Supplementary Discussion

Characterization of stereoprobe targets mapped by both cysteine- and protein-directed ABPP. The immune-restricted PH domain protein PLEK was stereoselectively liganded at C250 by (1*R*, 3*S*) stereoprobes 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[1, 2] 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 (IP_5) 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[3], was stereoselectively liganded by (1*S*, 3*R*) stereoprobes, including the alkyne/competitor pair WX-01-12/WX-02-46 as determined by both cysteine- (**Extended Data Fig. 5f**) and protein- (**Extended Data Fig. 5g**) 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[4]. We verified stereoselective labeling of recombinant NFU1 by WX-01-12 (**Extended Data Fig. 5h**) and stereoselective blockade of this interaction by WX-02-46 (**Extended Data Fig. 5i**), and mutagenesis supported that C210 was the direct site of engagement by the stereoprobes (**Extended Data Fig. 5h**). 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[5]. 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. 5j**). 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 (1*R*, 3*S*) stereoprobes[6].

In addition to NFU1, we noted several other instances of stereoselective liganding of conserved functional residues (**Supplementary Dataset 1**). For instance, (1*R*, 3*R*) 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. 5k-o**). TYMS is the primary target of anticancer agents such as 5-fluorouracil[7], 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 (1*S*, 3*S*) stereoprobes (**Extended Data Fig. 5p**). 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[8]. Four TMXs (TMX1-4) were quantified in our protein-directed ABPP experiments, but only TMX1 and TMX4 showed stereoselective interactions with the stereoprobes (**Extended Data Fig. 5p**). TMX1 and TMX4 share three cysteines, two of which are part of the catalytic CXXC motif conserved across all TMX proteins[9]. 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. 5q**), 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 (**Extended Data Fig. 5r, s**). We also verified with recombinantly expressed proteins that only TMX1 and TMX4, but not TMX2 and TMX3, react

with (1*S*, 3*S*) stereoprobes (**Extended Data Fig. 5t**). 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. 5u, v**). 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 1*R*, 3*S* (WX-01-10) and 1*S*, 3*S* (WX-01-11) alkyne stereoprobes, but not the corresponding non-alkyne competitors (**Extended Data Fig. 6a-c**), and this interaction profile was verified with recombinant LIMK1 expressed in HEK293T cells (**Extended Data Fig. 6d**). 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[10] (**Extended Data Fig. 6e,f**). LIMK1 displays very limited activity with commercial peptide substrates[11], so we instead evaluated stereoprobe effects using a NanoBRET assay measuring LIMK1 interactions with a general kinase inhibitor that targets the ATP-binding site[12]. Interestingly, both WX-01-10 and WX-01-11 stereoprobes enantioselectively *enhanced* NanoBRET signals (**Extended Data Fig. 6g, h**), and these effects were ablated in a C349A-LIMK1 mutant (**Extended Data Fig. 6i**). In contrast, an established ATP-binding pocket inhibitor of LIMK1 HG-9-91-01[13] decreased NanoBRET signals equivalently for both WT- and C349A-LIMK1 (**Extended Data Fig. 6i**). We confirmed that the stereoprobe-induced increase in NanoBRET signal was not caused by changes in LIMK1-Nanoluc expression (**Extended Data Fig. 6j**). 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 cysteines[14, 15]). 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 site[16, 17] (**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 a stereoprobe target liganded on a non-cysteine residue. We noticed 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 (1*S*, 3*S*) 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 (**Extended Data Fig.8f, g**). 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