Targeted Protein Degradation in the Mitochondrial Matrix and Its Application to Chemical Control of Mitochondrial Morphology

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Abstract

Dysfunction of mitochondria is implicated in various diseases, including cancer and neurodegenerative disorders, but drug discovery targeting mitochondria and mitochondrial proteins has so far made limited progress. Targeted protein degradation (TPD) technologies represented by proteolysis targeting chimeras (PROTACs) are potentially applicable for this purpose, but most existing TPD approaches

leverage the ubiquitin-proteasome system or lysosomes, which are absent in mitochondria, and TPD in mitochondria (mitoTPD) remains little explored. Herein, we describe the design and synthesis of a bifunctional molecule comprising TR79, an activator of the mitochondrial protease complex caseinolytic protease P (ClpP), linked to desthiobiotin. This compound successfully induced the degradation of monomeric streptavidin (mSA) and its fusion proteins localized to the mitochondrial matrix. Furthermore, in cells overexpressing mSA fused to short transmembrane protein 1 (mSA-STMP1), which enhances mitochondrial fission, our mitochondrial mSA degrader restored the mitochondrial morphology by reducing the level of mSA-STMP1. A preliminary structure-activity relationship study indicated that a longer linker length enhances the degradation activity towards mSA. These findings highlight the potential of mitoTPD as a tool for drug discovery targeting mitochondria and for research in mitochondrial biology, as well as the utility of mSA as a degradation tag for mitochondrial protein.

Introduction

In recent years, targeted protein degradation (TPD) technology has attracted great attention as a new drug discovery modality. TPD is a method to degrade and remove target proteins in cells by using compounds called degraders that utilize the endogenous protein degradation machinery. Given their unique mode of action, degraders are expected to be effective even against classically undruggable targets, such as nonenzymatic proteins and aggregation-prone proteins.[1–3] Ubiquitin-proteasome system (UPS)-harnessing degraders termed proteolysis targeting chimeras (PROTACs), which are chimeric molecules composed of a ubiquitin ligase ligand and a ligand for the protein of interest (POI), have been most actively studied degraders and many PROTACs have already entered Phase I to III clinical trials.[4,5] PROTACs are also used as chemical protein knockdown tools as alternatives to RNA interference, and have contributed to biological research.[6–8]

The emergence of PROTACs has already established that TPD technology is a promising approach, especially for hard-to-drug targets,[9] and has led to the development of other TPD technologies such as hydrophobic tagging,[10,11] which harnesses protein quality control machinery, and methods exploiting lysosomal degradation machineries,[12] including AUTACs,[13] ATTECs,[14] AUTOTACs,[15] and LYTACs.[16] In 2022, Clausen *et al*. developed BacPROTAC, a degrader utilizing unique degradation machinery in gram-positive bacteria, for TPD in bacteria.[17] This study pioneered the use of protein degradation machineries other than UPS and lysosomes.

Mitochondria are important organelles that produce about 90% of the ATP metabolized in cells. In

addition, they play multiple roles in processes such as ion homeostasis, cell growth, redox state maintenance, cell signaling, and stress response. Therefore, mitochondrial function is critical for the regulation of cell function, as well as being associated with the onset and progression of diseases such as cancer, neurodegenerative diseases, cardiovascular diseases, and metabolic diseases.[18] For example, short transmembrane protein 1 (STMP1), a 47-mer transmembrane micropeptide localized to the mitochondrial inner membrane, is upregulated in various cancer cells and is associated with cancer metastasis and recurrence. It has been reported that overexpression of STMP1 promotes mitochondrial fission and enhances tumor cell migration. Further, knockdown of STMP1 suppresses mitochondrial fission, suggesting it may be a potential target for cancer therapy.[19] For this reason, mitochondria and mitochondrial proteins that control mitochondrial function have attracted great attention as drug targets in recent years,[20–22] but few drugs or therapies have yet been approved.[22] The main reason for this is considered to be the complexity of mitochondrial biology.[23] Consequently, the achievement of TPD in mitochondria (mitoTPD) is expected to contribute greatly to the elucidation of mitochondrial biology, as well as expanding drug discovery options. Indeed, quite recently, TPD strategies for degrading mitochondrial proteins have begun to be developed.[24,25]

Herein, we describe the design and synthesis of a bifunctional molecule comprising TR79, an activator of the mitochondrial protease complex caseinolytic protease P (ClpP), linked to desthiobiotin, and we show that it can rapidly induce the degradation of monomeric streptavidin (mSA) and an mSA-fused protein of interest overexpressed in mitochondria. As proof of concept, we demonstrate that in cells

overexpressing mSA-STMP1 fusion protein, which enhances mitochondrial fission, our degrader restored the mitochondrial morphology by reducing the level of mSA-STMP1, thereby enabling control of mitochondrial morphology. We believe these results advance the prospects for employing mitoTPD as a modality to develop drugs targeting mitochondrial proteins, as well as validating the mSA-based degradation tag platform as a useful tool to study mitochondrial biology.

Results

Conceptual basis of this work

As mitochondria lack UPS and lysosomes, mitoTPD requires harnessing protein degradation machinery that is unique to mitochondria. Candidates include the following four ATP-dependent proteases: Lon, ClpXP (a complex of caseinolytic protease P (ClpP) and AAA+ATPase ClpX), the matrix-oriented AAA (m-AAA) protease, and the intermembrane space-oriented AAA (i-AAA) protease.[26–28]

ClpP is a serine protease resident in the matrix and is well conserved across species, being found in mitochondria, chloroplasts, and most bacteria.[26] We hypothesized that mitoTPD could be achieved by using a chimeric small molecule consisting of a ClpP activator and a ligand of the target protein to bring the target protein into close proximity with activated ClpP (Figure 1a). Numerous activators of ClpP have already been reported and shown to degrade mitochondrial proteins non-specifically.[29,30] We selected TR79, which has a primary amine in a pyrimidinedione scaffold, as a ClpP activator. Since this amino group has already been used to conjugate TR79 to agarose resin for ClpP pull-down,[31] we thought that it would be suitable as a linker site for chimeric compound design. As a POI, we selected monomeric streptavidin (mSA)[32] for the following two reasons: 1) biotin and desthiobiotin are well known and commercially available as mSA ligands with high affinity, and 2) mSA has a sufficiently small molecular weight (12 kDa) and has a track record as a fusion protein.[33] We selected desthiobiotin as a ligand for mSA because it is thought to be bioorthogonal in mammals that lack avidin, as discussed

later. Accordingly, we designed and synthesized compound **1**, which connects TR79 and desthiobiotin via a tetraethylene glycol linker (Figure 1b, Scheme S1, S2).

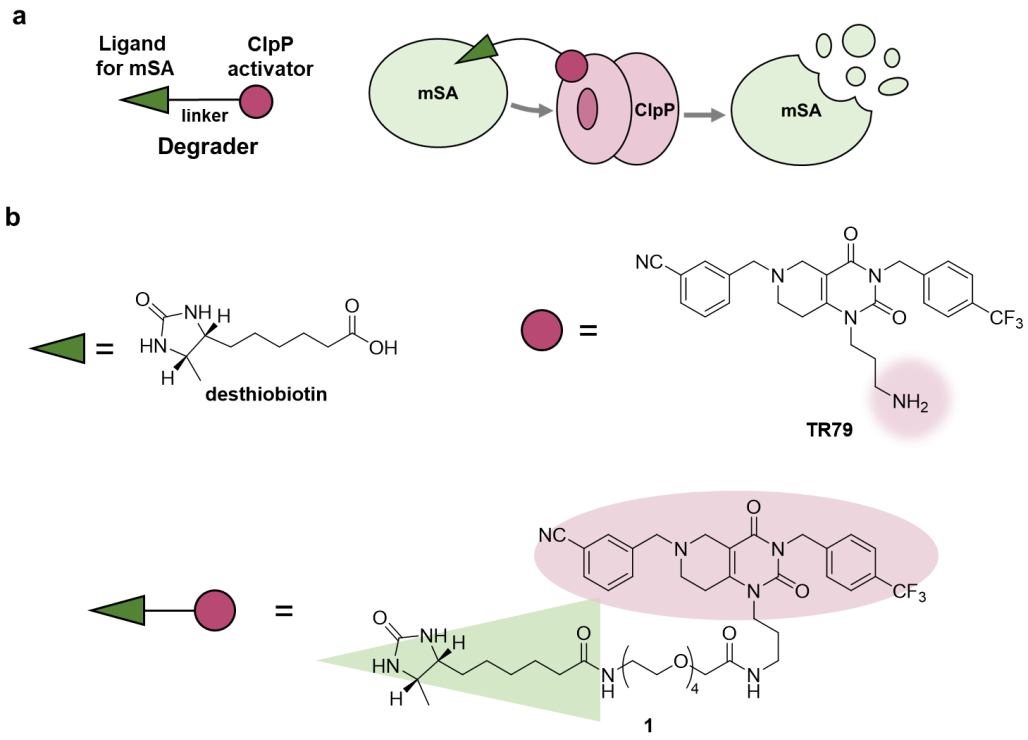

Figure 1. Conceptual basis of TPD using mitochondrial ClpP

a. Schematic illustration of mitoTPD by a chimeric compound consisting of a ClpP activator conjugated to an mSA ligand via a linker.

b. Structures of desthiobiotin (an mSA ligand), TR79 (a ClpP activator), and the chimeric compound **1**. The primary amino group of TR79 serves as the conjugation site for the PEG linker.

In vitro mSA degradation by 1

First, we conducted in vitro ClpP activation assay and pull-down assay using SA-conjugated beads to confirm that compound **1** is capable of ClpP activation and can form a [SA-**1**-ClpP] ternary complex (Figure S1, S2a). The results validated our molecular design. Next, in vitro mSA degradation assay revealed that compound **1** decreased mSA in a dose-dependent manner ($DC_{50,24h}$ = 197 nM under the conditions of 50 nM ClpP, 2.5 μM mSA) (Figure 2a). Excess desthiobiotin inhibited the mSA degradation activity of **1**, suggesting that the formation of the [mSA-**1**-ClpP] ternary complex is required for the activity of **1** (Figure 2b).

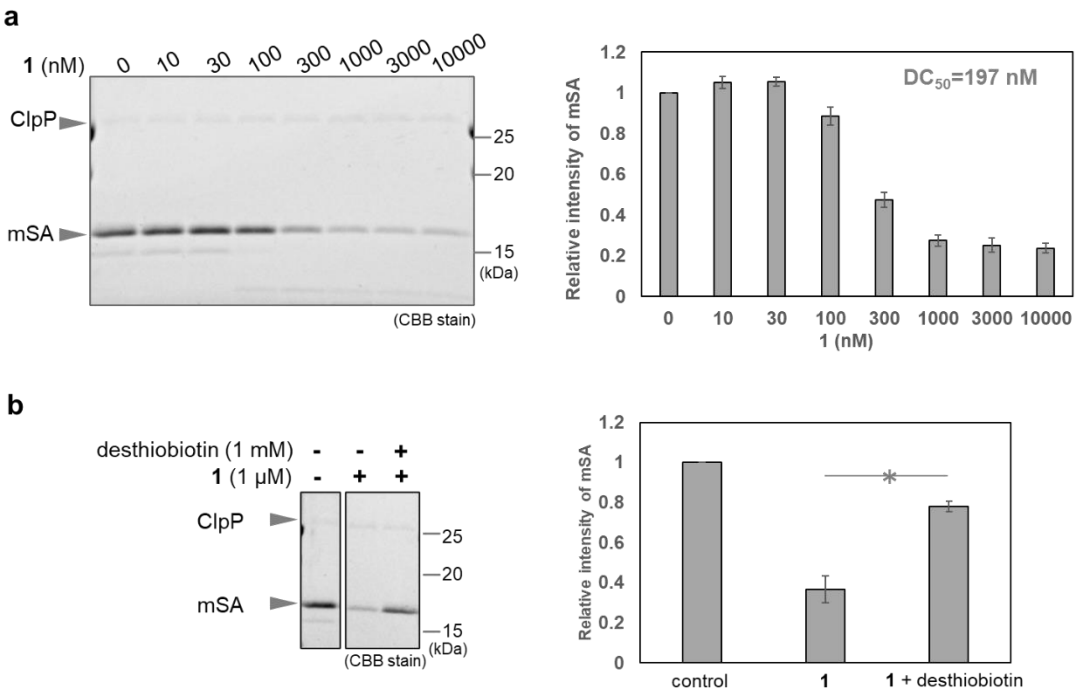

Figure 2. In vitro mSA degradation by 1

a. Degradation of mSA in vitro by treatment with various concentrations of **1** (SDS-PAGE/CBB

stain). The quantitative results are shown in the bar graph on the right. The intensity is normalized to that of the control sample (indicated as 0 nM), taken as 1.

b. Effect of desthiobiotin on mSA degradation mediated by **1**. The quantitative results are shown in the bar graph on the right. The intensity is normalized to the control sample as above.

Error bars indicate the SEM (n = 3 measurements). *: $p<0.05$, Student's t-test

Whereas ClpP activators promiscuously enhance protein degradation, chimeric compound **1** is expected to mediate selective degradation of the target protein. Indeed, in cell lysates supplemented with mSA and ClpP, compound **1** led to a decrease of mSA, while other proteins appeared to be unaffected as indicated by CBB staining (Figure S2b).

To further evaluate the selectivity, we used mass spectrometry-based proteomics analysis. Analysis of the sample derived from the above cell lysates supplemented with mSA and ClpP detected 1410 proteins. In the presence of compound **1**, mSA and 33 other proteins (2.4%) were decreased (Figure 3a and Table S1). Furthermore, in the presence of biotin as a competitor with **1**, the abundances of 4 proteins, including mSA, were increased compared to the sample prepared in the absence of biotin. These results indicate that only 3 proteins other than mSA are recruited to ClpP by compound **1** as off-targets, and the 30 other proteins are degraded non-specifically by **1**-activated ClpP (Figure 3b), suggesting that the degradation-inducing activity of **1** is highly selective for mSA.

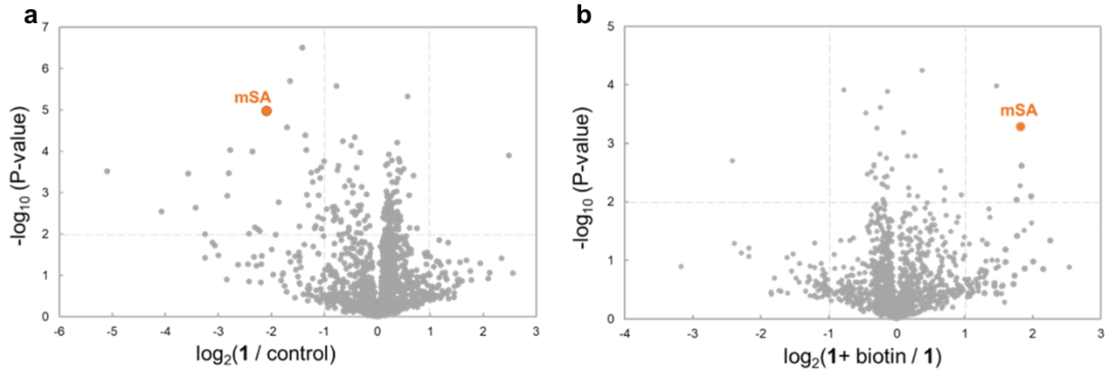

Figure 3. Impact of 1 on other cellular proteins.

Results of proteomics analysis. The vertical axis represents -$\log_{10}$ of the *p*-value (n = 3 measurements) (dotted lines: $p = 0.01$), and the horizontal axis represents the $\log_2$ fold change of protein abundance

(dotted lines: 0.5-fold and 2-fold changes, respectively).

a. The **1**-treated sample was compared to the control (DMSO-treated) sample.

b. The **1**-treated sample was compared to the **1**+biotin-treated sample.

Intracellular mSA degradation-inducing activity of 1

To evaluate the cellular activity of compound **1**, we next prepared HeLa cells transiently expressing mSA (cox8 (mitochondrial targeting signal sequence: MTS)[34]-His6-mSA-FLAG, hereafter cox8-mSA-FLAG or mSA) localized to the mitochondrial matrix (Figure S3a). Selective expression of cox8-mSA-FLAG in mitochondria was confirmed by isolation of the mitochondrial fraction followed by western blotting, which revealed three anti-FLAG-positive bands at 15-20 kDa. It is known that MTS is generally cleaved at the mitochondrial outer membrane or inner membrane when the protein translocates from the cytosol to the mitochondria. We speculated that the largest protein (about 20 kDa) was intact cox8-mSA-FLAG remaining on the outer membrane or in the intermembrane space of the mitochondria. The lowest-molecular-weight band remained mainly in the cytoplasmic fraction, and may consist of defective or misfolded molecules. We considered that the middle band was the desired mitochondrial mSA, and focused on this band in the following experiments (Figure S3b).

When HeLa cells transiently expressing mitochondrial cox8-mSA-FLAG were treated for 12 h with **1** (0-30 μM), we found that 10 μM and 30 μM **1** successfully reduced the mSA level with a DC_{50} value of 4.8 μM (Figure 4a). A significant reduction of mSA was also observed upon 4 h treatment with 10 μM **1** (Figure 4b). These results indicate that **1** reduces mSA in mitochondria in a dose- and time-dependent manner. We also performed similar experiments using MCF7 cells transiently expressing mitochondrial cox8-mSA-FLAG. After12 h treatment with 1 μM **1**, we observed a significant decrease of mSA abundance, and the DC_{50} value was 0.96 μM. In the case of treatment with 3 μM **1**, the mSA

level was significantly reduced within 2 h (Figure 4b). The reason for the difference in potency of compound **1** towards HeLa cells and MCF7 cells might be a difference in the expression levels of mSA (Figure S4).

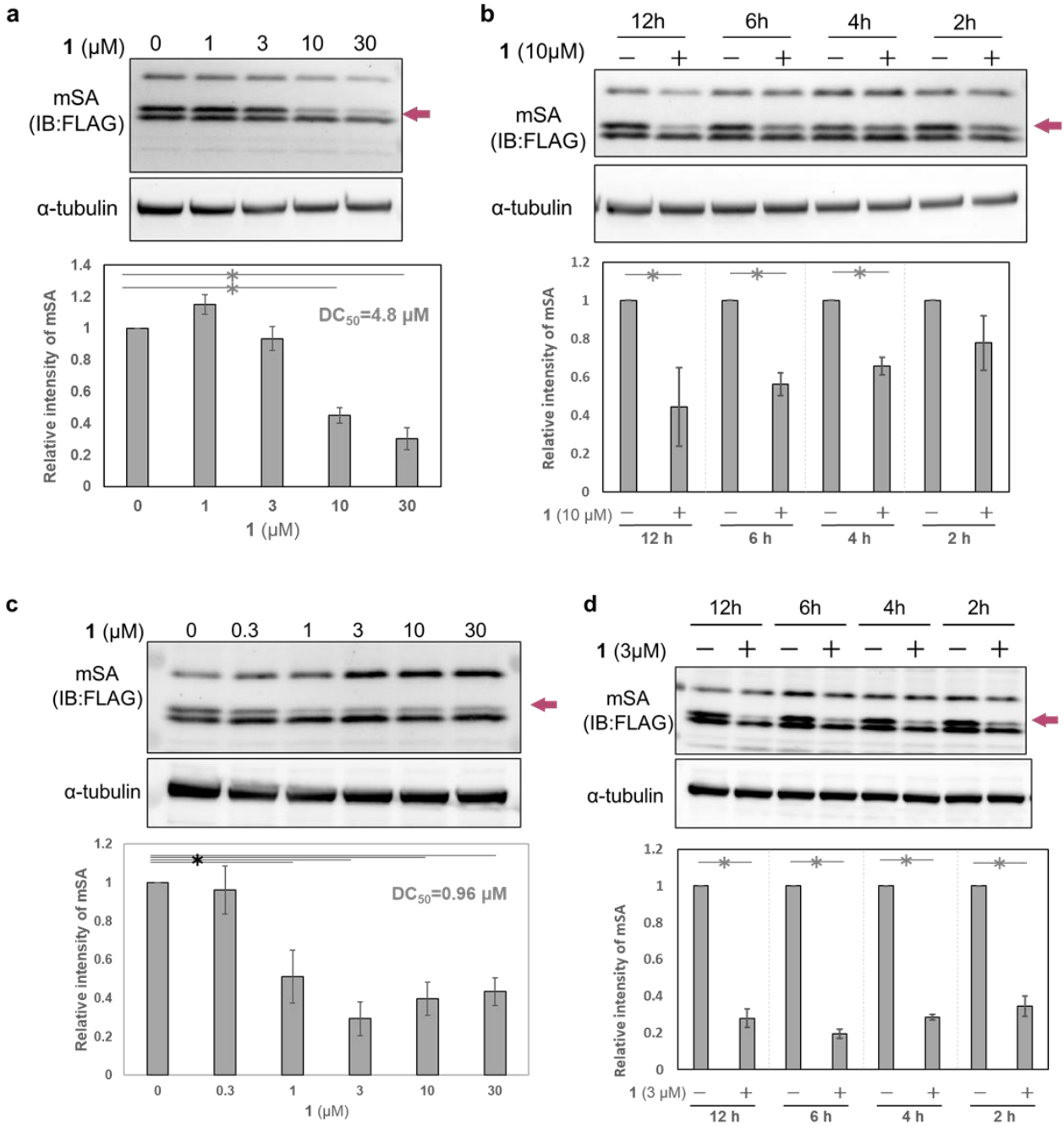

Figure 4. Degradation of mSA in mitochondria by treatment with 1.

a. Western blots and quantification of mSA in HeLa cells treated with various concentrations of **1** for 12 h.

b. Time-course of mSA level in HeLa cells treated with 10 μM **1**. Western blots and quantification results are shown.

c. Western blots and quantification of mSA in MCF7 cells treated with various concentrations of **1** for 12 h.

d. Time-course of mSA level in MCF7 cells treated with 3 μM **1**. Western blots and quantification results are shown.

Brown arrows on Western blot images indicate the putative mitochondrial mSA. Band intensities in the plots were normalized to the control band intensity, taken as 1. Error bars indicate SEM (n = 3 measurements). ∗: $p<0.05$, Student's t-test

To confirm the involvement of ClpP in the mSA-degradation-inducing activity of **1**, we carried out ClpP RNAi experiments in HeLa cells. The cells were first transfected with siRNA and incubated for 12 h, then transfected with mSA and treated with **1**. Western blot analysis showed that ClpP RNAi suppressed the **1**-induced degradation of mSA (Figure 5a, Figure S5). We also examined whether proteasomes and lysosomes are involved in **1**-induced mSA degradation. Pharmacological inhibition of proteasome or lysosome did not affect the mSA degradation induced by **1** (Figure 5b). These results suggest that the **1**-induced degradation of mSA depends on ClpP. Under conditions of proteasome inhibition, the intensity of the lowest-molecular-weight band related to mSA was increased, and this result is consistent with the idea that this band is due to mis-localized and/or mis-cleaved cox8-mSA-FLAG at least partially located in the cytosol.

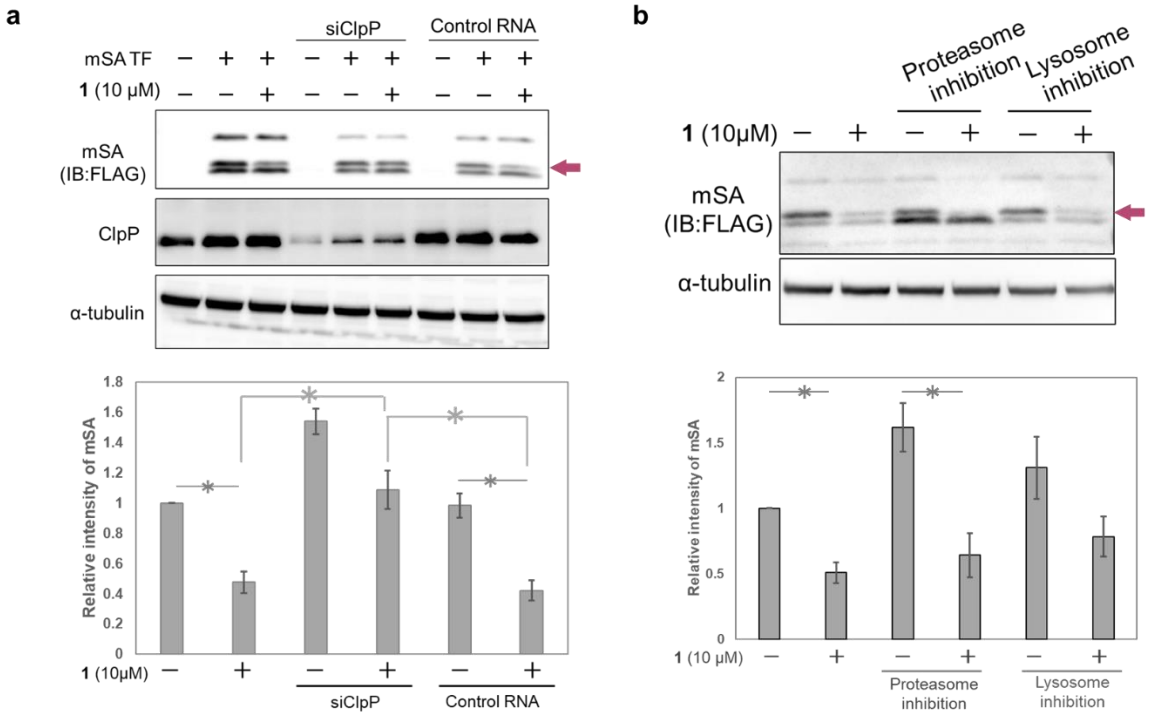

Figure 5. Analyses of mSA degradation pathway.

a. Degradation of mSA induced by 48 h treatment with **1** in HeLa cells with ClpP knockdown by transfection of siClpP. TF stands for transfection.

b. Degradation of mSA induced by 48 h treatment with **1** in HeLa cells in the presence of an inhibitor of proteasome or lysosome. Proteasome inhibition was performed by treatment with 300 nM bortezomib. Lysosome inhibition was performed by co-treatment with 10 μM leupeptin and 30 mM NH_4Cl.

Western blots and quantification results are shown. The data were normalized to the control value, taken as 1. Error bars indicate SEM (n = 3 measurements). ∗: $p<0.05$, Student's t-test

To investigate the impact of compound **1** on total cellular protein abundance, proteomics analysis was conducted on cells treated with compound **1** (Figure S6). LC-MS/MS analysis detected 3105 proteins, among which 26 proteins (0.8%) were significantly decreased . Of these, 77% (20 proteins) were mitochondria-localized proteins, supporting the idea that compound **1** functions within mitochondria (Table S2). Mitochondrial ClpP activator TR57, which shares the same pharmacophore as TR79, has been reported to decrease approximately 8% of detected proteins (686/~8000) in SUM159 cells.[35] Though these findings are not directly comparable, they suggest that compound **1** may reduce target proteins more selectively than ClpP activators.

Chemical control of mitochondrial morphology by mitoTPD.

We next focused on STMP1, a mitochondrial short peptide enhancing mitochondrial fission, as a target protein to investigate whether mitochondrial morphology could be controlled. Since a ligand and ligand binding site of STMP1 were not available, we utilized mSA as a degradation tag and examined the degradation of mSA-STMP1 fusion protein. Although STMP1 is a trans-inner membrane peptide, we anticipated that mSA fused to the matrix-oriented N-terminus of STMP1 would be exposed to the matrix and would be targetable to our mitoTPD. First, we confirmed that cox8-His6-mSA-STMP1-FLAG (hereafter mSA-STMP1) is expressed in mitochondria (Figure S7) and that treatment with compound **1** decreased mSA-STMP1 in a dose-dependent manner (Figure a). Next, we evaluated changes in mitochondrial morphology caused by the expression of mSA-STMP1 and treatment with

1, using fluorescence-microscopic images and an ImageJ/Fiji macro tool, Mitochondria Analyzer.[36] Analysis of the number of mitochondrial branches, branch length, number of branch junctions, and form factor, which indicates mitochondrial shape, showed that expression of mSA-STMP1 leads to mitochondrial fission, resulting in a smaller, less branched structure, in accordance with previous work.[19] Treatment of HeLa cells expressing mSA-STMP1 with **1** successfully restored the changes in mitochondrial morphology, as judged according to all the evaluated factors (Figure c). These results suggest that mitoTPD is able to control mitochondrial morphology.

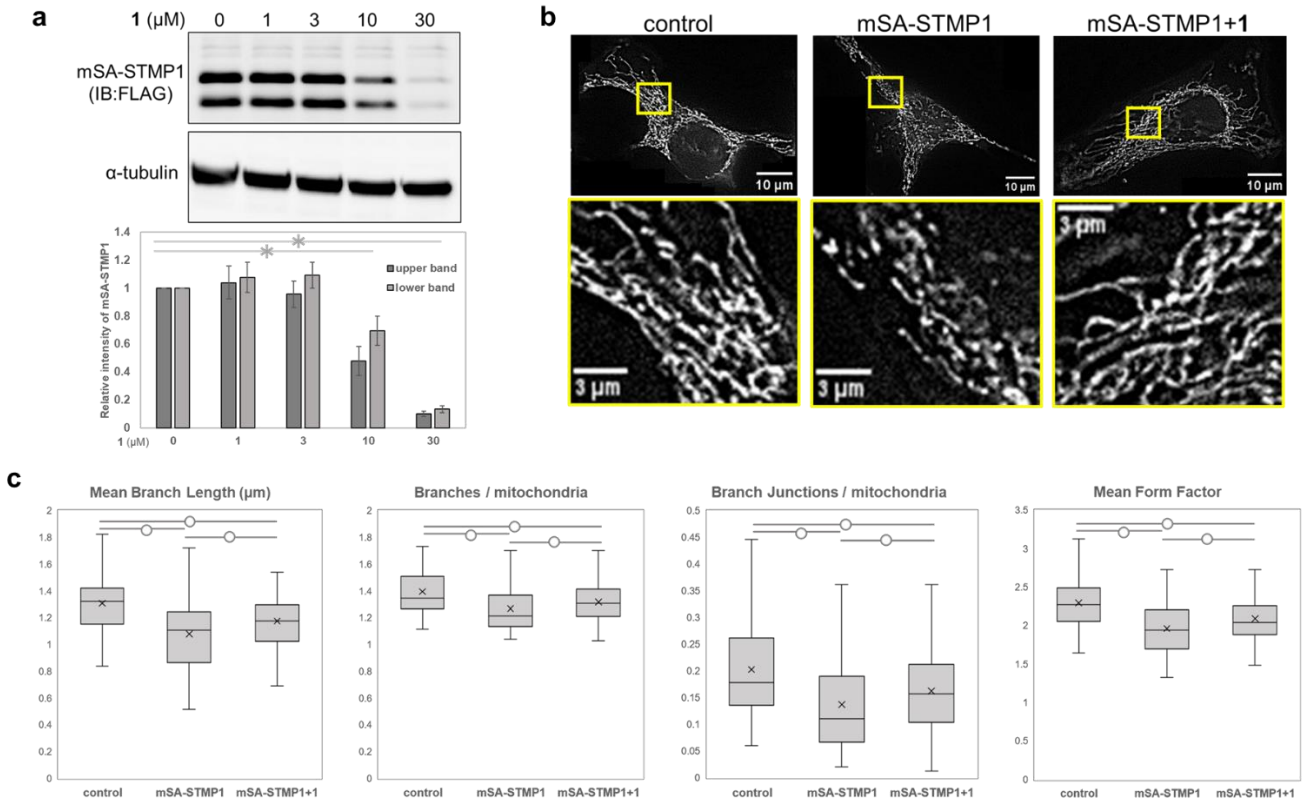

Figure 6. The impact of expression of mSA-STMP1 and its degradation by treatment with 1 on mitochondrial morphology.

a. Degradation of mSA-STMP1. Western blot and quantification results are shown.. The data was

normalized to the control, taken as 1. Error bars indicate SEM (n = 3 measurements). ∗: $p<0.05$, Student's t-test. The two anti-FLAG antibody-positive bands are considered to be due to mSA-STMP1 (lower band) and its processing variant (upper band).

b. Representative microscopic images of mitochondria in HeLa cells expressing mSA-STMP1 after MitoTracker staining (top scale bars: 10 µm, bottom: 3 µm) with or without 1 µM **1** treatment.

c. Box-and-whisker plot of mitochondrial morphology in microscopic images. Mean branch length: total branch length divided by number of branches. Branches/mitochondria: total number of branches in the image, normalized to mitochondria count. Branch junctions/mitochondria: number of junctions within all skeletons in the image, normalized to mitochondria count. Junctions are points where 2 or more branches meet. Mean form factor: a measure of shape, where the value 1 indicates a round object and increases with elongation. Control: 98 cells, mSA-STMP1: 92 cells, mSA-STMP1+compound **1**: 128 cells. Steel-Dwass test (○, $p<0.05$)

Effect of linker structure of the degrader 1

It is well established that the linker length and structure of PROTACs significantly affect their POI-degradation activity. To evaluate the structure-activity relationship of the linker in **1**, we synthesized two other compounds, **2** and **3**, with different PEG linker lengths. Their mSA degradation-inducing activity was assessed using HeLa cells expressing mitochondrial mSA. Western blotting results showed that the longer the linker length, the more effectively these compounds induce mSA degradation (Figure 7). In particular, 10 μM **3** reduced the mSA level to approx. 20%, suggesting that the longer linker improves not only the half-maximal degradation concentration (DC_{50}), but also the maximum degradation efficacy (D_{max}).[37]

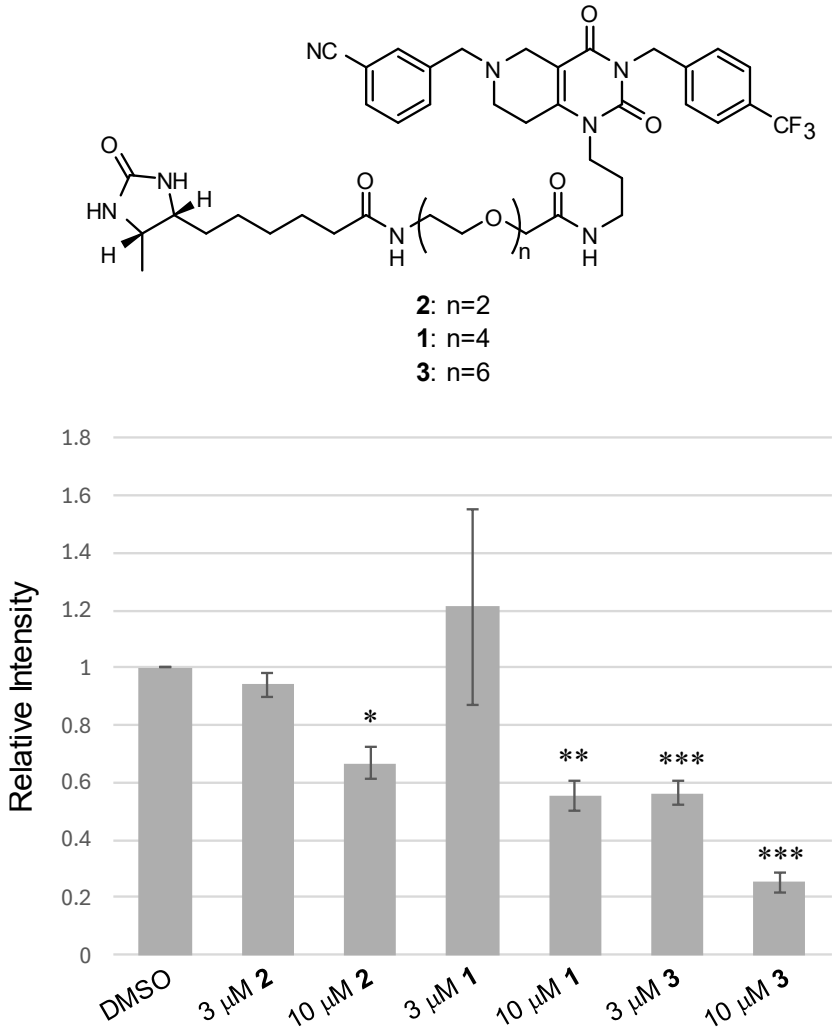

Figure 7. Structure-activity relationships for linker length of compound 1. Quantitative western blot results are shown as a bar graph. Values are normalized to that of the control DMSO sample, taken as 1, and are the mean ± SEM (n = 4 measurements). *, $p < 0.005$; **, $p < 0.0005$; ***, $p < 0.00005$; Student's t-test.

Discussion and Conclusion

In this study, we designed degraders for mitoTPD technology, comprised of a mitochondrial protease activator linked to a ligand for the POI. By applying this conceptual framework, we successfully

induced the degradation of mSA localized within mitochondria. Furthermore, by targeting STMP1, which is involved in mitochondrial fission, we demonstrated the feasibility of chemical control of mitochondrial morphology. Given the involvement of mitochondria and mitochondrial proteins in diseases such as cancer and neurodegenerative disorders, the TPD technology developed in this study has the potential to open up new options for drug discovery targeting mitochondria. Notably, our mitochondrial mSA degrader **1** exhibited degradation activity within 2-4 hours. Additionally, the degrader's potency could be adjusted by changing the linker length.

A limitation of our study is that we targeted artificially expressed mSA and its fusion protein within the mitochondria, but not endogenous mitochondrial proteins. Nevertheless, it is noteworthy that we achieved the degradation of the fusion protein of STMP1, a transmembrane protein localized to the mitochondrial inner membrane, suggesting the applicability of mitoTPD technology not only to the mitochondrial matrix, but also to inner membrane-localized proteins whose ligand binding site is exposed to the matrix. Various PROTACs targeting protein tags such as HaloTag and $FKBP12^{F36V}$ have been reported and have been employed as rapid knockdown tools for biological research.[7,38–44] Our mitochondrial mSA-tag degraders may therefore prove useful as research tools for mitochondrial biology. The use of biotin and its analogs in cells raises concerns about bioorthogonality, given that biotin functions as a coenzyme in cells. Indeed, to our knowledge, there has been no report of utilizing biotin-binding proteins, including mSA, as PROTAC degradation tags. However, in the case of bacteria, it has been reported that the affinity of desthiobiotin for protein biotin ligases is very low or

undetectable.[45] This implies that desthiobiotin would be a bioorthogonal molecule in mammals that lack avidin, and based on this, we employed the combination of mSA and desthiobiotin in this study, demonstrating for the first time its value as a cellular degradation tag.

Independently of our research, Wang et al. recently reported a TPD technology for mitochondrial proteins.[24] They employed the known compound ONC201[46] as a ClpP activator and developed a degrader targeting human mitochondrial RNA polymerase (POLRMT), an intramitochondrial protein. This degrader, 3B-1, consisted of ONC201 linked to a POLRMT inhibitor. However, 3B-1 required 16 hours at a concentration of 40 μM to induce degradation of POLRMT, whereas our degraders worked within only 2-4 hours, and the DC_{50} values were 4.8 μM (HeLa) and 0.96 μM (MCF7), respectively. This may be attributed to the weaker ClpP-activation potency of ONC201 as compared with TR79[31], though a difference in stability between POLRMT and mSA could also have contributed to the outcome. Interestingly, Wang et al. found that increasing the length of the ethylene glycol linker in 3B-1 resulted in loss of activity, whereas we found that a longer linker increased the degradation activity. Two possible explanations can be considered: 1) the potency of ClpP activators affects the potency of ClpP-based degraders, and 2) the optimal linker length varies depending on the target protein. The technology reported by Wang et al. and us differs from PROTACs in using degraders that directly recruit the protease complex. Therefore, various features, such as characteristics arising from ternary complex formation and degradation time course, are expected to differ from those of PROTACs. Further work will be needed to fully elucidate the characteristics of this technology.

Acknowledgement

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References

(1) Cromm, P. M.; Crews, C. M. Targeted Protein Degradation: From Chemical Biology to Drug Discovery. *Cell Chem. Biol.* **2017**, *24* (9), 1181–1190. https://doi.org/10.1016/j.chembiol.2017.05.024.

(2) Itoh, Y.; Ishikawa, M.; Naito, M.; Hashimoto, Y. Protein Knockdown Using Methyl Bestatin−Ligand Hybrid Molecules: Design and Synthesis of Inducers of Ubiquitination-Mediated Degradation of Cellular Retinoic Acid-Binding Proteins. *J. Am. Chem. Soc.* **2010**, *132* (16), 5820–5826. https://doi.org/10.1021/ja100691p.

(3) Tomoshige, S.; Nomura, S.; Ohgane, K.; Hashimoto, Y.; Ishikawa, M. Discovery of Small Molecules That Induce the Degradation of Huntingtin. *Angew. Chem. Int. Ed.* **2017**, *56* (38), 11530–11533. https://doi.org/10.1002/anie.201706529.

(4) Békés, M.; Langley, D. R.; Crews, C. M. PROTAC Targeted Protein Degraders: The Past Is Prologue. *Nat. Rev. Drug Discov.* **2022**, *21* (3), 181–200. https://doi.org/10.1038/s41573-021-00371-6.

(5) Chirnomas, D.; Hornberger, K. R.; Crews, C. M. Protein Degraders Enter the Clinic — a New Approach to Cancer Therapy. *Nat. Rev. Clin. Oncol.* **2023**, *20* (4), 265–278. https://doi.org/10.1038/s41571-023-00736-3.

(6) Erb, M. A.; Scott, T. G.; Li, B. E.; Xie, H.; Paulk, J.; Seo, H.-S.; Souza, A.; Roberts, J. M.; Dastjerdi, S.; Buckley, D. L.; Sanjana, N. E.; Shalem, O.; Nabet, B.; Zeid, R.; Offei-Addo, N. K.; Dhe-Paganon, S.; Zhang, F.; Orkin, S. H.; Winter, G. E.; Bradner, J. E. Transcription Control by the ENL YEATS Domain in Acute Leukaemia. *Nature* **2017**, *543* (7644), 270–274.

https://doi.org/10.1038/nature21688.

(7) Mehta, S.; Buyanbat, A.; Kai, Y.; Karayel, O.; Goldman, S. R.; Seruggia, D.; Zhang, K.; Fujiwara, Y.; Donovan, K. A.; Zhu, Q.; Yang, H.; Nabet, B.; Gray, N. S.; Mann, M.; Fischer, E. S.; Adelman, K.; Orkin, S. H. Temporal Resolution of Gene Derepression and Proteome Changes upon PROTAC-Mediated Degradation of BCL11A Protein in Erythroid Cells. *Cell Chem. Biol.* **2022**, *29* (8), 1273-1287.e8. https://doi.org/10.1016/j.chembiol.2022.06.007.

(8) Yamano, K.; Kikuchi, R.; Kojima, W.; Hayashida, R.; Koyano, F.; Kawawaki, J.; Shoda, T.; Demizu, Y.; Naito, M.; Tanaka, K.; Matsuda, N. Critical Role of Mitochondrial Ubiquitination and the OPTN–ATG9A Axis in Mitophagy. *J. Cell Biol.* **2020**, *219* (9), e201912144. https://doi.org/10.1083/jcb.201912144.

(9) Tomoshige, S.; Ishikawa, M. PROTACs and Other Chemical Protein Degradation Technologies for the Treatment of Neurodegenerative Disorders. *Angew. Chem. Int. Ed.* **2021**, *60* (7), 3346–3354. https://doi.org/10.1002/anie.202004746.

(10) Toure, M.; Crews, C. M. Small-Molecule PROTACS: New Approaches to Protein Degradation. *Angew. Chem. Int. Ed.* **2016**, *55* (6), 1966–1973. https://doi.org/10.1002/anie.201507978.

(11) Neklesa, T. K.; Tae, H. S.; Schneekloth, A. R.; Stulberg, M. J.; Corson, T. W.; Sundberg, T. B.; Raina, K.; Holley, S. A.; Crews, C. M. Small-Molecule Hydrophobic Tagging–Induced Degradation of HaloTag Fusion Proteins. *Nat. Chem. Biol.* **2011**, *7* (8), 538–543. https://doi.org/10.1038/nchembio.597.

(12) Li, X.; Liu, Q.; Xie, X.; Peng, C.; Pang, Q.; Liu, B.; Han, B. Application of Novel Degraders Employing Autophagy for Expediting Medicinal Research. *J. Med. Chem.* **2023**, *66* (3), 1700–1711. https://doi.org/10.1021/acs.jmedchem.2c01712.

(13) Takahashi, D.; Moriyama, J.; Nakamura, T.; Miki, E.; Takahashi, E.; Sato, A.; Akaike, T.; Itto-Nakama, K.; Arimoto, H. AUTACs: Cargo-Specific Degraders Using Selective Autophagy. *Mol. Cell* **2019**, *76* (5), 797-810.e10. https://doi.org/10.1016/j.molcel.2019.09.009.

(14) Li, Z.; Zhu, C.; Ding, Y.; Fei, Y.; Lu, B. ATTEC: A Potential New Approach to Target Proteinopathies. *Autophagy* **2020**, *16* (1), 185–187. https://doi.org/10.1080/15548627.2019.1688556.

(15) Ji, C. H.; Kim, H. Y.; Lee, M. J.; Heo, A. J.; Park, D. Y.; Lim, S.; Shin, S.; Ganipisetti, S.; Yang, W. S.; Jung, C. A.; Kim, K. Y.; Jeong, E. H.; Park, S. H.; Bin Kim, S.; Lee, S. J.; Na, J. E.; Kang, J. I.; Chi, H. M.; Kim, H. T.; Kim, Y. K.; Kim, B. Y.; Kwon, Y. T. The AUTOTAC Chemical Biology Platform for Targeted Protein Degradation via the Autophagy-Lysosome System. *Nat. Commun.* **2022**, *13* (1), 904. https://doi.org/10.1038/s41467-022-28520-4.

(16) Banik, S. M.; Pedram, K.; Wisnovsky, S.; Ahn, G.; Riley, N. M.; Bertozzi, C. R. Lysosome-Targeting Chimaeras for Degradation of Extracellular Proteins. *Nature* **2020**, *584* (7820), 291–297. https://doi.org/10.1038/s41586-020-2545-9.

(17) Morreale, F. E.; Kleine, S.; Leodolter, J.; Junker, S.; Hoi, D. M.; Ovchinnikov, S.; Okun, A.; Kley, J.; Kurzbauer, R.; Junk, L.; Guha, S.; Podlesainski, D.; Kazmaier, U.; Boehmelt, G.; Weinstabl,

H.; Rumpel, K.; Schmiedel, V. M.; Hartl, M.; Haselbach, D.; Meinhart, A.; Kaiser, M.; Clausen, T. BacPROTACs Mediate Targeted Protein Degradation in Bacteria. *Cell* **2022**, *185* (13), 2338-2353.e18. https://doi.org/10.1016/j.cell.2022.05.009.

(18) Andreux, P. A.; Houtkooper, R. H.; Auwerx, J. Pharmacological Approaches to Restore Mitochondrial Function. *Nat. Rev. Drug. Discov.* **2013**, *12* (6), 465–483. https://doi.org/10.1038/nrd4023.

(19) Xie, C.; Wang, F.-Y.; Sang, Y.; Chen, B.; Huang, J.-H.; He, F.-J.; Li, H.; Zhu, Y.; Liu, X.; Zhuang, S.-M.; Fang, J.-H. Mitochondrial Micropeptide STMP1 Enhances Mitochondrial Fission to Promote Tumor Metastasis. *Cancer Res.* **2022**, *82* (13), 2431–2443. https://doi.org/10.1158/0008-5472.CAN-21-3910.

(20) Javadov, S.; Kozlov, A. V.; Camara, A. K. S. Mitochondria in Health and Diseases. *Cells* **2020**, *9* (5), 1177. https://doi.org/10.3390/cells9051177.

(21) Malty, R. H.; Jessulat, M.; Jin, K.; Musso, G.; Vlasblom, J.; Phanse, S.; Zhang, Z.; Babu, M. Mitochondrial Targets for Pharmacological Intervention in Human Disease. *J. Proteome Res.* **2015**, *14* (1), 5–21. https://doi.org/10.1021/pr500813f.

(22) Singh, A.; Faccenda, D.; Campanella, M. Pharmacological Advances in Mitochondrial Therapy. *eBioMedicine* **2021**, *65*. https://doi.org/10.1016/j.ebiom.2021.103244.

(23) Delgado de la Herran, H. C.; Cheng, Y.; Perocchi, F. Towards a Systems-Level Understanding of Mitochondrial Biology. *Cell Calcium* **2021**, *95*, 102364. https://doi.org/10.1016/j.ceca.2021.102364.

(24) Wang, D.; Wang, W.; Fang, L.; Qi, L.; Zhang, Y.; Liu, J.; Liang, Y.; Yang, H.; Wang, M.; Wei, X.; Jiang, R.; Liu, Y.; Zhou, W.; Fang, X. Mitochondrial Protease Targeting Chimeras for Mitochondrial Matrix Protein Degradation. *J. Am. Chem. Soc.* **2023**, *145* (23), 12861–12869. https://doi.org/10.1021/jacs.3c03756.

(25) Sanyal, S.; Kouznetsova, A.; Ström, L.; Björkegren, C. A System for Inducible Mitochondria-Specific Protein Degradation in Vivo. *Nat. Commun.* **2024**, *15* (1), 1454. https://doi.org/10.1038/s41467-024-45819-6.

(26) Nouri, K.; Feng, Y.; Schimmer, A. D. Mitochondrial ClpP Serine Protease-Biological Function and Emerging Target for Cancer Therapy. *Cell Death Dis.* **2020**, *11* (10), 1–12. https://doi.org/10.1038/s41419-020-03062-z.

(27) Goard, C. A.; Schimmer, A. D. Mitochondrial Matrix Proteases as Novel Therapeutic Targets in Malignancy. *Oncogene* **2014**, *33* (21), 2690–2699. https://doi.org/10.1038/onc.2013.228.

(28) Wedam, R.; Greer, Y. E.; Wisniewski, D. J.; Weltz, S.; Kundu, M.; Voeller, D.; Lipkowitz, S. Targeting Mitochondria with ClpP Agonists as a Novel Therapeutic Opportunity in Breast Cancer. *Cancers* **2023**, *15* (7), 1936. https://doi.org/10.3390/cancers15071936.

(29) Bhandari, V.; Wong, K. S.; Zhou, J. L.; Mabanglo, M. F.; Batey, R. A.; Houry, W. A. The Role of ClpP Protease in Bacterial Pathogenesis and Human Diseases. *ACS Chem. Biol.* **2018**, *13* (6), 1413–1425. https://doi.org/10.1021/acschembio.8b00124.

(30) Mabanglo, M. F.; Houry, W. A. Recent Structural Insights into the Mechanism of ClpP Protease Regulation by AAA+ Chaperones and Small Molecules. *J. Biol. Chem.* **2022**, *298* (5). https://doi.org/10.1016/j.jbc.2022.101781.

(31) Graves, P. R.; Aponte-Collazo, L. J.; Fennell, E. M. J.; Graves, A. C.; Hale, A. E.; Dicheva, N.; Herring, L. E.; Gilbert, T. S. K.; East, M. P.; McDonald, I. M.; Lockett, M. R.; Ashamalla, H.; Moorman, N. J.; Karanewsky, D. S.; Iwanowicz, E. J.; Holmuhamedov, E.; Graves, L. M. Mitochondrial Protease ClpP Is a Target for the Anticancer Compounds ONC201 and Related Analogues. *ACS Chem. Biol.* **2019**, *14* (5), 1020–1029. https://doi.org/10.1021/acschembio.9b00222.

(32) Kroetsch, A.; Chin, B.; Nguyen, V.; Gao, J.; Park, S. Functional Expression of Monomeric Streptavidin and Fusion Proteins in Escherichia Coli: Applications in Flow Cytometry and ELISA. *Appl. Microbiol. Biotechnol.* **2018**, *102* (23), 10079–10089. https://doi.org/10.1007/s00253-018-9377-7.

(33) Demonte, D.; Dundas, C. M.; Park, S. Expression and Purification of Soluble Monomeric Streptavidin in Escherichia Coli. *Appl. Microbiol. Biotechnol.* **2014**, *98* (14), 6285–6295. https://doi.org/10.1007/s00253-014-5682-y.

(34) Yu, H.; Koilkonda, R. D.; Chou, T.-H.; Porciatti, V.; Ozdemir, S. S.; Chiodo, V.; Boye, S. L.; Boye, S. E.; Hauswirth, W. W.; Lewin, A. S.; Guy, J. Gene Delivery to Mitochondria by Targeting Modified Adenoassociated Virus Suppresses Leber's Hereditary Optic Neuropathy in a Mouse Model. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109* (20), E1238–E1247. https://doi.org/10.1073/pnas.1119577109.

(35) Fennell, E. M. J.; Aponte-Collazo, L. J.; Pathmasiri, W.; Rushing, B. R.; Barker, N. K.; Partridge, M. C.; Li, Y.-Y.; White, C. A.; Greer, Y. E.; Herring, L. E.; Lipkowitz, S.; Sumner, S. C. J.; Iwanowicz, E. J.; Graves, L. M. Multi-Omics Analyses Reveal ClpP Activators Disrupt Essential Mitochondrial Pathways in Triple-Negative Breast Cancer. *Front. Pharmacol.* **2023**, *14*.

(36) Chaudhry, A.; Shi, R.; Luciani, D. S. A Pipeline for Multidimensional Confocal Analysis of Mitochondrial Morphology, Function, and Dynamics in Pancreatic β-Cells. *Am. J. Physiol. Endocrinol. Metab.* **2020**, *318* (2), E87–E101. https://doi.org/10.1152/ajpendo.00457.2019.

(37) Bondeson, D. P.; Mares, A.; Smith, I. E. D.; Ko, E.; Campos, S.; Miah, A. H.; Mulholland, K. E.; Routly, N.; Buckley, D. L.; Gustafson, J. L.; Zinn, N.; Grandi, P.; Shimamura, S.; Bergamini, G.; Faelth-Savitski, M.; Bantscheff, M.; Cox, C.; Gordon, D. A.; Willard, R. R.; Flanagan, J. J.; Casillas, L. N.; Votta, B. J.; den Besten, W.; Famm, K.; Kruidenier, L.; Carter, P. S.; Harling, J. D.; Churcher, I.; Crews, C. M. Catalytic in Vivo Protein Knockdown by Small-Molecule PROTACs. *Nat. Chem. Biol.* **2015**, *11* (8), 611–617. https://doi.org/10.1038/nchembio.1858.

(38) Etersque, J. M.; Lee, I. K.; Sharma, N.; Xu, K.; Ruff, A.; Northrup, J. D.; Sarkar, S.; Nguyen, T.; Lauman, R.; Burslem, G. M.; Sellmyer, M. A. Regulation of eDHFR-Tagged Proteins with Trimethoprim PROTACs. *Nat. Commun.* **2023**, *14* (1), 7071. https://doi.org/10.1038/s41467-023-42820-3.

(39) Grohmann, C.; Magtoto, C. M.; Walker, J. R.; Chua, N. K.; Gabrielyan, A.; Hall, M.; Cobbold, S. A.; Mieruszynski, S.; Brzozowski, M.; Simpson, D. S.; Dong, H.; Dorizzi, B.; Jacobsen, A. V.; Morrish, E.; Silke, N.; Murphy, J. M.; Heath, J. K.; Testa, A.; Maniaci, C.; Ciulli, A.; Lessene, G.; Silke, J.; Feltham, R. Development of NanoLuc-Targeting Protein Degraders and a Universal Reporter System to Benchmark Tag-Targeted Degradation Platforms. *Nat. Commun.* **2022**, *13* (1), 2073. https://doi.org/10.1038/s41467-022-29670-1.

(40) Bond, A. G.; Craigon, C.; Chan, K.-H.; Testa, A.; Karapetsas, A.; Fasimoye, R.; Macartney, T.; Blow, J. J.; Alessi, D. R.; Ciulli, A. Development of BromoTag: A "Bump-and-Hole"–PROTAC System to Induce Potent, Rapid, and Selective Degradation of Tagged Target Proteins. *J. Med. Chem.* **2021**, *64* (20), 15477–15502. https://doi.org/10.1021/acs.jmedchem.1c01532.

(41) Okitsu, K.; Hattori, T.; Misawa, T.; Shoda, T.; Kurihara, M.; Naito, M.; Demizu, Y. Development of a Small Hybrid Molecule That Mediates Degradation of His-Tag Fused Proteins. *J. Med. Chem.* **2018**, *61* (2), 576–582. https://doi.org/10.1021/acs.jmedchem.7b00413.

(42) Nabet, B.; Roberts, J. M.; Buckley, D. L.; Paulk, J.; Dastjerdi, S.; Yang, A.; Leggett, A. L.; Erb, M. A.; Lawlor, M. A.; Souza, A.; Scott, T. G.; Vittori, S.; Perry, J. A.; Qi, J.; Winter, G. E.; Wong, K.-K.; Gray, N. S.; Bradner, J. E. The dTAG System for Immediate and Target-Specific Protein Degradation. *Nat. Chem. Biol.* **2018**, *14* (5), 431–441. https://doi.org/10.1038/s41589-018-0021-8.

(43) Tomoshige, S.; Naito, M.; Hashimoto, Y.; Ishikawa, M. Degradation of HaloTag-Fused Nuclear Proteins Using Bestatin-HaloTag Ligand Hybrid Molecules. *Org. Biomol. Chem.* **2015**, *13* (38), 9746–9750. https://doi.org/10.1039/C5OB01395J.

(44) Buckley, D. L.; Raina, K.; Darricarrere, N.; Hines, J.; Gustafson, J. L.; Smith, I. E.; Miah, A. H.; Harling, J. D.; Crews, C. M. HaloPROTACS: Use of Small Molecule PROTACs to Induce Degradation of HaloTag Fusion Proteins. *ACS Chem. Biol.* **2015**, *10* (8), 1831–1837. https://doi.org/10.1021/acschembio.5b00442.

(45) Purushothaman, S.; Gupta, G.; Srivastava, R.; Ramu, V. G.; Surolia, A. Ligand Specificity of Group I Biotin Protein Ligase of Mycobacterium Tuberculosis. *PLOS ONE* **2008**, *3* (5), e2320. https://doi.org/10.1371/journal.pone.0002320.

(46) Ishizawa, J.; Zarabi, S. F.; Davis, R. E.; Halgas, O.; Nii, T.; Jitkova, Y.; Zhao, R.; St-Germain, J.; Heese, L. E.; Egan, G.; Ruvolo, V. R.; Barghout, S. H.; Nishida, Y.; Hurren, R.; Ma, W.; Gronda, M.; Link, T.; Wong, K.; Mabanglo, M.; Kojima, K.; Borthakur, G.; MacLean, N.; Ma, M. C. J.; Leber, A. B.; Minden, M. D.; Houry, W.; Kantarjian, H.; Stogniew, M.; Raught, B.; Pai, E. F.; Schimmer, A. D.; Andreeff, M. Mitochondrial ClpP-Mediated Proteolysis Induces Selective Cancer Cell Lethality. *Cancer Cell* **2019**, *35* (5), 721-737.e9. https://doi.org/10.1016/j.ccell.2019.03.014.