Site-specific labeling of cell surface proteins with biophysical probes using biotin ligase

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We report a highly specific, robust and rapid new method for labeling cell surface proteins with biophysical probes. The method uses the *Escherichia coli* enzyme biotin ligase (BirA), which sequence-specifically ligates biotin to a 15-amino-acid acceptor peptide (AP). We report that BirA also accepts a ketone isostere of biotin as a cofactor, ligating this probe to the AP with similar kinetics and retaining the high substrate specificity of the native reaction. Because ketones are absent from native cell surfaces, AP-fused recombinant cell surface proteins can be tagged with the ketone probe and then specifically conjugated to hydrazide- or hydroxylamine-functionalized molecules. We demonstrate this two-stage protein labeling methodology on purified protein, in the context of mammalian cell lysate, and on epidermal growth factor receptor (EGFR) expressed on the surface of live HeLa cells. Both fluorescein and a benzophenone photoaffinity probe are incorporated, with total labeling times as short as 20 min.

Biophysical probes such as fluorophores, photoaffinity labels and spin labels have been extremely useful for investigating protein structure and function *in vitro*, but technological hurdles have limited their use in the heterogeneous context of the cytoplasm or cell surface. Green fluorescent protein (GFP) is an attractive option because of its high labeling specificity and ease of use, but it is a large tag (238 amino acids) and can be used only for fluorescence imaging. Recent protein-based tags that either react covalently or form high-affinity complexes with small-molecule probes circumvent the problem of versatility, as in principle probes of any structure can be introduced, but the problem of size remains[1–5]. In addition, tags based on endogenous mammalian proteins such as O^6-alkylguanine transferase (AGT) and dihydrofolate reductase (DHFR) produce background labeling of their endogenous counterparts in mammalian cells[1,2]. In addition, the noncovalent interactions on which DHFR and FK506 binding protein (FKBP) labeling rely dissociate over minutes to hours, causing signal deterioration[2,3].

Labeling approaches that use peptides rather than proteins as targeting sequences are less invasive but generally sacrifice specificity. For instance, the FlAsH technology, which targets arsenic-functionalized fluorophores to tetracysteine motifs displayed on recombinant proteins, requires complex washout procedures to remove the probe from monothiols inside cells[6]. Fluorophore-binding peptide aptamers similarly show reduced specificity compared to protein tag–based methods[7]. The recently developed hexahistidine-based labeling approach may offer higher specificity but also relies on a noncovalent interaction that deteriorates within minutes[8]. We have recently reviewed the merits and disadvantages of existing labeling methodologies elsewhere[9].

Thus, there is a need for new methodology that combines the minimal invasiveness of a small peptide tag with the excellent labeling specificity of GFP. Moreover, the attachment strategy should be covalent, so that probe dissociation is not a concern. To address this problem, we capitalized on the *E. coli* enzyme BirA, which catalyzes the biotinylation of a lysine side-chain within a 15-amino-acid consensus 'acceptor peptide' (AP) sequence[10]. BirA has already been used for the specific biotinylation of AP-fused recombinant proteins *in vitro* and in living cells, because of its excellent sequence specificity; endogenous mammalian proteins are not modified by BirA[11], and bacteria have only one natural substrate, biotin carboxyl carrier protein (BCCP)[10]. The mechanism of biotinylation involves activation of biotin as an adenylate ester, followed by its trapping by the lysine side-chain of the AP. To harness the specificity of BirA for ligation of other biophysical probes to proteins, we sought to re-engineer the biotin binding site to accommodate various biotin analogs. We were particularly interested in ketone 1, a biotin isostere with the ureido nitrogens replaced by methylene groups (**Fig. 1a**). Because the ketone group is absent from natural proteins, carbohydrates and lipids, it can be selectively derivatized on cell surfaces with hydrazide- or hydroxylamine-bearing probes under physiological conditions[12]. Here we describe the development of new protein labeling methodology (**Fig. 1**) based on the observation that wild-type BirA uses ketone 1 efficiently in place of biotin.

RESULTS

Wild-type BirA ligates ketone 1 to the AP

Racemic ketone 1 was synthesized in four steps from a known sulfoxide[13] in a route that recapitulates one of the known syntheses of biotin (**Fig. 1b** and **Supplementary Methods** online)[14]. We developed an HPLC-based assay to determine whether wild-type or

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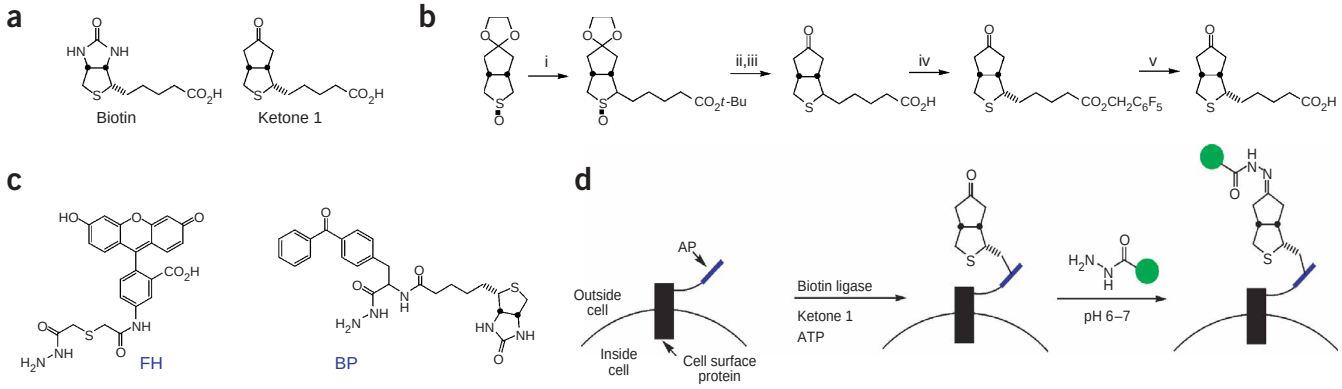

Figure 1 | Site-specific protein labeling using biotin ligase and a ketone analog of biotin. (**a**) Structures of biotin and ketone 1. (**b**) Synthesis of ketone 1. **i.** MeLi, THF/HMPA, −78 °C, then $I(CH_2)_4CO_2t$-Bu, −30 °C. **ii.** PPh_3, CCl_4, reflux. **iii.** AcOH, aq. HCl, reflux. **iv.** DIPEA, $C_6F_5CH_2Br$, CH_2Cl_2, then HPLC separation of diastereomers. **v.** LiOH, THF/MeOH/H_2O. (**c**) Structures of fluorescein hydrazide (FH) and benzophenone-biotin hydrazide (BP). (**d**) General method for labeling acceptor peptide (AP)-tagged recombinant cell surface proteins with biophysical probes. Biotin ligase (BirA) catalyzes the ligation of ketone 1 to the AP (blue); a subsequent bio-orthogonal ligation between ketone and hydrazide (or hydroxylamine) introduces the probe (green).

a mutant of BirA could catalyze the ligation of this probe to a synthetic acceptor peptide. When we combined wild-type BirA with synthetic AP, ketone 1, and ATP, a new product peak was observed by HPLC; omission of ATP or BirA from the reaction eliminated this peak (**Fig. 2a**). MALDI-TOF analysis confirmed that the product had the expected molecular weight for ketone 1 ligated to the AP (calculated (plus sodium ion) 2,541.3 g/mol; observed 2,542.3 g/mol) (**Fig. 2b**). Ketone 1 was also separated into its constituent enantiomers by chiral HPLC, and we verified that only one enantiomer was accepted by BirA (data not shown).

To quantitatively compare the rate of BirA-catalyzed ketone 1 ligation to that of biotin ligation, we measured the rates of product formation for both reactions under identical conditions (**Fig. 2c**). The initial rate for ketone 1 ligation (0.258 ± 0.024 μM/min) was lower by only a factor of 3.7 than that for biotin ligation (0.954 ± 0.018 μM/min, matching the previously reported rate for BirA biotinylation of BCCP[15]). However, whereas the biotin ligation rate remained constant until >50% conversion, the ketone ligation rate slowed markedly after ~100 enzyme turnovers, suggesting that product inhibition might be occurring. To avoid such inhibition, we used >0.01 equivalents of BirA relative to protein substrate in all subsequent labeling experiments.

In vitro labeling of recombinant proteins

To test the use of BirA and ketone 1 for labeling of a recombinant protein, we generated a test substrate by fusing the AP to the C terminus of cyan fluorescent protein (CFP-AP). Purified CFP-AP was first enzymatically labeled with ketone 1, and then fluorescein hydrazide was added to derivatize the ketone. The resulting hydrazone adduct was reduced with sodium cyanoborohydride to improve its stability and separated from excess fluorophore by SDS-PAGE. The fluorescence and Coomassie blue–stained images of the resulting gel are shown (**Figs. 3a,b**). Fluorescein is conjugated to CFP-AP only when ATP is present in the enzymatic ligation reaction, indicating that conjugation is dependent on enzyme activity. Point mutation of the CFP-AP acceptor lysine to alanine (CFP-Ala) also abolishes fluorescein conjugation, demonstrating that the labeling is site specific.

To test the specificity of the BirA-mediated labeling reaction, we expressed CFP-AP in human embryonic kidney 293T (HEK) cells and then subjected the cellular lysate to the same two-stage labeling procedure used before (**Figs. 3c,d**). Only CFP-AP is labeled with fluorescein, even in the presence of endogenous mammalian proteins at similar concentration. Again, labeling is dependent on the presence of ATP, and lysates from untransfected HEK cells are not labeled. Thus, wild-type BirA accepts ketone 1 as a cofactor without compromising its exceptional specificity for the peptide substrate.

We also assessed the sensitivity of our labeling method by comparing it to antibody detection. Lysate from CFP-AP-

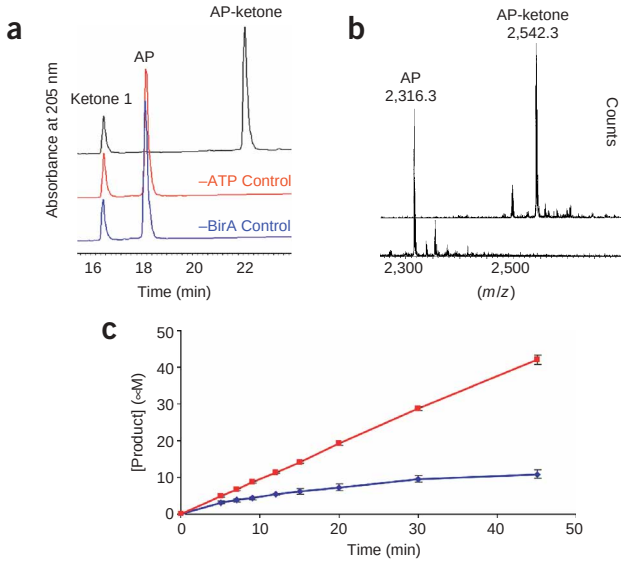

Figure 2 | BirA-catalyzed ligation of ketone 1 to the AP. (**a**) HPLC traces showing BirA- and ATP-dependent ligation of ketone 1 to a synthetic acceptor peptide (KKKGPGGLNDIFEAQKIEWH; acceptor lysine is underlined). (**b**) MALDI-TOF spectrum showing mass of purified AP-ketone conjugate. (**c**) Time course of biotin (■) and ketone 1 (◆) ligation to synthetic AP using 0.091 μM BirA. Each data point represents the average of three experiments. Error bars, 1 s.d.

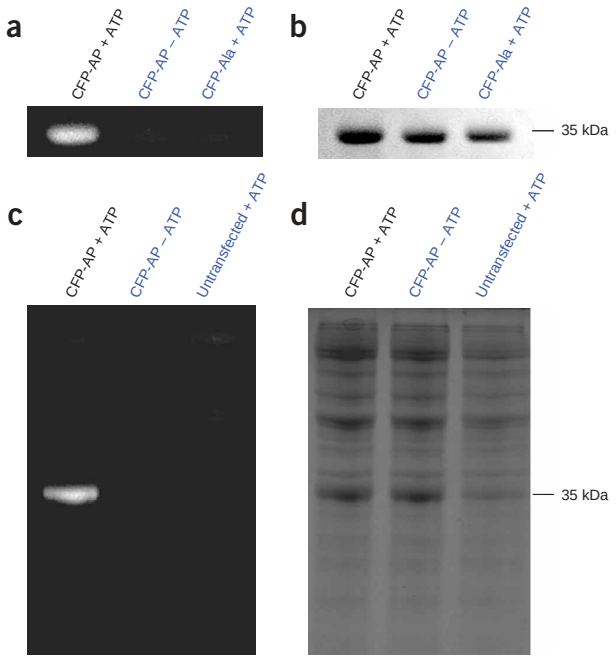

Figure 3 | Labeling of recombinant CFP-AP with fluorescein hydrazide. (**a**) Fluorescence gel image of denatured CFP-AP protein samples (fused AP sequence: GLNDIFEAQKIEWHE; acceptor lysine underlined) labeled by ketone 1 and fluorescein hydrazide. Omission of ATP (second lane) eliminates labeling, as does mutation of the acceptor lysine (CFP-Ala; third lane). (**b**) Coomassie blue–stained image of the gel in **a**. (**c**) Fluorescence image of CFP-AP–transfected HEK cell lysates labeled with ketone 1 and then with fluorescein hydrazide. Only the CFP-AP is labeled. Omission of ATP (second lane) eliminates labeling, and labeling is not seen when lysates are not transfected with the CFP-AP plasmid. (**d**) Coomassie blue–stained image of the gel in **c**.

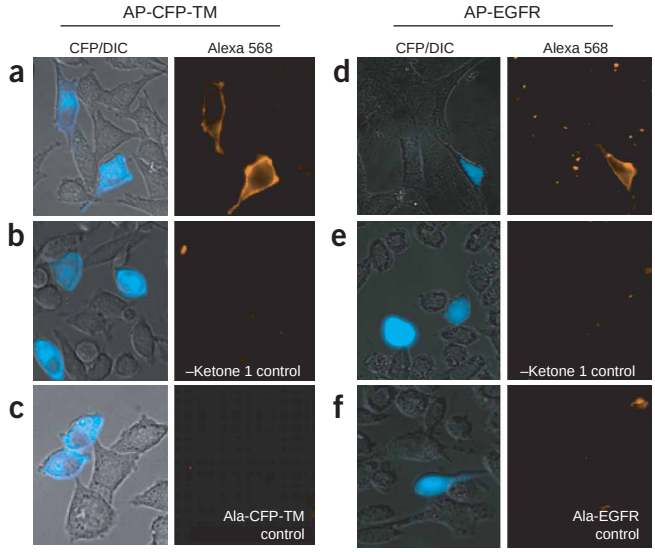

Figure 4 | Site-specific labeling of proteins expressed on the surface of live HeLa cells with BP. Cells expressing either the AP-CFP-TM construct (**a–c**) or AP-tagged EGFR (**d–f**) were labeled with ketone 1 for 60 min and then by BP for 60 min. The probe was then detected via its biotin handle using streptavidin-Alexa 568. The left columns show the CFP images (false-colored cyan) merged with the DIC images. The right columns show the Alexa 568 images (false-colored orange). (**a**) HeLa cells transfected with AP-CFP-TM are labeled with ketone 1 and BP, whereas untransfected neighboring cells are not. (**b**) Negative control without ketone 1. (**c**) Negative control with AP-CFP-TM replaced by the Ala-CFP-TM point mutant. (**d**) HeLa cells cotransfected with AP-EGFR and a cytoplasmic CFP marker plasmid show BP labeling, whereas untransfected neighboring cells are not labeled. (**e**) Negative control without ketone 1. (**f**) Negative control with AP-EGFR replaced by the Ala-EGFR point mutant.

transfected HEK cells was either treated with ketone 1 followed by fluorescein hydrazide, as above, or probed with anti-pentahistidine mouse antibody followed by fluorescein-conjugated secondary antibody (the CFP-AP construct bears an N-terminal hexahistidine tag). The fluorescence image of the resulting blot shows that our method is considerably more sensitive than antibody labeling for detection of CFP-AP in lysate (**Supplementary Fig. 1** online).

Labeling of cell surface proteins

Because ketones are absent from native cell surfaces, ketone 1 should permit the site-specific introduction of hydrazide or hydroxylamine probes onto AP-tagged cell surface proteins. To demonstrate this, we fused the AP to the N terminus of CFP and then attached this construct to the transmembrane (TM) domain of the platelet-derived growth factor receptor. The TM domain targets the entire construct to the cell surface, whereas CFP allows identification of transfected cells. This construct, called AP-CFP-TM, was efficiently expressed in HeLa cells 12–24 h after transfection. Direct enzymatic biotinylation with extracellular BirA confirmed that the AP tag was expressed on the cell surface and sterically accessible to BirA (data not shown). To highlight the scope of our methodology, we labeled AP-CFP-TM with a custom probe (benzophenone-biotin hydrazide, BP; structure shown in **Fig. 1c** and synthesis described in **Supplementary Methods** online), which bears a hydrazide for conjugation to ketone 1, a photocrosslinking-competent benzophenone moiety, and a biotin moiety to allow sensitive detection by streptavidin staining (separate experiments have shown that streptavidin does not bind to ketone 1 itself; data not shown).

To initiate labeling, the media was replaced with Dulbecco's phosphate buffered saline (DPBS), pH 7.4, containing BirA, ketone 1 and ATP. Because we were concerned about the possible toxicity of extracellular ATP, we separately examined the growth rate of HeLa cells incubated for 24 h with various amounts of ATP and observed no effect at concentrations below 5 mM; we therefore used 1 mM ATP for all our cellular experiments. The ketone ligation was allowed to proceed for 10–60 min. The cells were then rinsed to remove excess ketone, and BP was added in slightly acidic medium (DPBS, pH 6.2), which is known to accelerate hydrazone formation[16]. After cells were incubated for 10–60 min, streptavidin conjugated to the fluorophore Alexa 568 was used to detect the biotin handle of the BP probe. The fluorescence and differential interference contrast (DIC) images of the labeled cells are shown (**Fig. 4**). Cells transfected with AP-CFP-TM show distinct membrane labeling by the BP probe (as indicated by the streptavidin–Alexa 568 staining pattern), whereas neighboring untransfected cells remain unlabeled (**Fig. 4a**). Negative controls without ketone 1 (**Fig. 4b**) or with Ala-CFP-TM replacing AP-CFP-TM (**Fig. 4c**) show only background levels of staining, demonstrating that BP labeling proceeds via ketone 1 and is highly specific for the lysine of the AP tag. Despite the necessity of a two-stage labeling protocol, high levels of labeling

were achieved in as little as 20 min, which should allow our method to be used for the study of relatively fast biological processes, such as receptor trafficking.

We also used the AP-CFP-TM construct to quantify the sensitivity of BirA-catalyzed labeling. On a dish of cells expressing AP-CFP-TM at a range of concentrations (<10 μM to >1 mM as determined by comparison of fluorescence intensity to that of a wedge of purified CFP protein of known concentration; see **Supplementary Methods** online for details), labeling by the BP probe was clearly detectable, with a signal-to-background ratio $>4{:}1$, for cells expressing 10 μM or more of AP-CFP-TM. Based on deconvolution imaging, which shows approximately a 1:1 ratio of cell surface to intracellular AP-CFP-TM fluorescence (data not shown), and assuming an average cell volume of 1 pl, 10 μM expression level corresponds to $\sim 10^6$ copies of AP on the cell surface. Thus, our labeling method can detect cell surface proteins expressed at 10^6 copies per cell and perhaps even less.

To demonstrate BirA-mediated labeling of a natural protein, we selected the epidermal growth factor receptor (EGFR). The trafficking behavior and ligand-dependent dimerization, and possible higher-order oligomerization[17], of EGFR are of great biological interest. In addition, EGFR has proven intractable to study by extracellular GFP fusion, which severely impairs receptor expression and trafficking[18]. Thus, only minimal-sized probes and tags may be tolerated by the extracellular domain. We fused the AP to the N terminus of human EGFR, expressed the construct in HeLa cells and demonstrated both robust surface expression and steric accessibility of the AP tag using direct enzymatic biotinylation. We then used BirA- and ketone 1–mediated labeling to introduce the BP probe onto AP-EGFR. Cells cotransfected with AP-EGFR and cytoplasmic CFP (used as a transfection marker) show surface staining, whereas untransfected cells do not (**Fig. 4d**). Negative controls without ketone 1 (**Fig. 4e**) or with AP-EGFR replaced by Ala-EGFR (**Fig. 4f**) gave no labeling.

Because of its small size in comparison to the GFP tag, the 15-amino acid AP tag is less likely to alter the expression, trafficking or function of EGFR. We compared the distribution of AP-EGFR and untagged wild-type EGFR by immunofluorescence staining with anti-EGFR antibody and found no substantial difference (**Supplementary Fig. 2** online). In addition, EGF treatment elevates phosphotyrosine levels at the plasma membrane indistinguishably in wild-type EGFR- and AP-EGFR-transfected cells (**Supplementary Fig. 2** online), suggesting that the AP tag does not appreciably affect receptor function[19]. The AP tag at the N terminus thus seems to be minimally invasive and should allow the introduction of a range of probes with which to study EGFR trafficking and function in live cells.

DISCUSSION

We have developed new methodology for tagging cell surface proteins with biophysical probes using a 15-amino-acid AP sequence and the enzyme BirA. The method is highly specific because it capitalizes on the excellent sequence specificity of BirA, and it is versatile because the ketone platform allows the introduction of a wide range of probes. In these respects, BirA-based labeling compares favorably to existing labeling approaches, such as AGT[1], DHFR[2], FKBP[3] and ACP/PCP[4,5], which use large and hence more sterically invasive tags; and FlAsH[6], peptide aptamers[7] and Ni-NTA[8], which are less specific or rely on noncovalent interactions. Our method has disadvantages, however, and must therefore be seen as a complement to, rather than a replacement for, existing methodologies. The labeling is restricted to cell surface proteins because competing ketone- and aldehyde-containing small molecules inside cells most likely would prevent specific ligation of hydrazide- or hydroxylamine-functionalized probes to the target protein. It may be possible to surmount this problem through extensive washouts, as has been suggested[20], but our preferred approach will be to re-engineer BirA to accept other, more chemically orthogonal biotin analogs. We and others[11] have already shown that BirA can be expressed inside mammalian cells and retains its activity and specificity. Another limitation of our approach is the two-step labeling protocol, which limits the biological processes that can be studied based on their timescale. We report a minimal labeling time of 20 min, which is faster than that for techniques such as FlAsH, but slower than for AGT or ACP/PCP tagging strategies. Efforts are underway to accelerate the hydrazone formation reaction, in parallel with efforts to identify BirA mutants that accept probes of interest directly, providing a single-step labeling protocol.

Our method and the recently described ACP/PCP strategies represent, to our knowledge, the only reported examples of *in vivo* enzyme-mediated site-specific protein labeling (although *in vitro* labeling has been achieved using transglutaminase[21,22] and sortase[23] enzymes). A unique advantage of these methods is that labeling can be spatially controlled through genetic targeting of the labeling enzyme. For example, we are now working to target BirA to the presynaptic surface, to specifically label proteins at the postsynaptic surface of neurons. No other labeling methodology yet allows this degree of spatial control.

In conclusion, we have developed new protein labeling methodology that combines the specificity of GFP tagging with the minimal invasiveness of a small peptide. We demonstrated the labeling on a synthetic peptide, on purified protein, on mammalian cell lysate and on both an artificial construct and EGFR expressed on the surface of living mammalian cells. Both fluorophore and benzophenone attachment to EGFR should permit noninvasive study of receptor trafficking and oligomerization in live cells. Future efforts will focus on extending the methodology to intracellular labeling and improving the timescale of labeling, as well as on exploiting the enzyme dependence to spatially restrict labeling to interesting protein subpopulations.

METHODS

HPLC assay for probe ligation to synthetic AP. The synthetic AP with sequence KKKGPGGLNDIFEAQKIEWH was synthesized by the Tufts University Core Facility. The crude peptide was purified by reverse-phase HPLC (Microsorb-MV 300 C18 column, Varian; 10–39% acetonitrile in water with 0.1% TFA over 35 min, flow rate 4.7 ml/min); the desired peak had a retention time of 25.5 min. After lyophilization, the peptide was redissolved in water and the concentration was determined from the absorbance at 280 nm using the calculated extinction coefficient of 5690 $M^{-1}cm^{-1}$. Reaction conditions for the probe ligation to the AP were as follows: 50 mM bicine, pH 8.3, 5 mM magnesium acetate, 4 mM ATP, 100 μM AP, 1–2 μM BirA and 1 mM probe (either biotin or racemic ketone 1). Reactions were incubated at 30 °C for 1–2 h, then quenched through addition of 45 mM EDTA. Reactions were analyzed on a reverse-phase HPLC column

(Microsorb-MV 300 C18). Biotin ligation reactions were analyzed using a gradient of 10–43% acetonitrile in water with 0.1% TFA over 20 min (flow rate 1.0 ml/min); retention times were 8.2 min for biotin, 16.3 min for the AP and 17.7 min for the AP-biotin conjugate. Ketone 1 ligation reactions were analyzed using a gradient of 10–46% acetonitrile in water with 0.1% TFA over 25 min (flow rate 1.0 ml/min); retention times were 16.4 min for ketone 1, 17.9 min for the AP and 22.0 min for the AP–ketone 1 conjugate. For MALDI-TOF analysis, the product peak was collected, diluted with matrix solution (saturated α-cyano 4-hydroxycinnamic acid in 50% acetonitrile in water with 0.05% TFA) and spotted onto the sample target. Positive-ion MALDI-TOF data were acquired in reflector mode with external calibration.

Measurement of probe ligation kinetics. For kinetic measurements, the reaction conditions were the same as above except that 0.091 μM BirA was used, and for the ketone ligation reactions, 2 mM of racemic ketone 1 was used. A 400 μl reaction was initiated by addition of BirA and incubated at 30 °C. At various time points, a 40 μl aliquot was removed and quenched with EDTA. Reactions were analyzed by reverse-phase HPLC as described above. The area ratios of AP and AP-probe conjugate peaks were converted to concentrations of AP-probe conjugate using a calibration curve generated by mixing known ratios of AP and AP-probe conjugate. The concentration of AP-probe conjugate was plotted against time, and the reported initial rate was the slope of the line fit to the linear region of product synthesis.

Fluorescent labeling of CFP-AP. The reaction conditions for enzymatic ligation of ketone 1 to CFP-AP were as follows: 50 mM bicine, pH 8.3, 5 mM magnesium acetate, 4 mM ATP, 10–20 μM CFP-AP, 1.3 μM BirA and 100 μM racemic ketone 1. The reaction was incubated at 30 °C for 3 h, then 0.1 M HCl was added to adjust the pH to 6.2. Fluorescein hydrazide (Molecular Probes) was added to a final concentration of 1 mM, and the reaction was incubated at 30 °C for 12–16 h. Sodium cyanoborohydride (15 mM) was added to reduce the hydrazone for 1.5 h at 4 °C. The total protein was precipitated by addition of trichloroacetic acid (TCA) to a final vol/vol ratio of 10%. The protein pellet was redissolved in SDS-PAGE loading buffer, resolved by SDS-PAGE and visualized with the STORM 860 instrument (Amersham Biosciences).

Fluorescent labeling of CFP-AP in mammalian cell lysates. Human embryonic kidney 293T (HEK) cells were transfected with a pcDNA3 plasmid containing the CFP-AP gene (with an N-terminal hexahistidine tag) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Lysates were generated after 24–48 h at 70–80% confluence using a hypotonic lysis protocol to minimize protease release. Briefly, cells were concentrated by centrifugation and then resuspended in 1 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM PMSF, 1 mM EGTA and protease inhibitor cocktail (Calbiochem). After incubation at 4 °C for 10 min, the cells were lysed by vigorous vortexing for 2 min at room temperature. The crude lysate was clarified by centrifugation, then divided into aliquots and stored at −80 °C. The reaction conditions for enzymatic ligation of ketone 1 were as follows: 50 mM bicine, pH 8.3, 5 mM magnesium acetate, 4 mM ATP, 1 μM BirA, 200 μM racemic ketone 1 and lysate to a final vol/vol ratio of 82%. The reactions were incubated at 30 °C for 4 h, then 0.1 M HCl was added to adjust the pH to 6.2. Fluorescein hydrazide was added to a final concentration of 1 mM, and the reaction was incubated at 30 °C for 20 h. After reduction with sodium cyanoborohydride (15 mM), the total protein was precipitated by addition of trichloroacetic acid to a final vol/vol ratio of 10%. The protein pellet was redissolved in SDS-PAGE loading buffer, resolved by SDS-PAGE and visualized with the STORM 860 instrument.

Labeling of cell surface AP-CFP-TM and AP-EGFR expressed in HeLa cells. HeLa cells were transfected with the AP-CFP-TM or AP-EGFR plasmid using Lipofectamine 2000 according to the manufacturer's instructions. After 12–24 h at 37 °C, the cells were washed twice with DPBS, pH 7.4. Enzymatic ligation of ketone 1 to AP-CFP-TM was performed in DPBS, pH 7.4, with 5 mM $MgCl_2$, 0.2 μM BirA, 1 mM racemic ketone 1 and 1 mM ATP for 10–60 min at 32 °C. Cells were then washed twice with DPBS, pH 6.2, and incubated for 10–60 min at 16 °C (to reduce endocytosis) with 1 mM BP in DPBS, pH 6.2. The cells were washed twice with DPBS, pH 7.4 and incubated with streptavidin-Alexa 568 (3 μg/ml; Molecular Probes) in DPBS, pH 7.4, and 1% BSA for 10 min at 4 °C. The cells were washed twice with DPBS, pH 7.4, and imaged in the same buffer on a Zeiss Axiovert 200M inverted epifluorescence microscope using a 40× oil-immersion lens. CFP (420DF20 excitation, 450DRLP dichroic, 475DF40 emission), Alexa 568 (560DF20 excitation, 585DRLP dichroic, 605DF30 emission) and DIC images (630DF10 emission) were collected and analyzed using OpenLab software (Improvision). Fluorescence images were background-corrected. Acquisition times ranged from 0.2–2 s.

Note: Supplementary information is available on the Nature Methods website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Methods* website for details).

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