CRISPR genome-wide screening identifies dependence on the proteasome subunit PSMC6 for Bortezomib sensitivity in multiple myeloma

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Abstract

Bortezomib (BTZ) is highly effective in the treatment of Multiple Myeloma (MM), however emergent drug resistance is common. Consequently, we employed CRISPR targeting 19,052 human genes to identify unbiased targets that contribute to BTZ resistance. Specifically, we engineered an RPMI8226 MM cell line to express Cas9 and a lentiviral-vector CRISPR library and cultured derived cells in doses of BTZ lethal to parental cells. Sequencing was performed on surviving cells to identify inactivated genes responsible for drug resistance. From two independent whole genome screens, we selected 31 candidate genes and constructed a second CRISPR sgRNA library, specifically targeting each of these 31 genes with four sgRNAs. After secondary screening for BTZ resistance, the top 20 "resistance" genes were selected for individual validation. Of these 20 targets the proteasome regulatory subunit PSMC6 was the only gene validated to reproducibly confer BTZ resistance. We confirmed that inhibition of chymotrypsin-like proteasome activity by BTZ was significantly reduced in cells lacking PSMC6. We individually investigated other members of the PSMC group (PSMC1 to 5) and found that deficiency in each of those subunits also imparts BTZ resistance. We found 36 mutations in 19S proteasome subunits out of 895 patients in the IA10 release of the CoMMpass study (https://www.themmrf.org/). Our findings demonstrate that the PSMC6 subunit is the most prominent target required for BTZ sensitivity in MM cells and should be examined in drug refractory populations.

Keywords

Proteasome; PSMC6; multiple myeloma; Bortezomib resistance

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Introduction

Multiple myeloma (MM) is a plasma cell malignancy, which has seen significant improvements in outcomes following the introduction of proteasome inhibitors (PI), specifically bortezomib (BTZ), carfilzomib and ixazomib into routine clinical practice (1–6). PIs used in combination with other compounds, achieve initial response in nearly 90–100% of patients (7). Despite the high initial response rates, almost all patients develop drug resistance over time and eventually becoming refractory to PIs. The underlying mechanisms of such PI resistance are still incompletely understood (8). The genes and pathways most commonly associated with BTZ resistance are PSMB5 (9–13), the ERN1-XBP1 pathway (14, 15) as well as the IGF-1 axis (16). PSBM5 mutations have been identified frequently in MM cell line but are not found in newly diagnosed patients (17, 18) while XBP-1 mutations have also been seen but are also found in less than 1% of newly diagnosed MM cases. Indeed the mutation prevalence of these genes combined is only 2% in supposedly PI refractory patients (19). XBP1 knock down experiments have shown correlation with BTZ resistance (14, 20). Recently, Acosta-Alvear et al reported an shRNA screening and found that genetic depletion of the vast majority of the 19S proteasomal regulator subunits including PSMC1 and 6, conferred a marked resistance to PIs (21). In a separate study Tsvetkov et al using gene trap screening identified that compromising the 19S proteasome including PSMC2 - 6 protected cells from proteasome inhibitors.(22) Sheffer et al.(23) using CRISPR technology also identified loss of PSMC6 as inducing resistance although to date results are only published in abstract form.

CRISPR-Cas9 knockout is a precise and efficient genome-editing technology (24, 25). This simple gene-engineering tool consists of a 20-base pair single-guide RNA (sgRNA) and a Cas9 protein, which can specifically recognize and cut the sgRNA binding region (24). Recently, a genome-scale CRISPR-Cas9 library was developed (26, 27) including 19,052 human genes, each gene being targeted with six sgRNAs. We used this genome-wide library to identify genes that are potentially associated with BTZ resistance. Our screening identified absence of the proteasome regulatory subunit PSMC6 as gene most consistently conferring BTZ resistance in human multiple myeloma cells.

Materials and Methods

Cell lines and plasmids

Human multiple myeloma cell lines (HMCLs) RPMI8226, FR4, JJN3, KMS11, KMS18, MM1.R, OPM2, and U266 were maintained in RPMI1640 with 5% fetal bovine serum, 100u/ml penicillin and 100 ug/ml streptomycin. All MM cells were from Dr. Leif Bergsagel's lab. These cells were tested for mycoplasma negative and validated by finger printing develop by Dr. Bergsagel's lab (unpulished data). All experiments were performed on MM cells less than 20 passages after thawed from liquid nitrogen. Plasmids pCDHpuroCr expressing selection marker puromycin and pCDHgfpCr expressing a green fluorescent protein selection marker (GFP) for cloning sgRNAs were derived from pCDH-CMV-MCS-EF1-Puro and pCDH-CMV-MCS-EF1-copGFP (SBI, Mountain View, CA 94043) respectively by cloning the sgRNA expression cassette from plasmid lentiGuide-Puro (Addgene #52963).(28). Plasmid pCDHBlastCas9HA was derived from lentiCas9-blast

(#52962).(28) by cloning Cas9 gene (Flag tag was replaced with HA tag) into pCDHBlast under the control of CMV promoter, which was constructed by Shi et al. (C.X. Shi; unpublished observations).

Small Molecules

Tunicamycin was from Abcam (Cat. Ab120296, Cambridge, MA, USA 02139) and its structure was published by Takatsuki et al(29). Staurosporine was from ApexBio (Cat. A8192, Houston TX USA 77054) and its structure was published by Furusaki et al (30, 31). BTZ was from LC laboratories (Cat. B-1408, Wobum, MA USA 01801). Carfilzomib was from MCE Medchem Express (Cat. HY10455, Monmouth Junction, NJ USA 08852). Dexamethasone (Cat. D4902-25MG) and melphalan (Cat M2011-100MG) were from Sigma (St. Louis, MO USA, 63103).

Utilization of sgRNA

The GeCKO V2 library (Addgene #100000049)(28) was expanded by transforming the library into DH10B cells by electrophoresis with a total of $2.5X10^7$ clones produced. A sgRNA library for the second round screening was created by cloning sgRNAs synthesized by IDT technology (Coralville, Iowa USA 52241) individually, mixed manually and finally cloned into the vector pCDHgfpCr. All other individual sgRNAs were cloned either into the plasmid pCDHpuroCr or pCDHgfpCr. sgRNA sequences used for targeting PSMC1 to 6 are displayed in supplemental 1.

Lenti-vector packaging and infection

A modified Graham's calcium-phosphate method (32, 33) was used for packaging lentiviral vectors. Briefly, 293T cells were split into 100 mm tissue culture dishes one to four (total volume 15 ml medium, no more than 70% confluent before transformation). Transformation was performed 24 hours later. For construction, 2ml calcium phosphate-DNA co-precipitation solution was added to two packaging plasmids (18.34 ug for pSPAX2, 7.34 ug for pMD2.G) and a lenti vector (24.34 ug), 100 ul 2.5M CaCl2, add 1/10 TE, PH8.0 to total volume to 1 ml. Then one volume of 2xCa/DNA solution was added quickly to an equal volume of 2x HEPES solution. After 1 minute, 1.5 ml of the mixed solution was added to the cell culture medium. The next morning the medium was changed with 12 ml fresh DMEM medium with 10% FBS. The viruses were harvested 20 hours after medium change. For infection of MM cells one million cells were added into six well plates with 6 ul polybrene (5 mg/ml) and 1.5 ml lenti virus and added fresh medium to total volume 3ml. One hour later, 3 ml of fresh medium was added. Next morning the medium was changed to 6 ml fresh medium.

CRISPR sgRNA screening

RPMI8226Cas9 cells were infected with the CRISPR library. The infected cells were treated with BTZ at 7nM for seven days (changed medium with 7 nM BTZ at day 2 and day 4). Residual cells surviving BTZ treatment were expanded over 15 days without BTZ treatment and then subjected to DNA sequencing

DNA sequencing and data analysis

We screened the abundance of sgRNA targeting the ~19K genes in cells showing resistance to BTZ by next generation sequencing (Ion Torrent PGM, ThermoFisher). An *in house* algorithm was used to clean the gene-specific sgRNA by eliminating the surrounding adapters sequences. A script written in the Perl programming language was used to identify all instances of the "tag" sequence AAAGGACGAAACACC? that occur in the fasta files. The '?' can denote A, C, G, or T. The script also captures the next "target" sequence of twenty nucleotides downstream from the tag. The targets are stored in a hash table which counts the frequency with which each target appears adjacent to the tag. The gene-specific sgRNA were quantified and the 100 most abundant hits were subsequently blasted against the human genome.

Proteasome chymotrypsin-like activity, caspase 3&7 activity assay and 26S proteasome assay

Proteasome chymotrypsin-like activity was measured by cell based assay which was performed according manufacture description (Cat No. G8660, Promega. Madison, WI, USA 53711). Briefly, 2,000 cells were plated into 384-well plates. Cells were treated with different concentration (3, 4, 5, 6, 7, 8 and 9 nM) of BTZ for 24 hours. Then, proteasome-Glo reagent was added into the cells. Luciferase activity was assayed by luciferase reader Cytation 3 imaging reader (Winooski, VT 05404 USA). Caspase 3 & 7 activity assay was similar to proteasome chymotrypsin-like activity but using caspase 3 & 7 Glo (Cat No. G8091 Madison, WI, USA 53711) instead of proteasome-Glo. Total 26S proteasome assay was performed by ELISA kit according the manufacture instruction (Cat No. 353541, United States Biological, Salem, MA USA 01970). This kit is specific only for the detection of the intact 26S proteasome in cell lysates. The capture antibody is specific for the 20S particle while the detection antibody is specific for the 19S particle. This kit is not cross-reactive with the following: 19S particle, 20S particle, PSMB1, LMP2 and PSMA3.

Western Blot and MTT assay

We used standard Western Blot. HA antibody (Cat. No. MMS-101P Clone 16B12) was from Covance (Emeryville California USA 94608). Caspase 8 (Cat. 9746S), ERN1 (Cat. 9956), SQSTM1/p62 (D5E2)(Cat. 8025S) and VCP/p97 (Cat. 2648) antibodies were from Cell Signaling (Danvers MA USA 01923). UFD1 antibody was from Bethyl Laboratories Inc (Montgomery, TX Cat. A303-847A). Immunoblot quantification was performed by the software ImageJ (National Institutes of Health, Bethesda, MD.(34) MM cells viability was determined by MTT assay (Sigma-Aldrich) following the manufacturer's instructions.

Quantitative PCR

Total RNA was isolated using RNeasy Plus Mini kit (QIAGEN) and reverse-transcribed using QuantiTect Reverse Transcription kit (QIAGEN). Quantitative PCR was performed using TaqMan Universal PCR Master Mix with predesigned probe (Applied Biosystems), and the comparative CT method was used for relative quantification on an ABI 7900HT Fast Real-Time PCR system (Applied Biosystems).

Results

Targeting MM cells with CRISPR-sgRNA

We first tested infection efficiency in eight MM cell lines (RPMI8226, KMS11, KMS18, FR4, JJN3, MM1.R, OPM2 and U266) with a lentivirus bearing a GFP marker (packaged from pCDH-CMV-MCS-EF1-copGFP). Flow cytometry results indicated that all cell lines were sensitive to lentivirus infection (Figure 1A). We then engineered all eight cell lines to over express Cas9 with a HA tag using lentivectors packaged from plasmid pCDHBlastCas9HA under selection of blasticidin for two weeks. We analyzed Cas9 expression of these eight cell lines. RPMI8226, MM1.R and KMS11 expressed higher levels of Cas9 than the remaining five cell lines (Figure 1B). Next, we tested knockout efficiency using a sgRNA targeting ERN1 in all eight cell lines. ERN1 expression level was significantly reduced in the three cell lines showing higher Cas9 level (MM1.R, KMS11, and RPMI8226) (Figure 1C). RPMI8226 cells were selected for primary sgRNA whole genome library screening, because of their superior capability for colony formation when compared to MM1.R and KMS11.

PSMC6 was identified as the only reproducible gene conferring BTZ resistance

Experimental screening procedures are illustrated in figure 2A. The top 100 sgRNAs that featured in next generation sequencing of surviving cells following BTZ treatment are listed in supplemental 2. The 31 most abundant of these sgRNAs found in cells surviving BTZ treatment were selected for secondary screening (Figure 2B). Each of the 31 potential BTZ resistance inducing genes was targeted by four sgRNAs, including three sgRNAs from the GeCKO V2 library A and the fourth from GeCKO V2 library B (Supplement 3). In addition, 14 non-specific oligos with high copy number in the next generation sequencing were included in the secondary screening as controls (supplemental 3). We performed secondary screening in quadruplicate and next generation sequencing was again performed in the resistant cells. Sequencing results were listed at supplemental 4. The 20 most abundant sgRNAs (Figure. 2C) were then selected for individual validation. Four sgRNAs (the same sequence was used as in the secondary screen) for each of these 20 genes were individually cloned and mixed for virus packaging. Ultimately, only the *PSMC6* gene was identified as consistently inducing BTZ resistance in independent screenings (Figure.2D). Next, we infected RPMI8226 cells with individually packaged sgRNA targeting *PSMC6*. Three of four sgRNA were associated with resistance to BTZ (Figure. 2E).

PSMC6 was validated as a BTZ-sensitive gene

To confirm that *PSMC6* deficiency correlates with BTZ resistance, we designed a pair of sgRNA (sgRNA # 5 and 6) targeting intron regions flanking exon 1 of *PSMC6* (Figure 3A). This pair of sgRNA only deletes endogenous *PSMC6* exon 1, but not *PSMC6* cDNA introduced by a vector, thus allowing the rescue of any PSMC6 deficiency phenotype by over-expression of wild type *PSMC6*. These sgRNAs delete around 800 bps, allowing screening for PSMC6 knockout by PCR. We subsequently obtained two clones (clones A and B) from RPMI8226 cells with *PSMC6* deficiency validated by PCR, quantitative PCR and Sanger sequencing (Figure.3B-D). Both clones developed resistance to BTZ with an IC50 for clone A and B of 6.0 nm compared to parental cells in which the IC50 is 2.5 nM.

(Figure. 3E). Furthermore, MM cells lacking *PSMC6* also developed resistance against Carfilzomib (CAR) (Supplemental Figure 1A). These results were fully reproduced in cell line KMS11 (Supplemental Figure 1B&C).

To exclude *off target* effects, we re-introduced wild-type *PSMC6* with an HA tag in the two PSMC6 gRNA targeted clones (Figure 3F). In both cases, the expression of wild-type PSMC6 rescued most of the BTZ-sensitive phenotype (Figure 3G)(IC50 for parent cell is 3.7nM, IC50 for clone A was restored from 7.6 to 5.5 nM, and IC50 for clone B was restored from 6.4 to 4.6 nM).

PSMC6 deficiency was specific for BTZ resistance

In order to confirm that drug resistance induced by *PSMC6* deficiency was specific to proteasome inhibitors, we treated *PSMC6* deficient RPMI8226 cells with dexamethasone, melphalan, tunicamycin (an ER stress inducer) and staurosporine (a pan kinase inhibitor). Isogenic *PSMC6* wild-type and *PSMC6* knockout RPMI8226 (Figure 4) or similarly engineered KMS11 cells did not show any change in sensitivity to dexamethasone (supplemental Figure 2A) and melphalan (supplemental Figure 2B).

Mechanism of BTZ resistance induced by PSMC6 deficiency

The basal level of proteasomal chymotrypsin-like activity has no significant change after deletion of *PSMC6* (Figure 5A) In contrast, the proteasomal chymotrypsin-like activity in cells lacking *PSMC6* was significantly reduced following exposure to BTZ (Figure 5B). MM cells with *PSMC6* knockout were resistant to BTZ-induced apoptosis, which was confirmed by measuring Caspases 3/7 activity (Figure 5C) and Caspase 8 expression (Figure 5D). We checked expression levels of SQSTM1/p62, an autophagy pathway protein (35) and found no significant change in 8226 cells with or without PSMC6 protein, however the protein expression level was significantly increased in KMS11 cells after deletion of the PSMC6 gene.(Figure 6). Expression levels of the endoplasmic reticulum-associated protein degradation gene VCP/p97, (36), were reduced for both RPMI8226 and KMS11 cells after deletion PSMC6 (Figure 6). VCP/p97 expression was further inhibited in KMS11 cells with or without PSMC6 by BTZ treatment. UFD1, which forms a complex with VCP/97(36), has similar expression change as SQSTM1/p62 (Figure 6). We next measured the total 26S proteasome and demonstrate that RPMI8226 and KMS11 cells lacking PSMC6 have varying change in 26S when compared to parental cells. However, both RPMI8226 and KMS11 cells without PSMC6 continue to express significant amounts of 26S proteasome (Supplemental Figure 2C). This is important to note since other studies have demonstrated absence of the 19S proteasome to be lethal because its absence precludes formation of functional 26S proteasomes. It seems this no longer applies in the CRSIPR knockout MM cells.

Depletion of PSMC subunits PSMC1, PSMC2, PSMC3, PSMC4, and PSMC5 also results in resistance to BTZ

We tested the effect on BTZ resistance of the remaining PSMC subunits PSMC1 to PSMC5 using three sgRNAs for each gene, which demonstrated that each PSMC gene also caused BTZ resistance in RPMI8226 and KMS11, albeit their IC50 was less than that of *PSMC6* (Figure 7A-B, IC50 were calculated by PRISM, see supplemental table S1). Cross

experiment comparisons are not tested for statistical variability. The induction of resistance was confirmed by individual CRISPR sgRNAs (Supplemental Figure 2D&E). Notably, however these five subunits did not appear in our CRISPR library screening.

PSMC6 mutation profile in Myeloma patients

We analyzed the mutation profile of all proteasome subunits in the genomic sequences derived by whole exome sequencing of 895 patients in the IA10 release of the CoMMpass study (https://www.themmrf.org/). It is important to note that the vast majority of exomes in this dataset are in newly diagnosed patients unexposed to BTZ. In that dataset a total of 36 mutations in 19S proteasome subunits were identified (one patient has three PSMC5 mutations and one patient has two PSMD7 mutations) (supplemental 5). A total of 16 PSMC subunit mutations, were identified although none of them was in PSMC6. PSMB5 is most frequently associated with BTZ resistance in MM cell lines in vitro. Two patients with PSMB5 mutations are detected in the CoMMpass dataset. Functional confirmation of the significance of these mutations was not performed.

Discussion

The mechanisms of BTZ resistance have remained elusive in MM. The proteasome subunit PSMB5 is a major target of BTZ. Mutations and increased PSMB5 expression have been correlated with BTZ resistance in vitro (8, 10, 13), but not yet in vivo (17, 18). By performing whole exome sequencing on multidrug resistant MM patients (37) we have previously identified a missense mutation in PSMG2, another component of the proteasome, as well as in XBP-1 potentially suggesting their involvement in BTZ resistance. Recently Acosta-Alvear et al. (21) using a next generation shRNA library screening demonstrated that knocking down the vast majority of subunits of the 19S proteasomal regulator, especially D group subunits PSMD2, PSMD6, PSMD12 and PSMD13 induces BTZ resistance. In their validation screening PSMC1 and 6 also appeared in the list of genes which can induce resistance.

In the current study we applied a newly developed unbiased CRISPR genome-wide screening to identify potential gene impairment correlated with BTZ resistance. After performing two independent screenings and further validation, we identified the proteasome regulatory subunit PSMC6 as the most prominent gene associated with BTZ resistant. We further demonstrated that impairment of the remaining PSMC family members also caused BTZ resistance, albeit less potently than with PSMC6. Of note, the PI resistance we observed was not complete, however the same effect was found in cells treated with BTZ and CAR, suggesting that both drugs are affected by PSMC6. Further validating our finding PSMC6 was also listed in an independent CRISPR screening performed by Sheffer et al. using the same library, although no further validations were reported (23).

PSMC6 and other PSMC family member are located in the base region of the proteasome 19S regulatory particle. All PSMC subunits have an ATPase function, which is necessary to unfold the substrates and translocate them into the core particle (38). PSMB5 is a catalytic subunit, which has chymotrypsin-like activity and is part of the proteasome 20S core

particle. Our results show that PSMC6 depletion reduces the BTZ-induced chymotrypsin-like activity of PSMB5.

Acosta-Alvear et al. also performed whole genome screening using shRNA and selected PSMD12 from their top hits for validation(21). However, they found that knockdown PSMD12 did not affect the activity of the 20S subunits targeted by carfilzomib. They demonstrated that the mechanism of PSMD12 was the selective accumulation of protective factors, including SQSTM1/p62, and two subunits of a heterotrimeric complex functioning in endoplasmic reticulum-associated protein degradation UFD1L and the triple AAA ATPase VCP/p97. We therefore checked expression levels of SQSTM1/p62, VCP/p97 and UFD1 and also found significant changes in KMS11 cells deleted of PSMC6 for SQSTM1/p62 and UFD but could not demonstrate such findings in RPMI8266 cells. Since our primary screen and validation were performed on 8226 cells, the autophagy pathway seems not to be involved in BTZ resistant MM cells lacking PSMC6. In our experiments, expression levels of VCP/p97 were reduced for both RPMI8226 and KMS11 cells deleted PSMC6, a finding in contrast to the report by Acosta-Alvear et al. regarding PSMD12 (21). Franke et al. (10) found that BTZ binding to mutated PSMB5 was impaired in BTZ resistant MM cells, underlying the basis for BTZ resistance in this situation. Since mutations of PSMC6 have not yet been identified one possible explanation of our finding is that PSMC6 ablation changes 26 proteasome structure and influences bortezomib binding to PSMB5. However this needs to be addressed by crystal structure studies.

Using another approach (gene-trap) Tsvetkov et al. have reached a similar conclusion, to our work, demonstrating that a modest reduction in PSMC2 - 6 protected cells from proteasome inhibitors. (22) One of their findings was that strong 19S reduction was cytotoxic, for they were unable to isolate stable clones deleted of the 19S subunit from the resistant pools of cells, unable to recover diploid cell variants depletion of PSMD12 by CRISPR technology and unable to recover clones with inactivated PSMC2 and PSMD12 from haploid mouse embryonic stem cells that harbor reversible gene-trap induced by Cre-mediated inversion. However in our study we developed both RPMI8226 and KMS11 cells with homozygous deletion of PSMC6 successfully. The knockout cells were healthy under microscopy. and expressed 26S proteasomes. One possible explanation is that PSMC6 function is cell dependent, since Tsvetkov et al, using haploid and near-haploid cell lines (near-haploid human chronic myeloid leukemia cells (KBM7), near-haploid fibroblast cells (HAP1) and haploid mouse embryonic stem cells). (22) Whether other 19S proteasome subunits are also dispensable in MM cells needs further study in future.

Genome-wide sequencing data of PI resistant MM are currently not available. We did however analyze the 895 patients sequenced as part of the MMRF CoMMpass trial and found only one mutation in *PSMC6*, however a significant number of patients with other 19S subunits mutations (3.7%) are noted. Most of these patients are newly diagnosed and we hypothesize that mutations would be enriched in resistant/refractory cases to PIs, in line with our recent findings in patients that have relapsed after treatment with IMiDs (19). Thus, CRBN, the gene targeted by IMiDs, was found mutated in only 0.2% of newly diagnosed MM (https://research.themmrf.org/rp/mycommpass), but in 12% of patients refractory to IMiDs therapies with mutations in the CRBN pathway in at least 22% of the same patients

and probably higher since structural variations were not analyzed (19). Acosta-Alvear et al. (21) found that PSMC2 expression levels quantified by flow cytometry were significantly higher in the group of patients who achieved complete response than partial responders after treatment with carfilzomib-based therapy. A similar longitudinal and comprehensive screening is desired in patients treated with PIs in order to elucidate the mutation rate and expression levels of PSMC genes over time and their potential role in the development of PI resistance in human MM.

In summary, using comprehensive genome wide CRISPR screening we identified and validated *PSMC6* depletion as the strongest hit associated with BTZ resistance. PSMC 1–5 subunits also confer BTZ resistance in MM cells. *PSMC6* deficiency reduced the ability of BTZ to suppress the chymotrypsin-like activity of PSMB5. Three independent studies with different approaches have now reached the same conclusion that deletion or down regulation of the 19S proteasome subunits leads to resistance to 20S proteasome inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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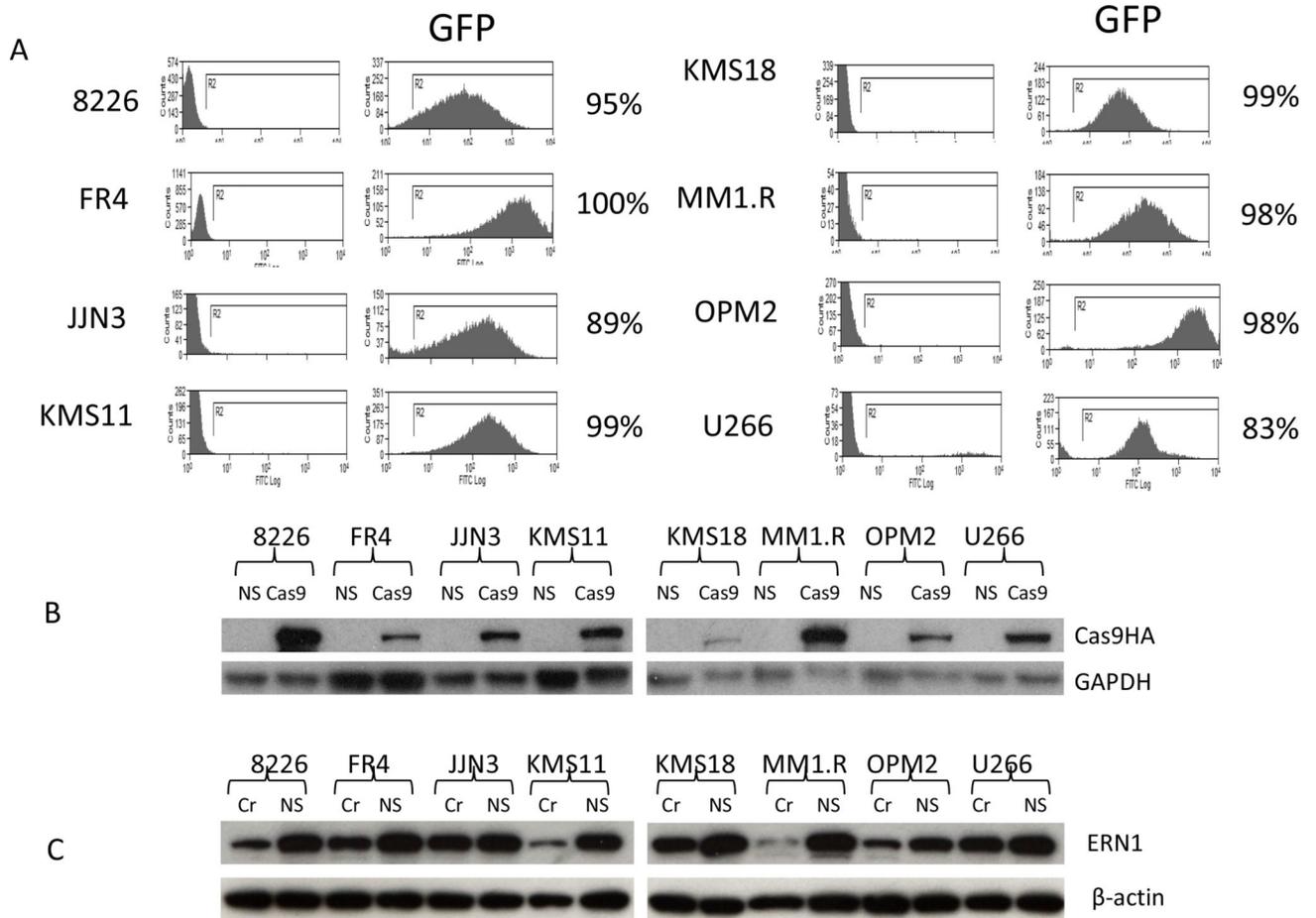
Figure 1.
Infecetion efficiency of lentivirus and targeting efficiency of sgRNA against ERN1 in MM cells. (A) Lenti virus infection efficiency to MM cells detected by flow cytometry. (B). Cas9 with HA tag was detected by Western Blot with HA antibody. (C) ERN1 knocking out efficiency by CRISPR technology in MM cells. Cr=ERN1 sgRNA. NS= non-specific sgRNA control.

A

RPMI8226 cells expressing Cas9

Crispr SgRNAs library
rescued into lentivirus

BTZ for 7days
Lethal BTZ for the control

Growth two weeks
without Bortezomib

Next generation
sequencing and analysis

Construct a library including
top 31 genes from the list
and 4 SgRNAs each gene

Repeat first round
screen procedure

Select top 20 genes
for individual verification

Individual gene
validation

B

C6orf70	ZNF33A	C6orf106
CCDC114	ANKEF1	ARHGEF15
PFAS	SSBP3	PHF21A
C11orf24	OR52N2	IFNB1
PSMC6	GPR155	AVPR2
SHANK1	CYB5R2	HINT3
ATP2A1	ANKS6	TRHDE
ATP5SL	PAOX	RPL39L
PBX3	NAV1	KHDC3L
HOXB1	TCP11L1	ZNF385B
EPDR1		

C

PBX3	CYB5R2
NAV1	TCP11L1
C6orf106	PHF21A
IFNB1	HINT3
RPL39L	CCDC114
ZNF385B	PFAS
HOXB1	C11orf24
PSMC6	ATP2A1
EPDR1	ATP5SL
GPR155	ARHGEF15

D

Exp. 1
Exp. 2
Exp . 3
Control

E

Crispr A
Crispr B
Crispr D
Control

Figure 2.
PSMC6 was the only gene producing resistance to BTZ after knocking out by CRISPR
sgRNA. (A) Schematic diagram for genome wide sgRNA screen. (B) Top gene list for first
round screen. (C) Top gene list for second screen. (D) Three independent experiments (MTT
assay) of RPMI8226 cells knocked out by a mix of four PSMC6 sgRNAs (A+B+C+D). (E)
PSMC6 knock out by three independent sgRNAs produces resistance to bortezomib (BTZ).
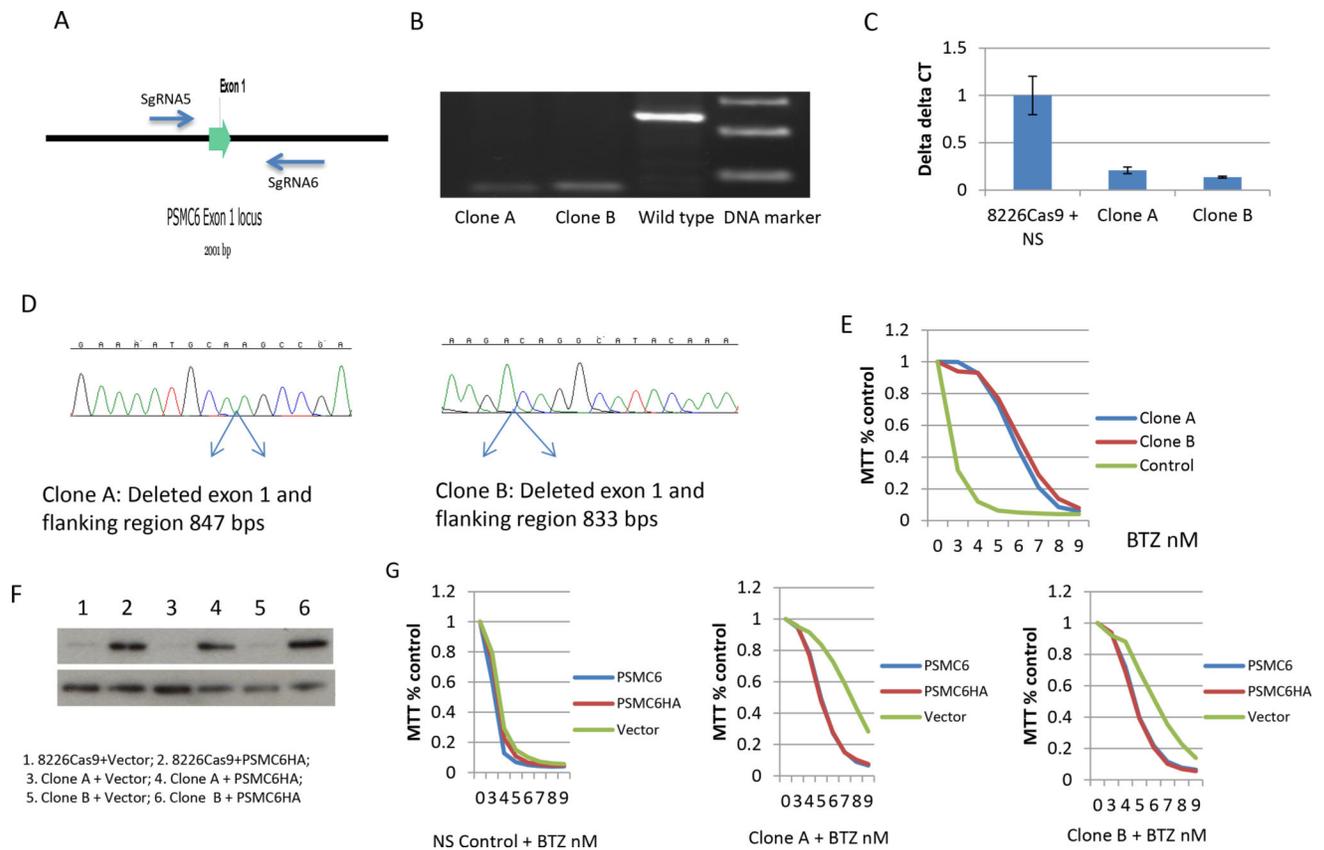
Figure 3.
PSMC6 was validated as a BTZ-sensitive gene. (A) A pair of sgRNAs targeting intron region flanking Exon 1. (B) PSMC6 exon 1 deletion was validated by PCR for two cloned cells. (C) PSMC6 expression levels detected by quantitative PCR. (D) Sanger sequencing analyze cloned RPMI8226 cells deleted PSMC6. PCR amplified PSMC6 exon 1 region and cloned into T-vector. Then purified plasmid was analyzed by Sanger sequencing. (E) MTT assay for cloned RPMI8226 cells (clone A and B) deleted PSMC6. The cells were treated with BTZ for 72 hours. Control=RPMI8226 cells infected with lenti-vector with non-specific sgRNA. (F) Over expression of PSMC6 with HA tag in cloned PSMC6 deficiency cells. (G) Over expression of PSMC6 wild type or with HA tag rescues the phenotype of PSMC6 deficiency. NS= non-specific sgRNA control. bortezomib (BTZ). Vector=empty lentivirus vector. PSMC6HA=lentivirus expressing PSMC6 with HA tag.
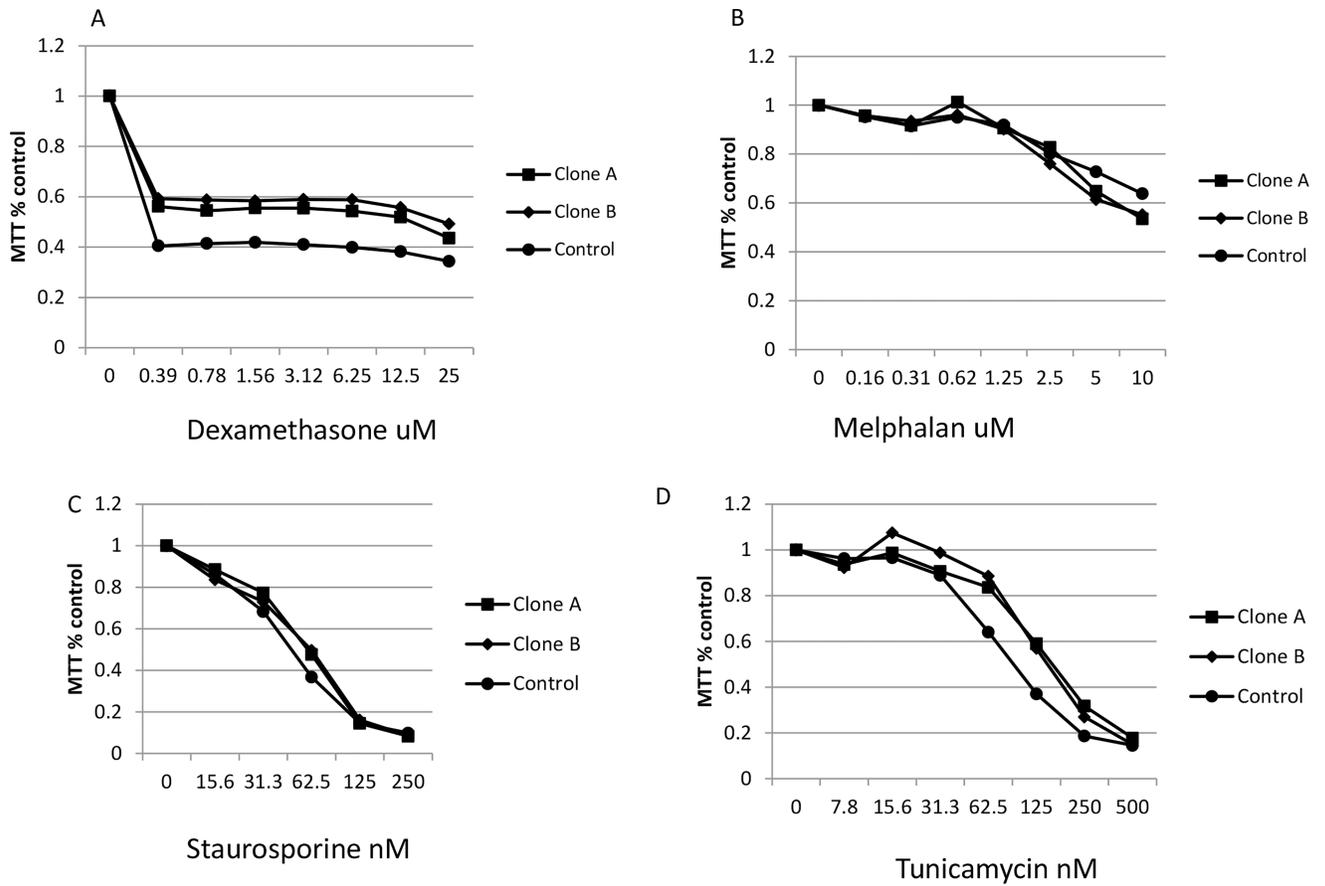
Figure 4.
MM cells without PSMC6 have no significant difference in sensitivity to others chemicals
tested. Cloned RPMI8226 deleted for PSMC6 by CRISPR sgRNA (PSMC6Cr5+6) were
tested for response to different chemicals assayed by MTT (72 hours treatment). (A)
Dexmethasone. (B) Melphalan. (C) Tunicamycin. (D) Staurosporine.

A

B

C

D
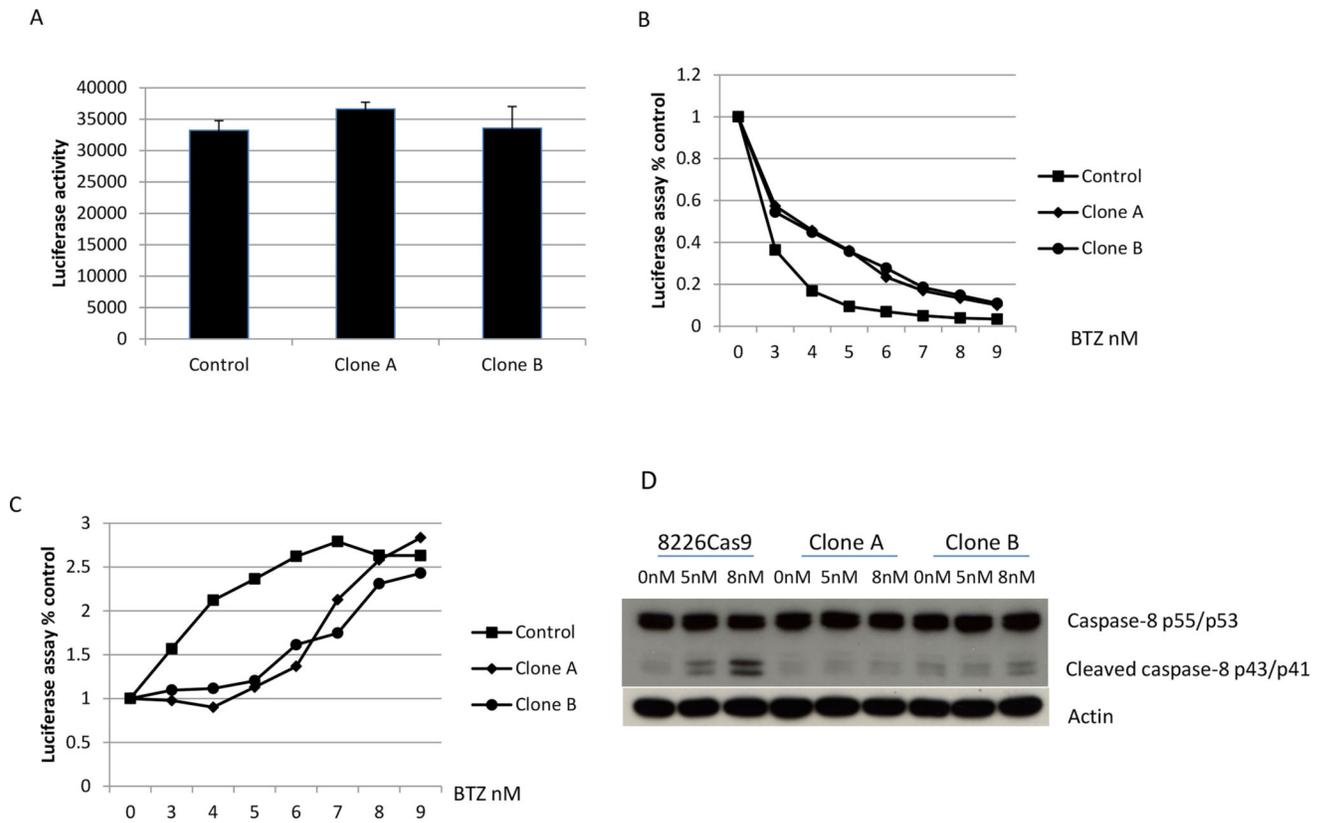
Figure 5.
MM cells without PSMC6 showed resistance to bortezomib (BTZ) induced apoptosis. (A) Cell-based, chymotrypsin-like activity assay on cell with or without PSMC6 without treatment with BTZ. (B) Cell-based, chymotrypsin-like activity assay. The cells were treated with BTZ for 24 hours. (C) Caspase 3/7 activity detected by luminescent assay. The cells were treated with BTZ for 24 hours. (D) Caspase 8 p55/53 and cleaved caspase 8 p43/41 detection by Western blot. The cells were treated with BTZ for 24 hours.
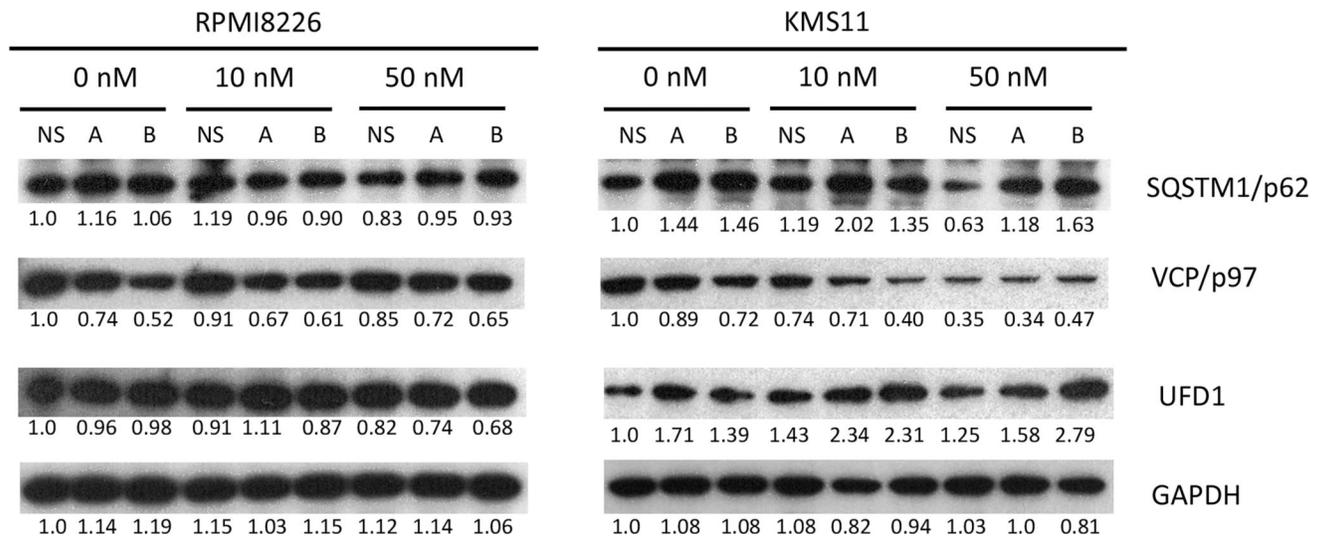
Figure 6.
Expression of SQTM1/p62, VCP/p97, and UFD1 in RPMI8226 and KMS 11 cells with or without PSMC6 treated with BTZ. Cloned RPMI8226 or KMS11 cells (clone A and B) deleted PSMC6 and Control cells infected with non-specific sgRNA (NS) treated with BTZ at 10 or 50 nM for 4 hours. The proteins expression was detected by western blot and analyzed by ImageJ software (numbers below the Figure 6 blots are normalized to GAPDH and untreated control).

A

RPMI8226Cas9

B

KMS11Cas9
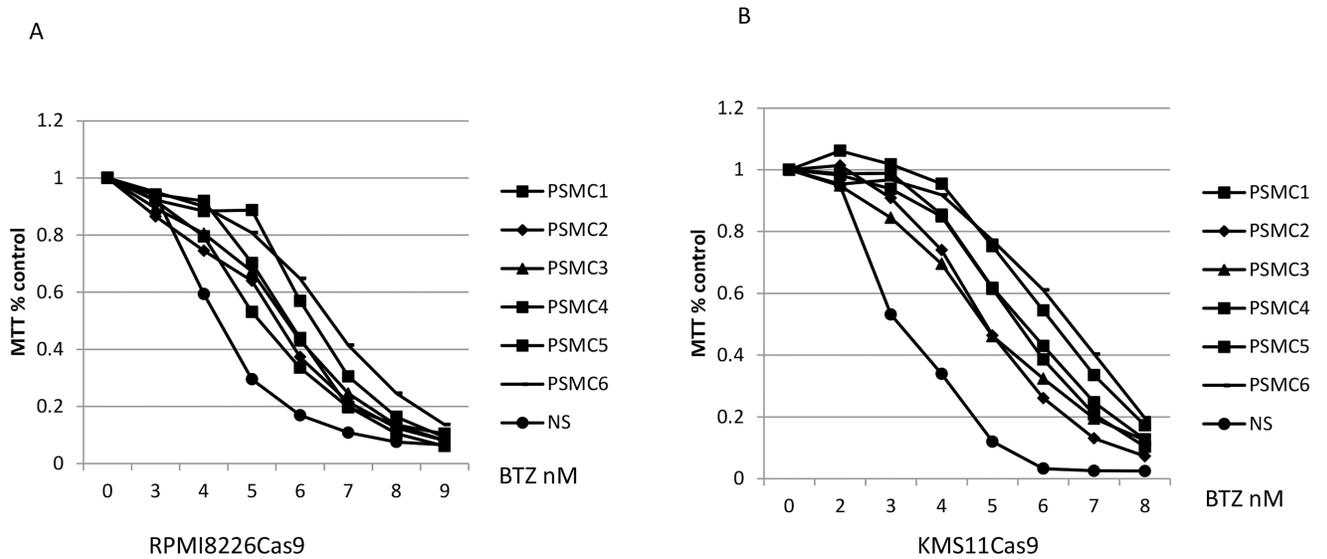
Figure 7.
Deficiency of PSMC1-PSMC5 also results in resistance to bortezomib (BTZ) treatment. The cells were infected with lentivirus including three sgRNAs for each gene. The infected cells were treated with BTZ at concentration of 4, 5, and 6 nM 72 hours after infection. The cells that survived to the highest concentration for drug treatment were tested for response to BTZ assayed by MTT (72 hours treatment). (A) RPMI8226 cells. (B) KMS11 cells.