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USP8 controls proteostasis pathways in B cells and multiple myeloma

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Ubiquitin-specific protease 8 (USP8) plays a pivotal role in the regulation of endosomal and lysosomal trafficking and is critically involved in the pathogenesis of various tumor entities. USP8 represents a vulnerability gene in multiple myeloma (MM), suggesting a functional role in the B- and plasma cell compartment. Here we analyzed mice with stage-specific *Usp8* deletion during B-cell development and investigated its role in patient-derived MM cells that are sensitive or resistant to the proteasome inhibitor Bortezomib (BTZ) using USP8 depletion and treatment with DUB-IN-2, a reported USP8 inhibitor. *Usp8* depletion in *Usp8^{fl/fl}Cd19-Cre* mice affected B-cell survival and development favoring immature, innate-like B cells, and germinal center and plasma cells, while also elevating immune-responses and causing Roquin depletion. Cells expressing catalytically inactive USP8 accumulated proteins modified with mixed ubiquitin/NEDD8 chains indicative of proteotoxic stress, which we identified as preferred USP8 substrates. In MM cells, USP8 knockdown reduced survival via lysosomal dysfunction. In contrast, DUB-IN-2 induced an enhanced ER stress response to treatment with BTZ questioning DUB-IN-2 function as a USP8 inhibitor, as confirmed by biochemical analysis. Thus, our results highlight the therapeutic potential of targeting USP8 and identify the combination of DUB-IN-2 and BTZ as a novel strategy for treating BTZ-resistant MM.

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INTRODUCTION

Targeting the ubiquitin proteasome system (UPS) represents an effective strategy in B-cell malignancies such as multiple myeloma (MM) [1]. Although proteasome inhibition by bortezomib (BTZ, Velcade®) or Carfilzomib (CFZ, Kyprolis®) improve patient outcome, relapse is common. More than 90 deubiquitinating enzymes (DUBs), including ~50 USPs, counteract ubiquitylation [2]. USP8 has a multi-domain structure and regulates endosomal sorting and transmembrane protein stability through interactions with endosomal sorting complexes required for transport (ESCRT) components, including STAM2 and charged multivesicular body (CHMP) proteins [3]. USP8's C-terminal catalytic domain is inhibited by phosphorylation-dependent 14-3-3 binding to its adjacent motif [4, 5]. Somatic mutations affecting the 14-3-3 BM

cause Cushing's disease (CD) leading to EGFR stabilization in ACTH-secreting pituitary adenomas [6, 7]. USP8 was also associated with autophagy and mitophagy regulation [3, 8, 9], and with the progression of many types of cancer including effects on the tumor microenvironment [10–14]. Multiple studies on USP8 in cancer have used DUB-IN-2, a reported USP8 inhibitor [10–12, 15–17], but its mechanism of action, specificity and kinetic parameters remain unclear [18, 19].

In T cells, USP8 interacts with GRB2-related adaptor protein downstream of Shc (GADS) and controls stability of the ESCRT component CHMP5 to stabilize the anti-apoptotic Bcl2 protein as a prerequisite for the positive selection of thymocytes [20]. T-cell-specific USP8 deletion impairs T-cell expansion and leads to spontaneous colitis with dysfunctional regulatory T cells [21].

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Ubiquitylation and deubiquitylation also regulate B-cell activation, proliferation, apoptosis, and their differentiation and function in innate and humoral immune responses [22]. USP8 was identified as a stimulation-dependent component of the B-cell receptor signalosome [23]. Moreover, an RNAi lethality screen identified *Usp8* as a key gene with increased vulnerability in MM compared to non-MM cells [24]. MM cells, which represent malignant plasma cells, have high protein synthesis rates, depend on UPS-mediated turnover, and are highly sensitive to disruptions in protein homeostasis. Inhibiting USPs like USP7, USP9X, and USP14 induces apoptosis in MM cells, underscoring the therapeutic potential of USP inhibitors [25–27]. Despite links to B-cell signaling and MM survival, USP8's role in B-cell development, immunity, and MM pathogenesis remains unclear.

Here, we investigate the role of USP8 in B-cell development and multiple myeloma (MM), including proteasome inhibitor-resistant disease. Using stage-specific USP8 deletion models, we uncover essential functions of USP8 in early B-cell development and immune homeostasis. Mechanistically, USP8 regulates proteotoxic stress responses linked to ubiquitin signaling. While DUB-IN-2 [18] resensitizes BTZ-resistant MM cells via ER stress, USP8 knockdown reduces survival independently of BTZ by inducing lysosomal dysfunction. Our findings highlight USP8 inhibition and DUB-IN-2/BTZ combination as potential strategies against BTZ-resistant MM, with DUB-IN-2 acting via USP8-independent effects.

METHODS

Supplementary Materials and Methods are provided in the Supplementary Data file on the Leukemia website.

Cell preparation and flow cytometry

Spleens, lymph nodes, and thymi were processed into single-cell suspensions in FACS buffer (PBS supplemented with 1–2% FCS). Bone marrow cells were obtained from tibias and femurs by flushing with FACS buffer. Peritoneal cavity cells were harvested post-euthanasia by i.p. injection of FACS buffer followed by gentle massage. Splenocytes and BM cells were depleted of red blood cells using RBC lysis buffer (Sigma or BioLegend). Cell suspensions were incubated with mouse Fc Block (BD or BioLegend) prior to staining. B cells were isolated from spleen using biotinylated anti-CD19 antibody (clone ID3, BD Pharmingen) or the BD IMag™ Biotinylated Mouse B Lymphocyte Enrichment Cocktail in combination with Streptavidin Particles Plus. T cells were isolated using the BD IMag™ Biotinylated Mouse CD4 T lymphocyte enrichment Cocktail with Streptavidin Particles Plus. DAPI or Zombie UV fixable viability dye (Biolegend) was used for live/dead discrimination. Surface and intracellular marker expression was analyzed using flow cytometers (Becton Dickinson FACSCalibur, FACSCanto II, or LSRFortessa SORP) and FlowJo software. Annexin V/PI staining was performed using the eBioscience Annexin V apoptosis detection kit (APC).

Cell culture, proliferation, activation, and viability

Purified B cells (5×10^4 /well) were stimulated with anti-IgM F(ab')₂ ($5 \mu\text{g ml}^{-1}$, 115-006-020, Jackson Immuno Research) $\pm$ anti-CD40 ($5 \mu\text{g ml}^{-1}$, 553787, BD Pharmingen), 100 ng/ml MPL-A (Invivogen) or 100 nM CpG (ODN 1668, Invivogen). Proliferation was measured after 36 h using a BrdU ELISA (Roche). For short-term stimulation, B cells were starved for 2 h in RPMI 1640 with 0.2% bovine serum albumin (no FCS), preincubated with anti-IgM ($10 \mu\text{g ml}^{-1}$) for 30 min on ice, and stimulated at 37 °C. T cells were starved for 2 h in RPMI 1640 without FCS, pre-incubated with soluble anti-mouse CD3 ϵ ($10 \mu\text{g ml}^{-1}$, 145-2C11, eBioscience) and anti-mouse CD28 ($2 \mu\text{g ml}^{-1}$, 37.51, eBioscience) for 30 min on ice, and stimulated at 37 °C with anti-hamster IgG1 ($5 \mu\text{g ml}^{-1}$, G94-56, BD Pharmingen). Ex vivo B cell expansion was performed using murine IL-4 (100 u ml^{-1} , Peprotech) and anti-mouse CD40 ($5 \mu\text{g ml}^{-1}$, HM40-3, Biolegend), or anti-IgM F(ab')₂ fragment ($5 \mu\text{g ml}^{-1}$) plus anti-CD40 ($2.5 \mu\text{g ml}^{-1}$). OHT ($1 \mu\text{M}$, Sigma) was added 24 h after isolation. DUB-IN-2 ($25 \mu\text{M}$, MedChemExpress), BTZ (5 to 25 nM , Selleckchem), CFZ (3 nM , MedChemExpress), and OTUB1/USP8 inhibitor 61 (Aobious) were added as indicated. Viability was assessed using the XTT cell proliferation kit II (Roche Diagnostics) and normalized to controls.

Cell lines, expression constructs, transfection, and inhibitor treatment

NP-specific mIgM-BCR-expressing transfectant K46L μm cells derived from the Balb C mouse B-cell lymphoma line K46 [28, 29] were obtained from W. Schamel. The human MM cell lines KMS-18 and KMS-27, as well as their BTZ resistant derivatives (T21A and A49V) expressing mutant PSMB5, have been described previously [30]. Cell lines were routinely tested to avoid mycoplasma contamination. Human and mouse USP8, C-terminally FLAG-tagged, were expressed using pFLAG-CMV5a and the retroviral expression vector MIEG3, respectively. Retroviral particles were generated in Platinum-E packaging cells using standard protocols. Mammalian expression vectors encoding HA-NEDD8 and HA-SEN8 were obtained from Addgene. Point mutations were introduced by site-directed mutagenesis using the QuikChange system (Stratagene). HEK293T cells were transfected with FuGENE HD (Promega) and harvested 36 h post transfection. MLN4924 (NAE inhibitor; LKT Laboratories), MLN7243 (UBA1 inhibitor; MedChemExpress), folimycin (MedChemExpress), bortezomib (BTZ; Selleckchem), and DUB-IN-2 (MedChemExpress) were used as indicated. ON-TARGETplus SMARTpool siRNA directed against human USP8 and CHOP, individual siRNAs directed against USP8 (Target Sequence siRNA_1: GGCAAGC-CAUUUAAGAUUA; siRNA_2: CCACUAGCAUCCACAAGUA), and a non-targeting (NT) control pool were purchased from Dharmacon. siRNA transfections were performed using Lipofectamine® RNAiMAX. For rescue experiments, the siRNA_2 target sequence in USP8 was mutated to CCCUAGCUUCCACAAGUA.

RESULTS

USP8 is essential for B-cell development

USP8 is critical for T-cell homeostasis where GADS acts as a binding partner [21]. However, B lymphocytes express only marginal levels of GADS (Fig. S1A), suggesting a different mechanism. To study USP8 function in B cells, we generated *Usp8^{fl/fl}Cd19-Cre* mice, which delete *Usp8* from the pre-B-cell stage, and compared them with *Usp8^{fl/fl}* controls (Fig. S1B). Residual USP8 protein in splenic B cells indicates a potential survival advantage of cells with incomplete deletion (Fig. S1C).

In contrast to T cells, *Usp8^{fl/fl}Cd19-Cre* mice exhibited reduced frequencies and numbers of splenic CD19⁺ B cells (Fig. 1A), with a stronger reduction in follicular than in marginal zone B cells (Fig. 1B), consistent with impaired development of mature (IgD⁺) splenic B cells (Fig. 1C). Similarly, percentages of CD19⁺ and mature IgM⁺IgD⁺ BM B cells were reduced, whereas early BM B cells were unaffected (Fig. 1D). Transitional B-cell development was impaired from the T2 stage onward (Fig. 1E). These findings were confirmed using *Usp8^{fl/+}Cd19-Cre* controls (Fig. S1D). B1 subsets showed a trend toward reduced numbers but increased frequencies within the IgM⁺ compartment (Fig. S1E). Splenic architecture reflected these defects, with reduced B220⁺ areas and primary follicles (FO), while T-cell zones remained unaffected (Fig. S1F). USP8-deficient B cells displayed impaired proliferation upon BCR or TLR stimulation, paralleled by reduced Ki67⁺ cells in vivo (Fig. S1G, H). To assess earlier developmental requirements, we generated *Usp8^{fl/fl}mb1-Cre* mice, which delete *Usp8* from the pro B-cell stage onward [31]. These mice exhibited a developmental block at the pre-B-cell stage, resulting in the absence of peripheral B cells (Fig. S1I).

Together, these data demonstrate that USP8 is essential for B-cell development at multiple stages.

Elevated Ig responses and robust PC numbers in *Usp8^{fl/fl}Cd19-Cre* mice

To further characterize consequences of USP8 depletion on B-cell development, we performed single cell RNA-seq analysis of splenic B cells from *Usp8^{fl/fl}* and *Usp8^{fl/fl}Cd19-Cre* mice. Clustering confirmed reduced follicular and transitional B cells, alongside relatively increased GC-like B cells and PCs (Figs. 1F, S2A–C). Total GC B-cell and PC numbers remained similar, but CD19⁺ PCs were underrepresented, suggesting a survival disadvantage of cells with efficient *Cd19-Cre*-mediated *Usp8* deletion (Fig. 1G). Differential

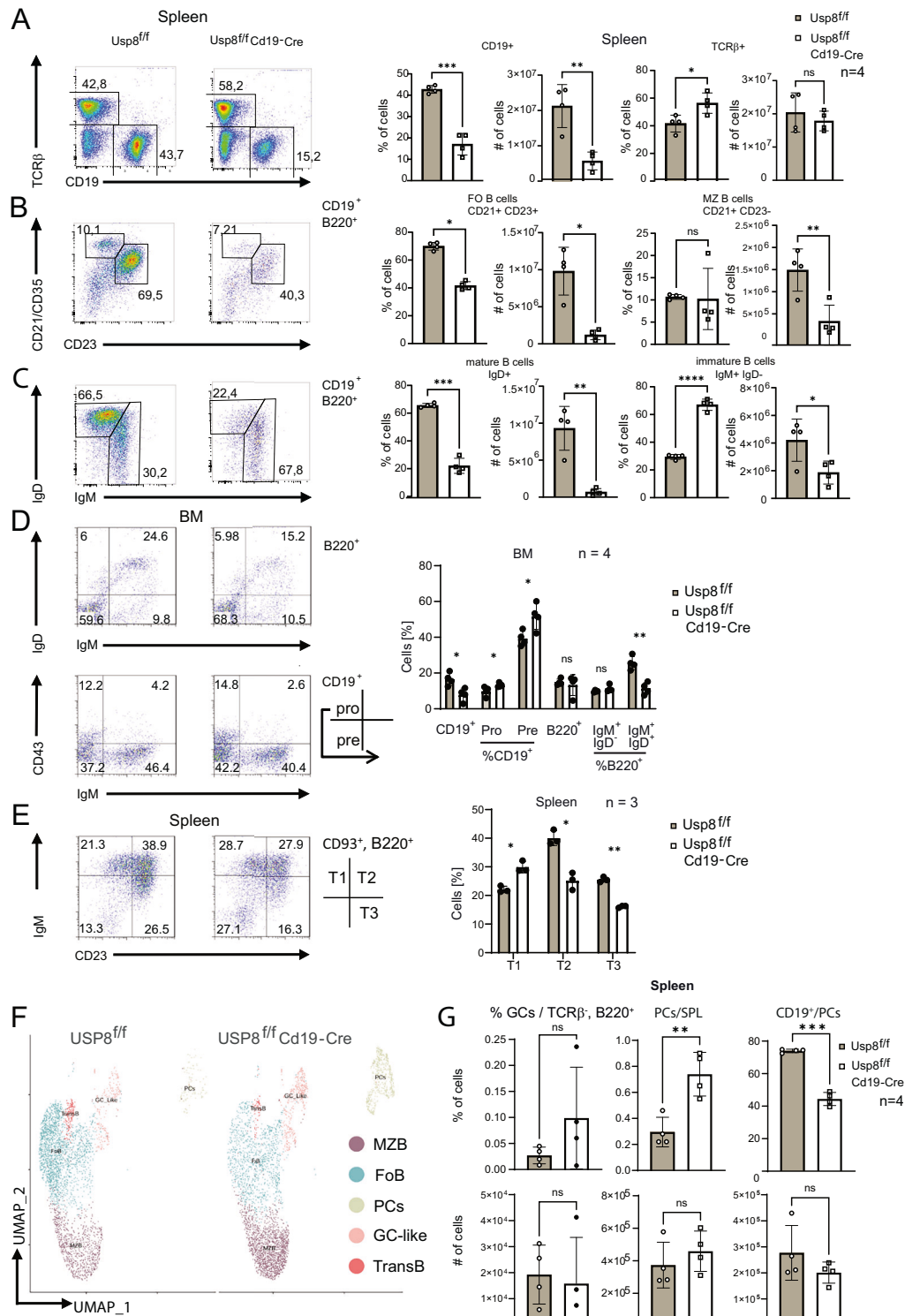
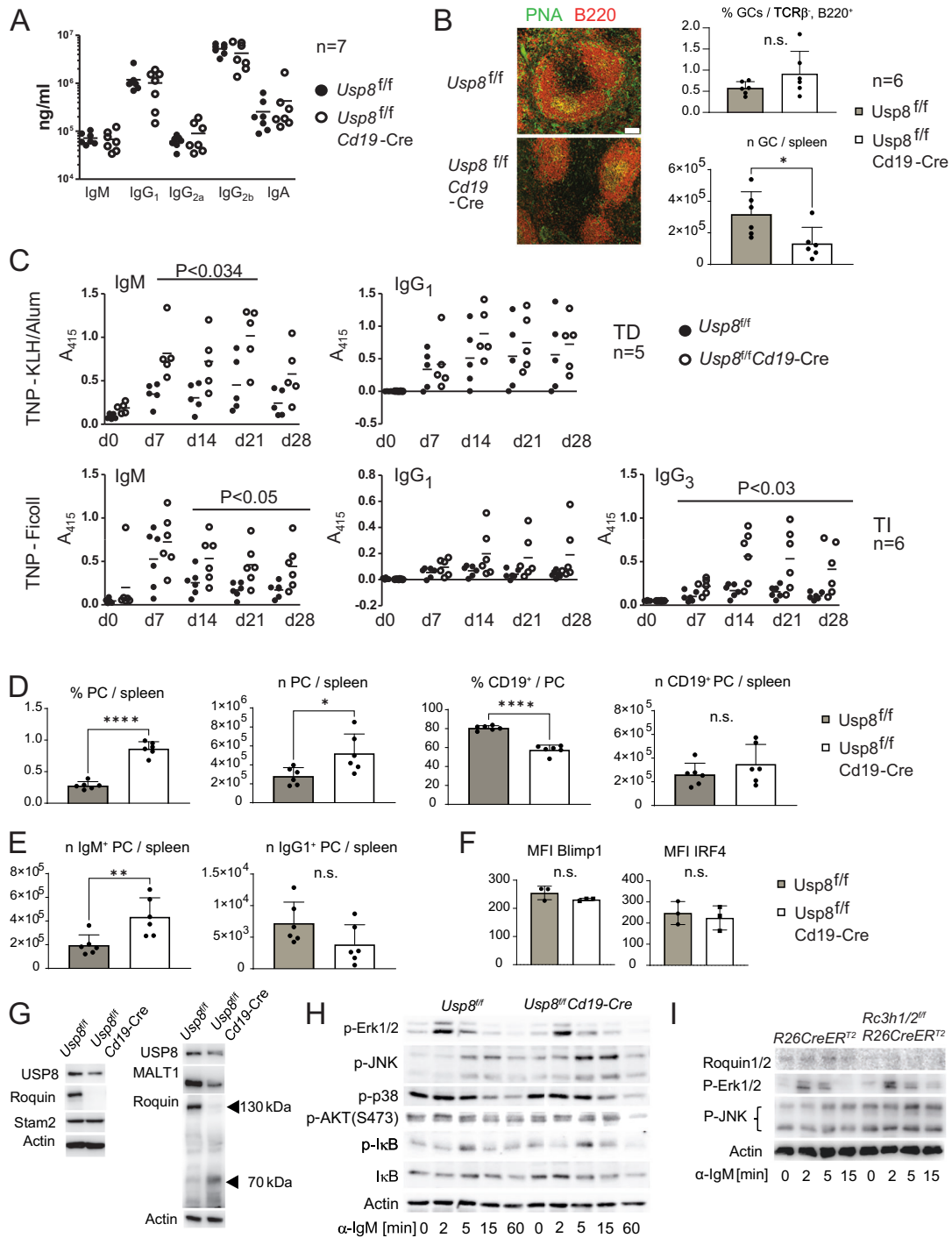


Fig. 1 Defect in B-cell development in *Usp8^{f/f}Cd19-Cre* mice. **A–C** Flow cytometry analysis of B-cell subsets in spleen of *Usp8^{f/f}* and *Usp8^{f/f}Cd19-Cre* mice ($n = 4$). Pre-gating is indicated at the top right of the FACS plots. Percentages and numbers of B-cell subsets are shown in the right panels. Unpaired 2-tailed t-test, mean $\pm$ s.d. **D** Flow cytometry analysis of *Usp8^{f/f}* and *Usp8^{f/f}Cd19-Cre* bone marrow (BM) ($n = 4$). Paired 2-tailed t-test, mean $\pm$ s.d. **E** Flow cytometry analysis of splenic transitional *Usp8^{f/f}* and *Usp8^{f/f}Cd19-Cre* B-cell subsets ($n = 3$). Paired, 2-tailed t-test, mean $\pm$ s.d. **F** Uniform Manifold Approximation and Projection (UMAP) of B cells enriched from spleens of *Usp8^{f/f}* and *Usp8^{f/f}Cd19-Cre* spleens. **G** Flow cytometry analysis of splenic *Usp8^{f/f}* and *Usp8^{f/f}Cd19-Cre* germinal center (GC; GL^{hi}, Fas^{hi}, TCR β ⁺, B220⁺) B cells and CD138^{hi} PCs, and CD19 expression on PCs ($n = 4$). Unpaired 2-tailed t-test, mean $\pm$ s.d. MZB marginal zone B cells, FoB follicular B cells, Pro pro-B cells, Pre pre-B cells, TransB Transitional B cells, T1, T2, T3 Transitional T1, T2, T3 B cells, BM bone marrow, PCs plasma cells, GC-like germinal center-like. Paired analysis was used, if sex-matched littermates were derived from n different litters. Source data are provided as source data file.



gene expression analysis showed increased IL-9R in FoB and MZB, higher expression of specific Ig variable regions in PCs, and reduced ZFP318, a transcriptional regulator of IgD expression [32], in GC-like B cells and FoB of $Usp8^{f/f} Cd19-Cre$ mice (Fig. S2D).

Basal serum Igs were unchanged (Fig. 2A). Although total GC B-cell numbers were reduced in spleens of TNP-KLH-immunized $Usp8^{f/f} Cd19-Cre$ mice, their frequencies among B cells were unchanged (Fig. 2B). After immunization, IgM and IgG3 levels and splenic PC numbers were increased (Fig. 2C). However, as in unimmunized mice, CD19 $^+$ PCs were underrepresented within the PC compartment (Fig. 2D). Consistent with elevated IgM responses, $Usp8^{f/f} Cd19-Cre$ mice had more IgM-positive, but not

IgG1 positive PCs per spleen (Fig. 2E). Key transcription factors remained unchanged (Fig. 2F). T-cell subsets and basal cytokines were unaffected (Fig. S2E, F).

Roquin proteins (Roquin-1 and -2) are essential for shaping T and B cell-dependent immune responses [33]. We therefore hypothesized that Roquin function might be impaired in $Usp8^{f/f} Cd19-Cre$ B cells. Indeed, Roquin-1/2 levels, but not STAM2, were reduced in USP8-depleted B cells, whereas short-term USP8 deletion ex vivo did not alter Roquin or CHMP5, indicating indirect regulation (Figs. 2G, S3A). Roquin cleavage is mediated by Malt1, whose protease activity is enhanced by mono-ubiquitylation at K644 or excess free ubiquitin [34, 35]. MALT1 ubiquitination was

Fig. 2 *Usp8^{fl/fl}Cd19-Cre* mice exhibit enhanced PC numbers and Ig responses upon T cell-dependent and T cell-independent immunization. **A** Normal basal immunoglobulin titers in *Usp8^{fl/fl}Cd19-Cre* mice ($n = 7$). **B** Control and *Usp8^{fl/fl}Cd19-Cre* mice were i.p. injected with 50 μ g TNP-KLH precipitated in alum and GC B cells were visualized by fluorescence imaging of frozen sections (scale bar, 100 μ m) and quantified by FACS analysis (GL7^{hi}, Fas^{hi}, TCR- β ⁺, B220⁺) at day 14 post injection ($n = 6$, unpaired 2-tailed t-test, mean $\pm$ /-sd). **C** Detection of TNP-specific Ig present in the serum of *Usp8^{fl/fl}* and *Usp8^{fl/fl}Cd19-Cre* mice that have been i.p. injected with 50 μ g TNP-KLH precipitated in alum (TD response, $n = 5$) or with 25 μ g TNP-Ficoll (TI response, $n = 6$) by ELISA. Significant differences are summarized collectively. Unpaired 2-tailed t-test, mean $\pm$ /-sd. *Usp8^{fl/fl}* and *Usp8^{fl/fl}Cd19-Cre* mice were treated as in (**B**) and spleen single cell suspensions were analyzed by FACS to determine frequencies of CD138^{hi} PCs and CD19 expression on PCs ($n = 6$) (**D**), frequencies of IgM⁺ and IgG1⁺ PCs ($n = 6$) (**E**), as well as the expression of Blimp1 and IRF4 in PCs ($n = 3$) (**F**). Unpaired 2-tailed t-test, mean $\pm$ /-sd. **G** Splenic *Usp8^{fl/fl}* and *Usp8^{fl/fl}Cd19-Cre* B cells were enriched and analyzed by Western blotting (WB). Results are representative of at least 2 independent experiments. **H** Splenic *Usp8^{fl/fl}* and *Usp8^{fl/fl}Cd19-Cre* B cells were starved for 2 h, stimulated for the indicated times with anti-IgM F(ab')₂ fragment antibody, and analyzed by WB. Results are representative of at least 2 independent experiments. **I** Upon oral gavage of tamoxifen, Roquin1/2 depleted *Rc3h1/2^{fl/fl} Rosa26-CreERT2* B cells and *Rosa26-CreERT2* control cells were enriched from spleens, stimulated as in (**H**), and analyzed by WB ($n = 3$). Source data are provided as source data file.

not detected, but gene expression analyses showed coordinated regulation of Usp8, Roquin, and Malt1 (Fig. S3B).

Mechanistically, USP8-depleted B cells showed enhanced JNK activation and Ca²⁺ mobilization upon BCR stimulation, while Erk1/2, p38, AKT, I κ B, and global tyrosine phosphorylation were unaffected (Figs. 2H, S3C, D). Ex vivo USP8 deletion did not reproduce these effects, whereas in vivo Roquin1/2 loss recapitulated increased JNK activation, implicating Roquin loss in this phenotype (Fig. 2I, S3E–G). Pathway analysis further suggested altered Rho-GTPase signaling and stress responses in USP8-deficient B-cell subsets (Fig. S3H, Table S1).

In summary, USP8 deficiency skews the splenic B-cell compartment toward GC-like B cells, PCs, immature, and innate-like B cells with a specific underrepresentation of CD19⁺ PCs, and enhances Ig and signaling responses, likely driven by indirect effects on Roquin and downstream pathways.

USP8 limits proteotoxic stress and cleaves ubiquitin/NEDD8 mixed chains

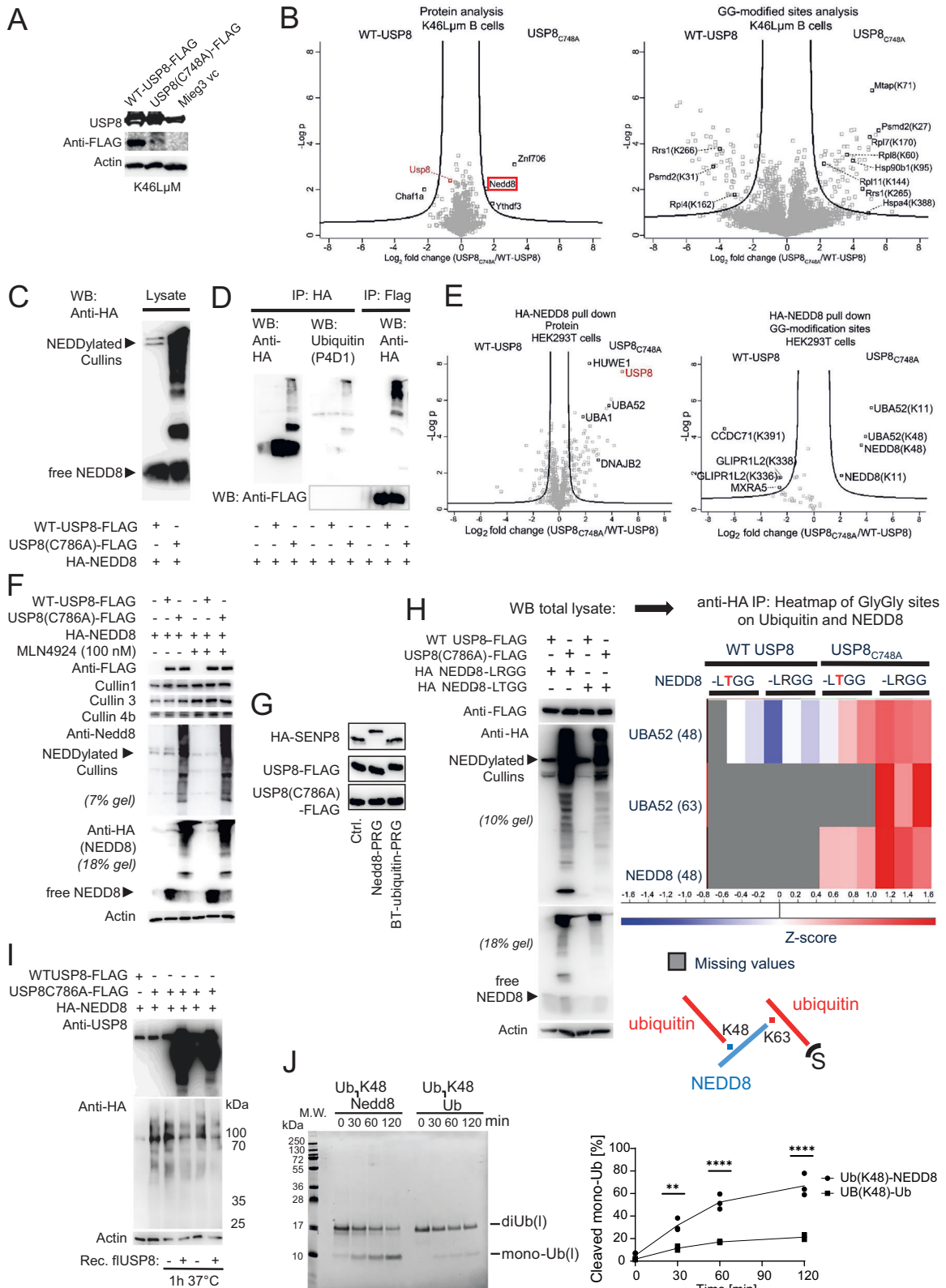
To further investigate USP8's role in B cell signaling, survival, and homeostasis, we stably expressed FLAG-tagged mouse wild-type (WT) USP8 and catalytically inactive USP8(C748A) in the murine K46L μ m B-cell lymphoma line (expressing NP-specific mIgM-BCR) [28, 29] (Fig. 3A) and analyzed the proteome and ubiquitin/ubiquitin-like modifications using the diGly enrichment (Ubiscan®) [36]. Overexpression of catalytically inactive USPs can trap their substrates [37]. Unexpectedly, NEDD8 levels were elevated in B cells expressing USP8(C748A) (Fig. 3B, left panel). NEDD8, the ubiquitin-like (ubl) modifier most similar to ubiquitin [38], carried multiple ubiquitin/ubl modified sites itself, including K11, K22, K33, K48, K54, and K60 (deposited in PRIDE database, identifier PXD024395; for accession details please see data availability section). These data suggest that USP8(C748A) increases levels of free or conjugated NEDD8, and that NEDD8 itself undergoes modification by either ubiquitylation or NEDDylation, which cannot be distinguished using diGly enrichment. Enhanced ubiquitin/ubl-modification was also observed on ribosomal proteins Rpl7, Rpl8, Rpl11, and the ribosome biogenesis regulator 1 (Rrs1) (Fig. 3B, right panel).

NEDDylation, like ubiquitin conjugation, is a sequential process mediated by the NEDD8 activating enzyme (NAE, a NAE1/UBA3 heterodimer), the conjugating enzymes UBE2M (UBC12) and UBE2F, and various E3 ligases. Its main targets are cullin proteins, whose mono-NEDDylation activates cullin-RING E3 ubiquitin ligases (CRLs). Under conditions of proteasome inhibition, the altered free ubiquitin-to-NEDD8 ratio triggers atypical conjugation of NEDD8 by the ubiquitin activating enzyme E1 (UBE1/UBA1) and the E3 ligase HUWE1, forming mixed ubiquitin/NEDD8 chains [39–44] that help counter proteotoxic stress [44]. Ribosomal substrates form nuclear aggregates when modified by ubiquitin/NEDD8 mixed chains shielding the nuclear UPS from excess proteins as a hallmark of proteotoxic stress [44].

Similar to atypical NEDDylation during proteotoxic stress, co-expressing HA-NEDD8 with human USP8(C786A)-FLAG in HEK293T cells strongly enhanced overall NEDDylation compared to WT USP8 (Fig. 3C). HA-NEDD8 pulldown contained ubiquitylated proteins, indicating mixed ubiquitin/NEDD8 chains (Fig. 3D), while USP8(C786A)-FLAG pulldowns captured NEDDylated substrates, but not free NEDD8, indicating that catalytically inactive USP8 traps them via ubiquitin in these mixed chains (Fig. 3D). Mass spectrometry (MS) of HA-NEDD8 pulldowns revealed increased accumulation of inactive USP8 versus WT with no ubl-modifications detected on USP8 itself (Fig. 3E, PRIDE PXD024755). Strikingly, UBA52 (ubiquitin), as well as UBE1 (UBA1) and HUWE1 were strongly enriched in the HA-NEDD8 pulldowns with USP8(C786A)-FLAG. Quantification of K- ϵ -GG modified peptides revealed K11- and K48-linked ubiquitin/NEDD8 modifications, indicating chains form mainly via these linkages (Fig. 3E). Mutating HA-NEDD8 K11/K48 to arginine reduced substrate NEDDylation and ubiquitylation (Fig. S4A). Mixed chains were further supported by the absence of K48/K63-specific ubiquitylation and significant sumoylation (Fig. S4B). NEDD8 (K22, K33) and ubiquitin (K63) modifications were detected only with USP8(C786A)-FLAG (PRIDE PXD024755). Consistent with non-canonical NEDDylation, the NAE1 inhibitor MLN4924 blocked Cullin NEDDylation, but not NEDDylation induced by USP8(C786A)-FLAG overexpression (Fig. 3F). In line with the lack of deNEDDylation activity of USP8, USP8 formed a covalent adduct with ubiquitin-propargylamide (PRG) [45], but not NEDD8-PRG, which selectively targeted the deNEDDylase SENP8 (Fig. 3G). These findings suggest that inactive USP8 induces proteotoxic stress, and that USP8 cleaves ubiquitin within mixed ubiquitin/NEDD8 chains.

Identification of non-cullin NEDD8 substrates has been limited by the lack of MS approaches that distinguish between ubiquitylation and NEDDylation. To confirm ubiquitin/NEDD8 hybrid chain formation (versus parallel chain formation or non-covalent interactions) and map reciprocal modification sites, we co-expressed USP8(C786A)-FLAG (or WT-USP8-FLAG) with HA-tagged WT NEDD8 or a C-terminal mutant (LRGG > LTGG) that remains conjugation-competent, but cannot generate the K- ϵ -GG remnants upon trypsin digestion [46] (Fig. 3H). MS analysis of HA-pulldowns detected K48 modifications on both, WT-NEDD8 and NEDD8(LTGG), identifying NEDD8(K48) as a ubiquitylation site. Conversely, K63 modifications occurred only in the presence of WT-NEDD8, indicating NEDDylation of ubiquitin at K63. Consistent with USP8-mediated cleavage of mixed chains, recombinant USP8 reduced substrate modifications (Fig. 3I). In vitro deubiquitination assays further showed that USP8 cleaves K48-linked ubiquitin/NEDD8 branches more efficiently than K48-diubiquitin (Fig. 3J).

These data indicate that loss of USP8 activity promotes proteotoxic stress and that USP8 directly cleaves mixed ubiquitin/NEDD8 chains.



USP8 controls proteostasis and survival of primary B cells

Consistent with proteotoxic stress induced by inactive USP8, OHT-mediated USP8 deletion in primary *Usp8^{fl/f}Rosa26-CreERT2* B cells increased overall ubiquitylation and accelerated apoptosis (Fig. 4A). A modest rise in JNK activation was observed, along

with cleavage of Caspase-8 and loss of the full-length Caspase-8 and Caspase-3 proteins (Fig. 4B).

Remarkably, hyperNEDDylation (indicative of mixed ubiquitin/NEDD8 chains) was not observed after USP8 deletion in primary B cells. Because hyperNEDDylation may require sufficient free

Fig. 3 USP8 regulates mixed ubiquitin/NEDD8 chain formation indicating proteotoxic stress. A, B NEDD8 levels and ribosomal protein modifications are elevated in B cells expressing USP8(C748A)-FLAG. **A** WT-USP8-FLAG and USP8(C748A)-FLAG expression in K46L μ m B cells analyzed by WB. **B** Volcano plots of USP8(C748A):WT-USP8 diGly proteomics experiments. Differentially expressed proteins (shotgun analysis) and regulated diGly sites in proteins derived from K46L μ m B cells ($n = 3$). Lysine positions of diGly sites are included in parentheses. **C–E** HEK293T cells transfected with WT-USP8-FLAG or USP8(C786A)-FLAG in the presence of HA-NEDD8. **C** Lysates analyzed by WB. **D** Anti-HA and anti-FLAG pulldowns analyzed by WB. **E** Anti-HA pulldowns analyzed by MS ($n = 3$) to quantify pulled down proteins and GlyGly-K sites. The lysine position of the identified di-glycine site is included in parentheses. **F** HEK293T cells transfected as in (C). 24 h prior to lysis cells were treated with MLN4924 (100 nM) as indicated and lysates were analyzed by WB. **G** HEK293T cells transfected with HA-SENp8, WT-USP8-FLAG or USP8(C786A)-FLAG. Lysates were incubated with buffer (ctr.), NEDD8-propargylamide (NEDD8-PRG), or ubiquitin-PRG and analyzed by WB. **H** HEK293T cells transfected with WT-USP8-FLAG or USP8(C786A)-FLAG in the presence of HA-NEDD8(LRGG) or HA-NEDD8(LTGG). Lysates were analyzed by WB (left panels). Anti-HA pulldowns were analyzed by MS (3 replicates/condition) and alterations of ubiquitin (UBA52) and NEDD8 diGly sites are shown in the heat map. Hybrid linkages between both modifiers are reconstructed in the scheme below. **I** Protein lysates from 293T cells transfected with USP8(C786A)-FLAG and HA-NEDD8 were incubated with recombinant USP8 and analyzed by WB. **J** DUB cleavage assays were performed with recombinant human full-length USP8 and synthetic K48-ubiquitinated-NEDD8 ($n = 3$) or K48-diubiquitin ($n = 2$) for the times indicated. Cleavage products were visualized after protein electrophoresis and protein staining of the gel. Statistical analysis after quantification utilized two-tailed unpaired t-test. Source data are provided as source data file.

NEDD8 [40], USP8-depleted cells were treated with MLN4924 to block canonical Cullin NEDDylation and increase free NEDD8. However, this also failed to induce hyperNEDDylation (Fig. 4C), suggesting that mixed chains form only in certain cell-types or may be removed by enzymes other than USP8.

USP8 depletion in primary B cells induced autophagy, indicated by increased lipidated LC3-II, but also impaired autophagic flux, as shown by accumulation of LC3 (Fig. 4C).

To assess ER stress, XBP1s expression was monitored [47]. ER stress occurred upon UBA1-inhibition with MLN7243, but not after USP8 depletion. Notably, UBA1 inhibition triggered USP8 cleavage, correlating with Caspase 3 activation (Fig. 4C).

Together these data indicate that loss of USP8 increases protein ubiquitylation and triggers proteotoxic stress-induced apoptosis in B-cells without inducing ER stress.

DUB-IN-2 controls proteostasis and survival of MM cells in synergy with BTZ

Given USP8's role in preventing proteotoxic stress and its identification as a vulnerability gene, we evaluated its function in MM, where malignant plasma cells are highly sensitive to disrupted protein homeostasis [1, 24]. We hypothesized that USP8 inhibition could enhance proteotoxic stress and resensitize BTZ-resistant MM cells. Using the reported USP8 inhibitor DUB-IN-2 [10–12, 18], we tested KMS-18 and KMS-27 cells, including BTZ-resistant variants carrying PSMB5 mutations (T21A or A49V) [30].

KMS-18 and KMS-27 MM cells, including BTZ-resistant variants, were treated with BTZ, DUB-IN-2, or both. Single-agent treatment was ineffective in resistant cells, and DUB-IN-2 was also ineffective in WT cells. However, the combination drastically decreased cell viability of both WT and BTZ-resistant cell lines (Fig. 4D). In KMS-18-T21A cells, 25 μ M DUB-IN-2 was required to overcome BTZ resistance (Fig. S5A). A similar synergistic effect was observed with CFZ and DUB-IN-2 in KMS-27 WT and CFZ-resistant KMS-27-A49V cells [30] (Fig. S5B). Synergy analysis using SynergyFinder Plus [48] confirmed positive interaction scores (Tables S2–S5). Combination treatments induced rapid accumulation of polyubiquitylated proteins within 6 h (Fig. 4E/F; Figure S5C). Increased NEDDylation was observed only in KMS-27/KMS-27-A49V cells after 24 h, indicating a cell type-specific response (Fig. 4F). Similar to UBA1 inhibition in primary B cells, 24 h BTZ treatment triggered USP8 degradation and caspase 3 activation in KMS-18 WT cells, effects enhanced by DUB-IN-2 co-treatment in all cell lines (Fig. 4E/F, right panels). Consistent with proteasome inhibition triggering apoptosis through ER stress in MM cells [49], the ER stress marker CHOP and its target, death receptor 5 (DR5) [47], were upregulated most strongly with combination treatment (Fig. 4E/F, Fig. S5C). XBP1s did not parallel CHOP upregulation, suggesting cell type-specific regulation of pro-apoptotic versus pro-adaptive stress responses

[50, 51]. Autophagy induction, indicated by LC3-II accumulation, was not observed (Fig. 4E/F, Fig. S5C).

To validate these findings in patient derived MM cells with heterogenous resistance mechanisms, BTZ/DUB-IN-2 treatment was tested in MM cells isolated from 9 BTZ-resistant and 6 BTZ-responsive patients. The two groups differed significantly in BTZ response ($p = 0.03$) (Fig. 4G). Combination treatment, using BTZ at the most discriminatory dose and DUB-IN-2 at the highest non-toxic concentration (Fig. S5D), reduced viability across samples (Fig. 4G/H, Table S6).

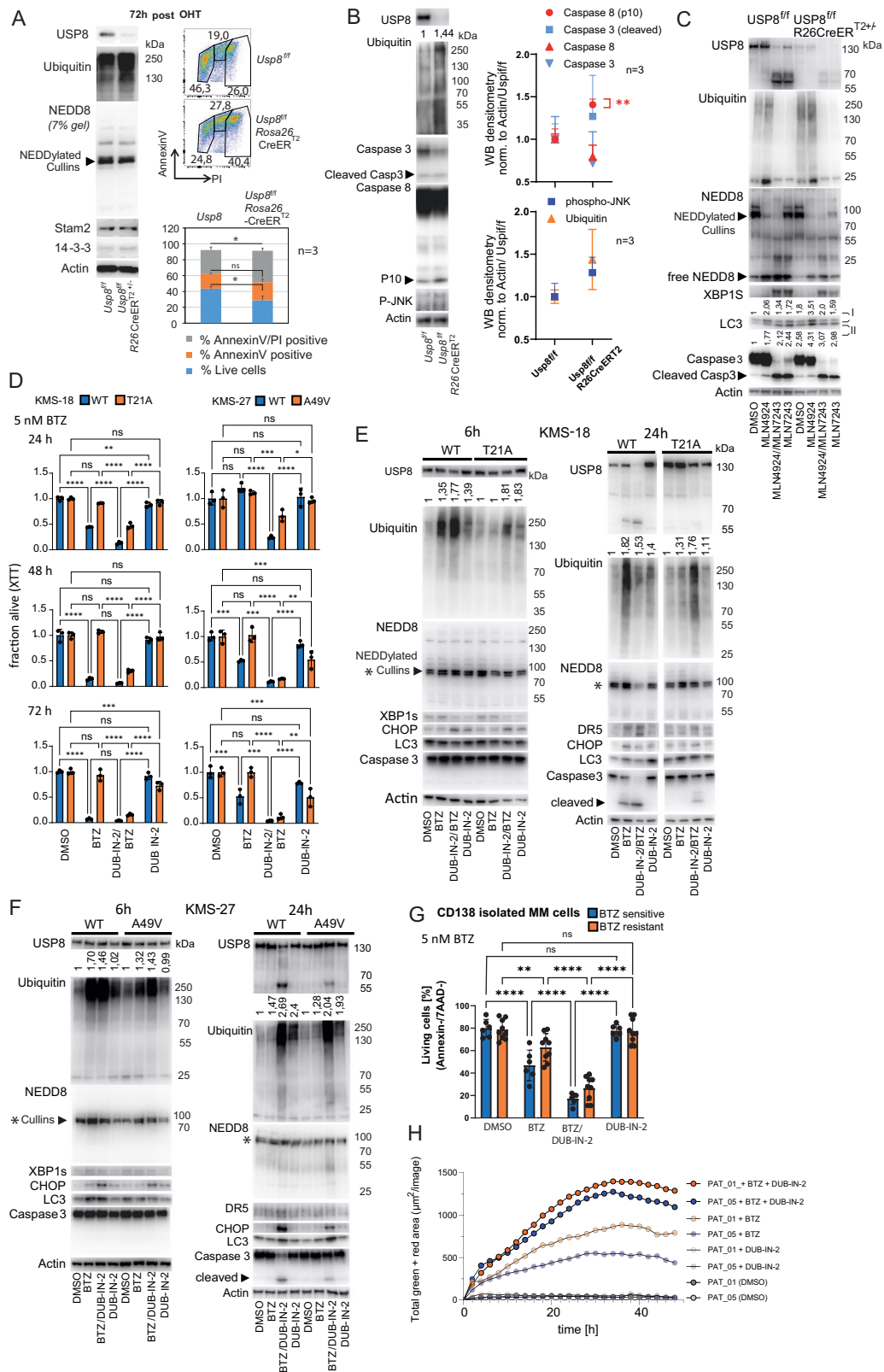
Overall, these data suggest that DUB-IN-2 can overcome BTZ/CFZ resistance by enhancing ER stress, highlighting its therapeutic potential.

DUB-IN-2 impacts MM cell viability independently of USP8

USP8 knockdown in KMS cells only partially mimicked DUB-IN-2 effects. Combined USP8 depletion and BTZ showed only additive effects or mild synergy, requiring 3-fold higher BTZ concentrations (Fig. 5A, Bliss scores [52] are listed in Table S7), with KMS-27-WT/A49V cells being more susceptible than KMS-18-WT/T21A cells. Unlike DUB-IN-2, USP8 depletion did not induce CHOP or enhance BTZ-induced CHOP as an indicator of ER stress, but activated Caspase 8 and Caspase 3 (Fig. 5B), suggesting DUB-IN-2 affects BTZ-resistant MM cells independently of USP8.

To test DUB-IN-2 specificity, USP8 was depleted in KMS-18-T21A cells before BTZ, DUB-IN-2, or combination treatment. Efficient USP8 knockdown (pooled siRNA or siRNA_2) still allowed DUB-IN-2 to further reduce viability, indicating its effects occur independently of USP8 (Fig. 5C).

As these results raised considerable concerns about DUB-IN-2's USP8 specificity, we further analyzed consequences of USP8 depletion. Although CHOP was not induced, its target DR5 was strongly upregulated, correlating with caspase 8 activation; partial USP8 knockdown with siRNA_1 was insufficient (Fig. 5D). 6 h DUB-IN-2 and/or BTZ treatment alone had no effect on DR5 expression or caspase activation, but enhanced protein ubiquitylation. Reexpression of USP8 in 293 T cells prevented hyper-ubiquitylation, DR5 upregulation, and JNK activation, confirming on-target effects (Fig. 5E). Of note, JNK activation has previously been shown to act upstream of DR5 transcriptional activation [53] and thus may represent a CHOP-independent pathway of DR5 induction. CHOP knockdown in KMS-18-T21A cells (to rule out transient CHOP overexpression) showed DR5 induction occurs independently of CHOP, which is also dispensable for BTZ/DUB-IN-2-induced cell death (Fig. 5F, G). USP8 depletion-induced JNK activation, which triggers apoptosis via both extrinsic and intrinsic pathways [54], was accompanied by caspase 3, 9, and 8 activation (Fig. 5F).



As an ESCRT component, USP8 regulates lysosomal trafficking [3]. To compare consequences of USP8 depletion with DUB-IN-2 treatment, lysosomal integrity was analyzed in KMS-18-T21A and KMS-27-A49V cells. Lysosome enlargement, indicative of impaired degradation, occurred only upon USP8 depletion, but not after

BTZ and/or DUB-IN-2 treatment (Figs. 5H and S5E). Pharmacological lysosomal inhibition with folimycin - similar to USP8 depletion - induced JNK phosphorylation and DR5 expression with little effects on CHOP or early ubiquitylation (Fig. 5I). Together, these results indicate that USP8 depletion impairs

Fig. 4 USP8 depletion and BTZ/DUB-IN-2 treatment induce proteotoxic stress and apoptosis in proliferating B and MM cells, respectively; DUB-IN-2 resensitizes resistant MM cells to BTZ. **A** *Usp8*^{fl/fl} and *Usp8*^{fl/fl}*Rosa26-CreERT2* splenic B cells were expanded in IL-4/CD40-containing medium for 24 h followed by OHT addition for 72 h. Lysates were subjected to WB analysis (left panels) and apoptosis was detected by FACS analysis (right panels, $n = 3$, two-tailed unpaired t-test, mean + sd). **B** *Usp8*^{fl/fl} and *Usp8*^{fl/fl}*Rosa26-CreERT2* splenic B-cell lysates generated as in (A) were subjected to WB and expression was quantified ($n = 3$, two-tailed unpaired t-test, mean + /-sd). **C** *Usp8*^{fl/fl} and *Usp8*^{fl/fl}*Rosa26-CreERT2* splenic B-cells were expanded as in (A) and treated with MLN4924 (NAE inhibitor, 500 nM) and/or MLN7243 (UBA1 inhibitor, 500 nM) for 16 h before lysis and WB analysis. LC3 I/II quantification is indicated. **D** Cell viability of KMS-18 or KMS-27 WT cells and BTZ-resistant mutant MM cells (KMS-18-T21A/KMS-27-A49V) treated with BTZ (5 nM), DUB-IN-2 (25 μ M), or both ($n = 3$, 2-way ANOVA, Tukey's post hoc test, mean + /-sd). WB analysis of KMS-18/KMS-18-T21A (**E**) or KMS-27/KMS-27-A49V (**F**) MM cells after exposure to BTZ (5 nM) and/or DUB-IN-2 (25 μ M). **G** BM cells from MM patients defined as "BTZ resistant" ($n = 9$) or "non-resistant" ($n = 6$) were incubated with solvent (DMSO), BTZ (5 nM), DUB-IN-2 (25 μ M), or BTZ + DUB-IN-2 for 24 h and viability was measured by AnnexinV/7AAD staining. (2-way ANOVA, Tukey's post hoc test, mean + /-sd; two sided t-test was used to compare the response to BTZ alone across groups, $p = 0.03$). **H** MM cells from selected patients were stained with CytoLight Rapid Green and treated with inhibitors as in (G). Ongoing staining of apoptotic cells was performed with Annexin V Red Dye. WB results in Fig. 4 are representative of at least 2 independent experiments. Quantification of ubiquitin modification shown on top of each blot represents the average of at least 2 independent experiments normalized to actin and DMSO controls. Source data are provided as source data file.

lysosomal degradation, a phenotype not recapitulated by DUB-IN-2 treatment, further questioning its specificity for USP8.

To analyze potential effects of DUB-IN-2 on autophagic degradation, KMS-18-T21A cells were treated with bafilomycin $\pm$ DUB-IN-2 and/or BTZ. No increase in LC3-II was detected, indicating no evidence for autophagy induction or altered autophagic flux. In line with pro-apoptotic IRE1 signaling [50, 51], BTZ/DUB-IN-2 co-treatment induced JNK and caspase 8 activation after 12 h, while DR5 expression remained unchanged (Fig. 5J). These results suggest that BTZ/DUB-IN-2-induced apoptosis is mediated by ER stress-driven JNK independently of DR5, whereas USP8 depletion promotes JNK activation, DR5 upregulation, and apoptosis primarily via lysosomal dysfunction [55] (Fig. 5K). The distinct responses, including lysosomal defects and lack of CHOP induction upon USP8 silencing, indicate that DUB-IN-2 acts independently of USP8.

The profound differences in cell death mechanisms prompted us to reevaluate DUB-IN-2 specificity in biochemical assays. A DUB profiling assay (Ubiquigent, >40 recombinant DUBs) showed no inhibition of USP8 or other DUBs across 30 nM–100 μ M (Fig. 6A/B). Consistently, in vitro assays with insect cell or bacterially expressed USP8 confirmed that DUB-IN-2, as well as DUB-IN-1, DUB-IN-3 [18], and DC-U4106 [19], did not inhibit USP8-activity (Fig. 6C–E). In contrast, the dual OTUB1/USP8 inhibitor 61 (OUI61) inhibited USP8 activity at ≥ 500 nM (Fig. 6E/F), but induced caspase-independent cell death without increased ubiquitylation, indicating off-target toxicity (Figure S6A/B). DUB-IN-2 also did not affect proteasome activity (Fig. S6C). Together, these data demonstrate that DUB-IN-2 does not inhibit USP8 and that its effects on MM viability are USP8-independent.

Although USP8 activity was unaffected, DUB-IN-2 treatment effectively overcame BTZ resistance in MM cells. We therefore evaluated BTZ/DUB-IN-2 co-treatment using NSG mice transplanted with KMS-18 or KMS-18-T21A cells. While BM infiltration was limited (Fig. S6D), stable spleen engraftment revealed reduced tumor expansion upon combination treatment (Fig. 6G). However, notable toxicity was observed under these conditions (Fig. 6H). To evaluate toxicity in a less vulnerable setting, treatment was tested in non-irradiated, immune-competent mice. Here, BTZ/DUB-IN-2 caused only transient weight loss without affecting survival or inducing gross pathological changes (Fig. 6I, Bienta Study Report T011125a). These findings support therapeutic efficacy of the combination and suggest a potentially manageable toxicity profile that may warrant further evaluation in humans.

To explore the potential clinical relevance of USP8 in MM, we next analyzed patient data from the MMRF-COMMPASS cohort. Lower USP8 expression at diagnosis was associated with a trend toward improved overall survival (Fig. S7A), and USP8 expression was significantly higher in stage III compared with stage II disease

(Fig. S7B). Notably, these analyses are based on RNA expression levels and may not fully reflect USP8 protein abundance.

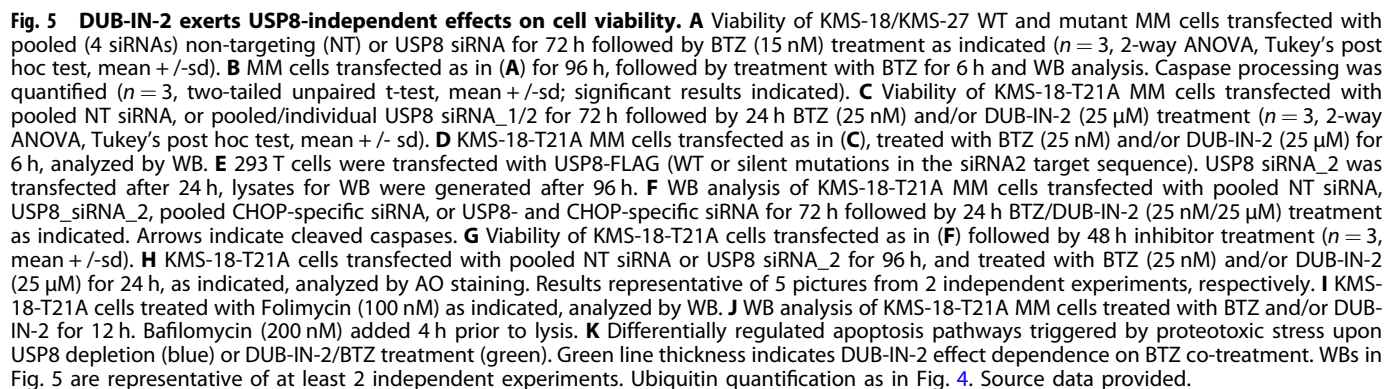
In summary, we define USP8 as a key regulator of proteostasis in B cells. Although DUB-IN-2 is not a specific USP8 inhibitor, this compound shows strong anti-MM activity in synergy with BTZ. These findings suggest that both USP8 inactivation and DUB-IN-2 treatment may be exploited therapeutically to overcome BTZ resistance and induce apoptosis in B-cell malignancies.

DISCUSSION

Our data show that USP8 is critical for B-cell development and survival. Deleting *Usp8* at later stages, as in *Usp8*^{fl/fl}*Cd19-Cre* mice, permits maturation beyond the pre-B-cell stage, resulting predominantly in immature, innate-like B cells, GC-like B cells, and PCs with reduced Roquin expression that drive normal (IgG₁) or enhanced (IgM and IgG₃) Ig production upon immunization. Roquin1/2, RNA-binding proteins with E3 ligase activity, shape T- and B cell responses by destabilizing mRNAs (e.g., *Ilfn*, *Icos*) and controlling mTor activity [33]. Roquin loss expands the B-cell pool and triggers spontaneous GC activation [56], as observed in *Roquin*^{san/san} (sanroque) mice, which develop spontaneous GCs and autoimmunity [57]. Preventing GC formation in these mice did not abolish autoimmune disease, but suggested that the expanded B1 B-cell population and extrafollicular PCs contributed to IgM autoantibody production [58]. Thus, loss of Roquin may contribute to the heightened activation potential of USP8-depleted splenic B cells, including increased JNK activation. Gene expression data further suggest altered Rho-GTPase signaling upon USP8 depletion, although the underlying mechanisms likely depend on the in vivo microenvironment and altered B cell composition. Notably, while *Cd19-Cre*-mediated recombination reduces *Usp8* expression across mature B-cell subsets at the transcript level, residual USP8 protein likely reflects delayed depletion kinetics after genomic deletion. Together with the phenotypic loss of mature B-cell populations observed in vivo, these findings support the conclusion that sustained USP8 expression is required for mature B-cell survival.

Proteotoxic-stress-induced B-cell defects in the absence of USP8 were first indicated in K46L μ m B cells expressing catalytically inactive USP8(C748A), where endogenous USP8 prevents cell death. USP8(C748A) traps substrates modified by mixed ubiquitin/NEDD8 chains [37], which are linked to aggresome-like body formation and autophagy [59], and nuclear protein aggregation under proteotoxic stress [44]. Consistent with prior studies [42, 43, 60], ubiquitin(K63) NEDDylation emerges as a proteotoxic stress signature, and we identify K48 ubiquitylation of NEDD8 as a USP8 target.

Our data indicate that disturbed proteostasis drives cell death in proliferating USP8-deficient B cells, explaining the developmental defects. Using MM as a model, we show that enhancing



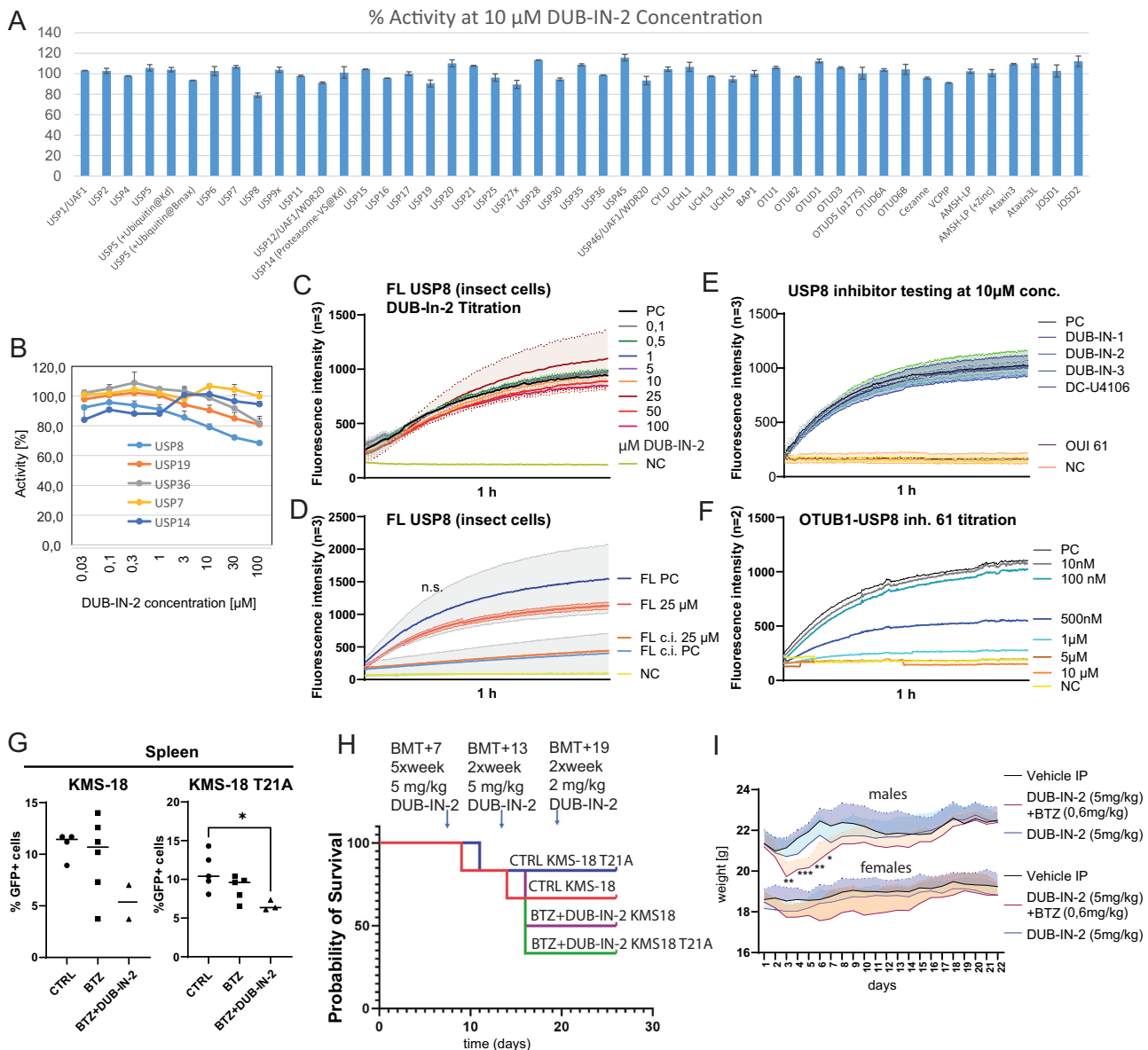


Fig. 6 Therapeutic potential of DUB-IN-2 in MM. **A** Evaluation of the selectivity of DUB-IN-2 (10 μ M) across the DUBprofiler panel (Ubiquigent). +Ubiquitin@kd/+Ubiquitin@Bmax: Enzyme activated half-maximally/maximally with additional ubiquitin; Proteasome-V5@kd: Enzyme activated with proteasome-V5. (Mean of duplicate experiments $\pm$ sd). **B** Effect of different DUB-IN-2 concentrations on selected DUBs. (Mean of duplicate experiments $\pm$ sd). **C** Evaluation of different DUB-IN-2 concentrations for inhibiting USP8 expressed in insect cells using ubiquitin-rhodamine as a substrate ($n = 3$; PC positive control, NC negative control (no enzyme)). **D** Assessment of DUB-IN-2 (25 μ M) effects on USP8 and a catalytically inactive (c.i.) variant (C786R) purified from insect cells ($n = 3$). **E** Assessment of commercially available USP8 inhibitors (10 μ M) for their effects on bacterially expressed USP8 ($n = 3$). **F** Effect of OTUB1/USP8 inhibitor 61 (OUI61) on bacterially expressed USP8 across different concentrations ($n = 2$). **G** Infiltration of MM cells in spleens of NSG mice treated with either control (0.9NaCl/PEG300 1:1 (v/v)), BTZ (BTZ, 0.6 mg/kg, twice a week), or BTZ + DUB-IN-2 ($n = 6$) using 5 mg/kg 5 times a week over a period of 13 days, followed by 2 mg/kg twice a week in spleen (1-way ANOVA, Tukey's post hoc test). **H** Kaplan-Meier plot showing the toxicity of the DUB-IN2/BTZ combination treatment (5 mg/kg). **I** Weight curves of C57BL/6 J mice ($n = 5$) IP injected with 0.9NaCl/PEG300 1:1 (v/v), BTZ (BTZ, 0.6 mg/kg twice a week), or BTZ combined with DUB-IN-2 using 5 mg/kg 5 times a week over a period of 22 days. (T-test: combination treatment compared to vehicle control). Source data are provided as source data file.

proteotoxic stress via DUB-IN-2 or USP8 knockdown has translational potential. The limited accumulation of mixed NEDD8/ubiquitin chains upon USP8 depletion appears cell type-specific and correlates with the increased sensitivity of primary B cells and MM cells to proteotoxic stress. A possible explanation is that endogenous mixed-chain intermediates are highly transient or efficiently cleared in these cells, thereby being preferentially captured under trapping conditions rather than accumulating upon genetic deletion alone.

BTZ-resistant MM cells were effectively targeted by combined DUB-IN-2/BTZ treatment, whereas DUB-IN-2 alone had minimal impact on viability. Interpretation of patient data is limited, as most "BTZ-resistant" cases received combination therapies, making true BTZ resistance difficult to define. However, given that BTZ/dexamethasone are typically used with modern agents, our cohort reflects current clinical practice.

MM cells rely on unfolded protein response (UPR) signaling to cope with high protein load [51], and both BTZ [49] and DUB-IN-2

synergistically induce apoptotic ER stress by targeting the UPS independently of USP8. In contrast, USP8 depletion impairs lysosomal function and reduces viability via a distinct mechanism. Emerging evidence suggests that severe disturbances in proteostasis can promote immunogenic cell death and enhance anti-tumor immune responses through the release of DAMPs and activation of type I interferon signaling [61, 62]. In MM, where plasma cells are highly dependent on proteostasis pathways, this raises the possibility that USP8 inhibition may not only induce tumor cell-intrinsic cytotoxicity but could also enhance tumor immunogenicity and thereby sensitize cells to T-cell-based immunotherapies. Recent work further supports a link between dysregulated deubiquitination pathways and interferon/ICD-like programs in MM [63]. However, multiple assays in this study document that DUB-IN-2 does not directly inhibit USP8, the proteasome, or other DUBs. However, overlapping effects previously reported upon USP8 depletion and DUB-IN-2 treatment may point to converging or partially shared pathways affected by these interventions. Thus, prior and future studies using DUB-IN-2 should be interpreted with caution, and conclusions drawn from its use may warrant reconsideration.

Despite these limitations, in vivo and patient-derived data shown here support further exploration of DUB-IN-2-based combination therapies. Identifying its molecular target(s) will be critical for further drug development. However, the current in vivo data indicate that anti-tumor efficacy and systemic tolerability are not yet fully aligned across model systems, highlighting that a defined therapeutic window will require further optimization in clinically relevant settings. Likewise, while targeting USP8 remains a promising strategy due to its role in proteostasis, cancer progression, and immune regulation, its essential functions in development and lymphocyte homeostasis warrant careful consideration of potential safety risks. Nevertheless, the accessibility of the hematopoietic system to small molecules and the potential for selective pharmacological modulation position USP8 as a highly promising therapeutic target.

DATA AVAILABILITY

All MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository. The Shotgun analysis and the analysis of GlyGly-K modification sites shown in Fig. 3B were registered under the data set identifier PXD024395 (Username: reviewer_pxd024395@ebi.ac.uk; Password: A6X1HcXc). The pull down-MS analysis shown in Fig. 3E was registered under the data set identifier PXD024755 (Username: reviewer_pxd024755@ebi.ac.uk; Password: Qgnx23Ev). The pull down-MS analysis shown in Fig. 3H was registered under the data set identifier PXD024429 (Username: reviewer_pxd024429@ebi.ac.uk; Password: FSW5Cl8e). Single cell RNA-seq analysis data are available at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE244334> (access token: xsxdsecavdyxbqj). Materials generated in this study are available upon request.

REFERENCES

- Robak P, Robak T. Bortezomib for the treatment of hematologic malignancies: 15 Years Later. *Drugs*. 2019;19:73–92.
- Clague MJ, Urbé S, Komander D. Breaking the chains: deubiquitylating enzyme specificity begets function. *Nat Rev Mol Cell Biol*. 2019;20:338–52.
- Dufner A, Knobeloch K-P. Ubiquitin-specific protease 8 (USP8/UBPY): a prototypic multidomain deubiquitinating enzyme with pleiotropic functions. *Biochem Soc Trans*. 2019;47:1867–79.
- Mizuno E, Kitamura N, Komada M. 14-3-3-dependent inhibition of the deubiquitinating activity of UBPY and its cancellation in the M phase. *Exp Cell Res*. 2007;313:3624–34.
- Kakihara K, Asamizu K, Moritsugu K, Kubo M, Kitaguchi T, Endo A, et al. Molecular basis of ubiquitin-specific protease 8 autoinhibition by the WW-like domain. *Commun Biol*. 2021;4:1272.
- Reincke M, Sbiera S, Hayakawa A, Theodoropoulou M, Osswald A, Beuschlein F, et al. Mutations in the deubiquitinase gene USP8 cause Cushing's disease. *Nat Genet*. 2014;47:31.

- Ma Z-Y, Song Z-J, Chen J-H, Wang Y-F, Li S-Q, Zhou L-F, et al. Recurrent gain-of-function USP8 mutations in Cushing's disease. *Cell Res*. 2015;25:306–17.
- Santelices J, Ou M, Maegawa GHB, Hercik K, Edelmann MJ. USP8 inhibition regulates autophagy flux and controls Salmonella infection. *Front Cell Infect Microbiol*. 2023;13:1070271.
- Mauri S, Bernardo G, Martinez A, Favaro M, Trevisan M, Cobraiville G et al. USP8 down-regulation promotes parkin-independent mitophagy in the drosophila brain and in human neurons. *Cells*. 2023;12. <https://doi.org/10.3390/cells12081143>.
- Xiong W, Gao X, Zhang T, Jiang B, Hu M-M, Bu X, et al. USP8 inhibition reshapes an inflamed tumor microenvironment that potentiates the immunotherapy. *Nat Commun*. 2022;13:1700.
- Yang H, Zhang X, Lao M, Sun K, He L, Xu J, et al. Targeting ubiquitin-specific protease 8 sensitizes anti-programmed death-ligand 1 immunotherapy of pancreatic cancer. *Cell Death Differ*. 2023;30:560–75.
- Xie F, Zhou X, Li H, Su P, Liu S, Li R, et al. USP8 promotes cancer progression and extracellular vesicle-mediated CD8+ T cell exhaustion by deubiquitinating the TGF- β receptor T β RII. *EMBO J*. 2022;41:e108791.
- Islam MT, Chen F, Chen H. The oncogenic role of ubiquitin specific peptidase (USP8) and its signaling pathways targeting for cancer therapeutics. *Arch Biochem Biophys*. 2021;701:108811.
- Tey PY, Dufner A, Knobeloch K-P, Pruneda JN, Clague MJ, Urbé S. Rapid turnover of CTLA4 is associated with a complex architecture of reversible ubiquitylation. *J Cell Biol*. 2025;224. <https://doi.org/10.1083/jcb.202312141>.
- Ke Z-B, Chen J-Y, Xue Y-T, Lin B, Huang Q, Huang X-Y, et al. Mechanical signal modulates prostate cancer immune escape by USP8-mediated ubiquitination-dependent degradation of PD-L1 and MHC-1. *Cell Death Dis*. 2025;16:413.
- Tang J, Long G, Xiao L, Zhou L. USP8 positively regulates hepatocellular carcinoma tumorigenesis and confers ferroptosis resistance through β -catenin stabilization. *Cell Death Dis*. 2023;14:360.
- Long Y, Hu Z, Yang D, Wang F, Zhao C, Zhang Y, et al. Pharmacological inhibition of the ubiquitin-specific protease 8 effectively suppresses glioblastoma cell growth. *Open Life Sci*. 2023;18:20220562.
- Colombo M, Vallese S, Peretto I, Jacq X, Rain J-C, Collard F, et al. Synthesis and biological evaluation of 9-Oxo-9H-indeno[1,2-b]pyrazine-2,3-dicarbonitrile analogues as potential inhibitors of deubiquitinating enzymes. *ChemMedChem*. 2010;5:552–8.
- Tian Y, Liu K, Liu R, Qiu Z, Xu Y, Wei W, et al. Discovery of potent small-molecule USP8 inhibitors for the treatment of breast cancer through regulating ER α expression. *J Med Chem*. 2022;65:8914–32.
- Adoro S, Park KH, Bettigole SE, Lis R, Shin HR, Seo H, et al. Post-translational control of T cell development by the ESCRT protein CHMP5. *Nat Immunol*. 2017;18:780–90.
- Dufner A, Kissler A, Niendorf S, Basters A, Reissig S, Schönle A, et al. The ubiquitin-specific protease USP8 is critical for the development and homeostasis of T cells. *Nat Immunol*. 2015;16:950.
- Zhang T, Sun J, Cheng J, Yin W, Li J, Miller H et al. The role of ubiquitinase in B cell development and function. *J Leukoc Biol*. 2020;n/a. <https://doi.org/10.1002/JLB.TMR0720-185RR>.
- Satpathy S, Wagner SA, Beli P, Gupta R, Kristiansen TA, Malinova D, et al. Systems-wide analysis of BCR signalosomes and downstream phosphorylation and ubiquitylation. *Mol Syst Biol*. 2015;11:810.
- Tiedemann RE, Zhu YX, Schmidt J, Shi CX, Sereduk C, Yin H, et al. Identification of molecular vulnerabilities in human multiple myeloma cells by RNA interference lethality screening of the druggable genome. *Cancer Res*. 2012;72:757.
- Lei H, Wang J, Hu J, Zhu Q, Wu Y. Deubiquitinases in hematological malignancies. *Biomark Res*. 2021;9:66.
- Xu Y-J, Zeng K, Ren Y, Mao C-Y, Ye Y-H, Zhu X-T, et al. Inhibition of USP10 induces myeloma cell apoptosis by promoting cyclin D3 degradation. *Acta Pharm Sin*. 2023;44:1920–31.
- Gao H, Xi Z, Dai J, Xue J, Guan X, Zhao L, et al. Drug resistance mechanisms and treatment strategies mediated by Ubiquitin-Specific Proteases (USPs) in cancers: new directions and therapeutic options. *Mol Cancer*. 2024;23:88.
- Kim KJ, Kanellopoulos-Langevin C, Merwin RM, Sachs DH, Asofsky R. Establishment and characterization of BALB/c lymphoma lines with B cell properties. *J Immunol*. 1979;122:549.
- Justement LB, Wienands J, Hombach J, Reth M, Cambier JC. Membrane IgM and IgD molecules fail to transduce Ca²⁺ mobilizing signals when expressed on differentiated B lineage cells. *J Immunol*. 1990;144:3272.
- Allmeroth K, Horn M, Kroef V, Miethe S, Müller R-U, Denzel MS. Bortezomib resistance mutations in PSMB5 determine response to second-generation proteasome inhibitors in multiple myeloma. *Leukemia*. 2021;35:887–92.
- Hobeika E, Thiemann S, Storch B, Jumaa H, Nielsen PJ, Pelanda R, et al. Testing gene function early in the B cell lineage in mb1-cre mice. *Proc Natl Acad Sci USA*. 2006;103:13789–94.

32. Enders A, Short A, Miosge LA, Bergmann H, Sontani Y, Bertram EM, et al. Zinc-finger protein ZFP318 is essential for expression of IgD, the alternatively spliced IgH product made by mature B lymphocytes. *Proc Natl Acad Sci USA*. 2014;111:4513–8.
33. Raj T, Negraschus A, Heissmeyer V. Roquin-dependent gene regulation in immune-mediated diseases and future therapies. *Int Immunol*. 2023;35:159–70.
34. Pelzer C, Cabalzar K, Wolf A, Gonzalez M, Lenz G, Thome M. The protease activity of the paracaspase MALT1 is controlled by monoubiquitination. *Nat Immunol*. 2013;14:337–45.
35. Schairer R, Hall G, Zhang M, Cowan R, Baravalle R, Muskett FW, et al. Allosteric activation of MALT1 by its ubiquitin-binding Ig3 domain. *Proc Natl Acad Sci USA*. 2020;117:3093–102.
36. Xu G, Paige JS, Jaffrey SR. Global analysis of lysine ubiquitination by ubiquitin remnant immunoaffinity profiling. *Nat Biotechnol*. 2010;28:868–73.
37. Morrow ME, Morgan MT, Clerici M, Growkova K, Yan M, Komander D, et al. Active site alanine mutations convert deubiquitinases into high-affinity ubiquitin-binding proteins. *EMBO Rep*. 2018;19:e45680.
38. Enchev RI, Schulman BA, Peter M. Protein neddylation: beyond cullin-RING ligases. *Nat Rev Mol Cell Biol*. 2015;16:30–44.
39. Kim W, Bennett EJ, Huttlin EL, Guo A, Li J, Possemato A, et al. Systematic and quantitative assessment of the ubiquitin-modified proteome. *Mol Cell*. 2011;44:325–40.
40. Hjerpe R, Thomas Y, Chen J, Zemla A, Curran S, Shpiro N, et al. Changes in the ratio of free NEDD8 to ubiquitin triggers NEDDylation by ubiquitin enzymes. *Biochem J*. 2012;441:927–36.
41. Hjerpe R, Thomas Y, Kurz T. NEDD8 overexpression results in neddylation of ubiquitin substrates by the ubiquitin pathway. *J Mol Biol*. 2012;421:27–9.
42. Leidecker O, Matic I, Mahata B, Pion E, Xirodimas DP. The ubiquitin E1 enzyme Ube1 mediates NEDD8 activation under diverse stress conditions. *Cell Cycle*. 2012;11:1142–50.
43. Lobato-Gil S, Heidelberger JB, Maghames C, Bailly A, Brunello L, Rodriguez MS, et al. Proteome-wide identification of NEDD8 modification sites reveals distinct proteomes for canonical and atypical NEDDylation. *Cell Rep*. 2021;34:108635.
44. Maghames CM, Lobato-Gil S, Perrin A, Trauchessec H, Rodriguez MS, Urbach S, et al. NEDDylation promotes nuclear protein aggregation and protects the Ubiquitin Proteasome System upon proteotoxic stress. *Nat Commun*. 2018;9:4376–4376.
45. Geurink PP, van der Heden van Noort GJ, Mulder MPC, Knaap RCM, Kikkert M, Ovaa H. Profiling DUBs and Ubl-specific proteases with activity-based probes. In: Hochstrasser M, editor. *Methods in Enzymology*. Academic Press; 2019. p. 357–87.
46. Impens F, Radoshevich L, Cossart P, Ribet D. Mapping of SUMO sites and analysis of SUMOylation changes induced by external stimuli. *Proc Natl Acad Sci USA*. 2014;111:12432.
47. Brancolini C, Iuliano L. Proteotoxic stress and cell death in cancer cells. *Cancers*. 2020;12:<https://doi.org/10.3390/cancers12092385>.
48. Zheng S, Wang W, Aldahdooh J, Malyutina A, Shadbahr T, Tanoli Z, et al. SynergyFinder Plus: toward better interpretation and annotation of drug combination screening datasets. *Genom Proteom Bioinform*. 2022;20:587–96.
49. Ri M. Endoplasmic-reticulum stress pathway-associated mechanisms of action of proteasome inhibitors in multiple myeloma. *Int J Hematol*. 2016;104:273–80.
50. Brewer JW. Regulatory crosstalk within the mammalian unfolded protein response. *Cell Mol Life Sci*. 2014;71:1067–79.
51. Xiong S, Chng W-J, Zhou J. Crosstalk between endoplasmic reticulum stress and oxidative stress: a dynamic duo in multiple myeloma. *Cell Mol Life Sci*. 2021;78:3883–906.
52. Fouquier J, Guedj M. Analysis of drug combinations: current methodological landscape. *Pharm Res Perspect*. 2015;3:e00149.
53. Chen L, Meng Y, Guo X, Sheng X, Tai G, Zhang F, et al. Gefitinib enhances human colon cancer cells to TRAIL-induced apoptosis of via autophagy- and JNK-mediated death receptors upregulation. *Apoptosis*. 2016;21:1291–301.
54. Dhanasekaran DN, Reddy EP. JNK-signaling: a multiplexing hub in programmed cell death. *Genes Cancer*. 2017;8:682–94.
55. Voronina MV, Frolova AS, Kolesova EP, Kuldyushev NA, Parodi A, Zamyatnin AAJ. The intricate balance between life and death: ROS, cathepsins, and their interplay in cell death and autophagy. *Int J Mol Sci*. 2024;25. <https://doi.org/10.3390/jms25074087>.
56. Bertossi A, Aichinger M, Sansonetti P, Lech M, Neff F, Pal M, et al. Loss of Roquin induces early death and immune deregulation but not autoimmunity. *J Exp Med*. 2011;208:1749–56.
57. Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. *Nature*. 2005;435:452–8.
58. Chevrier S, Kratina T, Emslie D, Karnowski A, Corcoran LM. Germinal center-independent, IgM-mediated autoimmunity in sanroque mice lacking Obf1. *Immunol Cell Biol*. 2014;92:12–19.
59. Kim S, Kwon M, Hwang Y, Yoon J, Park S, Kang HC. Stress-induced NEDDylation promotes cytosolic protein aggregation through HDAC6 in a p62-dependent manner. *iScience*. 2021;24:102146.
60. Vogl AM, Phu L, Becerra R, Giusti SA, Verschueren E, Hinkle TB, et al. Global site-specific neddylation profiling reveals that NEDDylated cofilin regulates actin dynamics. *Nat Struct Mol Biol*. 2020;27:210–20.
61. Galluzzi L, Buqué A, Kepp O, Zitvogel L, Kroemer G. Immunogenic cell death in cancer and infectious disease. *Nat Rev Immunol*. 2017;17:97–111.
62. Serrano-Del Valle A, Anel A, Naval J, Marzo I. Immunogenic cell death and immunotherapy of multiple myeloma. *Front Cell Dev Biol*. 2019;7:50.
63. Waad Sadiq Z, Brioli A, Al-Abdulla R, Çetin G, Schütt J, Murua Escobar H, et al. Immunogenic cell death triggered by impaired deubiquitination in multiple myeloma relies on dysregulated type I interferon signaling. *Front Immunol*. 2023;14:982720.

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AUTHOR CONTRIBUTIONS

A.D. and K.P.K. designed and supervised the study; F.I., M.F., W.W.S., H.O., P.P.G., H.B., and M.S. contributed to methodology. A.D., F.T., O.G., N.C., M.F., M.C.A., H.B., J.L., and G.J.H.N. carried out the investigation. A.D., G.M., F.T., F.I., and M.S. performed formal analysis. A.D. and K.P.K. wrote the original draft. K.P.K. acquired funding. M.P., H.O., P.P.G., B.J., W.W.S., O.Gr., H.B., K.A., M.S.D., and V.H. provided resources. A.D., F.I., M.S., and K.P.K. supervised the work. All authors approved the final version of the manuscript.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The authors confirm that all methods were performed in accordance with the relevant guidelines and regulations. Myeloma patients who were diagnosed and treated at the Department of Haematology at the University Hospital of Erlangen were included. Bone marrow cells were collected and used from left-over material of myeloma patients upon diagnostic bone marrow aspiration, which was approved by the local ethics committee of the University of Erlangen (219_14B, 19-56D). Animal experiments were performed in concordance with existing ethical guidelines and approved by the RP Freiburg (X -20/02 A and G-16/104) and the local authorities of North Rhine-Westphalia, Germany (LAVE AZ: 81-02.04.2021.A276). The authors

confirm that informed consent was obtained from all study participants. No identifiable images are included in this study.

ADDITIONAL INFORMATION

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