BRIEF COMMUNICATION
PUBLISHED ONLINE: 12 JUNE 2017 | DOI: 10.1038/NCHEMBIO.2406

nature chemical biology

Site-specific incorporation of phosphotyrosine using an expanded genetic code

Christian Hoppmann[1], Allison Wong[2], Bing Yang[1], Shuwei Li[3], Tony Hunter[4], Kevan M Shokat[2] & Lei Wang[1]*

Access to phosphoproteins with stoichiometric and site-specific phosphorylation status is key to understanding the role of protein phosphorylation. Here we report an efficient method to generate pure, active phosphotyrosine-containing proteins by genetically encoding a stable phosphotyrosine analog that is convertible to native phosphotyrosine. We demonstrate its general compatibility with proteins of various sizes, phosphotyrosine sites and functions, and reveal a possible role of tyrosine phosphorylation in negative regulation of ubiquitination.

Protein phosphorylation is a major post-translational modification (PTM) that plays a pivotal role in signal transduction and in the regulation of cellular events[1,2]. In particular, phosphorylation of tyrosine is involved in many processes including cell proliferation, cell cycle progression, metabolic homeostasis, transcriptional activation, neural transmission, differentiation, development, and aging[1,3]. Conversely, dysfunction of tyrosine phosphorylation results in various diseases, most prominently cancer[4–6]. Limited access to site-specifically phosphorylated proteins hampers the investigation of this major PTM in disease-relevant proteins[7].

Many methods have been developed to study protein phosphorylation. A generally useful approach is to substitute glutamate or aspartate for phosphoserine or phosphothreonine, despite the charge difference between carboxylate and phosphate monoester. Producing a mimic of phosphotyrosine (pTyr), however, is challenging, as there are no negatively charged aromatic residues among the 20 amino acids. Proteins can be phosphorylated with kinases, but *in vitro* kinase phosphorylation has limited site specificity and often results in substoichiometric phosphorylation[8]. To overcome this limitation, chemical approaches, including native chemical ligation and semisynthetic and cell-free methods, have been developed to introduce pTyr site selectively, but these methods are technically challenging and cannot be generally applied to all proteins[9–13]. Through the expansion of the genetic code[14], a chemical analog of pTyr has been incorporated into proteins in *Escherichia coli*[15], yet the analog cannot faithfully mimic the phosphate group and its negative charges. Direct incorporation of phosphorylated amino acids in live cells also has multiple challenges[16–20]: the phosphorylated amino acid enters cells poorly because of its negative charge, is unstable inside cells, and is incompatible with the elongation factor Tu (EF-Tu) for translation. Despite feeding excessive amounts of phosphorylated amino acids to cells, engineering EF-Tu, and reducing cellular phosphatase activity, these challenges still result in low incorporation efficiency and the presence of other amino acids at the phosphorylation site. To date, there is no general, efficient system available to prepare proteins with native pTyr introduced site specifically.

Here we developed an efficient and easily accessible method for the production of pure tyrosine-phosphorylated proteins. Our strategy circumvents the aforementioned critical problems: a charge-neutral and stable pTyr analog was genetically incorporated into proteins at the target phosphorylation site through the expansion of the genetic code, and was subsequently converted into a native pTyr by a facile pH shift (**Fig. 1a**). Using this approach we prepared multiple proteins with pTyr site-specifically introduced at the phosphorylation site, resulting in good yields without disrupting protein activity. We also investigated the effect of tyrosine phosphorylation on ubiquitin (Ub) structure and function, revealing a possible negative regulatory role of Ub phosphorylation.

Because the phosphate group of pTyr is labile to cellular phosphatases and its negative charge limits cell permeability, we genetically encoded the pTyr analog **1** containing a phosphoramidate group (**Fig. 1a**), which is stable and neutral and can be cleaved under acidic conditions to generate the native pTyr[21]. We synthesized unnatural amino acid (Uaa) **1** in 82% yield and high purity without the need for column chromatography (**Supplementary Results**, **Supplementary Note 1**). To optimize a cleavage condition suitable for proteins, we used low concentrations of HCl to remove the protecting group in **1**. At 0.04 M HCl (pH ~2), the protecting group was already cleaved completely within 36 h at room temperature to generate pTyr (**Supplementary Fig. 1**).

To genetically encode Uaa **1** in *E. coli*, we evolved the *Methanosarcina mazei* pyrrolysyl amber suppressor tRNA and cognate synthetase ($\mathrm{tRNA}^{\mathrm{Pyl}}_{\mathrm{CUA}}$–PylRS) pair[22] to be specific for this Uaa. A mutant library of PylRS was generated with residues 302, 309, 322, 346, 348, 401, 417, and 419 mutated as previously described[23,24]. From this library we identified a clone showing phenotypic dependence on Uaa **1** (**Supplementary Fig. 2**), which harbored a mutant (named MmNpYRS) containing Ser302, Met309, Leu322, Ala346, Gly348, Val401, Thr417, and Gly419 (**Supplementary Table 1**).

To investigate the efficiency and fidelity of MmNpYRS in incorporating **1**, we expressed in *E. coli* a gene for *Xenopus* calmodulin (CaM) that contained an amber codon TAG at site Met76 and a C-terminal 6× His tag, together with the $\mathrm{tRNA}^{\mathrm{Pyl}}_{\mathrm{CUA}}$–MmNpYRS. CaM consists mainly of α-helices and has low molecular weight (18 kDa) to facilitate MS characterization with high accuracy. Full-length CaM was obtained in good yield (~1.0 mg/ L^{-1}) in the presence of Uaa **1** (1 mM), but was undetectable in its absence (**Supplementary Fig. 3**). Analysis of the expressed CaM by ESI–MS confirmed incorporation of **1** into CaM (**Fig. 1b**). An observed peak at 18,103.8 Da corresponds to intact CaM containing Uaa **1** at site 76 (expected $[\mathrm{M+H}]^+$ = 18,104.0 Da); a second peak corresponds to the Uaa **1**-containing CaM lacking the initiator Met (expected

---

[1]Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, California, USA. [2]Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, California, USA. [3]Institute for Bioscience and Biotechnology Research, University of Maryland College Park, Rockville, Maryland, USA. [4]The Salk Institute for Biological Studies, Molecular and Cell Biology Laboratory, La Jolla, California, USA.
*e-mail: Lei.wang2@ucsf.edu

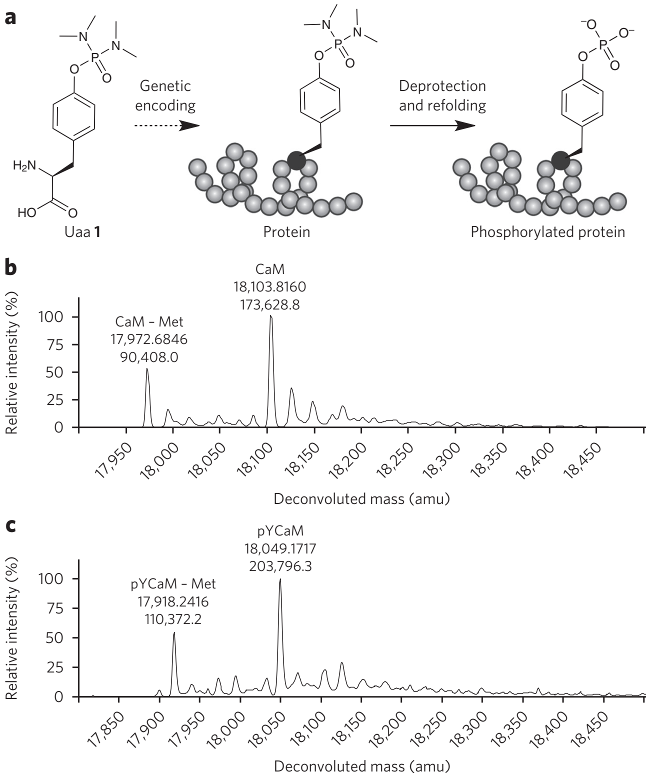

Figure 1 | Site-specific incorporation of phosphotyrosine into proteins. (**a**) Genetic encoding of the stable and neutral phosphotyrosine analog **1** into proteins and subsequent deprotection results in site-specific formation of a native phosphotyrosine. (**b**) ESI–MS spectrum of calmodulin (CaM) confirming the incorporation of Uaa **1** at site 76 (taller peak). (**c**) ESI–MS spectrum of CaM protein after HCl treatment confirming the conversion of Uaa **1** into phosphotyrosine (pYCaM; taller peak).

$[M\text{–}Met+H]^+$ = 17,972.8 Da; measured = 17,972.7 Da). No peaks were observed corresponding to CaM containing a natural amino acid at the TAG site. Additionally, the high fidelity of MmNpYRS was also confirmed by incorporating **1** into another protein, myoglobin (**Supplementary Fig. 4**). In contrast to the need of an engineered EF-Tu to incorporate phosphoserine[16], incorporation of **1** into proteins in *E. coli* suggests that wild-type (WT) *E. coli* EF-Tu is compatible with Uaa **1**–$tRNA^{Pyl}_{CUA}$.

To convert **1** into pTyr within proteins, we treated CaM solution (0.6 mg ml^{-1}) with HCl (final concentration of 0.4 M, pH~1) for 48 h at 4 °C. The sample was lyophilized to remove the acid and then dissolved in water. ESI–MS measurements clearly demonstrated the removal of the protecting group and formation of pTyr within CaM: A peak at 18,049.2 Da (**Fig. 1c**) corresponds to intact CaM with pTyr at site 76 (pYCaM; expected $[M+H]^+$ = 18,049.9 Da). A second peak corresponds to the pTyr-containing CaM lacking the initiator Met (expected $[M\text{–}Met+H]^+$ = 17,918.7 Da; measured = 17,918.2 Da).

We next introduced pTyr into green fluorescent protein (GFP), which has a different secondary structure β-sheet from the largely helical CaM. A GFP gene containing a TAG codon at Tyr182 and a C-terminal 6× His tag was expressed with the $tRNA^{Pyl}_{CUA}$–MmNpYRS in *E. coli*. The mutant GFP was purified, yielding 1.25 mg L^{-1}, corresponding to 31% of WT GFP purified under the same conditions. HCl (final concentration of 0.4 M, pH~1) was added to a diluted (0.1 mg ml^{-1}) solution of the mutant GFP; the mixture was incubated at 4 °C for 16 h and then readjusted to pH 7.5 with NaOH. Western blot analysis using anti-6× His antibody showed that full-length GFP was expressed only in the presence of Uaa **1** (**Supplementary Fig. 5**), confirming specific incorporation of Uaa **1** by the $tRNA^{Pyl}_{CUA}$–MmNpYRS. The GFP band up-shifted after HCl treatment, suggesting that deprotection generated pTyr, whose negative charge slowed phosphoprotein movement in SDS–PAGE. The pTyr-specific antibody detected a band in the HCl-treated GFP only, at the position colocalizing with the up-shifted GFP band in the anti-6× His blot, indicating the conversion of Uaa **1** into pTyr. The pTyr conversion efficiency was ~90% based on band intensities. Before and after acidic cleavage of Uaa **1**, the GFP proteins were both green fluorescent (**Supplementary Fig. 6**), indicating that Uaa **1** and its conversion into pTyr did not prevent GFP from folding into the correct fluorescent structure. To maximize deprotection of Uaa **1**, exposure time to HCl may depend on protein concentration and structure. Although we found 16 h sufficient for GFP and others (see below), CaM needed 48 h.

We next generated pTyr-containing Ub and investigated the impact of tyrosine phosphorylation on Ub conformation and function. Whereas serine phosphorylation has been extensively studied for Ub, phosphorylation of Tyr59, the only tyrosine residue of WT Ub, has been observed exclusively in cancerous tissue, though its biological relevance remains unknown[25,26]. In WT Ub, the hydroxyl of Tyr59 hydrogen bonds with the backbone amide of Glu51, forming a loop that is disrupted by mutation of Tyr59 (ref. 27). To provide direct evidence of whether phosphorylation of Tyr59 alters the Tyr59–Glu51 loop in Ub and affects Ub function, we incorporated Uaa **1** into Ub at site 59 and generated pTyr by acid cleavage followed by lyophilization. SDS–PAGE and MS confirmed the successful preparation of Tyr59-phosphorylated Ub (**Supplementary Fig. 7**; **Supplementary Fig. 10**). ESI–MS analysis of acid-treated Ub showed a peak at 9,468.1774 Da corresponding to intact Ub with pTyr at site 59 lacking the initial methionine (expected $[M+H\text{–}Met]^+$ = 9,468.6856 Da); trypsin digest and MS/MS sequencing of this protein confirmed that pTyr was incorporated at the TAG site 59.

To examine a potential conformational change induced by pTyr59, we measured the HSQC NMR spectra of WT Ub and the Tyr59-phosphorylated Ub (pTyrUb). The signals of Tyr59, Glu51, and other relevant amino acids in the loop shifted dramatically in pTyrUb compared to those of WT Ub (**Fig. 2a**). Most notably, the Glu51 signal showed a similar large shift to that observed previously in the Y59F Ub mutant[27]. In addition, most of the assigned and shifted residues were located in or near the Y59–E51 loop (**Fig. 2b**). To exclude possible shifts brought on by the acid treatment, we also subjected the WT Ub to the same treatment and detected no difference in the HSQC NMR spectra before and after treatment (**Supplementary Fig. 8**). These NMR data thus indicate that the Y59–E51 loop was indeed altered after phosphorylation of Tyr59.

To investigate whether pTyr59 would interfere with thioester formation between Ub and E2 conjugating enzyme, we studied the conjugation of pTyrUb to the E2 enzyme UBE2D3. In contrast to Ser65-phosphorylated Ub, which exhibits enhanced Ub–E2 conjugation[18,26], we found that pTyrUb dramatically decreased the Ub–E2 conjugation (**Fig. 2c**). To exclude any potential interference due to the acid treatment, we subjected WT Ub to the same treatment as the Uaa **1**-incorporated Ub, and found that the treated WT Ub still conjugated to UBE2D3 (**Supplementary Fig. 9**). Therefore, Tyr59 phosphorylation altered Ub conformation and decreased its ability to conjugate with the E2 enzyme UBE2D3, suggesting that Tyr59 phosphorylation on Ub could play a negative regulatory role in the ubiquitination process.

In summary, we have developed a new method to prepare phosphorylated proteins with pTyr site-specifically introduced in high quality and good yields. Combining genetic incorporation with facile pH conversion, this method enables pTyr to be introduced

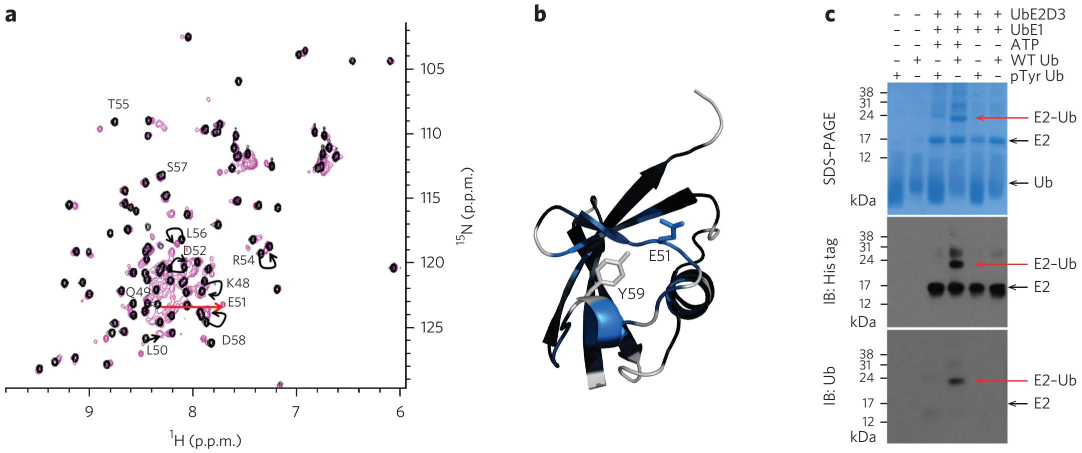

Figure 2 | Phosphorylation of Tyr59 in Ub impacts its conformation and function. (**a**) Overlay of ^{1}H–^{15}N HSQC spectra for WT Ub (black contours) and pTyr59 Ub (magenta contours). The amides located in the Lys48–Tyr59 loop of Ub are labeled. The resonance for the Glu51 shift is indicated in red. (**b**) Heatmap indicating residues shifted as a result of phosphorylation of Tyr59 in Ub. White, residues not assigned; black, residues with no shift; blue, residues that shifted. Tyr59 and Glu51 in the loop are shown in stick. (**c**) E2 charging is negatively regulated by phosphorylation of Tyr59 in Ub. SDS-PAGE of the E2-Ub conjugation assay showed that pTyrUb did not form the E2-Ub thioester while WT Ub did (red arrow), which was also confirmed by corresponding western blots immunoblotted (IB) with His-tag antibody and Ub antibody. All samples were loaded using nonreducing buffer to preserve the E2-Ub thioester linkage. By contrast, when the conjugated samples were treated with 2-mercaptoethanol under reducing conditions, the E2-Ub thioester is unstable and the corresponding E2-Ub band disappeared in the western blot (**Supplementary Fig. 9**), supporting its identity of E2-Ub.

in different secondary structures of various proteins with broad compatibility of protein type, size, and phosphorylation site. The method does, however, require the target protein to withstand mild acid treatment and to refold into the functional state upon acid removal. As the tRNA$^{Pyl}_{CUA}$–MmNpYRS was derived from the tRNA$^{Pyl}_{CUA}$–PylRS, which is orthogonal in prokaryotic and eukaryotic cells, we expect that the tRNA$^{Pyl}_{CUA}$–MmNpYRS can also be used in eukaryotic cells to prepare proteins difficult to express in bacteria. The technical simplicity of this approach should allow its general adoption, affording a robust method to facilitate the investigation of tyrosine phosphorylation in biology and diseases.

Received 21 September 2016; accepted 15 March 2017;
published online 12 June 2017

Methods

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

References

1. Hunter, T. *Curr. Opin. Cell Biol.* **21**, 140–146 (2009).
2. Tarrant, M.K. & Cole, P.A. *Annu. Rev. Biochem.* **78**, 797–825 (2009).
3. Lim, W.A. & Pawson, T. *Cell* **142**, 661–667 (2010).
4. Julien, S.G., Dubé, N., Hardy, S. & Tremblay, M.L. *Nat. Rev. Cancer* **11**, 35–49 (2011).
5. Blume-Jensen, P. & Hunter, T. *Nature* **411**, 355–365 (2001).
6. Tan, C.S. *et al. Sci. Signal.* **2**, ra39 (2009).
7. Humphrey, S.J., James, D.E. & Mann, M. *Trends Endocrinol. Metab.* **26**, 676–687 (2015).
8. Hornbeck, P.V. *et al. Nucleic Acids Res.* **40**, D261–D270 (2012).
9. Ottesen, J.J., Huse, M., Sekedat, M.D. & Muir, T.W. *Biochemistry* **43**, 5698–5706 (2004).
10. Oza, J.P. *et al. Nat. Commun.* **6**, 8168 (2015).
11. Jbara, M., Maity, S.K., Morgan, M., Wolberger, C. & Brik, A. *Angew. Chem. Int. Edn. Engl.* **55**, 4972–4976 (2016).
12. Serwa, R. *et al. Angew. Chem. Int. Edn. Engl.* **48**, 8234–8239 (2009).
13. Chen, Z. & Cole, P.A. *Curr. Opin. Chem. Biol.* **28**, 115–122 (2015).
14. Wang, L., Brock, A., Herberich, B. & Schultz, P.G. *Science* **292**, 498–500 (2001).
15. Xie, J., Supekova, L. & Schultz, P.G. *ACS Chem. Biol.* **2**, 474–478 (2007).
16. Park, H.S. *et al. Science* **333**, 1151–1154 (2011).
17. Pirman, N.L. *et al. Nat. Commun.* **6**, 8130 (2015).
18. George, S. *et al. FEBS Lett.* **590**, 1530–1542 (2016).
19. Fan, C., Ip, K. & Söll, D. *FEBS Lett.* **590**, 3040–3047 (2016).
20. Rogerson, D.T. *et al. Nat. Chem. Biol.* **11**, 496–503 (2015).
21. Chao, H.-G. *et al. J. Org. Chem.* **60**, 7710–7711 (1995).
22. Takimoto, J.K., Dellas, N., Noel, J.P. & Wang, L. *ACS Chem. Biol.* **6**, 733–743 (2011).
23. Hoppmann, C. *et al. Angew. Chem. Int. Edn. Engl.* **53**, 3932–3936 (2014).
24. Lacey, V.K., Louie, G.V., Noel, J.P. & Wang, L. *ChemBioChem* **14**, 2100–2105 (2013).
25. Yau, R. & Rape, M. *Nat. Cell Biol.* **18**, 579–586 (2016).
26. Wauer, T. *et al. EMBO J.* **34**, 307–325 (2015).
27. Chong, R.A. *et al. Proc. Natl. Acad. Sci. USA* **111**, 8434–8439 (2014).

Acknowledgments

We thank M. Kelly (UCSF NMR Core) for help with NMR measurements and helpful discussions. L.W. acknowledges the support of the NIH (R01GM118384).

Author contributions

C.H. conducted experiments and characterized data; A.W. assigned data for NMR; B.Y. prepared UBE2D3; S.L., T.H., and K.M.S. provided helpful discussions; L.W. conceived and directed the project; and C.H. and L.W. wrote the manuscript with inputs from S.L., T.H., and K.M.S.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Any supplementary information, chemical compound information and source data are available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. Correspondence and requests for materials should be addressed to L.W.

ONLINE METHODS

General methods. (*S*)-2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-3-(4(bis(dimethylamino)phosphoryloxy) phenyl)propanoic acid (Fmoc-Tyr(PO(NMe$_2$)$_2$) was obtained from EMD Millipore. All other chemicals were obtained from commercial sources and used without further purification. MS analysis of proteins was performed by JadeBio (San Diego) using methods previously described[28,29]. LC–MS measurement of Uaa **1** was performed on an AGILENT 1200 series LC system in combination with an Advion Expression CMS mass spectrometer. Oligonucleotides and primers were obtained from IDT. His-tag antibody (HRP) was from Invitrogen (MA-1-21315-HRP), Phospho-Tyrosine Mouse mAb (P-Tyr-100) was from Cell Signaling Technology (9411S) and IgG mouse secondary antibodies were from Santa Cruz Biotechnology (sc-2314). Ub-antibody (MAB1510) was from Millipore. All antibodies were used in a dilution 1:2,000. NMR spectra of Uaa **1** were recorded on Bruker 300 MHz. ^{1}H–^{15}N HSQC spectra for ^{15}N-labeled WT and pTyr59 ubiquitin (Ub) were acquired on a Bruker 800 MHz spectrometer and processed with Bruker Topspin. Samples were suspended at a concentration of 100 µM in 20 mM Na-phosphate (pH 6.0) 10% D$_2$O/90% H$_2$O buffer in a total volume of 300 µl. For all experiments, sample temperature was set to 300.7 K. Spectra and assignments were carried out in Collaborative Computational Project for NMR (CcpNMR) analysis by comparison to a standard assigned by HNCACB experiments. Chemical shift differences between WT and pTyr59 were calculated by taking the root-mean square of differences for a given residue.

Cleavage of the phosphoramidate group in Uaa 1. To a solution of **1** (1 mg ml^{-1}) in water was added hydrochloric acid to reach a final concentration of 0.04 M HCl (pH~2). The reaction mixture was incubated at 25 °C and the reaction was followed over time by successive LC–MS measurements at 1 h, 18 h and 36 h. After 36 h, ~95% of pTyr had been formed, confirming that even very low hydrochloric acid concentrations allowed cleavage the phosphoramidate group in **1**.

Selection for MmNpYRS. The library CRIZ previously designed for bulky amino acids was electroporated into DH10βT1-competent cells harboring pREP selection plas[14,30]. Selection was carried out on GMM-L plates containing 12.5 µg ml^{-1} tetracycline, 50 µg ml^{-1} kanamycin, 100 µg ml^{-1} chloramphenicol, and 1 mM Uaa **1** as previously described[23,24]. Single green colonies were picked and re-streaked on LB–tetracycline–kanamycine and on LB–tetracycline–kanamycine–chloramphenicol. One clone (B1) was sensitive to chloramphenicol selection in the absence of the Uaa on the latter (LB–T–K–Cm) plate, but was viable and nonfluorescent on the former (LB–T–K plate). This clone was characterized in detail and named as MmNpYRS.

Genetic encoding of Uaa 1 into proteins. To genetically incorporate Uaa **1** into proteins (calmodulin, myoglobin, GFP, Ub) in *E. coli*, BL21 cells were transformed with corresponding plasmids (pTak-CaM76TAG, pTak-Myo4TAG, pTak-GFP182TAG, or pTak-Ub59TAG with pBK-MmNpYRS). All the pTak plasmids[22] have a His-tag at the C terminus of the target protein. For the E2 charging assay, N-terminal His-tagged Ub was expressed. One colony was picked and grown overnight in 5 ml 2xYT supplemented with 30 µg ml^{-1} chloramphenicol and 50 µg ml^{-1} kanamycin at 37 °C. This starter culture was used to inoculate 100 ml of 2xYT-containing antibiotics. When optical density at 600 nm (OD$_{600}$) reached 0.5, 1 mM of Uaa **1** was added, and cells were induced for protein expression by adding 0.5 µM IPTG. After 16 h, cells were lysed and sonicated in 5 ml lysis buffer (50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole pH 8.0, 1% (v/v) Tween 20, 10% (v/v) glycerol and 0.5 mg ml^{-1} lysozyme). Lysed cells were centrifuged for 30 min at 14,000 *g*, and clarified supernatant was passed through a 0.1-ml column of Ni^{2+}-NTA agarose resin (Qiagen). The column was washed with 10 column volumes of wash buffer (lysis buffer without Tween 20 and lysozyme). Protein was eluted with 400 µl of elution buffer (wash buffer containing 250 mM imidazole, pH 8.0). The sample was concentrated using a Microcon Ultracel YM-10 (CaM, myoglobin, GFP) and YM-3 (Ub) spin column (Millipore) yielding concentrations of 1.0 mg L^{-1} of CaM, 1.25 mg ml^{-1} myoglobin, 1.25 mg ml^{-1} GFP, and 1.75 mg L^{-1} Ub. SDS–PAGE was 15% polyacrylamide, and His-tag or pTyr-specific antibodies were used for immunoblotting with a dilution of 1:2,000.

General procedures for acidic cleavage and formation of pTyr in proteins. To a diluted protein solution (0.1–1.0 mg ml^{-1}) in Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.5) was added HCl (4 M) to reach a final HCl concentration of 0.4 M and a pH ~1–2. Protein solutions of myoglobin, GFP, Ub were incubated at 4 °C for 16 h. Protein solution of CaM was incubated at 4 °C for 48 h. For western blot analysis, the pH of the samples was readjusted to pH 7.5 using NaOH (0.5 M). Subsequently, samples were loaded onto 15% gel for SDS–PAGE, blotted, and visualized using His-tag or pTyr-specific antibodies. Alternatively, HCl was removed by lyophilization of the reaction mixture. For recording MS spectra of CaM, HCl was removed by lyophilization and samples were redissolved in water. In addition, either C-terminally or N-terminally His-tagged Ub were freeze dried after HCl treatment and redissolved in water.

^{15}N labeling and ubiquitin expression (C-terminal His-tagged). To prepare ^{15}N-labeled Ub with pTyr at position 59 in *E. coli*, BL21 cells were transformed with plasmids pTak-Ub59TAG and pBK-MmNpYRS. One colony was picked and grown overnight in 5 ml 2xYT supplemented with 30 µg ml^{-1} chloramphenicol and 50 µg ml^{-1} kanamycin at 37 °C. This starter culture was used to inoculate 1,400 ml of 2xYT containing antibiotics. When OD$_{600}$ reached 0.6, cells were centrifuged for 5 min at 5,000 *g* and resuspended in 700 ml M9 media with antibiotics and 1 mM Uaa **1** and induced for protein expression by adding 0.5 µM IPTG. After 6 h, cells were lysed and sonicated in 10 mL lysis buffer (50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole pH 8.0, 1% (v/v) Tween 20, 10% (v/v) glycerol and 0.5 mg ml^{-1} lysozyme). Lysed cells were centrifuged for 50 min at 14,000*g*, and clarified supernatant was passed through a 1.4-ml column of Ni^{2+}-NTA agarose resin (Qiagen). The column was washed with 10 column volumes of wash buffer (lysis buffer without Tween 20 and lysozyme). Protein was eluted with 1,400 µl of elution buffer (wash buffer containing 250 mM imidazole, pH 8.0). The sample was concentrated using a Microcon Ultracel YM-3 spin column (Millipore). After diluting to a concentration of 0.5 mg ml^{-1} in Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.5), the Ub was treated with HCl (0.4 N) o/n, freeze dried and the buffer was exchanged to phosphate buffer (pH 5.8) yielding 1 mg ml^{-1} labeled Ub pTyr59. Labeled WT Ub was prepared accordingly. In brief, plasmid pTakWTUb was electroporated into BL21 cells and one colony was picked and grown overnight in 5 ml 2xYT supplemented with 30 µg ml^{-1} chloramphenicol at 37 °C. This starter culture was used to inoculate 600 ml of 2xYT containing antibiotic. When OD$_{600}$ reached 0.6, cells were centrifuged for 5 min at 5,000*g* and resuspended in 300 ml M9 media with antibiotic and induced for protein expression by adding 0.5 µM IPTG. After 6 h, cells were lysed and sonicated in 10 ml lysis buffer (50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole pH 8.0, 1% (v/v) Tween 20, 10% (v/v) glycerol and 0.5 mg ml^{-1} lysozyme). Lysed cells were centrifuged for 50 min at 14,000*g*, and clarified supernatant was passed through a 0.6 ml column of Ni^{2+}-NTA agarose resin (Qiagen). The column was washed with 10 column volumes of wash buffer (lysis buffer without Tween 20 and lysozyme). Protein was eluted with 600 µl of elution buffer (wash buffer containing 250 mM imidazole, pH 8.0) yielding 15 mg ml^{-1} WT Ub after buffer was exchanged to phosphate buffer (pH 5.8). As a control, we also treated the WT Ub with hydrochloric acid (0.4 N) for 16 h, freeze dried the sample, redissolved in buffer and measured HSQC NMR.

Ubiquitin expression (N-terminal His-tagged). To genetically incorporate Uaa **1** into ubiquitin in *E. coli*, BL21 cells were transformed with plasmids pTak-Ub59TAG_N-His and pBK-MmNpYRS. One colony was picked and grown overnight in 5 ml 2xYT supplemented with 30 µg ml^{-1} chloramphenicol and 50 µg ml^{-1} kanamycin at 37 °C. This starter culture was used to inoculate 200 ml of 2xYT containing antibiotics. When OD$_{600}$ reached 0.5, 1 mM of Uaa **1** was added, and cells were induced for protein expression by adding 0.5 µM IPTG. After 3 h, cells were lysed and sonicated in 10 ml lysis buffer (50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole pH 8.0, 1% (v/v) Tween 20, 10% (v/v) glycerol and 0.5 mg ml^{-1} lysozyme). Lysed cells were centrifuged for 50 min at 14,000*g*, and clarified supernatant was passed through a 0.2 ml column of Ni^{2+}-NTA agarose resin (Qiagen). The column was washed with 10 column volumes of wash buffer (lysis buffer without Tween 20 and lysozyme). Protein was eluted with 400 µl of elution buffer (wash buffer

doi:10.1038/nchembio.2406

containing 250 mM imidazole, pH 8.0). The sample was concentrated using a Microcon Ultracel YM-3 spin column (Millipore) yielding 1.0 mg L^{-1} of Ub. Buffer was exchanged to Tris-buffer (50 mM Tris, 150 mM NaCl, pH 7.5) before HCl treatment. Trypsin digest and MS/MS sequencing confirmed the pTyr at appropriate site.

E2 charging assay. Human E1 (UbE1) was obtained from Fisher Scientific (part number E304050). UBE2D3 (from Addgene 15784) was prepared by standard cloning procedures with His-tag. Reaction mixtures in Tris-buffer (50 mM Tris, 150 mM NaCl, pH 7.5) contained UBE1 (0.5 µM), UBE2D3 (2 µM), $MgCl_2$ (10 mM), ATP (10 mM) and the pTyrUb (10 µM) or WTUb (10 µM) at a total volume of 40 µl. As negative control, the same reaction was carried out without ATP. The mixture was incubated at 37 °C for 20 h. For SDS–PAGE, to 8 µl of each reaction 2 µl of nonreducing Laemmli buffer (Bio-Rad) was added, loaded onto a 15% SDS gel and stained with Coomassie. For western blot 1 µl of each reaction mixture was diluted with 5 µl Tris and 1.5 µl of nonreducing Laemmli buffer and loaded on 15% SDS gel, transferred to a membrane and visualized with His-tag (HRP) antibody. Subsequently, the membrane was stripped under mild conditions and visualized with anti-Ub (mouse) and IG-mouse antibody in 1:2,000 dilution. In parallel, sample were treated at 100 °C with reducing Laemmli buffer containing 2-mercaptoethanol, loaded onto SDS gel, transferred and immunoblotted with His-tag (HRP) antibody in 1:2,000 dilution. In addition, control experiments were conducted to exclude any interference of the E2 charging assay with acid treatment. WT Ub in Tris-buffer (50 mM Tris, 150 mM NaCl, pH 7.5) was diluted to 0.6 mg ml^{-1} and treated with hydrochloric acid (0.4 N) at 4 °C for 16 h. The sample was freeze fried and reconstituted with water. Western blot HRP antibody shows that the Ub-E2 thioester is formed.

Data availability. All data generated or analyzed during this study are included in this published article (and its supplementary information files).

28. Johnson, D.B. *et al. Nat. Chem. Biol.* **7**, 779–786 (2011).
29. Xiang, Z. *et al. Nat. Methods* **10**, 885–888 (2013).
30. Santoro, S.W., Wang, L., Herberich, B., King, D.S. & Schultz, P.G. *Nat. Biotechnol.* **20**, 1044–1048 (2002).

doi:10.1038/nchembio.2406