Comprehensive Mapping of Electrophilic Small Molecule-Protein Interactions in Human Cells

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Abstract

Covalent chemistry is a versatile approach for expanding the ligandability of the human proteome. Activity-based protein profiling (ABPP) can infer the specific residues modified by electrophilic compounds through competition with broadly reactive probes. Nonetheless, the extent to which such residue-directed ABPP platforms fully assess the protein targets of electrophilic compounds in human cells remains unclear. Here, we introduce a complementary approach that directly identifies proteins showing stereoselective reactivity with focused libraries of stereochemically-defined, alkynylated electrophilic compounds. Integration of protein- and cysteine-directed ABPP data from compound-treated human cancer cells revealed generally well-correlated target maps and highlighted specific features, such as protein size and the proteotypicity of cysteine-containing peptides, that help to explain gaps in each ABPP platform. The integrated ABPP strategy furnished stereoselective, high-engagement covalent ligands for > 300 structurally and functionally diverse human proteins, including compounds that modulate enzymes by canonical (active-site cysteine) and non-canonical (isotype-restricted and non-catalytic cysteines) mechanisms.
Introduction

Advances in our understanding of the genetic basis of human disease offer unprecedented opportunities for precision medicine. Nonetheless, many genotype-phenotype relationships lack mechanistic understanding and remain therapeutically unactionable due, in large part, to a dearth of selective pharmacological tools for studying disease-relevant proteins. Several innovative approaches have emerged to address this gap, prominent among which are covalent chemistry and chemical proteomics.

Covalent chemistry leverages electrophilic small molecules to modify nucleophilic amino acid residues (e.g., cysteines, lysines, serines, and tyrosines) in proteins. Advantages of covalent chemistry include – i) enhanced interactions with shallow pockets in proteins that are challenging to target by solely reversible binding; ii) improved selectivity by targeting isotype-restricted nucleophilic amino acids within sets of related proteins; and iii) sustained pharmacological activity that is dependent on protein turnover rather than the pharmacokinetics of small molecules.

Original covalent probes and drugs were mainly discovered serendipitously or by the incorporation of electrophilic groups into established reversible ligands, as has been shown for kinase inhibitors. More recently, the intentional pursuit of covalent chemical probes, either by targeting specific disease-relevant nucleophilic residues (e.g., G12C-KRAS) or by globally profiling the reactivity of structurally diverse electrophilic small molecules in cells has begun to showcase the broad potential of covalent chemistry to expand the small-molecule interactions (or ‘ligandability’) of the human proteome.
Global investigations of electrophilic small molecule-protein interactions frequently leverage the activity-based protein profiling (ABPP) technology, which infers the identity of nucleophilic residues modified by electrophilic compounds in biological systems through competition with broadly reactive amino acid-directed probes. For instance, we and others have used cysteine-directed iodoacetamide (IA) probes with alkyne or desthiobiotin (DTB) affinity handles to assess the proteomic reactivity of electrophilic fragments, natural products, and diversity-oriented synthesis-inspired compound collections. These cysteine-directed ABPP experiments have discovered covalent ligands that produce functional effects through modification of cysteine residues on diverse proteins, including DNA/RNA-binding proteins, metabolic and protein kinases, scaffolding/adaptor proteins, and E3 ligases. Nonetheless, in certain instances, cysteine-directed ABPP has fallen short of mapping biologically relevant protein targets of electrophilic compounds in human cells, reflecting gaps in its coverage of ligandable cysteines. Such gaps may reflect, for instance, cysteines residing on non-proteotypic peptides that fail to be detected by conventional liquid chromatography-mass spectrometry (LC-MS) protocol.

Understanding the proportion of ligandable cysteines that evade detection in original cysteine-directed ABPP platforms is important to minimize false-negative outcomes (i.e., electrophilic small molecule-cysteine interactions that are overlooked) and to better understand the proteome-wide selectivity of advance covalent chemical probes. Here, we describe a general strategy to address this question wherein focused libraries of stereochemically defined electrophilic compounds (or ‘stereoprobe’) are profiled for their proteomic reactivity in cells by two complementary approaches: 1)
cysteine-directed ABPP, wherein hit compounds are defined as those that stereoselectively block cysteine reactivity with an IA-DTB probe; and 2) protein-directed ABPP, wherein hit compounds are defined as those that block the stereoselective enrichment of proteins by stereochemically matched alkyne-modified probes. We show that each approach has distinct attributes, with cysteine-directed ABPP providing information on sites of covalent ligand engagement, and protein-directed ABPP sacrificing this site data in exchange for the simultaneous quantification of several peptides from each liganded protein. Integration of protein- and cysteine-directed ABPP data revealed generally good overlap in assignment of liganded proteins and showcase complementary strengths and limitations of each platform. In total, >300 stereoselective small molecule-protein interactions were identified from a library of only 28 stereoprobes. Follow up studies verified the site-specificity and stereoselectivity of covalent liganding events on diverse types of proteins, including those that engage conserved catalytic residues in diverse enzyme classes and affect enzymes through less canonical mechanisms involving non-catalytic and isotype-restricted cysteines. Our findings support that, when deployed together, cysteine- and protein-directed ABPP generate near-comprehensive maps of covalent liganding events for electrophilic small molecules in biological systems, thus offering a compelling approach to expand the ligandable proteome.

Results

Alkyne stereoprobe design and initial characterization
Previous cysteine-directed ABPP studies of a tryptoline acrylamide class of stereoprobes have identified stereoselectively liganded cysteines on a diverse array of proteins\textsuperscript{15, 20, 31}. We felt that this set of electrophilic compounds would be well-suited for comparatively evaluating cysteine- and protein-directed ABPP platforms, as the assignment of ligandability events with each platform could initially be restricted to interactions that display both high engagement and stereoselectivity. We accordingly synthesized a focused library of five sets of alkyne-functionalized tryptoline acrylamide stereoprobes — each set consisting of four stereoisomers (20 total compounds in black; **Fig. 1a**). By placing the alkyne handle at different positions on the tryptoline core, we hoped to account for potential effects of this group on proteomic interactions in comparison to the unmodified tryptoline acrylamide stereoprobes to be used as competitors in cysteine- and protein-directed ABPP experiments (eight total compounds in red; **Fig. 1a**).

We first assessed the overall proteomic reactivity of alkyne stereoprobes by gel-ABPP, where two human cancer cell lines – Ramos cells (a suspension B lymphocyte cell line) and 22Rv1 cells (an adherent human prostate carcinoma epithelial cell line) – were treated with 5 or 20 µM of each stereoprobe for 1 h, after which cells were lysed, and stereoprobe-reactive proteins visualized by conjugation to a rhodamine reporter tag using Cu(I)-catalyzed azide–alkyne 1,3-dipolar cycloaddition (CuAAC) chemistry\textsuperscript{32, 33}, SDS-PAGE, and in-gel fluorescence scanning\textsuperscript{34}. Each alkyne stereoprobe produced clear protein labeling at both 5 and 20 µM test concentrations, with expectedly stronger proteomic reactivity observed at 20 µM (**Fig. 1b, c and Extended Data Fig. 1a**). The trans stereoisomers generally showed greater proteomic
reactivity (Fig. 1b, c and Extended Data Fig. 1a), a property that was also observed in
cell lysates (Extended Data Fig. 1b), indicating it was not a consequence of greater cell
permeability. The trans stereoisomers also displayed greater reactivity with glutathione
(Supplementary Table 1). Even with the limited resolution afforded by one-dimensional
SDS-PAGE, several stereoselective stereoprobe-protein interactions were detected (red
asterisks, Fig. 1b, c and Extended Data Fig. 1a, b).

Having confirmed the concentration-dependent proteomic reactivity of alkyne
stereoprobes in cells, we next evaluated these compounds in protein-directed ABPP
experiments using multiplexed (tandem mass tagging, TMT) MS analysis.

Protein-directed ABPP with alkyne stereoprobes

We initially performed protein-directed ABPP of Ramos cells treated with 5 or 20 µM of
a representative set of alkyne stereoprobes (probe set 2). In this protocol, Ramos cells
were treated for 3 h with alkyne stereoprobes, after which cells were lysed, stereoprobe-
reactive proteins conjugated to azide-biotin by CuAAC, isolated with streptavidin beads,
trypsin digested, TMT labeled with TMT tags, and identified (MS1/MS2 analysis) and
quantified (MS3 analysis) by multiplexed (TMT10plex) MS (Extended Data Fig. 2a).
Proteins showing > 3-fold enrichment by one stereoprobe compared to its enantiomer
were assigned as stereoselective targets. More than 150 proteins were stereoselectively
enriched at 5 and/or 20 µM, and we noted that these proteins tended to show higher
stereoenrichment ratios in cells treated with 5 µM of the stereoprobes (Extended Data
Fig. 2b). This result could indicate that, at 20 µM, the stereoprobes begin to show low-
level labeling of multiple cysteines on proteins, which could in turn erode at the stereoenrichment values associated with specific engagement of individual cysteines. We therefore elected to perform future protein-directed ABPP experiments with 5 µM of the alkyne stereoprobes.

While the comparison of proteins enriched by alkyne stereoprobes provided an initial assignment of stereoselective targets, the stoichiometry of electrophile-protein interactions cannot readily be determined from such enrichment values. For our more in-depth analysis of covalently liganded proteins, we therefore performed competitive protein-directed ABPP experiments, where Ramos or 22Rv1 cells were first treated with DMSO or non-alkyne competitor stereoprobes (20 µM; WX-02-16/26/36/46 or WX-03-57/58/59/60; Fig. 1a) for 2 h, followed by treatment with corresponding alkyne stereoprobes (5 µM; Fig. 1a) for 1 h, and processing and analysis by multiplexed (TMT\(^{16}\)plex) MS (Fig. 2a). Proteins were designated as being stereoselectively liganded if they met the following criteria: 1) >3-fold enantioselective enrichment with at least one pair of alkyne stereoprobe enantiomers; and 2) >50% blockade of this enantioselective enrichment by the corresponding competitor stereoprobe.

Among >8000 quantified proteins, 271 proteins were stereoselectively liganded in protein-directed ABPP experiments performed in Ramos and 22Rv1 cells with five sets of alkyne stereoprobes (Fig. 2b and Supplementary Dataset 1). Each stereochemical configuration of the tryptoline acrylamide core liganded a distinct set of proteins, with the (S, R) stereoisomer engaging the greatest number of proteins (Fig. 2c). The (S, R) stereoisomer also stereoselectively engaged a greater number of proteins in cell lysates (Extended Data Fig. 2c). We more generally observed good
alignment between the cellular (*in situ*) versus cell lysate (*in vitro*) proteomic reactivity profiles of the stereoprobes (Fig. 2d), although there were exceptional proteins that were stereoselectively liganded only in cells or in lysates (Extended Data Fig. 2d). Despite the greater overall reactivity of the trans stereoprobes, most of the proteins enantioselectively liganded by the cis stereoprobes were not cross reactive with trans stereoprobes (Extended Data Fig. 2e), underscoring the importance of the absolute stereoconfiguration of the tryptoline core for stereoprobe-protein interactions.

We found that quadrant plots offered a way to visualize stereoselectively liganded proteins, where the positions of proteins on the x- and y-axes reflect enantioselective and diastereoselective enrichment, respectively, and the size of the dot represents the degree of competitive blockade of this enrichment by the corresponding non-alkyne stereoprobe for the designated quadrant (Fig. 2e). This presentation format also highlighted stereoselectively liganded proteins found in only one cell type, such as the immune-restricted proteins PLEK and MYO1G and the pioneering transcription factor FOXA1, which were stereoselectively engaged by (1R, 3S)-tryptoline acrylamides in Ramos and 22Rv1 cells, respectively (Fig. 2e and Extended Data Fig. 3). Finally, hierarchical clustering revealed similar target profiles for alkyne stereoprobes of the same stereochemistry, while also highlighting proteins that were engaged by only a single alkyne stereoprobe (Fig. 2f).

Integration of cysteine- and protein-directed ABPP data
As noted above, protein-directed ABPP does not generally provide information on the specific residues engaged by covalent ligands. We, therefore, next analyzed the tryptoline acrylamide stereoprobes (five sets of alkyne stereoprobes and two sets of non-alkyne competitor stereoprobes; Fig. 1a) by cysteine-directed ABPP in Ramos and 22Rv1 cells. Cysteine-directed ABPP was performed as described previously, where each multiplexed (TMT\textsuperscript{10plex}) experiment compared in duplicate all four stereoisomers of a given stereoprobe set alongside a DMSO control (20 µM compound, 3 h treatment; Extended Data Fig. 4a). Cysteines were assigned as stereoselectively liganded if they showed a > 66.7% decrease in IA-DTB reactivity in cells treated with a stereoprobe, and this decrease in IA-DTB reactivity was at least 2.5-fold greater than that observed for the enantiomer of the stereoprobe. We also required that at least one additional cysteine was unchanged (< 25% change in IA-DTB reactivity) on the parent protein harboring a stereoselectively liganded cysteine to avoid misinterpreting protein expression changes as cysteine liganding events. In total, > 38,000 cysteines on >9,000 proteins were quantified, of which 238 cysteines on 217 proteins were stereoselectively liganded (Fig. 3a and Supplementary Dataset 1). As was observed in the protein-directed ABPP experiments (Fig. 2c and Extended Data Fig. 2c), each stereochemical configuration of the tryptoline acrylamide core liganded a distinct set of cysteines, and the (S, R) stereoisomer engaged the greatest number of cysteines (Extended Data Fig. 4b).

Integration of data from the cysteine- and protein-directed ABPP experiments generated a total of 336 stereoselectively liganded proteins for the >10,000 proteins quantified across both Ramos and 22Rv1 cells (Fig. 3b and Supplementary Dataset
The stereoselectively liganded proteins were distributed across a wide range of functional protein classes, including enzymes, channels/transporters, transcription/translation factors, and adaptor/scaffolding proteins (Fig. 3c). About 40% (134) of these liganded proteins show essentiality (common or strongly selective) in the Cancer Dependency Map\(^3^6\) (Extended Data Fig. 4c). GO pathway analysis further revealed an enrichment of stereoselectively liganded proteins in processes involving RNA metabolism, autophagy, and cell cycle (Fig. 3d).

Nearly half of the stereoselectively liganded proteins (152 total) were identified by both ABPP platforms, with the remaining proteins distributed between those identified exclusively by cysteine- (65 total) or protein- (119 total) directed ABPP (Fig. 3e). In contemplating why some proteins might be identified as stereoselectively liganded by cysteine-, but not protein-directed ABPP, we noted proteins harboring two or more cysteines that were each liganded by stereoprobes of distinct stereochemistry. As one example, we show the ligandability map for HECTD4, an ~4000 amino acid E3 ligase, for which 10 cysteines were quantified and three were liganded by the stereoprobes, each with a distinct stereopreference (Fig. 3f). While such site-specific differences in stereoselective liganding can be resolved by cysteine-directed ABPP, they would be expected to confound stereoselective enrichment profiles in protein-directed ABPP experiments, and HECTD4 accordingly did not show enantioselective enrichment with either the cis or trans stereoprobes (Fig. 3g). PRKDC, a 4000+ amino acid protein kinase, provided a second case, where two cysteines among > 40 quantified cysteines were liganded by stereoprobes of distinct stereopreferences, thus hindering assignment of stereoselective enrichment by protein-directed ABPP (Extended Data Fig. 4d, e).
We finally observed an overall correlation between the size of proteins harboring stereoselectively liganded cysteines and their likelihood of being assigned as ligandable exclusively by cysteine-directed ABPP (**Fig. 3h**). While these results indicate that larger proteins may prove generally challenging to interpret by protein-directed ABPP due to the potential for harboring multiple electrophile-reactive cysteines, we did find exceptions (i.e., proteins > 2000 amino acids in length that were assigned as stereoselectively liganded by protein-directed ABPP (e.g., PIKFYVE; **Extended Data Fig. 4f**).[

When considering reasons why stereoprobe liganding events might be observed by protein-, but not cysteine-directed ABPP, we surmised that the proteotypicity of tryptic peptides containing liganded cysteines could be a contributory factor. Proteotypicity refers to the likelihood that a given peptide will be confidently and consistently identified in MS-based proteomics experiments and can be affected by physicochemical properties such as tryptic peptide length, hydrophobicity, and isoelectric point. Consistent with this hypothesis, we found that cysteine-containing tryptic peptides quantified in cysteine-directed ABPP experiments had higher predicted proteotypicity (DeepMSPeptide probability score for detection) compared to cysteine-containing tryptic peptides that were not quantified (**Fig. 3i**). Protein-directed ABPP, on the other hand, can better overcome challenges with peptide proteotypicity by quantifying multiple tryptic peptides per covalently liganded protein. We additionally found that predicted proteotypic peptides not quantified in our cysteine-directed ABPP experiments were enriched in peptides showing greater reactivity with the IA-DTB probe in denatured proteomes (**Extended Data Fig. 4g**), suggesting that some proteotypic
peptides may fail to be quantified in cysteine-directed ABPP experiments performed in native proteomes because they contain buried cysteines that are inaccessible to labeling by chemical probes\textsuperscript{38}.

We also observed another interesting category of stereoprobe liganding events that was exclusively identified by protein-directed ABPP due to interactions dependent on the alkyne modification itself. In these cases, as exemplified by STRBP (Fig. 3j, k) and FXR1 (Extended Data Fig. 4h, i), stereoselective enrichment was observed without competition in protein-directed ABPP experiments (Fig. 3j and Extended Data Fig. 4h), while cysteine-directed ABPP experiments revealed stereoselective liganding of a cysteine by the alkyne stereoprobe, but not its non-alkyne counterpart (Fig. 3k, Extended Data Fig. 4i, and Supplementary Dataset 1). Finally, integration of protein- and cysteine-directed ABPP data facilitated interpretation of other categories of stereoprobe-protein interactions, including: i) proteins that were stereoselectively enriched, but not competed, and for which liganding of a cysteine by the alkyne stereoprobe was not observed (Fig. 3l and Supplementary Dataset 1); and 2) proteins that were uniformly enriched and competed by all stereoisomers of one or more stereoprobe sets (73 total proteins in this category; Extended Data Fig. 4j and Supplementary Dataset 1). We interpret the first category as mostly reflecting low-stoichiometry, but nonetheless stereoselective stereoprobe-protein interactions, and the second category as a less frequent group of liganded cysteines residing in pockets that non-stereoselectively bind each of the stereoprobes.
Having mapped >300 stereoselective, covalent small molecule-protein interactions in human cancer cells by integrated cysteine- and protein-directed ABPP, we next aimed to verify and characterize a representative set of these interactions.

**Characterization of stereoprobe targets**

We initially focused on confirming covalent liganding events that were mapped by both cysteine- and protein-directed ABPP. We selected liganded proteins with diverse structural and functional annotations, including a pleckstrin homology (PH) domain-containing protein (PLEK), an iron-sulfur cluster scaffolding protein (NFU1), integral membrane proteins (TMX1, TMX4), and various classes of enzymes (TYMS (metabolic enzyme); LIMK1 (kinase); STAMBP (deubiquitinase)). Each protein and the corresponding mutant(s) where the liganded cysteine was converted to alanine were expressed as Flag epitope-tagged proteins by transient transfection in HEK293T cells. The transiently transfected cells were subsequently treated for 1 h with alkyne stereoprobe (5 μM, except where noted), and analyzed by an established gel-ABPP protocol involving conjugation of stereoprobe-labeled proteins to an azide-rhodamine reporter tag by CuAAC followed by visualization by SDS-PAGE and in-gel fluorescent scanning. We also performed competitive gel-ABPP experiments, where transfected cells were pretreated with non-alkyne competitor stereoprobe (20 μM, 1 h, except where noted) prior to exposure to alkyne stereoprobe.

The immune-restricted PH domain protein PLEK was stereoselectively liganded at C250 by (1R, 3S) stereoprobe in Ramos cells, including the alkyne/competitor pair
WX-01-06/ WX-02-26 (Extended Data Fig. 5a-c), and these stereoprobe interactions were recapitulated with recombinant WT-PLEK, but not C250A-PLEK, in transfected HEK293T cells (Extended Data Fig. 5d). While the three-dimensional structure of full-length PLEK has not yet been determined, an AlphaFold predicted structure identified a candidate small-molecule binding pocket adjacent to C250 that is also proximal to the D-myo-inositol 1, 2, 3, 5,6-pentakisphosphate (IP₅) binding site (Extended Data Fig. 5e), suggesting that covalent ligands targeting PLEK_C250 may have the potential to modulate the function of PLEK.

NFU1, a protein involved in transferring assembled [4Fe-4S] clusters to client proteins in the mitochondria, was stereoselectively liganded by (1S, 3R) stereoprobes, including the alkyne/competitor pair WX-01-12/WX-02-46 as determined by both cysteine- (Fig. 4a) and protein- (Fig. 4b and Extended Data Fig. 5f) directed ABPP. The stereoprobe-liganded tryptic peptide in NFU1 mapped by cysteine-directed ABPP contains the conserved CXXC motif (C210, C213) involved in [4Fe-4S] cluster binding and transfer. We verified stereoselective labeling of recombinant NFU1 by WX-01-12 (Fig. 4c) and stereoselective blockade of this interaction by WX-02-46 (Extended Data Fig. 5g), and mutagenesis supported that C210 was the direct site of engagement by the stereoprobes (Fig. 4c). Recent studies have found that genetic disruption of NFU1 by CRISPR-Cas9 technology causes selective toxicity in human colon cancer cells when cultured at low pH. Consistent with this report, we found that WX-01-12, while generally nontoxic to the colon cancer cell line SW480 when cultured at pH 7.4, exhibited stereoselective growth suppression when the media pH was lowered to 6.6 (Extended Data Fig. 5h). In this study, neither diastereomer (WX-01-09, WX-01-11)
showed substantial cell growth effects, while the enantiomer (WX-01-10) suppressed cell growth similarly across the tested pH range presumably due to engagement of the spliceosome factor SF3B1, which we have previously identified as a target mediating anti-proliferative effects of (1R, 3S) stereoprobes\textsuperscript{20}.

In addition to NFU1, we noted several other instances of stereoselective liganding of conserved functional residues (Supplementary Dataset 1). For instance, (1R, 3R) stereoprobes stereoselectively liganded the catalytic nucleophile (C195) of the nucleotide biosynthetic enzyme thymidylate synthase (TYMS), and we confirmed these interactions with recombinant TYMS (Extended Data Fig. 5i-l). TYMS is the primary target of anticancer agents such as 5-fluorouracil\textsuperscript{44}, and our data indicate a potential to create structurally distinct covalent inhibitors of this enzyme.

The thioredoxin-related transmembrane proteins TMX1 and TMX4 were stereoselectively liganded by the (1S, 3S) stereoprobes (Fig. 4d). TMX1 and TMX4 belong to a family of thiol-disulfide oxidoreductases that localize to the endoplasmic reticulum and are thought to facilitate disulfide isomerization of membrane proteins\textsuperscript{45}. Four TMXs (TMX1-4) were quantified in our protein-directed ABPP experiments, but only TMX1 and TMX4 showed stereoselective interactions with the stereoprobes (Fig. 4d). TMX1 and TMX4 share three cysteines, two of which are part of the catalytic CXXC motif conserved across all TMX proteins\textsuperscript{46} (Extended Data Fig. 6a). For TMX4, cysteine-directed ABPP experiments identified the conserved catalytic CXXC motif C64/C67 as the likely site(s) of stereoprobe engagement (Extended Data Fig. 6b), while the corresponding residues in TMX1 were not quantified in these experiments. We next mutagenized each cysteine to alanine in TMX1 and TMX4, which revealed that, for
both proteins, mutation of the N-terminal cysteine of the CXXC motif (C56 of TMX1 and C64 of TMX4) blocked stereoprobe reactivity (Fig. 4e, f). We also verified with recombinantly expressed proteins that only TMX1 and TMX4, but not TMX2 and TMX3, react with (1S, 3S) stereoprobes (Fig. 4g). Finally, we determined IC$_{50}$ values for engagement of TMX1 and TMX4 by the non-alkyne stereoprobe competitor WX-02-16 of 7 and 2 µM, respectively, while the enantiomer WX-02-36 showed much weaker reactivity (IC$_{50}$ values > 30 µM) (Extended Data Fig. 6c, d). Thus, WX-02-16 may offer a useful active site-directed chemical probe for studying the functions of TMX1/TMX4.

We selected LIMK1 for further investigation because it represented one of several proteins containing a paralog-restricted ligandable cysteine (Supplementary Dataset 1). The stereoselectively liganded cysteine (C349) in LIMK1 showed an unusual SAR reflecting enantioselective reactivity with both the cis and trans alkyne stereoprobes, but not the corresponding non-alkyne competitors (Fig. 4h, i and Extended Data Fig. 6e), and this interaction profile was verified with recombinant LIMK1 expressed in HEK293T cells (Fig. 4j). LIMK1_C349 is in proximity to a pocket that is highly conserved in the paralog LIMK2, except that a phenyalanine (F341) is in place of C349; this pocket is also adjacent to the ATP-binding site of the kinase$^{47}$ (Fig. 4k and Extended Data Fig. 6f). LIMK1 displays very limited activity with commercial peptide substrates$^{48}$, so we instead evaluated stereoprobe effects using a NanoBRET assay measuring LIMK1 interactions with a general kinase inhibitor that targets the ATP-binding site$^{49}$. Interestingly, both cis (WX-01-11) and trans (WX-01-10) stereoprobe ligands enantioselectively enhanced NanoBRET signals (Fig. 4l and Extended Data Fig. 6g), and these effects were ablated in a C349A-LIMK1 mutant (Fig. 4m). In
contrast, an established ATP-binding pocket inhibitor of LIMK1 HG-9-91-01 decreased NanoBRET signals equivalently for both WT- and C349A-LIMK1 (Fig. 4m). We confirmed that the stereoprobe-induced increase in NanoBRET signal was not caused by changes in LIMK1-Nanoluc expression (Extended Data Fig. 6h). While we do not yet understand how these NanoBRET data may translate into effects of the stereoprobes on LIMK1 substrate interactions in cells, we believe the cooperative binding profile displayed by stereoprobes with ATP-binding pocket inhibitors is consistent with a potential functional consequence of covalent ligands engaging LIMK1_C349.

We also found cases where stereoprobes liganded non-catalytic cysteines that are conserved between paralogous enzymes (Supplementary Dataset 1), such as the deubiquitinases STAMBP (C264) and STAMBPL1 (C276) (Extended Data Fig. 7a-d). The stereoprobe interactions with STAMBP_C264 and STAMBPL1_C276 showed distinct SARs (Extended Data Fig. 7a-d), pointing to the potential for developing selective covalent ligands targeting each DUB (as has been previously achieved for kinases that share a ligandable cysteine51, 52). The ligandable cysteine in STAMBP(L1) is located near the N-terminus of the DUB catalytic domain, and structures of this region (a.a. 246-424) indicate the cysteine is distal to the zinc-bound active site53, 54 (Extended Data Fig. 7e). We verified stereoselective and site-specific reactivity of stereoprobes with C264 in full-length, recombinant STAMBP (Extended Data Fig. 7f), setting the stage for future studies aimed at understanding the structure and function of this covalent ligand-DUB interaction.
Characterization of stereoprobe targets assigned only by protein-directed ABPP

We next investigated a representative set of stereoprobe-protein interactions exclusively mapped by protein-directed ABPP. These interactions presented an additional technical challenge, as we needed to experimentally deduce the cysteines liganded by the stereoprobes. The expectation that stereoselectively liganded cysteines should be absent in the tryptic peptide maps of proteins enriched by stereoprobes assisted in elucidating some stereoprobe-cysteine interactions. For instance, the poorly characterized protein C15orf57 (or CCDC32) was stereoselectively enriched and competed by (1S, 3R) stereoprobes (Fig. 5a), and only one of the four cysteines in this protein (C111) was absent from the tryptic peptide maps from protein-directed ABPP experiments (Extended Data Fig. 8a). We confirmed stereoselective labeling of recombinant WT-C15orf57, but not the C111A mutant, by the (1S, 3R) alkyne WX-01-12 (Fig. 5b), as well as stereoselective blockade of this labeling by pre-treatment with the non-alkyne competitor WX-02-46 (Extended Data. Fig. 8b).

Integrating the tryptic peptide maps of stereoprobe targets with knowledge of paralog reactivity also proved helpful for identifying liganded cysteines. For instance, the protein kinase STK39, but not its closely related paralog OXSR1 (74% sequence identity), was stereoselectively enriched and competed by a subset of (1S, 3S) stereoprobes (Fig. 5c). Among the cysteines not found in the stereoprobe-enriched tryptic peptide map of STK39, only two residues (C59 and C334) were unique to STK39 compared to OXSR1. Mutagenesis of C334, but not C59, blocked stereoprobe reactivity with STK39 (Fig. 5d). We also confirmed by gel-ABPP that recombinant STK39 reactivity with alkyne WX-03-346 was stereoselectivity blocked by non-alkyne
competitor WX-03-57 ([Extended Data Fig. 8c]). A crystal structure of STK39 indicates that C334 is distal to the ATP-binding site of the kinase located in region that is generally well conserved in OXSR1 ([Extended Data Fig. 8d, e]), suggesting that compounds engaging this residue may have allosteric, rather than orthosteric potential. Of note, C15orf57_C111 and STK39_C334 are both found on small (non-proteotypic) tryptic peptides (2-5 amino acids in length; [Fig. 5b, d]), potentially explaining why they were not quantified in cysteine-directed ABPP experiments.

Finally, we draw attention to another likely rare, but intriguing source of stereoprobe targets that are exclusively mapped by protein-directed ABPP – proteins that reacted with the tryptoline acrylamides at non-cysteine residues. We first came to suspect this possibility by noticing that two of the stereoselectively enriched and competed proteins did not contain any cysteine residues (AK3, HMOX1). For adenylate kinase AK3, which was stereoselectively engaged by (1S, 3S) stereoprobes, we also found that a single quantified tryptic peptide (amino acids 27-34: R.ITTHFELK.N) consistently failed to show stereoselective enrichment in protein-directed ABPP experiments ([Fig. 5e, f]). This type of aberrant quantification profile for a tryptic peptide from a stereoselectively enriched protein could arise if background signals are detected for the protein due to low-level non-specific stereoprobe reactivity at other sites (or due to nonspecific binding of the protein in unreacted form to the streptavidin beads). In such instances, we surmised that the tryptic peptide failing to exhibit stereoselective enrichment may contain the site of stereoprobe engagement. Although infrequent, such corrupted stereoselective enrichment profiles were also observed for tryptic peptides containing stereoprobe-liganded cysteines ([Extended Data Fig. 8f, g]). Since
acrylamides and other Michael acceptor electrophiles have been periodically found to react with lysines\textsuperscript{56, 57, 58}, we compared the stereoprobe reactivity profile of recombinant WT-AK3 and a K34R mutant and found that only the WT protein was stereoselectively engaged by (1S, 3S) stereoprobes (Fig. 5g). We additionally found that (1S, 3S) stereoprobes stereoselectively blocked the labeling of K34 by an N-hydroxysuccinimide desthiobiotin (NHS-DTB) probe in lysine-directed ABPP experiments (Fig. 5h). These data support that AK3_K34 can be stereoselectively liganded by tryptoline acrylamides. K34 is distal to the catalytic site of AK3\textsuperscript{59}, indicating that stereoprobe engagement may occur at a separate pocket on the protein (Fig. 5i).

Taken together, the experiments described in this section offer a roadmap for identifying liganded cysteines that may elude detection in cysteine-directed ABPP experiments due to, for instance, their presence on non-proteotypic peptides.

Covalent inhibitors targeting the kynurenine pathway enzyme AFMID

Among the stereoprobe targets mapped by protein-directed ABPP, the arylformamidase (AFMID) caught our attention because this enzyme, through catalyzing the hydrolysis of N-formylkynurenine to kynurenine (Fig. 6a), plays an important role in the tryptophan-kynurenine metabolic pathway that is implicated in immunological/inflammatory disorders\textsuperscript{60, 61} and host response to infectious agents\textsuperscript{62, 63}. To our knowledge, selective inhibitors of AFMID are lacking.

AFMID is a serine hydrolase, and we were curious as to the mode of engagement of this enzyme by tryptoline acrylamides. Protein-directed ABPP experiments identified AFMID as being stereoselectively liganded by the (1R, 3R)
alkyne/competitor stereoprobe pair WX-01-03/WX-01-36 (Fig. 6b). We did not detect a stereoselectively liganded cysteine for AFMID in cysteine-directed ABPP experiments, but an AlphaFold\textsuperscript{39,40} structural model suggested that one of the four cysteines in AFMID (C28) was proximal to the active site serine (S164) (Fig. 6c), and docking studies supported a productive binding mode for WX-01-03 compared to the inactive enantiomer WX-01-01 in the AFMID active site (Extended Data Fig. 9a). Consistent with these modeling predictions, we found that recombinant WT-AFMID, but not the C28A-AFMID mutant stereoselectively reacted with WX-01-03 in HEK293T cells (Fig. 6d), and this interaction was stereoselectively blocked by pre-treatment with WX-02-36 (Fig. 6e). We further found that WX-01-03 stereoselectively inhibited the hydrolytic conversion of N-formylkynurenine to kynurenine catalyzed by recombinant WT-AFMID (Fig. 6f), while the C28A-AFMID mutant was insensitive to WX-01-03 and a C28W AFMID mutant showed partial loss in activity that was not further attenuated by WX-01-03 (Fig. 6f and Extended Data Fig. 9b). As anticipated, an S164A AFMID mutant displayed negligible catalytic activity (Fig. 6f). We also found that WX-01-03 stereoselectively blocked the catalytic activity of endogenous AFMID with an IC\textsubscript{50} value of 150 nM as measured in the human hepatocellular carcinoma line HepG2 (Fig. 6g) which expresses high levels of AFMID (Extended Data Fig. 9c). Being a serine hydrolase, AFMID reacts with fluorophosphonate (FP) ABPP probes\textsuperscript{64}, and we found that WX-01-03, but not WX-01-01, blocked labeling of recombinant WT-AFMID by an FP-rhodamine probe (Extended Data Fig. 9d). Finally, we noted that, in many other mammalian organisms, including mouse, C28 in AFMID is replaced by a serine (Fig. 6h and Extended Data Fig. 9e). Considering the importance of mouse models for studying
immune-relevant metabolic pathways, we generated a S26C mutant of mouse AFMID and found that it was stereoselectively engaged (Fig. 6i) and inhibited (Fig. 6j) by WX-01-03 with an IC\textsubscript{50} value of 1.8 µM. In contrast WT mouse AFMID was insensitive to WX-01-03 (Fig. 6i, j). These results, taken together, indicate that tryptoline acrylamides act as stereoselective and site-specific inhibitors of human AFMID by engaging a non-catalytic, active site-proximal cysteine, and that mouse AFMID can be engineered by site-directed mutagenesis to display sensitivity to these compounds.

**Discussion**

By generating global portraits of small molecule-protein interactions in native biological systems, ABPP has enriched our understanding of covalent chemistry as a means to expand the druggable proteome. An ideal ABPP platform would, in a single experiment, identify each amino acid residue on each protein that reacts with an electrophilic compound in cells and quantify the extent of these reactions (i.e., the stoichiometry of residue modification). However, technical limitations in MS-based proteomics prevent the full realization of this goal, and the number of covalent protein binding events that may be overlooked by current ABPP protocols remains an open and important question. Here, we have described the integration of two complementary approaches – residue (cysteine)- and protein-directed ABPP – that we believe, together, furnish near-comprehensive maps of electrophilic small molecule-protein interactions in human cells.

Our results indicate that gaps in the proteomic coverage of cysteine-directed ABPP originate in large part from covalent reactions occurring with cysteines on non-proteotyptic peptides. These reactions, especially those that occur with clear SAR (e.g.,
stereoselectivity) and high stoichiometry, can frequently be identified by protein-directed ABPP. While this approach does not generally identify the residues liganded by electrophilic compounds in each protein, we have provided guidance for how to deduce this information by, for instance, examining the tryptic peptide maps of alkyne probe-enriched proteins for the absence of cysteine-containing peptides and by comparing cysteine conservation in paralogous proteins that either share or do not share ligandability profiles. Then, mutagenesis of candidate cysteines can be used to infer the site of liganding by electrophilic compounds.

We conversely identified several stereoselective and high-stoichiometry electrophilic compound-cysteine reactions on proteins by cysteine-directed ABPP that did not show evidence of liganding by protein-directed ABPP. We found specific instances where this outcome could be explained by the presence of two or more ligandable cysteines on the same protein that exhibited different SARs across our stereoprobe library, thus confounding the whole protein enrichment profiles. More generally, these discoveries highlight that interpreting the SAR of covalent liganding events by protein-directed ABPP stipulates that a protein possesses predominantly a single site of covalent reactivity, and this requirement may not be met by some proteins, especially large, multi-domain proteins with several pockets capable of binding small molecules.

Are there covalent small molecule-protein interactions that might still be missed by the integrated use of cysteine- and protein-directed ABPP? While we do not anticipate many such overlooked interactions, we can imagine rare cases of liganded cysteines on non-proteotypic peptides from very large proteins continuing to present technical challenges. Alternative protease digests can provide complementary
proteotypic peptide maps to those generated by trypsin\textsuperscript{65} which should in turn increase coverage of cysteine-directed ABPP. We additionally cannot exclude that some cysteines may show low intrinsic reactivity with the IA-DTB probe used herein, and other broad-spectrum, cysteine-reactive probes may accordingly be employed alternatives\textsuperscript{66, 67}.

Our work, along with other recent studies\textsuperscript{15, 68, 69}, underscore how stereochemically defined compounds can facilitate the discovery of specific small molecule-protein binding events in chemical proteomic experiments. Stereoselectivity may not only also assist in prioritizing high, but also lower stoichiometry stereoprobe-protein interactions for future study. Toward this end, we observed numerous stereoselectively enriched proteins in our protein-directed ABPP experiments that were not blocked by competitor stereoprobes, and we speculate that many such interactions are evidence of nascent ligandability. And, as has been shown in previous studies, stereoselective small molecule-protein interactions, regardless of their fractional occupancy, frequently occur at functional sites\textsuperscript{15, 20, 21, 69}, and thus low-stoichiometry covalent compounds may find utility as tools to screen nascent ligandable pockets in proteins for higher affinity small-molecule binders.

From a modest-sized library of tryptoline acrylamide stereoprobes, we discovered stereoselectively liganded cysteines on a diverse array of proteins from different structural and functional classes. In each instance, a low-µM (< 20 µM IC\textsubscript{50}) chemical tool, and an associated target engagement assay, are now available for cell biological studies. While these initial chemical probes are not yet specific for individual proteins, the stereoselectivity and site-specificity of the probe-protein interactions
provide useful controls, such as inactive enantiomeric compounds and cysteine mutants (for non-essential cysteine residues), to facilitate interpretation of functional studies, as we have shown previously\textsuperscript{15, 20, 21} and herein for proteins like LIMK1 and AFMID. Nonetheless, we also acknowledge that understanding the functional effects of stereoprobe-protein interactions is often contingent on the availability of robust assays capable of measuring the direct biochemical activities performed by proteins in cells. Even if some of the stereoprobe interactions described herein are found to lack direct functional effects on proteins, the ligands could serve as starting points for the design of heterobifunctional compounds that mediate the degradation of the protein of interest\textsuperscript{70}.

We close by highlighting future insights that may be gained with integrated cysteine- and protein-directed ABPP. The frequency of paralog-restricted ligandable cysteines identified in our data underscores the potential for covalent chemistry to continue to provide a means for achieving pharmacological selectivity among highly related proteins. We further speculate, however, that paralogs lacking the liganded cysteine may, in many instances, maintain a pocket suitable for binding small molecules. This hypothesis could initially be tested by engineering a cysteine into such paralogs and screening for alkyne-stereoprobe reactivity, as we demonstrated for the mouse ortholog of AFMID. Covalent stereoprobes might even then form the basis for screening engineered proteins to discover reversible ligands that would in turn maintain affinity for the wild-type protein. In this way, the landscape of proteomic ligandability illuminated by cysteine- and protein-directed ABPP could prove extendable beyond covalent chemistry. We also anticipate that in-depth investigations of cysteine- and protein-directed ABPP datasets may uncover proteoform-restricted liganding events,
manifesting as high stoichiometry interactions in protein-directed ABPP experiments without a ligandable cysteine being mapped by cysteine-directed ABPP. This type of profile would emerge if the alkyne stereoprobe exclusively interacts with a rare proteoform, and the more generic IA-DTB probe lacks such discriminatory reactivity. Finally, the comparison of distinct cell types or states may identify context-dependent covalent liganding events dictated by differential post-translational modifications, biomolecular associations, and/or localization (rather than mere expression) of proteins.

Each of these opportunities for expanding the ligandable proteome should benefit from the rich maps of covalent small molecule-protein interactions afforded by integrated cysteine- and protein-directed ABPP.

Methods

Cell lines and cell culture

22Rv1 (ATCC, CRL-2505), Ramos (ATCC, CRL-1596), HEK293T (ATCC, CRL-3216) and SW480 (ATCC, CCL-228™) cells were grown in RPMI (22Rv1, Ramos, ), or DMEM (HEK293T, SW480), supplemented with 10% fetal bovine serum (FBS), 2 mM L-alanyl-L-glutamine (GlutaMAX, 22Rv1 only) or 2 mM L-glutamine, penicillin (100 U/mL), and streptomycin (100 μg/mL), in a humidified, 37 °C/5% CO₂ tissue culture incubator.

Reagents

Additional reagents, source and catalog numbers are found in Supplementary Table 2

Cell proliferation assay (pH-dependent compound toxicity)
SW480 cells were cultured in standard DMEM and then switched to NaHCO₃-free DMEM supplemented with 22 mM NaHCO₃/22 mM NaCl (pH 7.4), 5.5 mM NaHCO₃/38.5 mM NaCl (pH 6.9), or 2.75 mM NaHCO₃/41.25 mM NaCl (pH 6.6), 10% FBS and pen/strep for 3 days, to acclimatized to the pH conditions. Cells were then seeded in the corresponding pH medium at 5000 cells per well (50 μL of 1x10⁵ cells/mL) in 96-well flat bottom white wall plates. After 24 h, 50 μL of the corresponding pH medium containing DMSO or 2x compound dilutions (from 1000x DMSO stocks) were added to the wells and cultured for 72 h. Plates were brought to RT and 50 μL of CellTiter-Glo® (Promega, G7570) added to each well and vigorously shaken with a microplate orbital shaker for 20 sec and incubated on a gentle rotating platform for 30 min at RT. Luminescence readings were taken with CLARIOstar (BMG Labtech) plate reader. After background subtraction, each treatment was normalized to DMSO control at the corresponding pH and graphed with GraphPad PRISM software version 9.5. Data represents mean ±SEM for three independent experiments, each of which was set up in triplicates.

**Cloning and mutagenesis**

All full-length plasmids were obtained from either OriGene in pCMV6 vector with C-terminal Myc-DDK (FLAG) epitope tag or from GenScript in pcDNA3.1-C-(k) DYK (FLAG), as shown in **Supplementary Table 3**. Mutagenesis was carried out using Q5® Site-Directed Mutagenesis Kit (New England BioLabs, E0554S), using primers shown in **Supplementary Table 3**.

**Gel-ABPP for proteome-wide reactivity**
-In situ reactivity: Ramos (5 mL of 3 million cells/mL) or 22Rv1 (3 mL of 1 million/mL seeded in 6 cm dish overnight) cells were treated with 5 or 20 µM alkyne probes for 1 h. Cells were collected and washed 3x with chilled DPBS. Cell pellets were resuspended in 200 µL of cold DPBS and lysed by sonication (2x15 pulses, 10% power output). Total protein content of whole cell lysates was measured using Pierce™ BCA Protein Assay Kit. Samples were normalized to 1 mg/mL and 50 µL treated with 6 µL of click mix (45 µL of 1.7 mM TBTA in 4:1 t-BuOH:DMSO, 15 µL of 50 mM CuSO4 in H2O, 15 µL of 1.25 mM rhodamine-PEG-Azide in DMSO, 15 µL of freshly prepared 50 mM TCEP in DPBS) for 1 h at RT with vigorous vortexing every 20 min. Click reaction was quenched by the addition of 18 µL of 4x SDS gel loading buffer and samples resolved on 10% SDS-PAGE and image by in gel fluorescent scanning.

-In vitro reactivity: Cell lysates (50 µL) were treated with 1 µL of 50x alkyne probe for 1 h at RT and analyzed as above, following click reaction.

Gel-ABPP with recombinant proteins

HEK293T cells (3x10⁵) were seeded in 6-well plate overnight and transfected with 1-2 µg of FLAG-epitope tag plasmids (depending on difficulty of expression) using PEI at a ratio of 1:3 (DNA: PEI), for 48 h. Cells were treated with alkyne probe only for 1 h or with competitor probe for 1 h, followed by alkyne probe for a further 1 h, processed and analyzed by in gel fluorescent scanning as described above.

Glutathione (GSH) reactivity assay

In brief, GSH was diluted to a final concentration of 50 µM in buffer consisting of 0.1M Tris pH 8.8, 30% acetonitrile. In triplicate, 100 µL of the GSH solution was added to a
clear 384 well plate (Greiner 781101). Stereoprobes (5 µL of 10mM) were then added to the GSH solution to achieve a final probe concentration of 500 µM and the reaction incubated for 2 h and 6 h timepoints at RT. Ellman’s reagent (5 µL of 100 mM) was then added to the plate and absorbance read at 440 nm. The concentration of GSH remaining was derived from a standard curve and observed rate ($k_{obs}/[I]$) was calculated assuming pseudo first-order reaction kinetics from the following equations:

$$\frac{d[GSH]}{dt} = -k*[GSH], \quad [GSH]_t = [GSH]_{0}*e^{-kt}$$

**LIMK1 NanoBRET target engagement assay**

LIMK1 NanoBRET target engagement assay was carried out in HEK293T cells transiently transfected with WT or C349A LIMK1-NanoLuc® Fusion Vector (Promega, NV3391) using the NanoBRET™ TE Intracellular Kinase Assay kit, following the manufacturer’s protocol (Promega, N2640). Briefly, HEK293T cells were trypsinized and resuspended in assay medium (Opti-MEM without phenol red, 1% FBS) to a density of 2x10⁵ cells/mL. DNA mixtures (10 µg/mL) were prepared in 1 mL of serum free Opti-MEM at a ratio of 9 µg/mL of Transfection Carrier DNA (Promega, E4881) and 1 µg/mL of LIMK1-NanoLuc plasmid. FuGENE HD reagent (30 µL) was then added to the DNA mixture, mixed by inversion, and incubated at RT for 20 min to form lipid-DNA complexes. Lipid–DNA complexes were then mixed with the HEK293T cell suspension at a 1:20 ratio and 100 µL of the final mixture added to white 96-well tissue culture plates (Corning/Falcon, 353377). Transfected cells were incubated in a humidified, 37 °C/5% CO₂ tissue culture incubator for 20 h. A 100x solution of K-10 NanoBRET tracer in 100% DMSO was diluted in Tracer Dilution Buffer to generate 20x (10 µM)
Complete NanoBRET Tracer Reagent. The Complete NanoBRET Tracer Reagent (5 µL/well of the 20x stock) was added to the transfected cells and mixed by shaking at 900 rpm on an orbital shaker. Test compounds (1000x stock in DMSO) were diluted to 10x final concentration in Opti-MEM reduced serum medium, no phenol red and 10 µL added to the cells containing 1x NanoBRET Tracer Reagent. Plates were thoroughly mixed for 15 sec at 900 rpm and incubated in a humidified, 37 °C/5% CO₂ tissue culture incubator for 3 h. Assay plates were brought to RT and 50 µL of 3x complete substrate plus inhibitor solution added to each well and incubated at RT for 2 min. Each condition was set up in triplicate and untransfected cells were used as background control. Donor (450 nm) and acceptor (610 nm) BRET signals were measured using a CLARIOstar microplate reader (BMG Labtech). Background correction was performed by subtracting average BRET ratio in the absence of LIMK1 from BRET ratio of each sample and milliBRET (mBu) ratios calculated as follows: \(((\text{Acceptorsample/Donorsample} - (\text{Acceptoruntransfected control/Donoruntransfected control})) \times 1,000\). Results were normalized to DMSO control and graphed using GraphPad PRISM v.9.5.

**LIMK1 Nanoluc immunoblot**

Following transfection and treatment with stereoprobe (in 6-well plate) as described above in the NanoBRET section, cells were washed 2x with chilled DPBS and lysed with RIPA buffer supplemented with complete protease and phosphatase inhibitor cocktail. After protein quantification and normalization, samples were boiled in SDS loading buffer and immunoblotted with either mouse anti-Nanoluc (Promega, cat# N7000) or anti-actin (Cell signaling technology, cat# 4967), followed by anti-mouse HRP, and visualization with luminol reagent.
**AFMID enzyme assay**

AFMID activity was assayed in HepG2 cells (endogenous AFMID) or HEK293T cells over expressing AFMID (recombinant AFMID). For *in situ* treatment, HepG2 cells (1x10^6) were seeded in 6-well plates overnight and treated with compounds for 3 h. Cell pellets were collected, washed with DPBS, and lysed by sonication (3x 8 pulses, 10% output) in assay buffer (50 mM Na2HPO4, 1 mM EDTA). Samples were spun at 16,000 x g for 10 min, and supernatant collected for enzyme assay. The concentration of soluble proteome was measured by Pierce™ BCA Protein Assay Kit and samples normalized to 1 mg/mL for endogenous and 0.025 mg/mL for recombinant AFMID.

AFMID activity was initiated by the addition of 1 µL of 25 mM N-formylkynurenine (Millipore Sigma, cat# PHR8908) in DMSO (500 µM final) to 50 µL of the lysate in Eppendorf tubes (LC-MS) or in half-area clear bottom 96-well plate (absorbance measurement) and the reaction allowed to proceed for 30 min (recombinant AFMID) or for 2 h (endogenous AFMID) at RT. For LC-MS analysis, reaction was quenched with 150 µL methanol containing internal standard (5 µg/mL L-Kynurenine D6: Cambridge Isotope Laboratories Inc, cat# DLM-7842). Samples were vigorously vortexed, chilled at -80°C for 30 min, and centrifuged at 16,000 x g for 10 min at 4 °C to pellet precipitated proteins. Supernatant (100 µL) was transferred to an LC-MS/MS vial for metabolomic analysis. For *in vitro* compound treatment, 50 µL of 1 mg/mL (HepG2 soluble lysate) or 0.025 mg/mL of HEK293T cells overexpressing AFMID (WT or mutants) were treated with DMSO or compound for 1h prior to enzyme assay (2 h for endogenous AFMID and 30 min for recombinant AFMID). For Absorbance measurement, absorbance readings were taken at 365nm on a CLARIOstar microplate reader.
LC-MS/MS measurement of kynurenine

Metabolomic profiling of kynurenine was achieved in positive mode by LC/MS–based multiple reaction monitoring (MRM) methods with Agilent Technologies 6460 Triple Quad coupled to Agilent 1290 Infinity LC HPLC. Samples were injected onto an Agilent Eclipse XDB-C18 (4.6mm x 150mm x5 µm) reverse-phase analytical column with a flow rate of 0.25 mL/min. The column was eluted isocratically with 5% mobile phase A (10 mM ammonium formate with 0.1% formic acid in water) for 0.5 min followed by a linear gradient to 40% mobile phase B (methanol with 0.1% formic acid) over 20 min. The following ESI parameters were used for MS analysis: drying gas temperature, 350°C; drying gas flow, 9 L/min; nebulizer pressure, 45 Ψ; sheath gas temperature, 375°C; sheath gas flow, 12 l/min; fragmentor voltage, 100 V; and capillary voltage, 3.5 kV. The MRM transitions m/z for the targeted analytes were 209.1→146 for kynurenine, 215.6→ 152 for Kynurenine D6 (internal standard) and 237→136 for N-formylkynurenine. For each treatment condition, the kynurenine signal was divided by that of the internal standard and then expressed as a percentage of the DMSO control.


-In situ treatment and sample processing: Ramos cells (suspension cells:12.5 mL of 3 million cells/mL—seeded 3 h prior to treatment) or 22Rv1 cells (adherent: 20 mL of 1 million/mL in 15 cm dish—seeded 24 h prior to treatment) were treated with DMSO or 20 μM of the non-alkyne competitor stereoprobes (WX-02-16/26/36/46 or WX-03-57/58/59/60) for 2 h. Cells were further treated with 5 μM of stereochemically matched alkyne probes for 1 h. Cells were washed 3x with chilled DPBS, and immediately
processed or stored at -80 °C. For non-competitive protein-directed ABPP, cells were treated with 5 or 20 μM of the alkyne stereoprobe only, for 3 h. Cell pellets were resuspended in 500 μL of cold DPBS and lysed by sonication (2x15 pulses, 10% power output). Total protein content of whole cell lysates was measured using Pierce™ BCA Protein Assay Kit. Samples were normalized to 2 mg/mL and 500 μL (1 mg of proteome) treated with 55 μL of click mix (30 μL of 1.7 mM TBTA in 4:1 t-BuOH:DMSO, 10 μL of 50 mM CuSO4 in H2O, 5 μL of 10 mM Biotin-PEG4-azide (BroadPharm, cat# BP-22119) in DMSO, 10 μL of freshly prepared 50 mM TCEP in DPBS) for 1 h at RT with vigorous vortexing every 20 min. Proteins were precipitated out of solution by the addition of chilled methanol (600 μL), chloroform (200 μL) and water (100 μL), followed by vigorous vortexing and centrifugation at 16,000 × g for 10 min, to create a disk. Without disrupting the protein disk, both top and bottom layers were aspirated, and the protein disk resonicated in 500 μL of methanol and centrifuged at 16,000 × g for 10 min. After complete aspiration of the methanol, protein pellets were resuspended in 500 μL of freshly made 8 M urea in DPBS, followed by the addition of 10 μL of 10% SDS and probe sonicated to clarity. Samples were reduced with 25 μL of 200 mM DTT at 65 °C for 15 min, followed by alkylation with 25 μL of 400 mM Iodoacetamide at 37 °C for 30 min. Samples were quenched with 130 μL of 10% SDS, transferred to 15 mL tube and the total volume brought up to 6 mL with DPBS (0.2% final SDS). Washed streptavidin beads (Thermo cat # 20353; 100 μL 50% slurry/sample) was then added and probed labeled protein enriched for 1.5 h at RT with rotation. After incubation, beads were pelleted (2 min x 2000 g) and washed with 0.2% SDS in DPBS (2 x 10 mL), DPBS (1 x 5 mL, then transferred to protein low-bind eppendorf safe-lock tube), HPLC water (2 x 1
mL), and 200 mM EPPS (1x 1 mL), at RT. Enriched proteins were digested on-bead overnight with 200 μL of trypsin mix (2 M urea, 1 mM CaCl2, 10 μg/mL trypsin, 200 mM EPPS, pH 8.0). Beads were spun down, supernatant collected and 100 μL of acetonitrile (30% final) added, followed by 6 μL of 20 mg/mL (in dry acetonitrile) of the corresponding TMT\textsuperscript{16plex} tag (for competitive protein-directed ABPP or TMT\textsuperscript{10plex} for non-competitive protein-directed ABPP) for 1.5 h at RT with vortexing every 30 min. TMT labeling was quenched by the addition of hydroxylamine (6 μL 5% solution in H\textsubscript{2}O) and incubated for 15 min at RT. Samples were then acidified with 20 μL 100% formic acid, combined and SpeedVac to dryness. Samples were desalted with Sep-Pak column and then high pH fractionated into 10 fractions, using Peptide Desalting Spin Columns (as described below).

\textbf{-In vitro treatment and sample processing:} For \textit{in vitro} treatment, 500 μL (1 mg) of proteome were treated with 5 μL of 100x probe for 1 h at RT and processed for MS-analysis as described for \textit{in situ} treatment above.

\textbf{- Data processing:} Enrichment ratios (probe vs probe) were calculated for each peptide-spectra match by dividing each TMT reporter ion intensity by the sum intensity for all the channels. Peptide-spectra matches were then grouped based on protein ID and, excluding peptides with summed reporter ion intensities < 10,000, coefficient of variation of > 0.5, and < 2 distinct peptides. Replicate channels were grouped across each experiment, and average values were computed for each protein. A variability metric was also computed across replicate channels, which equaled the ratio of median absolute deviation to average and was expressed in percentage. A protein was considered enantioselectively liganded if the variability corresponding to the alkyne
probe leading to highest enrichment did not exceed 20%, and at least one of the following additional criteria were met: (i) the average enrichment by the alkyne probe was > 3-fold that of its enantiomer and > 2-fold the enrichment observed following treatment with a stereochemically matched non-alkyne competitor; (ii) the average enrichment by the alkyne probe was > 3-fold that of its enantiomer, and at least one site in the protein was deemed liganded only by the alkyne probe (not by non-alkyne competitors) in cysteine-directed ABPP experiments; (iii) the average enrichment by the alkyne probe was > 2-fold that of its enantiomer and > 2-fold the enrichment observed following treatment with a stereochemically matched non-alkyne competitor, and at least one site in the protein was deemed liganded (by any probe) in cysteine-directed ABPP experiments (vide infra).

Proteomic Platforms: Multiplexed cysteine-directed activity based-protein profiling.

**In situ treatment and sample processing:** Cysteine-directed ABPP was carried out as previously reported with slight modifications. In summary, Ramos cells (suspension cells: 12.5 mL of 3 million cells/mL—seeded 3 h prior to treatment) or 22Rv1 cells (adherent: 20 mL of 1 million/mL in 15 cm dish—seeded 24 h prior to treatment) were treated with DMSO or 20 μM of the stereoprobes for 3 h. Cells were washed 3x with chilled DPBS, and immediately processed or stored at -80 °C. Cell pellets were resuspended in 500 μL of cold DPBS and lysed by sonication (2x15 pulses, 10% power output). Total protein content of whole cell lysates was measured using Pierce™ BCA Protein Assay Kit. Samples were normalized to 2 mg/mL and 500 μL (1mg of proteome) treated with 5 μL of 10 mM iodoacetamide desthiobiotin (IA-DTB, in DMSO) for 1 h at
room temperature with occasional vortexing. Proteins were precipitated out of solution by the addition of chilled HPLC grade methanol (600 μL), chloroform (200 μL) and water (100 μL), followed by vigorous vortexing and centrifugation at 16,000 x g for 10 min, to create a disk. Without disrupting the protein disk, both top and bottom layers were aspirated, and the protein disk washed with 1 mL cold methanol and centrifuged at 16,000 x g for 10 min. The pellets were allowed to air dry (just enough get rid of methanol droplets), and then resuspended in 90 μL of denaturing/reducing buffer (9 M urea, 10 mM DTT, 50 mM triethylammonium bicarbonate (TEAB) pH 8.5). Samples were reduced by heating at 65 °C for 20 min, followed by the addition of 10 μL (500 mM) iodoacetamide for 30 min, at 37 °C to cap free cysteines. Samples were then centrifuged at maximum speed (16,000 x g for 2 min) to pellet any insoluble precipitate and probe sonicated once more to ensure complete resuspension, and then diluted with 300 μL 50 mM TEAB pH 8.5 to reach a final urea concentration of 2 M. Trypsin (4 μL of 0.25 μg/μL in trypsin resuspension buffer with 25 mM CaCl₂) was added to each sample and digested at 37°C overnight. Digested samples were then diluted with 300 μL wash buffer (50 mM TEAB pH 8.5, 150 mM NaCl, 0.2% NP-40) containing streptavidin-agarose beads (50 μL of 50% slurry/sample) and were rotated at room temperature for 2 h. Samples were centrifuged (2,000 x g, 2 min), and the entire content transferred to BioSpin columns and washed (3x1 mL wash buffer, 3x1 mL DPBS, 3x1 mL water). Enriched peptides were eluted from beads with 300 μL 50% acetonitrile with 0.1% formic acid and speedVac to dryness. IA-DTB labeled and enriched peptides were resuspended in 100 μL EPPS buffer (200 mM, pH 8.0) with 30% acetonitrile, vortexed, and water bath sonicated. Samples were TMT labeled by the addition of 3 μL of 20
mg/mL (in dry acetonitrile) of corresponding TMT\textsuperscript{10plex} tag, vortexed, and incubated at room temperature for 1.5 h. TMT labeling was quenched with the addition of hydroxylamine (5 μL 5% solution in H\textsubscript{2}O) and incubated for 15 min at room temperature. Samples were then acidified with 5 μL formic acid, combined and dried using SpeedVac. Samples were desalted with Sep-Pak and then high pH fractionated with HPLC (as described below) into 96-well plate and recombined into 12 fractions total.

**Cysteine-directed ABPP of denatured proteome**

Cysteine-directed ABPP of denatured proteome was performed as previously reported\textsuperscript{38}. Briefly, cell lysates from Ramos or 22Rv1 cells (500 μL of 2 mg/mL) was added to empty 1.5 mL low bind tubes (native sample) and kept on ice. For denatured samples, cell lysates were added to tubes containing 240 mg of urea (8 M final concentration) and boiled at 65°C for 15 min. Both native and denatured samples were equilibrated to RT and then treated with 5 μL of 10 mM IA-DTB at RT for 1 h. Proteins were precipitated out of solution and processed for proteomic analysis as described in the cysteine-directed ABPP section above.

**- Data processing:** Cysteine engagement ratios (DMSO vs compound) were calculated for each peptide-spectra match by dividing each TMT reporter ion intensity by the average intensity for the DMSO channels. Peptide-spectra matches were then grouped based on protein ID and residue number (e.g., NFU1 C210), excluding peptides with summed reporter ion intensities for the DMSO channels < 10,000, coefficient of variation for DMSO channels > 0.5. Replicate channels were grouped across each experiment, and average values were computed for each cysteine site. A variability metric was also computed across replicate channels, which equaled the ratio of median
absolute deviation to average and was expressed in percentage. A cysteine site was considered enantioselectively liganded if the variability corresponding to the probe leading to highest blockade of iodoacetamide-desthiobiotin (IA-DTB) did not exceed 20%, and at least one of the following additional criteria were met: (i) the average IA-DTB blockade by the probe was > 66.7% and > 2.5-fold that of its enantiomer, and either (a) the same probe led to < 25% IA-DTB blockade of at least one other cysteine in the same protein, or (b) the same cysteine site was deemed liganded in another cysteine-directed ABPP experiment in this study; (ii) the average IA-DTB blockade by a given probe was > 50% and > 1.5-fold that of its enantiomer, and the protein was deemed enantioselectively liganded by protein-directed ABPP in this study (vide supra).

Offline fractionation

- **High pH spin column fractionation**: High pH fractionation was carried out as previously reported\(^\text{15, 35}\) using Peptide Desalting Spin Columns (Thermo 89852). Samples (protein-directed ABPP samples) were resuspended in 300 µL of buffer A (5% acetonitrile, 0.1% formic acid) by water bath sonication and bound to the spin columns. Bound peptides were then washed 2x with water, 1x with 5% acetonitrile in 10 mM NH\(_4\)HCO\(_3\), and eluted into 30 fractions with increasing gradient of acetonitrile. Every 10\(^{th}\) fraction was combined (e.g., 1, 10, and 30) and SpeedVac to dryness. Each of the resulting 10 fractions were resuspended in buffer A (5% acetonitrile, 0.1% formic acid) and analyzed by mass spectrometry.

- **HPLC fractionation**: Samples (cysteine-directed ABPP samples) were resuspended in 500 µL buffer A and fractionated with Agilent HPLC into a 96 deep-well plate containing 20 µL of 20% formic acid to acidify the eluting peptides, as previously reported\(^\text{20}\) The
peptides were eluted onto a capillary column (ZORBAX 300Extend-C18, 3.5 μm) and separated at a flow rate of 0.5 mL/min using the following gradient: 100% buffer A from 0-2 min, 0%–13% buffer B from 2-3 min, 13%–42% buffer B from 3-60 min, 42%–100% buffer B from 60-61 min, 100% buffer B from 61-65 min, 100%–0% buffer B from 65-66 min, 100% buffer A from 66-75 min, 0%–13% buffer B from 75-78 min, 13%–80% buffer B from 78-80 min, 80% buffer B from 80-85 min, 100% buffer A from 86-91 min, 0%–13% buffer B from 91-94 min, 13%–80% buffer B from 94-96 min, 80% buffer B from 96-101 min, and 80%–0% buffer B from 101-102 min (buffer A: 10 mM aqueous NH₄HCO₃; buffer B: acetonitrile). The plates were evaporated to dryness using SpeedVac and peptides resuspended in 80% acetonitrile, with 0.1% formic acid and combined to a total of 12 fractions (e.g., fraction1= well 1A+ 1B…1H, fraction 2= well 2A+2B….2H) (3x300 μL/column). Samples were SpeedVac to dryness and the resulting 12 fractions were re-suspended in buffer A (5% acetonitrile, 0.1% formic acid) and analyzed by mass spectrometry.

**TMT liquid chromatography-mass-spectrometry (LC-MS) analysis**

Samples were analyzed by liquid chromatography tandem mass-spectrometry using an Orbitrap Fusion mass spectrometer (Thermo Scientific) coupled to an UltiMate 3000 Series Rapid Separation LC system and autosampler (Thermo Scientific Dionex), as previously reported¹⁵, ³⁵. The peptides were eluted onto a capillary column (75 μm inner diameter fused silica, packed with C18 (Waters, Acquity BEH C18, 1.7 μm, 25 cm)) or an EASY-Spray HPLC column (Thermo ES902, ES903) using an Acclaim PepMap 100 (Thermo 164535) loading column, and separated at a flow rate of 0.25 μL/min. Data was acquired using an MS3-based TMT method on Orbitrap Fusion or Orbitrap Eclipse.
Tribrid Mass Spectrometers. Briefly, the scan sequence began with an MS1 master scan (Orbitrap analysis, resolution 120,000, 400–1700 m/z, RF lens 60%, automatic gain control [AGC] target 2E5, maximum injection time 50 ms, centroid mode) with dynamic exclusion enabled (repeat count 1, duration 15 s). The top ten precursors were then selected for MS2/MS3 analysis. MS2 analysis consisted of: quadrupole isolation (isolation window 0.7) of precursor ion followed by collision-induced dissociation (CID) in the ion trap (AGC 1.8E4, normalized collision energy 35%, maximum injection time 120 ms). Following the acquisition of each MS2 spectrum, synchronous precursor selection (SPS) enabled the selection of up to 10 MS2 fragment ions for MS3 analysis. MS3 precursors were fragmented by HCD and analyzed using the Orbitrap (collision energy 55%, AGC 1.5E5, maximum injection time 120 ms, resolution was 50,000). For MS3 analysis, we used charge state–dependent isolation windows. For charge state z = 2, the MS isolation window was set at 1.2; for z = 3-6, the MS isolation window was set at 0.7. Raw files were uploaded to Integrated Proteomics Pipeline (IP2) available at (http://ip2.scripps.edu/ip2/mainMenu.html) and MS2 and MS3 files extracted from the raw files using RAW Converter and searched using the ProLuCID algorithm using a reverse concatenated, non-redundant variant of the Human UniProt database (release 2016-07). Cysteine residues were searched with a static modification for carboxyamidomethylation (+57.02146 Da). A dynamic modification for IA-DTB labeling (+398.25292 Da) was included with a maximum number of 2 differential modifications per peptide. N-termini and lysine residues were also searched with a static modification corresponding to the TMT tag (+229.1629 Da for 10plex and +304.2071 Da for 16plex). Peptides were required to be at least 6 amino acids long. ProLuCID data was filtered
through DTASelect (version 2.0) to achieve a peptide false-positive rate below 1%. The MS3-based peptide quantification was performed with reporter ion mass tolerance set to 20 ppm with Integrated Proteomics Pipeline (IP2).

**Proteomic Platforms: Multiplexed Lysine-directed activity based-protein profiling.**

Lysine-directed ABPP was carried out in the same way as cysteine directed ABPP, with the following modifications: (1) NHS-DTB (30 µM final) was used in place of IA-DTB and (2) a dynamic modification for NHS-DTB labeling (-33.04175 Da) was used in place of IA-DTB labeling (+398.25292 Da) during the search.

**Data and Code availability**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD042541. Processed proteomic data are provided in Supplementary Dataset 1. All other data that support the findings of this study and codes are available from the corresponding authors upon reasonable request.

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**Author contributions**
E.N., B.M., and B.F.C. conceived the study. E.N., R.E.H., and K.E.D. generated proteomic data. E.N., B.M., and B.F.C, performed analysis of proteomic data and wrote the manuscript. G.M.S. assisted with proteomic data analysis. E.N., and R.E.H, confirmed protein-stereoprobe interactions by gel-ABPP. E.N., performed all functional assays except for GSH reactivity which was carried out by T. N. and P.A. Compound synthesis and characterization was supervised by E.N., K.E.D., D.O., B.M., and B.F.C. Additional resources to the study were contributed by M.M.D., G.M.S, and S.L.S. All authors edited and approved the manuscript. B.F.C. supervised the study.

**Declaration of interests**

G.M.S., T. N., and P.A. are employees of Vividion Therapeutics, and B.F.C. is a founder and member of the Board of Directors of Vividion Therapeutics.

**Inclusion and Diversity**

One or more of the authors of this paper self-identifies as gender minority in their field of research.
Figure 1. Alkyne stereoprobe sets for mapping electrophilic small molecule-protein interactions in cells. a, Structures of alkynylated tryptoline acrylamide stereoprobes used in this study (black) and the corresponding non-alkyne stereoprobes (red) used as competitors in ABPP experiments. b, In situ reactivity of alkyne stereoprobes (5 µM, 1 h) in 22Rv1 cells as determined by gel-ABPP where stereoprobe-reactive proteins are detected by CuAAC conjugation to an azide-rhodamine reporter group, SDS-PAGE, and in-gel fluorescence scanning. c, Concentration-dependent in situ reactivity of alkyne stereoprobe set 1 in Ramos and 22Rv1 cells (tested at 5 µM and 20 µM, 1 h). Red asterisks mark examples of
stereoselective stereoprobe-protein interactions. b, c, data are from a single experiment representative of two independent experiments.
Figure 2. Protein-directed ABPP platform for mapping stereoselectively liganded proteins in human cells. 

a. Workflow for protein-directed ABPP experiments where the stereoselective enrichment of proteins by alkyne stereoprobes and blockade of this enrichment by corresponding non-alkyne competitor stereoprobes are determined by multiplexed (tandem mass tagging, TMT16plex) MS-based proteomics.

b. Pie chart showing total number of stereoselectively liganded proteins mapped for alkyne stereoprobe set 1-5 in Ramos and 22Rv1 cells (271 total liganded proteins among >8000 quantified proteins).

c. Number of proteins stereoselectively liganded in situ by each stereoconfiguration of tryptoline acrylamide stereoprobes.

d. Correlation plots comparing in situ (5 µM, 1 h) and in vitro (10 µM, 1 h) enantioselective protein enrichment values from protein-directed ABPP experiments performed in Ramos cells treated with stereoprobe set 3.

e. Quadrant plots highlighting stereoselectively liganded proteins for each stereoconfiguration of alkyne stereoprobe set 1 in Ramos (top) and 22Rv1 (bottom) cells. Enantioselectivity (x-axis) is the ratio of enrichment for one stereoisomer vs its enantiomer, and diastereoselectivity (y-axis) is the ratio of enrichment of one stereoisomer vs the average of its two diastereomers.

f. Unsupervised clustering of the full stereoprobe interaction profiles for each stereoselectively liganded protein, where
clustering was based on the magnitude of competitor stereoprobe blockade of stereoselective enrichment observed for each liganded protein. White spaces represent protein-stereoprobe interactions that did not qualify as stereoselectively enriched by the indicated alkyne stereoprobe. Protein-directed ABPP data represent average values from at least four biological replicates (two independent experiments each with two biological replicates), per cell line.
Figure 3. Integrated protein- and cysteine-directed ABPP to generate a near-comprehensive portrait of stereoselectively liganded proteins in human cancer cells. **a**, Total number of proteins/cysteines, as well as the fractions of stereoselectively liganded proteins/cysteines, quantified by cysteine-directed ABPP in Ramos and 22Rv1 cells. Stereoselectively liganded cysteines were determined by averaging values from at least four biological replicates (two independent experiments each with two biological replicates), per cell line. **b**, Pie chart showing total number of stereoselectively liganded proteins mapped by integrated cysteine- and protein-directed ABPP for alkyne stereoprobe set 1-5 and competitor stereoprobe set 1-2 in Ramos and 22Rv1 cells (336 total liganded proteins among >10500 quantified proteins). **c**, Functional class distribution of stereoselectively liganded proteins assigned by Gene Ontology ( Panther) and KEGG Brite databases. **d**, GO-term enrichment analysis for stereoselectively liganded proteins in Ramos and 22Rv1 cells. **e**, Pie chart showing fraction of stereoselectively liganded proteins mapped by cysteine-directed ABPP, protein-directed ABPP, or both platforms. **f**, Heatmap of quantified cysteines in HECTD4 showing multiple cysteines (C586, C1488, C1783) that display different stereoselective liganding profiles with stereoprobe set 3 in cysteine-directed ABPP experiments. **g**, Bar graph showing lack of enantioselective enrichment or competition for HECTD4 in protein-directed ABPP experiments. Data represent average values ± SD, n = 4. **h**, Box and Whisker plot showing the relationship between protein size and likelihood of being assigned as liganded in cysteine- or protein-directed ABPP experiments (One-way ANOVA with Tukey’s multiple comparison, ***p=0.0001). **i**, Violin plot showing MS detection probability (DeepMS score; an estimate of peptide proteotypicity) for cysteine-containing tryptic peptides that were quantified (right) or not quantified (left) in cysteine-directed ABPP experiments (unpaired two-tailed t-test, **** p=0.0001). For the purposes of this analysis, we focused on cysteine-containing
tryptic peptides from stereoselectively liganded proteins. j, Stereoselective enrichment of STRBP by the (1R,3S) alkyne stereoprobe WX-03-347 without blockade of this enrichment by the corresponding competitor stereoprobe WX-03-58. k, Cysteine-directed ABPP data showing stereoselective engagement of STRBP_C142 by WX-03-347 (left), but not WX-03-58 (right). For j, k, data represent average values ± SD, n = 4. l, Pie chart showing total number of stereoselectively enriched proteins in protein-directed ABPP experiments with alkyne stereoprobe sets 1-5 in Ramos and 22Rv1 cells, and the fraction of these proteins showing blockade of enrichment by non-alkyne competitor stereoprobes (yellow) or not showing blockade of enrichment by non-alkyne competitor stereoprobes, but possessing (red) or not possessing (blue) a cysteine that was stereoselectively liganded by alkyne stereoprobes in cysteine-directed ABPP experiments.
Figure 4. Confirmation and characterization of stereoselectively liganded proteins mapped by both cysteine-directed and protein-directed ABPP. 

a. Cysteine-directed ABPP data showing stereoselective liganding of C210/213 of NFU1 in Ramos and 22Rv1 cells by alkyne (WX-01-12) and competitor (WX-02-46) stereoprobes. 

b. Protein-directed ABPP data showing stereoselective enrichment of NFU1 by WX-01-12 and blockade of this enrichment by WX-02-46 in 22Rv1 cells. Data represent average values ± SD, n = 4. 

c. Gel-ABPP data demonstrating engagement of WT-NFU1 and the C213A-NFU1 mutant, but not the C210A-NFU1 mutant by WX-01-12. Data represent in situ stereoprobe treatment (5 µM, 1 h) of HEK293T cells transiently expressing Flag epitope-tagged WT-NFU1 or a C210A or C213A NFU1 mutant. ABPP signals were measured by CuAAC conjugation to rhodamine-azide tag followed by SDS-PAGE and in-gel fluorescence scanning. 

d. Bar graph showing enantioselective enrichment of TMX1/4, but not TMX2/3 by (1S,3S) alkyne stereoprobe WX-01-09 from protein-directed ABPP experiments in Ramos cells. For each TMX protein, the signal intensity in WX-01-11-treated cells was set to a value of 1. Data represent average values ± SD, n = 4. 

e, f. Gel-ABPP data demonstrating engagement of recombinant WT-TMX1 and C59A- and C205A-TMX1 mutants, but not C56A- or C56A/C59A TMX1 mutants (e), and recombinant WT-TMX4 and C67A- and C213A-TMX4 mutants, but not C64A- or C64A/C67A-TMX4 mutants (f) by WX-01-09 (5 µM, 1 h). Experiments were performed in
transfected HEK293T cells as described in (c). 

**g.** Gel-ABPP confirming stereoselective engagement of recombinant TMX1 and TMX4, but not TMX2 and TMX3, by WX-01-09. 

**h.** Cysteine-directed ABPP data showing enantioselective liganding of C349 of LIMK1 in Ramos cells by (1R,3S) and (1R,3R) alkyne stereoprobes WX-01-10 and WX-01-11, respectively. Data represent average values ± SD, n = 4. 

**i.** Protein-directed ABPP data showing enantioselective enrichment of LIMK1 by WX-01-10 and WX-01-11 that is not blocked by WX-02-26 and WX-02-36, respectively, in Ramos cells. Data represent average ± SD, n = 4. 

**j.** Gel-ABPP data demonstrating engagement of recombinant WT-LIMK1, but not the C349A-LIMK1 mutant by WX-01-11 (5 µM, 1 h). Experiments were performed in transfected HEK293T cells as described in (c). 

**k.** Crystal structure of LIMK1 (PDB: 8AAU) showing C349 (red) in a pocket adjacent to the ATP (blue) binding site. Highlighted in yellow are residues conserved between LIMK1 and LIMK2 located within 15 Å of LIMK1 C349. 

**l.** Enantioselective and concentration-dependent enhancement of BRET signal in an LIMK1 NanoBRET kinase assay by WX-01-10 and WX-01-11 compared to their respective enantiomers (WX-01-12 and WX-01-09). Data were generated in HEK293T cells transiently expressing LIMK1-nanoLuciferase fusion protein, where cells were treated with 0.5 µM of the NanoBRET K-10 tracer and different concentrations of stereoprobes for 3 h (Data represent average values ± SD for n = 1, setup in triplicates). 

**m.** Enantioselective enhancement of BRET signal in LIMK1 NanoBRET kinase assay by WX-01-11 in WT but not C349A mutant cells. In contrast, the ATP-binding pocket kinase inhibitor HG-9-91-01 decreases the NanoBRET signal for both WT- and the C349A-LIMK1 mutant (data represent average values ± SD, n = 3). For c, e-g, and j, IB = anti-Flag immunoblot. For gel-ABPP data shown in c, e-g, and j, data are from a single experiment representative of at least two independent experiments and UT= untransfected cells.
Figure 5. Confirmation and characterization of stereoselectively liganded proteins mapped exclusively by protein-directed ABPP. a, Protein-directed ABPP data showing stereoselective enrichment of C15orf57 by alkyne stereoprobe WX-01-12 and blockade of this enrichment by competitor stereoprobe WX-02-46 in Ramos cells. Data represent average values ± SD, n = 4. b, Gel-ABPP data demonstrating stereoselective engagement of recombinant WT-C15orf57, but not the C111A-C15orf57 mutant by WX-01-12 (5 µM, 1 h). Experiments were performed in transfected HEK293T cells as described in Fig. 4c. Shown above the gel is the C111-containing tryptic peptide of C15orf57. c, Protein-directed ABPP data showing enantioselective enrichment of STK39, but not the paralog protein OXSR1, by WX-03-346 in Ramos cells. For each protein, the signal intensity for the inactive probe (WX-03-348) was normalized to 1. Data represent average values ± SD, n = 4. d, Gel-ABPP data demonstrating engagement of WT-STK39 and the C59A-STK39 mutant, but not the C334A-STK39 mutant, by WX-03-346 (1 µM, 1 h). Experiments were performed in transfected HEK293T cells as described in Fig. 4c. e, Protein-directed ABPP data showing stereoselective enrichment of AK3 by alkyne stereoprobe WX-03-338 and blockade of this enrichment by competitor stereoprobe WX-03-57 in 22Rv1 cells. Data represent average values ± SD, n = 4. f, Tryptic peptide map of AK3 from protein-directed ABPP experiments showing stereoselective enrichment of all quantified AK3 peptides by WX-03-338 except for the peptide containing K34 (red). In the heat map display, tryptic peptide signal intensities were normalized to 100% for the WX-03-338 treatment group. g, Gel-ABPP data demonstrating stereoselective engagement of recombinant WT-AK3, but not the K34R-AK3 mutant by WX-01-05. Experiments were performed in transfected HEK293T cells as described in Fig. 4c. h, Lysine-directed ABPP showing stereoselective liganding of AK3_K34 by WX-03-57. Data represent average values
± SD, n = 2. i, Crystal structure of AK3 (PDB: 6ZJD) showing distal location of K34 relative to the enzyme active site. For b, d, and g, data are from a single experiment representative of at least two independent experiments; IB = anti-Flag immunoblot, UT= untransfected cells.
Figure 6. Stereoselective and site-specific covalent ligands that inhibit the kynurenine biosynthetic enzyme AFMID. a, Function of AFMID in the tryptophan-kynurenine metabolic pathway. IDO, indoleamine 2, 3-dioxygenase; TDO, tryptophan 2, 3-dioxygenase. b, Protein-directed ABPP data showing stereoselective enrichment of AFMID by alkyne stereoprobe WX-01-03 and blockade of this enrichment by competitor stereoprobe WX-02-36 in Ramos cells. Data represent average values ± SD, n = 2. c, AlphaFold-generated structural model of AFMID showing location of candidate liganded cysteine (C28 in red), the catalytic serine S164 (blue), and the HGGYW motif (green) involved in oxyanion formation during substrate cleavage. d, e, Gel-ABPP data demonstrating stereoselective engagement of recombinant WT-AFMID, but not the C28A-AFMID mutant by WX-01-03 (5 µM, 1 h) (d) and blockade of this engagement by WX-02-36 (20 µM, 1 h) (e). Experiments were performed in transfected HEK293T cells as described in Fig. 4c. f, N-formylamidase activity of recombinant WT-, C28A-, C28W-, and S164A-AFMID with or without exposure to DMSO or WX-01-03 (5 µM, 1 h pre-treatment). AFMID variants were transiently expressed in HEK293T cells, and lysates were incubated with N-formylkynurenine (500 µM, 30 min) and the kynurenine product measured by LC-MS/MS. g, Concentration-dependent inhibition of endogenous AFMID activity in HepG2 cells by WX-01-03. HepG2 cells were treated with the indicated concentrations of WX-01-03 or WX-01-01 for 3 h, after which cells were lysed and assayed for AFMID activity as described in f, with the exception that the reaction was allowed to proceed for 2 h. h, Sequence alignment of human and mouse AFMID. i, Gel-ABPP data showing stereoselective engagement of a recombinant mouse S26C-AFMID mutant, but not mouse WT-AFMID, by WX-01-03 (5 µM, 1 h). j, Concentration-dependent and stereoselective inhibition of the catalytic activity of recombinant mouse S26C-AFMID mutant, but not mouse WT-AFMID, by WX-01-03. For f, g and j, data represent mean ± SD for three independent experiments. For d, e, and i, data are from a single experiment representative of at least two experiments; IB = anti-Flag immunoblot, UT= untransfected cells.
Extended Data Fig. 1. Gel-ABPP of alkyne stereoprobes in human cancer cells. a, *In situ* reactivity of alkyne stereoprobes (5 µM, 1 h) in Ramos cells as determined by gel-ABPP. b, *In vitro* reactivity of probe set 1 (5 or 20 µM, 1 h) in Ramos and 22Rv1 cell lysate. Red asterisks mark examples of stereoselective stereoprobe-protein interactions. a, b, data are from a single experiment representative of at least two experiments.
Extended Data Fig. 2. Protein-directed ABPP platform for mapping stereoselectively liganded proteins in human cells. a, Workflow for protein-directed ABPP experiments where the stereoselective enrichment of proteins by alkyne stereoprobes is determined by multiplexed (tandem mass tagging, TMT10plex) MS-based proteomics. b, Comparison of enantioselective enrichment values in protein-directed ABPP experiments in Ramos cells treated with alkyne stereoprobe set 2 (WX-01-05/06/07/08) at 5 vs 20 µM (3 h). Proteins shown are: 1) designated as stereoselective targets in either the 5 or 20 µM data sets (> 3-fold enantioselective enrichment); and 2) quantified in both the 5 and 20 µM data sets. c, Number of proteins stereoselectively liganded by each stereoconfiguration of stereoprobe set 3 (WX-01-09/10/11/12; 10 µM, 1 h) in vitro. d, Examples of proteins showing preferential stereoselective enrichment by stereoprobe set 3 in situ (SF3B1, UBA3) or in vitro (CMPK1, GRHPR). In situ conditions: 5 µM stereoprobe, 1 h; in vitro conditions: 10 µM stereoprobe, 1 h. Data represent average values ± SD, n = 4. e, Pie chart showing fraction of proteins that were enantioselectively liganded by cis stereoprobes (cis-only), trans stereoprobes (trans-only), or both cis and trans stereoprobes (cis/trans).
Extended Data Fig. 3. Stereoprobe-protein interaction maps from protein-directed ABPP experiments in human cancer cells. a-d, Quadrant plots highlighting
stereoselectively liganded proteins for each stereoconfiguration of alkyne stereoprobe sets 2-5 in Ramos (left) and 22Rv1 (right) cells. Enantioselectivity (x-axis) is the ratio of enrichment for one stereoisomer vs its enantiomer, and diastereoselectivity (y-axis) is the ratio of enrichment of one stereoisomer vs the average of its two diastereomers.
Extended Data Figure 4. Integrated protein- and cysteine-directed ABPP for mapping stereoselectively liganded proteins in human cancer cells. a, Workflow for cysteine-directed ABPP experiments where stereoprobe reactivity with cysteines is determined by multiplexed (tandem mass tagging, TMT\textsuperscript{10plex}) MS-based proteomics, as described previously\textsuperscript{15}. b, Number of proteins stereoselectively liganded by one (single; black) versus multiple (red) stereoprobe core configurations. c, Proportion of stereoselectively liganded proteins showing essentiality in the Cancer Dependency Map. d, Heatmap of quantified cysteines in PRKDC showing two cysteines (C1229 and C1499/C1507) that display different stereoselective liganding profiles with stereoprobe set 2 in cysteine-directed ABPP experiments. e, Protein-directed ABPP data showing lack of enantioselective enrichment or competition for PRKDC in Ramos cells. f, Protein-directed ABPP data showing stereoselective enrichment of PIKFYVE by alkyne stereoprobe WX-03-338 in 22Rv1 cells and blockade of this enrichment by competitor stereoprobe WX-03-57. g, Violin plot showing native-vs-denatured reactivity ratios for cysteine-containing tryptic peptides with good predicted proteotypicity (DeepMS probability > 0.5) that were either quantified or not quantified in cysteine-directed ABPP experiments performed in native proteomes. For the purposes of this analysis, we used cysteine-containing tryptic peptides from stereoselectively
liganded proteins (unpaired two-tailed t-test, **** p=0.0001). h, Protein-directed ABPP data showing stereoselective enrichment of FXR1 by the (1S,3S) alkyne stereoprobe WX-03-346 without blockade of this enrichment by the corresponding competitor stereoprobe WX-03-57.

i, Cysteine-directed ABPP data showing greater stereoselective liganding of FXR1_C157 by WX-03-346 (left) versus WX-03-57 (right).

j, Pie chart showing fraction of proteins liganded in a stereoselective (blue) and non-stereoselective (green) manner, in protein directed ABPP experiments. For e, f, h, and i, data represent average values ± SD, n = 4.
Extended Data Fig. 5. Confirmation and characterization of stereoselectively liganded proteins mapped by both cysteine-directed and protein-directed ABPP. a, b, Cysteine-directed ABPP data showing stereoselective liganding of PLEK_C250 in Ramos cells by (1R,3S) alkyne stereoprobe WX-01-06 (a) and competitor stereoprobe (WX-02-26) (b). c, Protein-directed ABPP data showing stereoselective enrichment of PLEK by WX-01-06 and blockade of this enrichment by WX-02-26 in Ramos cells. d, Gel-ABPP data demonstrating stereoselective engagement of recombinant WT-PLEK, but not a C250A-PLEK mutant by WX-01-06 (5 µM, 1 h). e, AlphaFold-predicted structure of PLEK showing location of C250 (red) relative to the IP5 binding pocket (blue). f, Protein-directed ABPP data showing stereoselective enrichment of NFU1 by alkyne stereoprobe WX-01-12 (5 µM, 1 h) and blockade of this enrichment by competitor stereoprobe WX-02-46 in Ramos cells. g, Competitive gel-ABPP data showing stereoselective blockade of WX-01-12 reactivity with recombinant WT-NFU1 by WX-02-46 (20 µM, 1 h pre-treatment). h, CellTiter-Glo data showing pH-dependent impairment in cell growth by WX-01-12 (5 µM, 72 h) in SW480 cells. Data are mean ± SD from three independent experiments. One-way ANOVA with Dunnett’s multiple comparison, **p=0.0023, ***p<0.0001. i, Cysteine-directed ABPP data showing stereoselective liganding of TYMS_C195 by (1R,3R) alkyne stereoprobe WX-01-07 (left) and competitor stereoprobe WX-02-36 (right) in Ramos cells. j, Protein-directed ABPP data showing stereoselective enrichment of recombinant TYMS by WX-01-07 (5 µM, 1 h) and blockade of this enrichment by WX-02-36 (20 µM, 1 h pre-treatment) in Ramos cells. k, l, Gel-ABPP data showing stereoselective engagement of WT-TYMS, but not a C195-TYMS mutant by WX-01-07 (k) and stereoselective blockade of this engagement by WX-02-36 (l). d, g, k, l, experiments were performed in transfected HEK293T cells as described in Fig. 4c. For a-c, f, i, and j, data represent average values ± SD, n = 4. For d, g, k, and l, data are from a single
experiment representative of two experiments; IB = anti-Flag immunoblot, UT= untransfected cells.
Extended Data Fig. 6. Confirmation and characterization of stereoselectively liganded proteins mapped by both cysteine-directed and protein-directed ABPP. a, Sequence alignment of TMX1 and TMX4 with three conserved cysteines highlighted in yellow. Peptides quantified in protein-directed ABPP are shown in red.

b, Cysteine-directed ABPP data showing stereoselective liganding of TMX4_C64/67 by (1S,3S) alkyne stereoprobe WX-01-09.

c, d, WX-01-09.

e, WX-02-16

f, g, h, LIMK1_C349

i, j, k, m, n, LIMK1 (Nanoluc)
in Ramos cells. Data represent average values ± SD, n = 4. c, d, Competitive gel-ABPP data showing concentration-dependent and enantioselective blockade of WX-01-09 (5 µM, 1 h) engagement of TMX1 (c) and TMX4 (d) by WX-02-16 (1 h pre-treatment). Top, representative gel-ABPP data; bottom, quantification of gel-ABPP (mean ± SD, n = 2). e, Cysteine-directed ABPP data showing lack of engagement of LIMK1_C349 by competitor stereoprobes WX-02-26 and WX-02-36. Data represent average values ± SD, n = 4. f, Sequence alignment of LIMK1 and LIMK2 showing conserved residues (yellow) that are proximal (< 15 Å) to the LIMK1-restricted stereoprobe-liganded cysteine C349 (red). g, NanoBRET kinase assay showing that WX-01-11 increases signals for LIMK1 with the general kinase NanoBRET probe K-10, with the largest effect observed at lower concentrations of the NanoBRET probe. Data represent average values ± SD for n = 1, setup in triplicates. h, Immunoblot of LIMK1-Nanoluc protein recombinantly expressed in HEK293T cells showing that the alkyne stereoprobes do not affect LIMK1 expression under conditions where they increase NanoBRET signal for the kinase. For c and d, IB = anti-Flag immunoblot, and for h, UT= untransfected cells. The red asterisk in c represents alkyne liganded and rhodamine tagged species of TMX1 (this corresponds to the signal seen in gel-ABPP above the IB).
Extended Data Fig. 7. Stereoselective liganding of a conserved cysteine in the deubiquitinase paralogs STAMBP and STAMBPL1. a, Protein-directed ABPP data showing stereoselective enrichment of STAMBP by the (1S,3R) alkyne stereoprobes WX-01-08 and WX-03-341 without blockade of this enrichment by the corresponding competitor stereoprobes WX-02-46 and WX-03-60, respectively. b, Cysteine-directed ABPP data showing stereoselective liganding of STAMBP_C264 by WX-01-08, but not WX-03-60. c, Protein-directed ABPP data showing stereoselective enrichment of STAMBPL1 by the (1S,3R) alkyne stereoprobes WX-01-08 and WX-03-341 and blockade of this enrichment by the corresponding competitor stereoprobes WX-02-46 and WX-03-60, respectively. d, Cysteine-directed ABPP data showing stereoselective liganding of STAMBPL1_C266/C276 by WX-03-60. For a–d, data represent average values ± SD, n = 4. e, Left, sequence alignment of STAMBP and STAMBPL1 showing conservation of the stereoprobe-ligated cysteine (C264 in STAMBP and inferred as C276 in STAMBPL1) highlighted in yellow. Right, overlay of the crystal structures of STAMBP (green, PDB: 3RZV) and STAMBPL1 (blue, PDB: 2ZNV) showing location of the conserved cysteines C264/276 (yellow) with respect to the deubiquitinase active site). f, Gel-ABPP data (performed as in Fig. 4c) showing stereoselective liganding of recombinant WT-STAMBP, but not a C264A STAMBP mutant by WX-01-08 (5 µM, 1 h). Data are from a single experiment representative of two independent experiments. IB = anti-Flag immunoblot, and UT= untransfected cells.
Extended Data Fig. 8. Leveraging tryptic peptide maps from protein-directed ABPP experiments to deduce stereoselectively liganded cysteines. a, Sequence of human C15orf57 showing the three cysteines in the protein (yellow highlight), peptides that were quantified in protein-directed ABPP (red), and the tryptic peptide containing the liganded cysteine, C111 (underlined). b, Competitive gel-ABPP data showing stereoselective blockade of alkyne stereoprobe WX-01-12 (5 µM, 1 h) engagement of C15orf57 by competitor stereoprobe WX-02-46 (20 µM, 1 h pre-treatment). Data are from a single experiment representative of two independent experiments: UT= untransfected cells. c, Competitive gel-ABPP data showing concentration-dependent, enantioselective blockade of alkyne stereoprobe WX-03-346 (1 µM, 1 h) engagement of STK39 by competitor stereoprobe WX-03-59 (1 h pre-treatment) (left) and quantitation of these data (right). Data represent one experiment. d, e, Crystal structure of STK39 (PDB: 5D9H) showing location of stereoprobe-liganded cysteine C334 (red) distal to the ATP pocket and highlighted in yellow are residues around C334 (<15 Å) that are conserved between STK39 and its paralog OXSR1 (yellow-highlighted residues also shown in the sequence alignment in e). f, Left, cysteine-directed ABPP data showing stereoselective liganding of FOXA1_C258 by (1R,3S) stereoprobe WX-02-26 in 22Rv1 cells. Right, protein-directed ABPP data showing stereoselective enrichment of FOXA1 by WX-01-02 and blockade of this enrichment by WX-02-26 in 22Rv1 cells. Data represent average values ± SD, n = 4. g, Tryptic peptide map of FOXA1 from protein-directed ABPP experiments showing stereoselective enrichment of all quantified FOXA1 peptides by WX-01-02 except for the peptide containing C258 (red). In the heat map display, tryptic peptide signal intensities were normalized to 100% for the WX-01-02 treatment group.
Extended Data Fig. 9. Stereoselective and site-specific covalent ligands that inhibit the kynurenine biosynthetic enzyme AFMID. a, Molecular docking showing the preferred non-covalent binding pose for WX-01-03 to C28 of AFMID AlphaFold structure (AF-Q63HM1-F1). Highlighted is C28 (blue) of AFMID in which the sulfur atom and the beta carbon of WX-01-03 acrylamide group are 5.92 Å apart. Also highlighted is the catalytic serine, S164 (purple) and the HGGYW motif (green) that forms the oxyanion hole during substrate cleavage. b, Anti-Flag immunoblot showing expression of recombinant WT-, S164A-, C28A-, and C28W-AFMID in HEK293T cells used in enzyme assay in Fig. 6f; UT= untransfected cells. c, Public RNAseq data showing relative mRNA expression of AFMID in cell lines of different lineages (data source: the Cancer Dependency Map; https://depmap.org/portal, 23Q2 release). Highlighted in purple is the liver cell line HepG2 showing high AFMID expression. d, Competitive gel-ABPP data showing enantioselective blockade of fluorophosphonate-rhodamine (1 µM, 1 h) reactivity with WT-AFMID by WX-01-03 (1 h pre-treatment). Data are from a single experiment representative of two experiments. e, Alignment of AFMID sequences from different organisms showing that the residue corresponding to human AFMID_C28 (blue) is often substituted for serine in other species (red).


33. Tornoe, C. W.; Christensen, C.; Meldal, M., Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(i)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J Org Chem* 2002, 67 (9), 3057-64.


