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SUMOylation inhibition overcomes proteasome inhibitor resistance in multiple myeloma

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Abstract:
Proteasome inhibition is a highly effective treatment for multiple myeloma (MM). However, virtually all patients develop proteasome inhibitor resistance which is associated with a poor prognosis. Hyperactive SUMO signaling is involved in both cancer pathogenesis and cancer progression. A state of increased SUMOylation has been associated with aggressive cancer biology. We found that relapsed/refractory MM is characterized by a SUMO-high state, and high expression of the SUMO E1 activating enzyme (*SAE1/UBA2*) is associated with poor overall survival. Consistently, continuous treatment of MM cell lines with carfilzomib (CFZ) enhanced SUMO pathway activity. Treatment of MM cell lines with the SUMO E1 activating enzyme inhibitor subasumstat (TAK-981) showed synergy with CFZ in both CFZ-sensitive and CFZ-resistant MM cell lines, irrespective of the TP53 state. Combination therapy was effective in primary MM cells and in two murine MM xenograft models. Mechanistically, combination treatment with subasumstat and CFZ enhanced genotoxic and proteotoxic stress and induced apoptosis was associated with activity of the prolyl isomerase PIN1. In summary, our findings reveal activated SUMOylation as a therapeutic target in MM and point to combined SUMO/proteasome inhibition as a novel and potent strategy for the treatment of proteasome inhibitor-resistant MM.

Conflict of interest: COI declared - see note

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ORIGINAL ARTICLE

SUMOylation inhibition overcomes proteasome inhibitor resistance in multiple myeloma

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Data sharing: Data can be accessed via the European Nucleotide Archive accession ID: PRJEB51059 and the PRIDE database - project accession: PXD032316. Reagents, cell lines, etc. can be obtained from the corresponding author and are also available upon reasonable request: matthias.wirth@charite.de.

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ABSTRACT

Proteasome inhibition is a highly effective treatment for multiple myeloma (MM). However, virtually all patients develop proteasome inhibitor resistance which is associated with a poor prognosis. Hyperactive SUMO signaling is involved in both cancer pathogenesis and cancer progression. A state of increased SUMOylation has been associated with aggressive cancer biology. We found that relapsed/refractory MM is characterized by a SUMO-high state, and high expression of the SUMO E1 activating enzyme (*SAE1*/*UBA2*) is associated with poor overall survival. Consistently, continuous treatment of MM cell lines with carfilzomib (CFZ) enhanced SUMO pathway activity. Treatment of MM cell lines with the SUMO E1 activating enzyme inhibitor subasumstat (TAK-981) showed synergy with CFZ in both CFZ-sensitive and CFZ-resistant MM cell lines, irrespective of the *TP53* state. Combination therapy was effective in primary MM cells and in two murine MM xenograft models. Mechanistically, combination treatment with subasumstat and CFZ enhanced genotoxic and proteotoxic stress and induced apoptosis was associated with activity of the prolyl isomerase PIN1. In summary, our findings reveal activated SUMOylation as a therapeutic target in MM and point to combined SUMO/proteasome inhibition as a novel and potent strategy for the treatment of proteasome inhibitor-resistant MM.

KEY POINTS

1. The SUMO pathway is activated in multiple myeloma and its magnitude associated with progression and treatment resistance
2. SUMO inhibition overcomes proteasome inhibitor resistance by blocking myeloma stress resilience, irrespective of p53 state

INTRODUCTION

Multiple Myeloma (MM) is a genetically and clinically heterogeneous plasma-cell malignancy [1,2]. Genetic lesions that are associated with MM include the loss-of-function of tumor suppressors and cell cycle regulators (*p53*, *CDKN2C*, *RB*) as well as the activation of oncogenic signaling pathways (MYC, RAS, NFκB), leading to aberrant cell cycle checkpoint regulation and increased proliferation [3]. Although the introduction of new drugs improved outcome, MM remains incurable and most patients die from their disease [4]. Currently, the common treatment regimens for MM patients include proteasome inhibitors which are routinely combined with dexamethasone, chemotherapy, immunomodulatory drugs (IMiDs) or CD38-specific antibodies [5,6]. While such regimens can induce remissions for many years, the development of drug resistance remains a major clinical problem [7,8]. Therefore, new therapeutic strategies to overcome drug resistance are urgently needed.

Post translational modification (PTM) of proteins by small ubiquitin-like modifiers (SUMOs), termed SUMOylation, are involved in maintenance of genome integrity, and in regulation of gene expression and intra- and extra-cellular signaling [9]. Similar to ubiquitination, protein SUMOylation occurs via a tightly controlled enzymatic pathway controlled by a SUMO-specific E1 activating enzyme (SAE1/UBA2), an E2 conjugating enzyme (UBE2I) and a subset of E3 SUMO ligases [10]. Importantly, protein SUMOylation is fully reversible and executed by the SENP (sentrin-specific proteases) family of isopeptidases, making SUMOylation a finely-tuned molecular switch [11,12]. To date, only one heterodimeric E1 enzyme and one E2 enzyme have been identified in the SUMOylation pathway, suggesting that disruption of either will substantially inhibit global SUMO conjugation. Essential regulators mediating a plethora of tumor-suppressive functions are the members of the p53 family, which can be subdivided in p53, p63 and p73 [13]. They all can bind p53 consensus sequences, and share some common targets, but also have diverse roles in tumorigenesis [13-15]. SUMO E3-like ligases of the PIAS family have been described to transcriptionally repress the activity of the p53 tumor suppressor [16]. Similar to p53,

the other family proteins p63 and p73 are also SUMOylated. Whereas SUMOylation of p73 does not affect its transcriptional activity but rather changes its subcellular localization [17], p63 SUMOylation leads to an impaired transactivation activity [18,19].

To date, PIN1 is the only known peptidyl-prolyl cis–trans isomerase that can specifically recognize and isomerize phosphorylated-Serine/phosphorylated-Threonine-Proline motifs and thus change the stability of its target proteins [20]. Dependent on the molecular context, PIN1 acts either as an oncogene or a tumor suppressor [20]. PIN1 modulates many target proteins and also controls a variety of biological processes, including apoptosis [21]. For example, PIN1 has been shown to modulate p53, which was required for activation of the proapoptotic protein BAX [22]. PIN1 itself is also subject to various post-translational modifications, and SUMOylation of PIN1 has been shown to impair its activity [23].

Due to the wide array of effects elicited by SUMOylation, it is not surprising that deregulation of SUMOylation has been associated with tumorigenesis, tumor progression, and adverse patient outcome in MM and, therefore, SUMO inhibition can potentially represent a novel approach in cancer treatment [10,24-26]. These and further data have led to the development of SUMO inhibitors (SUMOi) like subasumstat (TAK-981) [27]. Subasumstat blocks the enzymatic cascade of SUMOylation by forming an irreversible SUMO-subasumstat adduct, which prevents the transfer of SUMO from the E1 ligase complex to UBC9. Subasumstat has shown pre-clinical activity in multiple malignancies, including colorectal carcinoma and lymphoma [27], and is currently being evaluated in clinical trials for hematological diseases (NCT03648372 and NCT04776018).

In this study, we identified synergistic effects between subasumstat and CFZ in MM, including in CFZ-resistant MM. Combination treatment resulted in proteotoxic and genotoxic stress and induced apoptosis in MM model cell lines independent of the cellular *TP53* state, but dependent on PIN1. A combinatorial treatment of subasumstat and CFZ therefore constitutes a potential novel therapeutic strategy for patients with MM.

MATERIAL AND METHODS

Cell lines and culturing

AMO1, OPM2, JJN3 and NCI-H929 cells were obtained from the DSMZ (Braunschweig, Germany). MM1S cells were purchased from ATCC (Manassas, Virgina, USA). Cells were cultured in RPMI-1640 (21875034, Thermo Fischer Scientific, Waltham, USA) supplemented with 10% fetal bovine serum (A4766801, Thermo Fischer Scientific) and 1% penicillin/streptomycin (15140122, Thermo Fischer Scientific). All cell lines were cultured at 37°C and 5% CO_2 in a humidified incubator. All cell lines tested negative for mycoplasma contamination as described [28] and were repeatedly STR profiled for authentication. Generation of carfilzomib resistant cell lines is described in the in Supplementary Material and Methods.

FACS analysis

Flow cytometry analysis was performed following standard protocols. Cell suspensions, treated as indicated, were directly labelled with fluorescently labelled antibodies against the following surface proteins: CD38 (APC), CD138 (PE) and Annexin-V (FITC). For primary MM patient samples, red blood cells were lysed for 20 minutes in ACK buffer (A1049201, Thermo Fischer Scientific) before antibody labelling.

Data were acquired using a Beckman Coulter CytoFLEX flow cytometer and analyzed by FlowJo software v10.1 (FlowJo LLC).

Compounds

Subasumstat (TAK-981) was provided by Takeda Pharmaceutical Company Limited (Cambridge, USA). Carfilzomib (S2853), bortezomib (S1013), pomalidomid (S1567), doxorubicin (S1208) and dexamethasone (S5956) were purchased from Selleckchem (Houston, USA). Sulfopin was purchased from MedChemExpress (Monmouth Junction, NJ, USA)

In vivo xenograft experiment

Xenograft experiments were performed as previously described [29]. In summary, 1.0×10^7 MM cells were resuspended in serum-free medium, mixed with Matrigel Basement Membrane Matrix

(Corning, Corning, USA) at 1:1 ratio and injected subcutaneously into the flanks of female NOD.CB17/AlhnRj-*Prkdc*^{SCID}/Rj mice 8–10 weeks of age (Janvier). After tumor engraftment, mice were randomly assigned to receive CFZ (2 mg/kg) i.v., subasumstat (25 mg/kg) i.v., a combination of both or vehicle control twice per week. Treatment was performed for a total of 7 days. Tumor growth was monitored by caliper measurements. Mice were housed under SPF conditions and animal experiments were conducted in accordance with the local ethical guidelines and approved by the responsible regional authorities (District Government of Upper Bavaria, application no.: ROB-55.2-2532.Vet_02-17-230).

Primary MM patient material

Primary human MM bone marrow samples were obtained from the Department of Hematology, Oncology and Cancer Immunology of Charité University Medicine. All patients gave written informed consent and protocols were approved by the local ethics committee of Charité (vote # EA2/142/20). Bone marrow cells were used directly to perform short-term *ex vivo* treatments with CFZ and subasumstat as indicated.

Proteomics, Transcriptomics, Cell viability, Immunoblotting

Detailed procedures are provided in Supplementary Material and Methods.

Statistical analysis

Statistical analyses were done using GraphPad Prism (GraphPad Software, La Jolla, CA). Error bars shown in the figures represent the standard deviation (SD). For each experiment, the statistical test that was used is indicated in the figure legend.

RESULTS

The SUMO pathway is activated in proteasome inhibitor-resistant MM cells

Dysregulation of the SUMO pathway has been associated with aggressive cancer biology and poor prognosis [10,24]. Using publicly available transcriptome data of MM patient samples and healthy donors [30], we found increased expression of the SUMO core components *SAE1, UBA2, UBE2I, SUMO1, SUMO2* and *SUMO3* in MM patients compared to $CD138^+$ cells from healthy donors (Fig. S1A). We performed hierarchical clustering based on the SUMO core components on two different datasets[31,32], which revealed $SUMO^{high}$ and $SUMO^{low}$ subgroups (Fig. 1A, Fig. S1B). Here, $SUMO^{high}$ status was associated with significantly lower probability of overall survival (Fig. 1B, Fig. S1C). Additionally, gene expression of the two components of the heterodimeric SUMO activating E1 complex alone, *SAE1* and *UBA2*, was associated with significantly lower probability of overall survival, too (Fig. S1D). Furthermore, immunoblotting analysis revealed that primary MM patient cells showed increased protein SUMOylation status compared to $CD138^+$ cells of healthy donors (Fig. 1C and Fig. S1E). To further evaluate a potential dysregulation of the SUMO pathway during progression of MM, we interrogated transcriptome data from patients sequentially biopsied at first diagnosis and after relapse [33] (Table S1). Reactome database [34] signatures containing components of the SUMO machinery were significantly upregulated upon relapse, indicating a possible role for SUMOylation in MM progression and drug resistance (Fig. 1D).

To inform whether SUMO pathway activity was associated with resistance to proteasome inhibitor treatment, we next analyzed data from an RNAi screen that was designed to identify genes or proteins associated with resistance or sensitivity to CFZ [35]. Gene set enrichment analysis (GSEA) showed that interference with SUMOylation-associated genes increased sensitivity to CFZ treatment (Fig. 1E). AMO-1 and JJN3 MM cells that were rendered proteasome inhibitor-resistant through exposure to increasing CFZ concentrations showed enhanced levels of SUMOylated proteins (Fig. 1F, Fig. S2A).

In summary, these results point to a prominent role of SUMOylation in MM and link activity of the SUMO pathway to proteasome inhibitor resistance.

Synergy of SUMO and proteasome inhibition in MM cell lines

Since we identified high expression of components of the SUMO machinery and a hyperSUMO state in MM patient samples, we next tested the efficacy of the small molecule SUMO E1 inhibitor subasumstat in MM cell lines. Subasumstat potently inhibited SUMOylation, evidenced by the decrease of SUMO2/3 protein modification upon treatment of MM cells, and, accordingly, the pool of free SUMO2/3 increased (Fig. 2A). To test the growth inhibitory effect of subasumstat as single agent, we treated a panel of MM cell lines with different expression levels of the SUMO core machinery (Fig. 2B, 2C). Strikingly, JJN3 cells, which exhibited very high SUMOylation, were most sensitive to subasumstat treatment. Next, we investigated which proteins are differentially expressed and which signaling pathways cause the subasumstat mediated loss of viability in MM cells. We therefore generated a proteome of the p53 mutant cell line OPM2 after 16h subasumstat treatment and observed an induction of a DNA repair signature (Reactome)[34] and an apoptosis signature (C2 MSigDb)[36] (Fig. 2D).

Since increased SUMOylation was associated with proteasome inhibitor resistance (Fig. 1F), we set out to explore potential synergistic action between pharmacological inhibition of SUMOylation and the established proteasome inhibitors CFZ and bortezomib (BTZ). Applying sensitivity data to SynergyFinder [37] and CompuSyn (Chou-Talalay Method)[38], we identified dose-dependent synergistic effects in all tested MM cell lines after subasumstat and proteasome inhibitor combination treatment (Fig. 3A, 3B and Fig. S2B, S2C). Importantly, this effect was observed in *TP53* wild type, *TP53* mutant and *TP53* null cells (Fig. 2B, 3C Fig. S2D). Irrespective of efficacy of single CFZ or subasumstat treatment, all MM cell lines tested showed significant response to the combination treatment (Fig. 3C, S2E). Additionally, cell lines of other entities also displayed a synergistic effect except for the CML cell line K562 (Fig. S2F).

Resistance to proteasome inhibitors is a major clinical challenge in the treatment of MM patients[39]. To test if resistance to CFZ can be overcome by simultaneous subasumstat treatment, we combined subasumstat and CFZ in CFZ-resistant JJN3 and AMO1 cells (Fig. S2G). Here, we observed a significant drop in viability upon combination treatment (Fig. 3D). To investigate whether the uniform synergy observed upon subasumstat and proteasome inhibitor treatment was a class effect, we also tested combinations of subasumstat with other drugs used to treat MM, specifically dexamethasone and doxorubicin, and the IMiD pomalidomide. Here, results were more ambiguous, i.e. in some cell lines a subasumstat-based combination worked antagonistically and in others synergistically (Fig. S2H, I).

In summary, subasumstat single agent treatment reduces viability of MM cell lines and is highly synergistic with proteasome inhibitors independent of the cellular *TP53* status.

Subasumstat and CFZ combination treatment increases cellular stress response and apoptosis in MM cells

Having identified a synergistic effect between subasumstat and CFZ, we next aimed to investigate the underlying mechanism. We selected 3 cell lines with different expression of the SUMO core machinery and *p53* status (Fig. 2B, Fig. S2D): JJN3 cells are characterized by a high expression of SUMO core machinery genes and a deletion of the *p53* gene ($p53^{null}$); OPM2 cells show a low expression of SUMO core machinery genes and a dominant-negative $p53^{R175H}$ mutation ($p53^{mut}$); AMO1 display an intermediate expression of the SUMO core machinery and expresses wild type p53 ($p53^{wt}$). To globally analyze subasumstat- and CFZ-mediated changes in RNA and protein expression levels we performed RNA sequencing as well as quantitative proteomics after CFZ and subasumstat single and combination treatment (Fig. 4A and 4B). Transcriptome analysis indicated enriched apoptosis signatures in all 3 cell lines (Fig. 4A). In addition, activation of the unfolded protein response (UPR) and cellular stress response pathways were detected (Fig. S3A, S3B). In the combination treatments, significantly more

genes were regulated in comparison to the single treatments, supporting an increased cellular stress response (Fig. S3C). Quantitative proteomics analysis indicated CFZ-triggered ER stress and UPR response (Fig. 4B). We observed a significant upregulation of proteins involved in the UPR pathway in the combination treatment (Fig. 4C, Fig. S3D). In addition, other proteins involved in UPR (CREB3L2, DNAJB1, GADD45A) were also significantly upregulated upon combination treatment as compared to CFZ single treatment (Fig. S3C).

To compare the obtained proteome and transcriptome data, we filtered out proteins that are induced in all three cell lines (log2FC>0.5). In addition, we added genes that can be synergistically activated from the transcriptome data and compared which candidates can be induced at both the transcriptional and protein levels isolated. This comparison resulted in a total of 19 genes/proteins (Fig. 4D). These 19 candidates were then examined in all three indicated cell lines for their significant expression in the combination treatment. We identified 10 overlapping candidates, all of which are part of the UPR pathway and can be significantly induced synergistically (Fig. 4D).

Since we found that proteins of the DNA damage response (DDR) pathway as well as apoptosis-related proteins were upregulated in subasumstat treated cells (Fig. 2D), and we found a massive increase of the UPR (Fig. 4B-D), we performed western blots of selected markers for the DDR, the UPR, and apoptosis in JJN3, OPM2 and AMO1 cells (Fig. 4E). Indeed, combination treatment with CFZ and subasumstat clearly enhanced the effect on these processes in comparison to the single treatments, evidenced by induction of γH2AX (DDR), phosphorylation of CHK1 (DDR), XBP1 (UPR), cleaved PARP (apoptosis) and cleaved caspase 3 (apoptosis) (Fig. 4E). Annexin V staining revealed that dual subasumstat and CFZ treatment significantly increased the percentage of apoptotic cells when compared to the single treatments (Fig. 4F).

In summary, combination treatment with subasumstat and CFZ enhanced cellular stress responses and resulted in subsequent induction of cell death.

Sulfopin antagonizes subasumstat and CFZ treatment

The initiation of apoptosis occurs through various signaling pathways and is often regulated by members of the p53 protein family[13,40]. Since the OPM2 and JJN3 cell lines lack intact p53, but significant p53 hallmark signatures were observed in transcriptomic data of the combination treatments (Fig. 5A, Fig. S4A), we assumed a possible association of SUMOylation of p53 family regulators. One important regulator of the p53 family is the prolyl isomerase PIN1, which has been shown to be responsible for efficient promoter loading of p53 target genes [41]. In breast cancer cells, it has been shown that in the presence of mutant p53, the transcriptional function of p63 is regulated by PIN1 [42]. PIN1 SUMOylation is induced by proteotoxic stress (Fig. 5B) [43] and is associated with decreased activity [44]. Since we observed activation of p53 target genes (Fig. 5A, Fig. S4A) and this activation can be PIN1-dependently regulated, we investigated whether p53 target gene associated apoptosis could be abrogated by inhibition of PIN1. Therefore, we combined the specific PIN1 inhibitor sulfopin [45] with subasumstat. Indeed, we observed antagonistic effects in the combination treatments in OPM2 cells (Fig. 5C, D). Additionally, combination of subasumstat, carfilzomib and sulfopin could abrogate caspase 3 cleavage and diminished CHK1 phosphorylation (Fig. 5E, Fig. S4B).

Taken together, we show on the one hand that combination treatment of subasumstat and carfilzomib leads to a p53-associated response in p53 deficient cells. On the other hand, we show that PIN1 inhibition in combination with SUMO inhibitor treatment has an antagonistic effect and points to a previously unknown potential resistance mechanism in MM.

Combined subasumstat and CFZ treatment inhibits MM tumor growth *in vivo* and induces apoptosis in primary MM cells

We next investigate the effects of dual proteasome and SUMO inhibition in JJN3 and OPM2 *in vivo* xenograft models. Whereas sub lethal doses of single CFZ and single subasumstat treatment showed only a limited effect on tumor growth, significantly reduced tumor volumes were observed in the combination treatment group (Fig. 6A). Of note, subasumstat treatment did not show measurable side effects upon short-term treatment, as judged by body weight assessment of the mice. Treatment with CFZ led to weight loss in a number of mice, which seemed not enhanced by the combination with subasumstat (Fig. 6B).

We then treated primary MM patient cells (Table S2) *ex vivo* and determined the fraction of apoptotic cells by Annexin V-FITC staining after 24 hours in $CD38^{+}/CD138^{+}$ MM cells (Fig. S5). In five out of seven patient samples (relapsed and newly diagnosed) measured, cells treated with both CFZ and subasumstat showed an increased apoptosis in comparison to the single treatments (Fig. 6C).

In summary, *in vivo* treatment of MM xenografts confirmed efficacy of the subasumstat and CFZ combination and we nominate this combination for further evaluation in prospective clinical trials.

DISCUSSION

SUMOylation is a posttranslational modification that affects oncogenic pathways and acts as a safeguard to maintain cellular functions in cancers cells [11]. This has led to the development of specific SUMO inhibitors like subasumstat which has entered clinical trials [27]. Here, we show that SUMOylation is hyperactivated in proteasome inhibitor-resistant patient MM samples and that high expression of core components of the SUMO machinery correlate with inferior prognosis. We demonstrate that combining the SUMO inhibitor subasumstat with CFZ efficiently kills MM cells *in vitro* and blocks tumor growth *in vivo*.

Proteasome inhibition is an effective and universally applied therapy in MM and has become a backbone of MM treatment. Inhibition of the proteasome results in proteotoxic and genotoxic

stress, driving apoptosis of MM cells [39]. We show here that the combination of subasumstat and CFZ induced elevated UPR and DDR which ultimately triggered apoptosis in MM cells. Since loss of p53 function is linked to drug resistance and disease progression [46], we investigated cell lines with genetic alterations in *p53* and found that the combination of CFZ and subasumstat consistently induced apoptosis irrespective of the *p53* status. SUMO conjugation is typically triggered in response to genotoxic or proteotoxic stress indicating that the pathway contributes to cellular stress resilience [47-49]. Therefore, we postulate that inhibition of SUMOylation exacerbates the apoptotic effects of proteasome inhibition in MM cells, culminating in synergistic lethality.

Molecular homeostasis from apoptosis-associated proteins and its PTMs is critical for tumor cell survival and therapy resistance. Despite a lack of p53 function, we observed p53 signatures in all cell models tested. All p53 family members (p53, p63 and p73) are able to bind p53 consensus sequences to induce p53 target gene expression [40]. *TP53* mutations are seen in only 13% of MM patients, and are associated with worse overall survival [50]. Activity of p53 family proteins is regulated by different factors affecting PTM. The prolyl isomerase PIN1 has become a focus of cancer research in recent years and a first specific PIN1 inhibitor (sulfopin) with anti-tumor activity in preclinical *in vivo* models could be developed [45]. PIN1 causes stabilization of phosphorylated target proteins (incl. p53 family proteins) through proline isomerization [51]. Depending on the cellular p53 status, PIN1 appears to regulate p63 activity in variable ways [42]. PIN1 is required for efficient binding of p53 on target genes [41]. Additionally, PIN1 enhances TAp63a mediated apoptosis [52] and is required for p73 dependent apoptosis [53]. In the $p53^{mut}$ cell line OPM2, we found that PIN1 inhibition abrogates the apoptotic effects of subasumstat. This points to a previously unknown resistance mechanism that may be relevant for future treatment strategies in MM, but needs to be further investigated in future studies.

Resistance to proteasome inhibition arises almost invariably after prolonged treatment and is a major limitation in the management of MM patients. Therefore, there is an urgent clinical need of

novel therapies restoring proteasome inhibitor sensitivity in relapsed patients [5]. For instance, the associated “BRCAness” induced by proteasome inhibition may be exploited by combination with additional DDR inhibiting agents like PARP inhibitors [54]. We detected increased levels of SUMOylation in MM cells that were resistant to proteasome inhibition. This is in line with the finding that inhibition of SENP2, a negative modulator of SUMOylation, promotes BTZ resistance and increases SUMOylation [55]. Fine-tuning of intracellular SUMOylation by the deSUMOylase SENP6 was recently shown to determine chromatin organization and DDR [56], and alterations in SENP6 contributed to increased genomic instability and lymphomagenesis in B-cell lymphoma [57]. Since SUMOylation of proteins regulates the DDR and other cellular stress response proteins [47,48], we hypothesize that MM cells activate the SUMO machinery to cope with proteasome inhibitor-induced proteotoxic and genotoxic stress, resulting in proteasome inhibitor tolerance. As a consequence, concurrent inhibition of SUMOylation could effectively counter proteasome inhibitor resistance, suggesting that SUMO inhibition could be effective as a combination therapy in patients who have developed resistance to proteasome inhibition.

Dexamethasone-resistant MM cells can be re-sensitized when combining dexamethasone with subasumstat [58], supporting the notion of SUMOylation as a safeguard to buffer the cellular stress response. Although we observed inconsistent results in terms of synergy when combining dexamethasone with subasumstat, this shows that subasumstat may have beneficial effects beyond the combination with proteasome inhibitors, supporting its potential for the management of MM. SUMO inhibition counteracts not only the tumor cell-intrinsic induction of cell death [59], but also immune evasion, e.g. by activating cytotoxic T cells [60]. This leads to massive tumor cell reduction in pancreatic ductal adenocarcinoma [61], which is known as an immune desert, making this therapy extremely promising. Further studies will show whether such a mode of action could also be transferred to MM. Additionally, by cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) we recently showed that subasumstat had only moderate effects on physiological cell populations and displays favorable tolerability [62].

In summary, we find that combination of the SUMO inhibitor subasumstat and proteasome inhibitors acts synergistically in MM models by interfering with the cellular stress response, which ultimately triggers intrinsic tumor cell death. A combination of classical proteasome inhibitor-containing regimens with inhibition of the SUMO pathway could be a promising approach to address the frequently emerging therapy resistance in MM.

AUTHOR CONTRIBUTIONS

Conception and design of the study: G.H., F.B., U.K., M.W.; Acquisition of data and/or analysis and interpretation of data: G.H., F.B., M.H., U.P., Ma.H., J.B., M.K., I.S., S.B., E.R., A.M., Y.N., U.D., D.L., S.L., J.K., M.J., A.N., M.S., P.M., S.M, Fl.B., Ja.K., U.K, M.W. Drafting of the manuscript: G.H., F.B., U.K., M.W.; All authors revised the manuscript for important intellectual content and approved the final version submitted for publication.

COMPETING INTERESTS

U.K. received speaker honorary/adivisory fees from Roche, Janssen-Cilag, Takeda, BMS, Gilead, Hexal, Pfizer, Astra-Zeneca, Pentixapharm, Amgen, Novartis, MSD. U.K. received clinical study support from Celgene, Takeda, BMS, Roche, Astra-Zeneka, Novartis, MSD, Janssen-Cilag, Pfizer. Takeda Pharmaceuticals provided subasumstat (TAK-981) for research purposes. F.B. received honoraria and research funding from BMS/Celgene. The other authors declare that no conflicts of interest exist.

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551

552 **FIGURE LEGENDS**

553 **Figure 1. SUMO pathway is activated in multiple myeloma and associated with poor**
554 **prognosis.**

555 (**A**) Heatmap and hierarchical clustering of the SUMO core components *SAE1, UBA2, UBE2I,*
556 *SUMO1, SUMO2* and *SUMO3* derived from transcriptome data from n=768 multiple myeloma
557 patients of MMRF-CoMMpass data with indicated clustering into $SUMO^{high}$ and $SUMO^{low}$ groups.
558 (**B**) Kaplan-Meier curves for probability of survival of $SUMO^{high}$ and $SUMO^{low}$ groups as
559 described in (A). Curve comparison by log-rank test with indicated p-value.
560 (**C**) Immunoblot depicting expression of SUMO2/3 and SUMO1 in healthy $CD138^+$ cells and
561 $CD138^+$ MM cells, which have been isolated from human specimen by MACS. β-Actin served as
562 loading control.
563 (**D**) Top: a cohort of n=13 MM patients, biopsied at diagnosis, subsequently treated and biopsied
564 again after disease relapse. Biopsied material was subsequently used for transcriptome profiling.
565 Bottom: GSEA using the FGSEA package reveals enrichment of indicated Reactome
566 SUMOylation signatures of relapsed vs. newly diagnosed MM patients. Adjusted p-value (false
567 discovery rate) *p<0.05, **p<0.01; NES: normalized enrichment score.
568 (**E**) Top: schematic depiction of the CFZ resistance RNAi resistance screen performed by
569 Acosta-Alvear *et al*. [35]. Bottom: Enrichment indicated SUMOylation signatures upregulated in
570 CFZ-resistant compared to CFZ-sensitive U266 cells, determined by FGSEA using the
571 Reactome knowledgebase. Adjusted p-value (false discovery rate) **p<0.01, ***p<0.001; NES
572 normalized enrichment score.
573 (**F**) Top: schematic depiction of the applied strategy to cultivate CFZ-resistance MM cells. CFZ-
574 sensitive cells were cultured in CFZ-containing medium, slowly increasing the concentration (1-2
575 weeks) CFZ until cells became resistant (total duration > 12 weeks). Bottom: immunoblot
576 showing expression of SUMO2/3 and SUMO1 in AMO1 and JJN3 CFZ-resistant cells (R) in
577 comparison to AMO1 and JJN3 parental (P) cells. Parental cells were treated with 1μM

subasumstat and/or 12nM CFZ for 4h; DMSO served as vehicle control (-).Resistant cells were cultured in presence of 12nM CFZ and co-treated with 1µM subasumstat as indicated. Co-treatment with subasumstat depletes SUMOylation.

Figure 2. Subasumstat induces cell death in multiple myelma cell lines.

(**A**) Treatment of five MM cell lines with 250 nM subasumstat (Suba) inhibits 2/3 SUMOylation and increases the pool of free SUMO 2/3 compared to DMSO treated control cells.

(**B**) Expression of indicated core SUMOylation machinery genes in a panel of MM cell lines (data derived from depmap.org). TP53 status is indicated for each cell line.

(**C**) Subasumstat monotreatment on a panel of five multiple myeloma cells lines. Cells were treated for 3 days with different concentrations of subasumstat, subsequently viability was measured. Results of three independent measurements are shown.

(**D**) Top: OPM2 cells were treated for 16 hours with DMSO or subasumstat and analyzed by quantitative proteomics. Bottom: FGSEA plots of quantitative proteomics data. DNA repair and apoptosis proteins are significantly upregulated in subasumstat treated OPM2 cells over DMSO treated control cells.

Figure 3. Combined SUMO and proteasome inhibition acts synergistically in MM cell lines.

(**A**) Combination of subasumstat with proteasome inhibitors BTZ and CFZ has a synergistic effect on the viability of indicated MM cells. Synergy score has been determined by SynergyFinder using the Zero Interaction Potency method (ZIP). The presented ZIP synergy scores are the average of n=3 independent biological experiments with n=3 technical replicates. Cells were treated for with single and combination treatments using a 4x6 matrix.

(**B**) Landscape plots depicting the synergistic area of concentrations for subasumstat and CFZ combination treatment in JJN3, OPM2 and AMO1 cells. Cells were treated for 72 hours with the

indicated concentrations (4x6 matrix) of subasumstat and CFZ and cell viability was measured by CellTiterGlo. Subsequently, cell viability data was used to generate landscape-plots using SynergyFinder.

(**C**) Bar diagrams showing the effect on cell viability after 72 hours of treatment with CFZ, subasumstat and the combination thereof in JJN3 (2nM CFZ, 200nM Suba), OPM2 (2nM CFZ, 200nM Suba) and AMO1 (4nM CFZ, 200nM Suba) cells. Statistical testing was determined by one-way ANOVA. *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001.

(**D**) Bar diagram showing the effect on cell viability after 72 hours of treatment with 6 nM (JJN3) or 12 nM (AMO1) CFZ, 200 nM (JJN3) or 1μM (AMO1) subasumstat and the combination thereof in parental (P) and CFZ-resistant (R) cells. Statistical testing was determined by one-way ANOVA. ****: p≤0.0001.

Figure 4. Subasumstat and CFZ combination increases cellular stress response and apoptosis.

(**A**) Top: OPM2, JJN3 and AMO1 cells were treated for 4 hours with DMSO, 250 nM subasumstat, 5 nM CFZ or the combination thereof and subsequently analyzed by RNA sequencing (n=3 biological replicates). Bottom: GSEA analysis by the FGSEA package of the combination treatment (4 h) versus DMSO control shows enriched apoptosis signatures of the Hallmark set from the molecular signature database. FGSEA p-values and adjusted p-values (false discovery rate) are indicated

(**B**) Top: OPM2 cells were treated for 4 hours with DMSO, 250 nM subasumstat, 5 nM CFZ or the combination thereof and analyzed by quantitative proteomics. Bottom: Enriched Gene Ontology signatures in CFZ vs DMSO treated OPM2 cells are displayed.

(**C**) Graphical representation of quantitative proteomics data of OPM2 cells that are treated for 4 hours with a combination of subasumstat and CFZ over DMSO treated control cells. Proteins are

ranked in a volcano plot according to their statistical *P*-value (y-axis) and their relative abundance ratio (log_2 fold change, x-axis).

(**D**) Top: Significantly synergistically induced genes from transcriptomic data (log2FC>0; Fig. 4A) and proteins significantly induced in the combination treatment of subasumstat and CFZ (log2FC>0.5; indicated in Fig. 4C) were extracted and matching genes and proteins (n=19) were subsequently analyzed in all three indicated cell lines. Bottom: Heatmap of the fold change of treatment versus control of the identified indicated mRNA expression in the cell lines JJN3, OPM2 and AMO1.

(**E**) Immunoblots on JJN3, OPM2 and AMO1 cell lysates treated for 4 hours with 250 nM subasumstat, 5 nM CFZ or the combination thereof. Protein expression of gH2AX (S139), p-CHK1 (S345), pan-CHK1 to determine DNA damage response, XBP1 (unfolded protein response) and the apoptosis markers cleaved PARP and cleaved caspase 3 have been analyzed. β-Actin served as loading control.

(**F**) FACS analysis of JJN3, OPM2 and AMO1 cells stained with Annexin V and DAPI to measure apoptosis after treatment for 48 hours treatment with 250 nM subasumstat, 5 nM CFZ or the combination thereof. p-values were determined by one-way ANOVA. *p≤0.05; **p≤0.01; ***p≤0.001

Figure 5. Induction of cell death upon combined SUMO / proteasome inhibition is associated with PIN1.

(**A**) OPM2 cells were treated for 4 hours with DMSO, 250 nM subasumstat, 5 nM CFZ or the combination thereof and subsequently analyzed by RNA sequencing. GSEA analysis by the FGSEA package of the combination treatment (4 h) versus DMSO control shows enriched p53 signatures of the Hallmark set from the molecular signature database. FGSEA p-values and adjusted p-values (false discovery rate) are indicated

(**B**) PIN1 SUMOylation upon 8h of 10μM MG132 treatment or 1h heat shock versus control with indicated p-values. Data was retrieved from the qPTM database http://qptm.omicsbio.info/ from the study.

(**C**) Determination of viability of OPM2 cells, treated with 2.5μM subasumstat or 1μM sulfopin or combination of both for 72 hours. Cell viability was measured by CellTiterGlo.

(**D**) Landscape plots depicting the antagonistic/additive or synergistic area of concentrations for subasumstat in combination with sulfopin treatment in OPM2 cells. Cells were treated for 72 hours with the indicated concentrations (4x6 matrix) of subasumstat and sulfopin and cell viability was measured by CellTiterGlo. Subsequently, cell viability data was used to generate landscape-plots using SynergyFinder.

(**E**) Immunoblots on OPM2 cell lysates treated for 4 hours with 250 nM subasumstat, 5 nM CFZ, 2μM sulfopin or the combination thereof. Protein expression of indicated proteins were detected using specific antibodies as indicated in the methods section. Actin served as loading control.

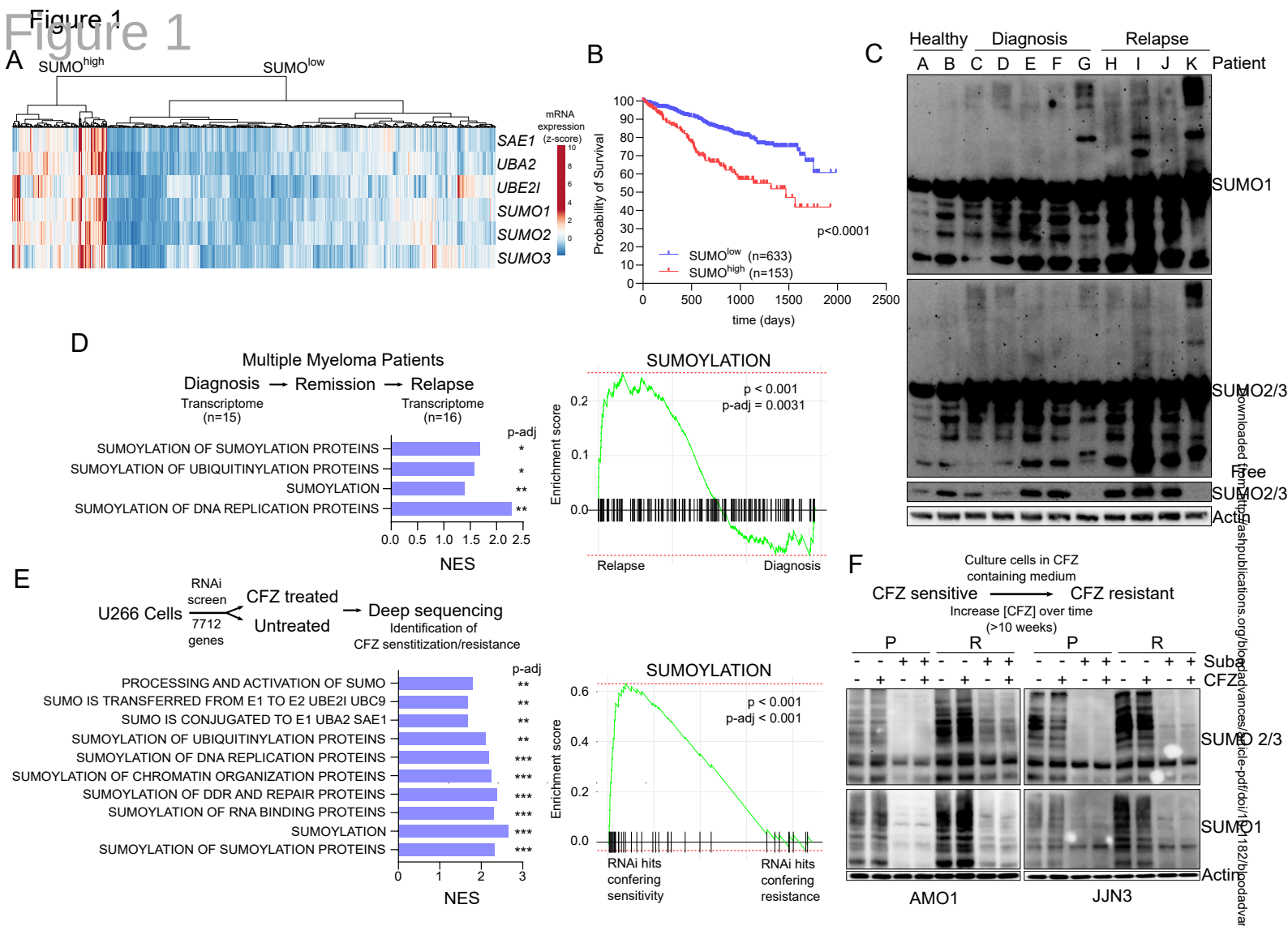
Figure 6. Efficacy of combined SUMO and proteasome inhibition in vivo and in primary multiple myeloma cells.

(**A**) Average tumor volume over time in nude mice injected with $1x10^7$ JJN3 or OPM2 cells. After tumor engraftment, mice were treated with either vehicle, subasumstat (25 mg/kg), CFZ (2 mg/kg) or the combination thereof for 7 days. p-values were determined by unpaired T-test. (*p≤0.05; **p≤0.01).

(**B**) Histogram showing the number of mice that lost >10% body weight (but less than 20%, which was the exclusion criterion) for each treatment group during the *in vivo* xenograft experiment.

(**C**) Bar diagram of Annexin V staining of five primary MM patient samples treated with DMSO, 250 nM subasumstat, 5 nM CFZ or the combination thereof.

Figure 1



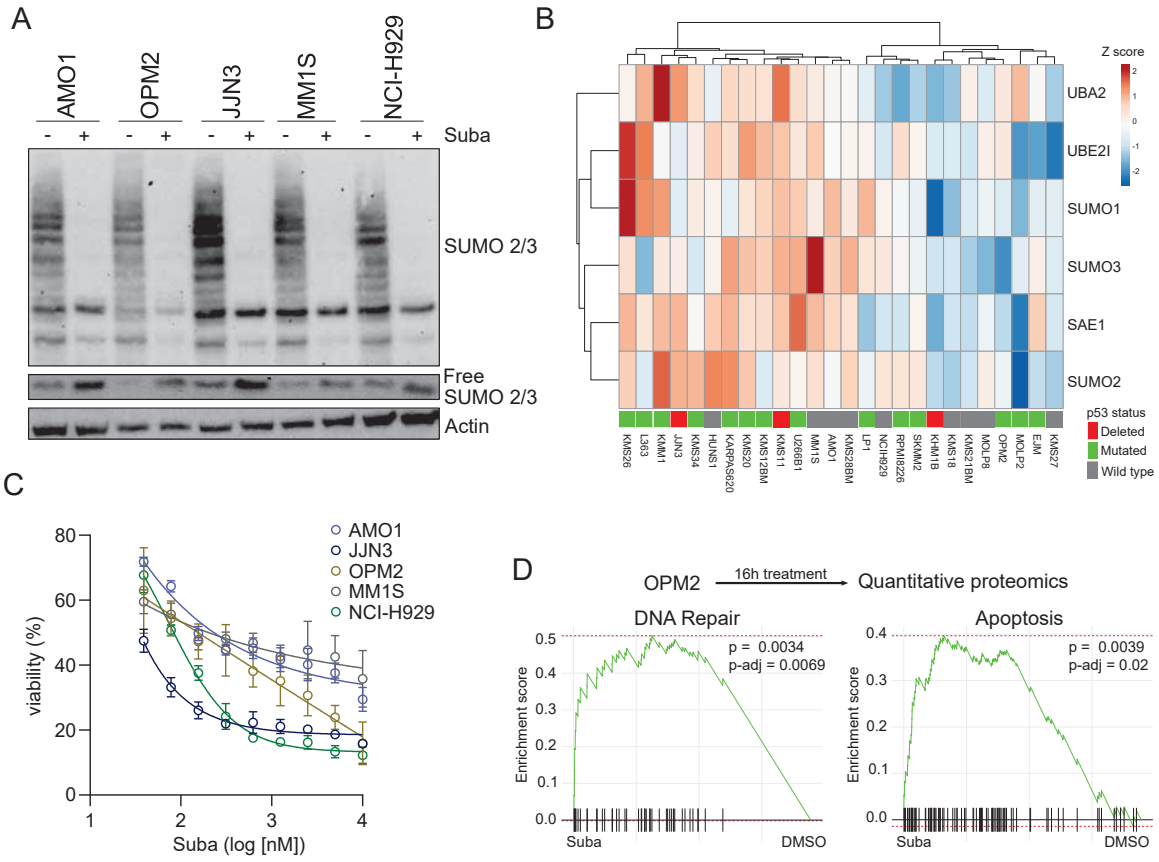
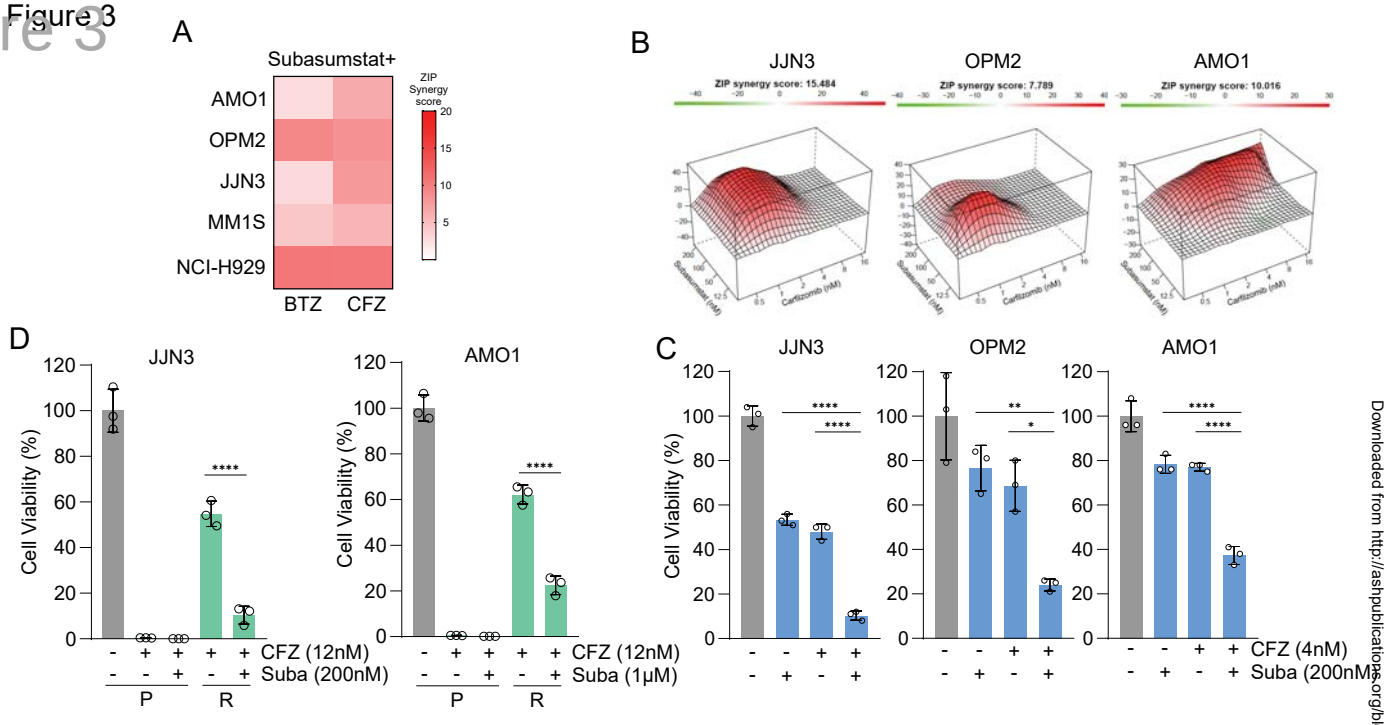
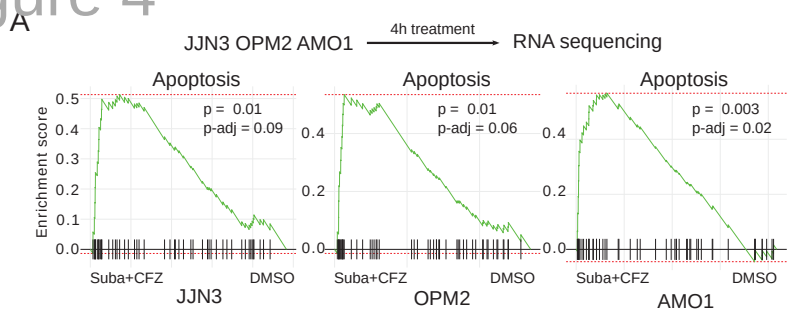
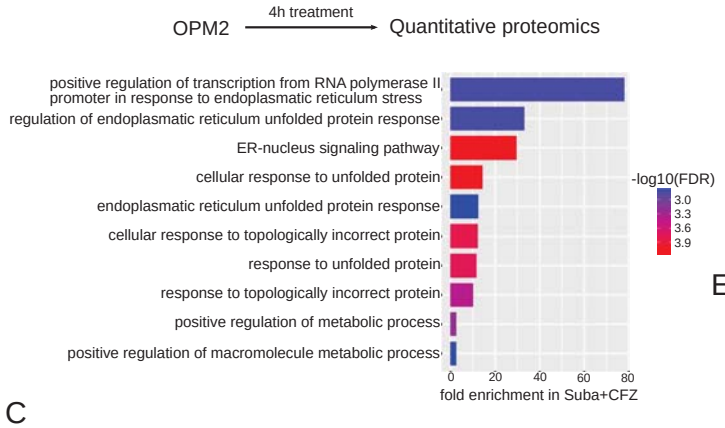
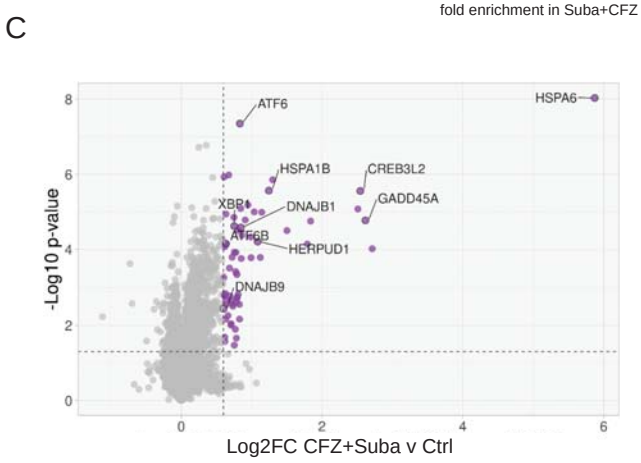


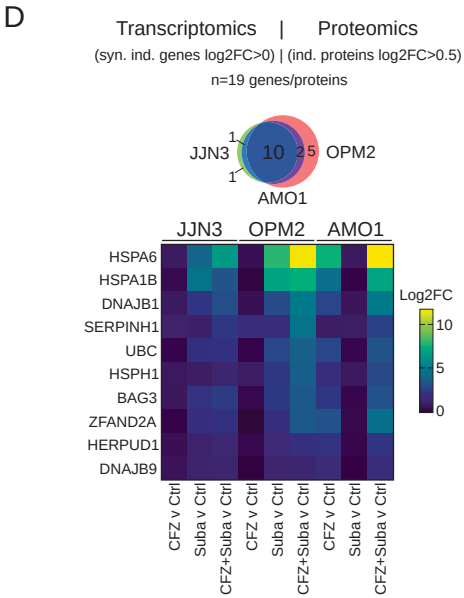
Figure 3

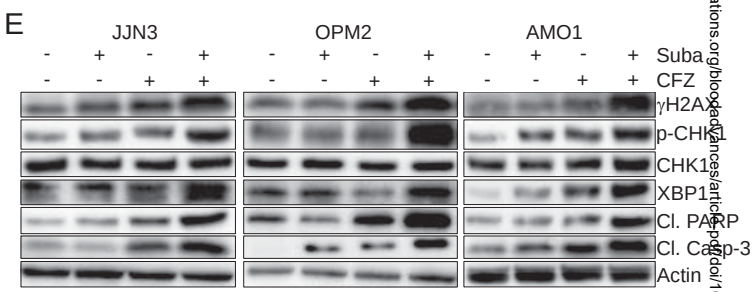












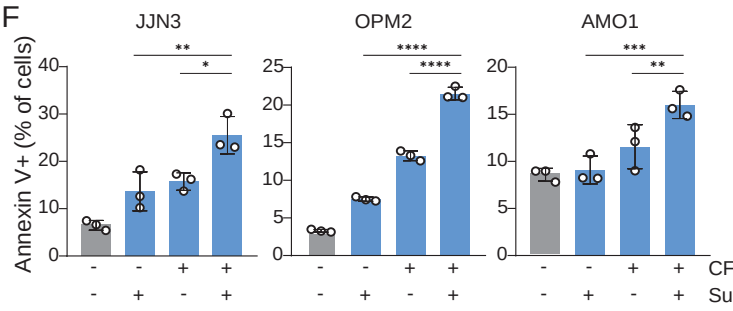


Figure 5

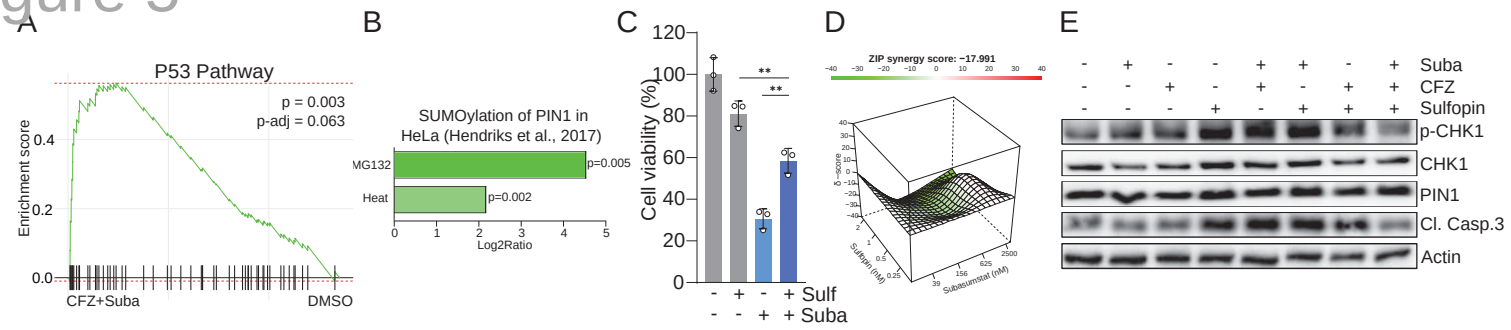


Figure 6