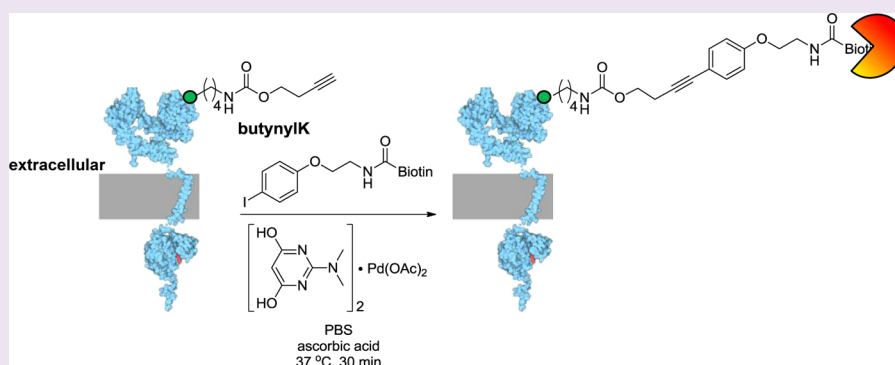# A Genetically Encoded Alkyne Directs Palladium-Mediated Protein Labeling on Live Mammalian Cell Surface

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S *Supporting Information*

ABSTRACT: The merging of site-specific incorporation of small bioorthogonal functional groups into proteins via amber codon suppression with bioorthogonal chemistry has created exciting opportunities to extend the power of organic reactions to living systems. Here we show that a new alkyne amino acid can be site-selectively incorporated into mammalian proteins via a known orthogonal pyrrolysyl-tRNA synthetase/tRNA$_{CUA}$ pair and directs an unprecedented, palladium-mediated cross-coupling reaction-driven protein labeling on live mammalian cell surface. A comparison study with the alkyne-encoded proteins *in vitro* indicated that this terminal alkyne is better suited for the palladium-mediated cross-coupling reaction than the copper-catalyzed click chemistry.

Complementary to direct fusion of fluorescent proteins, genetic encoding of unique functional groups[1] followed by their selective functionalization via bioorthogonal reactions[2] provides a powerful strategy to label proteins in their native environment. To increase the labeling efficiency in this two-step procedure, two strategies have been successfully developed: one involves genetic encoding of highly reactive, yet biocompatible, substrates based on the notion of substrate distortion/activation;[3,4] the other employs transition metal catalysts to accelerate the reactions.[5] Recent examples in the former include genetic encoding of the keto/aldehyde functionality via modification of the N-terminus of a protein,[6] and strained alkenes and alkynes such as cyclopropene,[7,8] spirohexene,[9] norbornene,[10−12] cyclooctyne,[13] trans-cyclooctene,[14,15] and bicyclononyne,[16,17] as well as latent reactive dienes and dipoles such as tetrazine[18] and tetrazole[19] for fast cycloaddition reactions. The latter includes the use of copper catalysts to dramatically accelerate azide−alkyne cycloaddition[20−22] and the use of biocompatible palladium catalysts for in-cell cross-coupling reactions.[23−27] Because there is no substrate preactivation involved, the labeling yields of the transition metal-catalyzed reactions depend critically on the labeling reagents and catalysts. In view of a growing number of hydrophilic ligands suitable for aqueous homogeneous catalysis[28] and rapid advance in designing biocompatible nanoparticle-based transition metal catalysts,[29,30] the transition

metal-based approach has become very attractive as it promises to bring a rich array of metal-catalyzed reactions to direct applications in biological systems.

We became interested in the use of palladium-mediated cross-coupling reactions for protein labeling in living cells because: (i) palladium is an exogenous metal absent from all known native living systems, (ii) palladium-mediated cross-coupling reactions are rich and show excellent functional group selectivity,[31] (iii) the palladium−ligand complexes can be cell permeable with low cytotoxicity,[32,33] and (iv) palladium-based reactions are potentially orthogonal to other bioorthogonal reactions, e.g., tetrazine ligation, so that simultaneous multi-target labeling can be performed.[34] Recently, we reported a copper-free Sonogashira cross-coupling reaction based on the 2-dimethylamino-4,6-dihydroxypyrimidine−palladium(II) complex that enabled the selective functionalization of proteins carrying metabolically incorporated homopropargylglycine (HPG) both in an aqueous medium and in *E. coli*.[25] To extend this Pd-mediated cross-coupling reaction to a mammalian system, herein we report the genetic encoding of an alkyne amino acid, N^ε-butynyloxycarbonyllysine (buty-

Scheme 1. Comparison of the Reactivity of Two Dipeptide Alkyne Substrates in Sonogashira Cross-Coupling Reaction and Click Chemistry
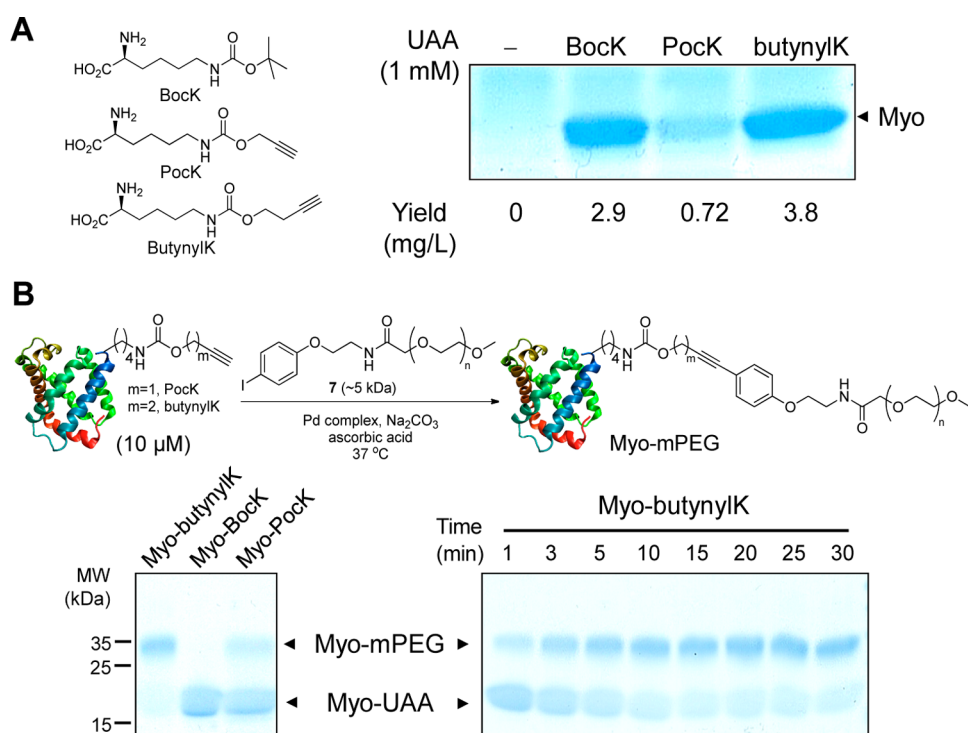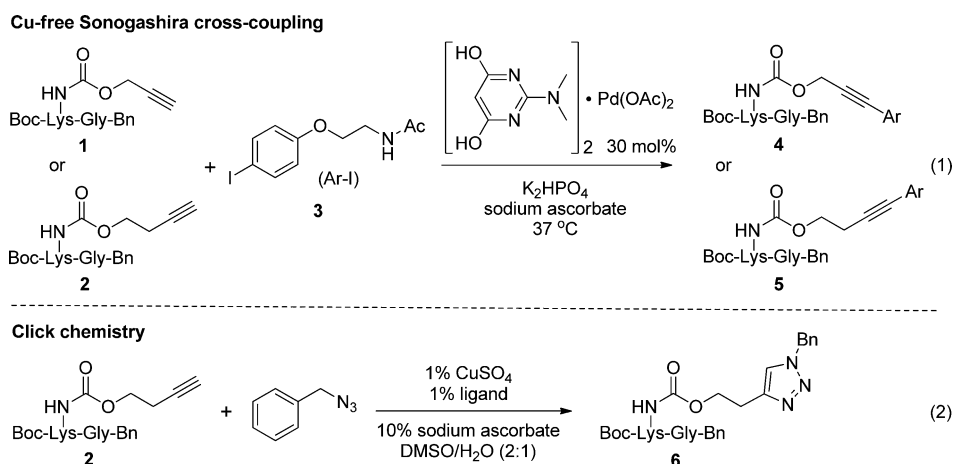

Figure 1. Genetically encoded alkyne amino acids direct selective PEGylation via Pd-mediated cross-coupling reaction. (A) Structures of the unnatural amino acids used in the study and SDS-PAGE of the purified myoglobin mutants incorporating each of the three UAAs. (B) Selective PEGylation of the alkyne-encoded myoglobin in vitro. For cross-coupling reactions, 10 μM of proteins were treated with 1 mM of Pd−7 complex at 37 °C for 30 min before addition of 0.5% 3-mercaptopropanoic acid and subsequent SDS-PAGE analysis (left). Time-course of PEGylation of the butynylK-encoded myoglobin is shown on the right.

nylK),[35] with a known orthogonal pyrrolysyl-tRNA synthetase/tRNA$_{CUA}$ pair, and its robust reactivity in directing Pd-mediated protein labeling in cell lysates, inside *E. coli*, and on live mammalian cell surfaces. A comparison study revealed that the Pd-mediated cross-coupling reaction exhibited higher efficiency than the Cu-catalyzed click chemistry in functionalizing the butynylK-encoded proteins *in vitro*.

Since genetic incorporation of N^{ε}-propargyloxycarbonylly-sine (PocK) has been reported in the literature,[36] our initial goal was to examine whether PocK could serve as a good substrate in the Pd-mediated Sonogashira cross-coupling reaction. To this end, we prepared a PocK-containing dipeptide **1**, along with a butynylK-containing dipeptide **2** expected to

exhibit similar reactivity as HPG—a methionine surrogate we used previously—[25] and tested their reactivity toward aryl iodide **3** (Scheme 1, eq 1). HPLC traces showed that compared to dipeptide **1**, dipeptide **2** gave a cleaner reaction with essentially quantitative conversion of aryl iodide when 2.4 equiv of **2** was used (Figure S1 in SI), indicating that butynylK is a better substrate for the Pd-mediated cross-coupling reaction. To examine how butynylK performs in Cu-catalyzed click chemistry, dipeptide **2** was reacted with benzyl azide using either L-histidine[37] or bathophenanthrolinedisulfonate disodium salt (BPS)[38] as the ligand by following a published procedure (Scheme 1, eq 2).[36] Unexpectedly, the reactions gave low yields of 46% and 73%, respectively, after 24 h (Figure S2),
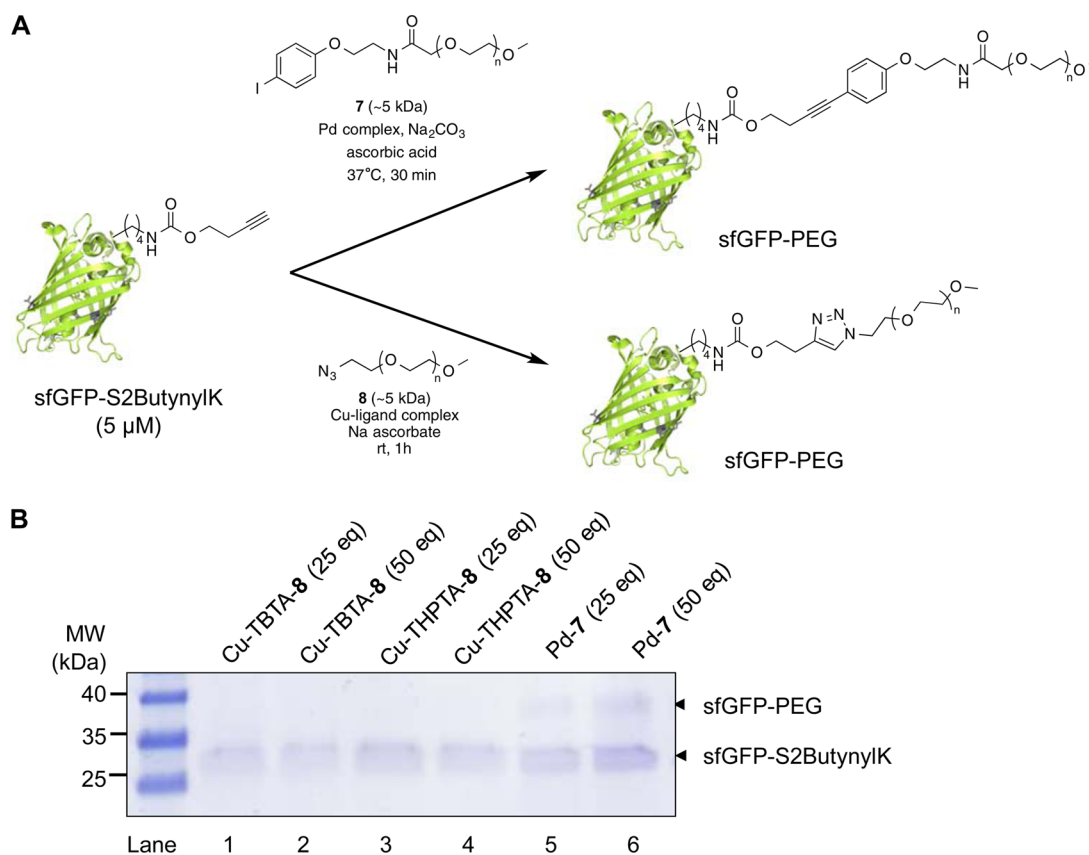
Figure 2. Selective PEGylation of butynylK-encoded sfGFP via Pd-mediated cross-coupling or Cu-catalyzed click chemistry. (A) Reaction scheme. (B) SDS-PAGE analysis of reaction mixtures showing PEGylation-induced gel shift. For click chemistry, 125 μM (25 equiv) or 250 μM (50 equiv) of azide **8**/Cu−ligand complex was used in the reaction; for Pd chemistry, 125 μM (25 equiv) or 250 μM (50 equiv) of the Pd−**7** complex was used in the reaction.

indicating that butynylK is not a good substrate for the click chemistry.

Given that butynylK is structurally analogous to PocK, we decided to examine whether the PocK-specific *M. barkeri* pyrrolysyl-tRNA synthetase (*Mb*PylRS)/*Mb*tRNA$_{CUA}$ pair is promiscuous enough to incorporate butynylK into proteins as well. To this end, we transformed *E. coli* DH10B cells with *p*BK-PylS plasmid, which encodes *Mb*PylRS, together with *p*Myo-4TAG-PylT-TEV-His6 plasmid, which encodes *Mb*tRNA$_{CUA}$ and C-terminally hexahistidine-tagged myoglobin with an amber codon at position 4. The transformed cells were allowed to grow in LB medium containing 50 μg/mL of kanamycin, 15 μg/mL of tetracycline, and 1 mM butynylK to OD$_{600}$ 0.6−0.8 before induction of protein expression with the addition of arabinose to a final concentration of 0.2%. For comparison, protein expression was also carried out in the presence of 1 mM N^ε-*tert*-butyloxycarbonyllysine (BocK) or PocK. The cells were harvested, and the myoglobin proteins were purified by Ni-NTA affinity chromatography. The identities of all three unnatural amino acid (UAA)-containing myoglobin proteins were confirmed by mass spectrometry (Figures S3−S5). The incorporation of UAA by the *Mb*PylRS/*Mb*tRNA$_{CUA}$ pair is specific as removing UAA abolished the myoglobin expression (Figure 1A). Among the three UAAs at 1 mM concentration, butynylK afforded the highest expression yield (3.8 mg/L, Figure 1A), presumably due to its large surface area and enhanced substrate properties. To examine whether the alkyne-containing proteins can serve as efficient substrates for Pd-mediated Cu-free Sonogashira cross-coupling, we treated

the butynylK- and PocK-encoded myoglobin with 100 equiv of the palladium−mPEG-substituted phenyl iodide (**7**, ~5 kDa) complex at 37 °C for 30 min and found that the PEGylation reaction proceeded more efficiently with butynylK-encoded myoglobin (73% yield based on gel shift assay in Figure 1B)[39] than with the PocK-encoded one (30% yield). Importantly, Pd-mediated PEGylation is highly selective as the BocK-encoded myoglobin did not produce any adducts under identical conditions (Figure 1B). A time-dependent PEGylation of Myo-butynylK was observed, with a second-order rate constant determined to be 5.2 M^{-1} s^{-1} (Figure 1B and Figure S7), comparable to that of Cu-catalyzed click chemistry with small-molecule substrates when BPS was used as a ligand (k_2 = 5.3− 38 M^{-1} s^{-1} at pH 8.0 depending on ligand concentration).[38]

Since Cu-catalyzed click chemistry has been widely employed in functionalizing alkyne-containing proteins both *in vitro* and *in vivo*,[22,36,40,41] we compared the efficiency of Cu-catalyzed click chemistry to that of the Pd-mediated cross-coupling reaction involving the butynylK-encoded proteins. Myo-butynylK was treated with either mPEG-azide **8** in the presence of the Cu−His or Cu−BPS complex[21] or the Pd−**7** complex (Figure S8A). Remarkably, PEGylation was observed only for the Pd-mediated reaction, but not for the click chemistry (Figure S8B). When 25 and 50 equiv of the Pd−**7** complex were used, the yields were calculated to be 58% and 60%, respectively (lanes 5 and 6 in Figure S8B). The results are consistent with our small-molecule studies in which butynylK showed higher reactivity in the Pd-mediated reaction than in click chemistry (Scheme 1). To further assess the reactivity of
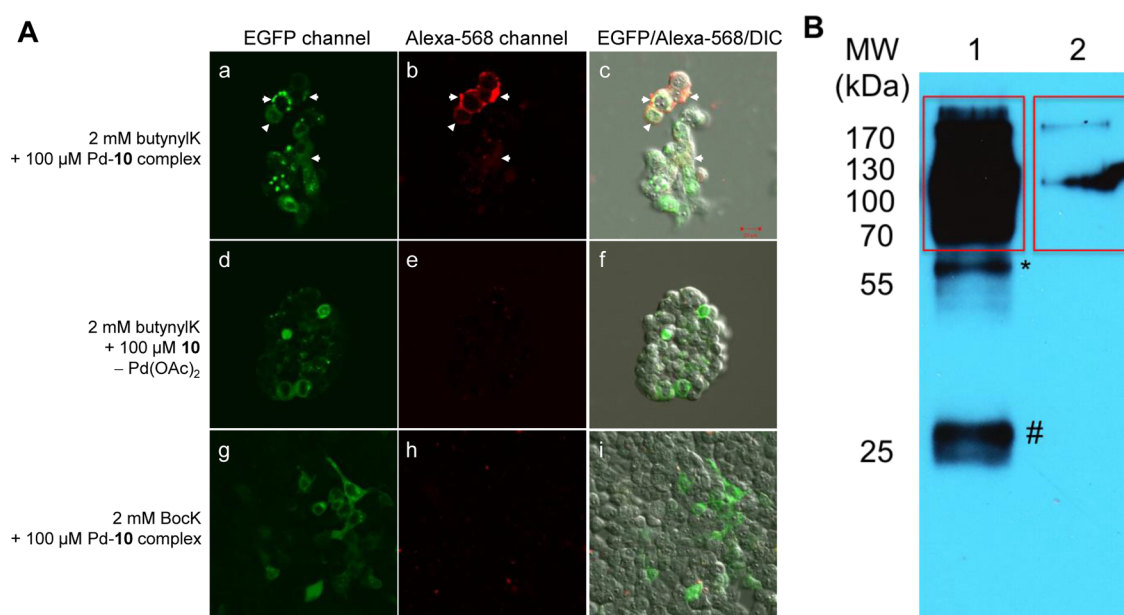
Figure 3. Selective labeling of butynylK-encoded EGFR−EGFP on cell surface by palladium-mediated cross-coupling reaction. (A) Confocal micrographs. For panels a−c, the transfected cells were cultured in the presence of 2 mM butynylK and treated with 100 μM of the Pd−**10** complex in PBS buffer for 30 min; for panels d−f, the transfected cells were cultured in the presence of 2 mM butynylK and treated with 100 μM of biotin-phenyl iodide **10** in PBS buffer alone for 30 min without Pd(OAc)$_2$; for panels g−i, the transfected cells were cultured in the presence of 2 mM BocK and treated with the Pd−**10** complex in PBS buffer for 30 min. Scale bar (shown in panel c) = 20 μm. (B) Immunoblotting analysis for determining the labeling yield on mammalian cell surface. Lane 1 = unreacted EGFR(128butynylK)-EGFP, Lane 2 = biotinylated EGFR(128TAG)-EGFP. HEK293 cells expressing EGFR(128butynylK)-EGFP were treated with 50 μM of biotin-phenyl iodide **10** in PBS buffer for 30 min at 37 °C before cell lysis. Labeling yield was estimated by densitometry comparing the signal intensity of lane 1 vs lane 2 (boxed area) using ImageJ software. * and # denote the heavy and light chains of the anti-EGFR polyclonal antibody, respectively.

butynylK-encoded proteins in Cu-catalyzed click chemistry, sfGFP containing butynylK at position 2 (sfGFP-S2ButynylK) was subjected to Cu-catalyzed click reaction with mPEG-azide **8** in the presence of the second-generation ligands, TBTA and THPTA[42] (Figure 2A). We found that 25 and 50 equiv of Cu-TBTA-**8** and Cu-THPTA-**8** did not afford the desired PEGylated products whereas the use of similar amounts of Pd-**7** gave 17% and 21% yield for 25 and 50 equiv, respectively (Figure 2B). However, increasing the Cu-TBTA-**8** complex to 500 equiv led to the formation of the PEGylated product in 39% yield (Figure S9B), considerably lower than Pd-**7** (68% yield) when used at the same amount (Figure S10B). On the other hand, increasing the amount of Cu-THPTA-**8** complex did not lead to significant product formation (Figure S11B).

To assess whether butynylK can direct Pd-mediated protein labeling inside *E. coli* cells, we treated Myo-butynylK or Myo-BocK-expressing DH10B cells with the Pd−fluorescein iodide complex and followed the reactions by SDS-PAGE and in-gel fluorescence. For comparison, we also subjected the purified proteins and the bacterial cell lysates to the same Pd-mediated reaction. Strong fluorescent bands were observed only for Myo-butynylK but not for Myo-BocK, in all three contexts (Figure S12), indicating that the Pd-mediated cross-coupling reaction is highly selective, tolerant of native biological functionalities, and suitable for whole-cell applications. The yield of the fluorescent labeling was determined to be 67% based on LC-MS analysis (Figure S13).

One of the limiting factors preventing wider use of Cu-catalyzed click chemistry is the toxicity of copper(I) salt toward mammalian cells.[37,43,44] Given the low toxicity of the palladium complexes toward *E. coli* cells,[25−27] we sought to determine the cytotoxicity of our arylpalladium complexes toward mammalian

cells. Thus, we treated human embryonic kidney (HEK) 293 cells with biotin-phenyl iodide (**10**), Pd(OAc)$_2$, 2-dimethyla-mino-4,6-dihydroxy-pyrimidine (DADHP) ligand, and the palladium−**10** complex for 3 h and determined their cytotoxicity using MTT assay. All the reagents showed essentially no cytotoxicity at concentrations $\leq$200 μM; however, at 400 μM, both biotin-phenyl iodide (**10**) and Pd−**10** complex showed considerable toxicity (Figure S14A). Much lower levels of cytotoxicity were observed, even at 400 μM of the reagents, with the Chinese hamster ovary (CHO) cells (Figure S14B) and HeLa cells (Figure S14C). A potential drawback about the Pd−aryl reagent is that the palladium may coordinate with cysteine, necessitating the use of an excess amount of the reagent in functionalizing the cysteine-containing proteins. However, no residual cytotoxicity was detected after washing away Pd−**10** complex and continuing the culture in a fresh medium (Figure S15).

To examine whether butynylK can direct Pd-mediated protein labeling in mammalian cells, we cotransfected HEK293 cells with *pMmPylRS*-EGFR(128TAG)-EGFP-HA plasmid (which encodes a C-terminally HA-tagged epidermal growth factor receptor (EGFR)−EGFP fusion protein with an amber codon at position 128 in the extracellular loop of EGFR and an *M. mazei* pyrrolysyl-tRNA synthetase)[10] and *p4CMVE*-U6-PylT plasmid (which encodes four copies of *M. mazei* tRNA$_{CUA}$). The transfected HEK293 cells were treated with 100 μM of the Pd−biotin-phenyl iodide **10** complexes for 30 min, and excess reagents were then removed with PBS washing. The biotinylation of EGFR−EGFP on the cell surface was detected by treating the cells with streptavidin−Alexa-568 for 20 min followed by washing with PBS before confocal laser-scanning microscopy. In the EGFP channel (ex 488 nm, em

493−574 nm), fluorescent cells were detected only when butynylK or BocK was present in the culture medium (Figure 3A, panels a, d, and g). In the Alexa-568 channel (ex 580 nm, em 585−712 nm), only the Pd−**10** complex-treated HEK293 cells expressing the butynylK-encoded EGFR-EGFP showed red fluorescence (compare panel b to e and h in Figure 3A). The fluorescence images were acquired in two separate channels with a single laser source exciting one wavelength at a time to avoid any possible fluorescence leakage to the unintended channel. As seen in the overlaid images, the red fluorescent cells coincided with cells that showed green fluorescence (indicated by white arrows in panels a−c), suggesting that the labeling reaction was indeed directed by butynylK. However, not all green fluorescent cells were labeled, indicating variability in reagent penetration into the clustered HEK293 cells. Similar results were obtained when only 50 μM of the Pd−**10** complex was used in the labeling reaction (Figure S16). It is noteworthy that our repeated attempts to label the PocK-encoded EGFR−EGFP protein on the cell surface under similar conditions were not successful, highlighting the necessity of using butynylK with enhanced reactivity in the palladium-mediated cross-coupling reaction.

To determine the labeling efficiency of the Pd-mediated cross-coupling on mammalian cell surface, HEK293 cells were cotransfected with *p*cDNA3-EGFR(128TAG)-EGFP and *p*CMV6-MmPylRS-U6-tRNA (which encodes an *M. mazei* pyrrolysyl-tRNA synthetase and *M. mazei* tRNA$_{CUA}$). The transfected HEK293 cells were treated with 50 μM of the Pd−**10** complex for 30 min at 37 °C and then washed with PBS to remove excess reagents. The biotinylated EGFR-(128butynylK)−EGFP protein was captured with immobilized streptavidin and detected by anti-EGFR polyclonal antibody. The signal intensity was compared to the immunoprecipitated unreacted EGFR(128butynylK)−EGFP on the same Western blot, giving an estimated labeling yield of ~12% (Figure 3B).

In summary, we have demonstrated the genetic incorporation of an alkyne amino acid, butynylK, which allows selective palladium-mediated protein labeling *in vitro*, inside *E. coli* cells, and on live mammalian cell surface. To our knowledge, this is the first example of applying palladium chemistry to protein labeling in a mammalian system. This bioorthogonal palladium-mediated cross-coupling reaction should allow us to study specific biological events, e.g., EGFR activation and trafficking,[45] in mammalian systems.

■ EXPERIMENTAL SECTION

Confocal Microscopy Study of the Labeling of the ButynylK-Encoded EGFR-EGFP in HEK293 Cells via Pd-Mediated Cross-Coupling. HEK293 cells were cultured on 35 mm tissue culture plates in 2 mL of DMEM medium supplemented with 10% FBS and allowed to grow to 60−70% confluency in a humidified 37 °C, 5% CO2 incubator. The cells were then cotransfected with two plasmids, *p*MmPylRS-EGFR(128TAG)-EGFP-HA and *p*4CMVE-U6-PylT using the Lipofectamine 2000 transfection reagent (Life Technologies) in DMEM supplemented with 0.1% FBS and 2 mM butynylK or BocK. After transfection, cells were allowed to grow for 16 h and then washed once with PBS. Following this, the membrane-bound EGFR−EGFP fusion protein was labeled with the freshly prepared palladium−**10** complex (final concentration = 50 μM or 100 μM) or biotin-phenyl iodide **10** only (final concentration = 100 μM) in PBS for 30 min. Afterward, cells were washed twice with PBS and treated with streptavidin-conjugated AlexaFluor 568 (500× dilution) for another 20 min. The treated cells were again washed with PBS (2×) before microscopy. Image acquisition was carried out using a Zeiss LSM 710 laser scanning microscope equipped with an EC Plan-Neofluar 20×/

0.80 M27 objective and 2× plane scan zoom. The acquired images were processed using the Zeiss ZEN 2011 Light Edition program.

■ ASSOCIATED CONTENT

⑤ Supporting Information
Supplemental figures, experimental procedures, and compound characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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