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Communication

μMap Photoproximity Labeling Enables Small Molecule Binding Site Mapping

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ABSTRACT: The characterization of ligand binding modes is a crucial step in the drug discovery process and is especially important in campaigns arising from phenotypic screening, where the protein target and binding mode are unknown at the outset. Elucidation of target binding regions is typically achieved by X-ray crystallography or photoaffinity labeling (PAL) approaches; yet, these methods present significant challenges. X-ray crystallography is a mainstay technique that has revolutionized drug discovery, but in many cases structural characterization is challenging or impossible. PAL has also enabled binding site mapping with peptide- and amino-acid-level resolution; however, the stoichiometric activation mode can lead to poor signal and coverage of the resident binding pocket. Additionally, each PAL probe can have its own fragmentation pattern, complicating the analysis by mass spectrometry. Here, we establish a robust and general photocatalytic approach toward the mapping of protein binding sites, which we define as identification of residues proximal to the ligand binding pocket. By utilizing a catalytic mode of activation, we obtain sets of labeled amino acids in the proximity of the target protein binding site. We use this methodology to map, in vitro, the binding sites of six protein targets, including several kinases and molecular glue targets, and furthermore to investigate the binding site of the STAT3 inhibitor MM-206, a ligand with no known crystal structure. Finally, we demonstrate the successful mapping of drug binding sites in live cells. These results establish μMap as a powerful method for the generation of amino-acid- and peptide-level target engagement data.

A significant proportion of contemporary first in class drugs have been discovered by phenotypic drug discovery (PDD).[1] The advantages of PDD have led to significant investments across biopharma, occupying up to 40% of project portfolios.[2] The widespread adoption of unbiased screening strategies requires robust methods for validation, as correct mechanistic assignment is a key determinant of clinical success.[3] However, target mischaracterization remains common, leading to misallocation of resources toward candidates that have little therapeutic efficacy.[4] Key to effective therapeutic development is the identification of not only the target but also the active binding site of a small molecule drug. Structural understanding is integral to rational optimization and aids in comprehensive assignment of ligand−target engagement.[5] This is especially important in cases where (1) no known binding site exists; (2) multiple binding sites are present; (3) allosteric or orthosteric inhibition prevails;[6] and (4) the ligand induces complexation between multiple protein targets.[7] Thus, the development of echnologies capable of giving insight into the location of drug binding sites and providing structural information remains a long-standing goal in drug discovery (Figure 1a).

Apart from computational tools, binding site determination has been classically achieved through three methods: X-ray crystallography (XRC), hydrogen−deuterium exchange mass spectrometry (HDX-MS), and photoaffinity labeling (PAL). XRC has revolutionized drug discovery by providing vast libraries of ligand binding events and binding pockets for rational drug design.[8] Nevertheless, there are many instances where crystallography is challenging or impossible.[9] Additionally, crystal structures may not accurately represent the solution-phase dynamics of the ligand−protein interaction.[10] And while HDX-MS has been enabling,[11,12] binding site characterization of low affinity ligands remains challenging.[13] Alternative approaches that leverage covalent chemical capturing of binding sites, such as photoaffinity labeling (PAL), are necessary in these cases.[14] PAL utilizes an affinity handle-bound cross-linking group, such as a diazirine, appended to the ligand of interest.[15] Following binding of the small molecule−PAL probe conjugate, UV irradiation activates the probe, which can covalently cross-link to proximal residues. Cross-linking events are then detected through chemoproteomic workflows to provide binding site structure and location information.[16] This approach has become routine in biopharma, and is highly enabling for target ID.[17] While many successful examples of the application of PAL to binding site mapping exist,[18−22] the stoichiometric nature of the cross-linker renders this method capricious, and significant optimization is often required to maximize labeling over off-

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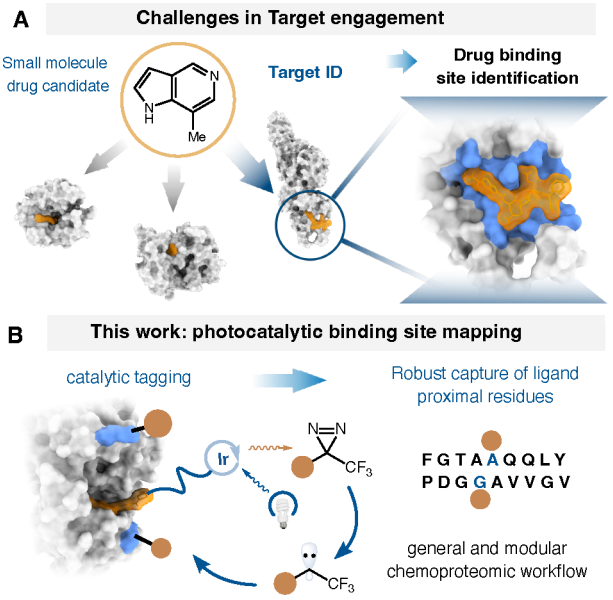

Figure 1. (a) Identification of drug candidate binding sites is a significant challenge in drug development. (b) This work: photocatalytic proximity labeling (μMap) as a platform for the mapping of drug-target binding sites.

target quenching.[23,24] Furthermore, the fragmentation of PAL probes can complicate mass spectrometry analysis, necessitating preliminary experimentation to determine potential adduct masses for each probe.[25,26] Recently, promising new approaches to experimental binding pocket discovery have been described. Both small molecule and RNA−protein interactions can be interrogated via reverse footprinting, cross-linking-isolation-pulldown (CLIP), and two-dimensional NMR techniques.[27−29]

Despite these advances, the binding site determination remains challenging. Thus, the development of a platform that allows robust mapping of ligand binding regions without the need for extensive optimization could greatly accelerate drug development.

We recently reported a method for small molecule target identification that utilizes a photocatalytic energy transfer mechanism.[30] Specifically, the iridium photocatalyst is appended to a small molecule ligand, allowing it to be localized to its target. Upon visible light irradiation, the probe generates carbenes from diazirine precursors only in the immediate vicinity of the ligand−catalyst conjugate. This enables the tagging of any protein associated with the small molecule ligand, which can be analyzed via chemoproteomics. Critically, the catalytic nature of this method allows multiple tagging events of the target, amplifying signal. Using this approach, we have demonstrated successful target identification of several membrane, cytosolic, and nuclear proteins, several of which could not be identified with classic PAL. We hypothesized that the tight labeling radius of the carbene would lead to the labeling of amino acid residues about the ligand binding site. Thus, we questioned if we could leverage the high on-target signal of this platform to achieve robust binding site mapping, which we define as labeling of residues proximal to the binding site of the ligand of interest (Figure 1b).

First, we first sought to use an in vitro selectivity assay to examine the ability of specific small molecule−iridium conjugates to direct labeling toward a given target protein. We chose bovine carbonic anhydrase (CA) as a model substrate, which has been shown as a robust target for ligand directed modification previously.[31] In the presence of an equimolar ratio of CA and a competitor protein, bovine serum albumin (BSA), we hypothesized that selective labeling of CA over BSA could be achieved with the sulfonamide-tethered photocatalyst **1** (Figure 2a). We anticipated that the sulfonamide moiety would preferentially bind to CA, and subsequent irradiation in the presence of a biotin-tagged diazirine, **2**, would lead to selective labeling of CA over BSA. Indeed, analysis of labeling ratios by immunoblotting with streptavidin demonstrated efficiency and selectivity (Figures 2b, S1).

Having confirmed selective labeling of CA via Western blot analysis, we then sought to develop a mass-spectrometry pipeline for the identification of labeled residues (Figure 2c). Thus, performing targeted labeling of carbonic anhydrase with catalyst **1**, protein was then reduced and alkylated, cleaned up using single-pot solid phase enhanced sample preparation (SP3),[32] and digested with trypsin. The sample was analyzed by MS2 under data-dependent acquisition (DDA). Resultant data were then processed in MSFragger[33] to reveal 75 carbonic anhydrase peptides, representing an 80% sequence coverage. To identify the highest confidence labeled residues, modified peptides were sorted by fold change in intensity vs free catalyst control. A cutoff of >2 Log2FC in peptide intensity over free photocatalyst control, with a −Log10 p-value of greater than 1.3, and at least 2 peptide spectral matches (PSMs) on average across replicates was applied. Visual analysis of the corresponding scatterplot clearly reveals two modified peptides (Figure S9). MS/MS spectra of these two labeled peptides confirmed carbene modification and identified the two highest confidence modified residues: H2 and Q135 (Figure 2D). Gratifyingly, examination of the crystal structure of CA bound to sulfonamide reveals the proximity (11 and 17 Å, respectively) of these residues to the sulfonamide photocatalyst binding site, as defined by the distance from labeled residue to drug molecule (Figure 2E, orange).

Having established a mass spectrometry workflow, we next sought to examine the generality and scope by applying this method to other protein targets. Thus, we questioned whether we could capture ligand-proximal residues of (+)-JQ-1 to bromodomain 4 (BRD4). Using both the active and inactive enantiomers of JQ-1, we prepared two PEG3-linked photocatalyst conjugates (Figure 3a, **3**, active (+) enantiomer shown). We then performed labeling of recombinant BRD4 (residues 44−168) comparing the active enantiomer catalyst (**3**) against the inactive enantiomer catalyst. Subsequent mass-spec analysis revealed four modified peptides, containing a total of six labeled residues, each directly proximal to the JQ-1 binding site. Residues H77, Q78, F79, A80, W81, and Q84 are all within 15 Å of the binding site. Notably, P82 is within the (+)-JQ-1 binding pocket.

We next examined dasatinib, a marketed treatment for myelogenous leukemia that displays nanomolar binding to Bruton's tyrosine kinase (BTK; kd = 5 nM). Mass spectrometry analysis of the modified peptides revealed several labeled residues within no more than 16 Å of the binding site, including residue Y551, which is positioned within the binding pocket (Figure 3b). With the goal of exploring the mapping of other kinase binding sites, we next employed Ir-conjugated AT7519 to the labeling of recombinant cyclin-dependent kinase, CDK2. MS/MS analysis of photolabeled CDK2 revealed three labeled peptides, all less than 15 Å from the AT7519 binding pocket (Figure 3c).

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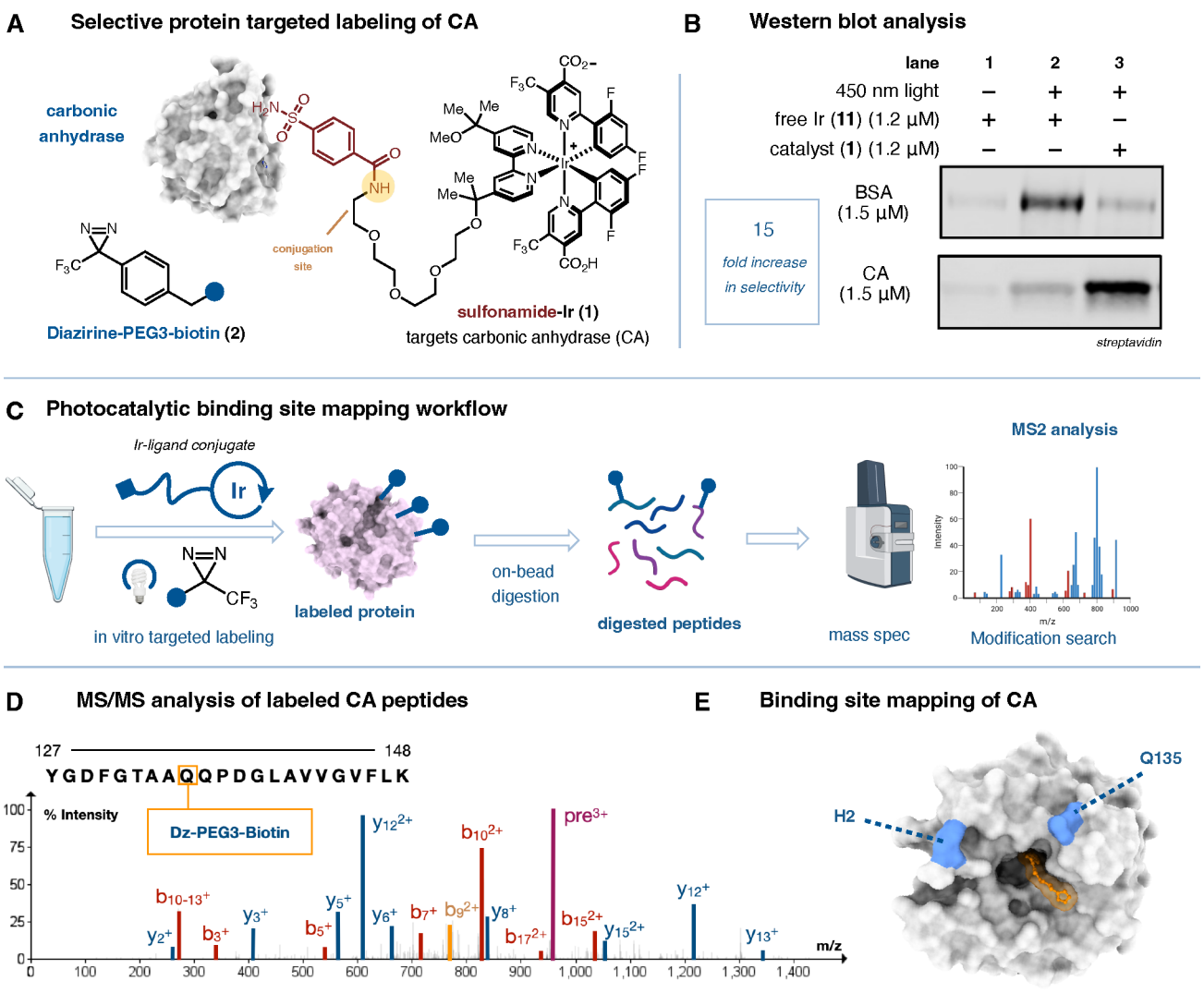

Figure 2. (a) in vitro targeted labeling of bovine carbonic anhydrase (CA) using benzenesulfonamide iridium (**1**). (b) Western blot analysis of in vitro labeling demonstrates the successful targeted labeling of CA over bovine serum albumin (BSA). (c) Workflow for label-free mass spec binding site identification. (d) Illustrative MS2 spectra for peptide YGDFGTAAQQPDGLAVVGVFLK showing the modification of glutamine 135. (e) Residues detected for the binding site mapping of bovine CA with sulfonamide photocatalyst (**1**) using a modified peptide cutoff of >2 Log2FC intensity vs free photocatalyst control. PDB: 6SKS.

Elucidating the target of a molecular glue has been historically challenging.[34,35] We hypothesized that our novel μMap platform might offer an efficient solution. We thus set out to map the binding site of the molecular glue lenalidomide to its target, E3 ligase cereblon (CRBN). Mass spec analysis of the photolabeling employing lenalidomide-PEG0-Ir (**6**) showed a molecular footprint of the small molecule binding site on CRBN, capturing seven binding site proximal residues within 16 Å of the lenalidomide docking site (Y349 not pictured as it lies within the binding pocket) (Figure 3d).

A method capable of simultaneously characterizing protein complexes and their ligand binding sites would greatly expedite the design of new molecular glues. Thus, we applied our platform to the mapping of the FKBP12-rapamycin-mTOR complex. We prepared the rapamycin−iridium conjugate (**7**) by esterification at the C40 alcohol, a well-established exit vector (Figure 3e).[36,37] Western blot verification revealed labeling of FKBP12 and mTOR only in the presence of photocatalyst with labeling ablated by the addition of excess rapamycin (Figure S6). We then performed our mass-spectrometry workflow to detect several labeled residues on both protein targets (Figure 3e). Gratifyingly, we observed three FKBP12 residues within 10 Å from rapamycin: K53, G52, and R58. Furthermore, our system successfully captured several mTOR residues. Notably, residues L2261, N2262, H2265, and H2277 were located within the "catalytic cleft" of mTOR, directly facing the proximal rapamycin−FKBP12 interface and photocatalyst exit vector. These data demonstrate the capacity of our platform to capture proteins in complex, as well as to detect "through space" residues proximal to the binding interface.

We next sought to map the binding site of a compound with no known crystal structure. Human signal transducer and activator of transcription 3 (STAT3) is aberrantly activated in numerous cancers, driving proliferation, metastasis, and chemoresistance.[38] Targeting of STAT3 presents significant therapeutic potential.[39] However, the lack of deep binding pockets across the protein surface renders STAT3 an "undruggable" target. This is exemplified by the few candidates to enter clinical trials over the past 20 years. Naphthalene-sulfonamides are one class of STAT3 inhibitors currently in clinical trials.[40,41] Originally

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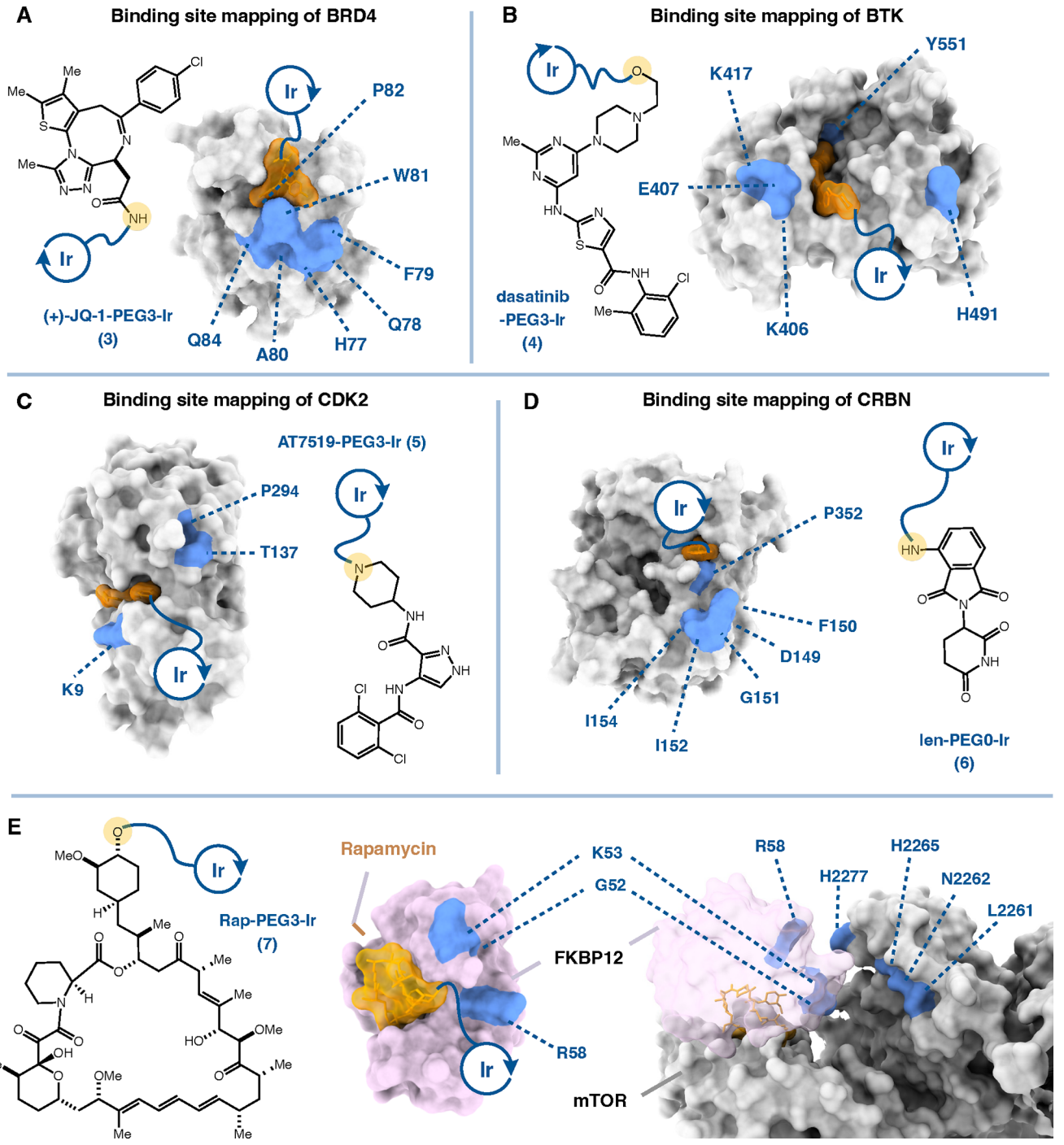

Figure 3. (a) Residues detected for the binding site mapping of recombinant BRD4 using the (+)-JQ-1 photocatalyst (**3**). Applied modified peptide cutoff at >4 Log2FC intensity vs inactive enantiomer control. PDB: 3MFX. (b) Binding site mapping of recombinant BTK using a dasatinib photocatalyst (**4**). Applied modified peptide cutoff at >3.85 Log2FC intensity vs off compete control. PDB: 6AUB. Ligand shown is CGI2815. Distances from the ligand binding site were calculated from PDB: 3K54 (c) Binding site mapping of recombinant CDK2 using AT7519 photocatalyst (**5**). Applied modified peptide cutoff at >2.5Log2FC intensity vs off-compete control. PDB: 2VU3 (d) Residues detected for the binding site mapping of recombinant CRBN using lenalidomide photocatalyst (**6**) using a modified peptide cutoff of >3 Log2FC intensity vs off compete control. PDB: 6BN7. (e) Binding site mapping of the FKBP12-rapamycin-mTOR ternary complex with rapamycin photocatalyst (**7**) using a modified peptide cutoff of >6 Log2FC intensity vs off compete control for mTor and >3.5 Log2FC intensity vs off compete control for FKBP12. PDB: 8ERA.

presumed to bind the orthosteric SH2 domain, studies from Ball and co-workers using a rhodium(II)-functionalized derivative suggest that these compounds instead bind allosterically to the distal coiled-coil domain (CCD).[40] MM-206 (**9**), a naphthalene sulfonamide inhibitor that has shown promising anti-STAT3 activity in preclinical models, is also suggested to inhibit STAT3 via this allosteric binding mode.[40,42] However, to date, there remains no bound crystal structure or mapping of the STAT3 binding site of MM-206. Thus, we questioned if our platform could be used to interrogate orthosteric versus allosteric for this STAT3 inhibitor.

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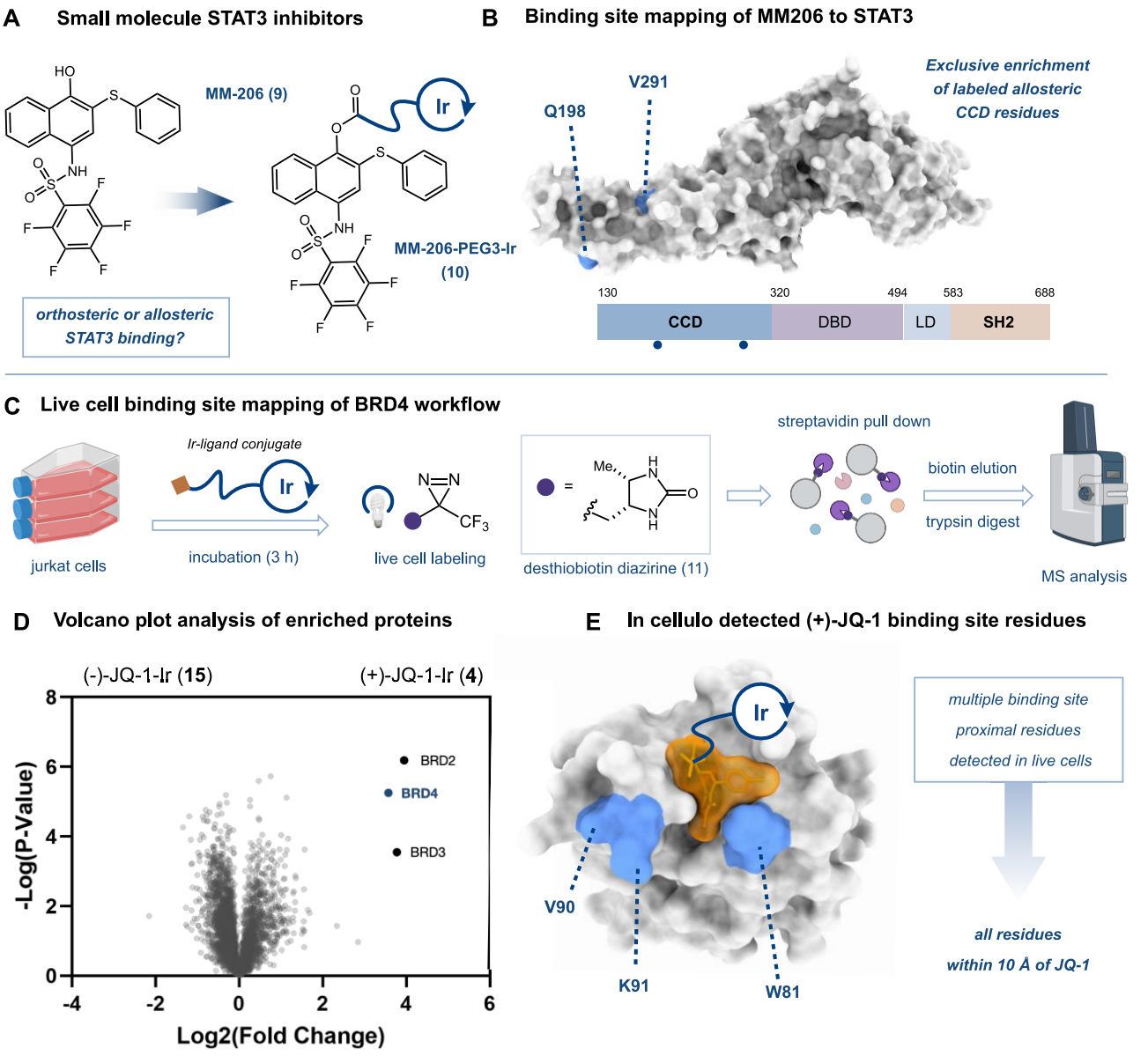

Figure 4. (a) Recent work suggests that MM-206 (**9**) may be an allosteric, rather than orthosteric, STAT3 inhibitor. We investigated this via the MM-206-catalyst conjugate (**10**) (b) Residues detected for the binding site mapping of recombinant STAT3 (130−688) with MM-206 photocatalyst (**10**) using a modified peptide cutoff across two replicates with >1.5 Log2FC intensity vs off compete control. PDB: 6NUQ. (c) Workflow for live cell binding site mapping in Jurkat cells. (d) Volcano plot showing enriched proteins resulting from μMap, showing exclusive selectivity of BRD proteins, including BRD4. (e) Modified residues detected in live cell binding studies of BRD4; all of which are within 10 Å of the small molecule.

To prepare an MM-206 STAT3 targeting conjugate, we chose to append the photocatalyst via esterification at the C1 alcohol (Figure 4a, **10**).[43] After validating the selective labeling of STAT3 with this catalyst via Western blot (Figure S8), we performed targeted labeling of recombinant STAT3 (residues 130−688) in vitro with excess free MM-206 as a negative control. Excitingly, all high-confidence residues detected were located on the CCD domain, providing direct experimental evidence for allosteric binding (Figure 4b). Notably, one of these modified residues (Q198) is in a region that has been demonstrated to be crucial for the CCD binder, K116.[44] These results showcase the capability of our method to study STAT3 inhibitor binding sites and their potential for informing the development of new allosteric inhibitors to undruggable targets.

Having demonstrated successful identification of binding-site proximal residues across a variety of protein targets, we questioned whether the robust on target labeling signal of μMap would enable us to achieve the direct binding site mapping of a protein target in living cells. Such in cellulo binding site mapping remains a longstanding challenge in the field, with few successful examples to date.[21,45] Thus, we sought to perform targeted binding site mapping of (+)-JQ-1 to BRD4 in live Jurkat cells. To confirm successful target engagement, we first performed live cell photolabeling using the cell permeable (+)-JQ-1-Ir (**3**) conjugate. Proteomics analysis of labeled cells following direct trypsinization of bead-enriched proteins revealed a high enrichment of several bromodomain reader proteins, including BRD4 (Figure 4d). Employing the desthiobiotin-PEG3-diazirine probe (**23**) permitted the biotin-based elution of modified peptides. Gratifyingly, analysis of these modified peptides revealed 3 covalently modified residues of BRD4, all of which are located less than 10 Å from the (+)-JQ-

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1 ligand pocket (Figure 4e), confirming the capacity of our platform to enable the binding site mapping of drugs in complex native proteomes.

While our method offers distinct advantages, each state-of-the art binding site mapping technology has unique benefits and limitations. Picotti and co-workers have previously investigated the binding interaction of rapamycin and FKBP12 using a machine-learning based chemoproteomic approach.[46] Compared to techniques such as μMap or PAL, this approach has the benefit of utilizing the unmodified drugs in analysis, allowing for higher throughput. While powerful, limited proteolysis mass spectrometry (LiP-MS) relies on changes in protein structure to alter degradation; therefore, care must be taken to ensure corresponding structural changes are a direct result of drug activity. In comparison to PAL, μMap differs in that it labels catalytically via proximity. This means that, in general, more distal residues are labeled than with PAL approaches, which directly link the drug to the protein of interest. However, the increase in signal allows for μMap to be employed in a cellular context without the need for isotopic tagging as well as yielding a higher number of labeling events in general, albeit requiring cutoffs to identify the highest confidence labeled residues. Furthermore, fragmentation of PAL−drug conjugates will vary based upon the drug used, and thus, our method allows for robust search of one modification mass across any drug−catalyst conjugate, as exemplified in our open-search data.

In conclusion, we have developed a robust and general method for small molecule binding site mapping by labeling proximal residues. This method has been shown to be amenable to a range of small molecule/protein pairs, multiprotein complexes, and "undruggable" targets. We envision that this method will find broad utility for practitioners in academia and industry who require urgent target engagement data.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c03325.

Additional experimental details, materials, methods, and supporting figures (PDF)

Table 1 - data analysis table for all in vitro modified peptides (XLSX)

Table 2 - full peptide data table for targeted replicates of each in vitro example (XLSX)

Table 3 - in vivo JQ1 Jurkat DIA proteomics Data. MS data has been uploaded to the MASSive repository (MSV000092043) (XLSX)

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Notes

The authors declare the following competing financial interest(s): D.W.C.M. declares an ownership interest in the company Dexterity Pharma LLC, which has commercialized materials used in this work.

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