An improved smaller biotin ligase for BioID proximity labeling

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**ABSTRACT** The BioID method uses a promiscuous biotin ligase to detect protein–protein associations as well as proximate proteins in living cells. Here we report improvements to the BioID method centered on BioID2, a substantially smaller promiscuous biotin ligase. BioID2 enables more-selective targeting of fusion proteins, requires less biotin supplementation, and exhibits enhanced labeling of proximate proteins. Thus BioID2 improves the efficiency of screening for protein–protein associations. We also demonstrate that the biotinylation range of BioID2 can be considerably modulated using flexible linkers, thus enabling application-specific adjustment of the biotin-labeling radius.

**INTRODUCTION** Identification of protein–protein associations (PPAs) is a fundamental approach to the study of protein function, subcellular proteomes, and biological mechanisms. The proximity-dependent biotin identification (BioID) method was developed to overcome barriers imposed by conventional screening methods for PPAs (Roux et al., 2012). The BioID method is based on proximity-dependent cellular biotinylation by a promiscuous bacterial biotin ligase (Escherichia coli BiA R118G, hereafter called BioID; Choi-Rhee et al., 2004; Cronan, 2005) fused to a bait protein to generate a history of PPAs over time in living cells (Roux et al., 2012). These biotinylated proteins can be selectively isolated by conventional biotin capture methods and identified using mass spectrometry (MS) analysis (Roux et al., 2012, 2013; Kim et al., 2014; Mehus et al., 2016). These candidates identified by the BioID method can represent direct interactors, indirect interactors, and/or vicinal proteins that do not physically interact with the fusion protein.

Owing to its applicability to insoluble proteins and weak/transient PPAs, the BioID method has rapidly become widely used to study PPAs in the nuclear envelope (Roux et al., 2012; Kim et al., 2014), centrosomes (Comartin et al., 2013; Firat-Karalar et al., 2014), cell–cell junctions (Van Itallie et al., 2013, 2014; Guo et al., 2014; Steed et al., 2014; Ueda et al., 2015), spatiotemporal dynamics of epigenetic factors (Lambert et al., 2015; Mulholland et al., 2015), cancer development (Elzi et al., 2014; Cole et al., 2015; Dingar et al., 2015), cross-talk between cytoskeletons (Jiu et al., 2015), mRNA decay (Nishimura et al., 2015), signaling pathways regulating various essential cellular mechanisms (Couzens et al., 2013; Cheng et al., 2015; Rodriguez-Fraticelli et al., 2015; Schumacher et al., 2015; Zhou et al., 2015), and ubiquitin metabolism (Coyaud et al., 2015; Yeh et al., 2015). BioID has also been applied to elucidate interplay mechanisms between host and virus or microorganisms for HIV (Kueck et al., 2015; Le Sage et al., 2015; Ritchie et al., 2015), *Chlamydia trachomatis* (Kabeiseman et al., 2014), *Chlamydia psittaci* ( Mojica et al., 2015), *Toxoplasma gondii* (Chen et al., 2015), *Trypanosoma brucei* (Morrisonwood et al., 2013; McAllaster et al., 2015), and herpes viruses (Lajko et al., 2015).

One challenge for any method that relies on expression of fusion proteins is proper subcellular targeting. Although BioID (321 amino acids) is slightly larger than green fluorescent protein, we have observed that fusion to BioID occasionally prevents efficient targeting of certain fusion proteins. In attempt to ameliorate that limitation, we report our development and analysis of a new, smaller promiscuous...
that this region is critical for BirA biotin ligase activity (Henke and Cronan, 2014). With our second approach, we used Uniprot to identify the smallest known biotin ligase, which is from Aquifex aeolicus. Based on the published protein crystallography, biotin ligases from A. aeolicus and E. coli share similarities in their overall structures (Wilson et al., 1992; Tron et al., 2009). However, the biotin ligase from A. aeolicus (233 amino acids) lacks the DNA-binding domain and is substantially smaller (Figure 1A). To create a smaller enzyme for proximity-dependent biotinylation, we humanized the biotin ligase of A. aeolicus and mutated a conserved residue within the biotin catalytic domain (R40G) in an attempt to enable promiscuous biotinylation (Choi-Rhee et al., 2004; Cronan, 2005). Called BioID2, this smaller ligase was tested for promiscuous biotinylation in live mammalian cells.BioID and BioID2 were each fused to the highly insoluble nuclear protein lamin A (LaA) and expressed in HEK293 cells. Both fusion proteins were predominantly localized at the nuclear envelope (NE), leading to biotinylation of proteins at the NE (Figure 1B and C). Thus the BioID2 appears at least fundamentally comparable to the original BioID in living mammalian cells. Because BioID2 is substantially smaller than BioID, we tested whether BioID2 improves localization of its fusion protein other than LaA. To this end, we transiently expressed human Sun2 with N-terminal BioID or BioID2 in a mouse fibroblast NIH3T3. Sun2 is a type II NE protein with an N-terminal nucleoplasmic domain. Like many other NE proteins, Sun2 is sensitive to endoplasmic reticulum mislocalization upon the fusion of bulky motifs to its N-terminus (Antonin et al., 2011; Ungricht et al., 2015). Compared with BioID, BioID2 enabled a more appropriate targeting of Sun2 to the NE when the proteins were expressed at comparable levels (Figure 1D). The relative mean intensity of the expressed Sun2 at the NE compared with the endoplasmic reticulum (ER) indicates that BioID2-Sun2 is approximately twofold more concentrated at the NE than BioID-Sun2 (Figure 1E). These results support the conventional wisdom that a smaller tag is less disruptive to a fusion protein. In addition, because A. aeolicus is a thermophilic bacterium, we predicted that the BioID2 might optimally function at higher temperatures. To monitor the influence of temperature on promiscuous biotinylation by BioID and BioID2, we performed in vitro biotinylation assays using bovine serum albumin (BSA) as substrate. Among the tested temperatures, biotinylation by BioID2 was optimal at 50ºC, whereas biotinylation by BioID was optimal at 37ºC. Both enzymes exhibited reduced activity below 37ºC (Supplemental Figure S1). These observations suggest that BioID2 would be the preferred ligase for thermophilic

RESULTS AND DISCUSSION
A smaller promiscuous biotin ligase, BioID2, improves localization of its fusion protein
We first attempted to reduce the size of BioID through targeted deletion of the N-terminal DNA-binding domain of the enzyme; however, the resultant products failed to retain enzymatic activity (unpublished data) as expected from subsequent studies showing

FIGURE 1: Promiscuous biotinylation by BioID2. (A) The dimensions of E. coli (left; PDB ID 1BIA) and A. aeolicus (right; PDB ID 2EAY) biotin ligases based on prior structural analyses. The catalytic (yellow), ATP-binding (green), and DNA-binding (red) domains. (B) BioID and BioID2 were fused with LaA and expressed in HEK293 cells. Fusion proteins were detected by specific antibodies against BioID or BioID2, respectively (red). Biotinylated proteins were labeled with streptavidin (green). DNA was labeled with Hoechst dye 33258 (blue). Scale bar, 10 μm. (C) Proteins biotinylated by BioID-LaA, BioID2-LaA, BioID-only, or BioID2-only were detected with HRP-conjugated streptavidin after SDS–PAGE separation. Expression of either promiscuous ligase leads to biotinylation of endogenous proteins (left). Fusion proteins were detected with anti-myc antibodies (right). (D) BioID-human Sun2 or BioID2-human Sun2 were transiently expressed in NIH3T3 cells. Fusion proteins were detected using an anti-Sun2 antibody incapable of detecting murine Sun2. Scale bar, 10 μm. (E) The NE/ER ratio of the mean intensity of BioID-human Sun2 or BioID2-human Sun2 detected with anti-human Sun2. Values are mean ± SEM. We measured 48 nuclei/condition.
with BSA for 16 h with 0.2–50 μM biotin. Biotinylation by BioID was markedly reduced as the concentration of biotin decreased below 50 μM, whereas BioID2 sustained maximum biotinylation through 3.2 μM biotin (Figure 2A). Similar results were observed in HEK293 cells stably expressing BioID-LaA or BioID2-LaA and incubated for 16 h with 0.001–200 μM biotin. BioID2-LaA exhibited saturation of biotinylation at 0.1 μM biotin; however, biotinylation by BioID-LaA required 1 μM biotin for maximum biotinylation. Of interest, we noted some basal biotinylation in the BioID2-LaA cells under conditions with no biotin supplementation (Figure 2B). The basal biotinylation is likely induced by BioID2-LaA responding to the low levels of biotin in serum included with the culture medium since it was inhibited by culturing cells for 72 h in medium with biotin-depleted serum. To rule out accumulation of basal biotinylation in normal medium, we monitored biotinylation by BioID2-LaA under biotin-depleted conditions. Under these conditions, BioID2-LaA generated detectable biotinylation from 0.01 μM biotin, whereas BioID-LaA required 0.1 μM biotin (Figure 2C). However, biotinylation by both BioID-LaA and BioID2-LaA reached maximal levels ∼16 h after addition of biotin under conditions of biotin saturation (50 μM; Supplemental Figure S2). This observation suggests that biotin depletion conditions might need to be used for time-sensitive studies using BioID2 (e.g., comparing drug-treated to control conditions) to more exclusively capture protein associations that occur during a discrete time frame. However, the higher efficacy of BioID2 with lower biotin concentrations would be a useful characteristic when performing BioID in model systems such as *Caenorhabditis elegans*, *Drosophila melanogaster*, or *Mus musculus* in which biotin supplementation might be more complicated than simple medium supplementation.

BioID and BioID2 are functionally comparable

To compare the practical efficacy of BioID2 to that of original BioID, we evaluated candidates identified by both enzymes when targeted to a highly stable and structurally well-characterized protein complex. To this end, we C-terminally fused BioID and BioID2 to Nup43, a constituent of the nuclear pore complex (NPC; Daigle et al., 2001; Loiodice et al., 2004; D’Angelo et al., 2009; Savas et al., 2012). When stably expressed in HEK293 cells, Nup43-BioID and Nup43-BioID2 were incubated with NPC, DS, and Mm proteins localized at the NPC when supplemented with conditions but is also highly efficient at biotinylating proximate proteins in live cells at 37°C.

**BioID2 requires less biotin than BioID**

We next used purified BioID and BioID2 to compare their requirements for biotin supplementation. BioID or BioID2 were incubated with BSA for 16 h with 0.2–50 μM biotin. Biotinylation by BioID was markedly reduced as the concentration of biotin decreased below 50 μM, whereas BioID2 sustained maximum biotinylation through 3.2 μM biotin (Figure 2A). Similar results were observed in HEK293 cells stably expressing BioID-LaA or BioID2-LaA and incubated for 16 h with 0.001–200 μM biotin. BioID2-LaA exhibited saturation of biotinylation at 0.1 μM biotin; however, biotinylation by BioID-LaA required 1 μM biotin for maximum biotinylation. Of interest, we noted some basal biotinylation in the BioID2-LaA cells under conditions with no biotin supplementation (Figure 2B). The basal biotinylation is likely induced by BioID2-LaA responding to the low levels of biotin in serum included with the culture medium since it was inhibited by culturing cells for 72 h in medium with biotin-depleted serum. To rule out accumulation of basal biotinylation in normal medium, we monitored biotinylation by BioID2-LaA under biotin-depleted conditions. Under these conditions, BioID2-LaA generated detectable biotinylation from 0.01 μM biotin, whereas BioID-LaA required 0.1 μM biotin (Figure 2C). However, biotinylation by both BioID-LaA and BioID2-LaA reached maximal levels ∼16 h after addition of biotin under conditions of biotin saturation (50 μM; Supplemental Figure S2). This observation suggests that biotin depletion conditions might need to be used for time-sensitive studies using BioID2 (e.g., comparing drug-treated to control conditions) to more exclusively capture protein associations that occur during a discrete time frame. However, the higher efficacy of BioID2 with lower biotin concentrations would be a useful characteristic when performing BioID in model systems such as *Caenorhabditis elegans*, *Drosophila melanogaster*, or *Mus musculus* in which biotin supplementation might be more complicated than simple medium supplementation.
Improved promiscuous biotin ligase of abundant candidates that account for \(\sim 90\%\) of total spectra counts are commonly detected by Nup43-BioID or Nup43-BioID2 (Supplemental Table S1). Similarly, 60% of detected background proteins are commonly identified from BioID-only and BioID2-only. Those commonly identified proteins are clearly abundant proteins since they account for \(\sim 87\%\) of total spectra counts (Supplemental Table S3).

50 μM biotin (Figure 3, A and B). To reveal the identity of these endogenous proteins, we followed large-scale pull downs of biotinylated endogenous proteins by MS analysis. These results revealed that \(\sim 40\%\) of spectral counts detected by Nup43-BioID are NPC-associated proteins (Nups), whereas \(\sim 60\%\) of spectral counts detected by Nup43-BioID2 are Nups (Supplemental Table S1). Of note, the vast majority of abundant candidates that account for \(\sim 90\%\) of total spectra counts are commonly detected by Nup43-BioID or Nup43-BioID2 (Supplemental Table S1). Similarly, 60% of detected background proteins are commonly identified from BioID-only and BioID2-only. Those commonly identified proteins are clearly abundant proteins since they account for \(\sim 87\%\) of total spectra counts (Supplemental Table S3).
In all, this comparative BioID analysis at the NPC demonstrates the specificity and functionality of BioID2 in screening for PPAs.

Within the Nup107–Nup160 complex, both Nup43-BioID and Nup43-BioID2 detected Nup96 and Nup107 (Figure 3C). Nup85, a direct interacting protein of Nup43, is not efficiently biotinylated by Nup43-BioID (Kim et al., 2014). However, Nup43-BioID2 was able to detect Nup85 (Figure 3C), suggesting that BioID2 might be more efficient at biotinylating proximate proteins. We also put a 5-nm linker consisting of three repeats of GGGGS between Nup43 and BioID to resolve any steric hindrance that potentially inhibits biotinylation of Nup85 by Nup43-BioID. However, Nup85 was not detected by Nup43-BioID with the 5-nm linker (unpublished data). Thus these observations suggest that BioID2 may be more efficient in biotinylation when examining the Nups outside of the Nup107–Nup160 complex that were detected by Nup43-BioID and Nup43-BioID2, we observed that Nups located proximate to the Nup107–Nup160 complex were commonly detected (Figure 3D). For instance, the proximate location between the Y-complex and nuclear basket Nups such as Nup153 has been documented by biochemical experiments, cross-linking-MS (XL-MS), and cryo-electron tomography (cryo-ET) studies (Vasu et al., 2001; Bui et al., 2013). In addition, the flexibility of nuclear basket proteins may also enable contact with Nup43 (Fahrenkrog et al., 2002). Nup43-BioID2 also detected hCG1, a cytoplasmic Nup associated with Nup214 through the direct interaction with Gie1 (Kendirgi et al., 2005; Folkmann et al., 2013), and central NPC constituents such as Nup62 and Nup93 (Figure 3D). This detection of distally located Nups by Nup43-BioID2 also suggests that the practical biotinylation range of BioID2 might be somewhat larger than that of BioID and/or that BioID2 is capable of detecting proteins refractory to detection by the original BioID.

The biotinylation range can be increased with a flexible linker

The practical labeling radius of promiscuous biotin ligases is an important experimental consideration since a large radius would predictably detect more proteins that may not be direct interactors of the bait, whereas, conversely, a smaller radius would potentially limit detection of relevant protein interactors for a larger protein or a protein complex. Our previous studies indicated the practical labeling radius of BioID is ∼10 nm (Kim et al., 2014). We hypothesized that the biotinylation range of BioID2 could be increased using a flexible linker. To test this hypothesis, we inserted a 25-nm linker consisting of 13 repeats of GGGGS (Amet et al., 2009) between Nup43 and BioID2 (Nup43-Linker-BioID2; Figure 4A). In HEK293 cells stably expressing Nup43-Linker-BioID2, the fusion protein was localized at the NPC (Supplemental Figure S3) and biotinylated proteins at the NPC (Figure 4C). The insertion of the linker did not lead to any abnormal proteolytic processing of the fusion protein and enabled biotinylating of endogenous proteins in live cells (Figure 4B). Given the size of the Nup107–Nup160 complex, Nup43-Linker-BioID2 can theoretically access all the constituents of the complex. Indeed, by MS analysis of a large-scale BioID pull down, Nup43-Linker-BioID2 detected all the constituents of the Nup107–Nup160 complex, including distal tip Nups such as Nup160 and Nup133 (Figure 4D). Outside the Nup107–Nup160 complex, central channel proteins Nup205 and Nup62 were also uniquely detected by the addition of the linker (Figure 4E). Of interest, in Nup43-Linker-BioID2, karyopherins that mediate transport across the NPC were also detected, presumably because the linker allows the ligase to occasionally contact the karyopherins in the central channel (Supplemental Table S2). Our data suggest that the biotinylation range of BioID2 can be increased using a molecular linker, thus enabling identification of proteins well beyond the range of the ligase alone. However, despite the detection of substantially more NPC proteins than Nup43-BioID2, Nup43-Linker-BioID2 did not dramatically increase the detection of candidates outside of the Nup107–Nup160 complex. This observation might indicate the preserved specificity to the region proximate to Nup43.

In summary, BioID2 is fundamentally comparable to BioID in its ability to promiscuously biotinylate proximate proteins but requires considerably less biotin, which may facilitate application of the BioID method to model systems for which biotin supplementation methods are limited. With its smaller size, BioID2 should enable a more appropriate targeting and thus function of fusion proteins. In addition, the biotinylation range and/or efficacy of BioID2 appear enhanced compared with those of BioID, thus enabling detection of proteins that are refractory to biotinylation with the original ligase. In addition, we demonstrated that the use of flexible linkers can increase the labeling radius of BioID2, thereby overcoming limitations inherent to the intrinsic biotinylation range of BioID ligases. This will provide users of the BioID method flexibility in experimental design when studying larger proteins whose spatial dimensions exceed the biotinylation range, probing the constituency of large protein complexes, or globally surveying the protein constituency of discrete subcellular domains.

MATERIALS AND METHODS

Plasmids

Humanized BioID2 was synthesized and amplified by PCR. The amplified BioID2 was inserted into the previously generated BioID-LaA pcDNA 3.1 vector (Kim et al., 2014) via Nhel and Xhol to replace BioID with BioID2. BioID-LaA and BioID2-LaA in pcDNA 3.1 were amplified by PCR and inserted into pBabe.puro using EcoRI and SalI. BioID2 was amplified by PCR and inserted into the previously generated Nup43-BioID pcDNA 3.1 vector (Kim et al., 2014) using BamHI and Pmel to replace BioID with BioID2. Nup43-BioID and Nup43-BioID2 in pcDNA 3.1 were subcloned to pBabe.puro using EcoRI and SalI. Linker-BioID was synthesized and amplified by PCR. The PCR products were inserted into the Nup43-BioID pcDNA 3.1 vector (Kim et al., 2014) via EcoRI and Pmel to replace BioID with Linker-BioID. BioID2 was amplified and inserted to Nup43-Linker-BioID using BamHI and Pmel to replace BioID with BioID2. Nup43-Linker-BioID and Nup43-Linker-BioID2 in pcDNA 3.1 vector were subcloned to pBabe.puro using EcoRI and SalI. To generate HA-BioID-LaA and HA-BioID2-LaA, BioID and BioID2 were amplified with primers containing sequences for HA. The PCR products were inserted into the previously generated BioID-LaA pcDNA 3.1 vector using Nhel and Xhol. To generate glutathione S-transferase (GST) fusion protein, BioID and BioID2 were amplified by PCR and inserted into pGex 4T-1 vector (GE Healthcare Life Sciences, Pittsburgh, PA) using EcoRI and Xhol.

Structural analysis

Previously published Protein Data Bank (PDB) structures of the biotin ligase from E. coli (DOI 10.2210/pdb1bia/pdb) (Wilson et al., 1992) and A. aeolicus (DOI 10.2210/pdb2eay/pdb) were analyzed by MacPyMOL (www.pymol.com).

Immunofluorescence

Stable cells were fixed in 3% (wt/vol) paraformaldehyde/phosphate-buffered saline (PBS) for 10 min. Cells were permeabilized by 0.4% (wt/vol) Triton X-100/PBS for 15 min, followed by 0.5% SDS/PBS for 5 min. Cells were labeled with the following primary antibodies: chicken polyclonal anti-BiA (1:2000, ab14002; Abcam, Cambridge, MA), rabbit polyclonal anti-myc (1:1000, ab9106; Abcam), rabbit polyclonal anti-hemagglutinin (HA; 1:1000, ab9110; Abcam), and other relevant antibodies.
After transient expression of BioID-Sun2 and BioID2-Sun2 in mouse NIH3T3 cells, the fusion proteins were detected using an anti–human Sun2 antibody (1:200, A001209; Sigma-Aldrich, St. Louis, MO) that does not detect murine Sun2. Images were taken using Nikon A1-confocal microscope. The mean intensity of the fusion proteins at the NE or ER was obtained using ImageJ software (National Institutes of Health, Bethesda, MD).

Immunoblot
To analyze total cell lysates using immunoblot, 1.2 x 10^6 cells were lysed in SDS–PAGE sample buffer, boiled for 5 min, and sonicated to shear DNA. Proteins were separated on 4–20% gradient gels (Mini-PROTEAN TGX; Bio-Rad, Hercules, CA) and transferred to nitrocellulose membrane (Bio-Rad). After blocking with 1% (wt/vol) BSA and 0.2% Triton X-100 in PBS for 10 min, membranes were
incubated with horseradish peroxidase (HRP)–conjugated streptavidin (1:40,000, ab7403; Abcam) for 40 min. The signals from antibodies were detected using enhanced chemiluminescence. After detecting biotinylated proteins, membranes were subjected to 1% (wt/vol) sodium azide and 1.5% (vol/vol) hydrogen peroxide for 10 min to quench the HRP activity from the previous analysis. After blocking with 10% (vol/vol) adult bovine serum and 0.2% Triton X-100 in PBS for 20 min, appropriate primary antibodies were added, including mouse monoclonal anti-tubulin (1:2000, T9026; Sigma-Aldrich). The primary antibodies were detected using HRP-conjugated anti-chicken (1:40,000, A9046; Sigma-Aldrich), anti-rabbit (1:40,000, G21234; Life Technologies), or anti-mouse (1:40,000, F21453; Life Technologies) antibodies.

Cell culture
HEK293 cells were obtained from the American Type Culture Collection, Manassas, VA (ATCC CRL-1573s), but not tested for contamination. Stable cell lines were generated using retroviral transduction. BiolID and BiolID2 in pBabe.puro vector were transfected into HEK293 Phoenix cells (National Gene Vector Biorepository, Indianapolis, IN) using Lipofectamine 2000 (Life Technologies) as recommended by the manufacturer. After overnight incubation at 37°C, the transfected cells were further incubated at 32°C for 36 h. The culture media were collected and centrifuged at 1000 × g for 5 min. The supernatant of the centrifuged media and Polybrene (4 μg/ml; Santa Cruz Biotechnology, Dallas, TX) were added to HEK293 cells. Puromycin (0.5 μg/ml; Fisher Scientific, Waltham, MA) was added to HEK293 cells 48 h after transduction. The expression of fusion proteins was further verified using immunofluorescence and immunoblotting. The stable cell lines were maintained in 5.0% CO2 at 37°C in DMEM (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS). To prepare biotin-depleted media, 50 ml of DMEM supplemented with 10% FBS was incubated with 1 ml of Dynabeads (MyOne Streptavidin C1; Life Technologies) overnight and filtered with a 0.22-μm filter. Cells were incubated with biotin-depleted media for 72 h before analysis. Cell growth under the biotin-depleted media was carefully monitored. Because the members of the Nup107–Nup160 complex are associated with mitotic proteins (Zuccolo et al., 2007; Chatel and Fahrenkrog, 2011), large-scale BiolID analyses for Nup43-BiolID2 or Nup43-BiolID were performed under growth-arresting low-serum conditions (DMEM supplemented with 0.1% FBS) to inhibit cell division.

Production of GST-fusion proteins
Expression of GST-fused BiolID and BiolID2 was induced with 1 mM isopropyl-β-D-thiogalactoside for 4 h at 37°C in a shaking incubator. The cell culture was then harvested by centrifugation at 6000 × g for 10 min. Harvested cells were resuspended in PBS and lysed by gentle sonication. Clarified lysate was incubated with glutathione resin (GBiosciences, St. Louis, MO) for 1 h with rotation and washed with five column volumes of PBS. Fusion proteins were eluted with three column volumes of 10 mM glutathione and 50 mM Tris, pH 8.0. Eluted fractions were then dialyzed against 1 l of PBS overnight. Protein concentration was measured based on ultraviolet absorbance at 280 nm.

In vitro assay
In vitro biotinylation reactions were performed in 500 nM BiolID-GST or BiolID fusion protein, 1.5 mM ATP, 1 mg/ml BSA, 1.8 mM β-mercaptoethanol, and a varied amount of biotin (50–800 μM) in PBS. Reactions were prepared in 0.5 ml and carried out overnight at 37°C. To investigate biotinylation in variable temperature, BiolID and BioID2 reactions were prepared as previously described. The reactions were supplemented with 800 μM biotin and incubated overnight. The 96-well PCR thermocyclers (Applied Biosystems, Foster City, CA) were programmed with a single stage to hold a specific temperature for the duration of the reaction. A sample volume containing 1 μg of BSA from each reaction was coated onto a 96-well polystyrene plate in 0.1 M citric acid and 0.2 M sodium phosphate, pH 5.0, for 1 h and blocked overnight in 5% nonfat dry milk. Plates were washed with PBS-T (PBS with 0.05% Tween-20) and developed with streptavidin-HRP (Invitrogen, Carlsbad, CA). Detection was determined by incubation with O-phenylenediamine substrate (Invitrogen) for 10 min and quenching with 2 M sulfuric acid before measurement of the absorbance at 495 nm in a SpectraMax MS plate reader (Molecular Devices, Sunnyvale, CA).

BioID pull down
For large-scale BioID pull down, 4 × 10^7 cells were incubated with 50 μM biotin for 16 h. After two times of PBS wash, the cells were lysed in 2.4 ml of lysis buffer containing 50 mM Tris, pH 7.4, 500 mM NaCl, 0.4% SDS, 1 mM dithiothreitol, and 1× Complete protease inhibitor (Halt Phosphatase Inhibitor Cocktail; Life Technologies). After collecting cells, Triton X-100 was added to 2% final concentration. After two times of sonication each for 1 min at 30% duty cycle and an output level of 4 (Sonifer-250; Branson), an equal volume of 50 mM Tris, pH 7.4, was added and cleaned using centrifugation at 16,500 × g for 10 min. The supernatant was collected to a 15-ml conical tube and incubated with 300 μl of Dynabeads overnight. Beads were collected using a magnetic stand and washed with twice with 2% (wt/vol) SDS, once with wash buffer containing 0.1% deoxycholate, 1% Triton X-100, 500 mM NaCl, 1 mM EDTA, and 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, once with wash buffer containing 250 mM LiCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, and 10 mM Tris, pH 8, and once with 50 mM Tris. Ten percent of the samples were saved for further analysis. The other 90% of samples were resuspended in 50 mM NH4HCO3 for MS analysis.

Mass spectrometry analysis and data analysis
Tryptic digestion of proteins isolated by BioID pull down was performed using an optimized method (Kim et al., 2014). Beads were resuspended in 50 μl of 8 M urea/50 mM ammonium bicarbonate and proteins and reduced by adding 2 μl of 0.5 M Tris (2-carboxyethyl)phosphine to 50 μl of beads–proteins suspension mix for 60 min at 30°C. The reaction was cooled to room temperature before alkylation with 4 μl of 0.5 M iodoacetamide at room temperature in the dark for 30 min. Sample volume was adjusted by adding 350 μl of 50 mM ammonium bicarbonate to dilute the 8 M urea to 1 M before trypsin digestion. MS-grade trypsin (Promega, Madison, WI) was added (1:20 ratio for beads, 5 μg of total trypsin for BioID samples) for overnight digestion at 30°C using an Eppendorf Thermomixer at 700 rpm. Digested peptides were isolated from the beads by centrifugation and magnetic separation, and peptide digests were transferred to a new tube and washed with 50 μl of 50 mM ammonium bicarbonate before pooling of digestions. Formic acid was added to the peptide solution (to 2%), followed by desalting by Microtrap (77720; Thermo Scientific, Waltham, MA), and then one-dimensional liquid chromatography (LC)–MS/MS analysis of peptides by a high-resolution, high-accuracy LC-MS/MS system consisting of an EASY-nLC 1000 HPLC, Acclaim PepMap peptide trap, 25 cm × 2 μm Easy-Spray C18 column, Easy Spray Source, and Q Exactive Plus mass spectrometer (all from Thermo
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Fisher Scientific. A 225-min gradient consisting of 5–16% B (100% acetonitrile) in 140 min, 16–28% in 70 min, 28–38% in 10 min, and 38–85% in 5 min was used to separate the peptides. The total LC time was 250 min. The Q Exactive Plus was set to scan precursors at 70,000 resolution, followed by data-dependent MS/MS at 17,500 resolution of the top 12 precursors.

The LC-MS/MS raw data of three technical replicates were combined and submitted to Sorcerer Enterprise, version 3.5 (Sage-N Research, Milpitas, CA) with SEQUEST algorithm as the search program for peptide/protein identification. SEQUEST was set up to search the target-decoy EBI:PI.HUAMAN database containing protein sequences, using trypsin for enzyme with an allowance of up to two missed cleavages, Semi Tryptic search, fixed modification of 57 Da for cysteine to account for carboxyamidomethylation, and precursor mass tolerance of 50 ppm. Differential search includes 16 Da for methionine oxidation and 226 on lysine for biotinylation. The search results were viewed, sorted, filtered, and statistically analyzed by using comprehensive proteomics data analysis software Peptide/Protein prophet, version 4.02 (ISB). The minimum transproteomic pipeline (TPP) probability score for proteins was set to 0.9 to assure very low error (much less than a false discovery rate of 2%) with reasonably good sensitivity. The differential spectral count analysis was done by QTools, an open-source in-house–developed tool for automated differential peptide/protein spectral count analysis (Brill et al., 2009). Proteins with fewer than three spectral counts or common MS background proteins, including keratins, histones, and ribosomal proteins, were removed due to the lack of confidence. Any candidate identified by Nup43-BioID or Nup43BioID2 was excluded if the relative percentage of total spectral counts was three-fold less than in the BioID- or BioID2-only, respectively. Statistical analysis

For the in vitro biotinylation assays, the biotinylation results are represented as mean ± SEM. Differences between multiple groups were compared by one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test multiple comparisons. To compare the targeting ability of BioID-Sun2 and BioID2-Sun2, the relative mean intensity of the fusion proteins was analyzed by an unpaired t test.

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