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Direct Proximity Tagging of Small Molecule Protein Targets Using an Engineered NEDD8 Ligase

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Supporting Information

ABSTRACT: Identifying the protein targets of bioactive small molecules remains a major problem in the discovery of new chemical probes and therapeutics. While activity-based probes and photo-cross-linkers have had success in identifying protein targets of small molecules, each technique has limitations. Here we describe a method for direct proximity tagging of proteins that bind small molecules. We engineered a promiscuous ligase based on the NEDD8 conjugating enzyme, Ubc12, which can be covalently linked to a small molecule of interest. When target proteins bind the small molecule, they are directly labeled on surface lysines with a biotinylated derivative of the small ubiquitin homologue, NEDD8. This unique covalent tag can then be used to identify the small molecule binding proteins. Utilizing the drug dasatinib, we have shown that dasatinib-directed NEDDylation occurs for known endogenous protein binders in complex cell lysates. In addition, we have been able to improve NEDDylation efficiency through rational mutagenesis. Finally, we have shown that affinity-directed NEDDylation can be applied to two other protein−ligand interactions beyond kinases. Proximity tagging using this engineered ligase requires direct binding of the target and, thus, provides a useful and orthogonal approach to facilitate small molecule target identification.

Small molecules are invaluable tools for the study of cellular signaling pathways and ultimately useful as therapeutics that aid in the treatment of human disease.[1] Identifying the protein targets of small molecules remains a major challenge in the field of drug discovery. Enrichment using small molecule affinity resins is a common technique for identifying protein interactions, but generally requires tight binding to sustain association during the isolation of the proteins. Other target identification methods include covalent enrichment strategies utilizing activity-based probes and photoreactive-cross-linking probes coupled with LC-MS.[2] However, activity-based probes require the binding site to contain a specific reactive residue. Photoreactive-cross-linkers allow for more promiscuous reactivity, but reaction yields are low and are sensitive to orientation.[3] Recently, several proximity-based enzymatic tagging technologies have been reported for identifying protein−protein interactions by transferring a covalent label from the "bait" protein to the "prey" partner proteins.[4] Two of these technologies, BioID and APEX, generate a diffusive reactive biotin derivative from the tagging enzyme active site which then labels proteins in the vicinity. However, the diffusive nature of this reactive probe does not ensure that tagged proteins actually make direct contact with the "bait" protein. Recently, we reported a direct enzymatic tagging technology based on an engineered form of the NEDD8 E2 conjugating enzyme, Ubc12, which we termed the NEDDylator.[4a] NEDD8 is a rare ubiquitin-like homologue that is part of a natural proximity-tagging system that catalyzes formation of an amide bond to surface lysines,[5] one of the most common amino acid in proteins, found at 7.2% abundance.[6] The NEDD8 protein tag can only be transferred to the prey protein by direct attack of the prey's lysine ε-amines upon the thioester in the active site of the Bait-NEDDylator, which ensures that direct contact must be made between the bait and prey proteins (Figure S1). The NEDDylator was previously used to identify >50 candidate protein substrates of the IAP family of ubiquitin E3 ligases.[4a] While we successfully applied the NEDDylator to E3 ligase interactions, a pathway with high homology to the NEDD8 pathway, we wondered if the NEDDylator could be used as a generic proximity-tagging enzyme and applied to proteins and ligands not co-evolved from ubiquitin or NEDD8 pathways.

To date, proximity-tagging enzyme systems have been utilized to identify protein−protein interactions. Here, we extend the use of the NEDDylator to find the protein targets of a bioactive small molecule. We produced a two-domain construct in which the small molecule is covalently attached and displayed via SNAP-tag[7] that is genetically fused to the NEDD8-E2 (Figure 1). We reasoned that once the target proteins bound the small molecule, they would be covalently

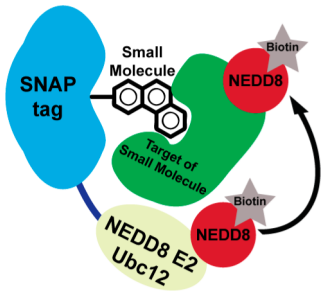

Figure 1. A proximity-tagging enzyme designed to label the protein targets of bioactive small molecules.

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labeled by our His_6-biotin tagged derivative of NEDD8 (HB-NEDD8, Figure S2). This tag allows facile purification and target identification by LC-MS/MS as described previously.[4a]

As a test case, we synthesized a derivative of the clinically approved tyrosine kinase inhibitor dasatinib, **DS1**, capable of being displayed from SNAP-tag (Figure 2a).[8] Dasatinib is

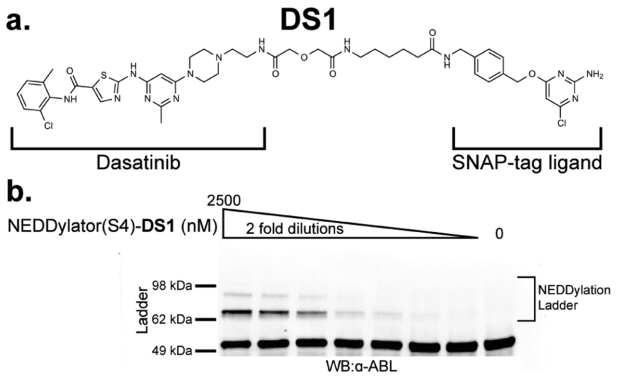

Figure 2. NEDDylator(S4) displaying dasatinib is capable of binding and NEDDylating ABL kinase. (a) **DS1**, a derivative of dasatinib capable of being displayed from NEDDylator(S4) and (S10). (b) Western blot showing dose-dependent NEDDylation of ABL-3D by NEDDylator(S4)-**DS1**.

approved for the treatment of chronic myelogenous leukemia and is a potent but somewhat promiscuous inhibitor of protein kinases, including ABL and SRC. After synthesis and purification, we measured the IC_{50} of **DS1** for a 3-domain construct (SH3-SH2-Kinase) of ABL (ABL-3D) and a kinase-domain-only construct of SRC (SRC-KD) in an *in vitro* activity assay and were pleased to find that it maintained an affinity in the low nanomolar range (Figure S3).

We hypothesized that linker length between the SNAP-tag and Ubc12 domains could affect labeling efficiency. Thus, we generated two constructs with varying serine-glycine linkers between SNAP-tag and Ubc12, NEDDylator(S4) and NEDDylator(S10) (Figure S4) and conjugated them with **DS1** to generate the corresponding protein-small-molecule chimeras, NEDDylator(S4)-**DS1** and NEDDylator(S10)-**DS1**. We tested the ability of the chimeras to inhibit ABL-3D and found each showed only a 3- to 6-fold reduction in potency when compared to free **DS1** (Figure S3). While a larger loss in affinity was observed for SRC-KD, the chimeras still inhibited at low nanomolar concentrations (Figure S3). Comparable reductions in potency have previously been reported when displaying kinase inhibitors from the active site of SNAP-tag.[9]

We next tested the ability of NEDDylator(S4)-**DS1** and NEDDylator(S10)-**DS1** to transfer the HB-NEDD8 tag to ABL-3D. Incubation of varying concentrations of NEDDylator-(S4)-**DS1** and NEDDylator(S10)-**DS1** with recombinant ABL-3D (200 nM, a physiologically relevant concentration)[10] in NEDDylation reaction buffer for 2 h resulted in NEDDylation of ABL-3D, as measured by laddering of ABL-3D on a Western blot (Figures 2b and S5). NEDDylation of ABL-3D was dependent on NEDDylator concentration, with no NEDDylation of ABL-3D observed in the absence of NEDDylator(S4)-**DS1** and NEDDylator(S10)-**DS1**. As the linker length between SNAP-tag and Ubc12 had little effect on NEDDylation efficiency, we chose to use the shorter NEDDylator(S4)-**DS1** for the remaining studies.

While performing the NEDDylation assays, we observed that self-NEDDylation of the NEDDylator protein was a common side reaction. We hypothesized that reducing self-NEDDylation may lead to improved trans-NEDDylation. To test this, we generated a mutant in which all 29 lysines of NEDDylator(S4) were mutated to arginine. Unfortunately, this mutant had poor solubility and did not retain SNAP-tag activity. For this reason, we decided to take a more targeted mutational approach. After several iterations we arrived at a mutant with three lysines removed from SNAP-tag and seven lysines removed from the N-terminal tail of Ubc12 (Figure S6a,b). This new construct, called NEDDylator(10ΔK), when compared to NEDDylator-(S4), showed a ∼50% decrease in self-NEDDylation and ∼25% increase in the trans-NEDDylation of ABL-3D (Figure S6c–f). While the percent of trans-NEDDylation was still not 100%, we felt the percent labeling was comparable to that observed for photoreactive probes[3] and was sufficient for application in protein interaction studies.

With our improved NEDDylator in hand, we performed additional NEDDylation reactions with ABL-3D and SRC-KD. NEDDylator(10ΔK)-**DS1** NEDDylated both ABL-3D and SRC-KD (Figure 3a). Importantly, if the NEDD8-E1, NAE1/

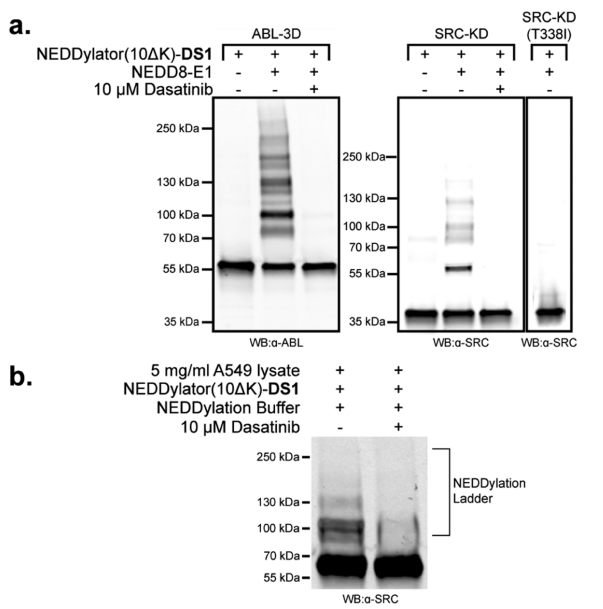

Figure 3. A 10-lysine-to-arginine/glutamine mutant of NEDDylator-(S4) labels ABL-3D and SRC-KD in a NEDD8-E1 and dasatinib-dependent manner. Western blots showing the NEDDylation (a) of ABL-3D, SRC-KD, and SRC(T338I)-KD by NEDDylator(10ΔK)-**DS1** and (b) of endogenous SRC by NEDDylator(10ΔK)-**DS1** in A549 cell lysate.

UBA3,[5] which is necessary for Ubc12-NEDD8 thioester formation, was not added to the reaction, then NEDDylation of ABL-3D and SRC-KD was not observed (Figure 3a). Additionally, competition with 10 μM of dasatinib inhibited NEDDylation of ABL-3D and SRC-KD (Figure 3a). NEDDylation experiments were also performed with SRC-KD(T338I), a mutation that is known to confer resistance to dasatinib.[11] As expected, no NEDDylation of SRC-KD(T338I) was observed (Figure 3a). We also NEDDylated a full-length SRC construct and observed increased NEDDylation relative to the kinase-domain-only SRC (Figure S7), suggesting that protein target length may affect NEDDylation efficiency. We

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next attempted to tag endogenous SRC kinase in cellular lysate from lung-cancer-derived A549 cells, a cell line known to express SRC. We incubated 5 mg/mL lysate with 1 μM NEDDylator(10ΔK)-**DS1** and NEDDylation buffer in the presence or absence of 10 μM dasatinib. Dasatinib-competitive NEDDylation of endogenous SRC was observed by Western blot (Figure 3b). Together, these results indicate that NEDDylation of ABL and SRC is dependent on active site binding to **DS1**. Thus, NEDDylation can be used as evidence of direct interaction between a small molecule and its protein target.

We next performed a stable isotope labeling in cell lysate (SILAC) mass spectrometry study to see if we could rapidly identify multiple targets of dasatinib in cell lysate (Figure S8).[12] Heavy-Lys/Arg-labeled A549 lysate was incubated with NEDDylator(10ΔK)-**DS1** plus DMSO, while light-Lys/Arg lysate was incubated with NEDDylator(10ΔK)-**DS1** plus competitive dasatinib. After labeling, the reactions were denatured with urea, combined, enriched on NeutrAvidin resin, washed, subjected to on-bead tryptic digestion, and analyzed using LC-MS/MS. This protocol was performed twice as two independent replicates. MS/MS spectra were searched and assigned to peptides using ProteinProspector search software (http://prospector.ucsf.edu/prospector/mshome.htm). A total of 864 proteins were identified in both replicates (Figure S9). The median SILAC heavy/light ratios of identified proteins from each replicate are plotted (Figure 4).

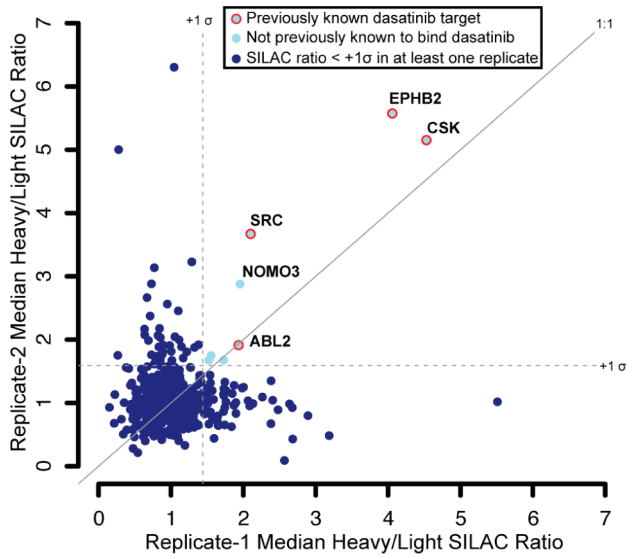

Figure 4. Identification of dasatinib targets in A549 lysate using NEDDylator(10ΔK)-**DS1** and a SILAC-based mass spectrometry method. Plotting the median heavy/light SILAC ratio from each NEDDylation replicate reveals eight proteins with ratios >+1σ in both replicates, four of which are previously known targets of dasatinib.

As expected, the majority of the 864 proteins identified were commonly observed background proteins[13] and native NEDD8 substrates that showed SILAC ratios centered around 1. Of the eight proteins with unique peptides that showed heavy/light ratios at least one standard deviation above the mean (+1σ) in both replicates, four were the previously known targets of dasatinib: ABL2, SRC, EPHB2, and CSK.[14] Due to the high homology between kinases, several of the peptides found for ABL2, SRC, and EPHB2 can also be assigned to other known dasatinib targets. With homology taken into account, we identified peptides that can be assigned to a total of 19 previously known targets of dasatinib (Table S1-Tab5). This number is in agreement with previously reported dasatinib enrichment experiments.[15] Of the four additional proteins with a heavy/light ratio >+1σ, three (HNRPF, ARPC3, and RL37A) were very close to the cutoff. The remaining protein, NOMO3, is a poorly annotated single-pass membrane protein that has not previously been reported to interact with dasatinib. We feel the ability of our SILAC-NEDDylator workflow to highlight 8 out of 864 identified proteins as possible targets, 4 of which are known to be true-positives, supports the utility of our methodology for identifying the protein targets of small molecules.

Based on our success in labeling dasatinib targets, we wondered if the NEDDylator could be applied to labeling the protein targets of other small molecules. To evaluate this, we generated a derivative of the small molecule methotrexate[16] capable of being displayed from NEDDylator(10ΔK), **MS1** (Figure S10a). When assayed, NEDDylator(10ΔK)-**MS1** showed methotrexate-dependent NEDDylation of the known target, dihydrofolate reductase (DHFR) (Figure S10b). To further evaluate the generality of our NEDDylator enzyme, we generated a NEDDylator construct with a Bcl-xL binding peptide[17] genetically fused to the N-termini of Ubc12, NEDDylator(7ΔK)-**BAD** (Figure S10c). Incubation of NEDDylator(7ΔK)-**BAD** with Bcl-xL resulted in NEDDylation of Bcl-xL (Figure S10d). Addition of the known Bad-like BH3 mimetic small molecules,[18] ABT-737 and ABT-263, inhibited NEDDylation. Together, these results support the generality of our NEDDylator proximity-tagging enzyme.

In summary, we have shown that small-molecule-directed NEDDylation of protein targets is possible and is dependent on binding of the small molecule to the target. Using rational mutagenesis, we have developed an enhanced NEDDylator protein that shows reduced self-NEDDylation and increased trans-NEDDylation of targets. We have further shown that small-molecule-directed NEDDylation can be performed in mammalian cell lysates, allowing for the enrichment and identification of protein targets using SILAC-based quantitative mass spectrometry. Because the small molecule portion of this technology is modular, we feel that this method can be readily applied to other small molecules of interest, so long as they can be derivatized without dramatically affecting binding, a limitation shared with other affinity enrichment approaches.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b06828.

Experimental details and data (PDF)

Peptide data (XLSX)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) (a) Frye, S. V. *Nat. Chem. Biol.* **2010**, *6*, 159. (b) Castoreno, A. B.; Eggert, U. S. *ACS Chem. Biol.* **2011**, *6*, 86.
(2) (a) Simon, G. M.; Niphakis, M. J.; Cravatt, B. F. *Nat. Chem. Biol.* **2013**, *9*, 200. (b) Mackinnon, A. L.; Taunton, J. *Curr. Protoc. Chem. Biol.* **2009**, *1*, 55. (c) Dorman, G.; Prestwich, G. D. *Trends Biotechnol.* **2000**, *18*, 64.
(3) Kawamura, A.; Hindi, S.; Mihai, D. M.; James, L.; Aminova, O. *Bioorg. Med. Chem.* **2008**, *16*, 8824.
(4) (a) Zhuang, M.; Guan, S.; Wang, H.; Burlingame, A. L.; Wells, J. A. *Mol. Cell* **2013**, *49*, 273. (b) Rhee, H. W.; Zou, P.; Udeshi, N. D.; Martell, J. D.; Mootha, V. K.; Carr, S. A.; Ting, A. Y. *Science* **2013**, *339*, 1328. (c) Lam, S. S.; Martell, J. D.; Kamer, K. J.; Deerinck, T. J.; Ellisman, M. H.; Mootha, V. K.; Ting, A. Y. *Nat. Methods* **2015**, *12*, 51. (d) Roux, K. J.; Kim, D. I.; Raida, M.; Burke, B. J. *J. Cell Biol.* **2012**, *196*, 801. (e) Kim, D. I.; Birendra, K. C.; Zhu, W.; Motamedchaboki, K.; Doye, V.; Roux, K. J. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, E2453.
(5) Enchev, R. I.; Schulman, B. A.; Peter, M. *Nat. Rev. Mol. Cell Biol.* **2015**, *16*, 30.
(6) King, J. L.; Jukes, T. H. *Science* **1969**, *164*, 788.
(7) (a) Keppler, A.; Gendreizig, S.; Gronemeyer, T.; Pick, H.; Vogel, H.; Johnsson, K. *Nat. Biotechnol.* **2002**, *21*, 86. (b) Keppler, A.; Pick, H.; Arrivoli, C.; Vogel, H.; Johnsson, K. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 9955.
(8) McCormack, P. L.; Keam, S. J. *Drugs* **2011**, *71*, 1771.
(9) (a) Hill, Z. B.; Perera, B. G.; Maly, D. J. *J. Am. Chem. Soc.* **2009**, *131*, 6686.
(10) Beck, M.; Schmidt, A.; Malmstroem, J.; Claassen, M.; Ori, A.; Szymborska, A.; Herzog, F.; Rinner, O.; Ellenberg, J.; Aebersold, R. *Mol. Syst. Biol.* **2011**, *7*, 549.
(11) Li, J.; Rix, U.; Fang, B.; Bai, Y.; Edwards, A.; Colinge, J.; Bennett, K. L.; Gao, J.; Song, L.; Eschrich, S.; Superti-Furga, G.; Koomen, J.; Haura, E. B. *Nat. Chem. Biol.* **2010**, *6*, 291.
(12) Ong, S. E.; Mann, M. *Nat. Protoc.* **2007**, *1*, 2650.
(13) Mellacheruvu, D.; Wright, Z.; Couzens, A. L.; Lambert, J. P.; St-Denis, N. A.; Li, T.; Miteva, Y. V.; Hauri, S.; Sardiu, M. E.; Low, T. Y.; Halim, V. A.; Bagshaw, R. D.; Hubner, N. C.; Al-Hakim, A.; Bouchard, A.; Faubert, D.; Fermin, D.; Dunham, W. H.; Goudreault, M.; Lin, Z. Y.; Badillo, B. G.; Pawson, T.; Durocher, D.; Coulombe, B.; Aebersold, R.; Superti-Furga, G.; Colinge, J.; Heck, A. J.; Choi, H.; Gstaiger, M.; Mohammed, S.; Cristea, I. M.; Bennett, K. L.; Washburn, M. P.; Raught, B.; Ewing, R. M.; Gingras, A. C.; Nesvizhskii, A. I. *Nat. Methods* **2013**, *10*, 730.
(14) Karaman, M. W.; Herrgard, S.; Treiber, D. K.; Gallant, P.; Atteridge, C. E.; Campbell, B. T.; Chan, K. W.; Ciceri, P.; Davis, M. I.; Edeen, P. T.; Faraoni, R.; Floyd, M.; Hunt, J. P.; Lockhart, D. J.; Milanov, Z. V.; Morrison, M. J.; Pallares, G.; Patel, H. K.; Pritchard, S.; Wodicka, L. M.; Zarrinkar, P. P. *Nat. Biotechnol.* **2008**, *26*, 127.
(15) Golkowski, M.; Brigham, J. L.; Perera, G. K.; Romano, G. E.; Maly, D. J.; Ong, S. E. *MedChemComm* **2014**, *5*, 363.
(16) Rajagopalan, P. T.; Zhang, Z.; McCourt, L.; Dwyer, M.; Benkovic, S. J.; Hammes, G. G. *Proc. Natl. Acad. Sci. U. S. A.* **2002**, *99*, 13481.
(17) Petros, A. M.; Nettesheim, D. G.; Wang, Y.; Olejniczak, E. T.; Meadows, R. P.; Mack, J.; Swift, K.; Matayoshi, E. D.; Zhang, H.; Thompson, C. B.; Fesik, S. W. *Protein Sci.* **2000**, *9*, 2528.
(18) Delbridge, A. R.; Strasser, A. *Cell Death Differ.* **2015**, *22*, 1071.

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J. Am. Chem. Soc. XXXX, XXX, XXX−XXX