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Heat shock transcription factor δ^{32} is targeted for degradation via an ubiquitin-like protein ThiS in *Escherichia coli*

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ABSTRACT

The posttranslational modification of proteins with ubiquitin and ubiquitin-like proteins (UBLs) plays an important role in eukaryote biology, through which substrate proteins are targeted for degradation by the proteasome. Prokaryotes have been thought to degrade proteins by an ubiquitin independent pathway. Here, we show that ThiS, an ubiquitin-like protein, is covalently attached to δ^{32} and at least 27 other proteins, leading to their subsequent degradation by proteases, in a similar manner to the ubiquitin-proteasome system (UPS) in eukaryotes. Molecular biology and biochemical studies confirm that specific lysine sites in δ^{32} can be modified by ThiS. The results presented here establish a new model for δ^{32} degradation and show that *Escherichia coli* uses a small-protein modifier to control protein stability.

1. Introduction

The heat shock response (HSR) can be defined as the cellular response to increased temperature, during which a set of proteins termed heat shock proteins (HSPs) are up-regulated. These HSPs are usually regulated by the heat shock factor sigma 32 (δ^{32}) in *Escherichia coli* [1].

Following exposure to increased temperature, the intracellular level of δ^{32} transiently increases, leading to the transcription of heat shock related genes, with the ultimate aim of restoring the intracellular milieu to an environment appropriate for correct protein folding. Whilst the degradation process of δ^{32}, the global regulator in *E. coli*, has been unraveled, the mechanism how does δ^{32} recognize by protease remains elusive. To date, only a revised model for the activity and control of δ^{32} degradation has been reported [2]. The first step in this model involves the transport of δ^{32} to the inner membrane by the co-translational protein targeting machinery, composed of the SRP (Signal Recognition Particle) and the SRP receptor. Following localization in the inner membrane, δ^{32} is subject to chaperone-mediated activity control [3,4] and degradation, mediated by the essential protein FtsH [5]. Among a group of five purified ATP-dependent proteases (Lon, ClpAP, ClpXP, FtsH, and ClpYQ) [6], ClpYQ was also shown to directly degrade δ^{32} [7].

To improve our understanding of the recognition mechanism in the process of δ^{32} degradation, we investigated small-protein modifiers that can target substrate proteins for degradation. Two ubiquitin-like proteins (UBLs), ThiS and MoaD, have been identified in *E. coli* [8]. In organisms, modification by ubiquitin or UBLs targets substrates for degradation by the proteasome, a process which is crucial for proper cellular function [9]. However, relatively little is known about the *E. coli* ubiquitin-like proteins ThiS and MoaD. Here, we sought to investigate the function of ThiS and MoaD as small-protein modifiers. Biochemical studies have demonstrated that UBL proteins in eukaryotes, such as SUMO, modify a range of proteins involved in diverse cellular processes, including transcriptional regulators [10]. Recent studies of the UBL-like proteins Pup in *Mycobacterium tuberculosis* and Samp in *Haloferax volcanii* have elaborated the function of these UBLs in prokaryotes [11,12]. Pup and Samp can been specifically conjugated to proteasome substrates. Furthermore, in the yeast *Saccharomyces cerevisiae* the cell type-specific transcriptional repressor MATα2 has been reported to be ubiquitinated [13]. Taken together, these results suggest a rationale to further investigate the modification of δ^{32}, with a specific view to understanding the actions of ThiS and MoaD on δ^{32}.

To gain further insights into δ^{32} modification in *E. coli*, we purified His tagged δ^{32} and analyzed the relationship between δ^{32} and ThiS or MoaD by mass spectrometry. We observed that the heat

Abbreviations: IPTG, isopropyl-β-d-thiogalactoside; UPS, ubiquitin-proteasome system; UBLs, ubiquitin-like proteins; HSR, heat shock response; CBB, coomassie Brilliant Blue; Co-IP, Co-Immunoprecipitation..

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shock transcription factor δ^{32} was modified *in vivo* and *in vitro*. Surprisingly, we discovered that the signature tag of ThiS was covalently linked to δ^{32} and we also identified 27 ThiS conjugates. These results imply that δ^{32} is likely to be degraded by an ubiquitin-like modification pathway and the control of protein stability by ubiquitin-like proteins, such as ThiS, does occur in *E. coli*.

2. Materials and methods

2.1. Strains, plasmids, primers and culture conditions

Strains, plasmids and primer sequences are listed in Supplementary Table S4. *E. coli* strains were grown in Luria–Bertani broth or on Luria–Bertani-agar plates (Difco, NJ, USA). Antibiotic concentrations were as follows for *E. coli*: 100 μg/ml kanamycin and ampicillin, 25 μg/ml for chloramphenicol. Isopropyl-b-D-thio-galactopyranoside was used at 0.5 mM.

rpoH, thiS, moaD and *clpX* were sequentially cloned into pET28a(+). In order to construct plasmid for co-purification of δ^{32}~ThiS complex, *rpoH* was cloned into multiple cloning sites-1 of pETDuet-1 digested with *BamH*I and *Sal*I, then *this* was cloned into multiple cloning sites-2 with *Nde*I and *Xho*I digesting. To construct plasmid for bacterial two hybrid system, *thiS* and *moaD* were cloned into the *BamH*I and *Xho*I sites of pTRG; *clpA*, *clpE*, *clpP*, *clpX*, *clpY*, *hslU* an *ftsH* were cloned into pBT using *Not*I and *Xho*I.

For analysis of *E. coli* lysates, 10 ml cultures were grown to an optical density at 600 nm of approximately 0.4–0.6. Volume of 1 ml bacteria was collected at indicted time point after IPTG added. Centrifuged at top speed for 30 s at 4 °C, cells were boiled in 50 μl of 2xSDS-PAGE loading buffer.

2.2. SDS-PAGE and western blotting analysis

For western blotting analysis, proteins were separated on 12% SDS–polyacrylamide gel electrophoresis (PAGE) gels. Proteins were transferred onto 0.22 μm Polyvinylidene Fluoride using a wet transfer system (Bio-Rad, CA, USA), and incubated with antibodies to δ^{32}, Flag-His_6-ThiS, Flag-His_6-MoaD or His_6-clpX. Horseradish peroxidase-coupled rabbit secondary antibodies were used according to the manufacturer's instructions (Santa cruz, TX, USA). Horseradish peroxidase was detected using Immobilon Western Chemiluminescent HRP Substrate (Millpore, MA, USA).

2.3. The bacterial two-hybrid system

For confirmation the interaction between thiS, moaD and clpX , the bacterioMatch® II Two-Hybrid System Vector Kit (Stratagene, CA, USA) was used. Fifty ng pBT bait vector plus pTRG target vector was then co-transformed to report strain, plated 20 μl aliquots of the cell suspension on nonselective screening medium (no 3-AT) and 200 μl on selective screening medium (5 mM 3-AT), respectively. Incubating the plates at 37 °C for 24 h, the apparent colonies were grown in $M9^+$ His-dropout broth with Tetracycline/Chloramphenicol at 37 °C overnight. To verify the interaction between the bait and target proteins, the cultures of putative positive colonies were streaked on dual selective screening medium (5 mM 3-AT + 12.5 μg/ml streptomycin).

2.4. Modification in E. coli

To examine the modification of proteins in *E. coli*, strains were incubated at 37 °C with shaking until an optical density of 0.6 at 600 nm was reached. Cultures were then induced with 0.5 mM IPTG and incubated for two hours. A volume of 1 ml of the induced cultures was collected and bacteria cells were isolated by centrifugation for 1 min at top speed in microfuge tubes at 4 °C. Bacteria were resuspended in 30 μl sterile water, mixed with 5xsample buffer and boiled for 10 min before analysis by SDS–PAGE and western blotting.

2.5. Proteomic analysis

Purified proteins were visualized on a 12% SDS–PAGE gel and excised from the gel for MS/MS analysis (Done in Center of Biomedical Analysis, Tsinghua University, BJ, China). The gel slice was divided into 4 bands, each of which was cut into cubes of approximately 2 mm and transferred into 1.5 ml Eppendorf tubes.

3. Results and discussion

3.1. Modification of δ^{32} in vitro and in vivo

Proteins can be modified by Pup in *E. coli* in a reconstituted system [14], but the pupylation of transcription factors has not yet been reported. We noticed that δ^{32} could be modified in *E. coli*, which suggested that an undiscovered degradation pathway for δ^{32} existed in *E. coli*. To begin with, we expressed δ^{32} in *E. coli* from the vector pET28a using the IPTG inducible T7 promoter system. After the addition of IPTG, we used western blotting to detect δ^{32} at indicted time points with an anti-δ^{32} monoclonal antibody (Neoclone). Two μg whole protein was loaded for western blotting analysis. As expected, modified δ^{32} was observed as distinct bands after 10 min (Fig. 1A upper panel). And at 0 min (without IPTG), modified δ^{32} was observed as well, which may be because of the basal expression. Accordingly, the amount of HSPs was slightly increased after the induction of δ^{32} expression from the T7 promoter (Fig. 1A lower panels). We subsequently purified δ^{32} from *E. coli*, followed by western blotting analysis, where we were able to detect higher-molecular-weight δ^{32} as well (Fig. 1B). Additionally, previous study showed that δ^{32} might be phosphorylated because two forms of δ^{32} were observed in the isoelectric dimension of two-dimensional gels, but phosphorylation of δ^{32} was not able to be detected [15,16]. To confirm that the higher-molecular-weight proteins were related to δ^{32}, purified δ^{32} was separated by SDS-PAGE and stained with CBB (Fig. 1C). The bands corresponding to the different forms of δ^{32} were excised from the gels, trypsin digested and identified by mass spectrometry (MS). Using this approach, we confirmed that all of the higher-molecular-weight proteins were related to δ^{32}. Surprisingly, the ubiquitin-like proteins ThiS and MoaD [17], and homologues of enzymes associated with sulfur-activation, such as MoaB, ThiF and ThiE, were also identified (Supplementary Table S1). ThiS and MoaD, which are closely related to the eukaryotic URM1, harbor sulfur in the form of a thiocarboxylate on the terminal glycine, just as the thioester linkages of UB/UBLs are formed during the conjugation process [8]. Furthermore, both ThiS and MoaD are adenylated by the enzymes ThiF and MoeB, respectively, prior to the acceptance of sulfur from the donor cysteine [18]. ThiF and MoeB are related to the Ub-conjugating E1 enzymes, which exhibit a characteristic architecture [19]. Thus, we speculated that δ^{32} was modified by ThiS or MoaD.

Our MS analysis also detected the proteins GshA and BirA. GshA belongs to the PafA family of proteins [11] that are related to the γ-glutamyl-cysteine synthetases and glutamine synthetases [20], whilst BirA is a Group II biotin protein ligase [21]. The activities of these identified enzymes suggests that they are responsible for conjugating activated ThiS or MoaD to δ^{32}. The potential ThiS or MoaD modification sites of δ^{32} were analyzed by tandem mass spectrometry (MS/MS) using established methods [22]. We identified one lysine site in δ^{32} that appeared to be attached to the ThiS peptide fragment N-EQWAQHIVQDGDQILLFQVIAGG-C, although

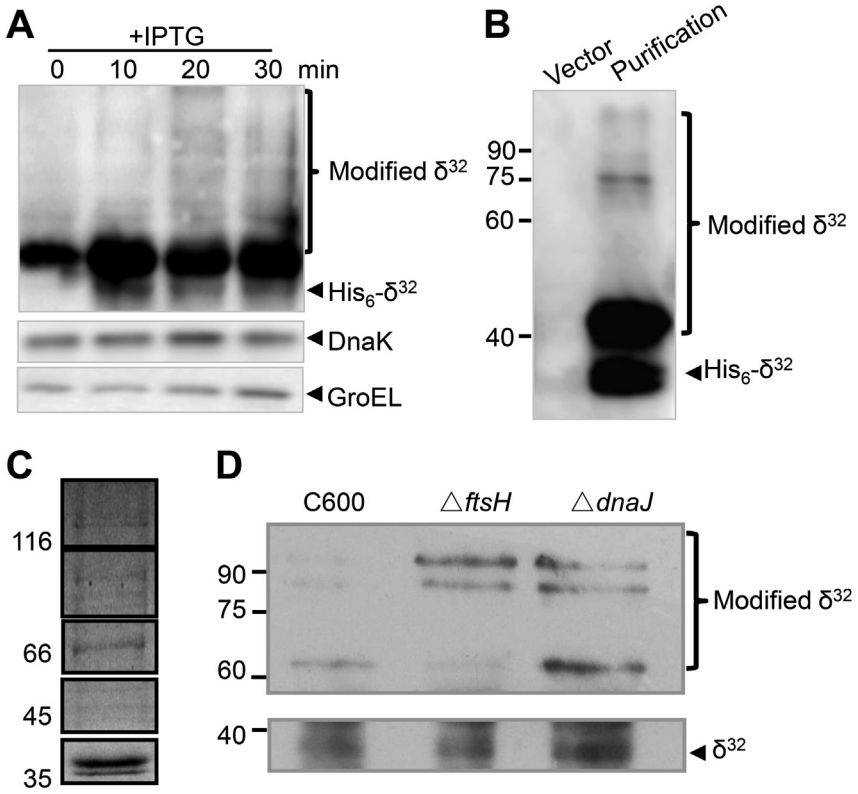

Fig. 1. δ^{32} could be modified *in vitro* and *in vivo*. (A) Western blotting analysis of whole cell extracts from strain *E. coli* Rosetta (DE3) transformed with pET28a-δ^{32} grown in the presence of 0.5 mM IPTG, Samples were taken at indicted time. (B) Following purification of δ^{32} from Rosetta (DE3), δ^{32} and high molecular mass forms were detected with δ^{32} monoclonal antibody. (C) 12% SDS–PAGE and staining for purified δ^{32} proteins by CBB. Molecular mass standards and range of gel slices excised for MS/MS-analysis are indicated on left. (D) *In vivo* immunoprecipitation was performed from C600, △*ftsH*, △*dnaJ* cell extracts using an anti-δ^{32} antibody. Protein complexes were revealed following western blotting analysis using anti-δ^{32} antibody. CBB; Coomassie brilliant blue.

the E-value was high, suggesting a high level of uncertainty in the identification. This may be due to the presence of a 23 amino acid signature sequence that remained attached to the protein, instead of the terminal two glycine residues after trypsin digestion. Thus, it was difficult to accurately determine the site at which δ^{32} was modified. The anti-δ^{32} monoclonal antibody was then used in immunoprecipitation experiments, followed by western blotting analysis, where we observed three slower-migrating bands, suggesting δ^{32} was modified *in vivo* (Fig. 1D). To further discern the nature of the modified δ^{32}, cells were analyzed following heat shock treatment. After treatment, more modified bands were observed (asterisk, Supplementary Fig. S1). Taken together, the results suggested that δ^{32} could be modified by UBL protein ThiS in *E. coli*.

3.2. The interaction of δ^{32} with ThiS in vivo and in vitro

Next, we wondered if δ^{32} had a direct interaction with ThiS. As expected, the interaction between δ^{32} and ThiS *in vivo* was verified in an *E. coli* bacterial two hybrid system (Fig. 2A). Simultaneously, we tested the interaction between δ^{32} and ThiS using co-immunoprecipitation *in vivo* and *in vitro*. ThiS was able to bind to δ^{32} (Fig. 2B and C), however, the interaction between ThiS and δ^{32} was extremely weak *in vitro*. This may be due to the absence of certain cofactors that were necessary to promote interaction between these proteins.

3.3. Modification of δ^{32} by ThiS

As we had identified an interaction between ThiS and δ^{32}, we wondered if δ^{32} was modified by UBL proteins. We expressed ThiS in *E. coli* Rosetta (DE3) and collected samples at defined time points, followed by western blotting analysis with the anti-δ^{32} antibody. The results showed that the overexpression of ThiS could initiate δ^{32} modification and that modified δ^{32} was degraded in the presence of tetracycline (due to the inhibition of prokaryotic protein translation) (Fig. 2D). Intriguingly, further clearly modified bands were observed when cells were grown in $M9^{+}$ high-salt broth (asterisk, Supplementary Fig. S2), suggesting that the relatively complex regulatory network of ThiS responded to environmental changes. To confirm a role for ThiS in endogenous δ^{32} degradation, we determined the level of δ^{32} in wild-type, △*thiS* and △*ftsH* cells after a temperature shift from 42 °C to 30 °C by western blotting (Supplementary Fig. S3). δ^{32} was degraded rapidly in wild-type cells, however, the deletion of *thiS* or *ftsH* strongly stabilized δ^{32}. Moreover, modified δ^{32} generated by its overexpression, was similarly degraded in the presence of tetracycline (Fig. 2E). In summary, these results suggested that the small modifier ThiS could control the stability of δ^{32}.

The ability of DnaK to control both the activity and stability of δ^{32}, facilitating its degradation by FtsH has been well studied [23]. Additionally, δ^{32} can be controlled by HslUV and Lon proteases [24,25], and there are corresponding proteases in the UPL degradation system. We speculated that, in *E. coli*, δ^{32} is degraded following modification by other proteases. Therefore, we looked for the presence of AAA + proteases [25] interacting with both ThiS and δ^{32}. Interestingly, ClpX, a hexametric AAA + protein, was found in a bacterial two hybrid screen (Fig. 2F). ClpX is modulated by three adaptor proteins SspB, UmuD and RssB [26], and uses ATP as an energy source to both unfold protein substrates and to translocate the unfolded polypeptides [27]. Using co-immunoprecipitation

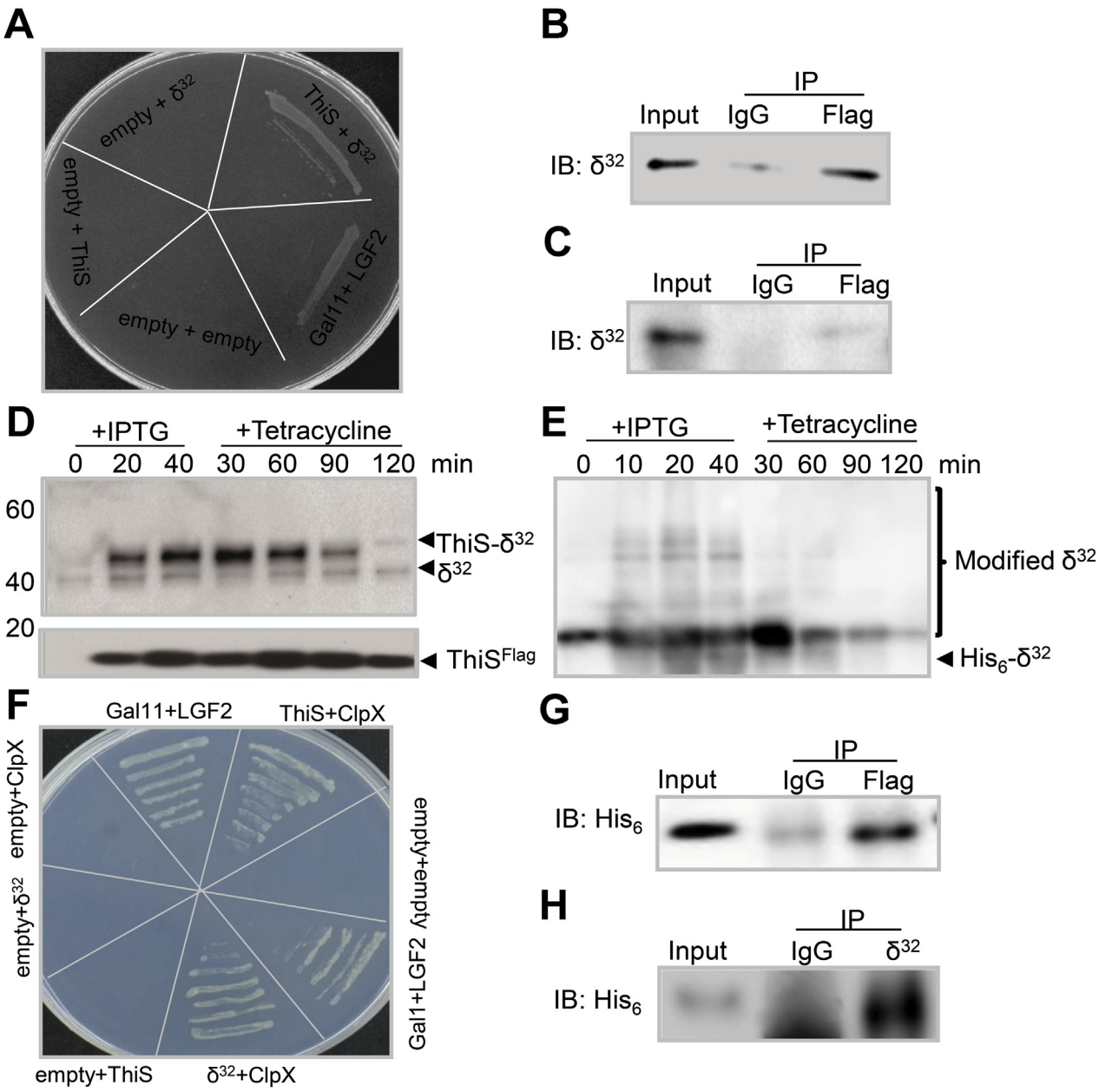

Fig. 2. Overexpression of ThiS generated modified δ^{32} significantly. (A) ThiS interacting with δ^{32} in a BacterioMatch *E. coli* two-hybrid system (Stratagene). *E. coli* report strain was transformed with combinations of plasmids encoding either of λcI on pBT or RNAP on pTRG, fused to test proteins. pTRG-GalII and pBT-LGF2 were used to positive control. All strains grew on selective screening Medium (5 mM 3-AT + 12.5 μg/ml streptomycin). (B) *In vivo* Co-immunoprecipitation of endogenous δ^{32} and pET28a-based Flag-tagged ThiS protein expressed in Rosetta (DE3) at 37 °C in presence of 0.5 mM IPTG. Immunocomplexes were revealed following western blotting analysis using anti-δ^{32} antibody. (C) Co-immunoprecipitation of *vitro*-purified δ^{32} and Flag-tagged ThiS, performed with an anti-Flag antibody. Immunocomplexes were separated by 12% SDS-PAGE, and analyzed by anti-δ^{32} western blotting. (D) Western blotting analysis (probed with anti-δ^{32} and anti-Flag) of whole cell extracts from strain Rosetta (DE3) transformed with pET28a-$ThiS^{Flag}$. Detailed operation was described as follow. (E) Analysis of modified δ^{32} degradation by western blotting. Cells transformed with pET28a-δ^{32} were grown to OD600 of 0.6 and samples were collected at indicted time points after adding 0.5 mM IPTG. Forty minutes later, the cell culture were collected with adding 200 μg/ml tetracycline at indicted time. (F) Verification of the interaction of δ^{32} or ThiS with ClpX in bacterial two hybrid system. (G) and (H) Co-immunoprecipitation of *vitro*-purified ClpX and ThiS or δ^{32}, performed with an anti-Flag antibody or anti-δ^{32} antibody, respectively. Immunocomplexs were detected by western blotting using an anti-His_6 antibody.

in vitro, we determined the interaction of ClpX with ThiS and δ^{32} (Fig. 2G and H, respectively). These data suggested that modified δ^{32} was degraded by ClpXP protease.

3.4. Identification of ThiS conjugates

In contrast to ubiquitinylated proteins, trypsin digested ThiS conjugates possess a large signature tag (23 amino acids) attached to a modified lysine residue. The tag generates complex ion patterns during MS/MS fragmentation [28]. To circumvent this problem, an alanine residue at position 64 of ThiS (immediately before the C-terminal GG motif) was mutated to lysine. In order to detect this mutant more easily, we constructed a His_6-Flag–$ThiS^{A64K}$ fusion and overexpressed this protein in *E. coli* Rosetta (DE3) following purified by Ni-NTA chromatography. Analysis of purified conjugates using an anti-Flag antibody enhanced detection of a diverse range $ThiS^{A64K}$ conjugates (Fig. 3A left panel). To confirm the identity of these conjugates, protein

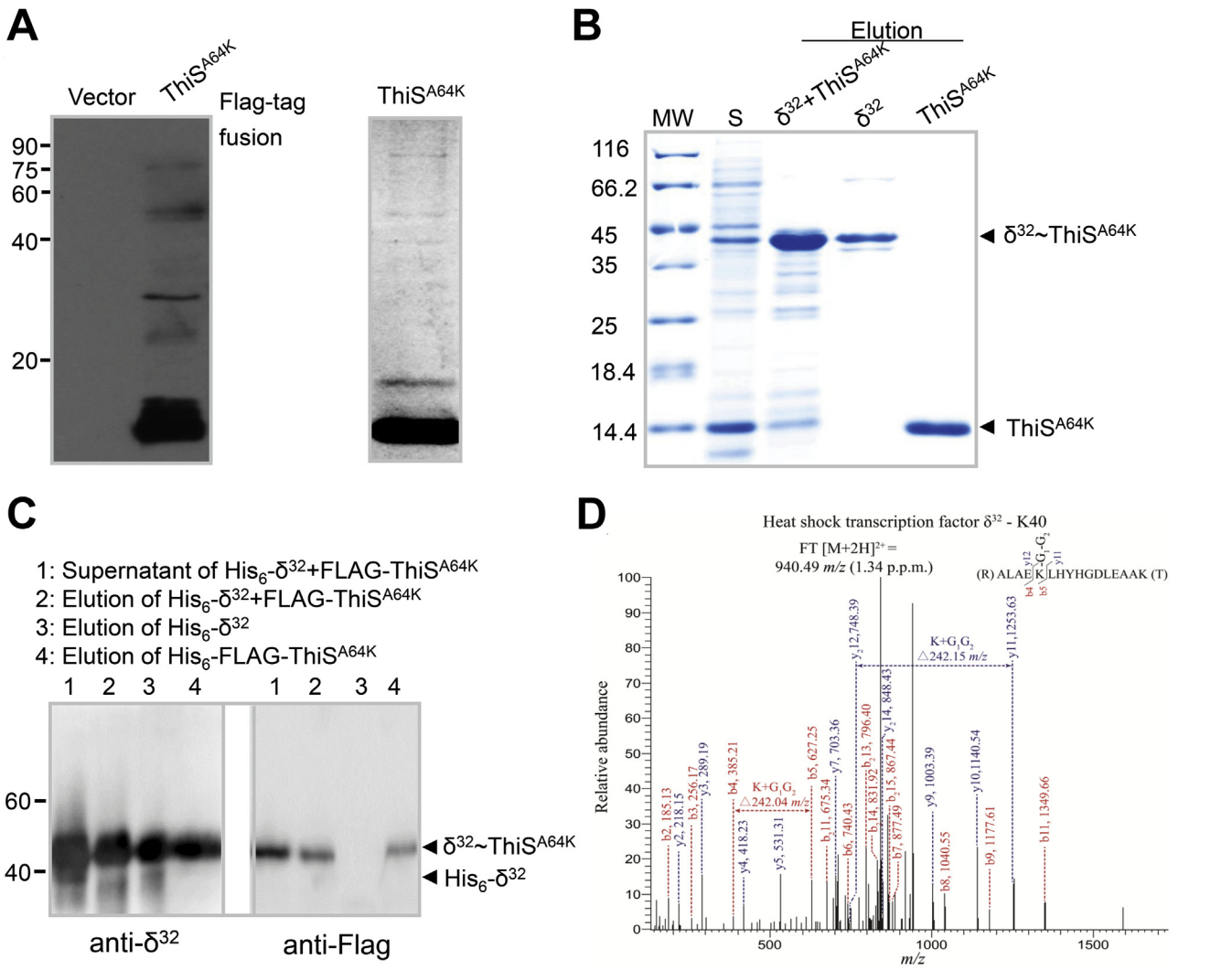

Fig. 3. ThiS was conjugated to δ^{32}. (A) Reveal of purified His-Flag-ThiS^{A64K} by western blotting using anti-Flag antibody (Left panel). Analysis of conjugated proteins with ThiS^{A64K} by MS/MS. 12% SDS–PAGE and staining for purified ThiS^{A64K} proteins by CBB (Right panel). (B) Purification of the His$_6$-δ^{32}~Flag-ThiS^{A64K} complex. Rosetta (DE3) was transformed with plasmid encoding His$_6$-δ^{32} and FLAG-ThiS^{A64K} in pETDuet-1. His$_6$-δ^{32}~FLAG-ThiS^{A64K} was purified sequentially with Ni-NTA agarose, His$_6$-Flag-ThiS^{A64K} and His$_6$-δ^{32} were purified separately as control. Proteins were analyzed by 12% SDS-PAGE and visualized with CBB. (C) Analysis of His$_6$-δ^{32}~Flag-ThiS^{A64K} complex by western blotting. The monoclonal mouse antibody was used to detect a relevant protein as indicated. (D) Lysine 40 in δ^{32} was identified as ThiS^{A64K} attachment site by MS/MS analysis. Peptide fragment was identified as *b*- or *y*-type ions. Other MS/MS spectra, Supplementary Fig. S4. CBB; Coomassie brilliant blue.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bands were excised from SDS-PAGE gels, digested with trypsin and identified by MS/MS (Fig. 3A right panel). Using this approach, we were able to identify at least 48 protein conjugates (Supplemental Table S2), including synthases, membrane fusion proteins, and an integrase. Consistent with its role as small modifier protein, ThiS^{A64K} was identified in SDS–PAGE gel slices across a wide-range of molecular masses (14–116 kDa). To exclude the effect of mutation, we analyzed wild-type ThiS conjugates as well (Supplementary Table S3). We identified 27 protein conjugates that were modified when the wild-type ThiS protein was used, however, the ThiS and ThiS^{A64K} conjugates did not overlap.

In order to find the exact modification sites of δ^{32}, we used tandem affinity chromatography to purify a His$_6$-δ^{32}~Flag-ThiS^{A64K} complex from *E. coli* Rosetta (DE3). We cloned His$_6$-δ^{32} into the first multiple cloning site of the plasmid pETDuet-1, and the gene for Flag-ThiS^{A64K} into multiple cloning site 2 of the same plasmid. Then, we purified the complex by Ni-NTA chromatography. We characterized the interaction between these proteins using MS/MS [12]. Upon analysis by SDS-PAGE the δ^{32}~ThiS^{A64K} complex was seen at the expected molecular weight of approximately 44 kDa (Fig. 3B). To verify the complex, we detected the purified proteins by western blotting. As expected, we observed the protein complex in the elution from cells expressing His$_6$-δ^{32} + Flag-ThiS^{A64K}, His$_6$-δ^{32} or His$_6$-Flag-ThiS^{A64K} at 44 kDa using an anti-δ^{32} monoclonal antibody (Fig. 3C left panel). Furthermore, the His$_6$-δ^{32}~Flag-ThiS^{A64K} complex was also detected in the elution of His$_6$-δ^{32} + Flag-ThiS^{A64K} and His$_6$-Flag-ThiS^{A64K} at the same molecular mass using an anti-Flag monoclonal antibody (Fig. 3C right panel), confirming the interaction between these two proteins and further illustrating that ThiS can be covalently attached to δ^{32}.

Five lysine sites in δ^{32} modified by ThiS were mapped by MS/MS fragmentation using collision-induced dissociation (CID) (Supplementary Fig. S4 A). The identification of these sites was based on the mass differences between the *y* and *b* ion series containing the ThiS^{A64K}-derived GG-footprint on lysine residues. The identification of three of these lysine sites shown in the mass spectrum (Fig. 3D and Supplementary Fig. S4 B and C).

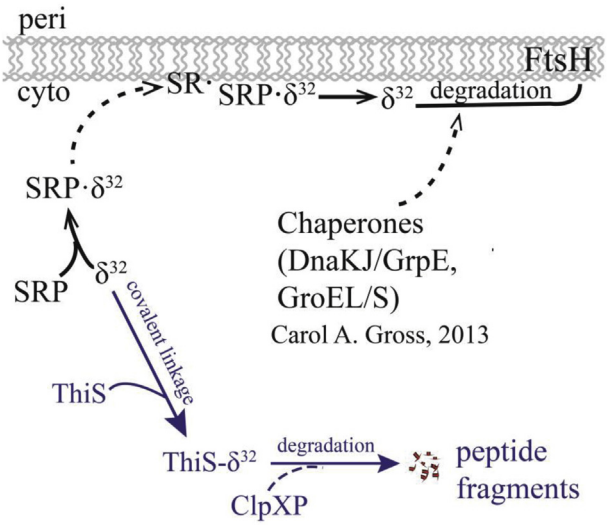

Fig. 4. A replenished model for δ^{32} degradation. Upper part represents the classical model for δ^{32} degradation. The replenished model incorporates. ThiS-dependent targeting δ^{32} to degrade. ThiS is attached to δ^{32} by unknown factors and then modified δ^{32} is degraded by ClpXP protease.

In bacteria, AAA + proteases play an important role in general protein maintenance and also in the regulation of cellular development and division [26]. These proteases act through use of adaptor proteins [29] or specific substrate recognition sequences, called degrons [30], to identify targets. As such, it is believed that a modified protein degradation pathway, where small protein modifiers target substrates for degradation, is uncommon in bacteria. However, we have discovered that the *E. coli* heat shock transcription factor δ^{32} was modified by the UBL protein ThiS, after which it was degraded (Figs. 1 and 2). Despite intensive study, how δ^{32} is specifically targeted for degradation is unclear. Our results show that δ^{32} is first modified by ThiS and subsequently recognized by ClpXP, a previously unknown route towards the degradation of δ^{32} (Fig. 4). Moreover, the identification of ThiS conjugates in *E. coli* (Fig. 3) indicates that a UBL pathway may function in the routine degradation of proteins, in a similar fashion to pupylation in *M. tuberculosis* [31] and sampylation in *Haloferax volcanii* [12].

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.087.

Transparency document

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