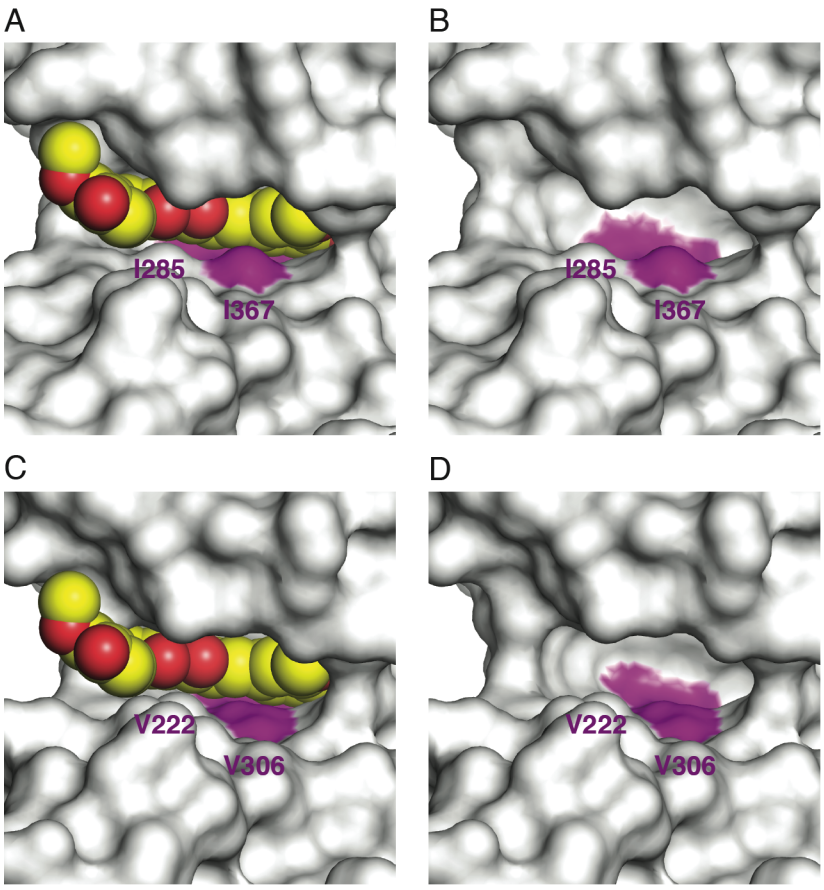

Fig. S2. Comparison of curcumin docking site between DYRK2 and DYRK1. (A) Curcumin binding to the DYRK2 ATP binding site as seen in the co-crystal structure, (**B)** Ile285 and Ile367 forms hydrophobic interactions with curcumin. **(C)** Curcumin modeled into the DYRK1 ATP-binding pocket. **(D)** DYRK1 has Vals instead of Iles at the same positions as in DYRK2.

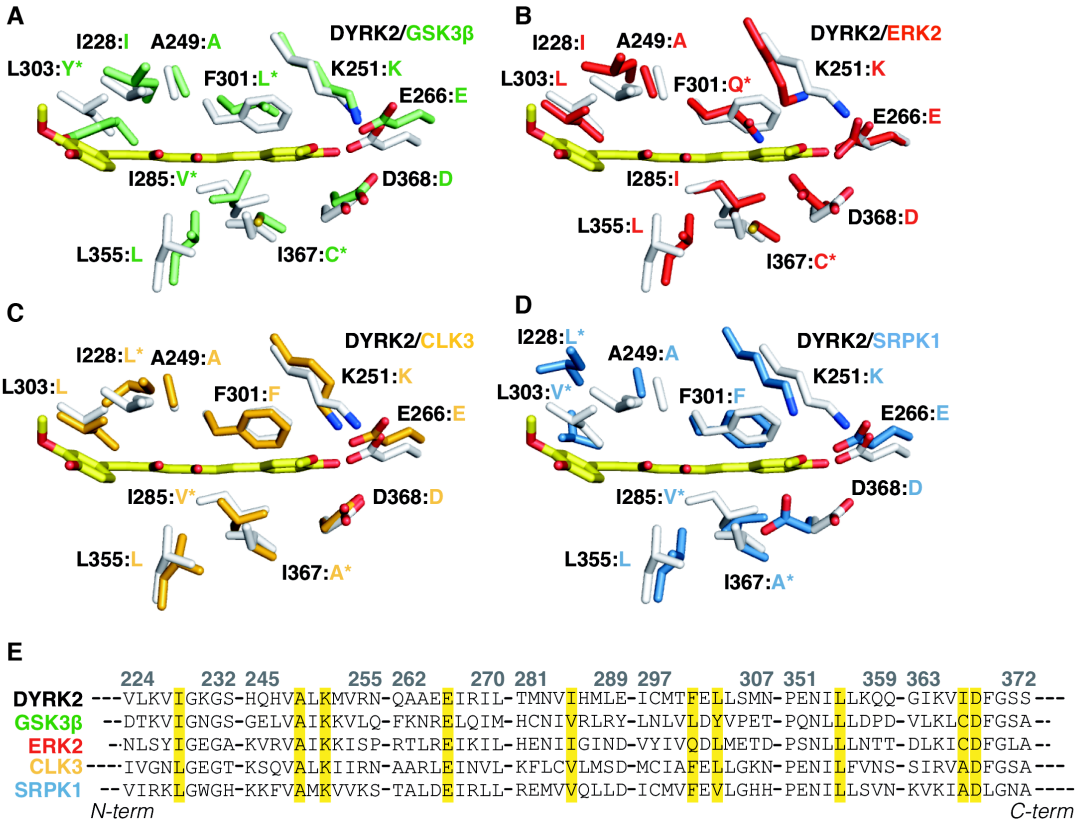

Fig S3. Hydrophobic interactions are key to curcumin specificity. Alignments of DYRK2 (white) and curcumin (yellow) complex with distantly related, GSK3β (**A**, green, PDB ID: 4ACC) and ERK2 (**B**, red, PDB ID: 2ERK), and more closely related protein kinases, CLK3 (**C** gold, PDB ID: 2EU9) and SRPK1 (**D**, blue, PDB ID: 1WAK). Structurally aligned residues indicated. Mismatched residues denoted by an asterisk (*). **(E)** Sequence alignment of DYRK2 and structurally aligned kinases in panels A-D. Curcumin interacting residues and their aligned equivalents are highlighted in yellow.

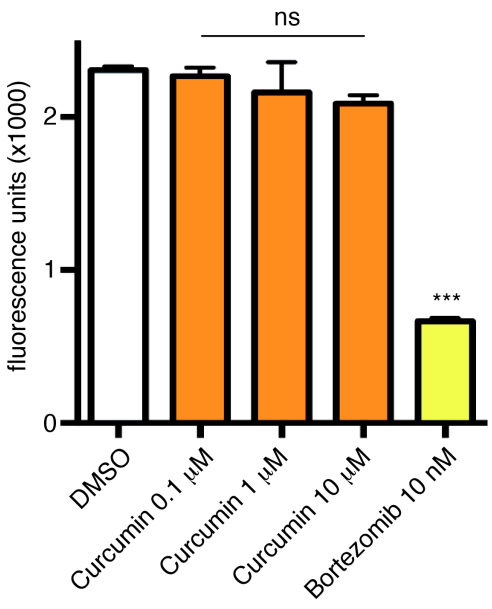

Fig. S4. Presence of 10 μM Curcumin does not affect proteasome activity *in vitro*. Affinity-purified 26S proteasome was assayed using bodipy-casein substrate in the presence of DMSO control or indicated concentrations of curcumin or 10 nM bortezomib. Fluorescence units are plotted on the Y-axis. (ns= not significant; ***p<0.001)

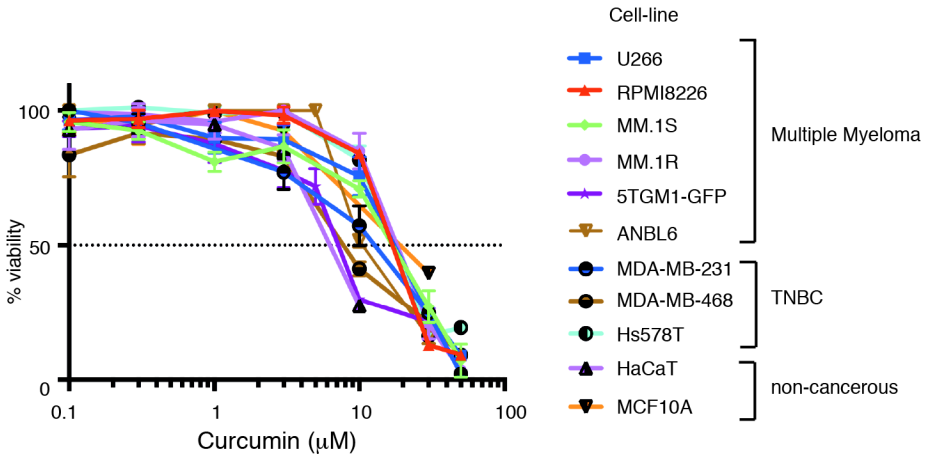

Fig. S5. Curcumin induces cytotoxicity in all cell lines tested with EC50 between 6-12 μM. Proteasome-addicted TNBC and myeloma cells as well as non-cancerous MCF10A and HaCaT cells were used for the assay. Curcumin treatment was carried out for 36 hrs for this experiment only.

Table S1. Kinase profiling data for 1 μM curcumin

Kinase	% activity	SD
MNK2	129	16
TLK1	128	6
EPH-A4	118	65
TTBK2	115	5
PRK2	114	8
TIE2	112	32
MNK1	112	10
EPH-B2	111	23
DDR2	109	18
JNK3	109	0
ZAP70	108	5
PAK5	108	10
CDK2-Cyclin A	107	0
TSSK1	106	8
PKCa	106	3
CK2	106	2
PINK	106	1
CK1δ	105	19
ULK2	105	18
GSK3b	105	7
p38d MAPK	105	1
EPH-B4	105	31
JNK2	104	1
p38a MAPK	104	2
EPH-A2	104	12
JAK3	104	16
OSR1	104	0
GCK	103	8
MKK6	102	19
TAK1	102	4
TTBK1	102	11
PKBa	102	11
FGF-R1	102	16
p38b MAPK	101	24
TESK1	101	24
PRAK	101	1
p38g MAPK	101	18
ASK1	101	3
EPH-B1	100	4
JNK1	99	4
NEK2a	99	9
IGF-1R	99	8

WNK1	98	21
EF2K	98	10
IRAK1	97	0
NEK6	97	1
EIF2AK3	95	11
LKB1	95	10
PKCz	94	9
PKA	94	15
ERK1	94	10
TrkA	93	25
EPH-B3	93	11
AMPK (29)	93	4
DAPK1	93	8
MKK1	93	17
MSK1	92	1
PDGFRA	92	0
BRSK1	92	3
SYK	92	16
MST3	91	1
MPSK1	91	16
SIK3	91	9
MKK2	91	20
BTK	90	4
MAPKAP-K2	90	20
PDK1	90	5
MARK3	90	1
ROCK 2	90	13
MEKK1	89	8
ULK1	88	10
CDK9-Cyclin T1	88	15
HIPK3	88	19
BRSK2	88	4
MAP4K5	87	13
MAP4K3	87	18
CAMK1	86	21
Aurora A	85	11
BRK	85	18
PKCγ	85	1
TAO1	84	18
PAK2	84	12
IRR	84	16
SIK2	83	3
ABL	82	8
TGFBR1	82	24
PAK6	82	2

SGK1	82	17
Src	81	7
Lck	80	2
Aurora B	79	8
MARK1	79	21
PKD1	79	5
MINK1	78	14
CSK	78	2
PAK4	78	8
MARK2	78	11
CK1γ2	77	19
ERK8	77	4
MAPKAP-K3	76	24
MST4	75	12
PLK1	75	19
ERK2	75	15
MELK	74	2
PIM2	74	2
TBK1	74	9
MARK4	74	9
IKKe	72	17
HER4	71	24
IRAK4	71	10
SmMLCK	70	16
PKBb	70	3
IKKb	69	11
CHK2	68	4
VEG-FR	67	6
RSK2	66	19
YES1	62	10
ERK5	62	7
CHK1	57	1
SRPK1	57	2
NUAK1	55	7
HIPK2	54	11
RSK1	53	2
TTK	51	9
CLK2	48	5
S6K1	46	16
IR	46	0
PIM1	46	6
HIPK1	44	7
CAMKKb	44	2
MST2	42	14
STK33	25	2

DYRK1A	20	2
RIPK2	16	3
MLK3	14	1
MLK1	14	0
PIM3	12	0
DYRK3	11	1
PHK	10	0
DYRK2	5	1

Table S2. Data collection and refinement statistics.

	DYRK2-curcumin
Data collection	
Space group	$P4_2$
Cell dimensions (Å)	83.659, 83.659, 149.127
Wavelength (Å)	1.0000
Resolution (Å)	2.5
R_{merge}	0.128 (1.569)
I / sI	21.8 (2.4)
Completeness (%)	99.8 (99.9)
redundancy	7.1 (7.1)
Wilson B-factor	54.5
Refinement	
No. reflections	35490
R_{work} / R_{free}	0.197 / 0.244
No. of atoms	
Protein	6432
Ligand	54
B-factors	
Protein	62.0
Ligand	88.9
R.m.s deviations	
Bond lengths (Å)	0.009
Bond angles (°)	1.246
Ramachandran	
Favored (%)	94.32
Outliers (%)	0

The dataset was collected from a single crystal. Values in parentheses are for highest-resolution shell.

Table S3. Combination indices for curcumin-carfilzomib combination on cell viability

Cell-line	Cz (nM)	Cur (μM)	CI
RPMI8226	3	5	0.4
MM.1R	1	5	0.5
MM.1S	1	5	0.5
5TGM1-GFP	5	5	0.6
MDA-MB-231	10	10	0.8
HCC1937	5	10	0.7
HCC1187	10	10	0.3
Hs578T	10	10	0.2

Cz: Carfilzomib
Cur: Curcumin
CI: Combination index (CI<1: synergism; CI=1: additive; CI>1: antagonism) (CompuSyn; Paramus NJ, USA)

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