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An Activity-Based Oxaziridine Platform for Identifying and Developing Covalent Ligands for Functional Allosteric Methionine Sites

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An Activity-Based Oxaziridine Platform for Identifying and Developing Covalent Ligands for Functional Allosteric Methionine Sites

By
Audrey Glynn Reeves

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Chemistry in the Graduate Division of the University of California, Berkeley

Committee in charge:
Professor Christopher J. Chang, Chair
Professor F. Dean Toste
Professor Daniel K. Nomura
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Abstract

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The rational design of compounds to covalently label natural amino acids has become a popular method in both therapeutics design and chemical biology exploration. The field is saturated with rich chemistry for the targeting of nucleophilic amino acids, with chemical advances in recent years opening the door to the targeting of additional amino acids. To further expand the realm of targetable amino acids, we have developed and assessed oxaziridines as covalent methionine-targeting reagents via Redox-Activated Chemical Tagging (ReACT) chemistry. This thesis describes work on establishing a platform for covalent methionine screening using cyclin-dependent kinase 4 (CDK4) as a model protein. Additionally, this thesis explores efforts at improving equity and inclusion within the College of Chemistry at UC Berkeley via a graduate student-led mentorship program aimed at improving the number of undergraduate transfer students in research labs as a method for mitigating the effects of transfer shock and lessening barriers to advanced education in STEM.
For Christopher, who shared my passion for learning
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Chapter 1:

Advances in activity-based protein profiling beyond cysteine
Introduction

With a limited twenty natural amino acids, nature must get creative in order to yield its richly diverse set of independently functioning proteins. One manner in which this is achieved is via the unique reactivity bestowed on each individual amino acid, in particular the amino acids that can readily perform chemical reactions. By nature of external aspects including their solvent accessibility, electrostatics, or modification status, the ability of these amino acids to perform their intended role(s) can be dramatically modulated. The method by which we as chemists can use specifically designed probes to parse out these reactive residues has been coined “activity-based protein profiling,” or ABPP.

Although bioconjugation chemistry centered on the reactivity of certain residues has been observed as early as the field of chemistry itself, the phrase ABPP was coined by Ben Cravatt and coworkers in the context of identifying reactive residues within a lysate proteome in the late 1990’s.\textsuperscript{1,2} ABPP can be broadly defined, with an extensive literature in the realm of specific proteases.\textsuperscript{2–10} Of interest to us is its application in the assessment of individual amino acid reactivity. With the thiolate ion among the most nucleophilic of the amino acids, the field has exploded with a deep study of cysteine as a target for both strong and mild electrophiles, in the context of protein targeting for therapeutics and broad biochemical investigation.\textsuperscript{11} Therefore, this review will focus on chemically reactive amino acids beyond cysteine. Among these are methionine, tryptophan, tyrosine, arginine, aspartic/glutamic acid, histidine, and lysine.

This review expands upon existing reviews that lay out chemical methods to target canonical amino acids.\textsuperscript{3,12–16} Where this review differs is its focus on the application of such methods to ABPP. We define a chemical probe as amenable to ABPP if it a) reacts with fewer than three amino acids under biocompatible conditions, b) yields an adduct stable to associated biochemical workflows including but not limited to gel electrophoresis and/or mass spectrometry, and c) contains (or has a structure amenable to the addition of) a moiety that can serve as a reporter group, either for visualization or for further pulldown. These factors are necessary to visualize and/or quantify the (re)activity of these individual amino acids within their native systems. Chemical probes that are important in labeling their respective amino acids but that are not amenable to ABPP are included and denoted as such.
Results and Discussion

Methionine

One of only two sulfur-containing amino acids, methionine is known for its role as the initiating amino acid in protein translation. As an aliphatic residue, methionine is generally found buried within the core of proteins and plays an important structural role in protein folding. These factors, combined with its overall low expression levels (since methionine is generally post-translationally excised by MAP), result in very few surface-accessible methionines, a potentially alluring quality for drug-discovery chemists designing covalent therapeutics from the perspective of minimizing off-target effects. The primary post-translational modification (PTM) of methionine is its oxidation to methionine sulfoxide which can be reversed by methionine sulfoxide reductase (Msr) eraser proteins. By analogy with phosphorylation installation and removal via kinases and phosphatases, context-dependent methionine oxidation and methionine sulfoxide reduction plays important roles in signaling pathways relevant to protein and cellular regulation.

Methods to alkylate methionine have been utilized since cyanogen bromide’s role in investigating disulfide bond locations in protein structures, but are poorly amenable to ABPP due to requirements for low pH, which perturbs native protein function. More recent methods to target methionine residues take advantage of its mild nucleophilicity and redox potential (Figure 1.1). Importantly, methionine lacks acidic protons under physiological pH ranges and so reacts in a pH-independent manner, a quality that sets it apart from acidic and basic amino acids. In 2017, our lab collaborated with Dean Toste and coworkers to develop Redox-Activated Chemical Tagging (ReACT) which utilizes oxaziridines to perform biomimetic oxidative nitrene transfer to the sulfethers of methionine under biocompatible conditions. Another useful method is that of alkylation by epoxides, which have been utilized to alkylate methionines in addition to other amino acids under biocompatible conditions, although with poorer selectivity for methionine than ReACT reagents. Both ReACT and epoxide-modification of methionine have been further demonstrated by us and others to be amenable to chemical biology techniques, establishing them as effective ABPP probes in this area. In addition to these two methods, Gaunt and MacMillan and coworkers have reported effective methionine-targeting chemical probes for peptide and protein modification outside of ABPP.

Tryptophan

Tryptophan fills a unique niche as the only indole-containing amino acid, allowing for distinct bonding interactions including hydrogen bonding, π-π stacking, and cation-π interactions. These interactions are often important at the active-site of enzymes, where tryptophan is frequently enriched. Accounting for only 1% of amino acids observed in proteins, it is an attractive target for site-specific protein modification. Its further metabolism is required for the generation of numerous essential metabolites, and it plays unique roles in various diseases, leading to its supplementation or sequestration as methods of treatment.
Inspired by analogous progress in the realm of synthetic organic chemistry, tryptophan has been targeted with transition metal-catalyzed C-H activation. Additionally, with it having the largest molar absorptivity among the natural amino acids, chemists have frequently targeted tryptophan via its ability to undergo photoinduced electron transfer (PET). These methods are highly efficient in modifying peptides, but incompatible with ABPP in complex lysates due in part to high doses of probe required for modification and/or the necessary addition of additional reaction components incompatible with native protein folding.

Two methods have arisen as compatible for ABPP, both relying on photocatalysis to generate reactive intermediates capable of generating covalent modification with tryptophan (Figure 1.2). It should be noted that while these labeling methods provide efficient routes for modifying tryptophans within a semi-native context, significant reactivity with other amino acids including histidine, cysteine, and tyrosine have also been observed and should be considered in the design of any experiment.

Tyrosine

As the only phenol-containing naturally occurring amino acid, tyrosine provides a scaffold for a diverse set of PTMs essential for communications. This reactive moiety has long been a structural target for biocompatible functionalization (Figure 1.3). Early work with Mannich-type reactions and palladium-catalyzed alkylation yielded efficient modification in peptides but were incompatible with complex lysate labeling due in part to the necessary conditions for reactivity resulting in protein unfolding and/or large deviation from native conditions. Early work applied diazonium salts for modifications of aryl amino acids, which yielded modest modification of tyrosine among other amino acids and, though biocompatible, its high propensity for modifying cysteine, histidine, and phenylalanine thus exclude it from our definition of ABPP-compatible. Apart from this, two ABPP-compatible methods have been developed to target tyrosine residues. Diazidocarboxamides, while challenging to generate synthetically, proved highly selective for modification of tyrosines under biocompatible conditions. While both these methods yielded observable modifications at tyrosines in the context of shotgun proteomics, SuFEx, developed by Hsu and coworkers, yielded over twice the modifications under similar conditions, though with a higher observation of modifications at lysines.

Arginine

Arginine is involved in numerous biological processes, namely metabolic pathways, enzymatic active sites, and as a substrate in the generation of reactive oxygen species such as nitric oxide. One of its most frequent PTMs is its enzyme-mediated methylation, which plays a key role in gene expression on histones. Arginine contains a guanidine group, which is less nucleophilic than its other primary amine-containing counterpart, lysine, but is capable of bidentate engagement of certain electrophiles. Acylation with activated esters is an effective method to modify arginine-containing peptides but requires reagents incompatible with ABPP (Figure 1.4).
bioconjugation method utilizes glyoxal-based chemistry,\textsuperscript{52} which was expanded to application for broader chemoproteomics applications by Dawson and coworkers.\textsuperscript{53} In experiments performed by Hacker and coworkers at probe concentrations approaching the millimolar range, these phenylglyoxal probes were able to successfully identify arginines via chemoproteomics.\textsuperscript{12}

**Aspartic/Glutamic Acid**

Aspartic and glutamic acid both contain a carboxylic acid moiety in addition to their C-terminus that is generally negatively charged at physiological pH (with frequent exceptions including buried residues, H-bonded residues, highly negatively-charged environments, etc.). These charges lead to their enrichment on the surface of proteins, aiding protein folding, and playing roles in enzymatic active sites as a base or hydrogen bond acceptor.\textsuperscript{48} Aspartic acid plays roles in the urea cycle and as a mild neurostimulator, while glutamic acid is a key component of metabolism, and is itself a potent neurotransmitter. These residues make enticing targets for covalent inhibition in bacterial samples due to their enrichment compared to humans and general surface accessibility.\textsuperscript{54}

The labeling of the carboxylic-acid containing amino acids aspartic acid and glutamic acid is challenging due the combination of their lower reactivity than cysteine and lysine and their inherent competition with terminal carboxylic acids. Targeting moieties must be carefully designed to obtain high selectivity. Isoxazolium compounds have been observed to react with glutamic acid,\textsuperscript{55} and the Raines group applied stabilized diazo compounds to target carboxylic acids (see also their application for tyrosine targeting), however these methods have only been applied to peptides in isolated solutions (Figure 1.5).\textsuperscript{56} Light-activated tetrazoles have been observed to modify carboxylic acids under biocompatible conditions by Hacker and coworkers.\textsuperscript{57} Azirines have also been used to target these amino acids.\textsuperscript{58} These methods have been utilized for proteome-wide ABPP including chemoproteomics.

**Histidine**

Histidine contains an imidazole side-chain, which frequently serves as a coordinating ligand or proton-shuttle in the active site of proteins.\textsuperscript{48} It’s both a powerful leaving group and good nucleophile, which engenders flexibility in its roles in biological systems but also introduces challenges when trying to develop methods to chemically target it for ABPP.

Thiophosphorodichloridates have been demonstrated as highly selective for histidine modification, however their resulting modifications are too labile to be amenable for further ABPP (Figure 1.6).\textsuperscript{59} To our knowledge, only one probe has been demonstrated as adept for ABPP, and it is that which also modifies tryptophan in equivalent amounts.\textsuperscript{12,39} The targeting of histidine for selective ABPP remains an unmet challenge in the field.
Lysine

With its primary amine that is charged at biological conditions, lysine is commonly found on protein surfaces and has been frequently observed as playing numerous roles including among protein-protein interactions and at enzyme active sites. It can undergo various PTMs, serving an essential role in cell communication.

With its nucleophilic primary amine, lysine is a popular target for protein and peptide modification. Acylation is an effective method to covalently modify lysine in a biocompatible manner and is compatible with ABPP, with many probes designed to achieve this purpose (Figure 1.7). Additional methods involve squalene derivatives, imine formation, and photo-catalyzed reactions. All of these methods listed displayed impressive selectivity for lysine over other comparable reactive amino acids, including the N-terminus, in work by Hacker et al. Iminoborinate chemistry also allows for site-selective lysine modification, with recent work applying this reaction in the design of reversible lysine-targeting covalent inhibitors.
Figure 1.1. Chemical probes for modification of methionines and their ABPP compatibility.
**Figure 1.2.** Chemical probes for modification of tryptophans and their ABPP compatibility.
Figure 1.3. Chemical probes for modification of tyrosines and their ABPP compatibility.
Figure 1.4. Chemical probes for modification of arginines and their ABPP compatibility.
Figure 1.5. Chemical probes for modification of aspartic/glutamic acids and their ABPP compatibility.
Figure 1.6. Chemical probes for modification of histidines and their ABPP compatibility.
Figure 1.7. Chemical probes for modification of lysines and their ABPP compatibility.
References


Chapter 2:

An activity-based oxaziridine platform for identifying and developing covalent ligands for functional allosteric methionine sites: redox-dependent inhibition of cyclin-dependent kinase 4

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#These authors contributed equally to this work
Abstract

Activity-based protein profiling (ABPP) is a versatile strategy for enabling identification and characterization of new functional protein sites and discovery of lead compounds for therapeutic development. Yet, the vast majority of ABPP methods applied for covalent drug discovery target highly nucleophilic amino acids such as cysteine or lysine. Here, we report a methionine-directed ABPP platform using Redox-Activated Chemical Tagging (ReACT), which leverages a biomimetic oxidative ligation strategy for selective methionine modification. Application of ReACT to the cancer-driver protein cyclin-dependent kinase 4 (CDK4) as a representative high-value drug target identified three new hyperreactive, ligandable methionine residues, including an allosteric M169 site that is proximal to an activating T172 phosphorylation site. With this information in hand, we designed and synthesized a new methionine-targeting covalent ligand library based on oxaziridine fragments bearing a diverse array of heterocyclic, heteroatom, and stereochemically-rich substituents. ABPP screening of this focused library against a clickable broad-spectrum ReACT probe identified 1oxF11 as a covalent modifier of the CDK4/Cyclin-D1 heterodimer at the M169 site. This compound inhibited CDK4 kinase activity in a dose-dependent manner on purified protein and in live cells. Further biochemical analyses with a phospho-specific CDK4 antibody revealed crosstalk between M169 oxidation and T172 phosphorylation upon 1oxF11 treatment, where M169 oxidation prevented phosphorylation of the activating T172 site on CDK4 and blocked cell cycle progression at the S-phase checkpoint. By identifying a new mechanism for allosteric methionine redox regulation on CDK4 and developing a unique modality for its therapeutic intervention, this work showcases a generalizable platform that provides a starting point for engaging in broader chemoproteomics and protein ligand discovery efforts to find and target previously undruggable methionine sites.
**Introduction**

Covalent small molecules that target specific amino acid residues are powerful chemical tools that can reveal fundamental new protein function and identify lead candidates for accelerating drug discovery.\(^1\)–\(^8\) Indeed, led by advances across broad fields encompassing organic chemistry, chemical biology, cell biology, and bioinformatics, covalent therapeutics now constitute approximately 30% of enzyme-targeting FDA-approved drugs.\(^9\) In this context, activity-based protein profiling (ABPP), where chemical probes measure protein function rather than protein abundance,\(^10\),\(^11\) has enabled new modalities for fragment-based drug discovery by applying small-molecule screening efforts in conjunction with chemoproteomics for target and site identification and characterization.\(^12\)–\(^35\) These technologies rely on residue-specific covalent warheads that can be used from proteins to proteomes,\(^5\),\(^36\)–\(^42\) yet the majority of reactive probe development to tackle this vast undruggable space has targeted cysteine\(^43\),\(^44\) or lysine,\(^21\),\(^24\),\(^26\),\(^45\) with relatively limited expansion of this chemical toolbox to other nucleophilic residues like tyrosine\(^46\) and glutamate/aspartate.\(^47\),\(^48\)

Motivated to contribute to this area as part of a larger program in our laboratories to create and use activity-based chemical probes for biological applications,\(^49\)–\(^52\) we and others have developed bioconjugation methods for methionine,\(^53\)–\(^59\) one of two privileged sulfur-containing amino acids along with cysteine. Methionine is distinguished by its characteristic thioether moiety, which endows this hydrophobic amino acid with high redox activity and low nucleophilicity relative to its highly redox-active and nucleophilic cysteine congener. The methionine sulfur atom enables not only greater rotational freedom through lower strain gauche interactions but also provides the opportunity for unique single-atom post-translational modifications (PTM) through a reversible two-electron oxidation to generate both \((R)\) and \((S)\)-methionine sulfoxide products.\(^50\) Akin to kinase writer and phosphatase era pairs for installing and removing phosphate PTMs, respectively, stereospecific reduction of methionine sulfoxide sites catalyzed by methionine sulfoxide reductase (Msr) eraser proteins can remove these single-oxygen PTMs. This reversible methionine thioether/sulfoxide cycle plays an integral part in the redox regulation of cell signaling events, antioxidant function, and other forms of protein regulation.\(^61\)–\(^63\)

In contrast to cysteine and other protein nucleophiles, the reactivity of protein-bound methionine is dictated by its redox potential rather than by its pKa. As such, we developed Redox-Activated Chemical Tagging (ReACT), a versatile bioconjugation method that targets methionine through the use of oxaziridine reagents that promote selective nitrène fragment transfer reactivity that is isoelectronic to native methionine oxidation by oxygen atom transfer.\(^53\) Methionine functionalization with ReACT proceeds selectively and rapidly at physiological pH and generates stable, mass-spectrometry compatible sulfimine adducts, enabling further chemoproteomic characterization of putative protein targets and sites of modification. Indeed, ReACT has found utility in the context of synthesis of stapled cyclic peptides,\(^56\) production of antibody-drug conjugates (MetMAb),\(^57\) proximity-activated
imaging reporters for protein function (PAIR),\textsuperscript{58} \textsuperscript{18}F radioimaging tracers and probes for protein and nucleic acid biotinylation (BioReACT).\textsuperscript{64} Here, we present the development of ReACT as a versatile methionine-targeting ABPP platform for covalent ligand discovery. We highlight its application to the study of the cancer-driver protein cyclin-dependent kinase 4 (CDK4), a serine/threonine kinase which serves as a master regulator of mitogenic signaling responsible for G1-S phase progression of the cell cycle.\textsuperscript{65–67} CDK4 is a high-value therapeutic target that is commonly misregulated in a variety of cancers and is one of many CDKs targeted in cancer drug therapy efforts.\textsuperscript{68–70} ReACT ABPP identified three new hyperreactive, ligandable methionine residues on CDK4, including an allosteric M169 site that is proximal to an activating T172 phosphorylation site. We then designed and synthesized a focused 179-member oxaziridine fragment library featuring a diverse array of heterocyclic, heteroatom, and stereochemically-rich substituents. Gel-based ABPP screening and chemoproteomic characterization identified 1oxF11 as a covalent modifier of the CDK4/Cyclin-D1 heterodimer at the M169 site. This compound inhibited the kinase activity of CDK4 in a dose-dependent manner on purified protein and in live cells. Interestingly, further biochemical studies using 1D- and 2D-western blot analyses with a phospho-specific pT172 CDK4 antibody revealed reciprocal crosstalk between M169 oxidation and T172 phosphorylation, where M169 oxidation by 1oxF11 prevented phosphorylation of the activating T172 site, thus blocking cell cycle progression at the S-phase checkpoint. The collective data identify a new mechanism for allosteric methionine regulation on CDK4 and a unique redox vulnerability for therapeutic intervention. Owing to the generality of the ReACT ABPP workflow, this platform provides a starting point for expanding the repertoire of chemical tools for identifying and characterizing new functional methionine sites and developing methionine-targeting covalent ligands for accelerating drug discovery.
Results and Discussion

ReACT Identifies New Ligandable Methionine Sites on the Cancer-Driver Protein CDK4. As a starting point to develop a methionine-targeting ABPP platform for covalent ligand discovery, we chose to study CDK4 as a representative kinase and high-value drug target. Kinases present a unique challenge in the design of selective therapeutics as they share a highly conserved ATP-binding active site amongst other common domains.\textsuperscript{71,72} Indeed, off-target binding of promiscuous kinase inhibitors, many of which are substrate analogs of ATP, has led to the failure of numerous drug candidates in clinical trials due to undesirable side effects.\textsuperscript{73} As such, identification of allosteric sites distal from the ATP-binding active site pocket provides a promising alternative approach for tuning kinase selectivity.

Against this backdrop, combining the synergistic approaches of fragment-based drug discovery\textsuperscript{74–76} and covalent ligand development\textsuperscript{2,5} can expand the scope of proteins that can be targeted by small molecules. This bottom-up approach involves screening of covalent ligand fragment libraries against broadly reactive, but amino acid-specific, bioconjugation warheads to discover novel binding pockets for covalent modification.\textsuperscript{77} Structure-activity relationships can be interrogated to further optimize selectivity and potency after identification of promising fragment leads. To identify potential new ligandable methionine sites in CDK4, we applied a set of three unique ReACT oxaziridine probes to this target: Ox1-alkyne, Ox1-azide, and Ox32-alkyne (Figure 2.1a). Interestingly, each ABPP probe showed a different pattern of covalent methionine labeling on our protein of interest, presaging that these sites can be preferentially targeted. Ox1-azide modifies three reactive sites: M169, M264, and M275 (Figure 2.1b, Figure 2.S1). In contrast, Ox1-alkyne labeled only M169, whereas Ox32-alkyne engaged only M264. Sites of modification were determined via shotgun proteomics (Figure 2.1c). The frequency of modifications observed on each methionine site correlated with the solvent accessibility of that residue. M169 displayed the highest reactivity, followed by M264, with M275 being the least solvent accessible and least reactive. Notably, the newly identified hyperreactive M169 site is proximal to a known T172 phosphorylation site on CDK4 that activates protein function.\textsuperscript{78,79}

Design and Synthesis of a Methionine-Targeting Covalent Ligand Library. After identifying hyperreactive methionine residues as potentially new ligandable sites for CDK4, we next sought to develop a methionine-directed covalent ligand platform to engage this protein target through the design and synthesis of a focused oxaziridine fragment library. To this end, we first optimized a suite of synthetic routes to such products, which included three methods for imine synthesis followed by oxidation via mCPBA (Scheme 1). As oxaziridine synthesis limited available functional groups to those stable to the final oxidation of the imine intermediate, and the oxaziridine itself can show sensitivity to acid, base, reductants, metals, and thiols, we sought to prepare a focused library to achieve sufficient structural diversity under these constraints.
The design of the oxaziridine library was initiated through a triaging selection of the key diversity element, namely the corresponding amine component. 43,950 amines were available for selection (Enamine); utilizing ICM Chemist Pro (Molsoft LLC) sub-structure selections were made driven by learnings from our initial study.\(^{56}\) Clustering analysis for each selected amine set was conducted using ICM Chemist Pro (Molsoft LLC) generating the detailed clusters for each sub-type (see Supplementary Information). Thereafter, rounds of selections based on diversity and removal of compounds containing foreseen chemoselectivity issues resulted in a preferred set of 234 amines. These amines were derivatized to the corresponding sulfimine or urea in silico and a 3-D conformational search allowed C=O stretching frequencies to be determined. In order to ensure formation of $N$-transfer sulfimine over $O$-transfer sulfoxide products upon reaction with methionine, we employed principle component analysis (PCA) methods to optimize this parameter and found that rates of sulfimine hydrolysis correlated with the calculated sulfimine adduct $\nu_{C=O}$ values (Figure 2.2a). The finalized library of 179 unique oxaziridine fragments featured a diverse array of functional groups, including spirocycles, halogens, azoles, ethers, and amides (Figure 2.2S2).

Methionine-Directed ABPP Screen Against CDK4 Identifies Lead Compound Fragment 1oxF11. With this oxaziridine fragment library in hand, we proceeded to screen for methionine-directed modifiers of CDK4 via a gel-based ABPP platform (Figure 2.2b). We chose the Ox1-azide ReACT probe as it was the most promiscuous and could engage three hyperreactive methionine sites in competitive binding assays with potential covalent oxaziridine ligands. Briefly, isolated CDK4 was treated either with DMSO (vehicle) or a covalent ligand from the oxaziridine library (ligand-treated). Samples were then treated with Ox1-azide probe followed by a quench step with $N$-acetyl methionine (NAM) to remove any excess oxaziridine. DBCO-Cy3 was then introduced by strain-promoted click chemistry to provide a fluorescence readout. The samples were subsequently separated via SDS-PAGE and fluorescence signals were normalized via silver stain to triage any covalent ligands that induced general protein aggregation, which would generate a false positive Cy3 signal (Figure 2.2b). The library contained fragments bearing a variety of functionalities, including spirocycles, halogens, azoles, ethers, and amides (Figure 2.2c).

Using ReACT, ABPP screening of the focused oxaziridine fragment library on CDK4 revealed 1oxF11 as a candidate for further study (Figure 2.2d, Figure 2.S3). This fragment showed competition with the Ox1-azide probe for CDK4 binding in a dose-dependent manner. We then tested the isoform specificity with two closely-related congeners, CDK1 and CDK6. CDK1 is ubiquitously expressed and essential for healthy cell function, and CDK6 shows the highest structural similarity to CDK4.\(^{80}\) Indeed, all CDK4 inhibitors currently in therapeutic use also display activity against CDK6. Along these lines, we were pleased to observe that 1oxF11 displayed no dose-dependent labeling against either CDK1 or CDK6 by gel analysis (Figure 2.2e). Additionally, we confirmed that this compound did not induce general aggregation in cell lysates (Figure 2.S4).
1oxF11 is a Covalent Modifier of CDK4 at the M169 Site and Inhibits Activity on Purified Protein. We next characterized the interaction between 1oxF11 and CDK4 in vitro. To start, we performed shotgun proteomics with 1oxF11 on purified CDK4, CDK1, and CDK6 proteins. We identified that the primary site of modification on CDK4 by 1oxF11 was M169, with minor labeling at M264 (Figure 2.3a, Figure 2.S5). Notably, no modifications by 1oxF11 were observed on either CDK1 or CDK6, further supporting the isoform specificity of this oxaziridine fragment. Finally, we showed that 1oxF11 inhibited activity of purified CDK4 protein in a dose-dependent manner using a luciferase-based activity assay as a proxy for kinase activity, observing an IC₅₀ around 200 nM (Figure 2.3b).

1oxF11 Decreases Cell Viability and Inhibits Cellular CDK4 Activity. Turning our attention to cellular studies, we sought to identify a model cell line that displayed heightened sensitivity to 1oxF11. We screened 1oxF11 across a small panel of cell lines with sensitivity to ribociclib, a clinically-approved CDK4/6 inhibitor (Figure 2.4a). The line most sensitive to 1oxF11 treatment, the human breast adenocarcinoma line MCF-7, was selected as a model for further study. Additionally, the contributions of CDK4 to uncontrolled cell division in the MCF-7 line have been well-documented. Indeed, we observed a dose-dependent decrease in cell viability of MCF-7 cells in response to 1oxF11, with an EC₅₀ of 330 µM (Figure 2.4b). This lower EC₅₀ observed in cells compared to in vitro is likely the result of several factors including cell permeability, non-productive consumption by thiols, and/or off-target effects.

To further probe how 1oxF11 contributes to the observed cellular phenotype, we synthesized a 1oxF11 analogue, 1oxF11yne, containing an alkyne handle for detection and enrichment using click chemistry (Figure 2.4c). Shotgun proteomics experiments revealed that the 1oxF11yne probe modifies CDK4 selectively at the same M169 site as the parent 1oxF11 fragment (Figure 2.4d). We then sought to test the effects of both probes on CDK4 activity within a cellular context. To achieve this goal, we turned to western blot analysis, monitoring the phosphorylation status of retinoblastoma protein (Rb), the main cellular substrate of CDK4. When active, CDK4 in complex with its cognate cyclin partner phosphorylates Rb at one of 14 sites (Figure 2.4e). We used phospho-responsive antibodies specific for three of these sites, S780, S807, and S811, as a method to assess CDK4 activity in cellulo. Indeed, incubation of serum-synchronized MCF-7 cells with either 1oxF11 or 1oxF11yne at a dose of 500 µM resulted in a marked decrease in signal from the pRb antibodies, suggesting a decrease in cellular CDK4 activity under these conditions (Figure 2.4f). Finally, we confirmed CDK4 target engagement in cells using a competition binding assay between 1oxF11 and 1oxF11yne (Figure 2.4g). To overcome detection challenges with the low endogenous expression levels of CDK4, even in cells known to upregulate the protein such as MCF-7, we performed transfection to achieve transient overexpression of CDK4 in these models. Treatment of cells with varying amounts of 1oxF11 competing ligand prior to incubation with 500 µM 1oxF11yne probe showed a dose-dependent decrease in signal in eluted proteins and a corresponding increase in signal in the respective supernatant, indicating
competition between the two compounds and engagement with the CDK4 target in a cellular context (Figure 2.S6). Additionally, 1oxF11yne displayed lower reactivity in lysate compared to Ox32-alkyne, further suggesting its heightened selectivity (Figure 2.S7).

Biochemical Studies of CDK4 Inhibition by 1oxF11 Reveal Reciprocal Crosstalk Between M169 Oxidation and T172 Phosphorylation. With data establishing 1oxF11 as a covalent modifier of CDK4 at a newly identified allosteric M169 site, along with its ability to inhibit CDK4 activity on purified protein and in cells and decrease cell viability in a dose-dependent manner, we sought to further interrogate its potential mechanism of action at the biochemical level. In this context, CDK4 plays a key role in the cell cycle in clearing the cell for division, only allowing for passage through the S-phase checkpoint when properly activated. In particular, this signaling pathway relies on proper binding of CDK4 to its respective cyclin, as well as phosphorylation at T172 by cyclin-dependent activating kinase (CAK) to activate the protein.\textsuperscript{84,85} Owing to the proximity of M169 to this activating T172 phosphorylation site, we hypothesized that M169 acts as an allosteric redox regulatory switch at this S-phase checkpoint. Oxidation can transform the normally hydrophobic methionine residue into a more hydrophilic and sterically-demanding methionine sulfoxide congener, which could block access of CAK to T172 and prevent its phosphorylation, thus causing the cell to fail the S-phase checkpoint (Figure 2.5a). This type of crosstalk between methionine oxidation and adjacent phosphorylation sites has been reported for other systems.\textsuperscript{86,87} Indeed, M169 and T172 lie in a flexible region of CDK4 that can come within 7 angstroms of each other, a distance observed to undergo this phenomenon previously (Figure 2.5b).\textsuperscript{88} Utilizing a custom pCDK4-Thr172 antibody, we observed that treatment with 1oxF11 was indeed able to diminish T172 phosphorylation status on CDK4 in MCF-7 cells in a dose-dependent manner (Figure 2.5c). Spot 3 corresponds to CDK4 in its T172 phosphorylated state, and spot 1 to unphosphorylated CDK4. Samples were separated via 2D SDS-PAGE, and spot 3 signal was normalized to that of spot 1. These data, along with supporting evidence showing engagement of CDK4 and inhibition of its activity in these same MCF-7 cell models, support a model in which M169 oxidation/T172 phosphorylation crosstalk offers a potential new redox vulnerability where oxidative modification of CDK4 at an allosteric M169 by 1oxF11 inhibits CDK4 activity by hindering phosphorylation at its activating T172 site.
Conclusion

We have presented a methionine-directed ABPP platform for identifying and developing covalent ligands for new functional methionine sites. We highlighted the potential value of this approach using CDK4 as a representative high-value target to showcase the application of ReACT probes to reveal fundamental new chemical function on proteins and accelerate drug discovery efforts by expanding covalent ligand development beyond the more common cysteine and lysine protein nucleophiles. Indeed, we applied methionine-directed ReACT probes with broad reactivity to identify novel hyperreactive, ligandable methionine sites on CDK4. We then optimized the synthesis of oxaziridine probes and used these methods to design and synthesize a focused covalent ligand library of ca. 180 oxaziridine fragments bearing chemically diverse functional groups, including spirocycles, halogens, azoles, ethers, and amides. Synthesis of the fragment library was guided by computational design to ensure efficient N-transfer rates and sulfimine stability of the subsequent products with methionine. We moved on to establish that this ReACT ABPP platform was useful for fragment-based screening efforts against the representative oncoprotein CDK4. Chemoproteomic experiments revealed that fragment 1oxF11 was a covalent modifier of CDK4 that selectively labeled its allosteric M169 site with isoform specificity over CDK1 and CDK6. Biochemical and cell-based assays showed that 1oxF11 can inhibit CDK4 activity on purified protein and in cells and decrease cell viability in a dose-dependent manner, with detection of target engagement in cells enabled by the synthesis of a 1oxF11yne probe bearing an alkyne handle for detection and enrichment. Further biochemical studies uncovered a novel redox regulatory mechanism for kinase inactivation through reciprocal oxidation/phosphorylation crosstalk between proximal M169 and T172 residues in CDK4, where M169 oxidation hinders phosphorylation at the protein-activating T172 site. Indeed, use of a phospho-specific pT172-CDK4 antibody in 1D- and 2D-western blot analyses established that treatment with the M169-modifying 1oxF11 covalent ligand diminished T172 phosphorylation and CDK4 activity. The resulting loss of CDK4 function prevented downstream Rb phosphorylation at S780 and S807/811, leading to cell cycle arrest by failure at the S-phase checkpoint. Our findings thus support a role for M169 as a redox sensor site at the S-phase checkpoint, preventing cell division under highly oxidative conditions by sterically preventing phosphorylation at T172. This newly discovered redox vulnerability in CDK4 provides an alternative modality for therapeutic intervention. Current and future efforts are geared to increase the size and diversity of methionine-directed activity-based probes and covalent ligand platforms to improve potency and selectivity, pursue other high-value targets and therapeutic modalities, as well as expand these approaches to other native amino acids in the proteome beyond cysteine, lysine, and methionine.
Materials and Methods

Reactions using moisture- or air-sensitive reagents were carried out in flame-dried glassware under an inert atmosphere of N₂. Solvent was passed over activated alumina and stored over activated 3 Å molecular sieves before use when dry solvent was required. All other commercially purchased chemicals were used as received (without further purification). SiliCycle 60 F254 silica gel pre-coated sheets (0.25 mm thick) were used for analytical thin layer chromatography and visualized by fluorescence quenching under UV light. Silica gel P60 (SiliCycle) was used for column chromatography. ¹H and ¹³C NMR NMR spectra were collected at 298 K in CDCl₃ or CD₃OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C using Bruker AV300, AVQ-400, AVB-400, AV-500, or AV-600 instruments at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard notation of δ parts per million relative to the residual solvent peak at 7.26 (CDCl₃) or 3.31 (CD₃OD) for ¹H and 77.16 (CDCl₃) or 49.00 (CD₃OD) for ¹³C as an internal reference. Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets. Low-resolution electrospray mass spectral analyses were carried out using LC-MS (Agilent Technology 6130, Quadrupole LC/MS and Advion Expression-L Compact Mass Spectrometer). High-resolution mass spectral analyses (ESI-MS) were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley. Ox1-alkyne, Ox32-alkyne, and Ox1-azide were synthesized using published procedures. All aqueous solutions were prepared using Milli-Q water, and all in vitro experiments were carried out in PBS, pH 7.4, unless otherwise noted. All biological experiments were prepared using freshly prepared aliquots.

Design methodology for diversity selection of oxaziridine library. The design of the oxaziridine library was initiated through selection of the diversity element, namely the corresponding amines required to synthesize the desired oxaziridine products. There were 43,950 available amines for selection (Enamine); utilizing ICM Chemist Pro (Molsoft LLC) an initial sub-structure selection was made: azetidine (286), pyrrolidine (2711), piperidine (3889), cycloheptylamines (328), iso-propylamines (4452), iso-butylamines (627), morpholines (409), ethylamine (6057), cyclo-heptylamines (328), 2,3,4,5-tetrahydro-1H-benzo[d]azepine (19) and iso-indolene (37). These choices were driven by learnings from our initial study to identify oxaziridine reagents that produced more stable methionine adducts (Table 2.S1).

Clustering analysis for each selected amine set was conducted using ICM Chemist Pro (Molsoft LLC) using (Tanimoto <0.4 as noted) generating the detailed clusters for each sub-type. Thereafter, 2 rounds of selections based on diversity and selecting the square root of the population of each cluster were conducted followed by a visual inspection to remove compounds containing foreseen chemoselectivity issues based reactivity with mCPBA. The resulting composite set was stripped of duplicate selections from within the sub-sets and the resultant 234 amines were then derivatized to the corresponding oxaziridine (from benzaldehyde), with the logP and MW distributions shown for the
products; it should be noted that the library also was analyzed showing all compounds
with HBD ≤1, the number of rotatable bonds was ≤8 with the majority being 3-5, and tPSA
distribution was 27-78Å (Table 2.S8). These properties were not constraints for the
library as the initial selections were made for structural diversity. All compounds were all
selected for conformational analysis.

Each of the base 234 structure coded as smiles was converted to 3D using Corina with
the following flags "-d wh, stagger, axchir, preserve, msc=2". Symmetry equivalents were
removed, then conformers were constructed using omega2[omega2] on default
settings. The conformers were optimized and IR frequencies computed using
Gaussian09[G09] with the keywords “opt(maxcycle=250) freq pop=nbo def2tzvp
m062x". For each conformer, the most intense frequency F between 1600.0 and 1740.0
cm-1 for the sulfimines, and 1700.0 and 1950.0 cm-1 for ureas, and the sum of the
electronic and thermal Free Energies were collected. The final figures are derived from
a Boltzmann weighted average of all the conformers of a structure.

Automated Parameter Extraction: The automated computational workflow was validated by obtaining the stretching frequencies from the set of compounds used to generate the reported hydrolysis kinetic model, and compared to the frequencies obtained manually (Table 2.S2). It was found that the automatically calculated stretching frequencies correlated very strongly with those obtained manually (Figure 2.S9), with a slope and R² = 0.98. As a further point of validation, the published rates of hydrolysis were plotted against the automatically calculated stretching frequency, which produced a similar model to the one obtained from manual calculated stretching frequencies. With this validation in hand, the stretching frequencies of a novel library of untested oxaziridines were extracted, to predict their respective sulfimine adduct stabilities towards hydrolysis.. The predicted Kobs are computed as 0.0595*(vC=O)-112.8 for sulfimines, and 0.0498*(vC=O)-102.8 for ureas. Equivalent structures in the two series are paired up in the output. Of the 234 input pairs, 1 sulfimine and 1 urea failed to converge, even after restarting, possibly due to unresolved steric crowding. The full list of sulfimine and urea stretching frequencies, as well as predicted stabilities can be seen in the attached Excel file (CDK4_SI_OxaziridineLibraryFrequenciesAndStabilities).

Gel-based ABPP. CDK4 was diluted in PBS to 50 nM, then 50 µL was added to each
well of a 96-well PCR plate (ThermoFisher Scientific). Ligands were dissolved fresh in
DMSO to 5 µM, and 1 µL was added so each well contained a unique ligand at the
indicated concentration. Wells were mixed and allowed to incubate 1 h at 23 °C. Ligands
were then chased with fresh Ox1-alkyne with 1 µL of 5 µM added to each well (100 nM
final), wells mixed and allowed to incubate 1 h at 23 °C. Excess oxaziridine was quenched
with N-acetyl methionine (Sigma; 01310) via addition of 1 µL of 10 mM DMSO stock (200
µM final), wells mixed and allowed to incubate 1 h at 23 °C. A stock was prepared 1:5 of
12.5 µM DBCO-Cy3 (Click Chemistry Tools; A140) in DMSO : 1.2% SDS/PBS. To each
well was added 6 µL of this stock, the wells mixed and allowed to incubate overnight
protected from light at 23 °C. The next morning 30 µL of 4X Laemmli’s buffer (Bio-Rad
Laboratories, Inc.; 1610747) containing 10% BME was added to each well. The plate was sealed and brought to 95 °C for 6 min. Samples were loaded and separated on precast 4–20% TGX gels (Bio-Rad Laboratories, Inc.) and scanned by ChemiDoc MP (Bio-Rad Laboratories, Inc) for measuring in-gel fluorescence. After that, the total protein level on the gel was assayed by silver staining (ThermoFisher Scientific; 24612) according to the manufacturer’s protocol and scanned by ChemiDoc MP.

**Shotgun proteomics.** To more accurately assess residue reactivity of oxaziridines on CDK4, 10 µg of CDK4 was diluted with 90 µg whole cell extract derived from mouse liver to a total volume of 100 µL in PBS. Protein mixture was treated with 50 µM oxaziridine (DMSO) and allowed to incubate at 23 °C for 30 min. Labeled protein was precipitated via addition of 900 µL MeOH at -80 °C overnight. The next day, sample was spun at max speed at 4 °C for 10 min. The pellet was gently washed 3 times with a solution of ice cold MeOH. The supernatant was then removed, and the pellet resuspended in 30 µL freshly prepared 8 M urea/PBS. A 5X stock of ProteaseMAX (Promega; V2071) was prepared by dissolved the pellet in 100 µL ammonium bicarbonate. To the protein mixture was added 30 µL 1X ProteaseMAX, 40 µL ammonium bicarbonate, and 10 µL of 110 mM freshly prepared TCEP (Pierce; 20490). The sample was then incubated at 60 °C for 30 min. To the sample was then added 2.5 µL of 500 mM freshly prepared iodoacetamide (Sigma Aldrich; I1149), and the sample was incubated protected from light at 23 °C for 30 min. 120 µL of PBS was then added, followed by 1.2 µL 5X ProteaseMAX. The sample was vortexed thoroughly. Sequencing grade Trypsin/Lys-C mix (Promega; V5071) was reconstituted in 40 µL trypsin buffer, and 4 µL was added to the sample. The sample was allowed to digest at 37 °C overnight. The next day the sample was acidified with 12 µL formic acid and spun at max speed for 30 min. The supernatant was taken to a low-adhesion tube and stored at -80 °C until MS analysis.

**Mass spec analysis.** Peptides from all experiments were analyzed on a Q Exactive Plus mass spectrometer (ThermoFisher Scientific); pure proteins were analyzed on a C18 only column, while complex proteomes were analyzed via a five step Multidimensional Protein Identification Technology (MudPIT). In both cases, Inlines (IDEX; M-520) were fitted with ~20 cm of 250 µm tubing and prepacked with 4 cm of Aqua C18 reverse-phase resin (Phenomenex; 04A-4299). Columns were made from 100 µm tubing pulled with a P-2000 laser (Sutter Instruments Co.) and packed with either 13 cm of Aqua C18 reverse-phase resin (pure proteins) or packed first with 10 cm of Aqua C18 reverse-phase resin followed by 3 cm of strong-cation exchange resin (Phenomenex; 04A-4398; MudPIT). Both inlines and columns were equilibrated after packing prior to use on an Agilent 1260 HiP AIS coupled to an Agilent 1260 Quat Pump using the following gradient: 100% buffer A to 100% buffer B over 10 min, hold at buffer B for 5 min, finish with a wash with 100% buffer A over 15 min (where buffer A is 95.5 H₂O:MeCN/0.1% formic acid, and buffer B is 80:20 H₂O:MeCN/0.1% formic acid). For best performance, columns were equilibrated the day of sample run.
Peptides were pressure-loaded onto an equilibrated inline until dry. The tubing was cut to ~2 cm above the resin bed and an appropriate equilibrated column was attached to the opposite end of the inline. The sample was attached to the LC-MS/MS via a MicroTee PEEK 360μm fitting (ThermoFisher Scientific; p-888) and the column tip aligned with the MS source opening. Data was collected in positive-ion mode using data-dependent acquisition mode with dynamic exclusion enabled (60 s) between 400 and 1800 m/z and a mass resolution of 70,000, with one MS1 scan followed by 15 MS2 scans of the nth most abundant ions. Nanospray voltage was set at 2.75 kV and heated capillary temperature at 200 °C. The MudPIT program utilized for all samples consists of five separate programs run sequentially, where each begins with either 0, 25, 50, 80, or 100% salt bump (buffer C, 500 mM ammonium acetate/H2O) followed by a gradient of 5-55% buffer B in buffer A. Pure proteins were run on only the first program (0% salt bump) from the MudPIT program. The flow was kept at 0.1 mL/min throughout.

Data were analyzed with Byologic (Protein Metrics Inc.). Raw files were searched directly against the Uniprot human or mouse database using the Byos HCP workflow, with decoys and common contaminants added. Peptides were assumed fully tryptic. All searches included the following modifications: Acetyl (+42.010565; Protein N-term; variable - rare1), carbamidomethyl (+57.021464; C; Fixed), and oxidation (+15.994915; M; variable - common1). Other modifications to methionine depended on the probe added to the sample and were all treated as variable - common1: 1oxF11yne (+235.09569), 1oxF11 (+154.07423), 1oxH2 (+168.08988). Peptides with b and y ions flanking the site of modification were assumed modified.

**Solvent Accessibility.** Residue solvent accessibility calculations of methionines on CDK4 protein were computed using the Discovery Studio 2021 platform from Dassault Systemes BIOVIATM. The 2W9Z pdb file for CDK4 was utilized and submitted to a “Solvent Accessibility” calculation. The software was set up with grid points per atom at 240 and probe radius at 1.4 Å.

**in vitro activity assay.** Effects of 1oxF11 on in vitro activity of CDK4 were determined via commercially available ADP-Glo assay (Promega; V6930). Isolated CDK4 was provided by Novartis. Retinoblastoma protein (aa 773-928) was obtained from commercial sources (Millipore Sigma; 12-439). The assay kit protocol was followed as directed, with the addition of a quench step (addition of 1 µL of 25 µM N-Acetyl methionine) after incubation of protein with oxaziridine ligand.

**Cell culture.** Cells were maintained by the UC Berkeley Tissue Culture Facility. All cells were maintained as a monolayer in exponential growth at 37 °C in a 5% CO2 atmosphere. MCF-7 and HepG2 were maintained in Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS, Seradigm). HT-29 and SW-48 were maintained in RPMI 1640 Medium (Gibco) supplemented with 10% FBS.

**Cell synchronization.** Cells were synchronized to G1 via serum starvation. Cells were plated at 50% confluency and allowed to adhere overnight in serum-containing media.
The next morning, the cells were gently washed twice with HBSS, and serum-free media was added. Cells were allowed to incubate an additional 24 h, for a total of 48 h without serum. Final cell confluency should remain below 75% for optimal results.

**Cell treatment.** All cells dosed with oxaziridine were treated using a 2% DMSO/media solution. Oxaziridines were dissolved in DMSO and used the same day as treatment. Media was removed from cells and replaced with fresh media containing oxaziridine in DMSO for a final concentration of 2% DMSO. Control wells were treated with 2% DMSO/media. Cells were incubated at 37 °C in a 5% CO₂ atmosphere.

**Cell viability assay.** Commercially available Cell Counting Kit-8 Assay (Dojindo) was used to investigate cell viability after treatment with oxaziridines. Cells were plated in 96-well plates (black/clear bottom; ThermoFisher) and grown to 75% confluency. Media was removed and replaced with media containing 2% DMSO and indicated compound concentration. Cells were allowed to incubate 24 h. To assess viability, media was removed and replaced with 100 µL media containing 10% CCK-8 assay solution. Plates were incubated at 37 °C in a 5% CO₂ atmosphere for 1-4 h until an orange color was visible. Viability was quantified via plate reader (monofilter) with absorption at 450 nm.

**Western blot analysis.** Cells were seeded in full media at 8e5 cells/well in a 6-well plate. They were then serum-synchronized and treated with compound as described previously. Cells were monitored by eye for cell death and harvested once high-dose cells were round but not detached. Cells were transferred to ice and washed twice gently with 1 mL ice-cold PBS. 10 mL of lysis buffer was prepared by dissolved a protease-inhibitor table (Pierce; A32953) and a phosphatase-inhibitor tablet (Roche; 4906845001) in 10 mL PBS (1% Triton X-100). Cells were scraped to harvest, transferred to 1.5 mL Eppendorf tubes, and incubated at 4 °C for 30 min on a rotator. Samples were spun at 5000 x g for 10 min to clarify lysate. Supernatant was transferred to a new tube and protein concentration quantified via BCA assay (Pierce; 23225). Samples were normalized to the lowest concentration using chilled lysis buffer. Samples were diluted with 4X Laemmlli’s buffer (10% BME) (Bio-Rad; 1610747) and loaded at 25 µg per lane on a 4-20% Tris-Gly SDS-PAGE gel. The gel was run at 160 V for 80 min and semi-dry electrotransferred to a PVDF membrane at 25 V, 2.5 Å, for 10 min. Blots were blocked with 5% BSA/TBST for 1 h, then washed 2 X TBST for 5 min, and cut for incubation with separate antibodies. Antibodies used were rabbit anti-pRb Ser807/811 (CST; 9308), rabbit anti-pRb Ser780 (CST; 3590), rabbit anti-β actin (CST; 4970), mouse phospho-T172 CDK4 (NB8-AD9), and rabbit anti-CDK4 (CST; 12790). All antibodies were diluted at 1:1000 in 5% BSA/TBST, with the exception of AD9 diluted at 1:500, at 4 °C overnight. The next morning the blots were washed 3 X with TBST prior to incubation with anti-rabbit IgG HRP conjugated secondary (CST; 7074) (1:3000 TBST) or anti-mouse IgG HRP conjugated secondary (CST; 7076S) (1:3000 TBST) for 2 h at room temp. Blots were quickly washed 3 X with TBST prior to incubation with ECL western blotting substrates (Promega; W1001) for 1 min and imaging with ChemiDoc MP.
2D gel electrophoresis. Serum-synchronized MCF-7 cells were treated with compound according to method outlined. Cells were monitored by eye for cell death and harvested once high-dose cells were round but not detached, about 2 hours. Media was removed and cells washed with HBSS. Trypsin was added to detach cells. Cells were transferred to a falcon tube containing 7 mL of complete DMEM and centrifuged at 1200 rpm for 2 min. Supernatant discarded and pellet gently resuspended in 1 mL PBS and transferred to an Eppendorf tube. Sample was spun once more, after which the pellet was quickly rinsed with ice cold MQ and spun a final time. Pellet was aspirated, flash frozen with LN2, and stored at -80 °C.

Samples were shipped on dry ice to K. Coulonval who proceeded with analysis. Cell pellets were solubilized in cold 30 mM Tris buffer pH 8.5 containing 7 M urea, 2 M thiourea and 4% CHAPS with continuous vortexing until unfrozen and then kept agitated for 20 min. After centrifugation at 15,700 g for 10 min at 4°C, proteins were quantified. An equal volume of 2-D-sample buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.4% 3-10 Pharmalytes, and 0.4% DTT) was added to samples normalized to 150 µg proteins. Proteins were separated by isoelectrofocusing on immobilized linear gradient (pH 5 to 8 [11 cm], Bio-Rad) strips, separated by SDS-PAGE and immunoblotted with antibodies against CDK4 (D9G3E, rabbit monoclonal) or phospho-T172 CDK4 (NB8-AD9, mouse). Secondary antibodies were coupled to horseradish peroxidase (Cell Signaling Technology). The proteins were detected using Western Lightning Plus ECL (Perkin Elmer) and viewed in Fusion FX gel documentation system using the Solo7S camera (Vilber Lourmat, France).

Expression of CDK4 in MCF-7 cells. MCF-7 cells were grown to 40% confluency in 3 mL DMEM media (Gibco) containing 10% (v/v) FBS in a 6-well chamber at 37°C, 5% CO₂. Transfection was then performed as per Lipofectamine 2000 protocol (Invitrogen). Briefly, 0 or 2.5 µg of pcDNA3.1(+)-FLAG-TEV-CDK4 expression construct was introduced at 0:0, 2:1 and 3:1 transfection reagent:DNA. The lipid-DNA complex was incubated for 30 mins at 23°C in Opti-MEM media (Gibco). Then, 250 µL complex was added to 2.75 mL DMEM containing no FBS. 3 mL DMEM + DNA was added to each well, while control wells received DMEM only. Cells were incubated for 6 H at 37°C, 5 % CO₂. The media was then aspirated, and cells were given 3 mL DMEM + 10% FBS and left to incubate for an additional 30 H at 37°C, 5 % CO₂. Cells were washed 3X in 500 µL ice-cold PBS then harvested by scraping in 200 µL ice-cold PBS + 1% Triton X-100 containing EDTA-free protease inhibitors (Roche). Cells were lysed at 4°C for 30 mins while rocking. Lysate was clarified by centrifugation at 10,000 x g at 4°C for 15 mins. The supernatant was then transferred to a fresh prechilled 1.5 mL microcentrifuge tube. Protein concentration was normalized to 2.0 mg/mL via BCA assay (Pierce; 23225). Samples were denatured in 4X Laemmlis’s buffer + 10% BME (Bio-Rad; 1610747) and boiled at 95°C for 8 mins. 30 µg protein was loaded onto a 4-20% Tris-Gly SDS-PAGE gel run at 160 V for 70 mins. Proteins were then electro-transferred to a PVDF membrane (25 V/2.5 A for 10 mins). Membranes were then blocked in a solution of TBST + 5% BSA (w/v) and rocked at 23°C for 1 H. Membranes were washed 3X in TBST for 5 mins each while rocking and cut using
a razor blade along the protein ladder for separate antibody incubation. Membranes were then blotted with primary rabbit anti-CDK4 (CST; 12790) or rabbit anti-β actin (CST; 4970) (both as 1:1000 TBST + 5% BSA suspensions) at 4°C overnight. The following morning membranes were washed 3X in TBST for 5 mins then incubated with anti-rabbit IgG HRP conjugated secondary (1:3000 TBST) (CST; 7076S) for 2 H at 23°C. Blots were quickly washed 3 X with TBST prior to incubation with ECL western blotting substrates (Promega; W1001) for 1 min and imaging with ChemiDoc MP. CDK4 expression was quantified by densitometry (ImageJ) and normalized to the actin loading control.

**Competition and pulldown of 1oxF11yne/1oxF11.** MCF-7 cells were expressed with CDK4-TEV-FLAG and normalized to 2 mg/mL as described. To 50 µL of this lysate was added 1 µL of a stock of 1oxF11 in DMSO for a final concentration of 500 µM, 250 µM, or 0 µM 1oxF11. A sample was set aside as “just lysate,” which was not treated with any compounds. After incubation with 1oxF11 for 1 hour at 22 °C, 1 µL of 25 mM 1oxF11yne was added to each sample as indicated for a final concentration of 500 µM 1oxF11yne. Samples were incubated again for 1 hour at 22 °C. DTB-N₃ was added to each compound with TBTA, TCEP, and Cu₂(SO₄), and CuAAC was allowed to proceed for 1 h at 22 °C. 450 µL of MeOH was added to each sample and proteins were precipitated at -80 °C for 12 h. Samples were then spun at max speed for 10 min at 4 °C. Pellet was resuspended in cold MeOH and samples spun again. Pellet was washed once more before resuspension in 150 µL 0.2% SDS/PBS. Samples were then boiled for 5 min and spun at 6500 x g for 5 min. Meanwhile, high-capacity streptavidin beads (10 µL per sample) (ThermoFisher Scientific; 20357) were washed 2X in PBS and 2X in MQ. The supernatant of the samples was added to 10 µL of the washed beads and allowed to incubate at 4 °C overnight. The next morning the samples were allowed to rock at 22 °C to resolubilize the SDS. The supernatant was collected and set aside for analysis. The beads were washed thoroughly with 2X PBS and 2X MQ and transferred to micro bio-spin columns (Bio-Rad; 7326204). Peptides were eluted from beads via addition of 2X 75 µL 0.1% FA (50% MeCN/MQ). Eluent was collected and beads washed once more with 20 µL of elution buffer. Columns were spun at 3000 x g for 3 min. Eluent was lyophilized to remove MeCN. After samples were dry, they were reconstituted in 75 µL PBS. Protein was diluted with 4X Laemmli’s buffer (Bio-Rad Laboratories, Inc.; 1610747) containing 10% BME and brought to 95 °C for 6 min. Samples were loaded and separated on precast 4–20% TGX gels (Bio-Rad Laboratories, Inc.) The gel was run at 160 V for 80 min and semi-dry electrotransferred to a PVDF membrane at 25 V, 2.5 Å, for 10 min. A separate gel was run and stained for total protein via Coomassie. Blot was blocked with 5% BSA/TBST for 1 h, then washed 2 X TBST for 5 min. Rabbit anti-CDK4 (CST; 12790) diluted at 1:1000 in 5% BSA/TBST was used to blot for CDK4 signal at 4 °C overnight. The next morning the blot was washed 3 X with TBST prior to incubation with anti-rabbit IgG HRP conjugated secondary (CST; 7074) (1:3000 TBST) for 2 h at 22 °C. Blots was quickly washed 3 X with TBST prior to incubation with ECL western blotting substrates (Promega; W1001) for 1 min and imaging with ChemiDoc MP.
General imine synthesis 1. Route is based off work by Lillo et al. Desired urea (10 mmol) and PhSO\(_2\)Na (2 eq) were dissolved in 20 mL of water. Then benzaldehyde (2 eq) in MeOH (8 mL) was added thereto followed by aqueous 80% HCOOH (0.8 mL). The reaction was stirred at room temperature overnight. If a precipitate was formed it was filtered, washed with water, hexanes, and dried. If no precipitate was observed, HCl was added until the reaction mixture reached pH 3. In our cases, after overnight stirring the precipitate was formed and collected after filtration, washing with water and hexanes, and dried.

General imine synthesis 2. To a solution of corresponding urea (115 mmol) and benzaldehyde (127 mmol) in dry CH\(_2\)Cl\(_2\) (20 mL) was added Ti(PrO)\(_4\) (140 mmol) dropwise at room temperature. The mixture was stirred at room temperature 23 h. The solvent was removed in vacuo to afford crude imine which was used immediately in the next step. The reaction was controlled by \(^1\)H NMR via appearance of the imine proton signal intensity.

General imine synthesis 3. To a flamed dried 3-neck round bottom equipped with a Dean-Stark trap and reflux condenser was added urea (10 mmol, 1 equiv.) and toluene (200 mL). To this mixture was added benzaldehyde (25 mmol, 2.5 equiv.) and pTsOH\(\cdot\)H\(_2\)O (2 mmol, 0.2 equiv.). The mixture was refluxed for 12 hours. The mixture was then concentrated under reduced pressure and was used in the next step without further purification.

General oxaziridine synthesis. In a 25 mL round-bottom flask, mCPBA (75%, 538 mmol) was pre-stirred in 1:1 CH\(_2\)Cl\(_2\)/sat. aq. K\(_2\)CO\(_3\) (140 mL) at room temperature for 10 minutes. A suspension formed. A solution of imine was added dropwise, but rapidly, using CH\(_2\)Cl\(_2\). Intense stirring at this step was observed to be essential. Additionally, if a partial dissolution of solids was observed after imine addition, sat. K\(_2\)CO\(_3\) was added to reaction mixture immediately. After 2 h, the reaction was diluted with water (150 mL) and extracted with CH\(_2\)Cl\(_2\) (3 x 100 mL). The organic layer was separated, washed with diluted aq. K\(_2\)CO\(_3\) (3 times) and water (2 times), dried over Na\(_2\)SO\(_4\), filtered, and concentrated in vacuo. The residue was purified by column chromatography.

1oxF11 urea characterization. Compound provided by Enamine. \(^1\)H NMR (400 MHz, DMSO-d6) \(\delta\) 5.72 (br s, 2H), 3.75 (dd, J = 8.8, 6.7 Hz, 2H), 3.45 – 3.31 (m, 4H), 3.10 (dd, J = 10.8, 3.3 Hz, 2H), 2.82 (dq, J = 7.2, 3.8 Hz, 2H). \(^{13}\)C NMR (101 MHz, DMSO-d6) \(\delta\) 157.90, 73.48, 50.73, 43.42. HRMS (ESI + TOF) calcld for C\(_7\)H\(_{13}\)N\(_2\)O\(_2\) [MH\(^+\)]: 157.0977. Found 157.0793.

1oxF11 characterization. Compound provided by Enamine. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.45 (m, 5 H), 5.23 (s, 1 H), 3.90 (m, 2 H), 3.77 (m, 2 H), 3.66 (m, 2 H), 3.50 (m, 2 H), 2.99 (br s, 2 H). \(^{13}\)C NMR (126 MHz, CDCl\(_3\)) \(\delta\) 160.14, 160.05, 133.08, 133.03, 130.91, 128.79, 128.05, 78.02, 77.96, 73.75, 73.59, 73.46, 73.29, 51.71, 51.44, 51.11, 50.83, 44.29, 44.25, 42.51. HRMS (ESI) calcld for C\(_{14}\)H\(_{16}\)N\(_2\)O\(_3\) [MH\(^+\)]: 261.1234. Found 261.1504.
Full name of chemical (number). methods. Characterization.

**Boc 1oxF11yne.** A solution of amine (3.0 g, 14.1 mmol, 1.0 equiv.) in CH$_2$Cl$_2$ (31 mL) was cooled to 0 °C. Triethylamine (2.9 mL, 21 mmol, 1.5 equiv.) was added at 0 °C, followed by the dropwise addition of propargylchloroformate (1.5 mL, 15.5 mmol, 1.1 equiv.). The solution was warmed to room temperature overnight. After 16 h, the reaction mixture was diluted with CH$_2$Cl$_2$ (50 mL), washed with 1M aq. HCl (20 mL), sat. aq. NaHCO$_3$ (20 mL), and brine (20 mL). The organic layer was dried over Na$_2$SO$_4$, filtered and concentrated in vacuo to afford the desired product as a pale yellow oil which solidified upon standing to a pale yellow solid (4.2 g, >95%) containing traces of CH$_2$Cl$_2$.

$^1$H NMR (500 MHz, CDCl$_3$) δ 4.66 (t, J = 2.6 Hz, 2H), 3.58 (m, 4H), 3.36 – 3.13 (m, 4H), 2.83 (s, 2H), 2.44 (t, J = 2.4 Hz, 1H), 1.42 (s, 9H).

$^{13}$C NMR (126 MHz, CDCl$_3$) δ 154.5, 153.9, 79.7, 78.6, 74.6, 52.8, 50.2, 49.7, 49.5, 42.5, 41.6, 40.7, 28.5. HRMS (ESI): calcd for C$_{15}$H$_{22}$N$_2$O$_4$Na [M+Na$^+$]: 317.1472. Found 317.1479.

**1oxF11yne urea.** To a solution of Boc-protected amine (2.0 g, 6.8 mmol, 1.0 equiv.) in CH$_2$Cl$_2$ (13 mL) was added HCl (2 M in Et$_2$O, 12 mL, 24 mmol, 3.5 equiv.) at room temperature. After 48 h, the solvent was removed in vacuo to afford the desired product containing ~10% remaining starting material. This material was resubjected to the same conditions using HCl (2 M in Et$_2$O, 6 mL, 12 mmol, 1.8 equiv.). After 16 h, the solvent was removed in vacuo to afford the corresponding amine hydrochloride salt (1.5 g, 96%) as a white solid which was directly used in the next step.

To a solution of the amine hydrochloride salt (1.45 g, 6.3 mmol, 1.0 equiv.) in water (6.3 mL) was added KOCN (1.5 g, 19 mmol, 3 equiv.) at room temperature. The reaction was sealed and stirred at 60 °C. After 24 h, the reaction was cooled to room temperature and extracted with EtOAc (5 x 100 mL). The combined organic layers were dried over Na$_2$SO$_4$, filtered and concentrated in vacuo to afford the desired urea product (1.3 g, 88%) as a white solid. $^1$H NMR (500 MHz, DMSO) δ 5.74 (s, 2H), 4.65 (t, J = 2.9 Hz, 2H), 3.53 (m, 2H), 3.48 (t, J = 2.4 Hz, 1H), 3.43 (m, 2H), 3.10 (m, 4H), 2.92 – 2.74 (m, 2H). $^{13}$C NMR (126 MHz, DMSO) δ 157.4, 153.2, 79.3, 77.3, 52.2, 50.2, 49.6, 49.4, 41.6, 40.6. HRMS (ESI): calcd for C$_{11}$H$_{16}$N$_3$O$_3$ [M+H$^+$]: 238.1186. Found 238.1199.

**1oxF11yne.** To a solution of corresponding urea (237 mg, 1.0 mmol, 1.0 equiv.) and benzaldehyde (0.12 mL, 1.2 mmol, 1.2 equiv.) in dry THF (3 mL) was added Ti(\text{IPrO})$_4$ (0.42 mL, 1.4 mmol, 1.4 equiv.) dropwise at room temperature. The mixture was stirred at room temperature 23 h. The solvent was removed in vacuo to afford crude imine which was used immediately in the next step.

In a 25 mL round-bottom flask, mCPBA (75%, 692 mg, 3.0 mmol, 3 equiv.) was presterried in 1:1 CH$_2$Cl$_2$/sat. aq. K$_2$CO$_3$ (8 mL) at room temperature for 10 minutes. A solution of crude imine from the previous step (in 1 mL CH$_2$Cl$_2$) was added dropwise, using additional CH$_2$Cl$_2$ (2 x 1 mL) rinses for a quantitative transfer. After 1 h, the reaction was diluted with water (30 mL), and extracted with CH$_2$Cl$_2$ (3 x 30 mL). The combined organic layers were washed with brine (30 mL), dried over Na$_2$SO$_4$, filtered and concentrated in vacuo.
Purification by column chromatography (75% to 85% EtOAc/Hexane) afforded the corresponding oxaziridine (194 mg, 57%) as a white foam. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.44 (m, 5H), 5.22 (s, 1H), 4.78 – 4.60 (m, 2H), 4.05 (m, 0.4H, minor rotamer), 3.84 – 3.64 (m, 4H), 3.50 – 3.29 (m, 3.6H), 3.01 – 2.87 (m, 2H), 2.46 (m, 1H). $^{13}$C NMR (126 MHz, CDCl$_3$) $\delta$ 160.4, 153.9, 130.9, 128.8, 128.03, 128.00, 78.5, 78.0, 74.7, 53.0, 50.7, 50.6, 50.1, 50.0, 41.1, 40.1. HRMS (ESI): calcd for C$_{18}$H$_{20}$N$_3$O$_4$ [M+H]: 342.1449. Found 342.1442.
Figure 2.1. Activity-based protein profiling (ABPP) using oxaziridine probes for Redox-Activated Chemical Tagging (ReACT) identifies new hyperreactive, ligandable methionine sites on CDK4. (a) Structures of Ox1-alkyne, Ox32-alkyne, and Ox1-azide ABPP probes. (b) Ribbon diagram of CDK4 (2W9Z). Methionine residues modified by oxaziridines are highlighted, with colored squares representing correspondent ReACT reagents found to modify each site. Each residue is additionally labeled with its calculated solvent accessibility. (c) Shotgun proteomics general workflow. Isolated protein is first incubated with compound. After excess compound is neutralized, protein is tryptic-digested and analyzed via MS/MS to reveal compound site(s) of modification.
Scheme 2.1. General synthetic routes to oxaziridine compounds for creating a focused covalent ligand fragment library. All syntheses began with primary or secondary amine synthons. Three routes were utilized to generate imine intermediates depending on starting amine. All imines were converted to oxaziridines using the same convergent method outlined.
Figure 2.2. Design, synthesis, and evaluation of an oxaziridine-based covalent ligand library for targeting methionine sites and identification of 1oxF11 as a covalent modifier of CDK4 via gel-based ABPP screening. (a) Design of oxaziridine fragments that favor formation of N-transfer sulfimine over O-transfer sulfoxide products upon reaction with methionine using principle component analysis (PCA). Rates of sulfimine hydrolysis correlate with calculated νC=O values. (b) Schematic of gel-based ABPP screening workflow. The protein target is preincubated with covalent ligand followed by treatment with Ox1-azide probe. Excess oxaziridine is then quenched with N-acetyl methionine (NAM) and the sample treated with DBCO-Cy3 overnight for fluorescence detection. Samples are finally separated by SDS-PAGE. Loss of fluorescent signal suggests competitive ligand binding to the protein. Silver stain is used to identify and exclude samples where signal loss corresponds to overall protein loss, likely due to aggregation induced by the ligand. (c) Representative structure types within the oxaziridine fragment library, organized by common functional groups. (d) Representative data from gel-based ABPP screen. CDK4 and ligand incubated at equimolar doses. (e) Structure of 1oxF11 fragment identified in gel-based ABPP screens as a competitive ligand for CDK4. Dose-dependent treatment of 1oxF11 against CDK4, CDK1, and CDK6 shows selective loss of fluorescent signal only with CDK4, suggesting isoform-specific engagement of this target.
Figure 2.3. 1oxF11 is a covalent modifier of CDK4 at M169 and inhibits its activity on purified protein. (a) MS/MS spectrum of 1oxF11-modified CDK4 showing functionalization at M169. (b) Activity assay on purified CDK4 protein showing dose-dependent inhibition in response to 1oxF11 treatment.
Figure 2.4. Decrease in cell viability and inhibition of cellular CDK4 activity by 1oxF11 and its 1oxF11yne analog in various cancer cell models. (a) Treatment of ribociclib-sensitive cell lines with 500 µM 1oxF11, demonstrating its ability to decrease cell viability. The MCF-7 line displays the highest sensitivity across the lines screened. Error bars represent standard deviation of at least three biological replicates. (b) Decrease in cell viability of MCF7 cells to increasing doses of 1oxF11. Error bars represent standard deviation of at least three biological replicates. (c) Synthesis of the 1oxF11yne probe bearing an alkyne synthetic handle for detection and enrichment purposes. (d) MS/MS data showing that 1oxF11yne is a covalent modifier of CDK4 at the same M169 site as the parent 1ox1F11 fragment. (e) Schematic of how measurements of cellular CDK4 activity were assessed, using Rb as a native CDK4 substrate. Western blot analysis of the extent of Rb phosphorylation provides a method for product detection. (f) Western blots assessing changes in cellular CDK4 activity with increasing added 1oxF11 and 1oxF11yne concentrations. Treatments of MCF-7 cells with 1oxF11 and 1oxF11yne result in lower signals at Rb sites phosphorylated by active CDK4. (g) Competition binding assay between 1oxF11 and 1oxF11yne for CDK4, providing evidence for target engagement in cells. MCF-7 lysates with CDK4-FLAG plasmid overexpression were pretreated with varying concentrations of 1oxF11 as indicated, followed by a 500 µM treatment of 1oxF11yne. All samples were then subjected to CuAAC to DTB-N3 and pulled down onto high-capacity streptavidin agarose beads. Supernatant was collected, and bound beads eluted with 50% MeCN/0.1% FA. Eluted sample was lyophilized, reconstituted in PBS, and separated via SDS-PAGE. Gel was transferred to PVDF membrane, and CDK4 signal was assessed via western blot using anti-CDK4 antibody.
Figure 2.5. The ReACT covalent ligand probe platform enables discovery of a reciprocal oxidation/phosphorylation crosstalk pathway in CDK4 through proximal allosteric M169 and T172 sites, where M169-targeted oxidation can inhibit CDK4 activity by preventing phosphorylation at T172. (a) Schematic outlining oxidation/phosphorylation crosstalk between CDK4 M169 and T172. Under low oxidative conditions, T172 of CDK4 is phosphorylated by cyclin-dependent activating kinase (CAK) as part of a critical activating step leading to cell division to pass the S-phase checkpoint. High oxidative conditions can lead to oxidation at M169, which blocks the T172 phosphorylation site, thus preventing cell division via S-phase checkpoint failure. This crosstalk identifies a methionine redox-dependent vulnerability for potential therapeutic intervention. (b) Ribbon structure (2W9Z) highlighting proximity of M169 and T172. (c) Monitoring CDK4 phosphorylation status using 2D-western blot analyses with phospho-specific CDK4 antibodies. Phosphorylation at T172 decreases with increasing concentrations of added 1oxF11 in MCF-7 cells. Spots 1, 2, 3, and 4 represent different phosphorylation states of CDK4, with spot 1 being unphosphorylated and spot 3 being monophosphorylated at T172. Peaks represent changes in intensity of spots normalized to peak 1 intensity. A clear decrease in spot 3 is observed upon treatment with increasing doses of 1oxF11, consistent with a model where this covalent ligand inhibits CDK4 activity by promoting M169 oxidation to block T172 phosphorylation.
**Figure 2.S1.** MS/MS of Ox1-azide and Ox32-alkyne modification on CDK4. All samples represent 50 µM probe treatment in 10 µg CDK4/CCND1 spiked into 90 µg mouse liver lysate. (a) Ox32-alkyne modification at Met264 of CDK4. (b) Ox1-azide modification at Met169 and (c) Met264 of CDK4.
Figure 2.S2. Oxaziridine fragment library structures.
**Figure 2.S3.** Oxaziridine library screen against CDK4-CCND1. Samples were treated according to gel-ABPP method outlined at 50 nM compound and 50 nM CDK4-CCND1.
Figure 2.S4. Aggregation test with 1oxF11. Lysates were treated with varying doses of 1oxF11 according to gel-ABPP method outlined. (a) Cy3 channel image, (b) silver stain image.
Figure 2.S5. Additional MS/MS spectrum. MS/MS of CDK4, modified with 1oxF11 at M264. Isolated CDK4-CCND1 spiked in mouse liver lysate was treated with 1oxF11 as shotgun proteomics method outlined.
Figure 2.S6. Competition of 1oxF11 and 1oxF11yne by gel. Lysate was collected from MCF-7 cells overexpressed with CDK4 and treated with DMSO or 1oxF11 as indicated. Then, all samples were treated with 500 μM 1oxF11yne, followed by a click step to DTB-N3 and subsequent pulldown onto high-capacity streptavidin agarose beads overnight at 4 °C. Supernatant was saved and run as “S” lanes, proteins bound to resin were eluted and run as “E” lanes. Samples were separated by SDS-PAGE and bands visualized by Coomassie staining. CDK4 appears at 36 kDa.
Figure 2.S7. Reactivity comparison of Ox32-alkyne and 1oxF11yne. Lysate was collected from MCF-7 cells overexpressed with CDK4 and treated with either 10 µM Ox32alkyne or 10 µM 1oxF11yne. Then, Alexa488-azide was appended to tagged proteins via CuAAC. Samples were boiled, separated by SDS-PAGE, and visualized.
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Table 2.S1. Selection of compatible derivatives toward more stable methionine adducts.
Figure 2.S8. LogP and MW distributions of products chosen to encompass the oxaziridine library.
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Table 2.S2. Sulfimines used for validating the automated workflow for obtaining C=O stretching frequencies. Manually calculated parameters and rate of hydrolysis obtained from previous report$^{2}$ (*denotes predicted hydrolysis rates)
Table 2.S9. a) Comparison of νC=O obtained from automated vs. manual workflows and b) recreated kinetic model using the automatically calculated νC=O with predicted hydrolysis rates of select sulfimines (data points in blue)
References


(89) Corina version 4.2.0, Molecular Networks GmbH, [www.mn-am.com](http://www.mn-am.com).


Chapter 3:

Development, Implementation, and Evaluation of a Graduate Student-Led Transfer Student Mentorship Program

This chapter was originally published on ChemRxiv (2022) by Audrey G. Reeves,# Amanda J. Bischoff,# Brice Yates, Daniel D. Brauer, and Anne M. Baranger.

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Abstract

The undergraduate transfer process has well-documented challenges, especially for those who identify with groups historically excluded from science, technology, engineering, and mathematics (STEM) programs. Because transfer students gain later access to university networking and research opportunities than first-time-in-college students, transfer students interested in pursuing post-baccalaureate degrees in chemistry have a significantly shortened timeline in which to conduct research, a crucial component in graduate school applications. Mentorship programs have previously been instituted as effective platforms for the transferal of community cultural wealth within large institutions. We report here the design, institution, and assessment of a near-peer mentorship program for transfer students, the Transfer Student Mentorship Program (TSMP). Founded in 2020 by graduate students, the TSMP pairs incoming undergraduate transfer students with current graduate students for personalized mentorship and conducts discussion-based seminars to foster peer relationships. The transfer student participants have access to a fast-tracked networking method during their first transfer semester that can serve as a route for acquiring undergraduate research positions. Program efficacy was assessed via surveys investigating the rates of research participation and sense of belonging of transfer students. We observed that respondents that participated in the program experienced an overall improvement in these measures compared to respondents that did not. Having been entirely designed, instituted, and led by graduate students, we anticipate that this program will be highly tractable to other universities looking for actionable methods to improve their students’ persistence in pursuing STEM degrees.
Introduction

Transferring from a community college to a four-year university provides a feasible route to an advanced degree for many students, primarily due to its affordability. In addition to entering a social sphere on average two years after first-time-in-college (FTIC) students, transfer students are disproportionally members of historically excluded groups from Science, Technology, Engineering, and Mathematics (STEM). Many transfer students also experience transfer shock, a decrease in academic performance after transferring to a baccalaureate-granting institution. The detrimental effects of transfer shock can be mitigated when students have a strong sense of community at their new institution. Accelerating the process of forging connections between transfer students and existing students and faculty at their transfer institution provides a direct, actionable method for improving equity and inclusion in the sphere of STEM academia.

College students’ persistence in STEM is enhanced by mentoring, advising, participation in research, participation in bridge programs, and many other factors. Programs combining multiple approaches to facilitating persistence in STEM have beneficial outcomes for a larger proportion of students than programs with a single component. For undergraduate transfer students, mentoring by peers and faculty can enhance enculturation into the university setting. Near-peer mentoring of undergraduates by more senior undergraduates or graduate students has also been demonstrated to enhance persistence in STEM and science identity. These mentoring relationships have demonstrated positive effects on the science identity, self-efficacy, and sense of belonging of the mentors involved as well as mentees.

Undergraduate research experience is critical for advancing through higher education in STEM. Strong connections have been made between undergraduate research experience and improved GPA or decision to continue to an advanced degree. This trend is augmented among undergraduates that identify with groups that have been historically excluded from STEM fields. The persistence of community college and transfer students in STEM has also been enhanced through participation in undergraduate research experiences. Formal instruction on how to apply for undergraduate research positions is limited or nonexistent at many institutions, and many principal investigators (PIs) find undergraduate students to fill these positions via networking within the institution. Furthermore, research experience and a letter of recommendation from a supervising PI are highly beneficial in applications for postgraduate degrees.

Through the graduate student-led development of the Transfer Student Mentorship Program (TSMP), we aimed to provide transfer students with information about undergraduate research and postgraduate degrees, build community among transfer students, and provide mentorship before and during the first transfer semester. This program would entail one-on-one mentorship of transfer students by graduate students and periodic group seminars and discussions on research-based topics with graduate and transfer student mentors. We sought to: (1) evaluate whether graduate student and
transfer student mentors and transfer student participants could be successfully recruited and participate in optional seminars and one-on-one mentoring sessions, and (2) provide a framework for the setup of similar programs for transfer students at comparable institutions with a heavy research focus but low faculty to undergraduate student ratio. We also aimed to understand whether this program would increase research participation among transfer students, and whether it would result in changes in sense of belonging among both transfer students and graduate student mentors.
Theoretical Framework

Community cultural wealth (CCW) theory postulates that sources of capital outside of monetary capital such as navigational, social, and familial capital can be beneficial in navigating institutions for underrepresented communities within larger power structures.\(^{22}\) In an academic sphere, an undergraduate student with high CCW might have a parent or sibling in STEM, whereas a student with low CCW might be the first in their family to attend college. Students with higher cultural capital as measured by familiarity with "rules of research" have been demonstrated to have greater success securing STEM undergraduate research positions.\(^{20}\) One method that has been previously assessed as effective in increasing these sources of capital and communicating the hidden curriculum instrumental to educational and career progression within an academic sphere is mentorship.\(^{23,24,25,26}\) In an academic context, deliberate pairing of new students (low capital) with established mentors (high capital) initiates the flow of community wealth downstream, providing a clear path to increase access to continuing higher education.

Underrepresented communities’ entrance and persistence in STEM also connects to social identity theory (SIT), which asserts that a portion of an individual’s sense of self derives from perceived membership in a relevant social group.\(^{27,28}\) The persistence of students from underrepresented groups in STEM has been correlated with higher science identity.\(^{17}\) In the context of mentorship, SIT offers a connection for an individual who perceives themselves in the out group, often a result of historical exclusion by an institution, with someone they perceive as being in the in group, a person who is already established in this community. With this connection, the individual can more easily visualize the transferal of themselves into the in group. In this work, the in group is generally defined as those in academic and/or industrial positions of power with high capital.

Where CCW highlights the transition of an individual more literally to a status of higher capital, SIT reflects how this individual perceives themselves as *belonging* in this group of higher capital. Together, the frameworks of CCW and SIT provide a lens to understand how a mentorship-centric program such as the TSMP may improve the transfer student experience within a STEM discipline.
Results and Discussion

Objectives. In the UC Berkeley College of Chemistry, all transfer students matriculate at the beginning of their junior year. As such, transfer students have a significantly shorter timeline for establishing professional connections and participating in research than FTIC students. A timeline comparing this difference between a transfer student and an FTIC student both interested in pursuing advanced degrees immediately after graduation is provided in Figure 3.1. This timeline outlines one example of how a student might find a research position through a connection made in a classroom setting. In this timeline, it is assumed that both students successfully acquire an undergraduate research position by the conclusion of their first semester.

Undergraduate research positions vary widely in their admissions processes, work styles, and availabilities, even within a single department, and there are no clear routes as to how to best obtain such positions. These positions are largely filled via informal networking, frequently a result of a graduate student meeting an interested undergraduate in a course they’re teaching. These factors place transfer students interested in performing undergraduate research at a significant disadvantage when applying to such positions. For a transfer student intending to apply for graduate programs during their senior year, even an ideal situation such as that outlined in Figure 3.1 would leave them with only one semester in a research lab before the graduate school application cycle opens.

The programmatic goals of the TSMP were threefold: (1) to instruct transfer students on many of the hidden curricula present in academic culture, (2) to provide fast-tracked networking to place interested transfer students in undergraduate research positions, and (3) to provide community for transfer students as they entered the College of Chemistry. Our three goals are reflected in the design of the TSMP, which consists of group seminars, one-on-one mentorship meetings with graduate student mentors, and small-group discussions involving senior transfer student mentors, all taking place during the transfer students’ first semester at UC Berkeley. Compared to a classroom setting where relationships with peers, graduate teaching assistants, and professors would typically be established over the course of a semester, these near-peer mentors can extend their personal network to their transfer student mentees based on their research interests as early as their first meeting at the beginning of their transfer semester.

Leadership and Participant Recruitment. The TSMP was designed entirely by graduate students and led by two co-directors, both graduate students. The co-directors were responsible for designing and presenting seminars, reserving venues for in-person meetings, matching graduate student mentors with transfer students, and coordinating graduate student mentors and transfer student mentors. Co-directors also provided guidance regarding mentorship topics and distributed information on open positions in research labs to the program. Transfer student mentors supported various administrative tasks under supervision of the co-directors, which included sending emails regarding upcoming seminars to all transfer students, ordering and distributing food at seminars,
coordinating the virtual room held concurrently with the in-person seminars, and guiding small-group discussions.

Recruitment of undergraduate participants began immediately after students were admitted to the College of Chemistry in April. The program was advertised to all junior and senior transfer students in the Berkeley College of Chemistry via email at multiple points throughout the summer before commencement of the program. The program was first advertised as part of a panel on programs available for transfer students at Berkeley, held during a recruitment weekend and open to all transfer students recently accepted to UC Berkeley. The main source of advertisement to the TSMP was through an email sent to all incoming transfer students to the College of Chemistry, describing the program and including a form for interested parties to sign up. This led to 35 junior transfer students and eight (8) senior transfer students signing up for participation in the TSMP. No application process was required, thus, all students who signed up were able to participate in the TSMP. All junior TSMP participants were matched with a graduate student mentor.

Graduate and transfer student mentors were also recruited via email. The TSMP was advertised to all graduate students in the Department of Chemistry via a weekly email sent out to the entire department for the duration of a month the summer before the TSMP began. The email briefly described the program and linked to a form where interested parties could sign up to become a mentor. In this manner, we successfully recruited 20 graduate student mentors and paired each with either one or two mentees. Transfer student mentors were recruited from the previous year’s program class of transfer students. Transfer students in the 2020 TSMP cohort were emailed and asked whether they were interested in providing mentorship for the new class of transfer students. In this manner, we successfully recruited four transfer student mentors.

**Program Description.** The TSMP began with a seminar open to all incoming transfer students on the subject “Finding an undergraduate research position” one month before their first transfer semester. This seminar was given both with the intention of recruiting students to the program and to help them understand how to approach obtaining a research position in an academic lab before they started their first semester at Berkeley. Following this seminar, transfer students and mentors were recruited. A one-hour introductory meeting and mentorship training was required for graduate and transfer student mentors before the program began, led by the program co-directors. Transfer students were matched to an appropriate mentor both by subfield of interest and specific identity whenever possible (e.g., mentors that matched the student’s gender or race, or who identified as first-generation college students). Mentors were matched with a maximum of two mentees and instructed to set up 3-5 meetings with their mentees over the course of the fall semester. Graduate student mentors were exclusively members of the chemistry graduate program, while transfer students were split evenly between chemistry and chemical engineering majors. A general timeline of the fall semester program scheduling is provided in Figure 3.2.
During the fall semester, the TSMP programming consisted of both one-on-one meetings between graduate student mentors and transfer students, and discussion-based small group seminars. Group discussions were divided into thirds: beginning with an unstructured social period over a provided dinner, followed by the indicated seminar presented by one of the co-directors (or a transfer student mentor on one occasion), and concluding with small-group discussions led by a combination of graduate student mentors and transfer student mentors. The discussion portion provided a valuable source of structured peer-to-peer mentorship between incoming transfer students and senior transfer student mentors. While these discussions were optional for all transfer student participants, all transfer student mentors and at least six graduate student mentors were required to attend each discussion. The topics for the small group seminars are detailed in Table 3.1.

Seminar topics were selected to communicate some of the hidden curricula involved in pursuing advanced graduate degrees or job opportunities to those unfamiliar with the process. Through a CCW lens, many transfer students would be described as entering academia with low capital. For instance, many students without family or community academic connections may be unaware that in the United States, many STEM PhD programs provide a livable stipend and tuition for the duration of study, information that can critically alter an individual's career plans. These seminars were structured to familiarize transfer students with this helpful information that is often not taught in their classes. One-on-one mentorship meetings were largely unstructured, though suggested topics for discussion at individual meetings were included in group discussion slides and in follow-up emails after group discussions. Additional details on these facets of the TSMP, their availabilities, and offerings is provided in Table 3.2.

Program Participation. Of the transfer students that signed up for the TSMP, all 35 juniors were matched with a graduate student mentor for the semester. Based on graduate student mentor survey responses, approximately 85% of mentees met with their mentors at least once over the course of the fall semester. Among both graduate and transfer student respondents, the median number of individual meetings per student-mentor pair over the course of the semester was three with a median absolute difference of 1 (Figure 3.S1). Of the mentors in our program responding to the survey, nine of the thirteen graduate student mentors had previous one-on-one mentorship experience, which included instances such as mentorship of an undergraduate and/or a more junior graduate student in their research lab, tutoring, and participation in a formalized mentorship program such as the ChemMentor Program at UC Berkeley, which pairs incoming graduate students with current graduate students in small mentorship groups. All mentors were expected to have had experience as a teaching assistant based on UC Berkeley’s graduate student teaching requirement. Of the six total seminars hosted, both transfer student and graduate student mentor respondents reported attending a median of three seminars with a median absolute difference of zero for graduate student mentors and 1.5 for transfer students.
Survey Design and Study Populations for Sense of Belonging Investigation. We invited participants in the TSMP program as well as transfer students not in the TSMP to engage in a survey-based investigation of sense of belonging. TSMP students, non-TSMP transfer students, and mentors were invited to participate in the survey. Surveys were approved by the UC Berkeley Institutional Review Board (IRB Protocol ID: 2021-07-14517) and distributed electronically via Qualtrics at the beginning and end of the program, corresponding to August and December of 2021 and March of 2022.

Inspiration for a survey-based assessment on sense of belonging drew from previous work in the Berkeley Department of Chemistry. Ten questions asking for a Likert scale-type response to cartoons paired with statements reflecting sense of belonging in a graduate program were used to assess sense of belonging of both transfer and graduate students. While the statements used had previously been validated as measuring sense of belonging in a graduate student population, they had not previously been used to measure sense of belonging in undergraduates. To probe the internal consistency of our scale when measuring sense of belonging of undergraduates, we calculated Cronbach’s $\alpha$ for both the beginning and ending Likert-type survey responses after inverting responses to statements corresponding with a negative sense of belonging so that a high score always indicated higher sense of belonging. The responses were found to be consistent with the exception of the phrase “I’m grateful to have a supportive social network” for both transfer and graduate student respondents, with Cronbach’s $\alpha$ values of 0.72 or higher for all populations (Table 3.S1-3.S4). We additionally included qualitative questions to allow respondents to further clarify feelings of sense of belonging and science identity in their own words. These questions along with a selection of responses are outlined in Table 3.3 and Table 3.4. All statistical analyses were performed in Microsoft Excel for Mac, Version 16.59.

Ten (10) TSMP undergraduates and 13 graduate student mentors completed the August survey, 12 TSMP undergraduates and 13 graduate student mentors completed the December survey, and 16 graduate student mentors completed the follow-up survey in March. This corresponds to 29% of the TSMP participants and 65-80% of the graduate student mentors. In addition, we recruited eight (8) and seven (7) students from the general transfer student population to participate in the initial and final surveys, respectively, as a comparison group (non-TSMP). Nine (9) transfer students and 10 graduate students participated in both the initial and final surveys; comparisons of survey responses over time are displayed only for these students. The majority of transfer student respondents were juniors (15, 90%). Of the 14 transfer student respondents who answered the optional demographic questions, the majority were first in their immediate family to attend college (10, 71%).

Exploration of Sense of Belonging. To probe the sense of belonging of survey respondents, we used a summated scale of responses to the nine internally consistent Likert-type questions. This scale added the 0-10 rankings of students’ agreement with statements related to their sense of belonging in the College of Chemistry. A maximum
score of 90 on the summated scale correlates to the highest sense of belonging. The sense of belonging of respondents in general improved over the semester if they participated in the program (Figure 3.3). Students 1, 2, and 5 reported a -20% change or greater in sense of belonging by the concluding survey, with the remaining students experiencing changes from -7% to as high as +34%.

Students 1 and 2, who provided the only matched responses from transfer students that did not participate at all in the TSMP, both exhibited a marked decrease in sense of belonging from the beginning to end of their first transfer semester. A decrease in belongingness amongst transfer students is consistent with previous research indicating that many transfer students experience “transfer shock” during their first semester, reporting lower confidence in their institutional knowledge after the first semester begins than before they have attended the first semester at their new institution.30 The fact that the majority of respondents who participated in the TSMP report either little change or bettering of their sense of belonging over the course of the first semester suggests that the TSMP may be mitigating the effects of transfer shock on this population.

We additionally collected qualitative answers to a question investigating belongingness in the College of Chemistry (Table 3.4). Responses draw from all populations across both the initial and final surveys, with individual responses available in the supplementary information (Table 3.S5). The five responses highlighted here were chosen to reflect a variety of reasons why respondents felt they did or did not belong, as well as to point out trends that were observed. Many respondents used words like “accepted”, “fit-in”, or “like-minded” to describe instances where they felt high belongingness, and some specified that these applied only within the sphere of other transfer students or their previous college. Some respondents that felt low belongingness mentioned feeling tested by their peers or that they didn’t belong. Interestingly, several respondents clarified that while they didn’t currently feel a high sense of belonging in the College of Chemistry, they were hopeful that they one day would.

Though this program provided a channel to improve connectivity between transfer students, graduate student mentors, and senior transfer student mentors, it did not introduce FTIC students in any manner, a group many respondents highlighted as the “in” group in their responses regarding belongingness. It follows that the TSMP would do little to affect how participants view themselves as part of this in group, as the program’s design does not incorporate FTIC undergraduate students.

**Exploration of Science Identity.** Akin to sense of belonging, we collected responses to a qualitative short response question asking students to first define a scientist or engineer (depending on their major), as well as whether they identified as meeting this definition themselves. The responses of six students, separated by major, are highlighted in Table 3.3 because they each included both a definition of a scientist or engineer as well as whether the respondent felt that they met this definition. All responses are available in the Supplementary Information (Table 3.S6). Chemistry-major respondents regardless of
TSMP participation tended to identify themselves as scientists, while the majority of chemical engineering-major respondents did not identify themselves as engineers.

**Rates of Research Participation.** Undergraduate research participation is known to correlate with a higher sense of belonging and science identity. We sought to improve science identity and sense of belonging of transfer students via increasing rates of research participation through connections made in the TMSP.

Graduate student mentors were contacted three months after the conclusion of the TSMP to comment on the status of their mentee(s) and their research participation. We received responses from 16 mentors (80% response rate), corresponding to the status of 27 mentees (Figure 3.4). While only one mentee was known to have definitively had a research position prior to the program, seven mentees were known to have such positions at its conclusion. The number of "unsure" responses from mentors increased largely between the two timepoints, which correlates with separate data collected on the number of meetings between each mentor and mentee and may indicate an attrition of mentor-mentee relationships after the conclusion of the program.

Our research additionally identified an unmet need in the department regarding chemical engineering students and undergraduate research. Survey respondents in this population report high interest in performing undergraduate research, yet consistently struggled to find these positions (Figure 3.S2). These numbers reflect the high number of undergraduate chemical engineering majors and low number of available research positions in the Department of Chemical and Biomolecular Engineering in the College of Chemistry (Figure 3.S3).

**Exploration of Graduate Student Experiences.** Peer mentorship among graduate students has been demonstrated to academically and professionally benefit both mentored and mentoring students. We sought to further understand how participating as a mentor in the TSMP affected graduate student mentors’ sense of belonging and professional development. To this purpose, we administered surveys to graduate students at the beginning and conclusion of the TSMP asking for short answer responses about their experiences and level of agreement with cartoons assessing sense of belonging.

In general, graduate student mentor respondents self-reported little change in their sense of belonging between the beginning and end of the program (Figure 3.S4). Answers to response questions about graduate student mentors’ professional development yielded an interesting pattern in responses (Table 3.5). Some respondents suggested that the program had built their confidence in mentoring. Several respondents also used these questions as an opportunity to discuss how they learned about the transfer student experience or how they would like to support transfer students in pursuing research positions in the future. While the graduate student mentors did not provide responses indicating that they thought their future careers may benefit from their participation in the
program, they did seem to have more ideas about how to help transfer students in their career trajectories after being part of the TSMP.

Limitations. The low response rate observed as well as self-selection for TSMP and non-TSMP groups rather than random assignment are contributing limitations in this study. Improved responses across all populations would provide a more generalizable picture of changes in research participation, science identity, and belongingness. One way we are working to assess research participation amongst these populations more accurately is via the addition of a question surveying this experience in a survey that all undergraduate students in the College of Chemistry must complete prior to graduation.
Conclusions and Implications

This work demonstrates that a graduate student-designed and led mentorship program can be successfully implemented at a large research institution. Graduate student mentors consistently participated in group seminars and initiated one-on-one meetings with their transfer student mentees over the course of the program. Although signing up for and subsequently participating in the program was completely optional for transfer student participants, the majority met one-on-one with their mentors multiple times throughout the fall semester.

Transfer student respondents in the belongingness survey generally reported higher agreement with metrics measuring their sense of belonging by the conclusion of the program if they had participated in the TSMP. Though further analysis is necessary, our program may be serving to mitigate transfer shock for many transfer students. Further analysis as to which aspects of the program are responsible for these changes would benefit the community in the design of future programs aimed at smoothing the transition of transfer students during their first semester.

A strength of a mentorship program fitting the TSMP model is that it requires few resources and can easily be replicated at other institutions. The program was designed and directed by graduate students, with periodic faculty advising. Mentor and participant recruitment was accomplished via email and an information session during the summer before the program. While time commitment was minimal for students and mentors (approximately 10 hours over the course of the semester), it provided mentorship and peer connections for nearly half of the incoming class of transfer students to the UC Berkeley College of Chemistry. The TSMP provides a framework by which transfer students to four-year institutions, a population that often includes groups historically underrepresented in STEM, can be connected with mentors and resources to help them in their STEM educational and career pursuits.
Figures

Figure 3.1. A timeline comparison between a first-time-in-college student (FTIC) and transfer student in the UC Berkeley College of Chemistry, where both find undergraduate research positions as a result of a connection made in the classroom, both graduate in four years, and both are interested in attending graduate school immediately after graduation. Under these circumstances, the FTIC student has 2.5 years of performing research before preparing graduate school applications (orange dashed), while the transfer student only has one semester and summer (maroon dashed).
Figure 3.2. Timeline of administrative and programmatic events for the TSMP alongside academic and survey timelines.
<table>
<thead>
<tr>
<th>Seminar Topics</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applying for Research Positions</td>
<td>How to contact research groups for the greatest chance of acquiring an undergraduate research position</td>
</tr>
<tr>
<td>Navigating Classes in the CoC</td>
<td>Advice on building a schedule most efficiently; led by a senior transfer student (TSM)</td>
</tr>
<tr>
<td>Introduction to Research Groups</td>
<td>Description of various research groups in the CoC; choosing which subdiscipline is best for you</td>
</tr>
<tr>
<td>Careers in Chemistry</td>
<td>Career paths available with a bachelors, masters, or doctorate in chemistry</td>
</tr>
<tr>
<td>Applying for Funded Research Opportunities</td>
<td>How to apply for external funding in graduate programs</td>
</tr>
</tbody>
</table>

Table 3.1. Seminar topics covered in TSMP meetings in Fall 2021 and their respective descriptions.
<table>
<thead>
<tr>
<th></th>
<th>Seminars</th>
<th>One-on-one mentorship</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>available to:</strong></td>
<td>all transfer students</td>
<td>incoming transfer students only</td>
</tr>
<tr>
<td><strong>offerings:</strong></td>
<td>• structured mentorship</td>
<td>• unstructured, personalized mentorship</td>
</tr>
<tr>
<td></td>
<td>• community building</td>
<td>• fast-tracked networking</td>
</tr>
</tbody>
</table>

**Table 3.2.** Aspects of the TSMP, their offerings, and which students they were available to.
Figure 3.3. A comparison of matched student responses to the belongingness survey, sorted by participation in the program followed by major. In general, respondents that participated in the TSMP experienced improvement or consistency in their sense of belonging compared to respondents who did not participate in the TSMP. $n = 9$
<table>
<thead>
<tr>
<th><strong>What does it mean to feel “at home” somewhere? How does this feeling apply to your experience in the College of Chemistry?</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>“The College of Chemistry is very competitive and I constantly feel like I’m being ‘tested’ about whether or not I’m worthy of being here and it’s generally very stressful.”</td>
</tr>
<tr>
<td>“Home is...where I feel accepted, seen, and valued...my advisor in the College of Chemistry has made me feel this way and so have the students I am surrounded by.”</td>
</tr>
<tr>
<td>“As a new transfer student,... I still feel very overwhelmed with the coursework. However, I do feel as though I fit in among the other transfer students in my courses/major.”</td>
</tr>
<tr>
<td>“...a network of amicable and [like-minded] peers [would] make me feel much more at home...”</td>
</tr>
<tr>
<td>“I don’t really typically feel at home at the [CoC] because I don’t really think I’m like a lot of the students here.”</td>
</tr>
</tbody>
</table>

**Table 3.3.** Responses to the questions “What does it mean to feel “at home” somewhere? How does this feeling apply to your experience in the College of Chemistry?”
Table 3.4. Select responses to the questions “What to you constitute a scientist/engineer? Do you describe yourself as a scientist/engineer? Why or why not?”. Responses are sorted according to major, with (a) being the chemistry majors and (b) the chemical engineering majors. Asterisks denote students that did not participate in the TSMP.
“My mentee had a research position prior to [Pre-TSMP] or after [Post-TSMP] participating in the TSMP.”

Figure 3.4. Responses of TSMP mentors to questions asking whether their mentee had a research position before or after participating in the TSMP. Data was collected three months after conclusion of the program for 27 mentees. $n = 16$ graduate student mentor respondents
Table 3.5. Graduate student mentor responses to the questions “What has the impact of the TSMP been on your professional development?” and “Which TMSP seminar did you find the most useful and why?”
Figure 3.S1. Distribution of number of individual mentorship meetings per mentee. Graduate student mentors were surveyed on how many mentorship meetings they had with each of their mentees, and 80% of graduate students (16 of 20) responded, corresponding to 77% (27 of 35) mentees who signed up for the program. The number of mentorship meetings exhibits a bimodal distribution with maxima at 1 and 4 meetings, suggesting that program participants settled into two primary groups; one group that engaged continuously with the program over the course of the fall semester, and a second group that did not connect with or lost interest in the program after initial meetings.
Figure 3.S2. Research position status of transfer student study participants over time. A greater proportion of chemical engineering transfer student respondents reported searching for a research position than chemistry transfer student respondents in general, and a greater proportion of TSMP participant respondents reported obtaining research positions both during the pre-program and post-program surveys. This data includes respondents who answered both the August and December surveys in addition to respondents who only answered one of the two surveys, so August and December results are not directly comparable.
The Berkeley College of Chemistry departmental demographics (Chemistry versus Chemical and Biomolecular Engineering).

![Chart showing student and faculty numbers for Chemistry and Chemical Engineering and Chemistry and Chemical Biology]

*using Spring 2021 enrollment numbers
**only faculty with active research groups are included

**Figure 3.S3.** The Berkeley College of Chemistry departmental demographics. The ratio of the College of Chemistry student body that is in the Department of Chemical and Biomolecular Engineering (CBE) versus Chemistry or Chemical Biology (Chem) is much higher than the ratio of faculty in CBE versus Chem. These numbers were collected from Spring 2021 enrollment numbers for students and collected from the College of Chemistry faculty page for faculty with active research groups. Because CBE has a much lower faculty to student ratio than Chem, it is expected that there are fewer research positions available per CBE student than per Chem student.
Figure 3.S4. Summed belongingness scores of graduate student mentors. The ratings to Likert-type survey questions of graduate student mentors who completed both the August and December surveys were summed to produce an overall sense of belonging score, with the exception of the survey question “I am grateful to have a supportive social network” which was not internally consistent with the other questions. While one student (Student G6) showed a marked increase in sense of belonging score over time, most students did not display large changes in their sense of belonging score. The prefix G is used to clarify that this data displays graduate student responses.
<table>
<thead>
<tr>
<th>Agreement item number</th>
<th>Caption for Transfer Students</th>
<th>Caption for graduate students</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>My classmates probably get much better grades than I do...</td>
<td>My classmates probably get much better grades than I do...</td>
</tr>
<tr>
<td>2</td>
<td>I’m definitely not smart enough to be here... (in chemistry class)</td>
<td>I’m definitely not smart enough to be here... (in chemistry class)</td>
</tr>
<tr>
<td>3</td>
<td>I wish there were more faculty I could talk to who would understand the hardships I face.</td>
<td>I wish there were more faculty I could talk to who would understand the hardships I face.</td>
</tr>
<tr>
<td>4</td>
<td>I am grateful to have a supportive social network</td>
<td>I am grateful to have a supportive social network</td>
</tr>
<tr>
<td>5</td>
<td>I feel so happy and accepted here!</td>
<td>I feel so happy and accepted here!</td>
</tr>
<tr>
<td>6</td>
<td>Other students are more productive and scientifically successfully than I am</td>
<td>Other grad students are more productive and scientifically successfully than I am</td>
</tr>
<tr>
<td>7</td>
<td>I feel like my audience sees me as a serious scholar!</td>
<td>I feel like my audience sees me as a serious scholar!</td>
</tr>
<tr>
<td>8</td>
<td>I feel like an outsider. (next to chemistry building)</td>
<td>I feel like an outsider. (next to chemistry building)</td>
</tr>
<tr>
<td>9</td>
<td>My instructor values my ideas and respects me.</td>
<td>That was a productive meeting... I’m so glad my advisor values my ideas!</td>
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<td>I am an independent, confident scientist!</td>
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**Table 3.S1.** Agreement item numbering for sense of belonging scale.
**Table 3.S2.** August survey levels of agreement with sense of belonging statements on a 1-10 scale.

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* "T" prefix stands for transfer student
** "G" prefix stands for graduate student
Table 3.S3. December survey levels of agreement with sense of belonging statements on a 1-10 scale.

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* "T" prefix stands for transfer student
** "G" prefix stands for graduate student
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**Table 3.S4.** Cronbach's $\alpha$ values for sense of belonging scale.
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<td>&quot;I feel like I can comfortable express the way I feel and act when involved with others in the college of Chemistry.&quot;</td>
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<tr>
<td>&quot;I wouldn't quite say I feel at home at Berkeley yet, but I am definitely getting there. Once I can establish a network of amicable and like minded peers I believe I will feel much more at home at Cal.&quot;</td>
<td>no</td>
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<td>&quot;When I was at my community college, I had my own community over there. I want to have the same kind of community, but even better at the College of Chemistry.&quot;</td>
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<td>&quot;There is a good amount of support here, with the different programs such as TSMP itself or CAMP.&quot;</td>
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<tr>
<td>&quot;To me, feeling &quot;at home&quot; somewhere means I am comfortable enough to say what's on my mind with less of a filter than I would normally have in something like an academic setting. The College of Chemistry at UC Berkeley is very competitive and I constantly feel like I'm being &quot;tested&quot; about whether or not I am worthy of being here and it's generally very stressful.&quot;</td>
<td>no</td>
<td>ChemE</td>
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<tr>
<td>&quot;I don't think that my experience in the CoC at Cal is quite at &quot;home&quot; level. It could definitely get there! It's just not been enough time. Through spending more time on campus, making more friends, having set study spots, and getting to know faculty more, I think campus could feel like home to me.&quot;</td>
<td>no</td>
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</tr>
<tr>
<td>&quot;To feel &quot;at home&quot; somewhere, I need to be comfortable with my surroundings. Applying to my experience in the College of Chemistry, it has been a bit difficult adjusting, but I value making use of resources given such as the College of Chemistry and Chemical Engineering Library, as well as the Peer Tutoring Center.&quot;</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>&quot;As a new transfer and less than a month into my first semester, I still feel very overwhelmed with the coursework. However, I do feel as though I fit among the other transfers in my courses/major.&quot;</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>&quot;I'm not there yet. I would probably feel at home after struggling through classes with other people who I become friends with.&quot;</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>&quot;To me, feeling at homes comes with a sense of safety and protection. Home is a place where I feel accepted, seen, and valued. I feel like, thus far, my advisor in the college of chemistry has made me feel this way and so have the students I am surrounded by.&quot;</td>
<td>yes</td>
<td>ChemE</td>
</tr>
</tbody>
</table>

### December survey

<table>
<thead>
<tr>
<th>Responses</th>
<th>TSMP</th>
<th>Major</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Feeling &quot;at home&quot; at UC Berkeley means to adjust to how to succeed in class and to feel like a part of the community of the college.&quot;</td>
<td>no</td>
<td>Chem</td>
</tr>
<tr>
<td>&quot;Have a support network, meaningful activities to do and people to do them with. No need to feel I have to act a certain way.&quot;</td>
<td>no</td>
<td>ChemE</td>
</tr>
<tr>
<td>&quot;The staff has been very friendly. I feel like I can go up to anyone and ask for help.&quot;</td>
<td>no</td>
<td>ChemE</td>
</tr>
<tr>
<td>&quot;I feel like a valued member of my classes and the lab I am performing research in.&quot;</td>
<td>yes</td>
<td>Chem</td>
</tr>
<tr>
<td>&quot;I don't really typically feel at home at the college of chemistry because I don't really think I'm like a lot of the students here. But honestly the majority of the students here are insecure and have toxic competitive mindsets.&quot;</td>
<td>yes</td>
<td>Chem</td>
</tr>
<tr>
<td>&quot;There are many supportive students if you make the effort to maintain good [relationships].&quot;</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>&quot;I feel like coc isn't home yet. I haven't made friends yet. The ones I thought I made would just take homework answers from me and use me. I asked if I can join their study group and they said yes but never invited me. They talked about how they met up ever Wednesday and Thursday at the end of the semester but never asked me to join. They would just ask me for answers and never help me if I was stuck on an answer.&quot;</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>&quot;Having faculty and classmate/friends to work with homework and other problems on. I have sort of experience it but it's sort of halfway.&quot;</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>&quot;I haven't felt at home yet, I think it is until we find our social circle both in friends and mentors that we start feeling at home and supported but I haven't found any of that yet&quot;</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>&quot;Unsure- to me it's just school. I go and do work then go home.&quot;</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>&quot;My first semester was tough which really impacted the way I view myself at Berkeley. Unfortunately, I did not feel so confident when beginning the semester but I hope feel more &quot;at home&quot; next semester!&quot;</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>&quot;Feeling at home to me means a place where I can feel comfortable to be myself. To be accepted for who am and respected for the things I stand for.&quot;</td>
<td>yes</td>
<td>ChemE</td>
</tr>
</tbody>
</table>

**Table 3.55.** Transfer student responses to experience question 1: “Think about what it means to you to feel "at home" somewhere. How does this feeling apply to your experience in the College of Chemistry at UC Berkeley?
Table 3.S6. Transfer student responses to experience question 2: “What to you constitutes a scientist? Do you describe yourself as a scientist? Why or why not? (For engineering students, please answer these questions for “engineer” rather than “scientist”).

<table>
<thead>
<tr>
<th>Responses</th>
<th>TSMP</th>
<th>Major</th>
</tr>
</thead>
<tbody>
<tr>
<td>“patient, creative”</td>
<td>no</td>
<td>Chem</td>
</tr>
<tr>
<td>“A true scientist is someone that is driven by curiosity. Instead of doing what most people do and watch movies for fun, scientists spend a lot of time thinking and using the laws of science to better understand why something is a certain way.”</td>
<td>no</td>
<td>Chem</td>
</tr>
<tr>
<td>“What defines a scientist is how they think on a daily basis. A scientist would often use the knowledge they acquire from school and apply it to everything around them (even if they aren’t in the lab or in a classroom).”</td>
<td>no</td>
<td>Chem</td>
</tr>
<tr>
<td>“A scientist is anyone that systematically attempts to understand a process. I would describe myself as a scientist as I constantly attempt to understand how things work in my daily life, eg, how is coffee brewed?”</td>
<td>no</td>
<td>Chem</td>
</tr>
<tr>
<td>“I think I can describe myself as a scientist. Whenever I get approached with something, I will always have questions about it, even if I do not ask about it in person, and will do my best to look up on that topic for answers later.”</td>
<td>no</td>
<td>ChemE</td>
</tr>
<tr>
<td>“For me, someone who is an engineer is someone with experience and education designing and analyzing processes and applying the science of a subject to the real world on a large scale. I don’t really consider myself a full engineer yet because I don’t have the same hands-on experience as someone I would consider a full engineer would.”</td>
<td>no</td>
<td>ChemE</td>
</tr>
<tr>
<td>“I think an engineer is someone with ideas and is able to execute such ideas. I only consider myself an engineer when I am working an internship that I am hands on with or even writing code. Currently, since I am not working in either of those circumstances, I don’t consider myself an engineer, maybe a student-engineer? Unsure”</td>
<td>no</td>
<td>ChemE</td>
</tr>
<tr>
<td>“I think anyone can be an engineer, but I don’t think engineering is for everyone. It’s for the student who wants to be challenged and is willing and able to take on that challenge. I’m not sure if I would feel confident enough to describe myself as an engineer yet. I don’t feel confident enough in the, what feels like, small amount of knowledge I have regarding the subject.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“I consider myself an Engineer as I work to understand concepts and look for solutions in places that may not be the most evident. I am proud to be studying as a minority in the Engineering field, from all aspects of my background.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“An engineer is one who is curious, passionate, and enthusiastic about exploring the unknown through the use of STEM. I do not feel as though I have enough knowledge to be considered an engineer however I do aspire to be one or try my best to be one.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“Engineers are problem solvers who utilize math and other knowledge respective of their particular fields. I don’t think I’m that much of an engineer since I’m not particularly interested in solving problems more than wanting to build things that I find interesting or are just new ways of doing things.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Responses</th>
<th>TSMP</th>
<th>Major</th>
</tr>
</thead>
<tbody>
<tr>
<td>“I think a scientist is someone who strives to discover ways to improve the world. I think I’m a scientist because I have a passion for using science to improve my community.”</td>
<td>no</td>
<td>Chem</td>
</tr>
<tr>
<td>“An engineer is someone who can break a problem down, understand the needs of the problem, and come up with ways to meet those needs as simply and as efficiently as possible. I would like to be there one day, but currently, I do not feel I have enough knowledge to break down many of the problems.”</td>
<td>no</td>
<td>ChemE</td>
</tr>
<tr>
<td>“I feeling I am learning to be one, but I am not one yet. I think an engineer is someone who works to solve problems and gets paid for that work.”</td>
<td>no</td>
<td>ChemE</td>
</tr>
<tr>
<td>“I would describe myself as a scientist. A scientist is someone whose curiosity leads to discovery”</td>
<td>yes</td>
<td>Chem</td>
</tr>
<tr>
<td>“Someone who utilizes math and various science principles to develop solutions to various problems.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“After taking my first chemical engineering course I am more confident in saying that I am an engineering in the making. I understand what engineers do, and how engineering relates to everything I have learned before”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“I do because I am constantly learning and constantly curious of my surroundings.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“not sure”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“I believe an engineer is driven, curious, enthusiastic, and passionate about STEM. I believe I am an engineer in training, however I would have liked to have a stronger STEM background.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“I describe myself as an engineer because I have always been good at analyzing and interested in innovative thinking and having an understanding of how things work. I believe I can develop on this skill and interest, and use them to solve problems and improve the way of life of others.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
</tbody>
</table>
Table 3.S7. Transfer student responses to experience question 3: “Which TSMP seminar(s) did you like best? What about the seminars did you find useful?”

<table>
<thead>
<tr>
<th>Responses</th>
<th>TSMP</th>
<th>Major</th>
</tr>
</thead>
<tbody>
<tr>
<td>“How to get involved with research. Had many good tips. Also liked the funding one.”</td>
<td>yes</td>
<td>Chem</td>
</tr>
<tr>
<td>“2 [Applying for research positions] and 3 [Introduction to research groups]. There were a lot of helpful resources for finding research and how to write an email to a PI for research opportunities.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“I liked the more industry focused ones”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“The applying for Research Seminar’”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
</tbody>
</table>
### Table 3.S8. Transfer student responses to experience question 4: “Did you feel that your TSMP graduate student mentor was well matched to you and your research interests? Please describe why or why not.”

<table>
<thead>
<tr>
<th>Responses</th>
<th>TSMP</th>
<th>Major</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Yes, she was currently working in a field I was interested in.”</td>
<td>yes</td>
<td>Chem</td>
</tr>
<tr>
<td>“Yes. They helped me improve my resume to send to professors and grad students when applying to research.”</td>
<td>yes</td>
<td>Chem</td>
</tr>
<tr>
<td>“My graduate student mentor is in a different field of study but his advice and guidance with applying for research was still helpful in planning my application.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“Somewhat, she was a chemistry major”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“Not really, but I learned about life after grad from them.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“Sort of. She was pretty helpful in looking for research opportunities but there was just a general disconnect because I am in chemical engineering and she was in chemistry.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“Yes, my mentor helped as much as possible. She advised me on how to get research positions and offered me resources.”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“they are great but they are not in the same major(chemE)”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
<tr>
<td>“Yes because it was in the College of Chemistry, but I am interested in exploring chemical engineering and she didn't have a lot of input about that since her expertise is chemistry. I still valued her advice and we talked about other things”</td>
<td>yes</td>
<td>ChemE</td>
</tr>
</tbody>
</table>
Table 3.9. Graduate student responses to experience question 1: “Think about what it means to you to feel “at home” somewhere. How does this feeling apply to your experience in the College of Chemistry at UC Berkely?”
<table>
<thead>
<tr>
<th>August survey</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Responses</strong></td>
</tr>
</tbody>
</table>
| "A scientist is someone that uses the scientific method to generate knowledge. I consider myself to be a scientist because I actively do this in my research."
| "A scientist asks questions and then seeks to answer them in a methodical way. I do see myself as a scientist"
| "A scientist spends time thinking about and working on scientific problems. My work focuses on solving scientific problems, which I believe qualifies me to the title of scientist."
| "Someone conducting research to answer a scientific question that will further our understanding of the world. Yes, I describe myself as a scientist because this is what I'm actively doing with my graduate work."
| "A person that is conducting research (or learning to conduct research) that may contribute to overall scientific progress. I feel I fit that bill."
| "I think someone who is funded by some entity to perform experiments that answer a particular question in a field. I guess can be poorly interpreted by someone who gets paid by shady alt-right/evangelical/etc. groups to do this poorly and fudge data or something malicious, but in my eyes those people are still technically "scientists", just not "good people". I do describe myself as a scientist because I feel like that is what I'm getting paid to do as a GSR."
| "The ability to conduct very evidence-based experiments to reach the sought-after non-ambiguous truth as well as the ability to critique other science independently. I am slowly becoming a scientist as I am nearing the end of my graduate school as I am learning these skills as I go. Many people graduate not knowing how to think independently which I think is [problematic]."
| "A scientist is someone who works in any kind of STEM job, comes up with hypotheses and/or tests them via experiments. I would describe myself as a scientist as I spend all my time doing this as a grad student."
| "A scientist is someone who does (or knows) science professionally. Since my income comes from working in a lab (as opposed to undergrad) I consider myself a scientist."
| "A scientist is a person who is thinking critically about problems related to science and working to solving them. As a graduate student, I believe I am a scientist and working towards being a better scientist"

<table>
<thead>
<tr>
<th>December survey</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Responses</strong></td>
</tr>
</tbody>
</table>
| "I guess? A scientist is someone who does science and since I get paid to do science I am a scientist. I usually call myself a chemist instead but I suppose yes, I am a scientist."
| "I am a frustrated scientist, but a scientist nonetheless. I don't think I'm particularly good at my job, especially the troubleshooting aspect of it, but I do it daily and try to make the best of the most interesting results of my work."
| "Curiously. Yes."
| "Yes, I do describe myself as a scientist, because I'm curious about the world and do research to find answers to my questions. I used to consider myself more of a "student" than a scientist, but that has changed recently"
| "I think a scientist is someone that applies the scientific method to things, which is usually in a formal setting like a lab, but I think that the scientific method can be applied to many aspects of life. I do consider myself a scientist both in occupation and in other things."
| "Someone who conducts experiments to answer a question of scientific relevance. I describe myself as a scientist because this is what I have aimed to do since I started studying science."
| "In a general sense, I think a scientist is anyone who is curious about the world and tries to understand it more, in whatever way. Yes, I describe myself as a scientist, because my work involves trying to explain something (very specific) about the world."
| "I'd probably consider just about anyone that asks critical questions about the world around them and does something to try to answer those questions. I'd consider myself a scientist" |

Table 3.510. Graduate student responses to experience question 2: “What to you constitutes a scientist? Do you describe yourself as a scientist? Why or why not?”
<table>
<thead>
<tr>
<th>December survey</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Responses</strong></td>
</tr>
<tr>
<td>“career paths—probably the most neglected part of my education until late undergraduate studies. I had a very one-track mind and wish I had explored more options earlier”</td>
</tr>
<tr>
<td>“Ih I didn't really find them useful”</td>
</tr>
<tr>
<td>“Applying to research positions. This is the biggest hurdle for a lot of the undergraduates.”</td>
</tr>
<tr>
<td>“How to apply to research positions”</td>
</tr>
<tr>
<td>“Intro to Research Groups”</td>
</tr>
<tr>
<td>“Navigating classes in the College of Chemistry, because I personally did not previously know much about the different options and structure of undergraduate programs here”</td>
</tr>
<tr>
<td>“Getting into a research lab”</td>
</tr>
<tr>
<td>“Finding a research group: helpful as a mentor for giving advice to my mentee”</td>
</tr>
<tr>
<td>“I only saw the full seminar for the one on finding research groups at Berkeley, but it definitely seemed helpful for students. My mentee was a bit unaware of how to look into research positions on campus before that.”</td>
</tr>
</tbody>
</table>

**Table 3.S11.** Graduate student responses to experience question 3: “Which TSMP seminar did you find the most useful and why?”
Table 3.S12. Graduate student responses to experience question 4: “What has the impact of the TSMP been on your professional development?”

<table>
<thead>
<tr>
<th>Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>“It’s been a really good experience to learn more about the transfer student experience at Berkeley, and adapting advice and mentorship to different student’s needs. The group discussions at the TSMP meetings have been particularly valuable.”</td>
</tr>
<tr>
<td>“none? I met some nice people though!”</td>
</tr>
<tr>
<td>“It was helpful to discuss research with undergrads and get their perspectives”</td>
</tr>
<tr>
<td>“It’s helped me understand what goes into a mentorship role,”</td>
</tr>
<tr>
<td>“minimal, to be honest. I was unable to attend most sessions in person but would have liked to form better ties with fellow mentors/mentees”</td>
</tr>
<tr>
<td>“It was fun to chat with my mentee about his interests, since they were much more engineering focused and he taught me about some of the engineering clubs/projects on campus that I hadn’t heard of before”</td>
</tr>
<tr>
<td>“Conversations with my mentees taught me more about the issues transfer students can face in joining research groups, forming relationships with peers/instructors, and navigating course requirements. This has motivated me to consider transfer students in particular if I end up recruiting or choosing another undergrad to work with on my research, and I plan to encourage my peers to do the same.”</td>
</tr>
</tbody>
</table>
**Table 3.S13.** Graduate student responses to experience question 5: “Did you feel that you and your transfer student mentee were well matched? Please outline why or why not.”

<table>
<thead>
<tr>
<th>December survey</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Responses</strong></td>
</tr>
<tr>
<td>“My first mentee did not respond to any of my emails. My second mentee stopped responding after our first meeting. So maybe we were not well matched in my mentees’ minds. The second mentee was a Chem Eng student with different research interests and career goals.”</td>
</tr>
<tr>
<td>“I did! As a transfer student myself during undergrad I found the pain of the ‘first semester’ very relatable.”</td>
</tr>
<tr>
<td>“Yes, one of them had similar interests and the other one was interested in med school which I knew about that whole application process.”</td>
</tr>
<tr>
<td>“Yes, one of them in particular was interested in the same field as me and had previous experience in it.”</td>
</tr>
<tr>
<td>“I didn’t feel particularly well-matched to my mentee because she is a chemical engineering major, and the job path for ChemE vs chemistry tends to be much”</td>
</tr>
<tr>
<td>“Kind of! I got along well with both of my mentees, but they were both studying chemical engineering and I’m not as well-informed about the specifics of chemical engineering courses, research groups, or internships. This meant that I didn’t know the answers to some of their questions and was only able to give them more general answers.”</td>
</tr>
<tr>
<td>“Yes, for the most part. We got along fairly well, and I felt that I was able to point her in the direction of some resources that appealed to her interest in energy specifically, and we were also able to discuss biotech options etc. However, she was in chemical engineering, and did have some questions that were more engineering-specific that I couldn’t particularly help her with.”</td>
</tr>
<tr>
<td>“Yes, I think me and my transfer student mentee were well matched. We have somewhat similar research interests and personalities.”</td>
</tr>
<tr>
<td>“Unfortunately not applicable. The first mentee I was paired with never responded after 3 attempts to contact them. Later I was given a second mentee that seemed enthusiastic but when we tried to meet they canceled and then asked to have my social media contacts and didn’t want to email and I thought that was a little odd.”</td>
</tr>
<tr>
<td>“Yes and no- one of my mentees was a good match and I felt like I could talk to him about classes and research groups in a way that he found helpful; my other mentee was an engineering major and I was very unfamiliar with his areas of interest.”</td>
</tr>
<tr>
<td>“Not necessarily, since the student was a chemE major and we had relatively different interests both in research and outside of school. But it wasn’t an issue at all really, I still think it went well and was (hopefully) quite helpful for the student.”</td>
</tr>
</tbody>
</table>
TSMP Transfer Student Program Survey

Note: This is the post-program survey, which differs from the pre-program survey by the addition of a few questions which have been highlighted here.

1. What year are you?
   - Sophomore
   - Junior
   - Senior
   - Other ________________________________

2. Have you ever been involved as an active member of the Transfer Student Mentorship Program (TSMP)?
   - Yes
   - No
   - Other ________________________________

3. If you were involved in the TSMP, which year did you first join?
   - 2020
   - 2021

4. Please describe how you would go about applying for an undergraduate research position in a campus lab (please answer even if you already have a research position).

_________________________________________
5. Please choose the option below that best describes your research experience.

- [ ] I am working as an undergraduate researcher in a campus lab
- [ ] I am working as an undergraduate researcher in an LBNL lab
- [ ] I am performing research through an established undergraduate research program (i.e. BLUR, URAP). Please include the program below:
  __________________________________________
- [ ] I am or have performed research through an established summer research program (i.e. Amgen scholars, SURF, REU). Please include the program below:
  __________________________________________
- [ ] I am interested in performing research but have not found a position yet.
- [ ] I have performed research in the past but am not currently in a research position
- [ ] I am not interested in performing research
- [ ] Other __________________________________________

6. What is your major?

________________________________________________
7. (If applicable) Approximately how many times did you meet with your TSMP graduate student mentor? *(Post-program survey only)*

________________________________________________________________________

8. (If applicable) Did you feel that your TSMP graduate student mentor was well matched to you and your research interests? Please describe why or why not. *(Post-program survey only)*

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

9. (If applicable) Approximately how many TSMP meetings did you attend? *(Post-program survey only)*

   - [ ] 1
   - [ ] 2
   - [ ] 3
   - [ ] 4
   - [ ] 5
   - [ ] 6
10. (If applicable) Do you have an ongoing relationship with other participants you met through the TSMP? (Post-program survey only)

○ Yes

○ No

○ Other ________________________________

11. (If applicable) Which TSMP seminar(s) did you like best? What about the seminars did you find useful? (Post-program survey only)

___________________________________________________________________________

___________________________________________________________________________
12. How strongly do you identify with the character on the right in the cartoon?

- 0 – Do not identify
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10 - Very strongly identify
13.  

0 – Do not identify

1

2

3

4

5

6

7

8

9

10 - Very strongly identify
I wish there were more faculty I could talk to who would understand the hardships I face.

- 0 – Do not identify
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10 - Very strongly identify
15. How strongly do you identify with the character on the left of the cartoon?

- 0 – Do not identify
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10 - Very strongly identify
16.

0 – Do not identify

1

2

3

4

5

6

7

8

9

10 - Very strongly identify
Other grad students are more productive and scientifically successful than I am.

- 0 – Do not identify
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10 – Very strongly identify
0 – Do not identify
1
2
3
4
5
6
7
8
9
10 - Very strongly identify
◯ 0 – Do not identify
◯ 1
◯ 2
◯ 3
◯ 4
◯ 5
◯ 6
◯ 7
◯ 8
◯ 9
◯ 10 - Very strongly identify
20.

○ 0 – Do not identify

○ 1

○ 2

○ 3

○ 4

○ 5

○ 6

○ 7

○ 8

○ 9

○ 10 - Very strongly identify
21. 

- 0 – Do not identify
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10 - Very strongly identify
22. Think about what it means to you to feel "at home" somewhere. How does this feeling apply to your experience in the College of Chemistry at UC Berkeley?

________________________________________________________________
________________________________________________________________
________________________________________________________________
________________________________________________________________
________________________________________________________________
________________________________________________________________

23. What to you constitutes a scientist? Do you describe yourself as a scientist? Why or why not? (For engineering students, please answer these questions for "engineer" rather than "scientist").

________________________________________________________________
________________________________________________________________
________________________________________________________________
________________________________________________________________
________________________________________________________________
________________________________________________________________

Your data will be kept private and only accessible by the study directors via password-protected computers. Please keep in mind that while your data is kept as secure and private as possible, no guarantees can be made against the low risk of an inadvertent breach in confidentiality. For this reason, if there is any risk that a breach of your responses would lead to any damage or discomfort we encourage you to please leave the question blank.

________________________________________________________________

24. (Optional) Please state the gender you best identify with.

________________________________________________________________
25. (Optional) Please state the sexuality you best identify with.
____________________________________________________

26. (Optional) Please state the race/ethnicity you best identify with.
____________________________________________________

27. (Optional) Are you the first in your immediate family to attend college?

○ Yes

○ No
TSMP Graduate Student Program Survey

*Note:* This is the post-program survey, which differs from the pre-program survey by the addition of a few questions which have been highlighted here.

1. What year are you?
   - [ ] 1st year
   - [ ] 2nd year
   - [ ] 3rd year
   - [ ] 4th year
   - [ ] 5th year
   - [ ] 6th year
   - [ ] Other _______________________

2. Have you previously worked with the Transfer Student Mentorship Program (TSMP)?
   - [ ] Yes
   - [ ] No
   - [ ] Other _______________________

3. Please briefly describe any previous experience you've had mentoring (can simply list mentorship positions).
   ____________________________________________________________________________
4. Approximately how many times did you meet with your transfer student mentee? *(Post-program survey only)*


5. Did you feel that you and your transfer student mentee were well matched? Please outline why or why not. *(Post-program survey only)*


6. How many TSMP meetings did you attend? *(Post-program survey only)*

   - [ ] 1
   - [ ] 2
   - [ ] 3
   - [ ] 4
   - [ ] 5
   - [ ] 6
7. Do you have an ongoing relationship with any other participants you met through the TSMP? (Post-program survey only)

________________________________________________________________

8. Which TSMP seminar did you find the most useful and why? (Post-program survey only)

________________________________________________________________

9. What has the impact of the TSMP been on your professional development? (Post-program survey only)

________________________________________________________________
10. How strongly do you identify with the character on the right in the cartoon?

- 0 – Do not identify
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10 – Very strongly identify
11.  

- 0 – Do not identify
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10 - Very strongly identify
I wish there were more faculty I could talk to who would understand the hardships I face.

- 0 – Do not identify
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10 - Very strongly identify
13. How strongly do you identify with the character on the left of the cartoon?

- 0 – Do not identify
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10 - Very strongly identify
14.

- 0 – Do not identify
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10 - Very strongly identify
15.

Other grad students are more productive and scientifically successful than I am.

- 0 – Do not identify
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10 – Very strongly identify
16.

- 0 – Do not identify
- 1
- 2
- 3
- 4
- 5
- 6
- 7
- 8
- 9
- 10 - Very strongly identify
17.

- I feel like an outsider.

  - 0 – Do not identify
  - 1
  - 2
  - 3
  - 4
  - 5
  - 6
  - 7
  - 8
  - 9
  - 10 - Very strongly identify
0 – Do not identify
1
2
3
4
5
6
7
8
9
10 - Very strongly identify
20. Think about what it means to you to feel "at home" somewhere. How does this feeling apply to your experience in the College of Chemistry at UC Berkeley?
21. What to you constitutes a scientist? Do you describe yourself as a scientist? Why or why not?

Your data will be kept private and only accessible by the study directors via password-protected computers. Please keep in mind that while your data is kept as secure and private as possible, no guarantees can be made against the low risk of an inadvertent breach in confidentiality. For this reason, if there is any risk that a breach of your responses would lead to any damage or discomfort we encourage you to please leave the question blank.

22. (Optional) Please state the gender you best identify with.

23. (Optional) Please state the sexuality you best identify with.
24. (Optional) Please state the race/ethnicity you best identify with.

........................................................................................................................

........................................................................................................................

25 (Optional) Are you the first in your immediate family to attend college?

○ Yes

○ No
1. To the best of your knowledge, did your first mentee have a research position before the beginning of the program?

- Yes
- No
- I don't know
- Other ______________________________

2. To the best of your knowledge, does your first mentee have a research position now?

- Yes
- No
- I don't know
- Other ______________________________
3. Approximately how many times did you meet with your first mentee?

- □ 0
- □ 1
- □ 2
- □ 3
- □ 4
- □ 5
- □ 6
- □ Other

The following questions are for mentors who had more than one mentee. If you had only one mentee, please skip to the end of the survey and click the forward arrow to finish.

4. To the best of your knowledge, if you had a second mentee, did your second mentee have a research position before the beginning of the program?

- □ Yes
- □ No
- □ I don’t know
- □ Other
5. To the best of your knowledge, if you had a second mentee, does your second mentee have a research position now?

- Yes
- No
- I don't know
- Other ________________________________

6. If you had a second mentee, approximately how many times did you meet with your second mentee?

- 0
- 1
- 2
- 3
- 4
- 5
- 6
- Other ________________________________
References


(7) Sto. Domingo Mariano R.; Sharp Starlette; Freeman Amy; Freeman Thomas; Harmon Keith; Wiggs Mitsue; Sathy Viji; Panter Abigail T.; Oseguera Leticia; Sun Shuyan; Williams Mary Elizabeth; Templeton Joseph; Folt Carol L.; Barron Eric J.; Hrabowski Freeman A.; Maton Kenneth I.; Crimmins Michael; Fisher Charles R.; Summers Michael F. Replicating Meyerhoff for Inclusive Excellence in STEM. *Science* 2019, 364 (6438), 335–337. DOI: 10.1126/science.aar5540.


Appendix A

Development and characterization of a fluorescent probe selective for iron (II)

Portions of this work were performed in collaboration with the following people:
ResFe-1 and ResFe-2 and their corresponding syntheses were designed by Allegra Aron.
Synopsis

Iron is an essential molecule in biological systems with a carefully balanced dichotomy (Figure A.A.1). At physiological concentrations, iron facilitates oxygen transport, cell growth regulation, and general redox for a variety of substrates. However, aberrant iron levels are implicated in the pathologies of disease states such as cancer, prion diseases, and aging. Interestingly, research over the past decade has implicated that labile iron is responsible for a novel method of cell death called “ferroptosis”, which causes cell death through lipid reactive oxygen species produced via ferrous iron’s catalytic transformation of water into hydroxyl radical via the Fenton reaction. Labile iron levels have also been intricately tied to cancer, with the exact pathologies remaining enigmatic. Thus, new tools are needed to elucidate iron’s careful role in disease pathologies and metabolism.

Fluorescent probes have been shown to successfully detect redox-active metals, such as iron, non-invasively in biological systems. However, the handful of ferrous iron probes currently published face issues regarding cell permeability, ferrous iron specificity, and/or poor time-scale resolution. With these issues in mind, this project involved the synthesis of a library of specific, reactivity-based iron probes for improved detection and selectivity. In this work, I synthesized two such probes and characterized them in vitro and in cellulo for their efficacy.
Reactions using moisture- or air-sensitive reagents were carried out in flame-dried glassware under an inert atmosphere of N₂. Solvent was passed over activated alumina and stored over activated 3 Å molecular sieves before use when dry solvent was required. All other commercially purchased chemicals were used as received (without further purification). SiliCycle 60 F254 silica gel pre-coated sheets (0.25 mm thick) were used for analytical thin layer chromatography and visualized by fluorescence quenching under UV light. Silica gel P60 (SiliCycle) was used for column chromatography. ¹H and ¹³C NMR spectra were collected at 298 K in CDCl₃ or CD₃OD (Cambridge Isotope Laboratories, Cambridge, MA) at 25 °C using Bruker AV300, AVQ-400, AVB-400, AV-500, or AV-600 instruments at the College of Chemistry NMR Facility at the University of California, Berkeley. All chemical shifts are reported in the standard notation of δ parts per million relative to the residual solvent peak at 7.26 (CDCl₃) or 3.31 (CD₃OD) for ¹H and 77.16 (CDCl₃) or 49.00 (CD₃OD) for ¹³C as an internal reference. Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets. Low-resolution electrospray mass spectral analyses were carried out using LC-MS (Agilent Technology 6130, Quadrupole LC/MS and Advion Expression-L Compact Mass Spectrometer). High-resolution mass spectral analyses (ESI-MS) were carried out at the College of Chemistry Mass Spectrometry Facility at the University of California, Berkeley. All aqueous solutions were prepared using Milli-Q water, and all in vitro experiments were carried out in PBS, pH 7.4, unless otherwise noted. All biological experiments were prepared using freshly prepared aliquots.

Milli-Q water was used to prepare all aqueous solutions. All spectroscopic measurements were performed in 20 mM HEPES, pH 7.4. Absorption spectra were recorded using a Varian Cary 50 spectrophotometer, and fluorescence spectra were recorded using a Photon Technology International Quanta Master 4 L-format scan spectrofluorometer equipped with an LPS-220B 75- W xenon lamp and power supply, A-1010B lamp housing with integrated igniter, switchable 814 photocounting/analog photomultiplier detection unit, and MD5020 motor driver. Samples for absorption and emission measurements were contained in 1-cm × 1-cm quartz cuvettes (1.4-mL volume, Starna).

A.A.1 Synthesis. To a flask unprotected from atmosphere or water were added adamantane (36.6 mmol), methoxyamine HCl (40.3 mmol), and pyridine (40.3 mmol) in 3.25 mL EtOH. The mixture was brought to 80 °C and allowed to stir for four hours. Reaction was then concentrated and dissolved in 50 mL 1 M HCl, extracted with CH₂Cl₂, brine, dried over Na₂SO₄, filtered and concentrated to provide pure white crystals with a blue tint at 88% yield. No further purification was necessary, product confirmed via ¹H NMR (CDCl₃). ¹H NMR (400 MHz, CDCl₃) δ 3.75 (s, 3H), δ 3.46 (s, 1H), δ 2.49 (s, 1H), δ 2.11 - 1.48 (m, 12 H).

A.A.2 Synthesis. To a flask protected from atmosphere and water were added A.A.1 (5.58 mmol) and 1,4-cyclohexanediene (11.2 mmol) to a solution of very dry pentane: CH₂Cl₂ (1:1 ratio). The mixture was brought to -78 °C, upon which ozone was bubbled
through for half an hour and which turned the mixture a vivid sapphire blue. After the ozone was removed, the mixture was concentrated and purified via column chromatography (1:3 EtOAC:Hex) and confirmed via $^1$H NMR (CDCl$_3$) to give the desired product in 27% yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 2.46 (t, $J = 7.1$ Hz, 4H), $\delta$ 2.08 (t, $J = 6.9$ Hz, 4H), $\delta$ 2.02 - 1.58 (m, 14 H).

A.A.3 Synthesis. To a flask unprotected from atmosphere or water were added A.A.2 (0.781 mmol), mCPBA (1.72 mmol), and sodium carbonate (1.56 mmol) in CH$_2$Cl$_2$ (10 mL). The reaction was brought to 40 °C and allowed to stir overnight. The next day the reaction was diluted in water, washed 3x with CH$_2$Cl$_2$, The organic layer was neutralized with a solution of sodium carbonate followed by brine, dried over Na$_2$SO$_4$, filtered, and concentrated. Residue was purified via column chromatography (1:10 MeOH:CH$_2$Cl$_2$) and confirmed via $^1$H NMR (CDCl$_3$) to provide the desired product in 54% yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 4.33 - 4.22 (m, 2H), $\delta$ 2.78 - 2.60 (m, 2H), $\delta$ 2.24 - 1.60 (m, 18H).

A.A.4 Synthesis. To a flask protected from atmosphere and water were added A.A.3 (0.91 mmol) and propylamine (15.477 mmol) in toluene (1.27 mL). The mixture was stirred at room temperature overnight, upon which it was diluted with water, washed 3x with CH$_2$Cl$_2$, dried over Na$_2$SO$_4$, filtered, and concentrated. A light orange oil resulted through for half an hour and which turned the mixture a vivid sapphire blue. After the ozone was removed, the mixture was concentrated and purified via column chromatography (1:4 EtOAC:Hex) and confirmed via $^1$H NMR (CDCl$_3$) as requiring no further purification, corresponding to a 55% yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.58 (m, 14 H).

A.A.5 Synthesis. To a flask protected from atmosphere and water were added A.A.4 (0.504 mmol) in DMF (1 mL). The mixture was brought to 50 °C and allowed to stir overnight. The next day, the reaction was diluted in water, washed 3x with CH$_2$Cl$_2$, once with brine, dried over Na$_2$SO$_4$, filtered, and concentrated. Residue was purified via column chromatography (1:1 EtOAC:Hex) and confirmed via $^1$H NMR (CDCl$_3$) to provide the desired product in 54% yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.80 (d, $J = 8.4$ Hz, 2H), $\delta$ 7.38 (d, $J = 8.0$ Hz, 2H), $\delta$ 5.82 (br s, 1H), $\delta$ 4.22 - 4.18 (m, 2H), $\delta$ 3.21 (q, $J = 6.3$ Hz, 2H), $\delta$ 2.47 (s, 3H), $\delta$ 2.31 - 2.23 (m, 2H), $\delta$ 2.20 - 1.61 (m, 18H), $\delta$ 1.59 - 1.47 (m, $J = 7.1$ Hz, 2H), $\delta$ 0.93 (t, $J = 7.3$ Hz, 3H).

ResFe-1 Synthesis. To a flask protected from atmosphere and water were added A.A.5 (0.0747 mmol), resorufin sodium salt (0.0583 mmol), and cesium carbonate (0.0747 mmol) in CH$_2$Cl$_2$ (1 mL). The mixture was brought to 40 °C and allowed to stir overnight. The next day, the reaction was diluted in water, washed 3x with CH$_2$Cl$_2$, once with brine, dried over Na$_2$SO$_4$, filtered, and concentrated. Residue was purified via column chromatography (1:10 MeOH:CH$_2$Cl$_2$) and confirmed via $^1$H NMR (CDCl$_3$) to provide the desired product in 54% yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.06 (s, 1H), $\delta$ 7.74 (d, $J = 9.0$ Hz, 1H), $\delta$ 7.47 (d, $J = 9.4$, 1H), $\delta$ 7.01 - 6.95 (m, 1H), $\delta$ 6.91 - 6.85 (m, 1H), $\delta$ 6.39 - 6.37
(m, 1H), δ 4.31 - 4.21 (m, 2H), δ 3.32 - 3.18 (m, 2H), δ 3.00 (s, 3H), δ 2.43 - 2.31 (m, 2H), δ 2.10 - 1.49 (m, 17H), δ 1.01 - 0.91 (m, 3H).

A.A.6 Synthesis. To a flask protected from atmosphere and water were added 4-nitrobenzyl bromide (1.852 mmol), potassium phthalimide (2.407 mmol), and TBAB (0.0056 mmol) in DMF (1.2 mL). The mixture was brought to 70 °C, upon which it turned a mild peach color, and was stirred for 8 hours. Crystals that had formed were filtered off and washed with EtOAc. Product confirmed via $^1$H NMR (DMSO-d$_6$), 92% yield.

$^1$H NMR (400 MHz, DMSO-d$_6$) δ 8.24 - 8.15 (m, 2H), δ 7.91 - 7.85 (m, 2H), δ 7.80 - 7.73 (m, 2H), δ 7.60 - 7.54 (m, 2H), δ 4.88 (s, 2H).

A.A.7 Synthesis. To a flask unprotected from atmosphere or water was added A.A.6 (1.701 mmol) to MeOH (1.5 mL). With the mixture stirring rigorously, hydrazine monohydrate was carefully added (6.803 mmol) and the mixture brought to 60 °C. After one hour, a white solid had crashed out from a pale yellow supernatant. The next day the reaction was stopped, was diluted in CH$_2$Cl$_2$, extracted with 1 M NaOH, dried over Na$_2$SO$_4$, filtered, and concentrated. Residue was confirmed via $^1$H NMR (DMSO-d$_6$) to provide the desired product in 31% yield with no further purification.

$^1$H NMR (400 MHz, DMSO-d$_6$) δ 8.13 (d, $J$ = 7.1 Hz, 2H), δ 7.67 (d, $J$ = 7.0 Hz, 2H), δ 3.78 (s, 2H).

A.A.8 Synthesis. To a flask protected from atmosphere and water were added A.A.3 (0.34 mmol) and A.A.7 (0.53 mmol) to toluene (1 mL). Solution was brought to 50 °C and stirred overnight. The next day the mixture was concentrated, and the residue diluted with CH$_2$Cl$_2$, washed three times with water, once with brine, dried over Na$_2$SO$_4$, filtered, and concentrated. Residue was purified via column chromatography (1:10 MeOH:CH$_2$Cl$_2$) and confirmed via $^1$H NMR (CDCl$_3$) to provide the desired product in 18% yield.

$^1$H NMR (400 MHz, CDCl$_3$) δ 8.16 (d, $J$ = 6.9 Hz, 2H), δ 7.49 (d, $J$ = 7.0 Hz, 2H), δ 6.28 - 6.20 (m, 1H), δ 4.56 (d, $J$ = 6.4 Hz, 2H), δ 3.87 - 3.65 (m, 2H), δ 2.49 - 2.40 (m, 2H), δ 2.36 - 2.13 (m, $J$ = 7.0 Hz, 2H), δ 2.01 - 1.62 (m, 15H).

A.A.9 Synthesis. To a flask protected from atmosphere and water was added A.A.8 (0.0942 mmol) to CH$_2$Cl$_2$ (0.8 mL), followed by the addition of Et$_3$N (0.188 mmol) and TsCl (0.141 mmol) slowly at 0 °C. The mixture was allowed to stir overnight. The next day, the reaction was diluted in water, washed 3x with CH$_2$Cl$_2$, once with brine, dried over Na$_2$SO$_4$, filtered, and concentrated. Residue was purified via column chromatography (1:10 MeOH:CH$_2$Cl$_2$) and confirmed via $^1$H NMR (CDCl$_3$) to provide the desired product in 72% yield.

$^1$H NMR (400 MHz, CDCl$_3$) δ 8.24 (d, $J$ = 7.1 Hz, 2H), δ 7.77 (d, $J$ = 7.2 Hz, 2H), δ 7.48 (d, $J$ = 8.2 Hz, 2H), δ 7.40 (d, $J$ = 8.0 Hz, 2H), δ 6.48 - 6.33 (m, 1H), δ 4.51 (d, $J$ = 6.3 Hz, 2H), δ 4.24 - 4.10 (m, 2H), δ 2.50 (s, 3H), δ 2.49 - 1.57 (m, 17H), δ 1.30 - 1.24 (m, 2H).

A.A.10 Synthesis. To a flask protected from atmosphere and water were added A.A.3 (0.151 mmol) and octylamine (0.303 mmol) in toluene (0.5 mL). The mixture was brought to 50 °C and allowed to stir overnight. The next day the mixture was concentrated, and the residue diluted with CH$_2$Cl$_2$, washed three times with water, once with brine, dried
over Na$_2$SO$_4$, filtered, and concentrated. A light orange oil resulted and confirmed via $^1$H NMR (CDCl$_3$) as requiring no further purification, corresponding to a 99% yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 5.92 (s, 1H), $\delta$ 3.77 - 3.62 (m, 2H), $\delta$ 3.30 - 3.12 (m, 2H), $\delta$ 2.70 - 2.52 (m, 2H), $\delta$ 2.50 - 1.58 (m, 17H), $\delta$ 1.51 - 1.47 (m, 4H), $\delta$ 1.46 - 1.22 (m, 8H), $\delta$ 0.93 - 0.82 (m, 3H).

A.A.11 Synthesis. To a flask protected from atmosphere and water was added A.A.10 (0.150 mmol) to CH$_2$Cl$_2$ (1.2 mL), followed by the addition of Et$_3$N (0.3 mmol) and TsCl (0.226 mmol) slowly at 0 °C. The mixture was allowed to stir overnight. The next day, the reaction was diluted in water, washed 3x with CH$_2$Cl$_2$, once with brine, dried over Na$_2$SO$_4$, filtered, and concentrated. Residue was purified via column chromatography (1:1 EtOAC:Hex) and confirmed via $^1$H NMR (CDCl$_3$) to provide the desired product in 31% yield. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.80 (d, $J$ = 8.2 Hz, 2H), $\delta$ 7.48 (d, $J$ = 8.1 Hz, 2H), $\delta$ 5.53 (s, 1H), $\delta$ 4.24 - 4.09 (m, 2H), $\delta$ 3.26 - 3.18 (m, 2H), $\delta$ 2.47 (s, 3H), $\delta$ 2.33 - 1.60 (m, 17H), $\delta$ 1.55 - 1.45 (m, 2H), $\delta$ 1.43 - 1.18 (m, 12H), $\delta$ 0.96 - 0.88 (m, 3H).

ResFe-2 Synthesis. To a flask protected from atmosphere and water were added A.A.9 (0.0678 mmol), resorufin sodium salt (0.0529 mmol), and cesium carbonate (0.0678 mmol) in DMF (0.94 mL). The mixture was brought to 40 °C and allowed to stir overnight. The next day, the reaction was diluted in water, washed 3x with CH$_2$Cl$_2$, once with brine, dried over Na$_2$SO$_4$, filtered, and concentrated. Residue was purified via column chromatography (1:10 MeOH:CH$_2$Cl$_2$) and confirmed via LC-MS to provide the desired product in an estimated 9% yield.

**in vitro characterization of ResFe probes.** All buffers and aqueous solutions were degassed prior to use to remove dissolved oxygen. In a glovebox were prepared the following: 1 mL of HEPES buffer with 0 µM, 10 µM, or 20 µM ResFe-1 or ResFe-2 solutions (1 µL of probe stock in DMSO was added). Samples were mixed well, transferred to quartz cuvettes, and sealed prior to removal from the glovebox for t=0. Emission spectra ($\lambda_{ex} = 573$ nm, $\lambda_{em} = 583$-650 nm) were collected at 0, 10, 20, 30, 40, 50, 60, and 120 min (see Figure A.A.2).

**in cellulo characterization of ResFe probes.** HEK293T cells were maintained in exponential growth as a monolayer in Dulbecco’s Modified Eagle Medium, high glucose, (DMEM, Invitrogen) supplemented with glutamax (Gibco), 10% fetal bovine serum (FBS, Hyclone) and 1% non-essential amino acids (NEAA, Gibco), and incubated at 37 °C in 5% CO$_2$. When cells had reached 75% confluency, media was replaced with fresh media, and the BPS well was dosed with 1 mM BPS. The cells were allowed to rest overnight. The next morning, the BPS solution was replaced with a fresh 1 mM BPS solution in media without FBS, the control well was replaced with media without FBS, and the Fe(II) well was dosed with a solution of 100 µM Fe(II) in media without FBS. The cells were allowed to rest overnight. The next day the media was removed from all wells, and all wells were given
a solution of 10 µM ResFe-1 or ResFe-2 in HBSS as indicated for half an hour prior to imaging at $\lambda_{ex} = 573$ nm.
Results and Discussion

Much of the synthetic route for each ResFe probe was derived from that of FIP-1, which utilizes a Griesbaum cooazonolysis to generate the trioxolane ring followed by a Baeyer-Villiger oxidation to generate an electrophilic center that can then be opened up with an appropriate nucleophile, here a primary amine (Scheme A.A.1). This ring opening results in an exposed alcohol, which after tosylation yields an appropriate leaving group for a substitution with a fluorophore. This route provides a facile method to swap out different fluorophores or additional moieties on scaffold. In this work, resorufin was utilized as the fluorophore for all ResFe probes, with differing primary amines used to introduce varying moieties to the probe scaffold.

ResFe-1 was synthesized following this synthetic route using propylamine and resorufin (Scheme A.A.1). ResFe-1 was confirmed to demonstrate reactivity with Fe(II) in vitro with a roughly 5-fold turn on of 1 µM ResFe-1 to 20 µM Fe(II) under anhydrous conditions (Figure A.A.2.a). However, in cellulo ResFe-1 resulted in very dim fluorescence with an observed trend inverse to our expectations. In HEK 293T cells, in the presence of Fe(II) ResFe-1 displayed dimmer fluorescence than the control, and in the presence of the iron chelator bathophenanthrolinedisulfonic acid (BPS) ResFe-1 displayed an increase in fluorescence (Figure A.A.3, top row). This trend implied that perhaps ResFe-1 was suffering from excess diffusion through the cell membrane. We turned to variations of the scaffold in order to improve these features.

ResFe-2 and ResFe-3 were designed for improved cell retention and turn-on (Schemes A.A.2 and A.A.3). ResFe-2 was synthesized and utilized for further studies analogous to those of ResFe-1. in vitro, ResFe-2 displayed poorer turn-on to Fe(II) than ResFe-1, with only a 1.2-fold turn-on, the result of significant autofluorescence of the probe (Figure A.A.2.b). In HEK 293T cells in a similar manner to ResFe-1, ResFe-2 displayed atypical behavior, with a decrease in fluorescence in both the Fe(II)-treated cells and the BPS-treated cells in comparison to the control (Figure A.A.3, bottom row).
**Conclusion**

ResFe-1 demonstrated good response *in vitro*, but poor response *in cellulo*. We hypothesized that the intermediate after reacting with iron diffused out of the cells, as demonstrated by the downward trend in fluorescence with increased ferrous iron concentration. *In vitro* response of ResFe-2 to ferrous iron was poorer than that of ResFe-1, however a response was still observed at 20 µM Fe(II). The *in cellulo* response was more promising with a decrease in fluorescence after the addition of the iron chelator BPS, though a decrease in fluorescence after the addition of 100 µM Fe(II) indicated that the probe was not working as intended.

The ease of synthetic alterations to the trioxolane scaffold allowed for flexibility in the attachment of a variety of fluorophores and cell-trappable moieties. The modular synthesis of these probes provides fine tuning of the emission wavelength with targeting group, valuable information in real time imaging of iron’s changing levels in the mitochondria, nucleus, and cytoplasm. Future efforts to expand a varied palette of probes would yield valuable tools for multi-organelle iron imaging as well as multi-analyte imaging in conjunction with other probes.
Iron homeostasis is highly regulated at both systemic whole body and cellular levels and proceeds in the following general sequence of pathways (a)–(c). Iron is first absorbed from the diet by the intestine (a), where it is then put into the circulatory system (b). Once in circulation, iron is bound to transferrin (Tf) as Fe3+ and can subsequently enter cells, which maintain iron homeostasis through a complex network of proteins (c). While Hepcidin (Hep) is a hormone that controls systemic homeostasis, cellular homeostasis is maintained through a dynamic network of import proteins including transferrin (Tf)/transferrin receptor (TfR) and divalent metal transporter-1 (Dmt1), storage proteins like ferritin (Ftn), and export proteins like ferroportin-1 (Fpn), in dynamic exchange with a central labile iron pool (LIP) that is comprised predominantly of Fe2+ coordinated to weakly bound ligands.
Figure A.A.2. *in vitro* characterization of ResFe probes with ferrous iron. Solutions of 1 µM of a) ResFe-1 and b) ResFe-2 were treated with varying concentrations of ammonium iron(II) sulfate in HEPES buffer devoid of oxygen. Samples were imaged periodically at $\lambda_{ex} = 573$ nm.
Figure A.A.3. *in cellulo* characterization of ResFe-1 and ResFe-2. a) Structures of ResFe-1 and ResFe-2. b) HEK 293T cells stained with ResFe-1 (upper) or ResFe-2 (lower) and either BPS, media control, or Fe(II). c) Quantification of b.
References


(2) Beard, J. L. ASNS. 2001, 131(2), 568S-580S.


Appendix B

Investigation of methionine hotspots within fractionated subcellular organelles derived from mouse liver

Portions of this work were performed in collaboration with the following people:

Livers were excised and perfused by Tong Xiao. Synthesis of Ox32-alkyne was performed by Steven Crossley. Portions of sample preparation and MS runs were performed by Angel Gonzalez-Valero.
Synopsis

The fractionation of a complex biological sample into multiple, less complex ones allows for enhanced and broader coverage in the realm of proteomics.\(^1\) This is commonly achieved by taking advantage of one of many pulldown techniques, for example after covalently labeling a sample with a biotin-containing molecule, these proteins are then pulled down and isolated using streptavidin resin.\(^2\) An additional method of fractionation is that of subcellular fractionation, where cells are separated into their subcellular organelles using either specialized kits or other fractionation methods. The combination of multiple fractionation techniques can allow for even more enhanced coverage and the identification of previously invisible modifications, in particular those of low abundance proteins.\(^3,4\) In this work, we utilize a published protocol to isolate five unique subcellular organelles from mouse liver tissue.\(^5\) These fractionated samples in turn were then subjected to isoTOP-ABPP with Ox32-alkyne to identify hyperreactive methionines within each organelle.
**Materials and Methods**

Fresh livers were perfused and acquired from Tong Xiao. All buffers were prepared and chilled 24-28 hours prior to use. Ox32-alkyne was synthesized by Steven Crossley.

**Subcellular fractionation of organelles from mouse liver.** Protocol adapted from previous work, written with Angel Gonzalez-Valero.\(^5,6\) Yields nucleus, mitochondria, plasma membrane, plasma membrane-associated organelles, endoplasmic reticulum, and cytosol with high purity from mouse liver tissue using density-medium fractionation.

**Buffers**

- Starting Buffer (SB) – 225 mM mannitol, 75 mM sucrose, 30 mM Tris-HCl pH 7.4
- Isolation Buffer (IB) – 225 mM mannitol, 75 mM sucrose, 0.5% (w/v) BSA, 0.5 mM EGTA, 30 mM Tris-HCl pH 7.4
- Nuclear Isolation Buffer 1 (NIB-1) – 300 mM sucrose, 50 mM Tris-HCl, 3 mM MgCl\(_2\) pH 7.4
- Nuclear Isolation Buffer 2 (NIB-2) – 1.98 M sucrose, 50 mM Tris-HCl, 1 mM MgCl\(_2\) pH 7.4
- Mitochondrial Isolation Buffer (MIB) – 250 mM sucrose, 10 mM HEPES-KOH, pH 7.2
- Plasma membrane resuspension buffer (PMRB) – 5 mM Bis-Tris, 0.2 mM EDTA pH 6.0
- Phosphate Buffered Saline (PBS)

**Tissue Homogenization**

1. Mice were raised and bred following AUP-2019-04-12038 approved by UC Berkeley ACUC. Strain C57BL/6J mice were used for the experiments. All mice were male and 6-8 months old at time of extraction.
2. Starve the mice overnight before tissue extraction. Sacrifice mice via cervical dislocation. Extract mice liver & immediately place in ice-cold SB. Perfuse liver to purge blood cells.
3. Obtain 9-10 g of wet tissue (2-3 mouse livers) & mince in ice-cold SB.
4. Transfer liver pieces to 40 mL glass Dounce homogenizer & add IB (4 mL/g tissue). Homogenize tissue (approx. 10-15 strokes).
   a. Homogenization & **all subsequent steps including centrifugations must be performed at 4°C**
5. Transfer the homogenate to a 50 mL conical tube & centrifuge at 1000 xg for 10 mins using FIBERLite F14-6x250 LE rotor. Eliminate upper fatty layer (if any). Transfer the supernatant (SN1) to a fresh 50 mL conical tube & keep both the pellet (P1, containing nuclei, cellular debris) & supernatant (SN1, containing cytosol, mitochondria, plasma membrane [PM], & endoplasmic reticulum [ER, microsomes]).
1. Precool ultracentrifuge & Beckman SW41 rotor to 4 °C
2. Gently resuspend pellet containing nuclei, cellular debris (P1) in 15 mL NIB-1. Load 10 mL of NIB-2 into six 13.2 mL polyallomer ultracentrifugation tubes. Carefully layer 3 mL of resuspension on top of the NIB-2 layer.
3. Obtain the mass of the six tubes & adjust the mass using NIB-1 until they match (within 0.01 g).
4. Ultracentrifuge at 70,900 xg for 90 mins using Beckman SW41 rotor (accel = 5, decel = 5)
   a. While this is spinning, can perform steps 6-11 to save time.
5. Carefully remove upper NIB-1 layer & band at interface containing crude membranous products. Discard remaining NIB-2 layer & gently resuspend pellet containing purified nuclei (P2) in 250 µL ice-cold PBS.
   a. Quantify nuclei concentration via Bradford assay & dilute to 2 mg/mL for proteomic/WB analysis. Flash freeze in liquid N2. Store at -80 °C.

**Mitochondria**
6. Transfer the supernatant (SN1) to a 50 mL conical tube & centrifuge at 1000 xg for 10 mins using FIBERLite F14-6x250 LE rotor. Discard pellet, collect supernatant (SN2) in a clean 50 mL conical.
7. Centrifuge supernatant containing cytosol, mitochondria, PM, & ER (SN2) at 10,000 xg for 10 mins using FIBERLite F14-6x250 LE rotor.
8. Transfer supernatant containing cytosol, PM, & ER (SN3) to fresh 50 mL conical tube. Resuspend pellet containing crude mitochondria (P3) in 10 mL of MIB.
9. Centrifuge mitochondrial resuspension (P3) at 10,000 xg for 10 mins using FIBERLite F14-6x250 LE rotor.
10. Discard mitochondrial supernatant. Resuspend mitochondrial pellet (P4) in 10 mL of MIB. Centrifuge mitochondrial resuspension at 10,000 xg for 10 mins using FIBERLite F14-6x250 LE rotor.
11. Discard mitochondrial supernatant. Gently resuspend purified mitochondria pellet (P5) in 1.5 mL PBS.
   a. Quantify mitochondria concentration via Bradford assay & dilute to 2 mg/mL for proteomic/WB analysis. Flash freeze & store at -80 °C.

**Cytosol, PM, & ER**
12. Centrifuge supernatant containing cytosol, PM, & ER (SN3) at 10,000 xg for 10 mins using FIBERLite F14-6x250 LE rotor.
13. Discard pellet containing mitochondrial contamination. Transfer supernatant containing cytosol, PM, & ER (SN4) to 13.2 mL polyallomer ultracentrifugation tubes.
14. Ultracentrifuge at 25,000 xg for 20 mins using Beckman 70Ti rotor. Transfer supernatant containing cytosol & ER (SN5) to fresh 50 mL conical tube. Resuspend pellet containing crude PM (P6) in 15 mL SB.
15. Ultracentrifuge resuspended pellet (P6) (again) at 25,000 xg for 20 mins using Beckman 70Ti rotor. Discard supernatant containing cytosol & ER contaminants. Resuspend pellet containing crude PM (P7) in 1.5 mL PMRB.

16. Create a discontinuous sucrose gradient. Start by adding 3 mL of 53% (w/w) sucrose gradient solution to a 13.2 mL polyallomer ultracentrifuge tube.

17. Layer 4 mL of 43% (w/w) sucrose gradient solution on top of 3 mL 53% (w/w) sucrose gradient solution.

18. Layer 4 mL of 38% (w/w) sucrose gradient solution on top of 4 mL 43% (w/w) sucrose gradient solution.

19. Layer suspension of crude PM (P7) on top of 4 mL 38% (w/w) sucrose gradient solution. Carefully layer ~ 1 mL PMRB to fill tube.

20. Transfer supernatant from step 14 (SN5) to a 13.2 mL polyallomer ultracentrifuge tube for cytosol and ER fractionation.

21. Ultracentrifuge both tubes for 2.5 H at 95,000 xgs using Beckman SW41 rotor (accel = 5, decel = 5).

22. From the discontinuous sucrose gradient, carefully remove the upper band containing plasma membrane-associating membranes (PAMs) at the top of the 38% (w/w) sucrose gradient solution using a Pasteur pipette and transfer to a fresh 50 mL conical. Discard the middle band containing mitochondrial contamination at the 38/43% (w/w) interface. Remove the band at the 43/53% (w/w) interface containing PM and transfer to a fresh 50 mL conical. Dilute the PAM and PM fractions in 10 mL SB.

23. From the cytosol & ER tube, collect 1.5 mL of supernatant for purified cytosol (SN6). Discard remaining supernatant and resuspend pellet containing ER (P8) in 10 mL SB.

   a. Quantify cytosol concentration via Bradford assay & dilute to 2 mg/mL for proteomic/WB analysis. Flash freeze & store at -80 °C.

24. Centrifuge the PAM and PM suspension at 10,000 xg for 10 mins using FIBERLite F14-6x250 LE rotor. Discard pellet containing mitochondrial contamination (if any).

25. Transfer PAM (SN7) and PM (SN8) supernatants to 13.2 mL polycarbonate ultracentrifuge tubes. Transfer ER resuspension (P8) to 13.2 mL polycarbonate tube.

26. Ultracentrifuge tubes for 60 mins at 95,000 xgs using Beckman SW41 rotor (accel = 5, decel = 5).

27. Discard all supernatants. Resuspend PAM (P9), PM (P10), and ER (P11) pellet in 50 µL, 50 µL, and 500 µL of PBS, respectively.

   a. Quantify PAM, PM, and ER concentration via Bradford assay & dilute to 2 mg/mL for proteomic/WB analysis. Flash freeze & store at -80 °C.
**Assessing fraction purity.** Fraction purity was assessed in two manners: (1) by western blot for proteins known to be overexpressed in particular organelles, and (2) by shotgun proteomics followed by analysis sorting proteins seen by organelle localization.

**Western blot**
1. Load 25 µg protein onto a 12.5% SDS-PAGE gel and run at 150 V for 80 mins.
2. Electrotransfer proteins to a PVDF membrane at 25 V, 2.5 A for 10 mins.
3. Block non-specific antibody binding by incubating membranes in TBST + 5% milk for 1 H.
4. Wash membranes 1x in TBST for 5 mins.
5. Incubate with appropriate antibody overnight at 4 °C.
   a. Rabbit anti-GAPDH (1:1000 TBST + 5% BSA) [Cytosolic marker, 37 kDa]
   b. Rabbit anti-CALR (1:1000 TBST + 5% milk) [ER marker, 55 kDa]
   c. Rabbit anti-Histone H3 (1:2000 TBST + 5% milk) [Nuclear marker, 17 kDa]
   d. Rabbit anti-ATPIF1 (1:1000 TBST + 5% BSA) [Mito marker, 12 kDa]
   e. Rabbit anti-TRPC3 (1:1000 TBST + 5% BSA) [PM/PAM marker, 105 kDa]
6. Wash membranes 3x with TBST for 5 mins.
7. Incubate membranes with anti-rabbit IgG HRP conjugated secondary (1:300 TBST) for 2 H at 23 °C.
8. Quickly wash membranes 3x with TBST and image with ECL western blotting substrates.

**Shotgun proteomics**
1. 10 µg of protein was diluted to a final volume of 100 µL with PBS.
2. Protein was precipitated via addition of 900 µL MeOH at -80 °C overnight.
3. The next day, sample was spun at max speed at 4 °C for 10 min. The pellet was gently washed 3 times with a solution of ice cold MeOH.
4. The supernatant was then removed, and the pellet resuspended in 30 µL freshly prepared 8 M urea/PBS. A 5X stock of ProteaseMAX (Promega; V2071) was prepared by dissolved the pellet in 100 µL ammonium bicarbonate.
5. To the protein mixture was added 30 µL 1X ProteaseMAX, 40 µL ammonium bicarbonate, and 10 µL of 110 mM freshly prepared TCEP (Pierce; 20490). The sample was then incubated at 60 °C for 30 min.
6. To the sample was then added 2.5 µL of 500 mM freshly prepared iodoacetamide (Sigma Aldrich; I1149), and the sample was incubated protected from light at 23 °C for 30 min.
7. 120 µL of PBS was then added, followed by 1.2 µL 5X ProteaseMAX. The sample was vortexed thoroughly.
8. Sequencing grade Trypsin/Lys-C mix (Promega; V5071) was reconstituted in 40 µL trypsin buffer, and 4 µL was added to the sample. The sample was allowed to digest at 37 °C overnight.
9. The next day the sample was acidified with 12 µL formic acid and spun at max speed for 30 min.
10. The supernatant was taken to a low-adhesion tube and stored at -80 °C until MS analysis.
11. After MS analysis, Subcellular content was determined using subcellular location data stored on UniprotKB and processed with an in-house python script written by Angel Gonzalez-Valero. The data were read and manipulated using the Python Data Analysis Library Pandas. Data were then filtered using textual patterns that relate to subcellular localization via regex. The data were then exported to Excel for subsequent analysis.

**Mass spec analysis.** Peptides from all experiments were analyzed on a Q Exactive Plus mass spectrometer (ThermoFisher Scientific); pure proteins were analyzed on a C18 only column, while complex proteomes were analyzed via a five step Multidimensional Protein Identification Technology (MudPIT).

1. Inlines (IDEX; M-520) were fitted with ~20 cm of 250 µm tubing and prepacked with 4 cm of Aqua C18 reverse-phase resin (Phenomenex; 04A-4299).
2. Columns were made from 100 µm tubing pulled with a P-2000 laser (Sutter Instruments Co.) and packed with either 13 cm of Aqua C18 reverse-phase resin (pure proteins) or packed first with 10 cm of Aqua C18 reverse-phase resin followed by 3 cm of strong-cation exchange resin (Phenomenex; 04A-4398; MudPIT).
3. Both inlines and columns were equilibrated after packing prior to use on an Agilent 1260 HiP AIS coupled to an Agilent 1260 Quat Pump using the following gradient: 100% buffer A to 100% buffer B over 10 min, hold at buffer B for 5 min, finish with a wash with 100% buffer A over 15 min (where buffer A is 95:5 H2O:MeCN/0.1% formic acid, and buffer B is 80:20 H2O:MeCN/0.1% formic acid). For best performance, columns were equilibrated the day of sample run.
4. Peptides were pressure-loaded onto an equilibrated inline until dry. The tubing was cut to ~2 cm above the resin bed and an appropriate equilibrated column was attached to the opposite end of the inline. The sample was attached to the LC-MS/MS via a MicroTee PEEK 360µm fitting (ThermoFisher Scientific; p-888) and the column tip aligned with the MS source opening.
5. Data was collected in positive-ion mode using data-dependent acquisition mode with dynamic exclusion enabled (60 s) between 400 and 1800 m/z and a mass resolution of 70,000, with one MS1 scan followed by 15 MS2 scans of the nth most
abundant ions. Nanospray voltage was set at 2.75 kV and heated capillary temperature at 200 °C. The MudPIT program utilized for all samples consists of five separate programs run sequentially, where each begins with either 0, 25, 50, 80, or 100% salt bump (buffer C, 500 mM ammonium acetate/H2O) followed by a gradient of 5-55% buffer B in buffer A. Pure proteins were run on only the first program (0% salt bump) from the MudPIT program. The flow was kept at 0.1 mL/min throughout.

6. Data were analyzed with Byologic (Protein Metrics Inc.). Raw files were searched directly against the Uniprot mouse database using the Byos HCP workflow, with decoys and common contaminants added. Peptides were assumed fully tryptic. All searches included the following modifications: Acetyl (+42.010565; Protein N-term; variable - rare1), carbamidomethyl (+57.021464; C; Fixed), and oxidation (+15.994915; M; variable - common1).

**isoTOP-ABPP for the assessment of hyperreactive methionines.** isoTOP-ABPP protocol adapted from literature. Residues with low ratios were presumed hyperreactive, while those with high ratios were presumed to be of low reactivity. A cutoff ratio of 2.5 was chosen to delineate between low and high reactivity and was chosen based on similar work by Weerapana and coworkers. Both light and heavy photocleavable biotin azide were purchased from Kerafast.

1. Dilute the desired proteome sample to a 2 mg/mL solution in PBS (total of 2 mL/sample)
   - If using cells: lyse with sonication and then spin at 1000 g/3000 rpm to remove debris
2. Add 500 µL of proteome solution (2 mg/mL) to 4 1.5 mL epis each (4 mg for one MS sample)
3. Add 5 µL of Oxs32-alkyne stock in DMSO to each sample for 500 µM final. After addition of the probe, vortex and incubate the reaction in RT for one hour.
4. During the incubation: Prepare a fresh solution of 50 mM TCEP in water (14.4 mg/ml), chill MeOH on ice.
5. To 2 epis, add 5 µL of the light 50 mM photocleavable biotin-azide (final 500 µM) and vortex. Add 5 µL of the heavy 50 mM photocleavable biotin-azide to the remaining 2 epis.
6. Add 10 µL of fresh 50 mM TCEP solution.
7. Add 30 µL of TBTA ligand solution and vortex (0.9 mg/mL in DMSO:t-butanol=1:4).
8. Add 10 µL of 50 mM Copper (II) Sulfate (12.5 mg/mL in water) and vortex.
9. Incubate the reaction at RT for one hour. At this stage, the proteins will start to precipitate, and the solution will turn cloudy.
10. Combine the tubes pairwise and centrifuge for 4 min at 6500 g. A protein pellet will form.
   ✓ Step 11-13 should be done at 4 °C or on ice
11. Remove the supernatant, add 500 µL cold methanol to each tube and sonicate for several seconds until pellet goes into solution completely. Combine tubes again into a 2 mL epi (start with 4 tubes and end with 1 tube).
12. Centrifuge tubes for 4 min at 6500 g at 4 °C.
13. Remove the supernatant and repeat the wash (steps 11 and 12) with 500 µl cold methanol.
14. Remove the supernatant, add 1 mL 1.2% SDS/PBS (w/v), sonicate for several seconds till the solution turns clear and heat to 90 °C for 5 min. Then, centrifuge for 5 min at 6500 g.
15. Take out the high-capacity streptavidin agarose beads slurry from 4 °C fridge and resuspend the slurry by rotating the bottle. Aliquot 170 µL slurry per sample.
16. Wash beads in the vacuum manifold with 1 mL PBS three times, then resuspend beads in the original volume taken from the avidin-agarose bottle.
17. Transfer roughly 200 µL washed beads into 15 mL conicals containing 5mL PBS (just try to get an equivalent volume of beads in each conical).
18. Transfer the 1 mL sample to the 15 mL conical tube which already contains 5 mL PBS and beads. The final concentration of SDS in the sample is 0.2%.
19. Incubate the labeled proteome sample with the beads in 15 mL conical tube on a rotator overnight at 4 C.
20. The next day, let the tubes rock at rt 1 h to warm them up.
21. Centrifuge the conical tubes at 1400 g for 3 min and remove the supernatant.
22. Wash the beads by adding 5 mL of 0.2% SDS/PBS (w/v), place on a rotator for 10 min, then spin at 1400 g for 3 min and remove the supernatant.
23. Transfer beads to Micro Bio-Spin column using two washes of 500 µL PBS.
24. Wash beads on vacuum manifold using three 1 mL washes of PBS.
25. Wash beads on vacuum manifold using three 1 mL washes with water.
26. Transfer the washed beads to epis using two 250 µL washes of fresh 6M Urea/PBS (1.8 g/5 mL).
27. Add 25 µL fresh-made TCEP solution (28 mg/mL in water = 20 mM) to each tube.
28. Incubate the tube at 65 °C for 20 min (avoid vortexing).
29. Cool the tube for several seconds and add 25 µL fresh-made 400 mM IA solution (74 mg/mL in water) to each tube. Incubate with agitation at 37 °C for 30 min.
30. Dilute the reaction by adding 950 µL PBS to each tube, centrifuge at 1400 g for 2 min and remove the supernatant.
31. Add a pre-mixed solution of 200 µL of 2M Urea/PBS, 2 µL of 100 mM Calcium Chloride (14.7 mg/mL) in water and 4 µL of Trypsin solution (20 µg lyophilized powder reconstituted in 40 uL Trypsin Buffer) to each tube.
32. Incubate the reaction at 37 °C incubate over-night in the shaking incubator.
33. The next day, transfer the supernatant and beads to a Bio-Spin column, elute supernatant using vacuum manifold.
34. Wash the beads with 3X 500 µL PBS and then 3X 500 µL water.
35. Transfer the beads with 300 µL water into a strip of PCR tubes, approximately 100 µL per tube.
36. Incubate beads under a 365 nm lamp at 500 mW/cm² for 30 min. Place samples 1-3 cm from lamp.
37. Transfer the supernatant and beads to a Bio-Spin column and elute into a low adhesion epi.
38. Wash the beads with 2 x 75 µL of water and combine the washings with the eluent.
39. Add 16 µL formic acid.
40. Store samples at -80 °C until mass spectrometric analysis.

Synthesis of Ox32-alkyne. Ox32-alkyne was synthesized by Steven Crossley.

A.B.1 Synthesis. To a flask unprotected from atmosphere was added 2-amino-2-methyl-1-propanol (75 mmol), Boc₂O (75 mmol), and EtN(Pr)₂ (82.5 mmol) to dry CH₂Cl₂ (37.5 mL). Reaction began as a white suspension, turned clear, and ended as a pale yellow. Reaction was stirred at room temperature overnight. The next day, the reaction was quenched with 40 mL H₂O and extracted with EtOAc, dried over MgSO₄, filtered, and concentrated. ¹H NMR (400 MHz, CDCl₃) δ 3.72 (s, 2H), δ 1.48 (s, 9H), δ 1.45 (s, 6H).

A.B.2 Synthesis. To a flask protected from atmosphere were added A.B.1. (75 mmol), KOH (150 eq), Et₄NI (7.5 mmol), NaI (7.5 mmol), and propargyl bromide (90 mmol) in THF (188 mL). The mixture yielded a yellow suspension reminiscent of orange juice, which was stirred rigorously at room temperature overnight. The next morning, the reaction was quenched with the addition of 190 mL H₂O and extracted 3 times with EtOAc, dried over MgSO₄, filtered, and concentrated. The residue was purified by column chromatography. ¹H NMR (400 MHz, CDCl₃) δ 4.09 (s, 2H), δ 3.51 (s, 2H), δ 2.49 (s, 1H), δ 1.46 (s, 9H), δ 1.35 (s, 6H).

A.B.3 Synthesis. To a flask protected from atmosphere were added A.B.2. (52.2 mmol) and 4 M HCl in dioxane (114.84 mmol) in dry MeOH (104 mL). The mixture was stirred at room temperature overnight. The solvent was then removed in vacuo and the solid suspended in Et₂O and solute filtered off. Off-white, crystalline needles were obtained and confirmed as the desired product. ¹H NMR (400 MHz, CDCl₃) δ 4.18 (s, 2H), δ 3.50 (s, 2H), δ 2.99 (s, 1H), δ 1.20 (s, 6H).

A.B.4 Synthesis. To a flask protected from atmosphere were added A.B.3 (47 mmol), KOCN (187 mmol), and water (50 mL). Mixture was brought to 60 °C, upon which it turned a clear yellow color. The mixture was stirred overnight. The next morning, the solution had turned opaque, and was cooled to room temperature prior to extraction with EtOAc and dried over Na₂SO₄. Product confirmed by TLC only.
**Ox32-alkyne Synthesis.** To a flask protected from atmosphere were added A.B.4. (4.37 mmol), PhCHO (5.24 mmol), and Ti(OPr)$_4$ (6.12 mmol) in dry THF (13.2 mL). Solution was a clear yellow color. The mixture was stirred at room temperature 23 h. The solvent was removed *in vacuo* to afford crude imine which was used immediately in the next step. The reaction was controlled by $^1$H NMR via appearance of the imine proton signal intensity.

A solution of mCPBA (13.11 mmol) in a 1:1 saturated K$_2$CO$_3$ (H$_2$O) : CH$_2$Cl$_2$ mixture (35 mL) was allowed to stir rapidly at room temperature for ten minutes. A suspension formed. A solution of imine in CH$_2$Cl$_2$ from the previous step was added dropwise, upon which the white emulsion turned yellow. After 2 h, the reaction was diluted with water (150 mL) and extracted with CH$_2$Cl$_2$ (3 x 100 mL). The organic layer was separated, washed with diluted aq. K$_2$CO$_3$ (3 times) and water (2 times), dried over Na$_2$SO$_4$, filtered, and concentrated *in vacuo*. The residue was purified by column chromatography. $^1$H NMR (400 MHz, CDCl$_3$) δ 7.43 (m, $J = 7.44$ Hz, 5H), δ 6.21 (s, 1H), δ 4.98 (s, 1H), δ 3.58 (d, $J = 8.9$ Hz, 1H), δ 3.48 (d, $J = 8.6$ Hz, 1H), δ 2.48 (s, 1H), δ 1.57 (s, 3H), δ 1.39 (s, 3H).
Results and Discussion

Isolation and purity assessment of fractionated subcellular organelles. Numerous sucrose-based fractionation protocols are available for the fractionation of a variety of subcellular organelles. By combining two such protocols, we were able to design a protocol that yielded the isolation of six unique subcellular organelles: nucleus, cytosol, microsomes, mitochondria, plasma membrane, and plasma-membrane associated membranes (Figure A.B.1).5,6 Certain steps required careful layering of multiple sucrose solutions to generate a differential sucrose gradient followed by a high-speed spin in a swinging bucket rotor. When performed correctly, this step yielded visible bands at each interface at the conclusion of the spin (Figure A.B.2). From 9 g of liver, we were able to isolate the following: 140 mg of cytosol, 14 mg of microsomes, 26 mg of mitochondria, 450 µg of plasma membrane, 25 µg of plasma-membrane associated membranes, and 850 mg of nucleus. The low amount of plasma-membrane associated membrane prevented us from performing analyses past its purity assessment.

Fractionation purity was assessed using two methods: (1) western blot analysis for marker proteins, and (2) shotgun proteomics followed by localization analysis of identified proteins. The following proteins were utilized in the western blot analysis: anti-GAPDH (cytosolic marker), anti-CALR (ER marker), anti-Histone H3 (nuclear marker), anti-ATPIF1 (mitochondrial marker), and anti-TRPC3 (PM/PAM marker). Analysis of the blots demonstrates that certain organelles were better enriched than others (Figure A.B.3). For instance, the cytosolic fraction returned only a single band across all blots for its respective marker, GAPDH. Compare this to the mitochondrial fraction which although enriched across all fractions the most strongly for its respective marker, ATPIF1, also displays bands across all other organelle markers. Assessing the fractionations in this manner, in order from most to least pure we observed plasma membrane, cytosol, plasma-membrane associated membrane, microsome (ER), nucleus, and mitochondria.

For purity assessment by shotgun proteomics, each sample was prepared for a MudPIT run on the hybrid quadropole-orbitrap mass spectrometer. After runs were analyzed for peptides observed via Byos, subcellular localization data was mined from Uniprot and processed with an in-house python script (written by Angel Gonzalez-Valero) to remove duplicates and further sort for analysis. This process was repeated with a sample of total liver homogenate that was subjected to the same protocol, as well as a published data set of proteins known in the mouse proteome derived from Uniprot (Figure A.B.4). The resulting analysis shows a slight enrichment in all fractionated organelles samples in comparison to the total liver homogenate sample. In particular, the cytosol and microsomes (ER) appear especially enriched. This aligns with the purity assessment via western blot, where the cytosol and microsome samples were among the most pure (Figure A.B.3). In the same vein, the nucleus and mitochondria remain among the lowest purity via shotgun proteomics, matching what was observed by western blot. Interestingly, the whole mouse proteome data collected directly from Uniprot and processed with our script yielded vastly different trends compared to the samples run on the mass spec.
Many factors could have attributed to this trend, though one of the most likely is that this dataset reflects proteins across the entire organism, while we worked solely with liver lysate.

Further analysis of the shotgun proteomics data collected both for the total liver homogenate and for the organelles confirmed the improvement in coverage that we had initially sought out. Consider the comparison between the total liver homogenate, cytosolic, and mitochondrial fractionations (Figure A.B.5). The total liver homogenate sample yielded 1,141 unique modified residues, the cytosolic sample 761, and the mitochondrial 107. Combining the cytosol and mitochondrial samples, these two fractionations increased the unique observed modified residues observed by approximately 30% compared to the homogenate alone. This dramatic increase validates the use of additional fractionation techniques to improve coverage in the realm of discovery chemoproteomics.

**isoTOP-ABPP for the identification of hyperreactive methionines.** With isolated subcellular fractionations in hand, we next turned to isotopic Tandem Orthogonal Proteolysis - Activity-Based Protein Profiling (iso-TOP ABPP) to identify which residues were of particular interest due to hyperreactivity. The methionine-reactive probe Ox32-alkyne was utilized to target such residues and was synthesized by Steven Crossley (Scheme A.B.1). Briefly, protein samples were divided in half, half treated with 50 µM of Ox32-alkyne (low dose), and the remaining half treated with 500 µM (high dose). The low dose was then labeled with a heavy-labeled azide, and the high dose with a light-labeled azide, after which samples were combined and prepared for mass spec proteomics. During analysis, residues that were (1) labeled with both a light and heavy modifier, (2) coeluted, and (3) yielded a light:heavy ratio below 2.5 were further investigated as potentially hyperreactive methionines.

One such protein, valacyclovir hydrolase (BPHL), arose from the mitochondrial fractionated sample with a light:heavy ratio of 1.47 (Figure A.B.6a). BPHL is a serine hydrolase primarily localized in the mitochondria and highly expressed in the liver and kidney. It plays a regulatory role in homocysteine metabolism and is involved in nucleoside prodrug activation. We observed a hyperreactive methionine at M52 of BPHL, which lies adjacent to the protein’s catalytic triad (Figure A.B.6b). Additionally, further analysis via Adaptive Poisson-Boltzmann Solver (APBS) revealed a highly negative electrostatic potential in this region (Figure A.B.6c). With the large difference in electrostatics between methionine and methionine sulfoxide, it is thus feasible that the oxidation status of M52 of BPHL could play a significant role in the accessibility of the protein’s catalytic triad, and thus play a role in regulating its activity.
Conclusion

Differential centrifugation is a powerful tool for subcellular organelle isolation. By combining established protocols, we successfully isolated six unique organelles - nucleus, cytosol, microsomes, mitochondria, plasma membrane, and plasma-membrane associated membranes - from a single sample of liver homogenate. The purity of each sample was assessed and demonstrated enrichment for the respective organelle. Comparison of shotgun proteomics runs of each organelle to a run of the initial liver homogenate revealed an approximately 30% increase in unique modified residues, validating this route as an effective method to improve proteomics coverage within complex protein samples. Further fractionation via isoTOP-ABPP allowed simultaneously for enhanced coverage as well as a ranking of methionine residues by their reactivity. Through this combination of fractionation methods, M52 of BPHL was identified as a potentially hyperreactive methionine. Investigation into the role this residue plays in native BPHL activity remains ongoing.
Figure A.B.1. Visualization of subcellular fractionation protocol, identifying how nucleus, mitochondria, endoplasmic reticula, plasma membrane, cytosol, and plasma-membrane associated membranes were isolated from a single homogenate derived from fresh rat liver.
Figure A.B.2. Image of discontinuous sucrose gradient containing separated plasma membrane, contaminating mitochondria, and plasma-membrane associated membranes (PAMs) corresponding to step 22 of the subcellular fractionation protocol.
Figure A.B.3. Assessment of purity of organelles by western blot. Horizontal labels correspond to isolated subcellular organelles at the completion of the fractionation protocol, where vertical labels correspond to antibodies of each blot and which organelle they respectively are utilized as a marker for.
Figure A.B.4. Assessment of organelle purity by shotgun proteomics. Subcellular protein percentages derived from mouse proteome (UniprotKB; UP000000589) and shotgun proteomics data from mouse liver homogenate and purified mouse liver fractions. Total MS-identified proteins and total proteins in the reference proteome are color-coded and labeled. Subcellular content was determined using subcellular location data stored on UniprotKB and processed with an in-house python script written by Angel Gonzalez-Valero. The data were read and manipulated using the Python Data Analysis Library Pandas. Data were then filtered using textual patterns that relate to subcellular localization via regex. The data were then exported to Excel for subsequent analysis.
Figure A.B.5. Comparison of unique residues observed between shotgun proteomics runs of total liver homogenate and isolated cytosolic and mitochondrial fractions.
Valacyclovir hydrolase (BPHL) contains a hyperreactive methionine residue. (a) MS2 spectra for the low dose- and high dose- treated mitochondrial fractionation samples demonstrating modification at M52 of BPHL for each. Peaks reflect ratio of XIC curves of coeluted BPHL peptides modified at M52 by Ox32-alkyne. A ratio of 1.47 light:heavy was observed, well below the 2.5 cutoff chosen to differentiate high-reactivity methionines from low-reactivity. (b) Highlighted on structure 2OCG, M52 of BPHL lies directly adjacent to the catalytic triad. (c) Electrostatic potential map of BPHL calculated via Adaptive Poisson-Boltzmann Solver (APBS). A highly negative electrostatic potential is observed in the region containing the catalytic triad/M52.
References

(1) Lee, Y. H.; Tan, H. T.; Chung, M. C. M. *PROTEOMICS* 2010, 10 (22), 3935–3956.


Appendix C

Q Exactive Plus maintenance, use, and troubleshooting
Synopsis

This appendix is provided as an overview of maintenance, use, and troubleshooting of the C. Chang lab Q Exactive Plus instrument. Many portions have been written with the intent to run samples via mudPIT, requiring an atypical set-up involving a custom-made stage and inline. These have been specified and will thus deviate from standard operating protocol of the instrument.
**Instrument Overview**

**Q Exactive Plus.** Offered by ThermoFisher, the Q Exactive Plus is a hybrid quadropole-orbitrap mass spectrometer with a detection range between 50 and 6000 m/z. It is especially powerful in the identification of modifications of specific amino acids within complex lysate mixtures. The ion source is an electrospray ionization (ESI) source, which the lab has connected to a custom-made stage, which in turn is connected to an Agilent LC (1260 series) and autosampler (1260 series). Our lab utilizes the Q Exactive Plus primarily for chemoproteomics experiments, running samples in positive mode and data-dependent acquisition (DDA).

**Pressure Cell.** The pressure cell, also referred to as the “bomb loader”, is utilized to both pack inlines and columns with resin and to load samples onto the packed inlines. The unit is connected to helium gas. Slurries of C18 resin and strong cation exchange (SCX) resin in methanol are loaded into glass tubing via the pressure cell.

**Column tip puller.** A Sutter P-2000 laser-based micropipette-puller is utilized in the pulling of columns from 100 µM tubing. The P-2000 utilizes a CO₂ laser-based heat source to generate column tips amenable for nanospray MS.
Experiment Overview

First reported by the Yates lab, Multidimensional Protein Identification Technology, or MudPIT, is a powerful technique allowing for on-line separation of peptides directly into a MS source.\textsuperscript{1} In order to utilize this technique in conjunction with the Q Exactive Plus, we ordered a custom stage built to allow for the attachment of the mudPIT set-up between the LC connection and open ion source. Pure proteins are analyzed on a C18 only column, while complex proteomes are analyzed via MudPIT.
Protocols

How to pack an inline. Inlines are packed using the pressure cell with 4 cm of C18 resin using 250 µM silicon-glass tubing.

1. Put together an inline by screwing 2 beige connectors fitted with green sleeves and a clean filter onto the body of the inline.
2. Cut approximately 25 cm of 250 µM tubing. Thread the tubing through the green sleeve of the beige connector that is connected to the filter. Reconnect the fitting and ensure that the tubing is snugly in place.
3. Prepare a slurry of C18 in methanol in an Eppendorf tube by adding approximately 10 mg of C18 in 150 µL methanol. The slurry should be opaque. Cut the lid of the Eppendorf tube off.
   a. Note: If you are reusing a slurry, check to make sure that it is clear of debris that could have been introduced by the tubing (bits of orange and/or black). If these are visible, prepare a fresh solution.
4. Shake the slurry to mix, then place it into the pressure cell and quickly seal the pressure cell. Thread the inline tubing that’s connected to the inline body into the pressure cell opening. You should be able to feel the bottom of the Eppendorf tube containing the slurry - when you’ve reaching this depth, raise the tubing about a centimeter.
5. Open the pressure cell to the pressurized gas.
   a. Note: When the pressurized gas is on, always keep a grasp on the tubing to ensure it does not eject from the pressure cell.
   b. You may need to tighten the nut that contains the opening for the inline tubing. Tighten this until you do not hear any escaping gas.
6. Pack resin until at least 4 cm of C18 is carried over to the inline body.
   a. Measure 4 cm from the bottom of the filter to the top of the packed resin.
   b. Note: If you have worked quickly enough and the slurry is still suspended, this should happen almost instantaneously.
   c. Note: If you spot any leaks at the green sleeves or body of the inline (other than through the exit green sleeve), address these leaks before further packing. Try replacing with a new green sleeve or using a new filter or beige connector.
7. When you have packed the proper amount of C18, lift the tubing out of the Eppendorf and into the portion of the pressure cell where it is no longer suspended in the slurry. Wait until the resin that has packed into the inline has dried completely.
8. Slowly release the pressure of the pressure cell. To do this, turn the knob until you just start to hear the hiss of gas releasing. Pause, and wait for the sound to cease. Then turn the knob the rest of the way.
9. The inline is now ready to be equilibrated.
   a. If the inline was overpacked, unscrew the beige connector that contains the packed inline tubing from the inline body. Push the tubing (containing the
excess resin) through the beige connector until only the appropriate amount of resin is left. Cut the excess tubing and resin and reattach the remaining tubing to the inline body.

How to pull columns. Columns are prepared by pulling 100 µM tubing using a Sutter P-2000 laser-based micropipette-puller.

1. Cut pieces of 100 µM tubing, each 30 cm long. Each cut piece will make two columns.
2. Prepare a flame. In the center of each piece of tubing, burn away the outer plastic of the tubing. The burned section should be approximately 5 to 10 cm.
3. Wipe away the burned resin using a clean paper towel and methanol.
   a. Note: To prevent blowing a fuse, lift the lid before turning it on.
5. Thread the tubing through the slot in the center of the Sutter P-2000. This is where the laser will separate the tubing and generate two column tips. Therefore, this is where the burned section of the tubing should be centered.
6. Bring both outer movable pieces into the center. With your fingers holding the tubing completely straight and lined up in the ridges of the outer mechanisms, gently tighten the two wheels that are close to where you have the burned tubing centered. These will keep the tubing in place.
   a. When done correctly, the tubing should lay completely horizontal throughout the entire setup. If a portion is raised or drooping, it is likely that the P-2000 will recognize an improper setup and will not pull the tip.
7. Close the lid and hit start. If the setup is correct, the red light should blink three to four times before pulling the column with an audible snap.
   a. If the setup is incorrect, the red light will turn on but will not blink, and no snap will be heard. If this happens, press “stop” and try to fix your setup starting from step 5.
8. The columns are now ready to be packed. Store them such that the column tips do not touch anything.

How to pack a column. Columns are packed using the pressure cell with either (1) 13 cm of C18 resin (for pure protein runs) or (2) 10 cm of C18 resin followed by 3 cm of SCX resin (for MudPIT runs; complex protein) in a 100 µM column prepared using the Sutter P-2000.

1. Pick up a column that has been pulled via the Sutter P-2000. Ensure the tip is clean of burned residue, wiping with a kimwipe and methanol if needed.
2. Gently scribe the column tip against the table using the glass scribe.
   a. Note: Better to under scribe here than over scribe. The tip should still have a well-defined point. If it looks more rounded, you may have over scribed and will need to start over.
3. Prepare a slurry of C18 in methanol in an Eppendorf tube by adding approximately 10 mg of C18 in 150 µL methanol. The slurry should be opaque. Cut the lid of the Eppendorf tube off.
   a. Note: If you are reusing a slurry, check to make sure that it is clear of debris that could have been introduced by the tubing (bits of orange and/or black). If these are visible, prepare a fresh solution.
4. Shake the slurry to mix, then place it into the pressure cell and quickly seal the pressure cell. Thread the column into the pressure cell opening (with the column tip facing up). You should be able to feel the bottom of the Eppendorf tube containing the slurry - when you’ve reaching this depth, raise the tubing about a centimeter.
5. Open the pressure cell to the pressurized gas.
   a. Note: When the pressurized gas is on, always keep a grasp on the tubing to ensure it does not eject from the pressure cell.
   b. You may need to tighten the nut that contains the opening for the inline tubing. Tighten this until you do not hear any escaping gas.
6. Check that the column is appropriately scribed. A properly prepared column should immediately yield droplets of methanol out the tip of the column.
   a. If no droplets are visible, turn off the flow of gas into the pressure cell and gently scribe the column tip again.
   b. If a spray is visible that is reminiscent of a fountain, you have over scribed and will need to start fresh from step 1 with a new column.
7. Pack the column with C18. Packing should be slower than with the inline, but not unbearably so. If packing is taking longer than 5 minutes or so to load just this initial C18, grab a fresh column and restart from step 1.
   a. For a C18 only column, pack at least 13.5 cm of C18.
   b. For a MudPIT column, pack 9 to 11 cm of C18. If you pack more than 11 cm, you will unfortunately need to throw the column out and restart the protocol from step 1.
   c. To aid in packing, periodically tap the bottom of the column tubing at the bottom of the Eppendorf containing the slurry to pick up some resin. I’ve also found that letting the resin dry out and then placing it back into the slurry to aid with packing.
8. When you have packed the proper amount of C18, lift the tubing out of the Eppendorf and into the portion of the pressure cell where it is no longer suspended in the slurry. Wait until the resin that has packed into the column has dried completely.
9. Slowly release the pressure of the pressure cell. To do this, turn the knob until you just start to hear the hiss of gas releasing. Pause, and wait for the sound to cease. Then turn the knob the rest of the way.
   a. Note: If you are making a MudPIT column and the resin unpacks at this step, you will need to ensure it is properly packed prior to the next step. To
do this, add an Eppendorf tube full of methanol to the pressure cell to wet and then dry the packed column.

10. For a C18 only column: Cut the column to 13 cm from column tip to end. The column is now ready to be equilibrated.

11. For a MudPIT column, continue further with this protocol.

12. Prepare a slurry of SCX in methanol in an Eppendorf tube by adding approximately 10 mg of SCX in 150 µL methanol. The slurry should be opaque. Cut the lid of the Eppendorf tube off.

   a. **Note:** If you are reusing a slurry, check to make sure that it is clear of debris that could have been introduced by the tubing (bits of orange and/or black). If these are visible, prepare a fresh solution.

13. Shake the slurry to mix, then place it into the pressure cell and quickly seal the pressure cell. Thread the column (packed with C18) into the pressure cell opening (with the column tip facing up). You should be able to feel the bottom of the Eppendorf tube containing the slurry - when you’ve reaching this depth, raise the tubing about a centimeter.

14. Pack with SCX until you have packed at least 4 cm. For example, if you packed 9 cm of C18, pack enough SCX so that the column is packed to at least 13 cm. It is ok to over pack here.

15. When you have packed the proper amount of SCX, lift the tubing out of the Eppendorf and into the portion of the pressure cell where it is no longer suspended in the slurry. Wait until the resin that has packed into the inline has dried completely.

16. Slowly release the pressure of the pressure cell.

17. Cut the tubing so that it contains only 3 cm of SCX. For example, if you packed 10.5 cm of C18, your column’s final length should be 13.5 cm.

18. The column is now ready to be equilibrated.

**How to equilibrate inlines and columns.** Before beginning, ensure solvents A and B are at appropriate levels. To avoid damaging or clogging the T-joint, remove any added components before stopping the flow of solvent (this prevents back-flow into the T-joint) - this is most important when equilibrating columns. Do not over-tighten T-joints.

1. Attach your sample to the T-joint of the Q Exac.

2. Open the XCalibur program.
   a. Ensure all components of the system are set to “Ready to download”, “Standby”, or “Off”. If this is not the case, refer to “Troubleshooting Guide: Run won’t start”.

3. We’re going to flow some solvent through the line to make sure everything’s connected properly before starting the equilibration sequence.
   a. Open “Instrument Configuration”, then “Agilent 1260”, and “Direct Control”.
   b. Type “0.1” into the flow rate (with solvent A at 100%) and hit enter.
   c. Let the pressure rise until you can see solvent coming out the end of your inline/column.
d. If you don’t see solvent coming out the end of your sample, check the pressure (lower left of the screen). If it’s lower than 30, look for leaks. If it’s greater than 70, the T-joints might be clogged and need to be replaced (refer to “Troubleshooting Guide: Low signal”).

4. Once we’ve confirmed everything is flowing nicely, we’ll get ready to start the run. Close out of the Direct control and Instrument Configuration windows. It will ask if you want to save changes, say no. The solvent will still be flowing.

5. Enter “Sequence View Setup”. Open the equilibration file “equilibration_agr_2020”. The standard equilibration run is as follows: 100% buffer A to 100% buffer B over 10 min, hold at buffer B for 5 min, finish with a wash with 100% buffer A over 15 min (where buffer A is 95:5 H2O:MeCN/0.1% formic acid, and buffer B is 80:20 H2O:MeCN/0.1% formic acid).

   a. If you are equilibrating a column longer than 13 cm, your equilibration method will need to be appropriately altered to compensate for the difference in pressure.

6. Highlight the run and click Run Sequence. A new window will pop up. Click ok. It will probably ask if you want to save changes, say no.

7. You will know that the run has started because the run time clock will start counting up. The equilibration selected runs for 31 minutes.

8. When the run is completed, the solvent will still be flowing and the MS will be on. First, remove your sample from the T-joint. Then turn off the solvent flow (see step 3 – type in “0” instead of “0.1” and hit enter).

9. And finally, from the Tune program, ensure that the MS is set to “standby”.

How to load a sample onto an inline. Prior to loading your sample, ensure that you have (1) an equilibrated inline, and that your sample is (2) appropriately resuspended and (3) free from debris. These latter two points can be achieved by properly sonicating your sample in the appropriate buffer (such as 0.1% formic acid in MQ water), followed by a hard-spin at max speed for 30 min and transfer to a new low-adhesion Eppendorf tube.

1. Insert your sample into the pressure cell (you will need to cut off the lid to the epi). Set an empty Eppendorf tube into the tray next to it. This will catch the solvent as it flows through the inline so you can keep track of how much sample has loaded.

2. Seal the pressure cell. Ensure that it has closed correctly with the O-ring in place before sealing the system. Tighten the bolts to ensure the pressure cell is properly sealed.

3. Insert your equilibrated inline into the pressure cell. You should be able to easily insert the tubing to reach the bottom of the epi. If you can’t, loosen the nut a bit. Gently raise and lower the tubing to verify you have it resting at the bottom of your epi. You should be able to “feel” the bottom of the epi. This will ensure that all of your sample will carry over. The body of the inline should be resting in the empty Eppendorf tube.

4. Tighten the nut with the wrench. There’s a bit of a balance here – too loose and your tubing will fly out of the pressure cell, but too tight and your sample will have
difficulty carrying over and onto the inline. I find that once I tighten it to meet resistance, then tap gently a couple more times for good measure, that I’ve reached the optimal balance.

5. While holding onto the tubing, turn the knob 180 degrees to open the line to the pressurized helium. Holding the tubing will help save your inline if you haven’t tightened the nut enough back in step 4. If the nut has been tightened correctly and the system is properly sealed, the tubing should stay in place on its own and the pressure cell shouldn’t make any noise.
   a. If once you’ve pressurized the pressure cell you notice the tubing start to slide upward, grab the tubing between your fingers, slowly release the pressure in the system, and start over from step 6 (this time tightening the nut a bit tighter).
   b. If you hear hissing and you’re still holding onto your inline, try tightening the nut that the tubing of your sample goes through. You don’t need to depressurize for this. You’ll know it’s tightened the right amount when you can no longer hear hissing.

6. A successful setup will show liquid starting to come out the end of the inline. If you do not see this, you can try starting over from step 3, ensuring the tubing is touching the bottom of the Eppendorf tube and that the nut is not overtightened. You can now walk away from the pressure cell as your sample loads.

7. You will know that your sample has completed loading when you see bubbles rapidly forming in the collection Eppendorf tube. Once you have reached this point, it’s time to depressurize the pressure cell and remove your inline. To do so, slowly turn the knob until you can hear a faint hissing. Once you hear this hissing, STOP TURNING THE KNOB. The hissing will stop after about 5-10 seconds. Once the hissing has stopped, turn the knob a little further. If you hear more hissing, wait again for it to subside. Otherwise, you can turn the knob completely to off.
   a. If you quickly turn the knob to off without gently depressurizing the system, your sample will backflow through the tubing and unpack the resin. It’s not the end of the world if this happens, but does have the possibility of affecting the integrity of your run. If this does happen, insert an Eppendorf tube filled with ~50 µL buffer A into the pressure cell. Run this buffer through your inline to repack the resin containing your sample. Make sure it is dry before removing, this time gently depressurizing first!

8. Samples can take from 20 min to 3 hours to load. If after 45 min or so I find that it’s still only ~50% or less loaded, I gently depressurize the system to check on my sample inside the pressure cell. This usually means that the sample has become filled with bubbles. I find that tapping the Eppendorf tube to rid the bubbles before continuing loading can help move it along. Otherwise just set aside much more time for the loading process to complete.

9. Once you have your loaded sample, be sure to clean up around the pressure cell. Throw out your collection Eppendorf tube, and tape your labeled sample up to the Q Exac for analysis.
How to set up a mudPIT run on the Q Exactive Plus.

1. Clean the source.
   a. Take the stage off. Use the source-remover gadget to remove the source, holding it with a folded-up paper towel to avoid burns. Be sure that the black O-ring stayed on the MS when you removed the source.
   b. Spray methanol through the front, then back, then front again of the source. You can remove the paper towel after the first spray. When spraying, you want to ensure that you can see a clear stream of MeOH coming out the other end. Replace the source on the MS.

2. Calibrate the instrument.
   a. Place the Hesi source on the instrument and connect the tubing attached to the syringe. The syringe contains +/− mode calibrant solution. There is more in the drawer under the computer if it runs empty.
   b. Using the instructions on the sheet in the drawer under the MS, change the settings in the Tune program for calibration. Scan range: 150 to 2000; Resolution: 70,000; Sheath gas flow rate: 8; Spray voltage: 5; Capillary temp: 275.
   c. Turn the MS on. Turn the injector on, at a rate of 2 µL/min. Check for characteristic peaks from the calibrant (see image). If peaks look ok, hit calibrate.
      i. If calibration fails and you’re in a pinch, try again this time injecting an additional ~100-200 uL. This can indicate a dirty ion source, so it’s likely you will need to clean the entrance to the MS (refer to “Troubleshooting Guide: Low signal”) or request a PM.

3. Replace the stage and save tune file.
   a. Remove the Hesi source and replace the stage. When replacing the stage, be sure to hold it up high while you connect it. It should drop down a little bit when you remove your hand.
   b. Change the Tune program values back to the run values according to the same sheet you used previously. Scan range: 400 to 1800; Resolution: 70,000; Sheath gas flow rate: 0; Spray voltage: 2.75; Capillary temp: 200. At the upper left of the program, click “save program”.

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4. Connect your sample and verify connection is good.
   a. Cut your inline down to about ~5-7 cm above the resin line. Thread an equilibrated column through the opposite green sleeve. For best results, calibrate this column the same day of the run.
   b. Attach this setup carefully to the T-joint on the stage, with the column tip facing the ion source. Tape the T-joint down gently, then attach more tape to the column. If the setup is too long (i.e. it cannot lay down at its furthest point without touching the source), then cut the inline tubing a bit shorter.
   c. Using direct inject in the XCalibur program, start the flow at 0.1 mL/min solvent A. The pressure should be between 30 and 70. If it is <30, there is likely a leak somewhere, and >70 implies a clog.
   d. Always check for leaks even if the pressure looks ok. If there is a leak, remove your setup from the T-joint but keep the solvent flow on to avoid clogging the T-joint.
   e. If there is a leak at the green sleeve attached to the column, try removing the green sleeve and snipping off the crimped portion. If the leak persists, your column might be clogged so try a different column.
   f. If there is a leak at the green sleeve attached to the inline, try the above regarding the green sleeve. After that, try replacing the filter and inline body.
   g. If there is a leak at the T-joint, remove and replace your sample, tightening the T-joint a bit further.
   h. Once you have your sample connected and can see solvent flowing through the column tip, turn the MS on via the tune program and carefully align your column tip with the source opening. You should see clear spectral lines (no large, gray boxes). If you do see large gray boxes, try taking the stage off and putting it on again.

5. Running your sample
   a. I like to start the run before I align everything to avoid any unnecessary sample loss. Once I’ve confirmed good connection and no leaks, I’ll start the run in XCalibur just like you would an equilibration. Open the desired file and name it accordingly. Highlight all the files you want to run and click run.
      i. The MudPIT program consists of five separate programs run sequentially, where each begins with either 0, 25, 50, 80, or 100% salt bump (buffer C, 500 mM ammonium acetate/H2O) followed by a gradient of 5-55% buffer B in buffer A.
      ii. Pure proteins run on only the first program (0% salt bump) from the MudPIT program. The flow is kept at 0.1 mL/min throughout.
   b. Now we need to align the stage so that the column tip rests ~0.5 cm from the source opening (should be far enough where a coin could slide between the two, but close enough that your finger wouldn’t fit). I first bring the stage ~5 cm from the source. Then I connect the long tape at the end of the stage to above the MS, near the syringe. Watch your sample as you do this to ensure it doesn’t smack into the source. This stabilizes it pretty well and
should put the column pretty accurately around the source tip. I then use the white knob on the stage to very gently bring the column to the desired ~0.5 cm distance. For additional adjustments you can use the tape strand that’s on the column.

c. You may or may not see solvent droplets appearing and disappearing on the column tip. Seeing them ensures your run is well set up and working, but sometimes I don’t see them and that’s fine too.

d. Open the realtime XIC tab on XCalibur. A good run will start to show a large spike in signal once solvent B reaches ~20%. Your initial signal should be in the 1e8 range, but I have had successful runs where I had lower than that at first but still saw good signal after solvent B started. Seeing good overall peak separation and strong signal (~1e10) on the XIC plot is a great indicator of whether your search will be successful or not. If this is only showing you a flat line, something has gone wrong somewhere because the MS is not seeing anything coming off. Refer to “Troubleshooting Guide: Low signal” for suggestions.

6. Cleaning up your run

   a. After your run is done, I try to remember to log back in a stop the solvent flow and turn the MS off. They will stay on perpetually if you don’t do this, which is wasteful of solvent and can be damaging to the MS. It’s ok at this point to turn off the solvent flow while your sample is attached - I’ve never seen it backflow at this point.

   b. Remove your sample from the stage. Throw the column and inline away into the glass waste and the filter away into the chemical waste. Replace the inline body to the rack.

7. Searching your run in Byos

   a. Raw files can be directly searched using the Byologic software.

   b. Open the Byos HCP workflow, and upload the appropriate Uniprot database.

   c. Peptides can be assumed fully tryptic. Common modifications to include are as follows: Acetyl (+42.010565; Protein N-term; variable - rare1), carbamidomethyl (+57.021464; C; Fixed), and oxidation (+15.994915; M; variable - common1). Other modifications are unique to the residue of study but are treated as “variable - common1” - be sure the modification mass is accurate.

   d. Peptides with b and y ions flanking the site of modification can be assumed modified.
**Instrument Maintenance**

To keep the instrument in good operating condition, the following tasks should be performed as indicated.

<table>
<thead>
<tr>
<th>Action:</th>
<th>Frequency:</th>
<th>Notes:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrate</td>
<td>Every 12 hours</td>
<td>Or immediately prior to run</td>
</tr>
<tr>
<td>Check solvent levels</td>
<td>Every run</td>
<td>Fill using HPLC grade solvents</td>
</tr>
<tr>
<td>Clean ion source, S-lens, and</td>
<td>Every 6 weeks</td>
<td>Sonicate in a 5% Alconox solution for 30 min, followed by sonication</td>
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<tr>
<td>exit lens</td>
<td></td>
<td>in a MQ solution for 30 min, and MeOH for 15 min. Dry with pressurized</td>
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<tr>
<td></td>
<td></td>
<td>nitrogen. Gently replace and bake out the system. Refer to manual for</td>
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<tr>
<td></td>
<td></td>
<td>additional details.</td>
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<tr>
<td>Bake out the system</td>
<td>After any shutdown</td>
<td>Examples of when system should be shut down include for cleaning, in</td>
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<tr>
<td></td>
<td></td>
<td>preparation for power outages, and in cases of incompatible room</td>
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<td></td>
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<td>temperatures (i.e., &gt; 35 °C).</td>
</tr>
<tr>
<td>Preventative Maintenance</td>
<td>Every 6 months</td>
<td>PM engineer will replace oil, clean ion source/S-lens/exit lens, and</td>
</tr>
<tr>
<td>(PM)</td>
<td></td>
<td>ensure instrument is properly functioning and calibrated.</td>
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</tbody>
</table>
### Troubleshooting Guide

<table>
<thead>
<tr>
<th>Problem:</th>
<th>Try:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low signal</td>
<td>• Check for leaks. Replace T-joint if necessary with a fresh T-joint.</td>
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<tr>
<td></td>
<td>• Clean source/ recalibrate. If needed sonicate ion transfer tube. (See “Instrument Maintenance”</td>
</tr>
<tr>
<td></td>
<td>• Make sure columns/inlines are properly equilibrated (no residual solvent B)</td>
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<tr>
<td></td>
<td>• Make sure the column is aligned to the source and the column is not clogged</td>
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<td></td>
<td>• Make sure the stage is properly connected and the voltage cable is not loose</td>
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<tr>
<td></td>
<td>• If signal is $&lt; e^8$ during calibration and cleaning ion transfer tube is not</td>
</tr>
<tr>
<td></td>
<td>sufficient, likely need PM</td>
</tr>
<tr>
<td></td>
<td>• Verify you’ve pulled down protein through protocol by confirming with Coomassie/western blot</td>
</tr>
<tr>
<td>Packing columns/inlines is very slow</td>
<td>• Solution is too dilute (should be opaque)</td>
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<tr>
<td></td>
<td>• Column tip is too narrow. Gently scribe tip with file</td>
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<tr>
<td></td>
<td>• Bits of tubing mixed in with resin. Always visually check resin before inserting into pressure cell to ensure cleanliness</td>
</tr>
<tr>
<td></td>
<td>• Low pressure of packing gas (unlikely)</td>
</tr>
<tr>
<td>Good signal but no mods</td>
<td>• Verify modification mass is correct</td>
</tr>
<tr>
<td></td>
<td>• Confirm modification via anti-biotin WB or something similar</td>
</tr>
<tr>
<td>Loading sample on Baume taking forever</td>
<td>• Check that bubbles haven’t accumulated in Eppendorf tube</td>
</tr>
<tr>
<td></td>
<td>• Make sure there are no clogs in the inline (usually the green sleeves)</td>
</tr>
<tr>
<td>Low pressure but no visible leaks</td>
<td>• Check all T-joints (the one directly attached to the sample and the one covered by the white box at end of stage)</td>
</tr>
<tr>
<td></td>
<td>• Make sure there is a back-spray flow</td>
</tr>
<tr>
<td></td>
<td>• If there is solvent B in the line from a previous run, the pressure will be</td>
</tr>
<tr>
<td></td>
<td>initially low (~38), but should come up after ~ 10 min of running solvent A though the lines</td>
</tr>
<tr>
<td>Run won’t start</td>
<td>• Ensure queue is cleared. If it is not, variate unpausing the queue followed</td>
</tr>
<tr>
<td></td>
<td>by stopping the items until all are cleared</td>
</tr>
<tr>
<td></td>
<td>• Ensure the autosampler and pump are connected to the system. To reconnect them, turn</td>
</tr>
<tr>
<td></td>
<td>them off, then remove them from the setup by using the Instrument Configuration</td>
</tr>
<tr>
<td></td>
<td>program. Restart the computer, add them back via the same program, and turn them back on.</td>
</tr>
</tbody>
</table>
## Replacement Parts & Consumables

<table>
<thead>
<tr>
<th>Part Number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-888</td>
<td>T-joint</td>
</tr>
<tr>
<td>M-120X</td>
<td>Filters</td>
</tr>
<tr>
<td>160-2635-10</td>
<td>Column tubing</td>
</tr>
<tr>
<td>160-2255-10</td>
<td>Inline tubing</td>
</tr>
<tr>
<td>M-520</td>
<td>Inline body</td>
</tr>
<tr>
<td>F-185X</td>
<td>Green sleeves for inlines</td>
</tr>
<tr>
<td>04A-4299</td>
<td>C18 resin</td>
</tr>
<tr>
<td>04A-4398</td>
<td>SCX resin</td>
</tr>
</tbody>
</table>
References