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Simultaneous capture of ISG15 conjugating and deconjugating enzymes using a semi-synthetic ISG15-Dha probe

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ISG15 is a ubiquitin-like (Ubl) protein attached to substrate proteins by ISG15 conjugating enzymes whose dysregulation is implicated in a multitude of disease processes, but the probing of these enzymes remains to be accomplished. Here, we describe the development of a new activity-based probe ISG15-Dha (dehydroalanine) through protein semi-synthesis. *In vitro* cross-linking and cell lysate proteomic profiling experiments showed that this probe can sequentially capture ISG15 conjugating enzymes including E1 enzyme UBA7, E2 enzyme UBE2L6, E3 enzyme HERC5, the previously known ISG15 deconjugating enzyme (USP18), as well as some other enzymes (USP5 and USP14) which we additionally confirmed to impart deISGylation activity. Collectively, ISG15-Dha provides a new tool that can simultaneously capture ISG15 conjugating and deconjugating enzymes for biochemical or pharmacological studies.

ISG15-Dha probe, semi-synthesis, activity based probe, proteomics, ISGylation

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1 Introduction

Human interferon-stimulated gene 15 (ISG15) is a ubiquitin-like (Ubl) protein induced by type I interferon or pathogens and implicated in host immune processes and the antiviral response [1,2]. Analogous to ubiquitin (Ub), ISG15 is covalently attached to the lysine residues of substrate proteins by ISG15 conjugating enzymes E1, E2, and E3 and removed by deconjugating enzymes, which together accomplish the reversible regulation of the ISG15 modification [3,4]. The abnormal regulation of these enzymes is closely associated with a variety of diseases, including COVID-19 [5–10]. Recent studies have showed that the levels of ISG15 conjugating and deconjugating enzymes are significantly increased upon SARS-CoV-2 infection [9].

Activity-based probes are powerful tools for proteomic profiling, mechanistic studies, and the screening of prototype drugs targeting Ub/Ubl conjugating and deconjugating enzymes [11–27]. Several probes for Ub conjugating and deconjugating enzymes have been developed and have greatly advanced biochemical and biophysical studies of Ub modifications [11–13,15–26]. In contrast, reported ISG15 probes (ISG15-PA, ISG15-VS, ISG15-VME, and ISG15-Br3) can capture ISG15 deconjugating enzymes (deISGylases) only (Scheme 1a), and no probes for ISG15 conjugating enzymes have been developed [7,27–34]. Here, we described the

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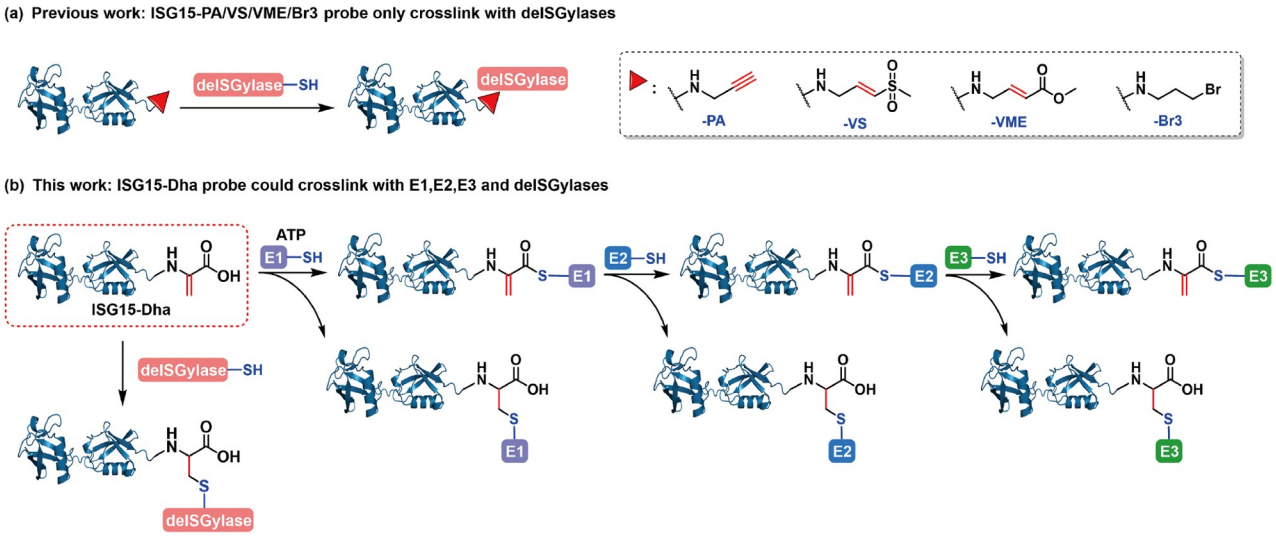

Scheme 1 (a) Reported ISG15 activity probes can only capture deISGylases; (b) the ISG15-Dha probe can capture ISG15 conjugating enzymes (E1, E2, E3) and ISG15 deconjugating enzymes (deISGylases) (color online).

development of ISG15-dehydroalanine (ISG15-Dha), a probe capable of capturing ISG15 conjugating enzymes [13,35]. Upon adenosine triphosphate (ATP)-dependent activation by UBA7 (E1), ISG15-Dha transferred downstream to UBE2L6 (E2) through sequential trans-thioesterifications *in vitro*, and proteomic profiling demonstrated that it could capture all the known Cys-based ISG15 conjugating enzymes in different cell lysates. Unexpectedly, ISG15-Dha also captured deISGylases, including USP18 (the known ISG15-specific deconjugating enzyme), USP5 and USP14. Therefore, ISG15-Dha constitutes a new tool for the capture of both ISG15 conjugating and deconjugating enzymes, and is expected to be used for the proteomic activity profiling of ISG15-regulation enzymes under different disease phenotypes.

2 Results and discussion

2.1 Design and synthesis of the ISG15-Dha probe

Ub-dehydroalanine (Ub-Dha) was recently reported to be capable of sequentially capturing Ub conjugating enzymes and has proved to be a valuable tool for proteomic profiling and mechanism studies of Ub conjugating enzymes [13]. We hypothesized that the ISG15-derived probe ISG15-Dha could capture ISG15 conjugating enzymes through cascade reactions (Scheme 1b).

To test this idea, we synthesized ISG15-Dha using a semi-synthetic strategy that entailed the reaction of recombinantly expressed mutant ISG15 ($ISG15^{C78S,\ G157C}$) with 2,5-dibromohexanediamide, where C78S mutation was introduced to avoid the reaction of Cys78 with 2,5-dibromohexanediamide (Figure 1a) [13,35]. Thiol **1** (40 mg) was obtained from 1 L lysogeny broth (LB) medium, by *E. coli* expression, which gave **2** (30 mg) after removal of the His tag and size exclusion chromatography (SEC) purification (Figure S1a–c, Supporting Information online). Subsequently, **2** was mixed with 2,5-dibromohexanediamide (20 eq.) at 42 °C in reaction buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*-ethane-sulphonicacid (HEPES), 150 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine (TCEP), and 4 M Gn·HCl, pH 9.0) to generate ISG15-Dha (**4**) in yields of 80% (by high performance liquid chromatography (HPLC)) and 60% (isolated) after 5 h (Figure 1b, Figure S1d). Prolonging the reaction time failed to further improve the yield. HPLC and electrospray ionization-mass spectrometry (ESI-MS) analyses confirmed that **4** had good homogeneity and correct molecular weight (Figure 1c, d). Refolding of **4** as previously described [36] proceeded in low yield (less than 10%); fortunately, this could be increased to 35% by slowly adding (drop-wise) the refolding buffer (50 mM HEPES, 150 mM NaCl, and 1 mM TCEP, pH 8.0) to a solution of **4** (in 50 mM HEPES, 150 mM NaCl, and 1 mM TCEP, 6M Gn·HCl, pH 8.0). The homogeneity of the refolded **4** was confirmed by SEC and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and its circular dichroism (CD) spectrum showed characteristic absorptions at 208 and 222 nm, consistent with the expressed ISG15 and $ISG15^{C78S,\ G157C}$, indicating that **4** was folded correctly (Figure 1e, f). Using this approach, 5 mg of well-folded ISG15-Dha (**4**) could be obtained from 40 mg of **1** within three days.

2.2 ISG15-Dha probe forms covalent bond with ISG15 conjugating enzymes *in vitro*

With ISG15-Dha in hand, we firstly investigated its

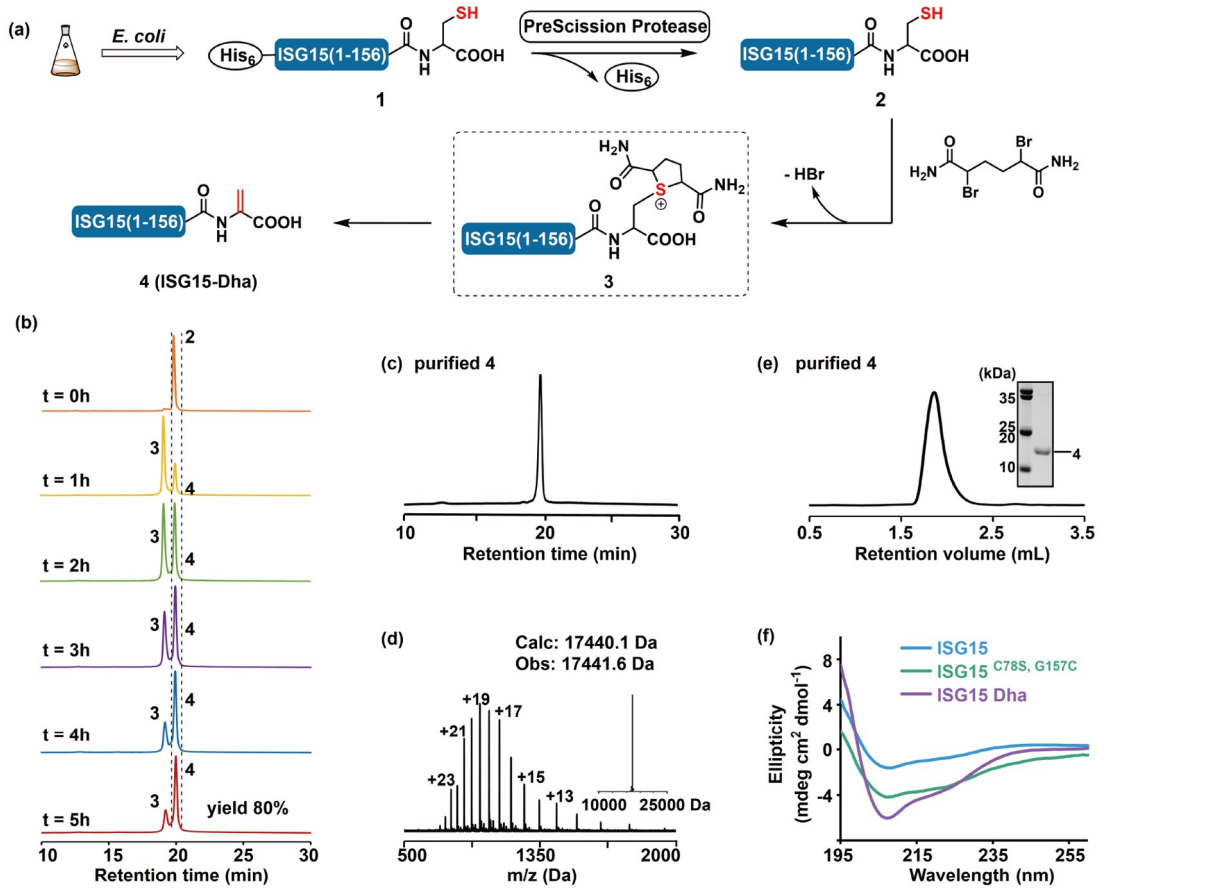

Figure 1 Design and synthesis of the ISG15-Dha probe. (a) Synthesis of ISG15-Dha *via* a semi-synthetic strategy; (b) reverse-phase (RP)-HPLC traces (214 nm) for the generation of probe **4** from **2**; (c) analytical RP-HPLC (214 nm) of probe **4**; (d) ESI-MS and deconvoluted spectra of probe **4**; (e) SEC and SDS-PAGE analysis of probe **4**; (f) CD spectra of probe **4** (color online).

crosslinking activity with ISG15 conjugating enzymes *in vitro* (Figure 2a) [13,37]. ISG15 activating enzyme (E1) UBA7 (0.5 μM) was incubated with 10 μM ISG15/ISG15-Dha at 37 °C and the reaction analyzed by SDS-PAGE, ISG15 was used as the control (Figure S2a, b). ISG15 reacted with UBA7 in the presence of ATP to generate UBA7~ISG15 thioester (Figure 2b, lane 5), which was reduced to UBA7 and ISG15 by the addition of dithiothreitol (DTT) (Figure 2b, lane 6). ISG15-Dha reacted with UBA7 to form an adduct having the same migration rate as UBA7~ISG15 thioester (Figure 2b, lane 8). Upon the addition of DTT, the intensity of the adduct band decreased whereas that of the UBA7 band increased (Figure 2b, lane 9), suggesting that ISG15-Dha reacts with UBA7 to generate two products, UBA7~ISG15-Dha thioester which is sensitive to added reducing agent, and UBA7-ISG15-Dha thioether which is stable to it (Figure 2b, c). The reaction of ISG15-Dha or ISG15 with UBA7 was ATP-dependent (Figure 2b, lane 4 and lane 7), indicating that the reaction proceeds *via* AMP intermediates. We also tested the crosslinking activities of ISG15-Dha towards Ub activating enzyme UBA1, small ubiquitin-like modifier (SUMO) activating enzyme AOS1-UBA2 and Nedd8 activating enzyme NAE1-UBA3, respectively. SDS-PAGE analysis showed that ISG15-Dha did not react with UBA1, UBA2 or UBA3, indicating that ISG15-Dha was highly selective for ISG15 activating enzyme UBA7 (Figure S3).

To test if UBA7~ISG15-Dha thioester could deliver ISG15-Dha to ISG15 E2, we added ISG15 E2 UBE2L6 to the mixture of ISG15-Dha and UBA7, and monitored it by SDS-PAGE (Figure 3a). UBE2L6 reacted with UBA7~ISG15 to generate UBE2L6~ISG15 thioester (Figure 3b, lane 5), which could be reduced by DTT (Figure 3b lane 6). In contrast, the conjugates generated by ISG15-Dha and UBE2L6 could be partially reduced (Figure 3b, lane7 and lane 8), indicating the formation of UBE2L6-ISG15-Dha and UBE2L6~ISG15-Dha. The presence of the UBE2L6-ISG15-Dha adduct was confirmed by tryptic MS/MS (Figure 3c). We also observed the formation of UBA7~ISG15-Dha-UBE2L6, a product of the E2 active site directly reacting with Michael acceptor on UBA7~ISG15-Dha which can be reduced to UBA7 and UBE2L6-ISG15-Dha by DTT under reducing conditions—analogous to Ub-Dha [13]. These assays suggested that ISG15-Dha probe can sequentially

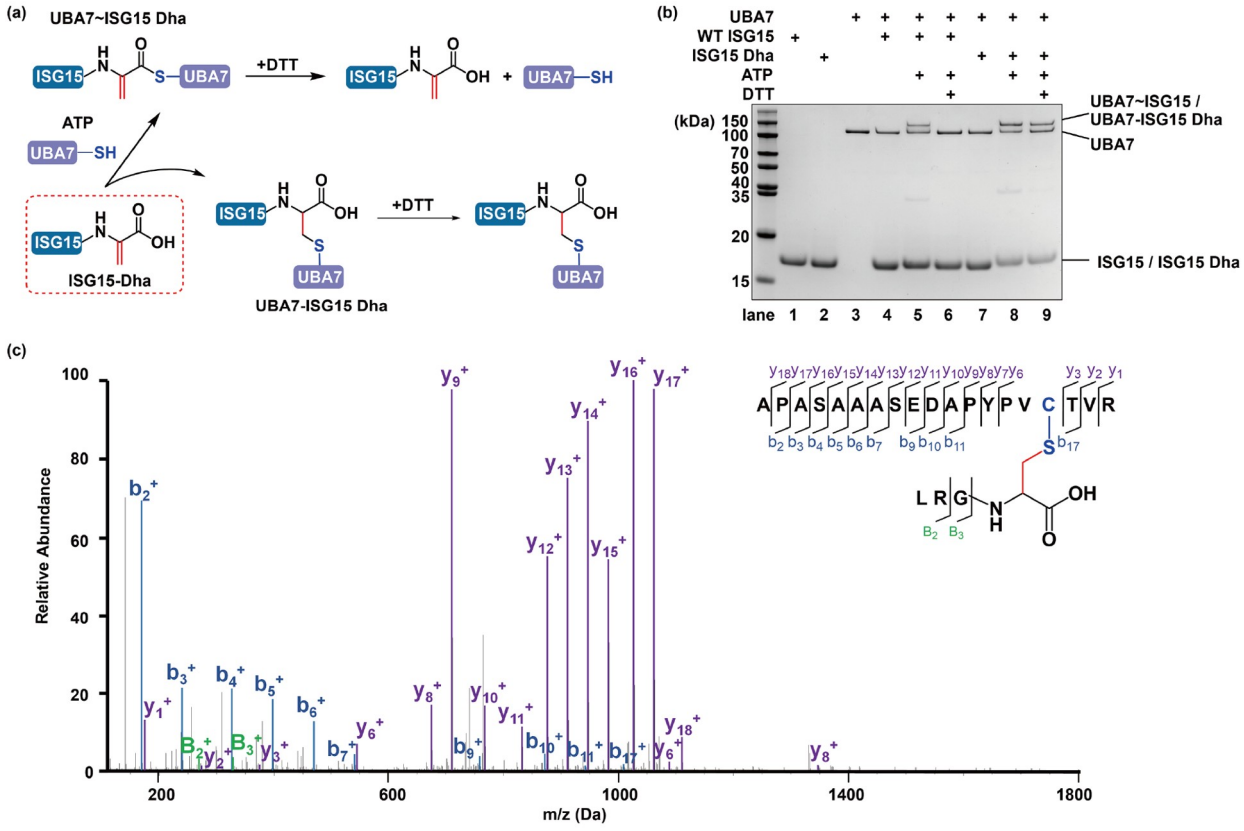

Figure 2 Capture of UBA7 *via* covalent thioether formation with ISG15-Dha. (a) Scheme depicting the reactivity of UBA7 with ISG15-Dha to give either the corresponding UBA7~ISG15-Dha thioester or UBA7-ISG15-Dha thioether; (b) SDS-PAGE analysis of the ATP-dependent labeling of UBA7 under reducing and non-reducing conditions; (c) tryptic MS/MS spectrum of the UBA7-ISG15-Dha thioether (UBA7~ISG15-Dha, thioester-linked conjugate; UBA7-ISG15-Dha, thioether-linked adduct) (color online).

capture ISG15 conjugating enzymes E1 and E2 in an activity-dependent manner.

2.3 ISG15-Dha probe as a proteomics tool to capture ISG15 conjugating enzymes

Having established that the ISG15-Dha probe could crosslink with ISG15 E1 and E2 enzymes *in vitro*, we tested its ability to capture ISG15 conjugating enzymes in biological samples. The probe biotin-ISG15-Dha was synthesized in a similar manner to ISG15-Dha (Figure 4a, Figure S4a). His-Avi-ISG15$^{C78S,\ G157C}$ (**5**) was expressed by *E. coli*, and then D-biotin was covalently linked to the Avi tag using the BirA enzyme in the presence of 5 mM ATP and 5 mM $MgCl_2$. The reaction proceeded with concomitant removal of the His tag. Biotin-Avi-ISG15$^{C78S,\ G157C}$ (**6**) was obtained in near-quantitative yield within 2 h (Figure S4b, c) [38]. Next, 0.1 mM **6** was mixed with 2,5-dibromohexanediamide (20 eq.) at 42 °C to generate biotin-ISG15-Dha (**8**), the yield of which could reach 70% after 5 h (Figure 4b, Figure S4a, d). Probe **8** was isolated in a yield of 40%. HPLC, ESI-MS and SDS-PAGE confirmed that **8** had good homogeneity and the correct molecular weight (Figure 4c). Probe **8** was then refolded as described above and its secondary structure was confirmed by CD spectroscopy (Figure 4d). *In vitro* crosslinking experiments demonstrated that biotin-ISG15-Dha probe (**8**) can also sequentially capture ISG15 conjugating enzymes (Figure S5a, b).

Next, we used the probe (**8**) for the affinity-based proteomic profiling of cell lysates. Interferon-β (IFN-β)-induced cell lysates (MCF-7, HeLa and HEK293T) were incubated with **8** in the presence of ATP, and apyrase-mediated ATP depletion was used as a control. The beads were washed and eluted in LDS loading buffer, and the samples were separated by SDS-PAGE, digested with trypsin, and finally analyzed by label free quantitation-mass spectrometry (LFQ-MS) (Figure 4e). After three biological replicates, the known ISG15-specific E1 enzyme UBA7 and E2 enzyme UBE2L6 were enriched in the lysates of MCF-7, HeLa and HEK293T cells in an ATP-dependent manner (Figure 4f–h, Tables S1–S3, Supporting Information online). ISG15 E3 enzyme HERC5 was also enriched in MCF-7 cell lysate, indicating that ISG15-Dha probe had the ability to capture ISG15 E1, E2, and E3 enzymes in cell lysates (Figure 4f). Several ubiquitin E3 ligases including HERC6 and RNF213 were also enriched, implicating their roles in the regulation of

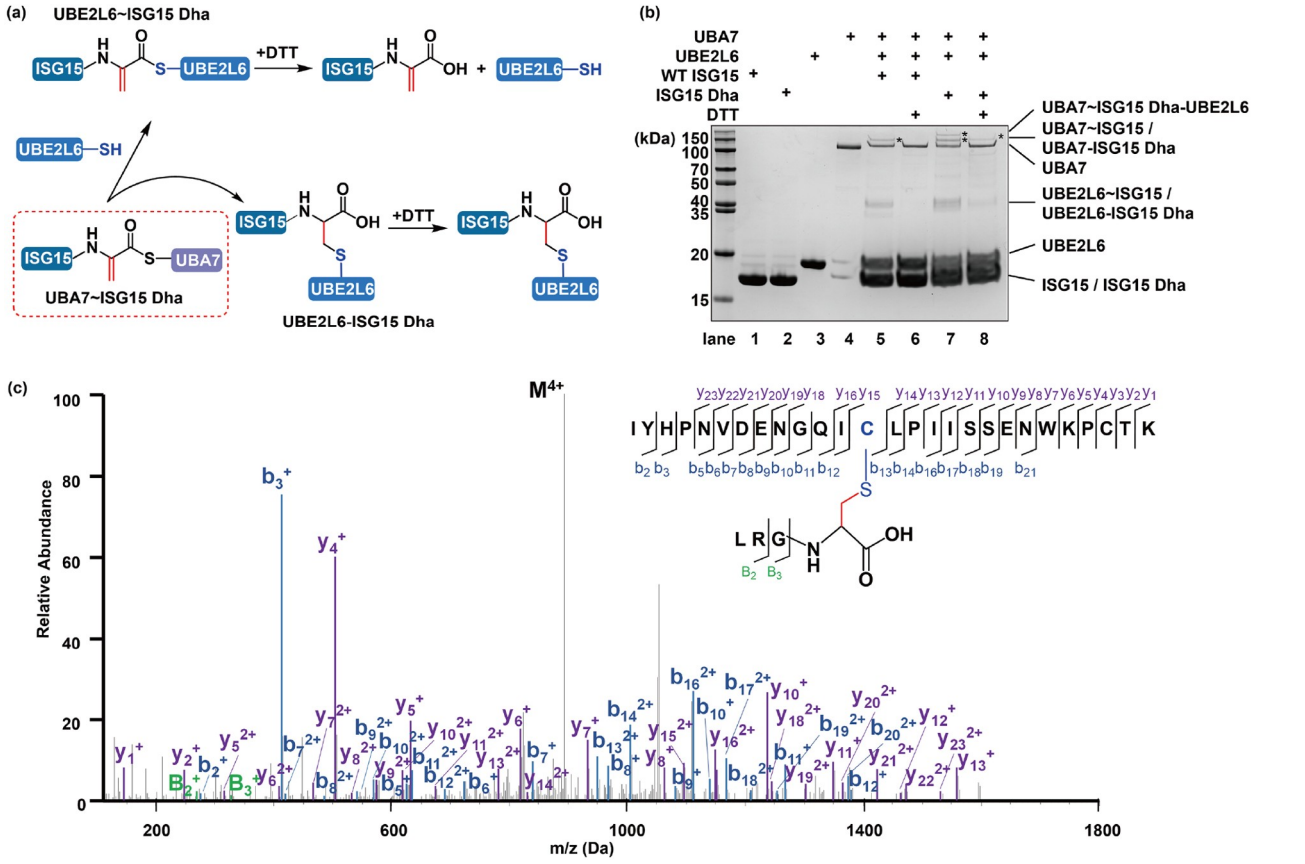

Figure 3 Capture of UBE2L6 *via* covalent thioether formation with ISG15-Dha. (a) Scheme depicting the reactivity of UBE2L6 towards UBA7~ISG15-Dha permitting the formation of either the UBE2L6~ISG15-Dha thioester or the UBE2L6-ISG15-Dha thioether; (b) reactivity of UBE2L6 towards ISG15 and ISG15-Dha under reducing and non-reducing conditions; (c) tryptic MS/MS spectrum of the covalent UBE2L6-ISG15-Dha complex (UBE2L6~ISG15-Dha, thioester-linked conjugate; UBE2L6-ISG15-Dha, thioether-linked adduct) (color online).

ISGylation after IFN stimulation (Figure 4f, g). For example, HERC6 has the highest sequence homology (49%) with the known ISG15 E3 enzyme HERC5 [39]. Previous studies have shown that the expression of HERC6 increased more than 10 times after IFN induction, and *in vitro* experiments showed that HERC6 can also activate ISG15, suggesting that human HERC6 may play a role in ISG15-mediated immune response [39,40]. RNF213 has recently been reported to oligomerize to bind ISG15 after IFN-β stimulation, thereby exerting antibacterial activity [41]. Neither the E1 nor E2 enzymes of Ub or other Ubls were enriched in our experiments, suggesting that biotin-ISG15-Dha is highly specific for ISG15 in cell lysates.

2.4 ISG15-Dha probe crosslinked with ISG15 deconjugating enzymes

Proteomic profiling showed that ISG15-specific deconjugating enzyme USP18 and other deubiquitinases (USP14, USP41, USP42) were enriched in the above cell lysates (Figure 4f–h), raising the possibility for ISG15-Dha to covalently capture deISGylases (Figure 5a). To test this, we expressed the catalytic domain of USP18 (USP18cat) and tested its reactivity with ISG15-Dha *in vitro*, and the previously reported ISG15-PA probe was used as a control [30]. Probe ISG15-PA efficiently crosslinked with USP18cat to generate an adduct band of about 70 kDa (Figure 5b, lane 2). ISG15-Dha also reacted with USP18cat to generate a 70 kDa covalent adduct whose formation was independent of ATP (Figure 5b, lane 3).

Having demonstrated that ISG15-Dha could react with USP18 *in vitro*, we then examined the ability of biotin-ISG15-Dha probe to capture other enzymes with deISGylation activity in cell lysates (Figure S6a). The biotin-ISG15-Dha probe was immobilized onto beads and incubated with IFN-β-induced cell lysates; the beads lacking immobilized probes were used as a negative control (NC). After elution, SDS-PAGE separation, trypsin digestion and LFQ-MS analysis, the proteomics results showed that biotin-ISG15-Dha could capture the known ISG15 deconjugating enzyme USP18 in MCF-7, HeLa and HEK293T cell lysates. In addition, deubiquitinase USP5, USP14, USP15, USP41 (with 82% sequence homology to USP18) and OTUB1 were significantly enriched in at least two cell lines (Figure 5c,

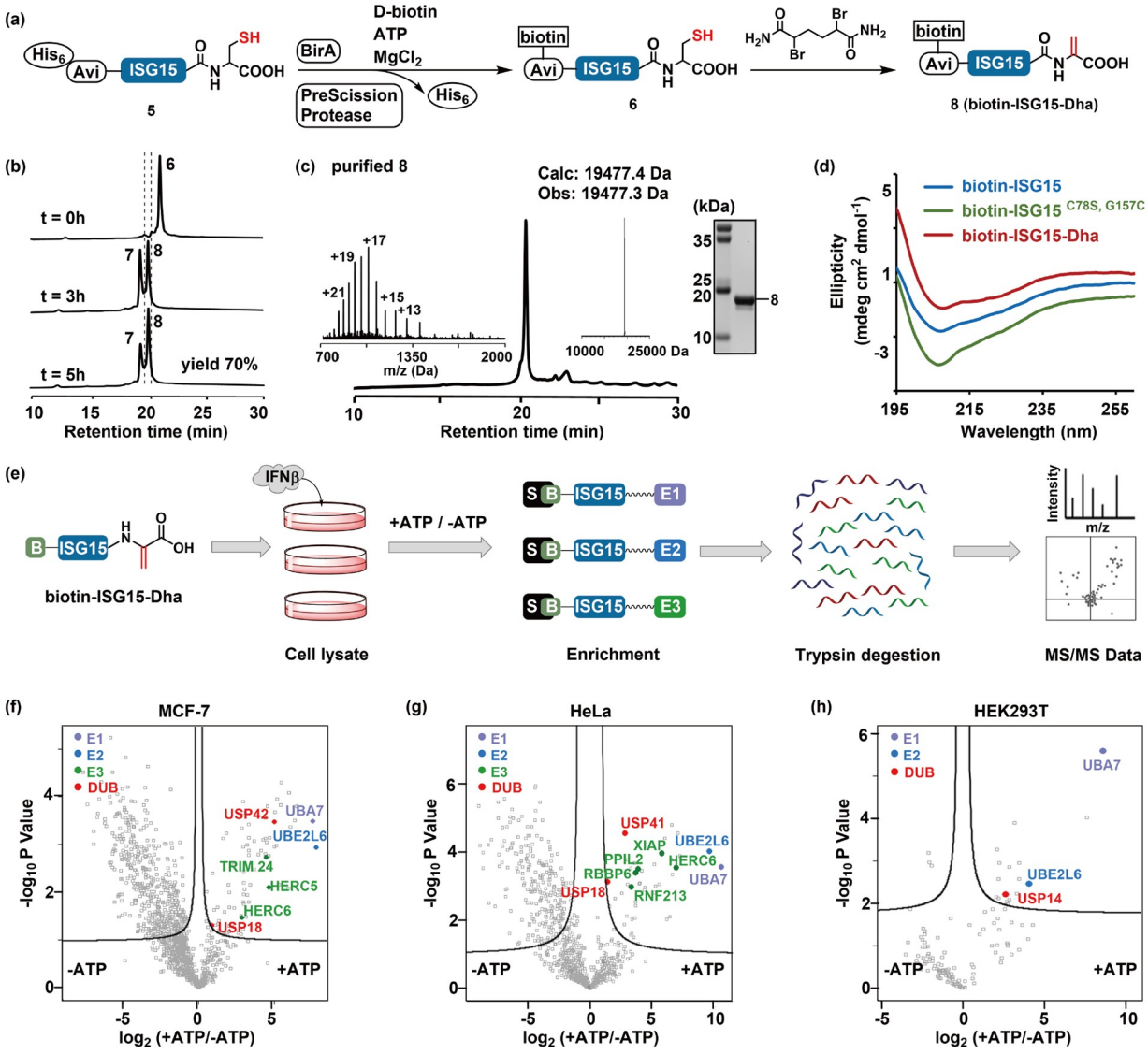

Figure 4 Proteome-wide activity profiling of ISG15-conjugation enzymes with biotin-ISG15-Dha probe (probe **8**). (a) Semi-synthesis of biotin-ISG15-Dha probe (**8**). (b) RP-HPLC traces (214 nm) showing the generation of probe **8** from **6**. (c) Analytical RP-HPLC (214 nm), ESI-MS and SDS-PAGE analysis of probe **8**. (d) CD spectra of probe **8**. (e) MS workflow schematic. Biotin-ISG15-Dha (probe **8**) was incubated with interferon-β (IFN-β)-induced MCF-7/HeLa/HEK293T cell lysates and conducted affinity-based proteomic profiling in the presence of ATP (as the experiment group) and with apyrase-mediated ATP depletion (as the control group). The enrichment was performed in parallel with three replicates. (f–h) Proteomic profiling of the ISG15 conjugating enzymes in IFN-β-induced MCF-7/HeLa/HEK293T cells. Volcano plot comparison of proteins captured by biotin-ISG15-Dha probe within ATP (+ATP) relative to without ATP (−ATP) in three biological replicates. Captured proteins (thresholds: false discovery rate (FDR)=0.05, artificial within groups variance (S0)=0.1) were selected for further analysis. Data processing and analysis were carried out with Proteome Discoverer (version PD1.4), and Perseus (version 1.5.8.5) software. The purple dot denotes significantly enriched E1 (UBA7), blue dot denotes significantly enriched E2 (UBE2L6), green dots denote significantly enriched E3, and red dots denote significantly enriched deISGylases (USP18) and deubiquitylases (color online).

Figure S6b–e and Tables S4–S6). To verify their deISGylation activity *in vitro*, we successfully expressed and purified USP5, USP14 and OTUB1. USP5 and USP14 efficiently hydrolyzed ISG15-AMC and crosslinked with ISG15-PA (Figure 5d–g), which was consistent with previous studies that USP5 and USP14 could react with ISG15-VS probe *in vitro* [42]. We further measured the kinetic parameters of USP5 and USP14. For USP5, the Michaelis constant (K_m) = 18.60 μM, the catalytic constant (K_{cat}) = 2.60 min^{-1}. For USP14, K_m = 1.18 μM, K_{cat} = 0.36 min^{-1} (Figure S7a, b). OTUB1 neither hydrolyzed ISG15-AMC nor crosslinked with ISG15-PA (Figure S8a, b). Taken together, these results showed that ISG15-Dha can also be a tool for deISGylases proteomic profiling.

A deubiquitinase of about 100 kDa may be a precursor of ISG15 (Pro-ISG15) processing enzyme [43]. To test whether USP5 (858 amino acids, 95.8 kDa) might be a Pro-ISG15 processing enzyme, we examined the ability of USP5 to process Pro-ISG15 *in vitro* (Figure S9a). HPLC results showed that USP5 could efficiently cleave Pro-ISG15 to

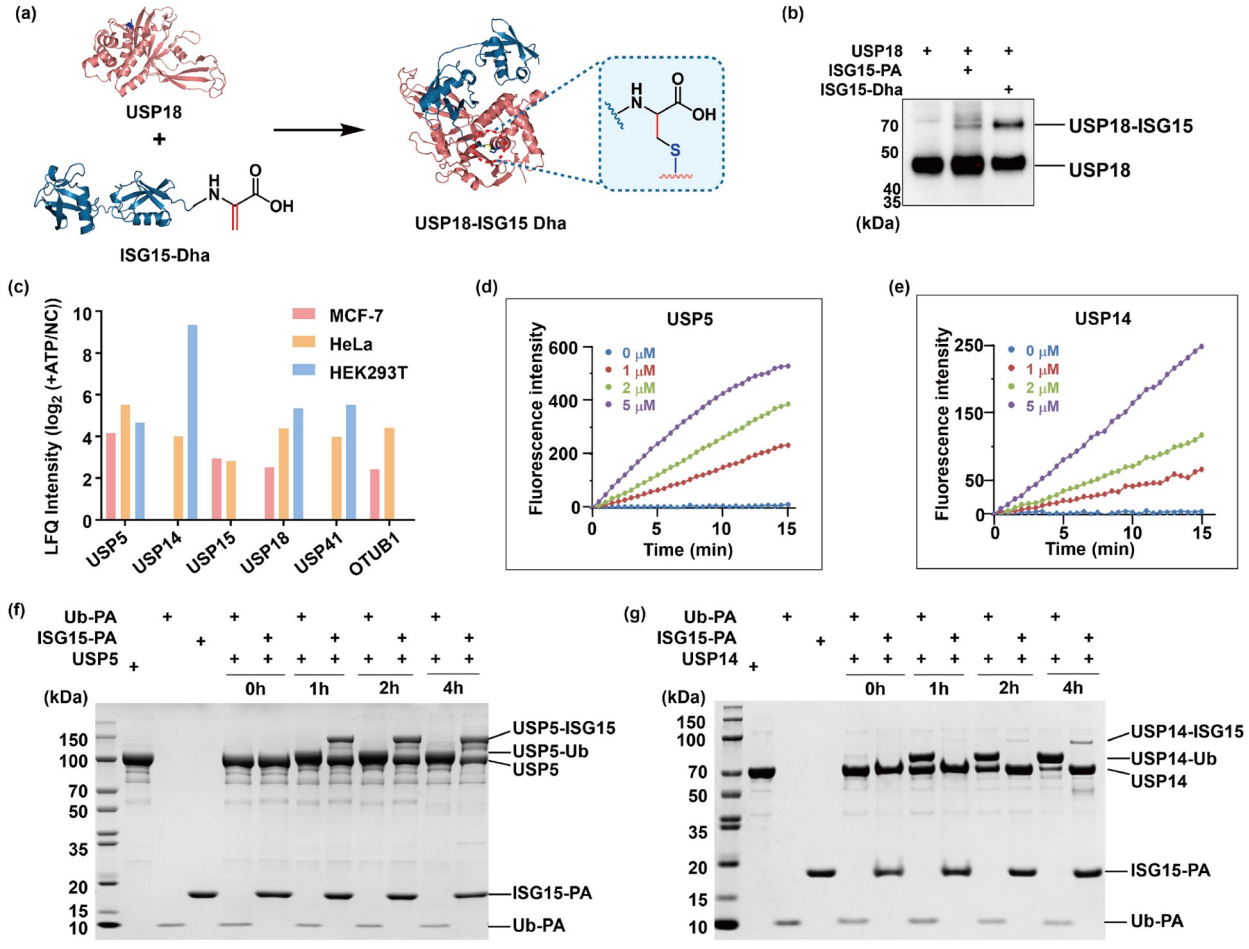

Figure 5 ISG15-Dha probe crosslinked with deISGylases. (a) Scheme depicting the reactivity of USP18 towards ISG15-Dha. (b) Western blotting analysis of ATP-independent reaction of USP18 towards ISG15-PA and ISG15-Dha. (c) Proteomics profiling of ISG15 deconjugating enzymes in IFN-β-induced MCF-7/HeLa/HEK293T cell lysates. Proteins captured by biotin-ISG15-Dha (probe **8**) bound to the streptavidin beads relative to beads lacking immobilized probes (NC) in three biological replicates. Captured proteins enriched at least two cell lines were selected for further analysis. (d, e) RFU detection for the reactivity of ISG15-AMC towards USP5 or USP14. (f, g) SDS-PAGE analysis for the reactivity of ISG15-PA (16 μM) towards USP5 (4 μM) or USP14 (4 μM) (color online).

generate mature ISG15 (Figure S9b, c). Therefore, we speculate that USP5 might also be a processing enzyme of Pro-ISG15 *in vivo*.

3 Conclusions

A probe for ISG15 conjugating enzymes, ISG15-Dha, has been designed and synthesized, and used to undertake *in vitro* crosslinking studies with ISG15 conjugating and deconjugating enzymes, and the proteomic profiling of cell lysates. This work provides a new tool for the study of ISG15 conjugating and deconjugating enzymes, including *in vitro* activity monitoring, proteomic profiling, drug screening, and structural determination.

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Conflict of interest The authors declare no conflict of interest.

Supporting information The supporting information is available online at http://chem.scichina.com and http://link.springer.com/journal/11426. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.

1 McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A. *Nat Rev Immunol*, 2015, 15: 87–103
2 Perng YC, Lenschow DJ. *Nat Rev Microbiol*, 2018, 16: 423–439
3 van der Veen AG, Ploegh HL. *Annu Rev Biochem*, 2012, 81: 323–357
4 Cappadocia L, Lima CD. *Chem Rev*, 2018, 118: 889–918
5 Honke N, Shaabani N, Zhang DE, Hardt C, Lang KS. *Cell Death Dis*, 2016, 7: e2444

6 Jiménez Fernández D, Hess S, Knobeloch KP. *Front Chem*, 2019, 7: 923
7 Shin D, Mukherjee R, Grewe D, Bojkova D, Baek K, Bhattacharya A, Schulz L, Widera M, Mehdipour AR, Tascher G, Geurink PP, Wilhelm A, van der Heden van Noort GJ, Ovaa H, Müller S, Knobeloch KP, Rajalingam K, Schulman BA, Cinatl J, Hummer G, Ciesek S, Dikic I. *Nature*, 2020, 587: 657–662
8 Liu GQ, Lee JH, Parker ZM, Acharya D, Chiang JJ, van Gent M, Riedl W, Davis-Gardner ME, Wies E, Chiang C, Gack MU. *Nat Microbiol*, 2021, 6: 467–478
9 Munnur D, Teo Q, Eggermont D, Lee HHY, Thery F, Ho J, van Leur SW, Ng WWS, Siu LYL, Beling A, Ploegh H, Pinto-Fernandez A, Damianou A, Kessler B, Impens F, Mok CKP, Sanyal S. *Nat Immunol*, 2021, 22: 1416–1427
10 Zhang M, Li J, Yan H, Huang J, Wang F, Liu T, Zeng L, Zhou F. *Front Cell Dev Biol*, 2021, 9: 788410
11 Lee BH, Lee MJ, Park S, Oh DC, Elsasser S, Chen PC, Gartner C, Dimova N, Hanna J, Gygi SP, Wilson SM, King RW, Finley D. *Nature*, 2010, 467: 179–184
12 Ekkebus R, van Kasteren SI, Kulathu Y, Scholten A, Berlin I, Geurink PP, de Jong A, Goerdayal S, Neefjes J, Heck AJR, Komander D, Ovaa H. *J Am Chem Soc*, 2013, 135: 2867–2870
13 Mulder MPC, Witting K, Berlin I, Pruneda JN, Wu KP, Chang JG, Merkx R, Bialas J, Groettrup M, Vertegaal ACO, Schulman BA, Komander D, Neefjes J, El Oualid F, Ovaa H. *Nat Chem Biol*, 2016, 12: 523–530
14 Mulder MPC, Merkx R, Witting KF, Hameed DS, El Atmioui D, Lelieveld L, Liebelt F, Neefjes J, Berlin I, Vertegaal ACO, Ovaa H. *Angew Chem Int Ed*, 2018, 57: 8958–8962
15 Pao KC, Wood NT, Knebel A, Rafie K, Stanley M, Mabbitt PD, Sundaramoorthy R, Hofmann K, van Aalten DMF, Virdee S. *Nature*, 2018, 556: 381–385
16 Bi X, Pasunooti KK, Liu CF. *Sci China Chem*, 2018, 61: 251–265
17 Si Y, Liang L, Tang S, Qi Y, Huang Y, Liu L. *Sci China Chem*, 2018, 61: 412–417
18 Hameed DS, Sapmaz A, Burggraaff L, Amore A, Slingerland CJ, Westen GJP, Ovaa H. *Angew Chem Int Ed*, 2019, 58: 14477–14482
19 Li YM, Li YT, Pan M, Kong XQ, Huang YC, Hong ZY, Liu L. *Angew Chem Int Ed*, 2014, 53: 2198–2202
20 Qi YK, Si YY, Du SS, Liang J, Wang KW, Zheng JS. *Sci China Chem*, 2019, 62: 299–312
21 Zuo C, Shi WW, Chen XX, Glatz M, Riedl B, Flamme I, Pook E, Wang J, Fang GM, Bierer D, Liu L. *Sci China Chem*, 2019, 62: 1371–1378
22 Lu L, Guo Y, Wang T, Liang L, Zhao S, Wang F, Liu L. *Sci China Chem*, 2020, 63: 237–243
23 Gui W, Shen S, Zhuang Z. *J Am Chem Soc*, 2020, 142: 19493–19501
24 Sui X, Wang Y, Du YX, Liang LJ, Zheng Q, Li YM, Liu L. *Chem Sci*, 2020, 11: 12633–12646
25 Liang LJ, Chu GC, Qu Q, Zuo C, Mao J, Zheng Q, Chen J, Meng X, Jing Y, Deng H, Li YM, Liu L. *Angew Chem Int Ed*, 2021, 60: 17171–17177
26 Wang Y, Chen J, Hua X, Meng X, Cai H, Wang R, Shi J, Deng H, Liu L, Li Y. *Angew Chem Int Ed*, 2022, 61:
27 Li Y, Varejão N, Reverter D. *Nat Commun*, 2022, 13: 1819
28 James TW, Frias-Staheli N, Bacik JP, Levingston Macleod JM, Khajehpour M, García-Sastre A, Mark BL. *Proc Natl Acad Sci USA*, 2011, 108: 2222–2227
29 Loch CM, Cuccherini CL, Leach CA, Strickler JE. *Mol Cell Proteomics*, 2011, 10: M110.002402
30 Basters A, Geurink PP, Röcker A, Witting KF, Tadayon R, Hess S, Semrau MS, Storici P, Ovaa H, Knobeloch KP, Fritz G. *Nat Struct Mol Biol*, 2017, 24: 270–278
31 Xin BT, Gan J, Fernandez DJ, Knobeloch KP, Geurink PP, Ovaa H. *Org Biomol Chem*, 2019, 17: 10148–10152
32 Clasman JR, Everett RK, Srinivasan K, Mesecar AD. *Antiviral Res*, 2020, 174: 104661
33 Ma C, Sacco MD, Xia Z, Lambrinidis G, Townsend JA, Hu Y, Meng X, Szeto T, Ba M, Zhang X, Gongora M, Zhang F, Marty MT, Xiang Y, Kolocouris A, Chen Y, Wang J. *ACS Cent Sci*, 2021, 7: 1245–1260
34 Wang T, Li C, Wang M, Zhang J, Zheng Q, Liang L, Chu G, Tian X, Deng H, He W, Liu L, Li J. *Angew Chem Int Ed*, 2022, 61: e202206205
35 Bernardes GJL, Chalker JM, Errey JC, Davis BG. *J Am Chem Soc*, 2008, 130: 5052–5053
36 Eid E, Boross GN, Sun H, Msallam M, Singh SK, Brik A. *Bioconjugate Chem*, 2020, 31: 889–894
37 Witting KF, van der Heden van Noort GJ, Kofoed C, Talavera Ormeño C, el Atmioui D, Mulder MPC, Ovaa H. *Angew Chem Int Ed*, 2018, 57: 14164–14168
38 Fairhead M, Howarth M. *Methods Mol Biol*, 2015, 1266: 171–184
39 Dastur A, Beaudenon S, Kelley M, Krug RM, Huibregtse JM. *J Biol Chem*, 2006, 281: 4334–4338
40 Dastur AR. Characterization of herc5: The major ligase for isg15, an antiviral ubiquitin-like protein. *Dissertation for the Doctoral Degree*. Austin: The University of Texas at Austin, 2007
41 Thery F, Martina L, Asselman C, Zhang Y, Vessely M, Repo H, Sedeyn K, Moschonas GD, Bredow C, Teo QW, Zhang J, Leandro K, Eggermont D, De Sutter D, Boucher K, Hochepied T, Festjens N, Callewaert N, Saelens X, Dermaut B, Knobeloch KP, Beling A, Sanyal S, Radoshevich L, Eyckerman S, Impens F. *Nat Commun*, 2021, 12: 5772
42 Catic A, Fiebiger E, Korbel GA, Blom D, Galardy PJ, Ploegh HL. *PLoS One*, 2007, 2: e679
43 Potter JL, Narasimhan J, Mende-Mueller L, Haas AL. *J Biol Chem*, 1999, 274: 25061–25068