PROGRAMMABLE PROTEIN STABILIZATION WITH LANGUAGE MODEL-DERIVED PEPTIDE GUIDES

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ABSTRACT

Dysregulated protein degradation via the ubiquitin-proteasomal pathway can induce numerous disease phenotypes, including cancer, neurodegeneration, and diabetes. Stabilizing improperly ubiquitinated proteins via target-specific deubiquitination is thus a critical therapeutic goal. Building off the incredible advances in targeted protein degradation (TPD), recently-described targeted protein stabilization (TPS) modalities rely on a limited set of chemical linkers and small molecule warheads, which do not exist for classically "undruggable" targets. Previously, we engineered ubiquibodies (uAbs) by fusing computationally-designed "guide" peptides to E3 ubiquitin ligase domains for modular, CRISPR-analogous TPD. Here, we engineer "deubiquibodies" (duAbs), via fusion of generated peptides to the catalytic domain of the potent OTUB1 deubiquitinase. In human cells, duAbs effectively stabilize exogenous and endogenous proteins in a DUB-dependent manner. To demonstrate duAb modularity, we swap in new target-binding peptides designed via our generative language models to stabilize unique target proteins, including a transcription factor and a kinase. In total, our duAb system represents a simple, programmable, genetically-encoded strategy for TPS.

1 INTRODUCTION

The ubiquitin-proteasomal pathway regulates critical processes, including protein folding, DNA repair, and cell differentiation, thus helping to maintain proteostasis (Zhao et al., 2022). Dysregulation of this pathway – such as improper degradation of tumor suppressors or mutant, misfolded proteins – can lead to severe pathogenic phenotypes, such as cancer, neurodegenerative disease, cystic fibrosis, and diabetes (Sabapathy & Lane, 2017; Ward et al., 1995; Liu et al., 2023; Ciechanover & Brundin, 2003). While targeted protein degradation (TPD) strategies (PROTACs, molecular glues, etc.) have been extensively engineered (Zhao et al., 2022), only recently has the reverse process, targeted protein stabilization (TPS), gained attention (Henning et al., 2022). Analogous to PROTACs, the current state-of-the-art TPS modality, termed deubiquitinase-targeting chimeras or DUBTACs, recruit endogenous deubiquitinases (DUBs) and thus rely on the design of chemical linkers, as well as the existence of small molecule warheads, which do not exist for classically "undruggable" proteins, those that lack putative or cryptic binding site accessibility, and are conformationally disordered (Henning et al., 2022).

In recent years, our team has described a novel TPD strategy, by genetically fusing target-specific short "guide" peptides, designed via both structure- and sequence-based algorithms, to the ubiquitin conjugation domain of the human CHIP E3 ubiquitin ligase, creating CRISPR-like "ubiquibodies" (uAbs) for TPD (Chatterjee et al., 2020; Palepu et al., 2022; Brixi et al., 2023; Bhat et al., 2023). Here, we design the analogous platform for TPS, termed deubiquibodies (duAbs), via fusion of generated guide peptides to the catalytic domain of the potent OTUB1 deubiquitinase. By first utilizing "off-the-shelf" binders that have been previously designed, our first-generation fusion architecture, when introduced into human cells, effectively stabilizes exogenous and endogenous proteins in a DUB-dependent manner. Finally, we showcase the inherent programmability of duAbs by simply swapping in new target-binding peptides designed via our recent generative language models, SaLT&PepPr and PepPrCLIP (Brixi et al., 2023; Bhat et al., 2023). These novel duAbs stabilize unique target substrates, including a transcription factor (FOXP3) and a kinase (WEE1), motivating rapid design of duAbs to diverse proteins in the proteome.

2 METHODS

Binder Design The βcat-SnP-7 peptide (Brixi et al., 2023), P60D2A peptide (Lozano et al., 2017), and YFP nanobodies (Kanner et al., 2020) were described in previous works and obtained from respective manuscript metadata. Novel binding peptides designed in this study were either generated by the previously-described SaLT&PepPr algorithm (Brixi et al., 2023) via input of an interacting partner sequence, or by the *de novo* PepPrCLIP algorithm (Bhat et al., 2023) via input of the target protein sequence. All binder sequences can be found in Supplementary Table A2.

Generation of Plasmids All duAb plasmids were generated from the standard pcDNA3 vector, harboring a cytomegalovirus (CMV) promoter and a C-terminal P2A-eGFP cassette. An Esp3I restriction site was introduced immediately upstream of the OTUB1 catalytic domain and flexible GSGSG linker via the KLD Enzyme Mix (NEB) following PCR amplification with mutagenic primers (Genewiz). For duAb assembly, oligos for candidate peptides were annealed and ligated via T4 DNA Ligase into the Esp3I-digested duAb backbone. Assembled constructs were transformed into 50 uL NEB Turbo Competent Escherichia coli cells, and plated onto LB agar supplemented with the appropriate antibiotic for subsequent sequence verification of colonies and plasmid purification (Genewiz).

Cell Culture and Flow Cytometry HEK293T and colorectal adenocarcinoma cell line, DLD1, were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 units/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (FBS). The hepatocellular carcinoma cell line, HepG2, was maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 100 units/mL penicillin, 100 mg/mL streptomycin, and 10% fetal bovine serum (FBS). The DLD1 and HepG2 cell lines were generous gifts from Dr. Matthew DeLisa. For duAb screening, pcDNA-duAb (500 ng) plasmids were transfected into cells as triplicates ($3x10^5$ cells/well in a 24well plate) with Lipofectamine 2000 (Invitrogen) in Opti-MEM (Gibco). After 2 days post transfection, 4 uM PR-619 (DUB inhibitor) was added to applicable cells (with equiv-

alent volume of media added to non-treated cells), and subsequently cells were harvested within 24 hours post-treatment and analyzed on a Attune NxT Flow Cytometer (ThermoFisher) for GFP fluorescence (488-nm laser excitation, 530/30 filter for detection) and mCherry fluorescence (561-nm laser excitation, 620/15 filter for detection). Viable, single cells were gated for data analysis based on default FSC/SSC parameters for the analyzed cells. Cells expressing eGFP were gated, and normalized values were calculated to a sample transfected with a non-targeting duAb using the FlowJo software (`https://flowjo.com/`). Representative flow cytometry gating strategies are indicated in Supplementary Figure A1.

For the TOPFlash assay, $3x10^5$ DLD1-TOP cells/well were seeded on a 24-well plate 20-24 hours prior to transfection. On the day of transfection, each well received the following plasmids: TOP-GFP plasmid (Addgene plasmid no. 35489) and a duAb plasmid. A total of 500 ng of plasmid DNA in a ratio of TOPFlash:duAb plasmids = 1:1 was mixed with Lipofectamine 2000 reagent in serum-free Opti-MEM medium and added dropwise to each well after incubation at room temperature for 20 min. After 72 h of incubation, cells were harvested and analyzed similarly as mentioned for duAb screening.

Immunoblotting On the day of harvest, cells were detached by addition of 0.05% trypsin-EDTA and cell pellets were washed twice with ice-cold 1x PBS. Cells were then lysed and subcellular fractions were isolated from lysates. Immunoblotting was performed according to standard protocols. Proteins were probed with rabbit anti-WEE1 antibody (Abcam, Cat no. ab137377, diluted 1:1000) or mouse anti-GAPDH (Santa Cruz Biotechnology, Cat no. sc-47724; diluted 1:1000) for overnight incubation at 4C. The blots were washed and then probed with a secondary antibody, goat anti-rabbit IgG (H+L), horseradish peroxidase (HRP) (ThermoFisher, Cat no. 31460, diluted 1:5000) or goat anti-mouse IgG (H+L) Poly-HRP (ThermoFisher, Cat no. 32230, diluted 1:5000) for 1-2 h at room temperature. Following three washes with 1X TBST for 5 min each, blots were detected by chemiluminescence using a Biorad ChemiDoc Touch Imaging System (Biorad). Densitometry analysis of protein bands in immunoblots was performed using ImageJ software as described here: https://imagej.nih.gov/ij/docs/examples/dot-blot/. Intensity data for the duAb bands was first normalized to band intensity of GAPDH in each lane then to the average band intensity for empty duAb vector control cases across replicates.

Statistical Analysis and Reproducibility To ensure robust reproducibility of all results, experiments were performed with at least three biological replicates and at least three technical measurements. Sample sizes were not predetermined based on statistical methods but were chosen according to the standards of the field (at least three independent biological replicates for each condition), which gave sufficient statistics for the effect sizes of interest. All data were reported as average values with error bars representing standard deviation (SD). For individual samples, statistical analysis was performed using the two-tailed Student's t test using GraphPad Prism 10 software, with calculated p values are represented as follows: *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$. No data were excluded from the analyses. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

3 Results and Discussion

duAb Engineering Recently, Kanner, et al. fused the OTUD1 deubiquitinase domain to YFP-targeting nanobodies to create enDUBO1 constructs that stabilize target-YFP fusion proteins (Figure 1A) (Kanner et al., 2020). We hypothesized that DUB domains exhibiting more potent deubiquitinase activity may improve TPS. To evaluate potential effectors for recruitment, Poirson, et al., conducted a proteome-scale induced proximity screen to rank both ubiquitinating and deubiquitinating enzymes in terms of catalytic activity (Supplementary Table A1) (Poirson et al., 2022). They isolated a subset of deubiquitinases, including OTUB1 and UCHL1, as well as a SUMOlase, UBC9, with potent stabilization activity (Poirson et al., 2022). Of note, OTUB1 is the endogenous deubiquitinase recruited by DUBTACs (Figure 1A) (Henning et al., 2022).

Using known domain annotations of these proteins in UniProt (Bateman et al., 2022), we isolated the catalytic domains of each enzyme and fused them to the aforementioned YFP-targeting nanobody utilized by Kanner, et al. via either the GAPGSG linker (used for enDUBO1) (Kanner et al., 2020), termed L1, or the flexible GSGSG linker already used in the uAb architecture, termed L2 (Supple-

mentary Table A1 and A2). We additionally wanted to validate that our DUB fusions act in a DUB-dependent manner by employing the pan-DUB inhibitor PR-619 (Hsu et al., 2021) when measuring KCNQ1-YFP potassium ion channel stabilization, as conducted in Kanner, et al. (Hsu et al., 2021). We show that YFP nanobody-L2-OTUB1 fusions demonstrate significantly increased KCNQ1-YFP stabilization vs. enDUBO1 (YFP nanobody-L1-OTUD1), YFP nanobody-L2-UCHL1, and YFP nanobody-L2-UBC9 in the presence of KCNQ1's E3 ubiquitin ligase NEDD4L, and that addition of PR-619 at a standard concentration (4 uM) to both samples abrogates stabilization, confirming the DUB-dependent mechanism of these constructs (Figure 1B).

We next sought to extend the usability of our duAbs by leveraging computationally derived target-recruiting peptides. Derived from our SaLT&PepPr algorithm (Brixi et al., 2023), we utilized our previously-designed βcat-SnP-7 peptide and compared the βcat-SnP-7-L2-OTUB1 and βcat-SnP-7-L2-OTUD1 fusions. Specifically, βcat-SnP-7 exhibits nanomolar binding affinity to β-catenin (Brixi et al., 2023), and we thus hypothesized that it would induce β-catenin-sfGFP stabilization when fused to DUB catalytic domains in HEK293T cells, which have an intact Wnt signaling pathway expressing β-catenin's natural E3 ubiquitin ligase, β-TrCP (Tan et al., 2014). We demonstrate that, when fused to βcat-SnP-7 via L2, the OTUB1 catalytic domain exhibits statistically significant stabilization of βcat-catenin-sfGFP proteins, as compared to OTUD1 (Figure 1C). We further corroborated this result by co-transfecting βcat-SnP-7-L2-OTUB1 fusions into DLD1 colorectal cancer cells alongside TOPFlash, a fluorescent reporter that serves as a reliable readout of β-catenin–dependent transcriptional activity (Horst et al., 2012). βcat-SnP-7-L2-OTUB1-treated cells exhibit significantly higher Wnt signaling than either untreated or βcat-SnP-7-L2-OTUD1-treated samples, indicating increased β-catenin levels (Figure 1D). Motivated by our results, we thus term binder-L2-OTUB1 fusions as duAbs (Figure 1A).

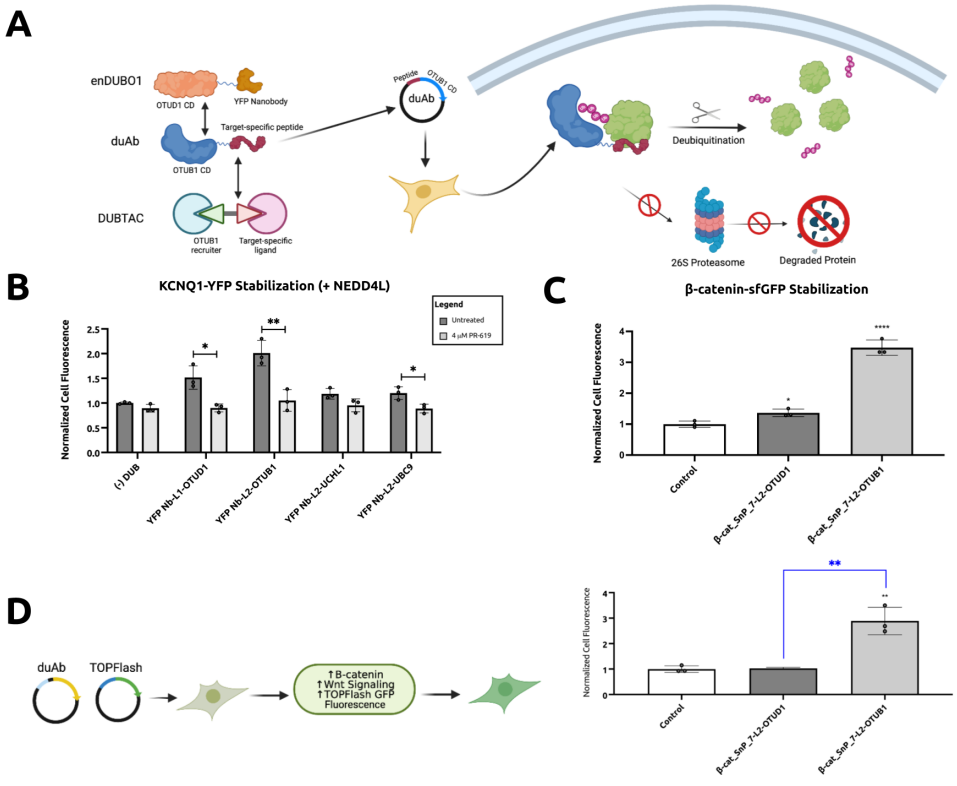

Figure 1: Engineering of the duAb architecture. A) Current targeted protein stabilization strategies. B) KCNQ1-YFP stabilization in HEK293T cells using different deubiquitinase catalytic domains. C) β-catenin-sfGFP stabilization in HEK293T cells. D) TOPFlash-GFP assay for measuring Wnt signaling in DLD1 cells.

duAb Programmability The enDUBO1 fusion described in Kanner, et al., relies on target proteins to be tagged with YFP to enable YFP nanobody binding and subsequent OTUD1-mediated deubiquitination, limiting the applicability of this genetically-encoded platform (Kanner et al., 2020). Having shown that our new duAb architecture can accept peptidic binder sequences for target-specific stabilization, we sought to demonstrate duAb programmability by designing these guide peptides to unique target proteins (Figure 2A). We first focused our attention on FOXP3, a classically undruggable transcription factor that plays a central role in the development and function of regulatory T cells (Tregs) (Hori, 2021). FOXP3 is naturally regulated by the CHIP E3 ubiquitin ligase, which is expressed in HEK293T cells (Barbi et al., 2015). We thus generated a FOXP3-mCherry HEK293T stable cell line, and applied our SaLT&PepPr interface-prediction algorithm to isolate guide peptides from its well-known interacting partner, NFAT (Supplementary Table A2) (Barbi et al., 2015; Wu et al., 2006). We demonstrate that SaLT&PepPr-derived duAbs induce statistically significant stabilization of FOXP3-mCherry in a DUB-dependent manner, and outperform that of the previously-designed P60D2A FOXP3-targeting peptide (Figure 2B) (Lozano et al., 2017).

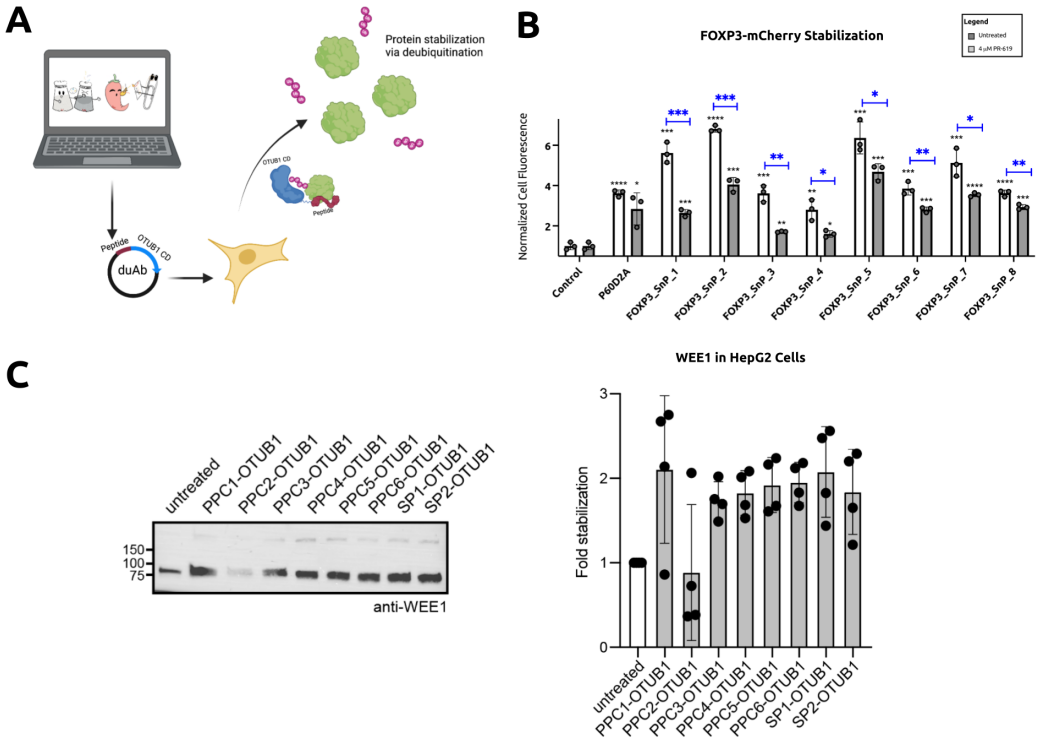

Figure 2: Programmable target stabilization via language model-derived peptides. A) Programmable target stabilization via language model-derived peptides. B) FOXP3-mCherry stabilization in HEK293T cells. C) Stabilization of endogenous WEE1 in protein extracts of HepG2 cells analyzed via immunoblotting.

Finally, we sought to stabilize a protein with a known small molecule ligand. Henning, et al. designed a DUBTAC to WEE1 (Henning et al., 2022), an inhibitor of tumor growth in non-cancerous eukaryotic somatic cells, which acts as a kinase to add phosphate groups to the cyclin-dependent kinase (CDK1)–cyclin B1 complex (Ghelli Luserna di Rorà et al., 2020). This addition of phosphate groups hinders the advancement of the cell cycle in the S and G2 phases of mitosis (Ghelli Luserna di Rorà et al., 2020). It has been established that WEE1 is regulated by the ubiquitin-proteasomal pathway in HepG2 hepatocellular carcinoma cell lines (Cruz et al., 2021), and thus, we designed 8 peptide-guided duAbs for WEE1, 2 via SaLT&PepPr (Brixi et al., 2023) and 6 via the *de novo* peptide design algorithm, PepPrCLIP (Bhat et al., 2023)), for subsequent transfection into HepG2

cells (Supplementary Table A2). Via Western blotting with an anti-WEE1 antibody, our results demonstrate that PepPrCLIP and SaLT&PepPr each prioritized peptides that induce statistically-significant endogenous stabilization of WEE1 when integrated into the duAb architecture (Figure 2C), motivating future comparison to DUBTACs for druggable and undruggable targets.

4 CONCLUSION

The CRISPR-Cas system is incredibly powerful for genome editing due to its modularity. The simple design of an sgRNA to a new genomic sequence enables Cas-mediated editing of that locus (Wang & Doudna, 2023). However, CRISPR is not limited to just gene disruption – the system has been extended to conduct single base editing, gene insertion, and even gene activation via fusion to transactivation domains (Wang & Doudna, 2023). Analogous to the original RNA-guided CRISPR nuclease system, our peptide-guided uAb degraders are genetically-encodable "off" switches for proteins. Here, we have created the respective "on" switch, by developing peptide-guided duAbs for TPS. Integrated with our rapid binder generation algorithms, our results demonstrate the simplicity and programmability of duAbs to stabilize diverse target substrates intracellularly.

As duAbs are 290 amino acids in length (Supplementary Table A2), their intracellular delivery, at first glance, poses a challenge for therapeutic application. However, with the rapid advancements of targeted lipid nanoparticle (LNP) platforms (Hou et al., 2021), duAbs can be readily encapsulated as mRNA and delivered to specific tissues of interest, as opposed to DUBTACs, which may home to any tissue, risking potential side effects and toxicity concerns. More interestingly, as a genetically-encoded tool, peptide-guided duAbs, alongside uAbs, may underlie a powerful proteome screening tool for drug discovery, allowing for combinatorial protein activation and inhibition screening, similar to the CRISPR-based Perturb-Seq platform (Dixit et al., 2016). Finally, with advancements in protein representation algorithms Chen et al. (2023), we envision that our language model-generated peptides can be augmented to specifically bind post-translational and mutant isoforms of target proteins, and can be fused to other PTM domains, including kinases, phosphatases, and deglycosylases, to name a few. This study, enabling modular peptide-guided protein stabilization, represents a next step towards this eventual goal of a fully programmable proteome editing system.

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A SUPPLEMENTARY FIGURES

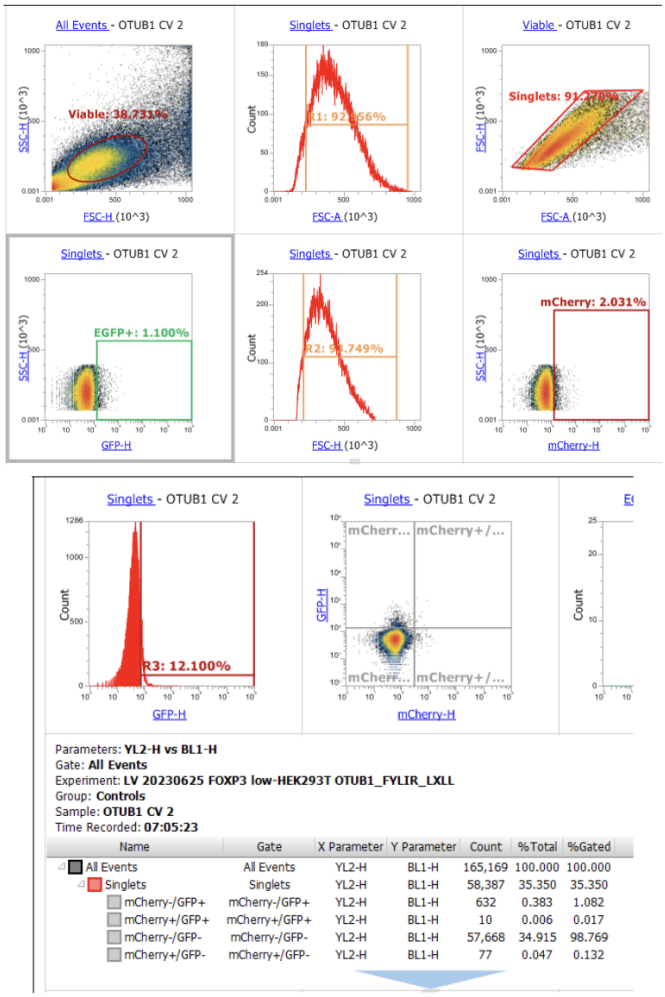

Figure A1: Example gating strategy for flow cytometry.

B SUPPLEMENTARY TABLES

Table A1: Linker and DUB catalytic domain sequences used in study.

Label/Name	Type	Amino Acid Sequence	UniProt ID
L1	Linker	GAPGSG	N/A
L2	Linker	GSGSG	N/A
OTUB1	DUB Catalytic Domain	AAEEPQQQKQEPLGSDSEGVNCLAYDEAIMAQQDRIQQEIAVQNP LVSERLELSVLYKEYAEDDNIYQQKIKDLHKKYSYIRKTRPDGNCFY RAFGFSHLEALLDDSKELQRFKAVSAKSKEDLVSQGFTEFTIEDFH NTFMDLIEQVEKQTSVADLLASFNDQSTSDYLVVYLRLLTSGYLQR ESKFFEHFIEGGRTVKEFCQQEVEPMCKESDHIHIIALAQALSVSIQ VEYMDRGEGGTTNPHIFPEGSEPKVYLLYRPGHYDILYK	Q96FW1
OTUD1	DUB Catalytic Domain	SDSEGVNCLAYDEAIMAQQDRIQQEIAVQNPLVSERLELSVLYKEYA EDDNIYQQKIKDLHKKYSYIRKTRPDGNCFYRAFGFSHLEALLDDS KELQRFKAVSAKSKEDLVSQGFTEFTIEDFHNTFMDLIEQVEKQTSV ADLLASFNDQSTSDYLVVYLRLLTSGYLQRESKFFEHFIEGGRTVKE FCQQEVEPMCKESDHIHIIALAQALSVSIQVEYMDRGEGGTTNPHIF PEGSEPKVYLLYRPGHYDILYK	Q5VV17
UCHL1	DUB Catalytic Domain	MQLKPMEINPEMLNKVLSRLGVAGQWRFVDVLGLEEESLGSVPAP ACALLLLFPLTAQHENFRKKQIEELKGQEVSPKVYFMKQTIGNSCG TIGLIHAVANNQDKLGFEDGSVLKQFLSETEKMSPEDRAKCFEKNE AIQAAHDAVAQEGQCRVDDKVNFHFILFNNVDGHLYELDGRMPFP VNHGASSEDTLLKDAAKVCREFTEREQGEVRFSAVALCKAA	P09936
UBC9	SUMOlase Catalytic Domain	MSGIALSRLAQERKAWRKDHPFGFVAVPTKNPDGTMNLMNWECAI PGKKGTPWEGGLFKLRMLFKDDYPSSPPKCKFEPPLFHPNVYPSG TVCLSILEEDKDWRPAITIKQILLGIQELLNEPNIQDPAQAEAYTIYCQ NRVEYEKRVRAQAKKFAPS	P63279

Table A2: Binder sequences used in this study.

Binder Name	Target UniProt ID	Target	Binder Sequence	Derivation
YFP Nb	N/A	YFP	MQVQLVESGGALVQPGGSLRLSCAASG FPVNRYSMRWYRQAPGKEREWVAGM SSAGDRSSYEDSVKGRFTISRDDARNT VYLQMNSLKPEDTAVYYCNVNVGFEYW GQGTQVTVSS	Kanner, et al.[11]
βcat_SnP_7	P35222	β-catenin	DPTAPPYDSLLVFDYEGS	Brixi, et al.[10]
P60D2A	Q9BZS1	FOXP3	RAFQSFRKMWPFFAM	Lozano, et al.[20]
FOXP3_SnP_1	Q9BZS1	FOXP3	YVINGKRK	SaLT&PepPr
FOXP3_SnP_2	Q9BZS1	FOXP3	NFYVINGKRK	SaLT&PepPr
FOXP3_SnP_3	Q9BZS1	FOXP3	KVNFYVINGKRK	SaLT&PepPr
FOXP3_SnP_4	Q9BZS1	FOXP3	VKVNFYVING	SaLT&PepPr
FOXP3_SnP_5	Q9BZS1	FOXP3	TPVKVNFY	SaLT&PepPr
FOXP3_SnP_6	Q9BZS1	FOXP3	FYVINGKRKRSQ	SaLT&PepPr
FOXP3_SnP_7	Q9BZS1	FOXP3	NGKRKRSQ	SaLT&PepPr
FOXP3_SnP_8	Q9BZS1	FOXP3	RSQPQHFTYH	SaLT&PepPr
WEE1_PpC_1	P30291	WEE1	VMVVYPPGSYLTASGSN	PepPrCLIP
WEE1_PpC_2	P30291	WEE1	SGGAGGYGFCTFSGSAL	PepPrCLIP
WEE1_PpC_3	P30291	WEE1	APMYRGYMRDSPGLPVK	PepPrCLIP
WEE1_PpC_4	P30291	WEE1	EMPGCKTHKGELVWETFNG	PepPrCLIP
WEE1_PpC_5	P30291	WEE1	YPGGGSWGQPHGGSWGQK	PepPrCLIP
WEE1_PpC_6	P30291	WEE1	RMPDDLYHTGELVQCTPHA	PepPrCLIP
WEE1_SnP_1	P30291	WEE1	DGAYFGTGFPHMLFMV	SaLT&PepPr
WEE1_SnP_2	P30291	WEE1	MSSSEEVSWISW	SaLT&PepPr