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Palladium-Triggered Chemical Rescue of Intracellular Proteins via Genetically Encoded Allene-Caged Tyrosine

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Supporting Information

ABSTRACT: Chemical de-caging has emerged as an attractive strategy for gain-of-function study of proteins via small-molecule reagents. The previously reported chemical de-caging reactions have been largely centered on liberating the side chain of lysine on a given protein. Herein, we developed an allene-based caging moiety and the corresponding palladium de-caging reagents for chemical rescue of tyrosine (Tyr) activity on intracellular proteins. This bioorthogonal de-caging pair has been successfully applied to unmask enzymatic Tyr sites (e.g., Y671 on *Taq* polymerase and Y728 on Anthrax lethal factor) as well as the post-translational Tyr modification site (Y416 on Src kinase) *in vitro* and in living cells. Our strategy provides a general platform for chemical rescue of Tyr-dependent protein activity inside cells.

Bioorthogonal cleavage reactions have been recently developed to rescue various small molecules and biomolecules *in vitro* and *in vivo*.[1−3] In particular, a panel of chemical de-caging strategies has been applied for gain-of-function, as opposed to the traditional loss-of-function, study of various proteins under living conditions.[2] However, as current bioorthogonal rescue strategies are largely centered on liberating the side-chain of lysine residue on a protein of interest, expanding this powerful tool to other amino acid side-chains is highly desired.[2] For example, Tyr is a catalytic residue in many enzymes including β-lactamase, cytochrome oxidase, polymerase, DNA topoisomerase as well as some effector proteins from human pathogens.[4] Tyr is also frequently subject to diverse post-translational modifications such as phosphorylation, sulfation, and nitration.[5] In particular, phosphorylation of Tyr by receptor Tyr kinases (e.g., Src, STAT4, and EGFR) plays crucial roles during signaling transduction.[6] Therefore, a biocompatible chemical de-caging strategy on Tyr would enable us to modulate the function, structure, and/or subcellular localization of proteins containing essential Tyr residue(s) under living conditions.

Herein, we developed an allene-based caging moiety and the corresponding palladium reagents for chemical de-caging of the phenol group under intracellular environment. A Tyr analogue containing an allene moiety was created and site-specifically incorporated into proteins via an evolved pyrrolysine amino acyl tRNA synthetase (PylRS)-tRNA pair. This allowed the blockage of Tyr-dependent protein activity that can be chemically rescued via the Pd-mediated de-allenylation reaction. We demonstrated the general applicability of this allene-Tyr/Pd catalyst de-caging pair for bioorthogonal rescue of essential Tyr activity on enzymes and effector proteins in living cells.

We started by searching for suitable caging moieties and de-caging reagents for Tyr. As demonstrated by several laboratories including ours, Pd-triggered de-caging reactions such as de-propargylation and de-allylation have shown broad applications in living cells, particularly for unmasking an array of chemically caged amine groups on prodrugs, surface glycans as well as the ε-amine on lysine from intact proteins.[1d,g,2a] Extending such Pd-triggered de-caging reactions to unmask the phenol group on Tyr is therefore worth exploring. The proposed mechanism for Pd-mediated depropargylation involves an allenylpalladium complex as the key intermediate, and the isomerization (allenylation) process during the intermediate formation may be the rate-limiting step[2a,7,8] (Figures S1 and S2). We envisioned that the allenyl group might be more sensitive to Pd-triggered de-caging than the propargyl group. To test this hypothesis, three different allene-containing caging moieties were designed: **1a** (1,2-allenyl ether), **1b** (2,3-allenyl ether), and **1c** (allenoate) (Figure 1a). As some allene-containing structures are highly reactive function groups in organic synthesis that may not be stable under intracellular conditions, we first examined the bioorthogonality of these caging moieties. We used the chemically caged fluorogenic coumarin analogues as the reporters to evaluate the ability of different allenes on caging the phenol group. Coumarin analogues with the phenol group caged by **1a**−**1c** were synthesized, and their fluorescence was all found to be efficiently quenched in compare with the de-caged coumarin molecule. Upon mixing of **1c**-caged coumarin with cell lysates, its fluorescence increased significantly, while that of **1a**- and **1b**-caged coumarins remain unchanged (Figure 1b). To further examine the bioorthogonality of these caging moieties, we subjected these coumarin analogues to LC-MS analysis before and after incubation with cell lysates. The absolute content of **1c**-caged coumarin was decreased after treatment with cell lysates, and a new product was formed which was later verified as the de-

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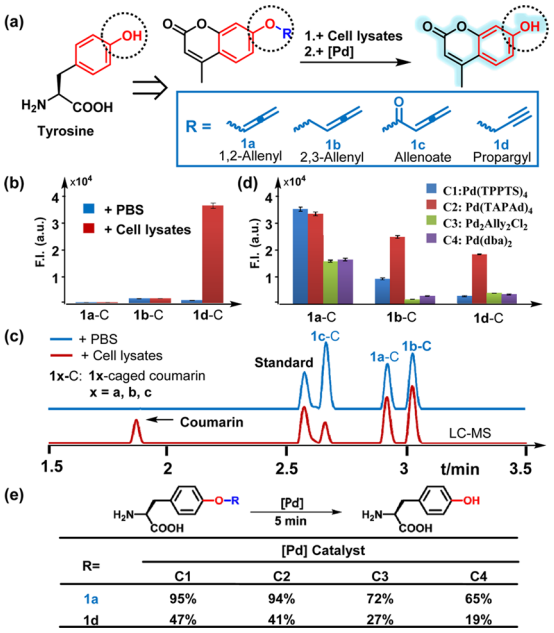

Figure 1. Development of an allene-based moiety and the corresponding palladium reagents as a chemical de-caging pair for Tyr. (a) A coumarin-based assay for examining the bioorthogonality and de-caging activity of different allene-based caging moieties for chemical rescue of Tyr. (b,c) Evaluating the bioorthogonality of caging moieties **1a**–**1c** by the fluorogenic assay (b) and LC-MS analysis (c). **1a**-C, **1b**-C, **1c**-C = **1a**-, **1b**-, **1c**-caged coumarin, respectively. (d) Evaluating the de-caging efficiency of **1a** and **1b** by different Pd reagents with **1d** as a control. TPPTS: tris(3-sulfophenyl) phosphine trisodium salt; TAPAd: 1,3,5-triaza-7-phosphaadamantane, $Pd_2Allyl_2Cl_2$: allylpalladium(II) chloride dimer; dba = dibenzylideneacetone. (e) The efficiency of different Pd reagents for unmasking the **1a**-caged Tyr as measured by LC-MS. **1d**-caged Tyr was used for comparison. All reactions were conducted with 100 μM compounds and Pd reagents in PBS buffer (20 mM, pH = 7.8, 5% DMSO) at 37 °C for 5 min. Error bars in (b) and (d) represent standard deviation from three replicates.

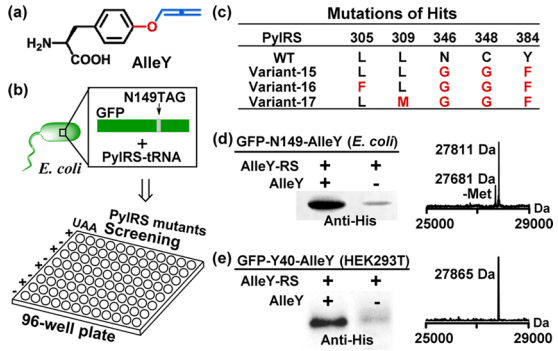

Figure 2. Developing a PylRS variant–tRNA pair to genetically encode AlleY in bacterial and mammalian cells. (a) Structure of **1a**-caged Tyr (AlleY). (b) GFP-based live cell screening assay for identifying AlleY-recognition PylRS variants. (c) Mutation sites on the identified AlleY-recognition PylRS variants. (d) Site-specific incorporation of AlleY into the GFP model protein in *E. coli* as verified by immunoblotting analysis and LC-MS. Expected MW of GFP-N149-AlleY, 27 810 Da; measured MW, 27 811 Da (major peak) and 27 681 Da (minor peak, with the N-Met cleaved). (e) Verification of the site-specific incorporation of AlleY into the GFP model protein in mammalian cells. Expected MW of GFP-Y40-AlleY, 27 864 Da; measured MW, 27 865 Da.

caged coumarin (Figure 1c). In contrast, the absolute content of **1a** and **1b** remained unchanged after treatment with cell lysates. The subsequent analysis indicated that de-caging of **1c**-caged coumarin was mainly caused by thiol-containing molecules such as cysteine in cell lysates (Figures S3 and S4). Therefore, we excluded **1c** and focused on the caging moieties **1a** and **1b** for further study.

The **1a**- and **1b**-caged coumarin analogues were next subject to Pd catalysts (**C1**–**C4**) with the propargyl-based caging moiety **1d** as a control (Figure 1d). The increase of fluorescent signal for each sample was monitored to compare the corresponding de-caging efficiency (Figures 1d and S5). Interestingly, **1a**-caged coumarin exhibited the fastest de-caging rate and the best de-caging efficiency compared to **1b**- and **1d**-caged coumarin with all four Pd reagents. Furthermore, we synthesized **1a**- and **1d**-caged Tyr analogues (named as AlleY and ProY respectively) and evaluated their de-caging efficiency in the presence of **C1**–**C4** by LC-MS (Figure 1e). Again, AlleY exhibited significantly higher de-caging efficiency than that of ProY by all four Pd reagents. Together, we demonstrated **1a**-caged Tyr (AlleY) and Pd reagents as an efficient de-caging pair for chemical rescue of Tyr.

Next, we genetically incorporated AlleY into proteins via the pyrrolysine-based genetic code expansion system, which has become a one-stop shop for encoding unnatural amino acids in diverse prokaryotic and eukaryotic species (Figure 2a).[9,10] Because the active-site pocket of PylRS has been previously shown to recognize an array of Tyr and Phe analogues,[11] we decided to create a rational designed library instead of the random mutagenesis to select the appropriate PylRS variant for AlleY incorporation. According to the co-crystal structure of *Methanosarcina mazei* PylRS (MmPylRS) mutant in complex with O-methyl Tyr (PDB: 3QTC, Figure S6), we designed 76 active-site mutants and combined them with 20 previously reported functional mutants to generate a set of 96 PylRS variants (all sequences are shown in Figure S7).[12] Each member of this 96-mutant library was co-transformed with a plasmid encoding the green fluorescent protein (GFP) bearing an in-frame amber mutation at residue N149 (GFP-N149-TAG) into *E. coli* DH10B cells and cultured in a 96-well plate with and without 1 mM AlleY (Figure 2b). The fluorescence of bacteria pellet from each well was recorded *E. coli* cells harboring PylRS variants 15, 16, and 17 were all found to give high fluorescence only in the presence of AlleY (Figure S8). In particular, variant-15 (N346G, C348G, Y384F) showed the highest incorporation efficiency among all these potential hits and was renamed as AlleY-PylRS (Figure 2c). Immunoblotting and MS analysis further confirmed the specificity and fidelity of AlleY incorporation into GFP-N149-TAG (Figures 2d and S9). The yield of the resulting GFP-N149-AlleY protein can reach 19 mg/L *E. coli* cells (Figure S10). The incorporation of AlleY was next demonstrated in mammalian cells by using GFP as the model protein. AlleY was efficiently incorporated into GFP-Y40-TAG in HEK293T cells to produce GFP-Y40-AlleY, which was verified by both immunoblotting and MS analysis (Figures 2e and S9). Finally, as AlleY and ProY have the same molecular weight (MW) that cannot be distinguished by MS, we further used the copper(I)-catalyzed alkyne–azide cycloaddition (CuAAC)-mediated labeling reaction to confirm that the incorporated AlleY was not converted to ProY after protein expression. Indeed, whereas the ProY-bearing GFP variant can be fluorescent labeled by Cy3-N_3 and Cu (I) due to the presence of the alkyne group, the AlleY-bearing GFP variant was not labeled under the same condition (Figure S11). Together, we successfully obtained

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the PylRS variant-tRNA pair to genetically encode AlleY in both bacterial and mammalian cells.

Pd-mediated de-allenylation reaction was then performed on AlleY-bearing proteins *in vitro* and in live cells. Purified GFP-N149-AlleY protein was incubated with Pd catalysts **C1**−**C4** for 5 min at 37 °C before the de-caging efficiency being measured by LC-MS. A 39 Da shift of the main peak was observed (calculated shift: 38 Da) after Pd-treatment, which corresponds to the removal of the allenyl group on GFP-N149-AlleY (Figure S12). Notably, consistent with the aforementioned de-allenylation results on AlleY, the de-protection reaction on the allenyl-caged group was also more efficient than that of the propargyl-caged group on intact proteins (Figure 3a). **C2** was found as the most

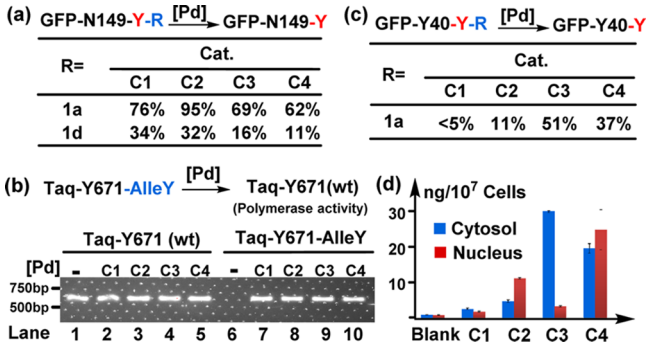

Figure 3. Pd-mediated chemical de-caging of AlleY-bearing proteins *in vitro* and in living cells. (a) The efficiency of different Pd reagents in de-caging the purified AlleY-bearing GFP protein as measured by LC-MS. ProY was used for comparison. The reaction was conducted in PBS buffer (20 mM, pH = 7.8, 5% DMSO) with 20 μM GFP and 100 μM Pd reagents for 5 min at 37 °C (b) Pd-mediated rescue of Taq enzyme activity. AlleY was incorporated at the active site (Y671) of Taq to block its polymerase activity (lane 6), which can be chemically rescued by Pd-reagents **C1**−**C4** (lane 7−10). Volume of reaction: 50 μL. [Taq enzyme] = 10 ng/μL; [DNTP] = 400 μM; [Primer-F] = 400 nM; [Primer-R] = 400 nM; [Template] = 0.2 ng/μL; [Pd] = 10 μM. (c) The efficiency of Pd-triggered Tyr de-caging on GFP protein in living cells. Cells were treated with Pd reagents (20 μM) in fresh DMEM for 2 h, and the de-caged products were purified and then subjected to LC-MS analysis. MS analyses of each sample were shown in Figures S14 and S15. (d) Cellular uptake and distribution of Pd reagents **C1**−**C4** as measured by ICP-MS analysis. Error bars represent the standard from three replicate experiments.

efficient de-caging reagent *in vitro* (95% de-caging yield) followed by **C1**, **C3**, and **C4**, with the de-caging yields between 60% and 80%. To further demonstrate our de-caging reaction on a protein other than GFP, we turned to *Taq* DNA polymerase (Figure S13). Previous study showed that Y671 was essential for the polymerase activity of Taq by wedging the dNTP and primer in the optimal positions.[4d,e,13] When AlleY was site-specifically incorporated at residue Y671, the generated Taq variant (Taq-Y671-AlleY) exhibited no polymerase activity as measured by polymerase chain reaction (PCR, Figure 3b, lane 6). To our delight, the addition of 10 μM Pd reagents (**C1**−**C4**) successfully rescued the polymerase activity (Figure 3b, lanes 7−10).

We next conducted the Pd-mediated de-allenylation on proteins within living cells. As catalysts **C1**−**C4** have all been previously utilized for intracellular protein de-caging with excellent biocompatibility, we directly applied these Pd catalysts for chemical rescue of Tyr.[1h] After expressing GFP-Y40-AlleY in HEK293T cells for 24 h at 37 °C followed by incubation with 20 μM **C1**−**C4** in fresh Dulbecco's modified eagle medium (DMEM) for another 2 h, the protein was purified and subjected to LC-MS analysis (Figure 3c). **C3** was found as the most efficient de-caging reagent under living conditions (51% de-caging yield), followed by **C4** (37%), **C2** (11%), and **C1** (<5%) (Figure S12). The discrepancy between the *in vitro* and *in vivo* de-caging efficiency was likely due to the different cellular uptake levels of these Pd catalysts. Indeed, our ICP-MS analysis showed that **C3** and **C4** have a higher cellular uptake efficiency than C1 and C2, which correlated with their higher de-caging activity inside cells (Figure 3d).

Finally, we applied our AlleY-Pd de-caging pair for gain-of-function study of proteins in living cells. We first demonstrated the chemical rescue of a Tyr phosphorylation site that plays essential roles in cell signal transduction. The oncogenic variant (Y527F) of Src kinase was first used as an example.[2b,14] This constitutive active form of Src auto-phosphorylates its Y416 residue and induces the hyperactive Tyr phosphorylation within the cell which is highly relevant to cancer. As the function of the constitutive active Src kinase also depends on the auto-phosphorylation at Y416, we replaced this essential Tyr by AlleY to block its phosphorylation. Cells expressing this caged Src variant (Src-Y527F-Y416-AlleY) were treated with 20 μM **C3** or **C4** for 3 h before being analyzed this protein by immunoblotting. As expected, the auto-phosphorylation at Y416 site was successfully rescued (Figure 4a, lanes 4 and 5).

We then demonstrated the chemical rescue of a Tyr-dependent enzyme inside live cells. The anthrax lethal factor (LF) was used for proof-of-concept, which is the key toxin effector from the notoriously known pathogen *Bacillus anthracis*.[15] After translocation into host cells upon infection, LF will cleave the N terminal amino acids of the MEK family

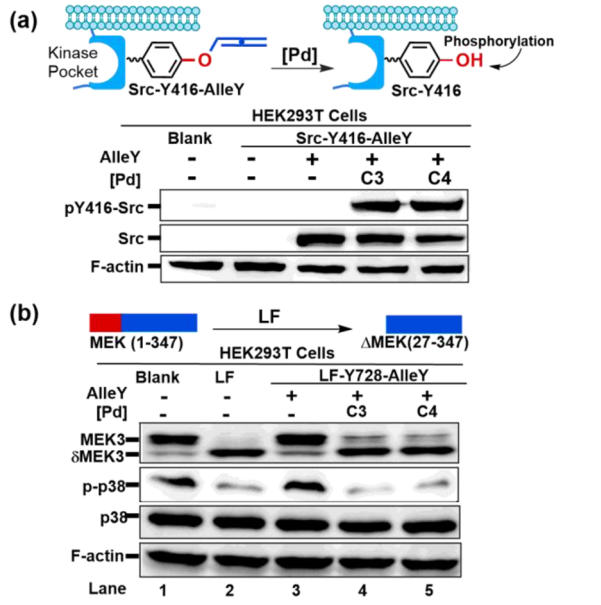

Figure 4. Pd-mediated chemical de-caging of Tyr-dependent enzymes in living cells. (a) Pd-mediated chemical de-caging of Y416 from Src-Y416-AlleY under living conditions. After expression and the de-caging reaction, the oncogenic Src variant was purified by Ni-NTA resin, and the subsequent self-phosphorylation activity on the rescued Y416 residue was examined by immunoblotting analysis. F-actin was used as the control. (b) Pd-mediated chemical de-caging of Y728 from LF-Y728-AlleY in living cells. The rescued LF protease activity was evaluated by the N-terminal cleavage of MEK3 as well as the decrease of phosphorylation level on p38 (downstream of MEK3).

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kinases which are all essential components in cell signaling transduction.[15] Y782 is a key residue near LF's catalytic triad that coordinates a water molecule to ensure efficient protease activity (Figure 4b, Figure S16).[4g,h] When replacing Y728 by AlleY, the resulting LF variant (LF-Y728-AlleY) exhibited no protease activity on MEK3 as opposed to wild-type LF (Figure 4b, lanes 2 and 3). To our delight, after incubation with 20 μM **C3** or **C4** for 3 h, the protease activity on MEK3 was efficiently restored (Figure 4b, lanes 4 and 5). To further verify the rescued activity from LF-Y728-AlleY, we examined the phosphorylation level of kinase p38 that is a downstream substrate of MEK3. Indeed, the phosphorylation of p38 was significantly decreased after our chemical rescue of LF that cleaves MEK3 (Figure 4b, lanes 4 and 5). These results all indicated that the protease activity of LF-Y728-AlleY can be chemically restored by Pd reagents.

In summary, we developed an allene-based caging moiety and Pd catalysts as a bioorthogonal de-caging pair for activation of Tyr-dependent proteins in live cells. The allene moiety served as a bioorthogonal caging group to mask the essential Tyr residue from an intact protein, while the Pd catalysts offer an effective approach to unmask this chemically caged Tyr analogue under living conditions. To our knowledge, this method for the first time demonstrated the small-molecule-based chemical de-caging strategy on a Tyr residue, which allows the gain-of-function study of Tyr-dependent protein activity in living cells. This de-allenylation chemistry may be generally applicable for rescue of diverse small molecules as well as biomolecules other than proteins that contain essential phenol groups. Furthermore, as allene is a highly useful functional group in organic synthesis, our study added a new and valuable bioorthogonol handle to the rapidly growing bioorthogonal chemistry toolkit.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b08933.

General considerations, supplementary methods, Figures S1−S16, protein sequences, and spectra (PDF)

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Notes

The authors declare no competing financial interest.

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