TITLE

Proteome-wide analysis of protein lipidation using chemical probes: in-gel fluorescence visualisation, identification and quantification of *N*-myristoylation, *N*- and *S*-acylation, *O*-cholesterylation, *S*-farnesylation and *S*-geranylgeranylation

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ABSTRACT

Protein lipidation is one of the most widespread post-translational modifications (PTMs) found in nature, regulating protein function, structure, and subcellular localisation. Lipid transferases and their substrate proteins are also attracting increasing interest as drug targets due to their dysregulation in many disease states. However, the inherent hydrophobicity and potential dynamic nature of lipid modifications makes them notoriously challenging to detect by many analytical methods. Chemical proteomics provides a powerful approach to identify and quantify these diverse protein modifications by combining bespoke chemical tools for lipidated protein enrichment with quantitative mass spectrometry-based proteomics. Here we report a robust and proteome-wide approach for the exploration of five major classes of protein lipidation in living cells, through the use of specific chemical probes for each lipid PTM. In-cell labelling of lipidated proteins is achieved by the metabolic incorporation of a lipid probe that mimics the specific natural lipid, concomitantly wielding an alkyne as bio-orthogonal labelling tag. After incorporation, the chemically tagged proteins can be coupled to multifunctional 'capture reagents' using click chemistry, allowing in-gel fluorescence visualisation or enrichment via affinity handles for quantitative chemical proteomics based on label-free quantification (LFQ) or tandem mass-tag (TMT) approaches.

In this protocol, we describe the application of lipid probes for *N*-myristoylation, *N*- and *S*-acylation, *O*-cholesterylation, *S*-farnesylation and *S*-geranylgeranylation, in multiple cell lines to illustrate both the

workflow and data obtained in these experiments. We provide detailed workflows for method optimization, sample preparation for chemical proteomics and data processing. A properly trained researcher (*e.g.* technician, graduate student, or postdoc) can complete all steps from optimizing metabolic labelling to data processing within three weeks. This protocol enables sensitive and quantitative analysis of lipidated proteins at a proteome-wide scale at native expression levels, which is critical to understanding the role of lipid PTMs in health and disease.

INTRODUCTION

Modification of proteins with lipids is fundamental to all forms of life, mediating functions including membrane binding, protein folding, and protein-protein interactions[1, 2]. Lipid post-translational modifications (PTMs) thereby regulate subcellular localisation, signalling networks, enzyme activity and cell-cell communication, and consequently lipid PTM dysregulation is implicated in disease states including cancer, neurodegeneration and infections[3]. Understanding the enzymes and substrates involved in lipid PTM pathways is therefore fundamental to cell biology and provides a wealth of potential drug targets. Indeed, several radioisotope-free biochemical assays have been developed for the identification of inhibitors of lipid transferase enzymes[4, 5]. For example, we recently reported the acylation-coupled lipophilic induction of polarization (Acyl-cLIP) assay as the first universally applicable biochemical assay that allows monitoring of any enzyme processing lipid PTMs[6]. Further, recent successes in structure determination of key enzyme classes responsible for lipid PTMs[7-13] makes these enzymes an increasingly tractable target class. However, the diversity in classes of lipid PTM, their large number of substrates, and dynamic reversible nature of certain lipid modifications can make it challenging to link a phenotype to a given enzyme or modification state. Here, we provide a protocol with detailed workflows to visualise, identify and quantify the proteins modified by different types of lipid PTMs, using in-gel fluorescence and chemical proteomics as readouts.

Chemical proteomic methods offer a powerful approach for the investigation of PTMs across whole proteomes, enabling identification and quantification of hundreds or thousands of proteins undergoing a specific modification of interest in complex biological system. Chemical probes based on lipid substrates incorporating a minimal bio-orthogonal alkyne handle have opened this challenging and diverse class of PTMs to a range of powerful modern analytical techniques[14] providing fundamental insights into the scope and dynamics of lipid PTM substrates, and validation of lipid transferase enzymes as drug targets[15-17].

Here, we provide a robust protocol for chemical biology analysis and chemo-proteomic profiling of multiple major classes of lipid PTM (**Figure 1a**)[14]:

1. *N*-Myristoylation: Co- and post-translational modification of N-terminal glycine residues with a saturated C14:0 fatty acid (*i.e.* myristic acid) by *N*-myristoyltransferases (NMT1 and NMT2 in mammals)[12, 15, 17-19].
2. Long chain *S*-acylation: Cysteine modification with long-chain fatty acids, often with a saturated C_{16} fatty acid, hence widely referred to as '*S*-palmitoylation', although other fatty acid chain lengths are also involved. Catalysed by a family of 23 zDHHC transferase enzymes in humans[20].

3. Long chain *N*-acylation: modification of an N-terminal cysteine residue with long-chain fatty acids (C_{14} or C_{16}), observed on Hedgehog (HH) proteins. Conducted by Hedgehog acyltransferase (HHAT) in an exclusive enzyme-substrate relationship, where HHAT modifies the cysteine thiol as a thioester, and subsequently rearranges it chemically to the N-terminal amide[2, 13, 21].
4. *S*-Farnesylation: Cysteine modification with farnesyl isoprenoid (C_{15}) at the C-terminal CAAX box motifs by farnesyl transferase (FTase), notably on RAS family GTPases[22].
5. *S*-Geranylgeranylation: Cysteine modification with geranylgeranyl isoprenoid (C_{20}) at the C-terminal CAAX box motifs by geranylgeranyl transferases (GGTase-I, II and III), notably on the RAB subfamily of GTPases, but also observed in 'prenyl switching' of key oncogenic RAS GTPases following FTase inhibition[16, 22]. GGTase-II does not use CAAX boxes, instead it uses RAB escort protein (REP) as a chaperone and often doubly *S*-geranylgeranylates at a CC or CXC motif[22].
6. *O*-Cholesterylation: Attachment of cholesterol uniquely to the C-terminus of Hedgehog (HH) protein signalling domain via an ester linkage, through an autocatalytic mechanism[23].

Each lipid PTM requires its own alkyne-functionalised probe which mimics the natural lipid substrate. For *N*-myristoylation and *S*- and *N*-acylation, lipid probes are commercially available, and we have reported the synthesis of lipid probes specific for *S*-farnesylation, *S*-geranylation and *O*-cholesterylation[15, 22, 23]. The here presented protocol can be extended to other similar PTMs, by preparing and validating corresponding chemical probes with similar bio-orthogonal labelling handles.

Methods for labelling protein lipidation in living cells

Historically, lipidation has been studied using radiolabelled lipids to detect modification of target proteins. Lipids containing radioisotopes are fed to cells and are incorporated alongside the native non-radiolabelled substrate, allowing PTM detection by means of gel electrophoresis followed by fluorographic or autoradiographic imaging[24]. However, such methods present numerous drawbacks including hazardous reagents, high cost, low sensitivity and lengthy detection periods, and environmental impact. Further, these methods were mostly limited to substrate-centric studies (*e.g.* by immunoprecipitation of specific substrates) rather than unbiased investigations on a whole-proteome level.

S-Prenylated substrates have been studied via selective solubilization of isoprenylated proteins into Triton-114 detergent layers[25]; however, robust application of this approach to whole-proteome profiling has not been demonstrated, and differentiation between *S*-farnesylation and *S*-geranylgeranylation is not possible.

Alternative proteomics approaches have exploited the labile nature of the thioester linkage in *S*-acylation, whereby unmodified cysteine residues are capped and then acylated cysteines are subsequently revealed by selective chemical cleavage of thioesters with hydroxylamine. Newly revealed cysteines are then functionalised with biotin (acyl-biotin exchange, ABE[26]) or bound to resins (acyl-resin assisted capture, Acyl-RAC[27]) for analysis. ABE and Acyl-RAC have allowed identification of thousands of *S*-acylated proteins; however, such specific chemical cleavage methods lack universal

applicability across the full range of lipid PTMs in nature[28, 29]. While ABE and Acyl-RAC are powerful methods to enable detection of *S*-acyl modifications in samples without metabolic labelling, technical drawbacks include high background and a lack of information about the chain length of the acyl modification. ABE and Acyl-RAC are thus complementary to metabolic tagging using chemical probes for *S*-acylation, thereby offering orthogonal validation strategies.

Overview of the procedure

The presented protocol applies chemical probes that mimic the natural lipid substrates of five major classes of lipid PTM: *N*-myristoylation (alkyne myristic acid, YnMyr **1**, **Figure 1b**), *N*- and *S*-acylation (both by using alkyne palmitic acid, YnPal **2**, **Figure 1c and 1d**, respectively), *S*-farnesylation (alkyne farnesol, YnF **3**, **Figure 1e**), *S*-geranylgeranylation (alkyne geranylgeraniol, YnGG **4**, **Figure 1f**) and *O*-cholesterylation (alkyne cholesterol, YnChol **5**, **Figure 1g**).

The lipid probes are supplemented from a DMSO stock solution directly into the standard cell culture medium, which may include standard serum. Here, defatted or low serum conditions are not required for metabolic labelling to occur. The lipid probes are internalized into the cell, activated through the native biosynthetic machinery, and incorporated into substrate proteins of the lipid PTM (See **Figures 1b-g**). Thereafter the cells are lysed, and the lipid probe-modified proteins are selectively functionalised (**Figure 2**).

The terminal alkyne present on each lipid probe affords bio-orthogonal modification of the lipid probe-labelled proteins, through copper(I) catalysed azide-alkyne cycloaddition (CuAAC), with multifunctional azide-containing 'capture' reagents. As shown in **Figure 2**, lipid probe-labelled proteins can be modified with AzTB **6**, to enable the fluorescent visualisation of lipid probe incorporation in for instance the entire proteome, directly in-gel after SDS-PAGE separation (*e.g.* for method optimization, see also **Figure 3a** for more detail). AzTB **6** also features a biotin moiety to enable selective enrichment of the lipid probe-labelled proteins on avidin beads after stringent detergent washing, and lipid PTM validation of specific proteins by means of fluorescence visualisation after enrichment or immunoblotting (overview in **Figure 2**, for more detail see **Figure 3b**).

Functionalizing lipid probe-labelled proteins with AzRB **7** (overview in **Figure 2,** for more detail see **Figure 4**) allows selective enrichment of lipid probe-labelled proteins on avidin beads after stringent detergent washing to remove non-lipid probe-modified proteins for mass spectrometry-based proteomic sequencing. AzRB **7** aids identification of the lipid probe-modified peptide via the presence of an internal arginine, which remains after tryptic digestion, thereby adding additional charge. Robust identification and quantification of lipid probe-modified proteins can be achieved by implementing isobaric labelling using tandem mass-tags (TMT, see **Figure 4b**) or label-free quantification (LFQ, see **Box 2**) approaches and powerful downstream data analysis pipelines (**Figure 4c**).

Development of the protocol

Terminally alkyne-tagged lipid probes and azide capture reagents have gained prominence over the corresponding reverse arrangement using azide probes and alkyne capture reagents. This preference is due to decreased non-specific background labelling with azide capture reagents, thus enhancing

identification of *bona fide* labelled substrates, and superior probe stability, amongst other factors[30, 31]. The present protocol details use of well-established and recently reported alkyne-tagged lipid probes for the study of protein lipidation. The chemical probes for *N*-myristoylation (YnMyr **1**) and *N*- and *S*-acylation (YnPal **2**) are widely available commercially, whilst syntheses for the more recently-developed probes for *S*-farnesylation (YnF **3**), *S*-geranylgeranylation (YnGG **4**) and *O*-cholesterylation (YnChol **5**) are fully detailed and annotated in the original reports[22, 23]. In all cases, lipid probe design was driven by insertion of the alkyne tag at a position minimally disruptive to the function of the lipid, and of the enzymes involved in metabolic activation and transfer onto protein substrates. In certain cases, for example for *O*-cholesterylation probe YnChol **5**, finding this position required experimental evaluation of different designs to identify the optimal lipid probe[23]. It is critical when assessing the validity of a new chemical probe that appropriate controls are included to demonstrate that the probe recapitulates the biochemical function of the native lipid substrate, for example through native substrate competition or selective chemical inhibition of target lipid transferase enzymes[15, 17, 22, 23, 32, 33]. For proteome-wide and substrate-specific studies on lipid modified substrates, we suggest analysing lipid probe labelling in the presence and absence of selective lipid transferase inhibitors, and subsequent validation of labelling sites by the inclusion of specific mutants[34, 35].

Applications

Alkyne-tagged lipid probes are applicable to a wide variety of biological questions and situations, and the presented range of lipid probes has allowed analyses of proteins undergoing *N*-myristoylation, *N*- and *S*-acylation, *S*-farnesylation, *S*-geranylgeranylation, and *O*-cholesterylation. This approach allows visualisation and analysis of lipidation for known substrates; for example, *N*-myristoylation of ARL1 with YnMyr **1**, and *O*-cholesterylation of HH with YnChol **5** can be quantified directly by an increase in molecular weight from the addition of the lipid probe and the multifunctional capture reagent[23, 33, 36].

By coupling this approach to chemical proteomics, novel lipid PTM substrates can be identified at endogenous expression levels without the need for genetic manipulation of cells (*e.g.* substrate overexpression) that may complicate biological interpretation. For example, use of YnMyr **1** enabled the identification of >100 *N*-myristoylated proteins at endogenous levels in cancer cells, including co-translationally *N*-myristoylated substrates as well as post-translational *N*-myristoylation of N-terminal glycine residues generated by caspase proteases during apoptosis[32, 33, 37].

The lipid probes presented here can also be used in combination with selective chemical inhibitors to provide fundamental insight into the scope of a lipid PTM of interest. For example, HH proteins are *N*-acylated on the N-terminal cysteine by the enzyme Hedgehog acyltransferase (HHAT), a structurally distinct enzyme to the zDHHCs responsible for the vast majority of cellular *S*-acylation. Quantitative analysis of metabolic labelling with YnPal **2** in response to selective HHAT inhibition indicated a reduction in HH *N*-acylation only, with no other substrate affected, thus supporting an exclusive enzyme-substrate relationship between HHAT and HH[23].

The use of alkyne-tagged lipid probes to determine the impact of small-molecule inhibitors across the cellular lipidated proteome is an essential step to demonstrate drug target engagement and link an observed phenotype to the proposed cellular mechanism of action. For example, several proposed

human NMT inhibitors have been invalidated through demonstration of a complete absence of selective inhibition of NMT in living cells[33]. Further, the range of lipid probes now available allows for analyses of cellular responses to inhibitors across multiple lipidated proteomes. This includes for instance the demonstration at the whole proteome level of the selective 'rescue' observed in *S*-prenylation of KRAS and NRAS by GGTase in response to inhibition of FTase[22].

Alkyne-tagged lipid probes can be readily applied to a variety of cell types and diseases states for comparative analysis of the lipidated proteome in a disease-specific manner, information critical for identifying enzymes responsible for lipid PTMs as potential drug targets. For example, in the rhinovirus responsible for the common cold, the human host NMT is hijacked to perform *N*-myristoylation of a virus-encoded protein for correct capsid assembly, making the host *N*-myristoylation machinery a potential drug target to protect against viral infections[17].

Bioprocessing of lipid probes also extends beyond lipid transferase enzyme activity, such as lipid probe activation and incorporation into other lipid processing pathways. Indeed, in the malaria parasite *Plasmodium falciparum*, YnMyr **1** is incorporated into both *N*-myristoylated proteins and via an ester into glycosylphosphatidylinositol (GPI)-anchored proteins, which can be differentiated by the sensitivity of the modification to base hydrolysis[38].

Metabolic tagging with YnMyr **1** has not only been used to profile *N*-myristoylated substrates present in human cell lines[15, 33], but also in the human pathogens *Leishmania donovani*[39], *Trypanosoma brucei*[40], Herpes simplex virus[41] and malaria parasites[35, 42], and in multicellular organisms such as zebrafish[43, 44]. In *Escherichia coli*, YnMyr **1** incorporation enabled the identification of bacterial lipoproteins[45], and in the bacterium *Clostridium difficile*, YnMyr **1** was used to metabolically label *S*-diacylglyceryl modified, surface-anchored proteins that play important roles at the host-pathogen interface[46].

Lipidation dynamics is an increasingly appreciated aspect of protein lipidation, particularly for *S*-acylation where the modification can be removed by distinct families of acyl-protein thioesterases. Metabolic labelling with YnPal **2** facilitates unique insights into *S*-acylation dynamics and temporal control of lipid probe incorporation through pulse-chase experiments in combination with broad-spectrum hydrolase inhibition[47, 48]. Alkyne-bearing lipid probes therefore empower a range of investigations into the diverse biological roles of lipid PTMs and their potential as drug targets. For these purposes, we have for instance employed metabolic labelling with YnPal **2** to identify *S*-acylated substrates expressed by human pathogens including Herpes simplex virus[41] and *Legionella pneumophila*[49].

Advantages and limitations of the approach

The panel of terminally alkyne-tagged lipid probes described here (**Figure 1b-g**) possess complementary advantages and limitations compared to some other chemo-proteomic methods to profile lipid PTMs.

A major advantage of metabolic labelling with lipid probes is its wider applicability compared to other approaches. These include: ABE[26] and Acyl-RAC[27] for *S*-acylation, *in vitro* isoprenoid affinity tagging[50, 51] for *S*-farnesylation and *S*-geranylgeranylation of non-prenylated substrates, and the *in vitro* Sortase A-tagging approach to selectively enrich N-terminal glycines revealed after pharmacological inhibition

of *N*-myristoylation[33, 36]. These methods can be performed on native cell lysate or tissue and require no metabolic labelling step, and have been used to successfully identify hundreds of substrate proteins in specific contexts. However, they can only be applied to a chemically compatible subset of lipid modifications and additionally do not allow detailed analysis of the temporal dynamics of the PTM[47, 48].
In addition, the herein described method using metabolic labelling with lipid probes allows for temporal control of PTM labelling, such as in pulse-chase experiments, which is driven by the capacity of the lipid probes to integrate into the metabolic pathways of the cell, including activation into lipid transferase substrates. This is advantageous as it allows the analysis of multiple steps in the formation of the lipid PTM of interest, and metabolic labelling provides exquisite temporal sensitivity to inhibitors added in the same time frame, the specific influence of which can be lost in methods which do not quantify the evolution of the system over time. The approach can readily be adapted to the use of other lipid probes, such as those reported earlier for *S*-prenylation[52].
The need for metabolic labelling is the most important limitation of lipid probes, meaning that any system which is not metabolically active cannot be analysed, and furthermore, the system will always be non-physiological as the system must be exposed to non-physiological lipid probes.
In addition, the fidelity of lipid probes for the full range of metabolic processes that the native lipid undergoes in the cell can add further complexity[38]. Metabolic incorporation of lipid probes is a multistep process (including *e.g.* conversion into the activated form (*N*-myristoylation, *N*-/*S*-acylation), recognition by the lipid transferase, transfer onto the protein), and the lipid probes may be recognized and transferred at lower efficiency rates than the endogenous substrate, as they are in competition with endogenous lipids. The stoichiometry and proportion of the protein lipidation process that is captured can be challenging to quantify and will likely vary between substrates, and lipid probes therefore should be used with caution when determining lipidation stoichiometry relative to other PTMs.
Moreover, owing to their excellent biomimicry, following prolonged cellular incubation, fatty acid- or prenyl-based lipid probes may undergo elongation or truncation to analogues of alternative lipid substrates[53], and whilst this potentially provides insights into interactions with cellular lipid metabolism, it also clouds PTM assignment. Appropriate controls are therefore desirable (*e.g.* selective lipid transferase inhibition, identification of modified peptides) to assign a lipid probe modification state to the proposed lipid PTM, particularly for novel substrates.
Major advantages of lipid probes are that the herein presented chemical probes for *N*-myristoylation, *S*-acylation and *S*-prenylation are currently the only approach that allows the direct identification of the lipid modification at a specific site, at the proteome level. Moreover, the probes YnF **3** and YnGG **4** facilitate the identification and separation of processing steps (proteolysis, methylation) downstream of *S*-farnesylation and *S*-geranylgeranylation directly in the mass spectrometric analyses[22].
Identification of a specific site of modification can however be challenging when the lipid probe is present on the peptide at the point of analysis. A substantial proportion of the modified peptide can remain bound to the avidin-bead during digestion and, because the lipidated peptide of interest has a hydrophobic character, it has low water solubility and reduced ionization in comparison to the non-lipidated counterpart. This effect is even more pronounced when using commercially available capture reagents such as biotin azide. While this effect plays a noticeable role, it is clear that these capture

reagents still support identification of the lipidated protein after protein-based pulldown. However, *S*-acylation remains very challenging to identify directly, presumably due to the lability of the thioester bond.

The use of enzyme-cleavable capture reagents such as AzRB **7** (**Figure 4a**) can partially overcome the latter problems by allowing a selective release of the modified peptide from the avidin-beads or resin, while also adding an additional charged residue to improve mass spectrometric detection[44].

In comparison, acyl-RAC using disulphide enrichment of revealed cysteines can also enable selective release of the modified peptide following digestion; however, analysis can be clouded by enrichment of other *S*-acylation sites, such as acyl carrier-proteins and ubiquitin ligases[54]. Identification of sites of lipidation should be treated with caution at least until orthogonally validated via mutagenesis of the proposed site, followed by *e.g.* immunoblotting and or by acyl-RAC analysis to confirm loss of the lipid PTM on the specific protein.

Experimental design

This protocol describes methods for the systematic study of protein *N*-myristoylation, *N*- and *S*-acylation, *S*-prenylation, and *O*-cholesterylation at a whole-proteome level in intact living cells. This method may be expanded to other lipid PTMs where alkyne-tagged lipid probes can be synthesized, following the same experimental steps described here. Depending on the target protein(s) of interest, the precise lysis buffer composition may need to be adapted to allow optimal isolation as its constituents may *e.g.* interfere with the CuAAC ligation[55]; it generally can be performed in any normal isotonic lysis buffer, and importantly in the absence of copper-chelating additives (*e.g.* EDTA), other than the selected ligand (*e.g.* TBTA). The biological question of interest will require the protocol to be scaled according to the number of samples required. It is therefore recommended to begin investigations using gel-based readouts for optimization (**Figure 3c**), prior to progressing to larger-scale chemical proteomics experiments with sufficient biological replicates to perform robust statistical tests on identified proteins (**Figure 4a-c**).

We suggest proceeding to chemical proteomics when the metabolic labelling with lipid probes has been optimized. The protocol described here offers chemical proteomics workflows suitable for label-free quantification (LFQ) and tandem mass-tag (TMT) methods of mass spectrometric detection. Combining all the different conditions tested within the same TMT multiplex improves accuracy, avoids the prevalence of missing values in case of low abundant proteins, and increases the identification rate of low abundance peptides enriched with lipid probes. It must be noted that multiplexing samples of very different profiles (*e.g.* proteins labelled with probe mixed with non-labelled samples) will reduce the chances of identification of peptides only found in a small percentage of the conditions. Here, label-free quantification can be more suitable as samples are not mixed.

With isobaric sets of either 6, 10 or more, the number of concomitantly TMT multiplexed samples has increased significantly. We suggest following one of two approaches. The first is to fit all conditions and replicates within the same TMT set, *e.g.* three biological replicates of the negative control (DMSO), positive control (with lipid probe), and a competition sample (lipid probe with inhibitor), which all fit in a TMT 10-plex. The second approach we suggest is for when there are more samples than that fit in the

multiplex, *e.g.* when the above example has four biological replicates. To allow multiplexing, we propose generating multiple TMT sets. For instance, here that would be two TMT 6-plexes containing two replicates of each biological condition, or four TMT 3-plexes containing one replicate of each biological condition. Regarding the choice of TMT channel, we advise to randomise TMT label allocations when possible, to counteract ratio-compression[56]. If not all the TMT reagents within a given set will be used (*e.g.* when having 9 samples when using a TMT 10-plex), then we recommend leaving out one of the 130 mass pair, since it has been shown that these have the highest ion coalescence[56].

Metabolic labelling with lipid probes using in-gel fluorescence as readout (Procedure 1)

Gel-based analysis requires smaller quantities of protein (~10 µg per sample) and thus minimizes lipid probe and capture reagent usage, as well as being less time-consuming and costly than chemical proteomics experiments. Moreover, this approach allows facile, rapid, and sensitive visualisation of the lipid PTM, in the proteome as a whole. While biotinylated capture reagents can be used for visualisation via immunoblot with avidin-conjugated horseradish peroxidase (HRP) reagents, in-gel fluorescence provides a superior linear detection range over the concentration of labelled protein and is therefore recommended for optimization. It is suggested to perform initial gel-based optimization using a capture reagent bearing a fluorophore (*e.g.* AzTB **6** or AzT **6b**, see **Figure 3a**), followed by SDS-PAGE separation and analysis by in-gel fluorescence. A caveat of this approach is that the fluorophore-wielding capture reagents might react differently compared to the biotinylated capture reagent (*e.g.* AzRB **7**) used for the chemical proteomic experiments. Design of proper controls in these analyses is critical to provide robust evidence for specific labelling by competing lipid probe labelling against the native lipid substrate (*e.g.* YnMyr **1** competing with myristic acid) as a negative control, or by using a concentration range of the lipid probe to demonstrate dose-dependent incorporation. We strongly recommend the following order of steps to optimise the maximal amount of metabolic labelling, in the absence of cytotoxicity and within the solubility limit of the lipid probe in the medium (**Figure 3c**).

1. Concentration-dimension: metabolic labelling for 18 h with different concentrations of lipid probe, across a range of, for example, 0, 0.1, 0.3, 1, 3, 10 and 30 µM. All lipid probes presented here label significant amounts of protein when present in the concentration range 3-30 µM. Within this range, increasing the lipid probe concentration can boost the metabolic labelling of proteins by stronger competition with the endogenous lipid substrate, but it is important to be aware that high concentrations can result in cytotoxicity and/or precipitation of the lipid probes. Lipids and lipid probes have limited solubility in aqueous matrixes and at higher concentrations they start to precipitate. The cytotoxicity of compounds can be rapidly assessed (see *e.g.* ref[33]) by using Cell Titer Blue and compound solubility can be detected by directly determined by measuring the optical density (OD) at 600 nm.
2. Time-dimension: metabolic labelling for different lengths of time, preferably across the range of 0, 2, 4, 8, 16 and 24 h, by using an optimized, non-toxic concentration of the lipid probe, that lies within the solubility limit of the lipid probe. Incubating for longer lengths of

time with lipid probe (up to 24 h) may enhance the total amount of metabolically labelled proteins but may lead to increased lipid metabolism or conversion of the lipid probe into related lipid species, and labelling may decrease after a certain time point, *e.g.* due to negative feedback[22]. The maximal amount of metabolic labelling is limited by the half-life of the modified proteins and the dynamic nature of the modification.

3. If the amounts of metabolic labelling remain insufficient, use more cells. This can be achieved by scaling up to 10 cm diameter dishes or T175 flasks, for example. In general, a 6-well plate should yield enough metabolically labelled proteins to visualise in-gel, either directly or after enrichment and in combination with Westernblotting (10-100 µg range), but conditions for chemical proteomics may require more material (100-300+ µg range, per sample).

With the metabolic labelling conditions thoroughly optimized, lipid probe labelling can now be investigated by titrating various concentrations of lipid transferase inhibitors or other perturbing agents (**Figure 3c**). Currently, we suggest IMP-1088 to inhibit *N*-myristoylation[17, 33], Tipifarnib to block *S*-farnesylation[22, 57], GGTI-2133 to abrogate *S*-geranylgeranylation[22] and IMP-1571 as on-target inhibitor of HHAT to block HH *N*-acylation[58]. No selective inhibitors are currently available for *S*-acylation by zDHHCs, RabGGTase-mediated *S*-geranylgeranylation, nor the auto *O*-cholesterylation of HH.
We recommend performing this competition analysis first before proceeding to chemical proteomics experiments of larger scale, as the in-gel visualisation method offers a faster turn-around of results than the chemical proteomics workflow while concomitantly being less expensive.

Whole proteome profiling of protein lipidation using chemical proteomics

Once optimal metabolic labelling conditions have been determined, the lipid probe-labelled proteins can be specifically enriched on avidin-coated beads followed by protease digestion for nanoLC-MS/MS analysis. For this purpose, a biotin-containing capture reagent must be used such as AzRB **7** (**Figure 4a**), which further contains an arginine residue to allow release of the modified peptide during on-bead trypsin digestion after enrichment.
Protein quantities required for chemical proteomics analysis are much higher than for in-gel analysis (generally >300 µg per sample), so there is a need to increase culture scale for these experiments. Further, at least three biological replicates are essential to provide sufficient statistical power in proteomic analysis. The use of properly designed controls is critical to allow clear differentiation between genuine lipidated proteins and non-specifically enriched proteins. Well-validated and highly selective lipid transferase inhibitors can be useful to reveal enzyme-mediated incorporation of the lipid probe.
This protocol has been written for use with a Q-Exactive mass spectrometer with an Orbitrap analyser, but it can be adapted to any spectrometer capable of high-resolution analyses. We list two general options for protein quantification: isobaric labelling using tandem mass-tags (TMT) or label-free quantification (LFQ) analysis. TMT and LFQ methods can be used for identification of novel substrates and the study of known substrates in new biological systems. TMT offers advantages in sample multiplexing, reducing machine time while improving accuracy and avoiding missing values where

proteins drop in abundance. Moreover, the multiplexing capability of TMT increases identification of low abundance peptides enriched with lipid probes. Since metabolic labelling with alkyne probes involves enrichment of a target subset of the proteome and generally results in low levels of protein after enrichment, high labelling efficiencies can be obtained using less TMT reagent per sample, thus decreasing costs. A 10-fold decrease in TMT reagents allows effective labelling; however even using such dilutions, costs involved in isobaric labelling cannot be overlooked. A cheaper LFQ protocol has therefore been provided in addition (**Box 2**). A disadvantage of TMT is that it has limited capabilities when comparing markedly different proteomes within the multiplex, for instance when identifying and quantifying lipid probe-modified proteins in the proteome of host cells with or without an infection by another organism or virus. Here, LFQ will be more effective (**Box 2**).

The use of dimethylated avidin beads (**Box 1**), instead of those with regular avidin, significantly improves the detection limit of low abundance peptides[36]. On-bead digestion of avidin-enriched proteins using trypsin yields not only peptides of enriched proteins, but also a prominent background of avidin-derived peptides. Dimethylation of the lysines of NeutrAvidin makes the biotin-binding protein proteolytically resistant to LysC, enabling a sequential on-bead (LysC)/off-bead (trypsin) digestion strategy thereby significantly boosting protein identification rates and the proteome sampling depth, without affecting LC-MS performance[36, 59].

The following practical aspects of sample preparation are essential to ensure reproducibility and consistency of results in proteomics experiments.

- Low protein-binding tubes, such as LoBind Eppendorf tubes, are recommended throughout proteomics sample preparation from the moment the labelled proteins undergo the CuAAC ligation, to Stage Tip elution. The sensitivity of mass spectrometry measurements means non-specific protein binding will significantly impact results.
- Use of LC-MS grade solvents is highly important to ensure that all solutions used are free of detergents and particulates that can interfere with liquid chromatography or the mass spectrometer; this is especially critical during the Stage Tip steps.
- The pH of buffers is also crucial: many sample preparation steps involve specific reactions with a complex protein mixture (*i.e.* reduction, alkylation, digestion, labelling) that have been optimized for maximal yield or recovery whilst also minimizing side-reactions. These reactions are generally pH-dependent; therefore, it is recommended to check the pH prior to use of the buffers and prepare them fresh when their pH has changed.

Target validation by immunoblotting

Once a known or novel protein substrate has been detected in the cell line of interest, further validation of this result can be achieved using pull-down assays and/or immunoblotting as described in **Figure 3b**. In certain cases, this will be limited by the availability of antibodies to detect the target protein at endogenous abundance. Overexpression of target protein fused to an affinity tag may aid detection; however, it should be appreciated that the overexpression may affect the lipidation status of the protein. Use of site-directed mutagenesis of the modified residue (for example, glycine to alanine for *N*-myristoylation, or cysteine to alanine for *S*-acylation) is important to confirm results from whole-

proteome profiling, and with recent advances in CRISPR-Cas9 and Prime Editing is achievable on the endogenous protein[60].

Expertise needed to implement the protocol

The optimization and validation steps can be performed by a researcher with proper training in biochemistry and cell biology techniques (*e.g.* technician, graduate student, or postdoc). Sample preparation for proteomic analysis is equally accessible to such a researcher following the presented protocol; however, training for running (nano)LC-MS/MS samples will be required specific to the equipment available and is best performed under guidance of an experienced researcher/technician in this area. Failure to accurately follow the proteomics sample preparation guidelines and incorrect equipment usage can be severely detrimental to high-sensitivity and expensive LC-MS/MS equipment.

MATERIALS

BIOLOGICAL MATERIALS

Cell lines

All cell lines were obtained from ATCC and verified by STR analysis by The Francis Crick Cell Services. All cells were cultured in humidified 37 °C incubators.

Numerous cell lines have been investigated in the past, including EA.hy926 (https://scicrunch.org/resolver/RRID:CVCL_3901), HeLa (https://scicrunch.org/resolver/RRID:CVCL_0030), HEK293A (https://scicrunch.org/resolver/RRID:CVCL_6910), HEK239T (https://scicrunch.org/resolver/RRID:CVCL_0063), MCF-7 (https://scicrunch.org/resolver/RRID:CVCL_0031), MDA-MB-231 (https://scicrunch.org/resolver/RRID:CVCL_0062), RPE-1 (https://scicrunch.org/resolver/RRID:CVCL_4388) cell lines and were cultured according to the guidelines from the ATCC. In addition, *in situ* labelling experiments have been successfully performed in living bacterial strains including *E. coli*, C. *difficile* and *L. pneumophila*, parasites such as *L. donovani*, *P. falciparum* and *T. brucei*, and in zebrafish embryos and larvae.

CRITICAL. Cell line authentication by STR analysis is crucial to verify the integrity of the model system. This authentication is generally supported by cell- or tissue-culture responsible core facilities or can be outsourced to *e.g.* ATCC amongst others.

REAGENTS

CAUTION. All the Material Safety Data Sheets for the reagents listed below should be read before use. Each reagent should be used and disposed of in accordance with the set guidelines. Appropriate personal safety protective equipment should always be worn.

CRITICAL. Unless otherwise noted, the reagents listed here can be obtained from any major chemical manufacturer.

Chemicals

For Procedure 1 – In-gel fluorescence visualisation of lipid probe-labelled proteins:

Sterile cell culture medium (appropriate for the specific cell line used, as indicated by *e.g.* ATCC)

Cell Titer Blue (G8080, Promega).

Phosphate-buffered saline (PBS, *e.g.* 806544-500ML, Sigma-Aldrich)

N-myristoyltransferase inhibitor IMP-1088 (25366, Cayman Chemical), and see for synthesis reference[17].

S-farnesyltransferase inhibitor Tipifarnib (SML1668-5MG, Merck Life Science)

S-geranylgeranyltransferase inhibitor GGTI-2133 (sc-221668, Insight Biotechnology)

N-acylation inhibitor IMP-1575, see for synthesis reference[58].

Myristic acid alkyne (YnMyr **1**) (13267, Cayman Chemical)

Palmitic acid alkyne (YnPal **2**) (13266, Cayman Chemical)

Farnesyl alkyne (YnF **3**), see for synthesis reference[22].

Geranylgeranyl alkyne (YnGG **4**), see for synthesis reference[22].

Cholesterol alkyne (YnChol **5**), see for synthesis reference[23].

Triton X-100 (*e.g.* T8787-100M, Sigma-Aldrich).

SDS (sodium dodecylsulphate) (*e.g.* 428023-500GM, Sigma-Aldrich).

cOmplete EDTA-free protease inhibitor tablets (11873580001, Sigma-Aldrich).

DC Protein Assay (5000112, Bio-Rad)

Water ice, (Polar G-Series countertop ice machine R600a, or equivalent)

DMSO (dimethylsulfoxide) (D8418-50ML, Sigma-Aldrich)

Ultrapure water (Milli-Q water purification system, 18.2 MΩ; Millipore or equivalent)

AzTB **6**, see for synthesis reference[44]. Alternatively, TAMRA biotin azide (*e.g.* 1048-25, Click Chemistry Tools) or AzT **6b**, (*e.g.* AZ109-5, Click Chemistry Tools)

Copper sulphate ($CuSO_4$) (451657-50G, Sigma-Aldrich)

TCEP (Tris(2-carboxyethyl)phosphine) (C4706-2g, Sigma-Aldrich)

TBTA (Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) (678937-50 MG, Sigma-Aldrich)

EDTA (ethylenediaminetetraacetic acid) (15575020, UltraPure™ 0.5M EDTA, pH 8.0, Invitrogen)

Methanol (analytical grade) (20847.307, VWR)

Chloroform (analytical grade) (C2432-1L, Sigma-Aldrich)

HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) (H4034-500G, Sigma-Aldrich)

NaOH (sodium hydroxide pellets) (S8045-500G, Sigma-Aldrich)

Streptavidin-coupled magnetic beads (S1420S, NEB)

BSA (bovine serum albumin) (A7906, Bio-Rad)

Skim milk powder (70166-500G, Sigma-Aldrich)

Precision Plus Protein™ All Blue Prestained Protein Standards (1610393, Bio-Rad)

ARL1 antibody (rabbit anti-human, 16012-1-AP, Proteintech, https://scicrunch.org/resolver/RRID:AB_2243131)

PRKACA antibody (rabbit anti-human, 4782S, Cell Signaling, https://scicrunch.org/resolver/RRID:AB_2170170)

IFITM3 antibody (rabbit anti-human, 59212S, Cell Signaling, https://scicrunch.org/resolver/RRID:AB_2799561)
CANX antibody (rabbit anti-human, 2679S, Cell Signaling, https://scicrunch.org/resolver/RRID:AB_2228381)
HRAS antibody (mouse anti-human, MAB3291, Millipore, https://scicrunch.org/resolver/RRID:AB_94790)
ULK3 antibody (rabbit anti-human, ab124947, Abcam, https://scicrunch.org/resolver/RRID:AB_10972508)
RHOA antibody (mouse anti-human, sc-418, Santa Cruz Biotechnology, https://scicrunch.org/resolver/RRID:AB_628218)
RAB8A antibody (rabbit anti-human, ab188574, Abcam, https://scicrunch.org/resolver/RRID:AB_2814989)
Hedgehog antibody. The shown results were with rabbit anti-human, sc-9024 (https://scicrunch.org/resolver/RRID:AB_2239216), Santa Cruz Biotechnology, but this has been discontinued; this product has been replaced by sc-365112 (https://scicrunch.org/resolver/RRID:AB_10709580), but we have not tested this antibody.
TUBA antibody (mouse anti-human, ab7291, Abcam, https://scicrunch.org/resolver/RRID:AB_2241126)
HSP90 antibody (mouse anti-human, sc-69703, Santa Cruz Biotechnology, https://scicrunch.org/resolver/RRID:AB_2121191)

Extra for Procedure 2 – Identification and quantification of lipid PTMs by chemical proteomics:
AzRB **7**, see for synthesis reference[44]. Alternatively, azide biotin (AzB, *e.g.* 1265-25, Click Chemistry Tools).
DTT (DL-Dithiothreitol) (43815, Sigma-Aldrich)
NeutrAvidin-coupled agarose beads (29201, Pierce)
CAA (2-chloroacetamide) (C0267-100G, Sigma-Aldrich)
Trypsin, Sequencing Grade (V5111, Promega Corporation)
Quantitative Fluorometric Peptide Assay (23290, Pierce).
Acetonitrile (anhydrous) (271004-100ML, Sigma-Aldrich)
TMT 10plex isobaric label reagent set (90406, ThermoFisher Scientific)
Hydroxylamine (50% (wt/vol), 467804-10ML, Sigma-Aldrich)
Ammonium formate (516961-100G, Sigma-Aldrich)
Formic acid (Y0001970, Sigma-Aldrich)
Ammonium hydroxide (338818-100ML, Sigma-Aldrich)
Acetonitrile (LC-MS grade) (83640.320, VWR)
Water (proteomic-grade, ultrapure) (1012620500, Supelco)
TFA (trifluoroacetic acid) (T6508-100ML, Sigma-Aldrich). **CAUTION.** Work in a fume-hood as TFA is toxic. **CRITICAL.** TFA is corrosive and degrades plastic. Avoid stock contamination from repeated or prolonged contact with plastics (including pipette tips, Eppendorf vials, etc.). As work around, we

suggest transferring a small volume of TFA (*e.g.* 200 µL) with a single-use glass pipette to a new glass vial. Usable for up to 1 week.

Extra for Box 1 – Bead derivatization:

TEAB (Triethylammonium bicarbonate buffer, 1.0 M, pH 8.4-8.6) (T7408-100ML, Sigma-Aldrich). **CRITICAL**. Store at 4 °C as TEAB is volatile and evaporates.

Endoproteinase LysC, Sequencing Grade (VA1170, Promega Corporation)

Formaldehyde (F8775-25ML, Sigma-Aldrich)

Sodium cyanoborohydride ($NaBH_3CN$) (42077-10G, Sigma-Aldrich)

Ethanolamine (411000-100ML, Sigma-Aldrich)

EQUIPMENT

For Procedure 1 – In-gel fluorescence visualisation of lipid probe-labelled proteins:

Pipettes (*e.g.* StarLab ErgoOne single-channel pipettes).

Pipette teat (*e.g.* BUL2000, Scientific Laboratory Supplies).

pH meter (*e.g.* FE20, Mettler-Toledo).

Multi-vortex mixer (*e.g.* Vortex IR, StarLab).

Vortex (*e.g.* ZX3 Vortex mixer, Fisher Scientific).

Refrigerated centrifuge suitable for 1.5 mL tubes (*e.g.* 5424 R centrifuge, Eppendorf).

Centrifuge suitable for 15 mL tubes and MS vials (*e.g.* 5910 R centrifuge, Eppendorf).

Water bath sonicator (*e.g.* Ultrasonic cleaner, VWR).

Magnetic rack (Magna GrIP rack (8-well), Millipore).

Heat block (*e.g.* Digital heat block, VWR).

UV spectrophotometer for protein estimation (*e.g.* EnVision Xcite Multilabel Reader, PerkinElmer).

SDS-PAGE system and power source (*e.g.* Mini-PROTEAN Tetra system and PowerPac, Bio-Rad).

Western blotting transfer system (*e.g.* Trans-Blot Turbo transfer system, Bio-Rad).

Fluorescence imaging system (*e.g.* Typhoon FLA 9500 Variable Mode Imager, GE Healthcare).

Chemiluminescence imagining system (*e.g.* LAS3000 imager, GE Healthcare).

Extra for Procedure 2 – Identification and quantification of lipid PTMs by chemical proteomics:

Heated shaker (*e.g.* FisherBrand Isotemp Shake Touch High Speed Shaker, Fisher Scientific).

Mass spectrometer-compatible HPLC (*e.g.* EASY-nLC 1000 Liquid Chromatograph, LC120, Thermo Scientific).

Mass spectrometer compatible with MaxQuant and Perseus (*e.g.* Q-Exactive Hybrid Orbitrap, IQLAAEGAAPFALGMAZR, Thermo Scientific).

ESI (*e.g.* Q-Exactive compatible EASY-Spray™ Source, ES081, Thermo Scientific).

Computer compatible of running MaxQuant, Perseus and/or PEAKS (*e.g.* based on Intel Core i7 9700K 8-core CPU, 32 Gb DDR4 DIMM memory, NVIDIA GeForce RTX2080 8Gb FH HDMI PCIe graphics card and 1 Tb SSD, running an updated version of Windows or Linux). **CRITICAL**. Analysis

and processing of proteomics data involving PTMs such as lipidation can be very demanding in terms of computer resource.

Materials

For Procedure 1 – In-gel fluorescence visualisation of lipid probe-labelled proteins:

Gloves (*e.g*. StarLab, SG-C-(XS/S/M/L)).

Pipette tips (*e.g.* StarLab TipOne pipette tips - S1111-3700-C, S1113-1706-C, S1112-1720-C (p10, p200, p1000 respectively)).

6-well culture plates (*e.g.* 391-8036, VWR).

15 mL conical centrifuge tubes (*e.g.* 339650, ThermoFisher Scientific).

50 mL conical centrifuge tubes (*e.g*. 339652, ThermoFisher Scientific).

Laboratory tissue paper (*e.g.* 115-1276, VWR).

Cell lifter (*e.g.* CLS3008-100EA, Sigma-Aldrich).

1.5 mL Eppendorf tubes (*e.g.* 211-0015, VWR).

96-well plates for Protein Concentration (*e.g.* 655101, Greiner).

1.5 mL LoBind Eppendorf tubes (Z666505-100EA, Sigma-Aldrich).

PVDF Durapore membrane filter, 0.1 µm pore size, hydrophilic (VVLP04700, Millipore).

Extra for Procedure 2 – Identification and quantification of lipid PTMs by chemical proteomics:

10 cm cell culture dishes (*e.g.* CLS430167-100EA, Sigma-Aldrich).

pH indicator paper (*e.g.* 85402.600, VWR).

SDB-RPS (polystyrene-divinylbenzene, reverse phase sulfonate) (66886-U, Supelco).

Glass Pasteur pipette (*e.g.* CLS7095D5X, Sigma-Aldrich).

Glass vial with screw cap (*e.g.* K60910C112, Scientific Laboratory Supplies).

Mass spectrometry vials (X100 Crimp to vial snap-ring micro-vial, CRV12-03P, Fisher Scientific).

Mass spectrometry caps (X1000 snap-cap red, septa black 11mm, CX11BLK-04, Fisher Scientific).

Software

Updated Microsoft Office (licensed, https://products.office.com/en-us/home) or Open Office (free, https://www.openoffice.org/).

MaxQuant v.1.6.4.0 or later (free, https://maxquant.net/maxquant/).

Perseus v.1.6.2.3 or later (free, https://maxquant.net/perseus/).

PEAKS v.8.0 or later (licensed, http://www.bioinfor.com/peaks-studio/).

REAGENT SETUP

For Procedure 1 – In-gel fluorescence visualisation of lipid probe-labelled proteins:

Inhibitor stocks

Weigh the inhibitor and dissolve in DMSO to obtain a 50 mM stock. Inhibitors can be stored at -80 °C for up to 6 months; however, this must be confirmed experimentally for novel inhibitors. **CRITICAL.** It is recommended to verify compound purity and integrity by LC-MS analysis, along with comparison of experimental data generated from across the storage period to detect any change in biological activity. **CRITICAL.** Avoid repeated freeze-thaw cycles by storing as *e.g.* 10 µL aliquots. **CRITICAL.** Ensure the stock concentration is high enough to not exceed the final, maximally tolerated DMSO concentration of the cells, at the end of the experiment (<0.1% (vol/vol) generally). **CRITICAL.** Ensure that once the top concentration of inhibitor is prepared in media (*e.g.* 50 µM, 0.1% (vol/vol) DMSO final), serial dilutions are prepared using media containing the same concentration of DMSO (*e.g.* 0.1% (vol/vol)), such that the DMSO concentration remains constant at all inhibitor dilutions.

Lipid probe stocks

Weigh the lipid probes (YnMyr **1**, YnPal **2**, YnF **3**, YnGG **4** or YnChol **5**) and dissolve in DMSO to obtain a 50 mM stock. Lipid probes can be stored at -80 °C for up to 6 months. **CRITICAL.** It is recommended to verify compound purity and integrity by LC-MS analysis. **CRITICAL.** Avoid repeated freeze-thaw cycles by storing as *e.g.* 10 µL aliquots. **CRITICAL.** Ensure the stock concentration is high enough to not exceed the final, maximally tolerated DMSO concentration of the cells, at the end of the experiment (<0.1% (vol/vol) generally).

Lysis buffer

This solution is 0.1% (wt/vol) SDS and 1% (vol/vol) Triton X-100 in phosphate-buffered saline (PBS), pH 7.4. Add 1 tablet of cOmplete EDTA-free protease inhibitor cocktail (Sigma-Aldrich) per 50 mL. Without protease inhibitors, it can be stored at RT for up to 6 months. **CRITICAL.** With protease inhibitors, discard within a day. Check the pH with a pH meter or with pH indicator paper.

Capture reagent AzTB 6 solution

This solution is 10 mM AzTB **6** in DMSO and can be stored at -20 °C for up to 6 months. **CRITICAL.** Avoid repeated freeze-thaw cycles by storing as *e.g.* 10 µL aliquots.

Capture reagent AzT 6b solution

This solution is 10 mM AzT **6b** in DMSO and can be stored at -20 °C for up to 6 months. **CRITICAL.** Avoid repeated freeze-thaw cycles by storing as *e.g.* 10 µL aliquots.

$CuSO_4$ solution

This solution is 50 mM $CuSO_4$ in ultrapure water and can be stored at -20 °C for up to 6 months. **CRITICAL.** Avoid repeated freeze-thaw cycles by storing as *e.g.* 50 µL aliquots.

TCEP solution

This solution is 50 mM TCEP in ultrapure water and can be stored at -20 °C for up to 6 months. **CRITICAL.** Avoid repeated freeze-thaw cycles by storing as *e.g.* 50 µL aliquots.

TBTA solution

This solution is 10 mM TBTA in DMSO and can be stored at -20 °C for up to 6 months. **CRITICAL.** Avoid repeated freeze-thaw cycles by storing as *e.g.* 25 µL aliquots.

Click mix

This solution is 1 volume of 10 mM capture reagent (AzTB **6**, AzT **6b** or AzRB **7** (see below for **Procedure 2**) solution, 2 volumes of 50 mM $CuSO_4$ solution, 2 volumes of 50 mM TCEP solution and 1 volume of 10 mM TBTA solution. **CRITICAL.** AzT **6b** only contains a fluorophore for in-gel visualisation, whereas AzTB **6** also contains a biotin amenable for enrichment using magnetic Streptavidin beads, or NeutrAvidin-coupled agarose beads. **CRITICAL.** Prepare freshly and 1.2× more than required. **CRITICAL.** Ensure the Click mix does not contain precipitates. Vigorously vortex the Click mix to dissolve everything, *e.g.* at 3,000 rpm for 10 sec.

EDTA solution

This solution is 500 mM EDTA in ultrapure water, pH 8.0, and can be stored at RT for up to 6 months. **CRITICAL.** This EDTA concentration is near the solubility limit, and therefore may be difficult to achieve, or may form precipitates over time. As alternative, this solution can be purchased (see **MATERIALS** section).

HEPES solution

This solution is 50 mM HEPES-NaOH, pH 8.0, in ultrapure water, and can be stored at RT for up to 1 month. **CRITICAL.** Check pH before use.

Protein pellet resuspension solution

This solution is 2% (wt/vol) SDS, 10 mM DTT in 50 mM HEPES, pH 8.0. Can be stored at RT for up to 1 month.

HEPES wash buffer A

This solution is 0.2% (wt/vol) SDS in 50 mM HEPES-NaOH, pH 8.0, in ultrapure water, and can be stored at RT for up to 1 month. **CRITICAL.** Check pH before use.

HEPES wash buffer B

This solution is 1% (wt/vol) SDS in 50 mM HEPES-NaOH, pH 8.0, in ultrapure water. It can be stored at RT for up to 1 month. **CRITICAL.** Check pH before use.

Laemmli sample buffer

This solution is 10% (wt/vol) SDS, 25% (vol/vol) β-mercaptoethanol and 0.02% (wt/vol) bromophenol blue in 50% (vol/vol) glycerol in 1 M Tris-HCl, pH 6.8. The solution can be stored at RT for 6 months,

but best results are obtained by freshly adding β-mercaptoethanol. **CAUTION.** Work in a fume-hood as β-mercaptoethanol is toxic.

Magnetic bead denaturation buffer

This solution is Laemmli loading buffer, diluted 2.5× in ultrapure water. **CAUTION.** Work in a fume-hood as β-mercaptoethanol is toxic.

Extra for Procedure 2 – Identification and quantification of lipid PTMs by chemical proteomics:

Capture reagent AzRB 7 solution

This solution is 10 mM AzRB **7** in DMSO and can be stored at -20 °C for up to 6 months. **CRITICAL.** Avoid repeated freeze-thaw cycles by storing as *e.g.* 10 µL aliquots.

Trypsin solution

This solution is 0.2 µg/µL trypsin in 50 mM HEPES-NaOH, pH 8.0, in ultrapure water and can be stored at -20 °C for up to 3 months. **CRITICAL.** Centrifuge the vial with 20 µg lyophilized trypsin at 1,000× *g* for 10 min at RT prior to dissolving in 100 µL HEPES solution. **CRITICAL.** Check the pH of the HEPES solution before dissolving lyophilized trypsin. **CRITICAL.** Avoid freeze-thaw cycles by storing as 5 µL aliquots.

Reduction and Alkylation solution

This solution is 55 mM TCEP and 165 mM CAA in 50 mM HEPES-NaOH, pH 8.0, in ultrapure water. **CRITICAL.** Prepare freshly and discard within a day.

Hydroxylamine solution

This solution is 5% (wt/vol) hydroxylamine in 50 mM HEPES-NaOH, pH 8.0, in ultrapure water. **CRITICAL.** Prepare freshly and discard within a day.

Peptide elution solution

This solution is 60% (vol/vol) acetonitrile in LC-MS grade water. **CRITICAL.** Prepare freshly and discard within a day.

Fractionation Tip loading solution

This solution is 1% (vol/vol) TFA in LC-MS grade water. **CAUTION.** Prepare in a fume-hood as TFA is toxic. **CRITICAL.** Prepare freshly and discard within a day.

Fractionation Tip wash solution

This solution is 0.2% (vol/vol) TFA in LC-MS grade water. **CAUTION.** Prepare in a fume-hood as TFA is toxic. **CRITICAL.** Prepare freshly and discard within a day. Check pH before use.

Fraction 1 solution

This solution is 100 mM ammonium formate in 40% (vol/vol) acetonitrile and 0.5% (vol/vol) formic acid in LC-MS grade water. **CRITICAL.** Prepare freshly and discard within a day.

Fraction 2 solution

This solution is 150 mM ammonium formate in 60% (vol/vol) acetonitrile and 0.5% (vol/vol) formic acid in LC-MS grade water. **CRITICAL.** Prepare freshly and discard within a day.

Fraction 3 solution

This solution is 5% (vol/vol) ammonium hydroxide in 80% (vol/vol) acetonitrile in LC-MS grade water. **CRITICAL.** Prepare freshly and discard within a day.

MS injection solution

This solution is 2% (vol/vol) LC-MS grade acetonitrile and 0.5% (vol/vol) TFA in LC-MS grade water. **CAUTION.** Prepare in a fume-hood as TFA is toxic. **CRITICAL.** Prepare freshly and discard within a day.

Extra for Box 1 – Bead derivatization:

LysC solution

This solution is 0.2 µg/µL LysC in 50 mM HEPES-NaOH, pH 8.0, in ultrapure water. It can be stored at -20 °C for up to 6 months. **CRITICAL.** Centrifuge the vial with 20 µg lyophilized LysC at 1,000× *g* for 10 min at RT prior to dissolving in 100 µL HEPES solution. **CRITICAL.** Check the pH of the HEPES solution before dissolving lyophilized LysC. **CRITICAL.** Avoid freeze-thaw cycles by storing as 5 µL aliquots.

Procedure 1 – In-gel fluorescence visualisation of lipid probe-labelled proteins. TIMING. Each iteration of the procedure takes 3-4 d.

This section describes the in-gel fluorescence visualisation of lipid probe-labelling and its application for optimizing the metabolic labelling conditions. The amount of proteins that are metabolically labelled with bio-orthogonally tagged lipid analogues depends on a variety of factors including, but not limited to, the abundance of both the lipid modification and the targeted proteins in the investigated cell type or cell line, the number of cells used, the degree of competition between the endogenous lipid and the analogue, and the duration of exposure to the lipid analogue. Due to this inherent variability, we strongly stress to optimize the metabolic labelling first on small scale, see **Figure 3**, in a manner tailored to the biological hypothesis being tested, before proceeding to chemical proteomics at a larger scale (**Figure 4**).

<CRITICAL> In **Step 10**, the CuAAC reaction either adds AzTB **6** or AzT **6b**. AzT **6b** only contains a fluorophore for visualisation, whereas AzTB **6** also contains a biotin amenable for enrichment of lipid probe-labelled proteins using magnetic Streptavidin beads.

<CRITICAL> In our lab we choose to use magnetic Streptavidin-beads for the optimisation workflow, because the magnetic separation is faster and method development benefits from fast turnaround times. The capture can also be done using NeutrAvidin beads by centrifuging at 3,000× *g* for 3 min at RT, instead of placing it on the magnet.

<CRITICAL> In general, 10 µg total protein is sufficient when analysing samples by in-gel fluorescence analysis and Western blot only, and 100-300+ µg total protein when pursuing enrichment of lipid probe-labelled proteins using magnetic streptavidin beads, followed by in-gel fluorescence analysis and Westernblotting.

Table 1. Suggested lipid probe labelling conditions as well as proteins suitable as PTM labelling controls.

Lipid PTM	**Lipid probe**	**Concentration**	**Time**	**Reported controls**
N-myristoylation	YnMyr **1**	20 µM	18 h	ARL1[32, 33, 36] and PRKACA[15, 32]
S-, *N*-acylation	YnPal **2**	20 µM	18 h	IFITM3[61] and CANX[62]
S-Farnesylation	YnF **3**	5 µM	18 h	HRAS[22] and ULK3[22]
S-Geranylgeranylation	YnGG **4**	5 µM	18 h	RHOA[22] and RAB8A[22]
O-Cholesterylation	YnChol **5**	5 µM	18 h	HH[23]

In-cell metabolic labelling. TIMING. 24 h or more.

1. Seed cells of choice in 6-well plates, in 3 mL media.
2. Grow the cell culture of choice to 70-80% of confluency. **CRITICAL STEP.** Cells should be in the exponential growth phase for optimal and reproducible metabolic incorporation of the tagged lipid probes. Cells should therefore not yet reach confluency at the end of the experiment as contact inhibition may alter protein synthesis and lipid metabolism, thereby affecting the results. This does not apply to non-proliferating cells.
3. Remove media, replace with 3 mL fresh media, pre-warmed to 37 °C, and return cells to incubator for 30 min. Optional: add Inhibitor stock(s) to the fresh media. **CRITICAL STEP.** Ensure the final concentration of DMSO in the medium will be less than what is maximally tolerated by the cells (<0.1% (vol/vol) generally) at the end of the experiment.
4. Add lipid probe stock to the media and incubate for 18 h. **CRITICAL STEP.** This is a step where optimisation is required. See **Table 1** for the suggested concentration and labelling times for the various PTM-specific lipid probes.
   CRITICAL STEP. Ensure the final concentration of DMSO in the medium will be less than what is maximally tolerated by the cells (<0.1% (vol/vol) generally) at the end of the experiment.
5. Aspirate the growth media and wash cells twice with an equal volume of PBS, pre-warmed at 37 °C.

6. Aspirate the PBS, add 150 µL Lysis buffer per well (6-well plate format) and harvest cells with a cell scraper while cooling the plates placed on crushed ice.
7. Transfer homogenate to a 1.5 mL microcentrifuge tube.
8. Centrifuge at 17,000× *g* for 10 min at 4 °C and transfer supernatant to a new tube. **PAUSE POINT.** Lysates can be kept at -80 °C for up to 6 months.

Bio-orthogonal tag functionalization with multi-functional capture reagents. TIMING. 2 h.

9. Determine the protein concentration of the cell lysate, using *e.g.* the DC Protein Assay f\Kit II and following the guidelines provided by the supplier. **CRITICAL STEP.** Make sure the BSA calibration curve is prepared in the same buffer as the samples.
10. Adjust the total protein concentration to 1-2 µg/µL with Lysis buffer.
    CRITICAL STEP. Total protein input should be 50 µg total protein or more to guarantee formation of a visible pellet during protein precipitation. For direct in-gel fluorescence analyses, without protein precipitation, 10 µg total protein is generally sufficient.
    CRITICAL STEP. The amount of protein required for visualisation depends on the metabolic labelling efficiency and the abundance of the modification, and may need further optimization.
11. Prepare the Click mix for copper(I)-catalysed azide-alkyne cycloaddition (CuAAC) by adding the reagents in the following order, with vigorously vortexing at 3,000 rpm for 3 s after each addition:
    1 volume of 10 mM capture reagent (AzTB **6** or AzT **6b**) solution, 2 volumes of 50 mM $CuSO_4$ solution, 2 volumes of 50 mM TCEP solution and 1 volume of 10 mM TBTA solution (final concentrations 100 µM, 1 mM, 1 mM and 100 µM, respectively).
    CRITICAL STEP. Prepare freshly and 1.2× more than required. **CRITICAL STEP.** Ensure the Click mix does not contain precipitates. Vigorously vortex the Click mix at 3,000 rpm for 10 sec to dissolve everything.
    CRITICAL STEP. AzT **6b** only contains a fluorophore for visualisation, whereas AzTB **6** also contains a biotin amenable for enrichment using magnetic Streptavidin beads.
    CRITICAL STEP. Prepare Click mix freshly and 1.2× more than required.
    CRITICAL STEP. Ensure the Click mix does not contain precipitates. Vigorously vortex the Click mix at 3,000 rpm for 10 sec to dissolve everything.
    CRITICAL STEP. Perform all following steps in the dark, by *e.g.* keeping tubes covered under foil, to minimize photobleaching of the fluorophore.
12. Add 6 µL Click mix per 100 µL sample and incubate for 1 h at RT while shaking vigorously on a multi-vortex mixer (700 rpm). **CRITICAL STEP.** This should drive the reaction to completion within 30 min at RT.
13. Stop the CuAAC reaction by adding EDTA stock solution to a final concentration of 5 mM. EDTA chelates the copper ions required for the CuAAC reaction, thereby terminating the ligation process, facilitating the efficient removal of copper ions from the mixture during the following precipitation steps. A blue-tinted pellet indicates residual copper ions

trapped in the precipitated pellet. **PAUSE POINT.** All samples can be kept at -80 °C for up to 1 week.

14. Optional. The metabolically labelled proteins can be visualised by in-gel fluorescence without removal of the excess AzTB **6** or AzT **6b** capture reagents, by denaturing the samples with 1/4th of the volume with Laemmli sample buffer and then analysed by SDS-PAGE (see **Step 33**).
    CRITICAL STEP. Residual capture reagent will result in a highly intense fluorescent band migrating at approximately 3 kDa and may thereby obscure the fluorescence emitted by metabolically labelled proteins, and therefore we suggest removal of the excess capture reagents prior to SDS-PAGE (next, **Step 15**).

Protein precipitation. TIMING. 1-2 h.

15. Precipitate proteins by adding 4 sample volumes of methanol, 1 volume of chloroform and 2 volumes of water, and vortex vigorously at 3,000 rpm for 10 sec. **CRITICAL STEP**. It is indispensable to keep the methanol:chloroform ratio of 4:1. Prior to addition of methanol, the sample volume can be increased with water to *e.g.* 100 µL to increase the ability to see and remove the aqueous and organic phase.
16. Centrifuge at 5,000× *g* for 2 min at RT. **CRITICAL STEP.** A white disk should form at the interphase while the two main phases are completely clear. If this does not happen, or one of the phases is still turbid, repeat the centrifugation step.
17. After centrifugation, remove the top layer, add 500 µL methanol and centrifuge at 17,000× *g* for 4 min at RT.
18. Discard the supernatant, add 500 µL methanol and vigorously vortex and sonicate until the pellet is completely resuspended, then centrifuge at 17,000× *g* for 4 min at RT. Perform this step twice in total. **PAUSE POINT.** All samples can be kept at -80 °C for up to 1 week.
19. Dry the pellet for a maximum of 5 min at RT by leaving the tube open, upside down on tissue paper. **CRITICAL STEP.** Do not allow the pellet to become fully dry, as this makes it more difficult to resuspend.
20. Resuspend pellet in 1/10th of the original sample volume of resuspension buffer, and vigorously vortex and sonicate until the pellet is completely dissolved. **CRITICAL STEP**. Ensuring complete re-solubilization of the protein in every sample is critical and will profoundly impact the success of the experiment.
21. Centrifuge at 17,000× *g* for 2 min at RT to ensure no undissolved material remains. **CRITICAL STEP.** If pellet forms, repeat the previous step.
22. Add 9/10th of the original sample volume of HEPES solution to reconstitute to a final concentration of 1 µg/µL protein and 0.2% (wt/vol) SDS. From here, AzTB **6**-functionalised samples can be submitted for biotin-enrichment (next, **Step 23**). Both AzTB **6** and AzT **6b**-functionalised samples can also be submitted for analysis through in-gel fluorescence (continue with **Step 33**).

Enrichment of biotin-functionalised proteins on magnetic Streptavidin-beads. TIMING. 5-8 h.

23. Cut the points off 200 µL pipette tips to allow transfer of solutions containing beads.
24. Using the modified tips, transfer 1.2× the total required volume of magnetic Streptavidin beads to a new 1.5 mL LoBind Eppendorf tube.
25. Equilibrate the magnetic Streptavidin beads with excess (*e.g.* 5 volumes) of HEPES wash buffer A, incubate for 2 min at RT with shaking on a multi-vortex mixer (700 rpm), centrifuge at 1,000× *g* for 10 sec and then place the tube on the magnetic rack for 2 min at RT, and discard the supernatant. Perform this step twice in total.
26. Resuspend the magnetic Streptavidin beads in the original volume from **Step 24** and distribute the suspension into new 1.5 mL LoBind Eppendorf tubes, using the modified 200 µL pipette tips.
27. Centrifuge the protein samples at 17,000× *g* for 5 min at RT and transfer the supernatant (containing 100-300+ µg total protein) to the magnetic Streptavidin bead-containing tubes. Take a 10 µg protein aliquot of the supernatant as 'input control' for SDS-PAGE analysis in **Step 33**. **CRITICAL STEP.** Do not transfer any pelleted material as this will negatively influence the pulldown.
28. Incubate the mixture for 3 h at RT while shaking (700 rpm). **CRITICAL STEP.** Ensure solution is sufficiently agitated to prevent the beads from forming a pellet.
29. Centrifuge at 1,000× *g* for 10 sec and place the Eppendorf tube on the magnetic rack for 2 min and transfer the supernatant to a new tube. Take a 10 µL aliquot of the supernatant as 'supernatant control' for SDS-PAGE analysis in **Step 33**), supplement with 1/4th of this volume of Laemmli sample buffer, mix and store at -20 °C. The remainder of the supernatant can be stored at -20 °C if required for other purposes.
30. Wash the beads with excess of HEPES wash buffer A, *e.g.* 1 mL per 30 µL beads, for 2 min at RT with shaking (700 rpm), then centrifuge at 1,000× *g* for 10 sec, place the Eppendorf tube on the magnetic rack for 2 min and discard the supernatant. Perform this step three times in total.
31. Wash the beads with excess of HEPES wash buffer B, *e.g.* 1 mL per 30 µL beads, for 2 min at RT with shaking (700 rpm), then centrifuge at 1,000× *g* for 10 sec and place the Eppendorf tube on the magnetic rack for 2 min and discard the supernatant. Perform this step three times in total.
32. Resuspend the beads in 30 µL magnetic bead denaturation buffer. **CRITICAL STEP.** Make sure all magnetic beads are flushed to the bottom of the Eppendorf tube in order to enable complete denaturation in the following step. **PAUSE POINT.** Samples can be kept at -20 °C for up to 1 week.

In-gel fluorescence visualisation of metabolic labelling. TIMING. 4-5 h.

33. For proteins labelled with AzT **6b**:
    - Denature the samples at 100 °C for 5 min on a heating block.

- Centrifuge at 5,000× *g* for 1 min.
- Load 12.5 µL of the mixture on SDS-PAGE gel (next, **Step 34**, see also **Figure 3a**).

For samples labelled with AzTB **6**:

- Denature the 'input' aliquot (from **Step 27**), 'supernatant' aliquot (from **Step 29**) and the magnetic beads suspension (the biotin-enrichment fraction, from **Step 32**) at 100 °C for 5 min on a heating block.
- Centrifuge at 5,000× *g* for 1 min.
- Place the tubes with magnetic beads suspension on the magnet for 2 min.
- Load 30 µL of the supernatant of the magnetic beads suspension on SDS-PAGE (next, **Step 34**), in parallel to 12.5 µL of the 'input' and 'supernatant' aliquots (see also **Figure 3b**).

CAUTION. In case of *S*-palmitoylation, boiling can be detrimental to labelling and may result in reduced in-gel fluorescence.

CRITICAL STEP. Denaturing the samples more than once increases protein degradation and smearing of the bands on the gel.

CRITICAL STEP. Avoid sample evaporation. Weigh-down the Eppendorf lids, *e.g.* with a heavy metal strip, to avoid pressure-assisted opening during denaturation.

CRITICAL STEP. Include a fluorescent molecular weight marker to enable comparison between the migration of labelled protein bands within, and across, gels and gel-derived Western blotting results.

34. Gel electrophoresis. Separate the denatured proteins by means of standard SDS polyacrylamide gel electrophoresis, using self-made or pre-cast SDS-PAGE gels of 12% (wt/vol) polyacrylamide, running at 90 V in the stacking gel (top) and 120 V in the resolving gel (bottom). **CRITICAL STEP.** The percentage of polyacrylamide directly affects the dynamic range at which proteins of different molecular weights can be separated; 12% (wt/vol) allows marked separation between 150 and 15 kDa, thereby suitable to visualise the majority of proteins metabolically labelled with lipid probes **1**–**5**. **CRITICAL STEP.** Shield the SDS-PAGE apparatus from light to minimize photobleaching of the fluorophore.
35. At the end of the gel electrophoresis step (approximately 90 min at 120 V), allow the front of bromophenol blue to briefly migrate from the bottom of the gel for 2 min. Then remove the gel from the apparatus and thoroughly rinse the still encased gels with ample tap water to remove any potential bromophenol blue from the outside casing. **CRITICAL STEP.** Failure to remove the bromophenol blue may result in highly fluorescent stains such as fingerprints on the gel.
36. Remove the gel from its casing and transfer to a tray holding ice-cold ultrapure water. **CRITICAL STEP.** Cooling the gel will reduce diffusion and keep protein bands sharper and defined during the upcoming scanning process. **CRITICAL STEP.** Shield the SDS-PAGE apparatus from light to minimize photobleaching of the fluorophore.

37. Scan the gels for fluorescence on a compatible imager (here a Typhoon Variable Mode Imager 9500 (GE Healthcare)), using a Cy3/TAMRA ($\lambda_{excitation}$ 532 nm, $\lambda_{emission}$ 610 nm) filter to detect the TAMRA fluorophore present on proteins labelled with the AzTB **6** or AzT **6b** capture reagents, and scan the gel at the appropriate wavelengths to visualise the molecular weight marker (such as Cy5 ($\lambda_{excitation}$ 635 nm, $\lambda_{emission}$ 670 nm) for the Precision Plus marker (1610393, Bio-Rad).
38. Western blotting. Immobilize the proteins in the gel onto a nitrocellulose membrane, using standard wet- or semi-wet blotting procedure. After blocking the membrane, incubate the membrane with antibodies against proteins of interest. In parallel, to validate the extent of enrichment, detect validated lipidated proteins using immunoblotting (see **Table 1** for suggested controls for each lipid PTM).

Procedure 2 – Identification and quantification of lipid PTMs by chemical proteomics

<CRITICAL> This is the main chemical proteomics workflow to detect lipid probe-modified proteins by mass spectrometry. For the visualisation of lipid probe-labelling and the optimization of the metabolic labelling approach, see **Procedure 1**.

In-cell metabolic labelling. TIMING. Variable, from several hours to days.

1. Seed cells of choice in 10 cm diameter culture dishes, each with 12 mL media, and at least in triplicate per condition when pursuing robust chemical proteomics.
2. Grow the cell culture of choice to 70-80% confluence. **CRITICAL STEP.** Cells should be in the exponential growth phase for optimal and reproducible metabolic incorporation of the tagged lipid probes. Cells should therefore not yet reach confluency at the end of the experiment as contact inhibition may alter protein synthesis and lipid metabolism, thereby affecting the results. This does not apply to non-proliferating cells.
3. Remove media, replace with 12 mL fresh media, pre-warmed to 37 °C, and return cells to incubator for 30 min. Optional: add Inhibitor stock(s) to the fresh media. **CRITICAL STEP.** Ensure the final concentration of DMSO in the medium will be less than what is maximally tolerated by the cells (<0.1% (vol/vol) generally) at the end of the experiment.
4. Add Lipid probe stock to the media and incubate for 18 h. **CRITICAL STEP.** The concentration and time required for metabolic labelling should be optimized. An optimization guide is suggested in **Procedure 1** and **Figure 3c**. **CRITICAL STEP.** Ensure the final concentration of DMSO in the medium will be less than what is maximally tolerated by the cells (<0.1% (vol/vol) generally) at the end of the experiment.
5. Aspirate the growth media and wash cells twice with an equal volume of PBS, pre-warmed at 37 °C.
6. Aspirate the PBS and add 400 µL Lysis buffer per 10 cm diameter plate, and harvest cells with a cell scraper while cooling the plates placed on crushed ice.

7. Transfer homogenate to a 1.5 mL microcentrifuge tube.
8. Centrifuge at 17,000× *g* for 10 min at 4 °C and transfer supernatant to a new tube. **PAUSE POINT.** Lysates can be kept at -80 °C for up to 6 months.

Bio-orthogonal tag functionalization with multi-functional capture reagents. TIMING. 1-2 h.

9. Determine the protein concentration of the cell lysate, using *e.g.* the DC Protein Assay Kit II and following the guidelines provided by the supplier. **CRITICAL STEP.** Make sure the BSA calibration curve is prepared in the same buffer as the samples.
10. Adjust the total protein concentration to 1-2 µg/µL with Lysis buffer.
11. Prepare the Click mix for copper(I)-catalysed azide-alkyne cycloaddition (CuAAC) by adding the reagents in the following order, with vigorous vortexing for 3 s after each addition:
    1 volume of 10 mM capture reagent AzRB **7** solution, 2 volumes of 50 mM $CuSO_4$ solution, 2 volumes of 50 mM TCEP solution and 1 volume of 10 mM TBTA solution (final concentrations 100 µM, 1 mM, 1 mM and 100 µM, respectively).
    CRITICAL STEP. Prepare freshly and 1.2× more than required. **CRITICAL STEP.** Ensure the Click mix does not contain precipitates. Vigorously vortex the Click mix to dissolve everything.
12. Add 6 µL Click mix per 100 µL sample and incubate for 1 h at RT while shaking vigorously on a multi-vortex mixer (700 rpm). **CRITICAL STEP.** This should drive the reaction to completion within 30 min at RT.
13. Stop the CuAAC click reaction by adding EDTA stock solution to a final concentration of 5 mM. EDTA chelates the copper ions required for the CuAAC reaction, thereby terminating the ligation process, facilitating the efficient removal of copper ions from the mixture during the following precipitation steps. A blue-tinted pellet indicates residual copper ions trapped in the precipitated pellet. **PAUSE POINT.** All samples can be kept at -80 °C for up to 1 week.

Protein precipitation. TIMING. 1-2 h.

14. Precipitate proteins by adding 4 sample volumes methanol, 1 volume of chloroform and 2 volumes of water, and vortex vigorously for 10 sec. **CRITICAL STEP**. It is indispensable to keep the methanol:chloroform ratio of 4:1. Prior to addition of methanol, the sample volume can be increased with water to *e.g.* 100 µL to increase the ability to see and remove the aqueous and organic phase.
15. Centrifuge at 5,000× *g* for 2 min at RT. **CRITICAL STEP.** A white disk should form at the interphase while the two main phases are completely clear. If this does not happen, or one of the phases is still turbid, repeat the centrifugation step.
16. After centrifugation, remove the top layer, add 500 µL methanol and centrifuge at 17,000× *g* for 4 min at RT.
17. Discard the supernatant, add 500 µL methanol and vortex and sonicate until the pellet is completely resuspended, then centrifuge at 17,000× *g* for 4 min at RT. Perform this step

twice in total. **PAUSE POINT.** Samples can be kept in methanol at -80 °C for up to 1 week.

18. Dry the pellet for a maximum of 5 min at RT by leaving the tube open, upside down on tissue paper. **CRITICAL STEP.** Do not allow the pellet to become fully dry, as this makes it more difficult to resuspend.
19. Resuspend pellet in 1/10th of the original sample volume of Protein pellet resuspension solution, and vigorously vortex and sonicate until the pellet is completely dissolved. **CRITICAL STEP**. Ensuring complete re-solubilization of the protein in every sample is critical and will profoundly impact the success of the experiment. **CRITICAL STEP**. Vigorous vortexing in the presence of SDS may produce significant amounts of foam, therefore it is suggested to keep the shaking under 700 rpm.
20. Centrifuge at 17,000× *g* for 2 min at RT to ensure no undissolved material remains. **CRITICAL STEP.** If a pellet forms, repeat the previous step.
21. Add 9/10th of the original sample volume of HEPES solution to reconstitute to a final concentration of 1 µg/µL protein and 0.2% (wt/vol) SDS. From here, samples can be submitted for biotin-enrichment on NeutrAvidin-coupled agarose beads (next, **Step 22**).

Enrichment of biotin-functionalised proteins and their on-bead proteolytic digestion. TIMING. 5-8 h.

<CRITICAL> On-bead digestion can either be performed in a single digestion step using trypsin (next, **Step 23**), or with a two-step digestion strategy described in **Box 1**. In the two-step digestion strategy, the lysine residues on the NeutrAvidin-coated beads are derivatized. This enables on-bead digestion with LysC, followed by off-bead digestion with trypsin in order to minimize the potential background resulting from avidin-borne peptides. For the preparation of derivatized NeutrAvidin-coupled agarose beads , see **Box 1, Step 1**.

<CRITICAL> As a general rule of thumb, use 30 µL of beads slurry per 1 mg of total protein (as per the manufacturers' instructions (Thermo Scientific), capable of binding >2 picomoles of biotinylated proteins per 30 µL of bead slurry).

22. Cut the point off 200 µL pipette tips to allow transfer of solutions containing beads.
23. Using the modified tips, transfer 1.2× the total required volume of beads to a new 1.5 mL LoBind Eppendorf tube.
24. Equilibrate the NeutrAvidin-coupled agarose beads, derivatized or not, with excess (*e.g.* 5 volumes) of HEPES wash buffer A, incubate for 2 min at RT with shaking on a multi-vortex mixer (700 rpm), then centrifuge at 3,000× *g* for 4 min at RT, and discard the supernatant. Perform this step three times in total.
25. Resuspend the beads in the original slurry volume from **Step 23** with HEPES wash buffer A and distribute the suspension into new 1.5 mL LoBind Eppendorf tubes, using the modified 200 µL pipette tips.
26. Centrifuge the protein samples at 17,000× *g* for 5 min at RT and transfer 90% of the supernatant to the bead-containing tubes. Optional: take an aliquot of the supernatant as 'input' control in SDS-PAGE analysis (see **Procedure 1**, **Step 33**). Generally, 10 µg

protein is sufficient at optimal labelling conditions. **CRITICAL STEP.** Do not transfer precipitates onto the NeutrAvidin-coupled agarose beads as thereafter it is impossible to removed them from the beads.

27. Incubate the mixture for 3 h at RT with shaking (700 rpm). **CRITICAL STEP.** Ensure that the solution is sufficiently agitated to prevent the beads from forming a pellet.
28. Centrifuge the mixture at 3,000× *g* for 4 min and transfer the supernatant into a new 1.5 mL LoBind Eppendorf tube. Optional: take an aliquot of the supernatant as 'supernatant' control in SDS-PAGE analysis (see **Procedure 1, Step 33**). Generally, 10 µg protein is sufficient for this purpose, at optimal labelling conditions.
29. Wash the beads with excess of HEPES wash buffer A, *e.g.* 1 mL per 30 µL beads, for 2 min at RT with shaking (700 rpm), then centrifuge at 3,000× *g* for 3 min, and discard the supernatant. Perform this step three times in total.
30. Wash the beads with excess of HEPES wash buffer B, *e.g.* 1 mL per 30 µL beads, for 2 min at RT with shaking (700 rpm), then centrifuge at 3,000× *g* for 3 min, and discard the supernatant. Perform this step three times in total.
31. Wash the beads with excess of HEPES solution, *e.g.* 1 mL per 30 µL beads, for 3 min at RT with shaking (700 rpm), then centrifuge at 3,000× *g* for 3 min, and discard the supernatant. Perform this step three times in total. **CRITICAL STEP.** This removes traces of SDS to ensure it does not inactivate LysC and trypsin and minimizes the amount of SDS that may interfere with the mass spectrometer later.

Protease digestion. TIMING. 18 h.

32. Resuspend the beads in 30 µL HEPES solution. In case of regular, underivatized NeutrAvidin-coupled agarose beads, proceed to the next **Step 33**. In case of derivatized NeutrAvidin-coupled agarose beads, see **Box 1**.
33. Add 1/10th of the volume (*e.g.* 3 µL to 30 µL) of Reduction and Alkylation solution and incubate for 10 min at RT while mildly shaking on a multi vortex shaker (700 rpm).
34. Digest proteins by adding 2 µL trypsin into the sample solution (0.02 µg/µL final concentration, generally 1 µg trypsin per 100 µg of total protein), carefully mix by pipetting up and down two times, and incubate overnight (>16 h) while vigorously shaking (1,100 rpm) on a heated thermo-shaker at 37 °C.
35. Centrifuge the samples at 3,000× *g* for 4 min and transfer to new LoBind tubes. Samples can now be processed for label-free quantification (LFQ, see **Box 2**) or for tandem mass-tag labelling (TMT, next, **Step 36**). **PAUSE POINT.** Evaporate all liquid in a centrifugal vacuum concentrator at 45 °C until dryness. Samples can be kept at -80 °C for up to 1 month.

TMT labelling of proteomics samples. TIMING. 4-6 h.

<CRITICAL> **Step 42** involves combining aliquots from all the different conditions tested within the same TMT multiplex. Accuracy is improved, because there are fewer missing m/z values for low abundant proteins, and more low abundance peptides enriched with lipid probes can be identified.

Multiplexing samples with very different profiles (*e.g.* labelled with probe and non-labelled) will reduce the chances of identification of peptides only found in a small percentage of the conditions; we suggest using the label-free quantification (LFQ) workflow instead (see **Box 2**).

36. Equilibrate dried TMT reagents to RT, resuspend each TMT labelling reagent aliquot by adding 40 µL acetonitrile and vigorously vortex for 5 min at RT. **CRITICAL STEP.** TMT labelling reagents are highly sensitive to moisture. **CRITICAL STEP.** To maintain reactivity, 0.8 mg TMT stock vials are dissolved in 400 µL anhydrous acetonitrile, split in 40 µL aliquots and evaporated in a centrifugal vacuum concentrator at 45 °C until dryness. TMT aliquots can be kept at -80 °C for up to 6 months.
37. Dissolve any dried sample from **Step 35** by in 40 µL HEPES solution, vortex for 2 min and sonicate for 2 min before continuing with the protocol. Centrifuge the samples at 17,000× *g* for 5 min at RT and transfer each supernatant to a new LoBind tube. Optional: Determine the peptide concentration of the samples using *e.g.* the Quantitative Fluorometric Peptide Assay Kit following the guidelines provided by the supplier. **CRITICAL STEP.** The peptide concentration should be approximately 1 µg/µL in the TMT labelling solution according to the manufacturer's instructions.
38. Verify the pH of the sample is pH 8–8.5 by testing 1 µL on pH paper. **CRITICAL STEP.** TMT labelling occurs optimally at pH 8–8.5 and does not work under acidic conditions. The pH can be adjusted by adding concentrated HEPES buffer (*e.g.* 0.5 M, pH 8.0).
39. Add 40 µL of the corresponding TMT reagent to each sample, reaching a 50% (vol/vol) acetonitrile in HEPES solution, and incubate for 2 h at RT while shaking on a multi-vortex mixer (700 rpm).
40. Add 1 µL hydroxylamine solution to quench the TMT labelling reaction.
41. Combine all TMT-labelled samples of one experiment in equal amounts into a single new 1.5 mL LoBind Eppendorf tube. Evaporate all liquid in a centrifugal vacuum concentrator at 45 °C until dryness, then continue with Stage Tip Fractionation. **PAUSE POINT.** Samples can be kept at -80 °C for up to 1 month.

MS sample clean-up. TIMING. 2-4 h.

42. Prepare Stage Tips for Fractionation (Fractionation Tips from here) by stacking 3 SDB-RPS disks in a 200 µL pipette tip, all at the same distance from the point of the tip.
43. Resuspend the protein digest in 150 µL Fractionation Tip loading solution and thoroughly vortex and sonicate the sample until the solution is homogeneous and clear. **CRITICAL STEP.** TFA acidifies the sample, thereby charging the peptides for Stage Tipping. Ensure pH < 3 using pH paper.
44. Transfer 90% of each supernatant to individual Fractionation Tips, and centrifuge at 2,000× *g* for 2 min. **CRITICAL STEP.** Ensure no aggregates are transferred to the Fractionation Tips; when in doubt leave 10% of the solution which will block the Fractionation Tips. **CRITICAL STEP.** Ensure all liquid has passed through, repeat the centrifugation step, or increase the centrifugation speed if necessary.

45. Add 60 µL Fractionation Tip wash solution, and centrifuge at 2,000× *g* for 2 min. Perform this step three times in total. **CRITICAL STEP.** This will desalt the samples. **CRITICAL STEP.** Ensure all liquid has passed through, repeat the centrifugation step, or increase the centrifugation speed if necessary.
46. Transfer the Fractionation Tip to a new 1.5 mL LoBind Eppendorf tube and elute peptides by adding 60 µL Fraction 1 solution to the Fractionation Tip and centrifuge at 2,000× *g* for 2 min. **CRITICAL STEP.** Ensure all liquid has passed through, repeat the centrifugation step, or increase the centrifugation speed if necessary.
47. Transfer the Fractionation Tip to a new 1.5 mL LoBind Eppendorf tube and elute peptides by adding 60 µL Fraction 2 solution to the Fractionation Tip and centrifuge at 2,000× *g* for 2 min. **CRITICAL STEP.** Ensure all liquid has passed through, repeat the centrifugation step, or increase the centrifugation speed if necessary.
48. Transfer the Fractionation Tip to a new 1.5 mL LoBind Eppendorf tube and elute peptides by adding 60 µL Fraction 3 solution to the Fractionation Tip and centrifuge at 2,000× *g* for 2 min. **CRITICAL STEP.** Ensure all liquid has passed through, repeat the centrifugation step, or increase the centrifugation speed if necessary.
49. Evaporate all solvent in a centrifugal vacuum concentrator at 45 °C until dryness. **PAUSE POINT.** The dried samples can be kept at -80 °C for up to 1 month.

Preparing samples for mass spectrometric analysis. TIMING. 1 h.

50. Add 15 µL MS injection solution to sample. **CRITICAL STEP**. The volume depends on the peptide concentration in the sample (optionally quantified in **Step 37**); we suggest as a rule-of-thumb to inject 0.1-1 µg of peptides and adjust the injection volume according to the maximal intensity observed chromatogram (important to avoid *e.g.* overloading the column or blocking the pre-column).
51. Dissolve the peptides by shaking (700 rpm) for 2 min, followed by sonicating for 3 min and shaking again for 5 min at RT.
52. Prepare Filtration Tips by inserting a 3-layer PVDF Durapore filter (0.1 µm) into a 10 µL pipette tip, and insert these in the septum of the MS vials. Filtration Tips do not need washing of equilibration before use. **CRITICAL STEP.** While the use of this filters is not essential, it provides an easy way of preventing the transfer of particulate contaminants into the MS vials, which are a major cause of blockage of nanoLC columns.
53. Transfer each sample into individual Filtration Tips and centrifuge at 400× *g* for 5 min directly into their corresponding MS vials. **CRITICAL STEP.** Ensure all liquid has passed through, repeat the centrifugation step, or increase the centrifugation speed if necessary.

Running samples on an Orbitrap mass spectrometer. TIMING. At least 8 h.

<CRITICAL> All the parameters listed here are for a Q Exactive mass spectrometer coupled to an EASY nLC-1000 via an EASY-spray source (all Thermo Fischer Scientific) but can be adapted to

other mass spectrometers of similar or superior performance. We recommend using an Orbitrap instrument due to its higher accuracy, especially with regard to measuring TMT labelled samples.

54. **Peptide separation**. In a typical experiment, peptides are separated in an Acclaim PepMap RSLC column of 50 cm× 75 µm inner diameter using a 2-3 h acetonitrile gradient with 0.1% (vol/vol) aqueous formic acid. We recommend using 2 h gradients for LFQ samples and a longer gradient (3 h) for TMT 10-plex samples.
55. **Data acquisition**. The mass spectrometer is operated in data-dependent mode, using a TopN approach for fragmentation peak selection.
    - The survey scan (or MS^1 scan) is set for acquisition from 350 to 1800 *m/z* at a resolution of 70,000 (measured at *m/z* 200), with a maximum injection time of 20 ms and an ion target of 10^6 ions.
    - Up to 10 of the most abundant peaks with charge +2 or higher and an intensity threshold greater than 8.3×10^2 are selected for fragmentation. For LFQ samples, we typically use a 2.0 *m/z* isolation window and a normalized collision energy of 25 for HCD fragmentation. For TMT samples, we typically narrow the isolation window to at least 1.6 *m/z* (instead of 2.0 *m/z*) and increase the normalized HCD collision energy to 31 (instead of 25). **CRITICAL STEP**. All these parameters should be tested and optimized depending on the performance of the mass spectrometer.
    - The MS/MS (or MS^2) scans are acquired with a resolution of 17,500 (at *m/z* 200) for LFQ samples and 35,000 (at *m/z* 200) for TMT samples. The maximum ion injection time is set to 120 ms and the ion target to 2×10^5 ions.

Processing and analysis of proteomics data with MaxQuant and Perseus. TIMING. Variable. Setup 30 min, run time hours to days.

<CRITICAL> MaxQuant and Perseus are under continuous development by Jürgen Cox and co-workers, who make it freely available for the scientific community. However, this also means some aspects might change from one version to the next (*e.g.* rearrangement of some specific tool groupings, novel additions that might streamline the process, etc.). When designing this protocol, we used MaxQuant version 1.6.4.0 and Perseus version 1.6.2.3. For data analysis using PEAKS software, see **Box 3**.

We recommend these guidelines are taken as a starting point of any data analysis performed for lipid probe experiments. However, for best performance and interpretation, data analysis must always be customized for each individual experiment, which is feasible once the basic principles of each step during the data processing are understood. We therefore explained the purpose of each of the steps during data analysis of a typical lipid probe experiment, in order to give the reader the freedom to choose alternative methods to achieve the same aim that might be better suited to the nature of their specific experiments. Notably, the workflow shown here will reveal the proteins reproducibly labelled with lipid probe, when compared to a DMSO control, or in the presence of an inhibitor for the involved lipid transferase (*e.g.* IMP-1088, a potent inhibitor for *N*-myristoyltransferase[17], in combination with metabolic labelling with YnMyr **1**).

Lipidation of proteins is validated by either the direct mass spectrometric evidence of the protein's amino acid being labelled with the lipid probe (*i.e.* high confidence) or by the observation that inhibition of the lipid transferase results in a significant decrease in enrichment of the protein (*i.e.* lower confidence). In a typical chemical proteomics experiment, we would recommend setting up the significance threshold (FDR corrected) to at 5% or smaller.
In addition, we typically consider Log_2 fold changes of above 0.5 and -0.5 as biologically relevant enrichments. It must be noted that these thresholds are arbitrary and should always be chosen in agreement with the precision of the measurements, *i.e.* the variability within replicates, and the sensitivity of the measurement. The latter involves the differences in dynamic range in MS^1 and MS^2-based quantification, which will result in completely different measurable Log_2 fold change ratio windows.

Preparation:

56. Add all the RAW files associated with the experiment in a single, appropriately named folder.
57. Start MaxQuant.
58. In 'Configuration', add the various lipid modifications via 'Add' and then typing the name of the modification, followed by updating the composition via 'Change' to:
    - TMT N-terminus: H(20)C(8)Cx(4)N(1)O(2)Nx(1), mass 229.162932141, with position 'Any N-term', type 'Standard' and Specificities ' - '.
    - TMT Lysines: H(20)C(8)Cx(4)N(1)O(2)Nx(1), mass 229.162932141, with position 'Anywhere', type 'Standard' and Specificities 'K'.
    - YnMyr **1** – AzRB **7**: H(37) O(4) C(22) N(7), with position 'G'.
    - YnPal **2** – AzRB **7**: H(43) O(4) C(22) N(7), with position 'C'.
    - YnF **3** – AzRB **7**: H(38) O(3) C(23) N(7), with position 'C'.
    - YnGG **4** – AzRB **7**: H(46) O(3) C(28) N(7), with position 'C'.
    - YnChol **5** – AzRB **7**: H(59) O(4) C(36) N(7), with position 'Anywhere'.
59. Save each modification individually by first clicking 'Modify table', followed by 'Save changes'.
60. When all modifications have been saved, restart MaxQuant. **CRITICAL STEP.** Restarting MaxQuant allows to check whether all inserted modifications have been saved by the program.
61. For the latest FASTA file with the matching proteome, for instance the human proteome for human material, visit UniProt.org (https://www.uniprot.org/proteomes/UP000005640) and download the 'canonical & isoform' database, and as 'uncompressed'.
62. From here, TMT experiments can be analysed by continuing with **Step 63** (next). For the analysis of samples via label-free quantification (LFQ), see **Box 2**.

Efficiency check of Tandem Mass-Tag (TMT) labelling. TIMING. Variable. Setup 30 min, run time hours to days. **CRITICAL STEP.** As TMT labelling efficiency can vary between TMT channels and

samples, we recommend to first verify the labelling is minimally 98%, as the chemical proteomics results might not correctly represent the biological condition.

63. Start MaxQuant.
64. In ‘Raw data’, load all the RAW files associated with the experiment (**Step 56**).
65. Select the RAW files belonging to the same TMT set and designate them as a single experiment through ‘Set experiment’.
66. Select the RAW files belonging to the same single experiment and set the number of fractions by clicking ‘Set fractions’.
67. In ‘Group-specific parameters’, ensure ‘Type’ is set as ‘Standard’ and ‘Multiplicity’ is set to ‘1’.
68. In ‘Modifications’, add ‘TMT N-terminus’ and ‘TMT Lysines’ in addition to the appropriate modifications (YnMyr **1** / YnPal **2** / YnF **3** / YnGG **4** or YnChol **5**, attached to AzRB **7**) as variable modifications. **CRITICAL STEP**. Ensure that at ‘Variable modifications’, there are already listed ‘Oxidation (M)’ and ‘Acetyl (Protein N-term)’, and at ‘Fixed modifications’, there is already ‘Carbamidomethyl (C)’. MaxQuant normally populates these fields automatically.
69. In ‘Digestion’, ensure the correct protease is selected (in case of our protocol, ‘Trypsin/P’ is sufficient as it includes also LysC).
70. In ‘Global parameters’, add the FASTA file containing the proteome of the investigated organism. After adding the FASTA file, select it and ensure ‘Identifier rule’ is set to ‘Uniprot identifier’, characterized by the specific parse rule “>.*\|(.*)\|“.
71. In ‘Protein quantification’, add the same modifications that were selected earlier as variable modifications. **CRITICAL STEP**. Ensure that ‘Oxidation (M)’ and ‘Acetyl (Protein N-term)’ are listed as ‘Modifications used in protein quantification’. MaxQuant normally populates this field automatically.
72. In ‘Identification’, check ‘Match between runs’ is turned on.
73. In the bottom of the program window, set the number of processors. **CRITICAL STEP.** The maximum number of processors selected for the analysis cannot exceed the number of cores of your computer.
74. Click ‘Start’ and MaxQuant will start analysing the RAW files. **CRITICAL STEP.** The time required for the analysis depends on the size and complexity of the experiment and the capabilities of the computer.
75. When finished, open Perseus.
76. Load two files from the MaxQuant search data into Perseus by clicking ‘Generic matrix upload’, then ‘select’ and find the correct file within the experiment folder following the path: ‘experiment name > combined > txt’. In the dialog on the left,
    - Load the ‘evidence.txt’ file: Transfer ‘Acetyl (Protein N-term)’, ‘TMT N-terminus’, ‘TMT Lysines’ to the ‘Main’ ‘dialog on the right with the ‘>’ button.
    - Transfer ‘id’ onto the ‘Text’ dialog on the right.

- Load the ‘peptides.txt’ file: Transfer ‘Evidence IDs’ onto the ‘Text’ dialog on the right.

77. At the ‘Multi-proc.’ section, select ‘Basic’, then ‘Matching rows by name’. Highlight ‘Base matrix’ and then select the ‘evidence’ data table. Then highlight ‘Other matrix’ and select the ‘peptides’ data table. A new dialog window will appear. Select ‘id’ in ‘Matching column in matrix 1’ and ‘Evidence IDs’ as ‘Matching column in matrix 2’. In ‘Numerical columns’, transfer ‘K count’ from the left to the right dialog. Click ‘OK’.
78. Clean up the data by removing false positives and contaminants. In ‘Filter rows’, choose ‘Filter rows based on categorical column’, and ‘Reverse’ and click ‘OK’. Return to this menu for one last time and choose ‘Potential contaminant’ and click ‘OK’.
79. Calculate the labelling efficiency at lysine residues.
    - In the ‘Basic’ tab from the main ‘Processing’ menu, select ‘Summary statistics (columns)’. On the ‘Columns’ dialog, transfer ‘TMT Lysines’ and ‘K count’ from the left to the right box. Transfer to the left box any other main column that might appear. On the ‘Calculate’ dialog, leave only ‘Sum’ on the right box.
    - Divide the number of all found lysine residues by the number of TMT labelled lysine residues to calculate the proportion of labelled lysines. For complete labelling and reproducibility of the results, it should be >98%.
80. Calculate the TMT labelling efficiency at the N-terminus.
    - In ‘Filter rows’, select ‘Filter rows based on numerical/main column’. Ensure ‘Number of columns’ is ‘1’ and that ‘Acetyl (Protein N-term)’ is selected as the column to be used in the filtering. ‘Number of relations’ should be set to ‘1’ and ‘Relation 1’ dialog set as ‘=0’. Click ‘OK’. This will remove all features detected with an acetylated N-termini.
    - On the ‘TMT N-terminus’ column, calculate the proportion of peptide N-termini that show labelling (represented by ‘1’) as compared to free N-termini (represented by ‘0’). Divide each of these numbers to the total number of features listed in the table. For complete labelling it should be >98%.

Data processing and quantification of Tandem Mass-Tag (TMT) samples in MaxQuant and Perseus. TIMING. Variable, 3 hours to days.

CRITICAL. This workflow can be used to identify and quantify proteins in samples generated with the main workflow (on-bead digestion with trypsin, **Step 34**), as well as those using the sequential on-bead (LysC), off-bead (trypsin) strategy using dimethylated NeutrAvidin-coupled agarose beads (**Box 1**, **Step 13**). In addition, identification and quantification of lipid probe-bound peptides can be achieved with this workflow (**Box 1**, **Step 10**). **CRITICAL**. Validating the TMT labelling efficiency is crucial (**Step 63**), as this directly impacts the validity of the acquired mass spectrometry data and subsequent proteomic analyses. **CAUTION**. Proceed with caution if TMT labelling is less than 98% as the chemical proteomics results might not correctly represent the biological condition.

81. Start MaxQuant.

82. In ‘Raw data’, load all the RAW files associated with the experiment as described in **Step 56**.
83. Select the RAW files belonging to the same TMT set and designate them as a single experiment through ‘Set experiment’.
84. Select the RAW files belonging to the same single experiment and set the number of fractions by clicking ‘Set fractions’.
85. In ‘Group-specific parameters’, ensure ‘Type’ is set as ‘Reporter ion MS2’. Choose the type of TMT employed (*e.g*. 6-plex or 10-plex) and adjust the correction factors for the used isobaric labels by selecting each isobaric label individually and clicking ‘Edit’. These correction factors are provided by the TMT kit manufacturer.
86. In ‘Modifications’, add the appropriate modifications (YnMyr **1** / YnPal **2** / YnF **3** / YnGG **4** or YnChol **5**, attached to AzRB **7**) as variable modifications. **CRITICAL STEP**. Ensure that at ‘Variable modifications’, there are already listed ‘Oxidation (M)’ and ‘Acetyl (Protein N-term)’, and at ‘Fixed modifications’, there is already ‘Carbamidomethyl (C)’. MaxQuant normally populates these fields automatically.
87. In ‘Digestion’, ensure the correct protease is selected (in case of our protocol, ‘Trypsin/P’ is sufficient as it includes also LysC).
88. In ‘Global parameters’, add the FASTA file containing the proteome of the investigated organism. After adding the FASTA file, select it and ensure ‘Identifier rule’ is set to ‘Uniprot identified’, characterized by the specific parse rule “>.*\|(.*)\|“.
89. In ‘Protein quantification’, add the appropriate modifications (YnMyr **1** / YnPal **2** / YnF **3** / YnGG **4** or YnChol **5**, attached to AzRB **7**) as modifications used in protein quantification. **CRITICAL STEP**. Ensure that ‘Oxidation (M)’ and ‘Acetyl (Protein N-term)’ are listed as ‘Modifications used in protein quantification’. MaxQuant normally populates this field automatically.
90. In ‘Identification’, check that ‘Match between runs’ is turned on.
91. In the bottom of the program window, set the number of processors. CRITICAL STEP. The maximum number of the processors selected for the analysis cannot exceed the number of cores of your computer.
92. Click ‘Start’ and MaxQuant will start analysing the RAW files. **CRITICAL STEP.** The time required for the analysis depends on the size and complexity of the experiment and the capabilities of the computer.
93. When finished, open Perseus.
94. Load the MaxQuant data in Perseus by clicking ‘Generic matrix upload’, then ‘select’ and find the ‘proteinGroups.txt’ file within the experiment folder made in **Step 56**, following the path: ‘experiment name > combined > txt’. In the dialog on the left, select all the ‘Reporter intensity corrected’ experiments and add them to ‘Main’ dialog on the right with the ‘>’ button. Finalize with ‘OK’.
95. Clean up the data by removing false positives and contaminants. In ‘Filter rows’, choose ‘Filter rows based on categorical site’, and ensure ‘Only identified by site’ is selected.

Click ‘OK’. Return to this menu and choose ‘Reverse’ and click ‘OK’. Return to this menu for one last time and choose ‘Potential contaminant’ and click ‘OK’.

96. Transform the data to Log_2. In ‘Basic’, go to ‘Transform’ and click ‘OK’.
97. Optional: rename the ‘Reporter intensity corrected *experiment name*’ through the ‘Rearrange’ menu, choosing ‘Rename columns’ or ‘Rename columns [reg. ex.]’ to modify multiple columns at the same time.
98. Designate biological conditions by clicking ‘Annot. rows’ and choosing ‘Categorical annotation rows’. Set ‘Row name’ to ‘Conditions’ (instead of Group1) and designate all conditions with a name (*i.e*. Ctrl, YnLipid, YnLipid+Inhibitor).
99. Group each TMT multiplex together by clicking ‘Annot. rows’ and choosing ‘Categorical annotation rows’. Set ‘Row name’ to ‘TMTplex’ and designate all replicates with numbers (*i.e*. 1, 2, 3, 4 for the individual samples within each 6- or 10-plex).
100. Normalize the data across all samples by subtracting the median across replicates within each TMT multiplex (‘Normalization’, then ‘Subtract’, select ‘Rows’, then ‘TMTplex’ and ‘Median’), followed by normalizing across the conditions (‘Normalization’, then ‘Subtract’, select ‘Columns’ and ‘Mean’).
101. Filter the data by choosing ‘Filter rows’, then ‘Filter rows based on valid values’, choosing ‘Number’, set ‘Mode’ to ‘In each group’ with ‘Groupin’ set to ‘Conditions’ and set at least a minimum of 2 valid values per triplicate. Click ‘OK’.
102. Generate a Volcano-plot by clicking the symbol in main toolbar or under the ‘Misc.’ menu.
103. Further analyse the data with t-tests (‘Tests’, then ‘Two-sample tests’ or ‘Multiple-sample tests’) and ANOVAs (‘Tests’, then ‘Two-way ANOVA’) to statistically mine differences between the various conditions.
104. Optional: add additional annotation, *e.g.* GOBP, GOMF or KEGG names to the proteins, by choosing ‘Add annotation’ in the ‘Annot. columns’ menu. This will allow further investigations in the role of identified proteins.

TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

Table 2: Troubleshooting

Procedure 1 – In-gel fluorescence visualisation of lipid probe-labelled proteins			
Step	**Problem**	**Possible reason(s)**	**Solution**
4	There is evidence of cytotoxicity, *e.g.* cells look morphologically different from untreated cells, display increased	1. The lipid probe concentration is too high. 2. Concentration of DMSO is too high.	1. Keep the lipid probe concentration below 30 µM. Start from **Step 1**. 2. Keep the DMSO concentration below

	apoptosis or necroptosis.		0.1% (v/v) in media. Start from **Step 1**.
15-17	Pellet is pink	Pellet still contains excess capture reagent.	Wash with MeOH again (repeat **Step 17** twice more). If it persists, it may indicate high levels of labelling (continue protocol).
15-17	Pellet is blue	The Click reaction was not quenched with EDTA, leaving the copper in the pellet.	Quench with EDTA. Repeat **Step 13**.
20-22	Cannot resuspend protein pellet	1. The pellet was dried for too long. 2. The pellet was not washed well enough in the previous steps.	1. Do not over dry the pellet. Leave to air-dry for 5 min only. Use a tip-probe sonicator to disrupt the pellet. If pellet size remains unaffected, start from **Step 10**. 2. Ensure the pellet breaks completely by thorough vortexing and sonication in all methanol washes. In normal situations, water-bath sonication is sufficient to resolubilize the pellet. However, tip-probe sonication can sometimes help bring harder pellets into solution.
37	Click reaction does not work *e.g.* no fluorescent bands in the gel.	1. Old reagents 2. The SDS concentration is too high. 1. Too low protein concentration.	**1.** Prepare fresh reagents solutions. Start from **Step 10**. 2. Ensure Lysis buffer does not contain over 0.1% (w/v) SDS. Start from **Step 1**. Verify protein concentration is 1-2 µg/µL. Start from **Step 9**.

37	Fluorescently labelled proteins visible in input, but none in the pulldown fraction	2. Avidin beads might be faulty	1. Use fresh batch of beads. Start from **Step 23**. 2. Do not freeze beads. Start from **Step 23**. 3. Ensure excess, unreacted capture reagent is removed from samples by precipitation. Start from **Step 10.**
Procedure 2 – Identification and quantification of lipid PTMs by chemical proteomics			
Step	**Problem**	**Possible reason(s)**	**Solution**
29-33	Constant loss of beads after wash steps	Withdrawing the supernatant with the pipette too close to the beads	1. Remove supernatant at a slower rate. 2. Remove supernatant with a 10 µL pipette tip stuck in front of the 100 µL or 1 mL pipette tip to be more precise. 3. Keep a 3-5 mm distance from the beads to not disturb the pellet. 4. Leave more supernatant behind and do more washes instead, to lose less beads per wash step. 5. If the amount of agarose varies greatly, start from **Step 23**.
37	No peptides after protease digestion	Proteases did not work	1. Use fresh batch of proteases. Start from **Step 10**. 2. Do not freeze/thaw proteases multiple times; make and use single-use aliquots. Start from **Step 10**.

44-55	Liquid does not go through Fractionation Tip	1. Column material is dirty. 2. Sample pellet blocks the column. Fractionation Tips are centrifuged too fast, packing the material too tight.	1. Store column material in a dust-free environment. 2. Centrifuge the sample before transferring the supernatant to the Fractionation Tip, and do not transfer any pelleted material. Centrifuge the Fractionation Tip at 2,000× *g* for 2 min.
80	TMT labelling is not complete (TMT labelling should be >98% to ensure proper quantification)	1. Incorrect pH *(see manufacturers manual for full troubleshooting Q&A)* 2. Incorrect buffer	1. Ensure labelling happens at pH 8-8.5. Start from **Step 10**. 2. TMT reagents are sensitive to moisture. Ensure aliquoting is performed with anhydrous acetonitrile and aliquots dried immediately after aliquoting without heating. Start from **Step 10**. 3. Avoid using buffers containing free amines, such as Tris. *(see manufacturer manual for full troubleshooting Q&A).* Start from **Step 10**.

ANTICIPATED RESULTS

This protocol can be applied to a broad range of living cells, and has already proven successful in various types of bacteria, mammalian cell lines and in zebrafish embryos and larvae. This section highlights the types of data and results that can be expected from common experiments.

Visualisation of protein lipidation using the presented lipid probes

In an experiment where primary human umbilical vein endothelial cells (EA.hy926) were used to investigate *S*-farnesylation, cells were incubated with a range of YnF **3** concentrations for 18 hours, washed, lysed and then the YnF **3**-labelled proteins were functionalised with AzTB **6**, allowing in-gel

fluorescence visualisation of the YnF **3**-labelled proteins present in the proteome. As shown in **Figure 5a**, there is a direct relationship between the number and intensity of YnF **3**-labelled protein bands and the concentration of lipid probe fed to the cells, up to a maximum of 10 μM. Importantly, feeding these cells with 25 μM YnF **3** resulted in a much lower number and intensity of labelled bands, illustrating the necessity of optimizing metabolic labelling conditions.
The lower labelling at higher concentrations can be caused by multiple factors, including feedback mechanisms related to the synthesis of YnF-PP (see also **Figure 5f**) and the solubility limit of the lipid probe. In the same cell type, labelling with YnGG **4** to study *S*-geranylgeranylation followed a similar optimum (**Figure 5b**).
Labelling of *N*-myristoylation with YnMyr **1** in MCF7 results in dozens of fluorescently identifiable bands across a broad molecular weight range including small *N*-myristoylated proteins as ARL1 (**Figure 5c**). Owing to the small size of ARL1, the attachment of YnMyr **1** and its functionalization with the AzTB **6** capture reagent significantly increases its molecular weight, causing a discernible molecular weight shift on the immunoblot between the native form of ARL1 and the YnMyr **1**-labelled protein, functioning by the same principle as the PEG shift assays introduced by the Hang lab[63].
Another small protein, Sonic hedgehog (SHH) overexpressed in HEK293A cells was investigated in a similar fashion via this molecular weight shift on the immunoblot after labelling *N*-acylation with YnPal **2** (**Figure 5d**) or after labelling of *O*-cholesterylation with YnChol **5** (**Figure 5e**). This also works for endogenously expressed SHH, further illustrating the versatility of the metabolic labelling approach[23].

Visualizing target engagement of lipid transferases via metabolic labelling with lipid probes.
Lipid probes are often used to study inhibition of the biological pathway(s) involved in the lipid PTM. For instance, EA.hy926 cells were incubated with YnF **3** to study *S*-farnesylation, but were co-incubated with different concentrations of farnesol, a natural competitor of YnF **3** as its structure is similar, only missing the terminal alkyne. As shown in **Figure 6a**, across a range of farnesol concentrations, the labelling of *S*-farnesylated proteins with YnF **3** was inhibited.
It should be noted that coupling metabolic labelling with a dose-response proteomics experiment is arguably superior as it enables approximate measurement of in-cell kinetic parameters analogous to Km (concentration of half maximal rate of probe incorporation) and Vmax (maximum rate of incorporation) for each individual substrate protein at once, as shown recently for YnF **3** and YnGG **4**[22].
The incorporation of lipid probes can also be elegantly used as a direct measure of the inhibitory potency of compounds on the targeted lipid transferase(s) within the intact living cell. For instance, as shown in **Figure 6b**, Tipifarnib is capable of inhibiting *S*-farnesylation with YnF **3** in EA.hy926 cells. Moreover, this approach reveals in unprecedented detail how YnF **3** labelling responds for each different *S*-farnesylated protein at varying Tipifarnib concentrations.
Besides fluorescent read-outs, multifunctional capture reagent AzTB **6** furthermore allows biotin-enrichment, as shown in **Figure 6c**, and as shown schematically in **Figure 3b**. HeLa cells were used to study the effect of NMT inhibitor IMP-1088, by reading out the total *N*-myristoylation activity with

YnMyr **1**. As shown in the input panel, IMP-1088 completely inhibited YnMyr **1**-incorporation to background levels. Enrichment of YnMyr **1**-labelled proteins not only allowed a significant fortification of fluorescent TAMRA signal in the pulldown panel, but also enabled the separation of lipid probe-labelled substrate proteins from the same proteins lacking lipid probe incorporation (**Figure 6c**, pulldown panel, and as schematically shown in **Figure 3b**). This is highlighted by the detection of ARL1, as the native unmodified protein remained in the supernatant fraction and only YnMyr **1**-labelled ARL1 was enriched, thereby validating that ARL1 is *N*-myristoylated. HSP90 is not *N*-myristoylated and thus also remains in the supernatant.

Identifying and quantifying target engagement of lipid transferase inhibitors using chemical proteomics.

Labelling with lipid probes bearing bio-orthogonal tags theoretically allows the detection of all such modified proteins through chemical proteomics by mass spectrometry. For instance, *S*-farnesylated proteins in EA.hy926 cells were metabolically labelled with YnF **3**, subsequently functionalised with AzRB **7**, enriched on NeutrAvidin-coupled agarose beads , digested with trypsin and the TMT-labelled peptides were measured by mass spectrometry (**Figure 7a**). In the resulting volcano plot, it can be seen that multiple known *S*-farnesylated proteins are significantly enriched in the YnF **3**-labelled sample compared to the DMSO (untreated) control. This includes the well-validated S-farnesylated protein KRAS[22] (**Figure 7a**, in purple), which is significantly enriched after YnF **3**-labelling. This approach also allows identification of proteins that were previously (and sometimes incorrectly) described as *S*-geranylgeranylated rather than *S*-farnesylated, and even the discovery of novel *S*-farnesylated proteins not previously described as *S*-prenylated but that do contain a CAAX-box. Moreover, other (potentially) *S*-prenylated proteins such as RAB proteins can also be identified, all in a single data-rich experiment. Lastly, this chemical proteomics approach facilitates studying the potency of lipid transferase inhibitors in living cells, with as read-out the reduction of lipid probe incorporation on all identified and quantified lipid PTM substrates. For instance, the inhibition of *S*-farnesylation in EA.hy926 cells with Tipifarnib can be identified using metabolic labelling with YnF **3**. As shown in **Figure 7b**, each line represents the response of individual proteins either *S*-farnesylated, *S*-geranylgeranylated, potentially *S*-prenylated (due to the presence of a CAAX box) and the RAB proteins. This type of profile plot can be generated in *e.g.* Perseus to show the differences and variability in abundance of each protein obtained in each replicate, such as for the known S-farnesylated substrate KRAS in the triplicate samples as shown in **Figure 7b**. However, replicates should not be connected by lines, and the overlaying, numerous lines hamper detailed perusal of the data. Instead, we recommend the use of volcano plots (**Figure 7c**), as these reveal the fold-change for each protein, as well as the significance of this fold-change between the different conditions and within the triplicates. The moderate reduction in KRAS *S*-farnesylation, as visible in the profile plot (**Figure 7b**) proved to be highly significant, as revealed by the volcano plot (**Figure 7c**) and further illustrating the importance of including significance in plots to scrutinize and potentiate chemical proteomics data.

AUTHOR CONTRIBUTIONS

Conceptualization, WWK, NP, TLH and EWT; Methodology, WWK, NP, AGG, JSM; Investigation, WWK, TLH, NP, AGG, JSM, PTC; Resources, EWT; Writing – Original draft, WWK and TLH; Writing – Review & Editing, all authors; Visualisation, WWK; Supervision, EWT.

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COMPETING INTERESTS

EWT is a director and shareholder of Myricx Pharma Ltd, and an inventor on a patent application describing NMT inhibitors including IMP-1088 (Bell, AS; Tate, EW; Leatherbarrow, RJ; Hutton, JA; Brannigan, JA, “Compounds and their use as inhibitors of *N*-myristoyl transferase”, PCT In Appl (2017) WO 2017001812).

DATA AVAILABILITY

The gel lanes depicted in **Figures 5** and **6** are shown uncropped **Supplemental Figures 1** and **2**. The mass spectrometry proteomics data used for **Figure 7** have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD022490.

FIGURE LEGENDS

Figure 1. Background: types of lipid PTMs covered and the corresponding lipid probes.

a. Types and structures of the different lipid PTMs covered by this protocol. Shown are the chemical structures of the lipid (black) and the type of residue to which the PTM is attached (green).
b. Metabolic labelling of *N*-myristoylation using the myristic acid analogue YnMyr **1**.
c. Metabolic labelling of *S*-acylation using the palmitic acid analogue YnPal **2**.

d. Metabolic labelling of *N*-acylation of Hedgehog proteins using YnPal **2**.
e. Metabolic labelling of *S*-farnesylation using farnesol alkyne YnF **3**.
f. Metabolic labelling of *S*-geranylgeranylation using geranylgeraniol alkyne YnGG **4**.
g. Metabolic labelling of *O*-cholesterylation using cholesterol alkyne YnChol **5**.

Figure 2. Schematic overview of the procedures presented.

Cells are metabolically labelled with lipid probe, lysed and the labelled proteins are modified with the capture reagent based on the application. Top: capture reagent AzTB **6** for analysis of probe-labelling by in-gel fluorescence and enrichment on avidin-beads to enable substrate validation by immunoblotting. Bottom: capture reagent AzRB **7** for enrichment on avidin-beads followed by release by trypsin to allow identification and quantification of probe-labelled proteins by chemical proteomics.

Figure 3. Procedure 1 – In-gel fluorescence visualisation of lipid probe-labelled proteins

a. Direct workflow. Cells are metabolically labelled with the alkyne lipid probe that corresponds to the lipid PTM of interest. Hereafter, cells are lysed, and the incorporated probes are ligated to capture reagent AzTB **6**, which facilitates fluorescent visualisation of lipid probe-labelled proteins directly in-gel after electrophoresis.
b. Enrichment workflow. AzTB **6**-functionalised, lipid probe-labelled proteins are enriched on Avidin-beads to increase detection sensitivity and lipid PTM substrate validation by immunoblotting.
c. Primary optimization workflow of metabolic labelling conditions. Sequential optimization of the lipid probe concentration, the labelling time, and if labelling is still below detection, the number of biological material can be scaled up for chemical proteomics.
d. Secondary optimization workflow of metabolic labelling conditions. After optimizations in **c**, metabolic labelling can be modulated with *e.g.* various concentrations of lipid transferase inhibitors, to facilitate identification of specific lipid transferase substrates.

Figure 4. Procedure 2 – Identification and quantification of lipid PTMs by chemical proteomics

a. Cells are metabolically labelled with the alkyne lipid probe that corresponds to the lipid PTM of interest. Hereafter, cells are lysed, and the incorporated probes are ligated to biotin-bearing capture reagent AzRB **7**, which facilitates enrichment on Avidin-beads. Captured proteins are then digested by trypsin and prepared for mass spectrometry.
b. Mass spectrometry sample preparation. Tryptic peptides from **a** are labelled with isobaric TMT tags, mixed with other TMT-tagged peptides from samples from the same experiment, fractionated and analysed by nanoLC-MS/MS, where precursor ions are selected in MS^1 and quantified and sequenced in MS^2.

c. Data analysis workflow. After a database search of the MS/MS files against the proteome of interest, peptides and proteins are identified and quantified, and the results are validated by statistical tests including Pearson correlation, Student's *t*-test and ANOVAs, and optionally, further integrative data analyses that utilize KEGG, STRING or GSEAs.
d. Data visualisation. Results from the data analysis can be visualised in a multitude of ways, including a profile plot showing the fold-change of the abundance per protein between biological replicates and conditions (left). Volcano plots showing the statistical significance of the fold-change per protein in addition to the fold-change between biological conditions (right).

Figure 5. Anticipated results – Visualisation of protein lipidation using the presented lipid probes

a. Visualisation of *S*-farnesylation and the effect of probe concentration on metabolic labelling. EA.hy926 cells were incubated with different YnF **3** concentrations for 18 h, then lysed and incorporated YnF **3** was functionalised with AzTB **6** to enable in-gel fluorescent visualisation of YnF **3-**labelled proteins. In this example, labelling is optimal at 5-10 µM YnF **3**, with multiple protein bands visible across a broad molecular weight range. Equal gel loading is shown by Coomassie (CBB) staining.
b. Visualisation of *S*-geranylgeranylation and the effect of probe concentration on metabolic labelling. EA.hy926 cells were incubated with different YnGG **4** concentrations for 18 h, then lysed and incorporated YnGG **4** was functionalised with AzTB **6** to enable in-gel fluorescent visualisation of YnGG **4**-labelled proteins. In this example, labelling is optimal at 10 µM YnGG **4**, with multiple protein bands visible at around 20-30 kDa. Equal gel loading is shown by Coomassie (CBB) staining.
c. Visualisation of *N*-myristoylation and discerning a molecular weight shift due to probe labelling. MCF-7 cells were incubated with 20 µM YnMyr **1** for 18 h, then lysed and incorporated YnMyr **1** was functionalised with AzTB **6** to enable in-gel fluorescent visualisation of YnMyr **1**-labelled proteins. In this example, labelling reveals dozens of protein bands spanning a broad molecular weight range. Immunoblotting for NMT substrate ARL1 reveals the mass shift caused by the added covalent attachment of YnMyr **1** and AzTB **6** ('Labelled') compared to the native ARL1 form ('Native'). Equal gel loading is shown by HSP90 detection.
d. Visualisation of *N*- and *S*-acylation and discerning a molecular weight shift due to probe labelling. HEK293A cells overexpressing SHH were incubated with 20 µM YnPal **2** for 18 h, then lysed and incorporated YnPal **2** was functionalised with AzT **6b** to enable in-gel fluorescent visualisation of YnPal **2**-labelled proteins. In this example, labelling reveals dozens of protein bands spanning a broad molecular weight range. Immunoblotting for SHH reveals the mass shift caused by the added covalent attachment of YnPal **2** and AzT **6b** ('Labelled') compared to the native SHH form ('Native'). Equal gel loading is shown by TUBA detection.

e. Visualisation of *O*-cholesterylation and discerning a molecular weight shift due to probe labelling. HEK293A cells overexpressing SHH were incubated with 5 μM YnChol **5** for 18 h, then lysed and incorporated YnChol **5** was functionalised with AzTB **6** to enable in-gel fluorescent visualisation of YnChol **5-**labelled proteins. In this example, labelling reveals only a single band corresponding to SHH. Immunoblotting for SHH reveals the mass shift caused by the added covalent attachment of YnChol **5** and AzTB **6** ('Labelled') compared to the native SHH form ('Native'). Equal gel loading is shown by TUBA detection.

Figure 6. Anticipated results – Visualizing target engagement of lipid transferases via metabolic labelling with lipid probes.

a. Inhibition of metabolic labelling with the natural substrate. EA.hy926 cells were incubated with 5 μM YnF **3** in combination with different concentrations farnesol for 18 h, then lysed and incorporated YnF **3** was functionalised with AzTB **6** to enable in-gel fluorescent visualisation of YnF **3**-labelled proteins. In this example, YnF **3** labelling is competed in a farnesol-concentration dependent manner. Equal gel loading is shown by Coomassie (CBB) staining.
b. Competition of metabolic labelling with a lipid transferase inhibitor. EA.hy926 cells were incubated with 5 μM YnF **3** in combination with different concentrations Tipifarnib for 18 h, then lysed and incorporated YnF **3** was functionalised with AzTB **6** to enable in-gel fluorescent visualisation of YnF **3-**labelled proteins. In this example, YnF **3** labelling is competed in a Tipifarnib-concentration dependent manner. Equal gel loading is shown by Coomassie (CBB) staining.
c. Enrichment of labelled proteins combined with small molecule inhibitor. HeLa cells were incubated with 20 μM YnMyr **1** in the presence or absence of IMP-1088, a small molecule inhibitor of NMTs, then lysed and incorporated YnMyr **1** was functionalised with AzTB **6** to enable in-gel fluorescent visualisation of YnMyr **1-**labelled proteins. In this example, labelling reveals dozens of protein bands spanning a broad molecular weight range, which is lost upon NMT inhibition. Immunoblotting for NMT substrate ARL1 reveals the mass shift caused by the added covalent attachment of YnMyr **1** and AzTB **6** ('Labelled') compared to the native ARL1 form ('Native'), which is lost upon NMT inhibition, and only the YnMyr **1**-labelled form is enriched via pulldown. Equal gel loading is shown by HSP90 detection.

Figure 7. Anticipated results – Identifying and quantifying target engagement of lipid transferase inhibitors using chemical proteomics.

a. Volcano plot showing the Log_2 fold-change and significance of proteins identified after enrichment of YnF **3**-tagged proteins and chemical proteomics. Depicted are individual *S*-farnesylated proteins (red triangle, *n* = 27), *S*-geranylgeranylated proteins (dark red triangle, *n* = 20), proteins with a CAAX-box (pink circle, *n* = 44), RAB proteins (black square, *n* = 32)

and other proteins (grey circle, *n* = 1480). The enrichment of KRAS, a known *S*-farnesylated protein and control for YnF **3** labelling, is highlighted in purple. A positive Log_2 fold-change indicates more protein identified in cells with YnF **3** than in cells without YnF **3** (control). Black lines depict the significance threshold at FDR = 0.05 and S0 = 0.1 .

b. Profile plot showing the Log_2 fold-change of proteins identified after inhibition of YnF **3** labelling with Tipifarnib. Depicted are individual *S*-farnesylated proteins (red, *n* = 27), *S*-geranylgeranylated proteins (dark red, *n* = 20), proteins with a CAAX-box (pink, *n* = 44), RAB proteins (black, *n* = 32) and other proteins (grey, *n* = 1480). The response of KRAS, a known *S*-farnesylated protein and control for YnF **3** labelling, is highlighted in purple. A negative Log_2 fold-change indicates less YnF **3**-labelled protein identified in cells treated with Tipifarnib than in cells with DMSO only.

c. Volcano plot showing the Log_2 fold-change of proteins shown in **b**, in addition to the significance of the fold-changes. The inclusion of significance visually separates Tipifarnib-inhibited substrates from non-affected PTM substrates. Depicted are individual *S*-farnesylated proteins (red triangle, *n* = 27), *S*-geranylgeranylated proteins (dark red triangle, *n* = 20), proteins with a CAAX-box (pink circle, *n* = 44), RAB proteins (black square, *n* = 32) and other proteins (grey circle, *n* = 1480). The response of KRAS, a known *S*-farnesylated protein and control for YnF **3** labelling, is highlighted in purple. A negative Log_2 fold-change indicates less YnF **3**-labelled protein identified in cells treated with Tipifarnib than in cells with DMSO only. Black lines depict the significance threshold at FDR = 0.05 and S0 = 0.1.

Box 1 Figure. Lysine dimethylation and two-step digestion strategy to minimize avidin-borne peptide background

a. Dimethylation of lysines makes NeutrAvidin proteolytically resistant against LysC, but not to trypsin as it can still cleave after unmodified arginine residues.

b. Two-step digestion strategy. After enrichment of biotinylated proteins on dimethylated NeutrAvidin-coupled agarose beads, proteins are digested on-bead with LysC, generating lysine-terminal peptides only, primarily from the enriched proteins and not the LysC-resistant NeutrAvidin. The supernatant is then transferred and digested off-bead with trypsin, to generate tryptic peptides ending in either lysine or arginine, which then can be submitted for mass spectrometric analyses. Biotinylated peptides captured by NeutrAvidin can be eluted and then similarly processed.

Supplementary Figure 1. Raw images used for Figure 5a-d.

a. Raw images used for **Figure 5a**.

b. Raw images used for **Figure 5b**.

c. Raw images used for **Figure 5c**.

d. Raw images used for **Figure 5d**.

Supplementary Figure 2. Raw images used for Figure 5e and Figure 6a-c.

a. Raw images used for **Figure 5e**.
b. Raw images used for **Figure 6a**.
c. Raw images used for **Figure 6b**.
d. Raw images used for **Figure 6c**.

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Box 1. Lysine dimethylation and two-step digestion strategy to minimize avidin-borne peptide background.

On-bead digestion of biotin/avidin-enriched proteins using trypsin yields not only peptides of enriched proteins, but also a prominent background of avidin-derived peptides. Dimethylation of the lysines of NeutrAvidin makes the protein proteolytically resistant to LysC, enabling a sequential on-bead/off-bead digestion strategy using LysC followed by trypsin, thereby significantly boosting protein identification rates[36]. As a rule of thumb, use 30 µL of NeutrAvidin-coupled agarose beads per 1 mg of total protein (as per the manufacturers' instructions (Thermo Scientific).

Additional Materials

TEAB solution

This solution is 100 mM TEAB, pH 8.5, in ultrapure water. **CRITICAL.** TEAB is volatile and will evaporate. Prepare freshly by dilution from commercial 1 M TEAB, and discard within a day. Check the pH before use.

Dimethylation solution

This solution is 0.2% (vol/vol) formaldehyde and 25 mM sodium cyanoborohydride dissolved in 100 mM TEAB, pH 8.5, in ultrapure water. **CAUTION.** Work in a fume-hood as formaldehyde, sodium cyanoborohydride and by-products are toxic. **CRITICAL.** Prepare freshly and discard within a day.

Dimethylation quenching solution

This solution is 1% (vol/vol) ethanolamine dissolved in 100 mM TEAB, pH 8.5, in ultrapure water. **CRITICAL.** Prepare freshly and discard within a day.

NeutrAvidin Elution solution

This solution is 150 µL 80% (vol/vol) acetonitrile, 0.1% (vol/vol) TFA and 0.2% (vol/vol) formic acid in ultrapure water. **CRITICAL.** Prepare freshly and discard within a day.

Procedure. TIMING. 4-6 h.

CAUTION. Perform the following steps in a fume-hood and always wear personal protective equipment. Formaldehyde, sodium cyanoborohydride ($NaBH_3CN$) and by-products are toxic. All waste should be handled accordingly and disposed of according to regulations.

1. Cut the point of a 1 mL pipette tip to allow transfer of solutions containing NeutrAvidin-coupled agarose beads.
2. Transfer 1.5× the total required volume of NeutrAvidin-coupled agarose beads to a conical 15 mL tube with screw cap. **CRITICAL STEP.** The volume of NeutrAvidin-coupled agarose beads per sample should be titrated for the abundance of biotinylated proteins within the samples. Generally, 30 µL derivatized NeutrAvidin-coupled agarose beads per 1 mg total protein is an effective starting point.
3. Wash 1 volume of beads with 5 volumes of TEAB solution for 1 min at RT with shaking (700 rpm), then centrifuge at 3,000× *g* for 3 min at RT, discard supernatant and resuspend pellet. Perform this step five times in total. **CRITICAL STEP.** Washes remove

traces of free-amine-containing components that will compete in dimethylation reaction. **CRITICAL STEP.** Ensure solution is sufficiently agitated to prevent pellet formation.

4. Dimethylate by resuspending the pelleted NeutrAvidin-coupled agarose beads with 5 volumes of dimethylation solution, incubate for 3 h at RT with shaking (700 rpm), then centrifuge at 3,000× *g* for 3 min at RT and discard supernatant. **CRITICAL STEP.** Ensure that the solution is sufficiently agitated to prevent pellet formation.
5. Resuspend beads with 5 volumes of dimethylation quenching solution and incubate for 2 min at RT with shaking (700 rpm), then centrifuge at 3,000× *g* for 3 min at RT, discard supernatant. Perform this step three times in total. **CRITICAL STEP.** Ensure solution is sufficiently agitated to prevent pellet formation.
6. Wash by resuspending the NeutrAvidin-coupled agarose beads with 5 volumes of HEPES buffer, then centrifuge at 3,000× *g* for 3 min at RT, discard supernatant. Perform this step three times in total. **PAUSE POINT.** Dimethylated NeutrAvidin-coupled agarose beads can be kept at 4 °C for up to 1 week.

On-bead digestion with LysC, off-bead digestion with trypsin. TIMING. 18-24 h.

7. Resuspend the dimethylated NeutrAvidin-coupled agarose beads in 30 µL HEPES solution, add 2 µL LysC protease directly into the solution, and carefully mix by pipetting up and down twice.
8. Incubate with LysC for 1 h while vigorously shaking (1,100 rpm) in a heated thermo-shaker at 37 °C.
9. Centrifuge the mixture at 3,000× *g* for 4 min and transfer 90% of the supernatant to a new 1.5 mL LoBind Eppendorf tube and continue to **Step 13** if isolation of probe-labelled peptides is not required. **CRITICAL STEP.** Do not transfer any dimethylated NeutrAvidin-coupled agarose beads. Trypsin can still digest NeutrAvidin by cleaving after arginine residues, thereby contaminating the sample with NeutrAvidin peptides. Thus, it is preferable to leave part of the supernatant behind than to transfer any NeutrAvidin-coupled agarose beads. **CRITICAL STEP.** Keep the beads after LysC digestion, as probe-labelled peptides functionalised with AzRB **7** (or AzB) still remain bound to the avidin beads. These will be eluted next (**Step 10**).
10. Elute AzRB **7** or AzB-functionalised, probe-labelled peptides from the beads with 150 µL NeutrAvidin Peptide elution solution. Incubate for 15 min at RT while mildly shaking on a multi-vortex shaker (700 rpm). Centrifuge the mixture at 3,000× *g* for 4 min and transfer 90% of the supernatant to a new 1.5 mL LoBind Eppendorf tube. Perform this step once more. **CRITICAL STEP.** Do not transfer any beads. Trypsin can still digest NeutrAvidin by cleaving after arginine residues, thereby contaminating the sample with NeutrAvidin peptides. Thus, it is preferable to leave part of the supernatant behind than to transfer any NeutrAvidin-coupled agarose beads. **CRITICAL STEP**. The eluted peptides can be analysed as separate samples, or added to the peptides from **Step 9**.
11. Evaporate all liquid in a centrifugal vacuum concentrator at 45 °C until dryness.

12. Dissolve the dried sample in 40 µL HEPES solution, vortex for 2 min and sonicate for 2 min.
13. To LysC supernatant (**Step 9**), eluted peptides (**Step 10**), or both, add 1/10th of the volume (*e.g.* 4 µL to 40 µL) of Reduction and Alkylation solution and incubate for 10 min at RT while mildly shaking on a multi-vortex shaker (700 rpm).
14. Digest proteins by adding 2 µL trypsin into the sample solution (0.02 µg/µL final concentration, generally 1 µg trypsin per 100 µg of total protein), carefully mix by pipetting up and down two times, and incubate overnight (>16 h) while vigorously shaking (1,100 rpm) on a heated thermo-shaker at 37 °C.
15. Samples can now be processed for label-free quantification (LFQ, see **Box 2**) or for tandem mass-tag labelling (TMT, **Procedure 2**, **Step 36**). **PAUSE POINT.** Evaporate all liquid in a centrifugal vacuum concentrator at 45 °C until dryness. Samples can be kept at -80 °C for up to 1 month. Dissolve sample by in 40 µL HEPES buffer, vortex for 2 min and sonicate for 2 min before continuing with the protocol.

Box 2. Label-Free Quantification (LFQ) – Sample preparation and chemical proteomics data analysis.

LFQ is an alternative for TMT labelling and continues from **Procedure 2**, **Step 37**. This workflow can be used to identify and quantify proteins in samples generated with the main workflow (on-bead digestion with trypsin, **Step 34**), as well as those using the sequential on-bead (LysC), off-bead (trypsin) strategy using dimethylated NeutrAvidin-coupled agarose beads (**Box 1**, **Step 13**). In addition, identification and quantification of lipid probe-bound peptides can be achieved with this workflow (**Box 1**, **Step 10**).

Procedure. TIMING. 1-2 h.

1. Centrifuge the samples obtained from **Procedure 2**, **Step 36** at 3,000× *g* for 4 min at RT and transfer the supernatant to a new LoBind tube.
2. Acidify the samples with TFA to reach 0.5% (vol/vol) and thoroughly vortex and sonicate the sample until the solution is homogeneous. **CRITICAL STEP.** TFA acidifies the sample, thereby charging the peptides homogeneously for Stage Tipping. Ensure that sample pH < 3.
3. Prepare Stage Tips for Desalting (Desalting Tips) by stacking 3 SDB-XC (or C_{18}) disks in a 200 µL pipette tip, all at the same distance from the point of the tip.
4. Activate the Desalting Tips with 150 µL methanol, and centrifuge at 2,000× *g* for 2 min. **CRITICAL STEP.** Ensure all liquid has passed through, repeat the centrifugation step, or increase the centrifugation speed if necessary.
5. Equilibrate the Desalting Tips with 150 µL proteomic-grade ultrapure water, and centrifuge at 2,000× *g* for 2 min. **CRITICAL STEP.** Ensure all liquid has passed through, repeat the centrifugation step, or increase the centrifugation speed if necessary.
6. Centrifuge the samples at 17,000× *g* for 5 min at RT and transfer 90% of each supernatant to individual Desalting Tips, and centrifuge at 2,000× *g* for 2 min. **CRITICAL STEP.** Leave 10% of the solution to avoid transferring any aggregates which will block the Desalting Tips. **CRITICAL STEP.** Ensure all liquid has passed through, repeat the centrifugation step, or increase the centrifugation speed if necessary.
7. Wash with 150 µL proteomic-grade ultrapure water, and centrifuge at 2,000× *g* for 2 min. **CRITICAL STEP.** Ensure all liquid has passed through, repeat the centrifugation step, or increase the centrifugation speed if necessary.
8. Transfer the Desalting Tip to new 1.5 mL LoBind Eppendorf tube and elute peptides by adding 60 µL Elution buffer to the Desalting Tip and centrifuge at 2,000× *g* for 2 min. **CRITICAL STEP.** Ensure all liquid has passed through, repeat the centrifugation step, or increase the centrifugation speed if necessary.
9. Place the samples into a centrifugal vacuum concentrator and incubate at 45 °C until dryness. **PAUSE POINT.** Samples can be kept at -80 °C for up to 6 months.
10. Samples can now be submitted for mass spectrometric analysis. Continue with **Procedure 2**, **Step 51**.

Data processing and analysis of Label-Free (LFQ) samples in MaxQuant and Perseus. TIMING. Variable, 3 h to more.

1. Start MaxQuant.
2. In ‘Raw data’, load all the RAW files associated with the experiment. Set ‘No fractions’, this will give each sample an individual Experiment name.
3. In ‘Group-specific parameters’, ensure ‘Type’ is set as ‘Standard’. In ‘Modifications’, add the appropriate modifications (YnMyr **1** / YnPal **2** / YnF **3** / YnGG **4** or YnChol **5**, attached to AzRB **7**) as variable modifications. **CRITICAL STEP**. Ensure that at ‘Variable modifications’, there are already listed ‘Oxidation (M)’ and ‘Acetyl (Protein N-term)’, and at ‘Fixed modifications’, there is already ‘Carbamidomethyl (C)’. MaxQuant normally populates these fields automatically.
4. In ‘Digestion’, ensure the correct protease is selected (in case of our protocol, ‘Trypsin/P’ is sufficient as it includes also LysC).
5. In ‘Label-free quantification’, select ‘LFQ’. All pre-set parameters are sufficient for the analysis.
6. In ‘Global parameters’, add the FASTA file containing the proteome of the investigated organism. After adding the FASTA file, select it and ensure ‘Identifier rule’ is set to ‘UniProt identified’, characterized by the specific parse rule “>.*\|(.*)\|“.
7. In ‘Protein quantification’, add the appropriate modifications (YnMyr **1** / YnPal **2** / YnF **3** / YnGG **4** or YnChol **5**, attached to AzRB **7**) as modifications used in protein quantification. **CRITICAL STEP**. Ensure that ‘Oxidation (M)’ and ‘Acetyl (Protein N-term)’ are listed as ‘Modifications used in protein quantification’. MaxQuant normally populates this field automatically.
8. In ‘Identification’, check that ‘Match between runs’ is turned on.
9. In the bottom of the program window, set the number of processors. **CRITICAL STEP.** The maximum number of processors selected for the analysis cannot exceed the number of cores of your computer.
10. Click ‘Start’ and MaxQuant will start analysing the RAW files. **CRITICAL STEP.** The time required for the analysis depends on the size and complexity of the experiment and the capabilities of the computer.
11. When finished, open Perseus.
12. Load the MaxQuant data in Perseus by clicking ‘Generic matrix upload’, then ‘select’ and find the ‘proteinGroups.txt’ file within the experiment folder, following the path: ‘experiment name > combined > txt’. In the dialog on the left, select all the ‘LFQ intensity’ experiments and add them to ‘Main’ dialog on the right with the ‘>’ button. Finalize with ‘OK’.
13. Clean up the data by removing false positives and contaminants. In ‘Filter rows’, choose ‘Filter rows based on categorical site’, and ensure ‘Only identified by site’ is selected.

Click ‘OK’. Return to this menu and choose ‘Reverse’ and click ‘OK’. Return to this menu for one last time and choose ‘Potential contaminant’ and click ‘OK’.

14. Transform the data to Log_2. In ‘Basic’, go to ‘Transform’ and click ‘OK’.
15. Optional: rename the ‘LFQ intensity *experiment name*’ through the ‘Rearrange’ menu, choosing ‘Rename columns’ or ‘Rename columns [reg. ex.]’ to modify multiple columns at the same time.
16. Designate biological conditions by clicking ‘Annot. rows’ and choosing ‘Categorical annotation rows’. Set ‘Row name’ to ‘Conditions’ (instead of Group1) and designate all conditions with a name (*i.e.*, Ctrl, YnLipid, YnLipid+Inhibitor).
17. Group replicates together by clicking ‘Annot. rows’ and choosing ‘Categorical annotation rows’. Set ‘Row name’ to ‘Replicates’ and designate all replicates with numbers (*i.e.*, 1, 2, 3, 4 for the individual samples within a quadruplicate).
18. Filter the data by choosing ‘Filter rows’, then ‘Filter rows based on valid values’, choosing ‘Number’, set ‘Mode’ to ‘In each group’ with ‘Groupin’ set to ‘Conditions’ and set at least a minimum of 2 valid values per triplicate. Click ‘OK’.
19. Generate a Volcano-plot by clicking the symbol in main toolbar or under the ‘Misc.’ menu.
20. Further analyse the data with t-tests (‘Tests’, then ‘Two-sample tests’ or ‘Multiple-sample tests’) and ANOVAs (‘Tests’, then ‘Two-way ANOVA’) to statistically mine differences between the various conditions.
21. Optional: add additional annotation, *e.g.* GOBP, GOMF or KEGG names to the proteins, by choosing ‘Add annotation’ in the ‘Annot. columns’ menu. This will allow further investigations in the role of identified proteins.

Box 3. Identification of NeutrAvidin-enriched proteins and the lipid probe modification sites using PEAKS studio software.

PEAKS studio software is developed by Bioinformatics Solutions Inc. (BSI) for proteomic analyses. A 30-day free trial is available for PEAKS studio, where after it will run in Viewer Mode only, unless a license is purchased.

Preparation. TIMING. 1 h or less.

1. Start PEAKS Studio.
2. In 'Window', choose 'Configuration'.
3. In the configuration window, click 'Enzyme' and designate the digestion method used (*e.g.* trypsin), or add a customised method with specific cleavage sites (for that, go to 'New', insert a name and then the required cleavage sites, save by clicking 'Add/Update'). **CRITICAL.** For studying *S*-prenylation with YnF **3** or YnGG **4**, create a modified trypsin method, containing cleavages after Lys (K), Arg (R) and Cys (C). The latter occurs as the cysteine at the C-terminus is cleaved by RCE1 during the prenylation process.
4. Then continue to 'PTM' and add the lipid modifications by clicking 'New' and then inserting the name of modification, along with the abbreviation and mono-isotopic mass. As the sample is a digest, make sure 'peptide' is ticked and insert the amino acid residues expected to be modified (*e.g.* G for glycine in *N*-myristoylation or C for cysteine in *S*-prenylation and *S*-palmitoylation) and the position in the peptide (anywhere, N- or C-terminus). Finally, insert the molecular formula of the modification. After adding all this information, click 'OK' to save. **CRITICAL.** The mono-isotopic mass of the modification can be calculated using *e.g.* ChemDraw using 'Analysis' and then clicking 'Exact mass'. **CRITICAL.** If it is unknown where the modification occurs, add 'X' to 'Anywhere' in the box next to 'Residues to be modified'.
    - YnMyr **1** – AzRB **7**: mass 463.2907, modified at N-terminus, formula $H_{37}O_4C_{22}N_7$.
    - YnPal **2** – AzRB **7**: mass 481.3376, modified at Cys (C), formula $H_{43}O_4C_{22}N_7$.
    - YnF **3** – AzRB **7**: mass 459.2958, modified at Cys (C), formula $H_{37}O_3C_{23}N_7$.
    - YnGG **4** – AzRB **7**: mass 527.3584, modified at Cys (C), formula $H_{45}O_3C_{28}N_7$.
    - YnChol **5** – AzRB **7**: mass 653.4638, modified anywhere (X), formula $H_{59}O_4C_{36}N_7$.
5. Then continue to 'Database'. In this window, go to 'FASTA format database' and choose 'UniProtKB/Swiss-Prot' from the drop-down menu. Then insert the name of the database, *e.g.* 'Homo sapiens proteome' and click 'Browse' to find the FASTA file saved on the computer. After all information has been added, click 'Add/Update' to save. **CRITICAL**. For the latest FASTA file with the human proteome, visit UniProt.org (https://www.uniprot.org/proteomes/UP000005640) and download the 'canonical & isoform' database, and as 'uncompressed'.
6. Under 'Instrument', choose the mass spectrometer setup used for the measurements. For instance, for a Q-Exactive setup, the following details can be used: Orbitrap ('Orbi-Orbi'), ESI (nano-spray) ion source, MS precursor scan with FT-ICR/Orbitrap and MS/MS

product scan with Linear Ion Trap, Precursor mass search using ‘monoisotopic’, parent mass error tolerance 15.0 ppm and fragment mass error tolerance 0.5 Da. If the program has the option to directly choose ‘Orbi-Orbi’, this will automatically update to the optimal Q-Exactive settings.

7. After all adjustments have been made, click ‘Close’ to return to the main PEAKS window.

For setting up PEAKS site identification search. TIMING. Variable, 3 h or more.

8. Start PEAKS Studio.
9. Click ‘File’ and then select ‘New project’.
10. There, designate a ‘Project name’ and choose the project location. **CRITICAL**. Ensure there is sufficient free space on the designated drive to allow the analysis to run. **CRITICAL.** The project location should be placed on the drive that allows the fastest read/write speeds; external USB drives may significantly slow down the analysis.
11. Load the RAW files into PEAKS by clicking ‘Add Data’, which loads all the data into the left window.
12. For TMT samples, select all the RAW files associated with the fractions from a single TMT run, and click ‘Create a new sample for selected files’, which will make PEAKS merge these files together in a new sample. Continue this process for the remaining RAW files obtained for the remaining fractionated TMT samples. For LFQ samples, select all the RAW files in the left window and click ‘Create a new sample for each file’, which will make PEAKS treat all files as individual samples.
13. In the column next to ‘Samples’, designate the enzyme (*e.g.* trypsin), then the instrument used (*e.g.* Orbitrap) and the employed fragmentation method (*e.g.* CID).
14. Click ‘Data Refinement >’ button to continue to the next window.
15. In the ‘Data Refinement’ window, top right, set the ‘Predefined parameters’ drop-down window to ‘default’ and make sure both ‘Correct Precursor’ and the ‘Mass only (recommended)’ are checked. **CRITICAL**. These pre-set parameters are sufficient for the analysis.
16. Next, click ‘Identification >’ to set up the parameters for the database search and the peptide identification.
17. In the following ‘PEAKS Search’ window, make sure that the ‘Predefined parameters’ are again set to ‘Default’ from the drop-down menu on the top right. Then, make sure that in ‘Error tolerance’, the ‘Precursor mass’ is set to 15.0 ppm using ‘monoisotopic mass’, and the ‘Fragment ion’ is set to 0.5 Da (If the program has the option to directly chose ‘Orbi-Orbi’, this will automatically update to the optimal Q-Exactive settings). Under ‘Enzyme’, choose ‘Specified by each sample’ from the drop-down menu, and set the program to ‘Allow non-specific cleavage at ‘one’ end of the peptide’, and set the maximum of ‘three’ missed cleavages (both to be chosen from drop-down menus). **CRITICAL**. These parameters are critical for the correct analysis.

18. In ‘PTM’, click ‘Set PTM’, and choose the modifications to be included in the analysis. Minimally, add ‘Carbamidomethylation’, ‘Oxidation’ and the appropriate lipid modification(s) (*i.e.* YnMyr **1** / YnPal **2** / YnF **3** / YnGG **4** or YnChol **5**, attached to AzRB **7**) to the ‘Selected Variable PTM’ window using the ‘=>’ button. When complete, click ‘OK’. Then, set the ‘Maximally allowed variable PTM per peptide’ to 5.
19. In ‘Database’, select the FASTA file containing the proteome information, and optionally, at ‘Contaminant database’, a FASTA file containing potential contaminant proteins. **CRITICAL.** These parameter settings can be saved for later analyses using the ‘Save’ option in the ‘Predefined parameters’ tab.
20. Lastly, under ‘General options’, ensure that ‘Estimate FDR with decoy-fusion’ is selected. **CRITICAL.** There are two more options: ‘Find unspecific PTMs and common mutations with PEAKS PTM’ and ‘Find more mutations with SPIDER’. In total, >485 already built-in modifications can be searched by PEAKS in these analyses, effectively exploding the search space. This number can be reduced by selecting only preferred modifications through clicking ‘Advanced’ settings. However, to minimise computer resource and the time required for the analysis purely based on the lipid modifications (*e.g.* YnMyr **1**), unselect all these options.
21. Click ‘Finish’ to start the PEAKS search. **CRITICAL.** The time required for the search is variable and may take up to multiple days.
22. When the search is finished, the ‘PEAKS identification’ window will appear in the Project tree, on the left of the PEAKS window. Click ‘PEAKS’ to open the identification result window, which includes ‘Summary’, ‘Protein’, ‘Peptide’, ‘De novo only’ and ‘LC/MS’. **CRITICAL.** Opening this window may take some time, depending on the speed of the computer.
23. Go to the ‘Summary’ window. At the top the window, set at ‘Peptides’ the FDR to 1.0% by clicking ‘FDR’ and choosing ‘1.0%’, which closes the FDR window. Then, at ‘Proteins’, set the ‘-10lgp≥’ to ‘20’ and unique peptides to ‘≥1’ unique peptides. Set ‘*de novo* only’ to ALC ≥50% and -10lgp ≤50. Click ‘Apply’ to filter the data. **CRITICAL.** Peptide FDR is set to 1.0% to filter out 99% of the false positive peptides from the analysis. **CRITICAL.** The settings for the protein analysis make sure that more than one unique significantly identified peptide is used to identify a specific protein. **CRITICAL.** All the data, including the analyses, can be exported by clicking the ‘Export’ button from the ‘Summary’ view.
24. In the ‘Summary’ window, the ‘Results statistics’ shows a summary of all the statistics and filter parameters used in the analysis and visualises a PTM profile.
25. The ‘Protein’ window shows the list of identified proteins, and the sequence coverage for each of the identified proteins. In the top row, proteins can be sorted by ‘PTM’, allowing quick perusal of proteins containing the PTM-modified peptide (*e.g.* YnMyr **1** attached to N-terminal glycine or YnPal **2** attached to a cysteine).
26. The ‘Peptide’ window shows the list of identified peptides and the corresponding spectrum, along with more detailed information. At the top of the window, again the

peptides can be sorted by ‘PTM type’, ‘PTM site’ and by the mass of the PTMs set for the PEAKS search (*e.g.* +459.30 for YnF **3** attached to AzRB **7**). Then, each peptide can be individually selected, revealing the spectrum of *b* and *y* ions. The PTM-modified amino acid will be shown with a small letter, and the unmodified amino acid by a capital letter. **CRITICAL.** The significance of the identified peptide, as per -10logP, as well as the complete sequencing of the peptide in terms of *b* and *y* ions is critical for designating the site of modification.

Figure 1

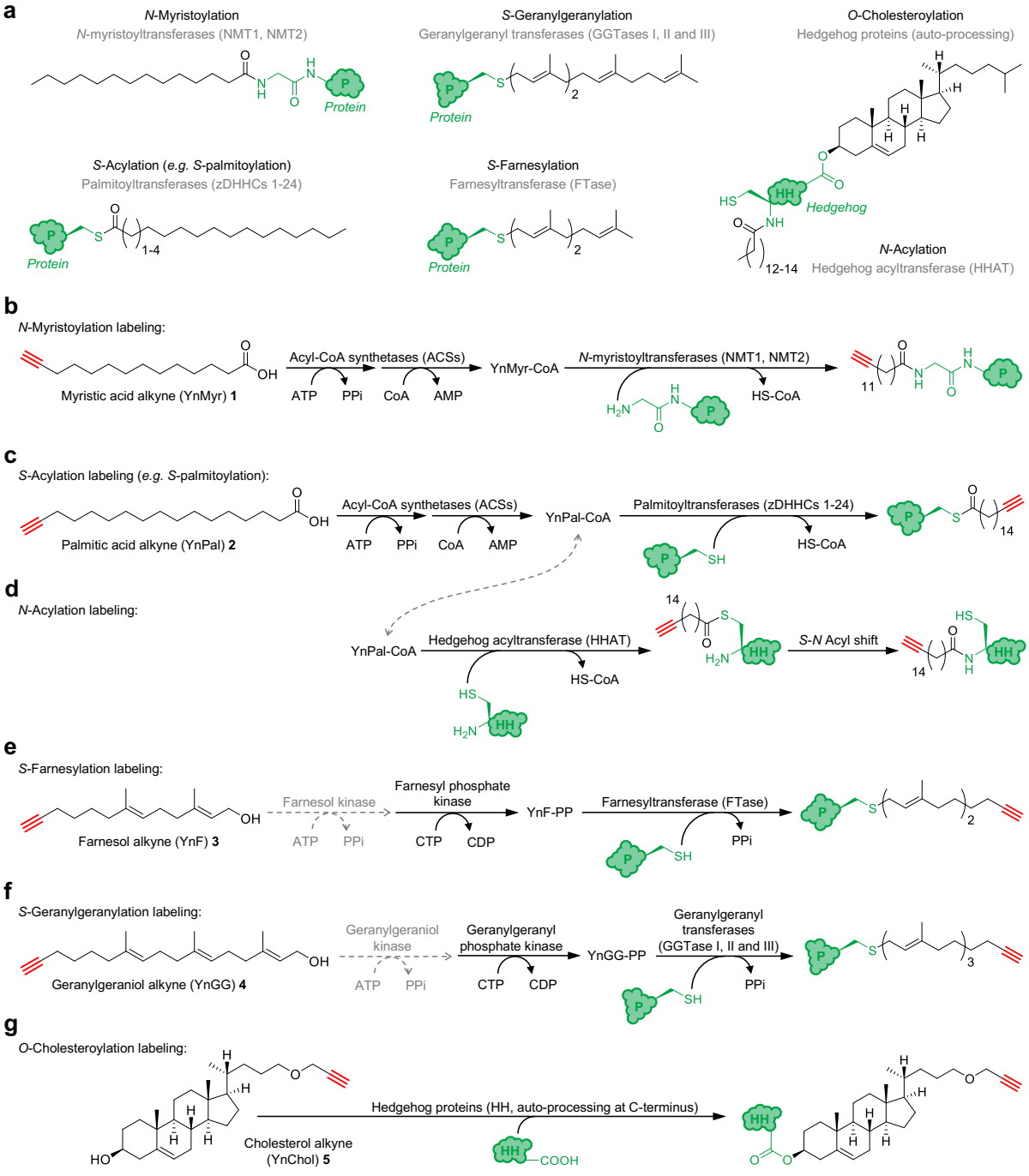

a
N-Myristoylation
N-myristoyltransferases (NMT1, NMT2)
Protein
S-Geranylgeranylation
Geranylgeranyl transferases (GGTases I, II and III)
Protein
O-Cholesteroylation
Hedgehog proteins (auto-processing)
Hedgehog
S-Acylation (e.g. S-palmitoylation)
Palmitoyltransferases (zDHHCs 1-24)
Protein
S-Farnesylation
Farnesyltransferase (FTase)
Protein
N-Acylation
Hedgehog acyltransferase (HHAT)
b
N-Myristoylation labeling:
Myristic acid alkyne (YnMyr) 1
Acyl-CoA synthetases (ACSs)
ATP
PPi
CoA
AMP
YnMyr-CoA
N-myristoyltransferases (NMT1, NMT2)
HS-CoA
c
S-Acylation labeling (e.g. S-palmitoylation):
Palmitic acid alkyne (YnPal) 2
Acyl-CoA synthetases (ACSs)
ATP
PPi
CoA
AMP
YnPal-CoA
Palmitoyltransferases (zDHHCs 1-24)
HS-CoA
d
N-Acylation labeling:
YnPal-CoA
Hedgehog acyltransferase (HHAT)
HS-CoA
S-N Acyl shift
e
S-Farnesylation labeling:
Farnesol alkyne (YnF) 3
Farnesol kinase
ATP
PPi
Farnesyl phosphate kinase
CTP
CDP
YnF-PP
Farnesyltransferase (FTase)
PPi
f
S-Geranylgeranylation labeling:
Geranylgeraniol alkyne (YnGG) 4
Geranylgeraniol kinase
ATP
PPi
Geranylgeranyl phosphate kinase
CTP
CDP
YnGG-PP
Geranylgeranyl transferases (GGTase I, II and III)
PPi
g
O-Cholesteroylation labeling:
Cholesterol alkyne (YnChol) 5
Hedgehog proteins (HH, auto-processing at C-terminus)

Figure 2

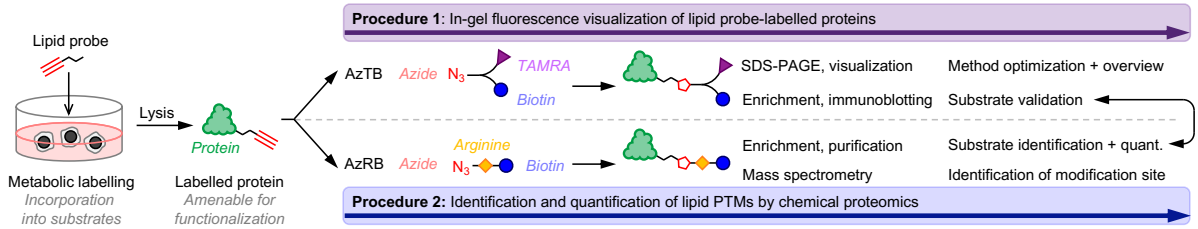

Procedure 1: In-gel fluorescence visualization of lipid probe-labelled proteins
Lipid probe
Lysis
Protein
Metabolic labelling
Incorporation into substrates
Labelled protein
Amenable for functionalization
AzTB
Azide
N3
TAMRA
Biotin
SDS-PAGE, visualization
Enrichment, immunoblotting
Method optimization + overview
Substrate validation
AzRB
Azide
Arginine
N3
Biotin
Enrichment, purification
Mass spectrometry
Substrate identification + quant.
Identification of modification site
Procedure 2: Identification and quantification of lipid PTMs by chemical proteomics

Figure 3

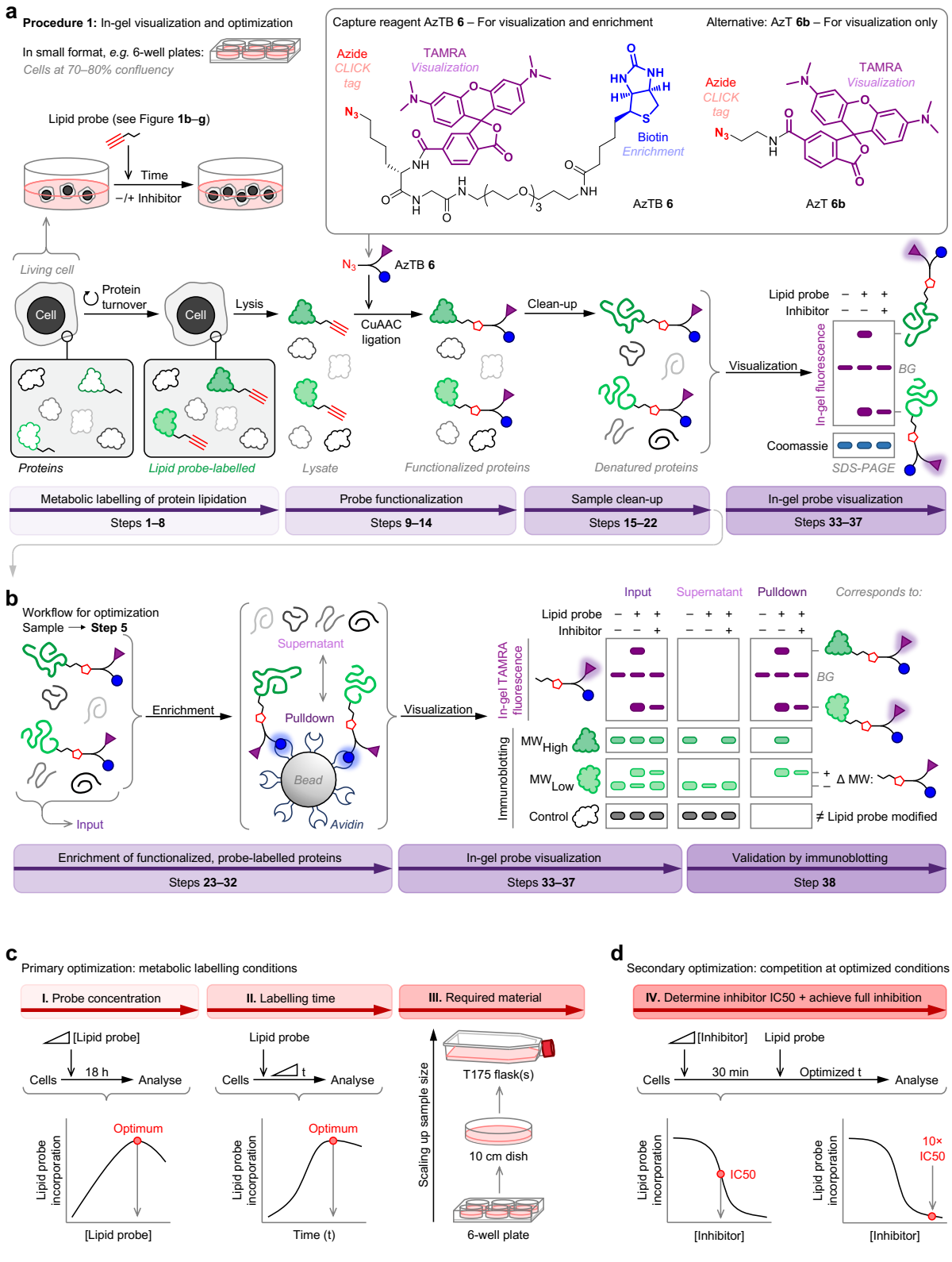

a
Procedure 1: In-gel visualization and optimization
In small format, e.g. 6-well plates:
Cells at 70–80% confluency
Lipid probe (see Figure 1b–g)
Time
–/+ Inhibitor
Living cell
Capture reagent AzTB 6 – For visualization and enrichment
Azide
CLICK tag
TAMRA
Visualization
Biotin
Enrichment
AzTB 6
Alternative: AzT 6b – For visualization only
AzT 6b
AzTB 6
Cell
Protein turnover
Lysis
CuAAC ligation
Clean-up
Visualization
Lipid probe
Inhibitor
In-gel fluorescence
BG
Coomassie
SDS-PAGE
Proteins
Lipid probe-labelled
Lysate
Functionalized proteins
Denatured proteins
Metabolic labelling of protein lipidation
Steps 1–8
Probe functionalization
Steps 9–14
Sample clean-up
Steps 15–22
In-gel probe visualization
Steps 33–37
b
Workflow for optimization
Sample → Step 5
Enrichment
Supernatant
Pulldown
Bead
Avidin
Input
Visualization
Input
Supernatant
Pulldown
Corresponds to:
Lipid probe
Inhibitor
In-gel TAMRA fluorescence
BG
Immunoblotting
MW High
MW Low
Control
Δ MW:
≠ Lipid probe modified
Enrichment of functionalized, probe-labelled proteins
Steps 23–32
In-gel probe visualization
Steps 33–37
Validation by immunoblotting
Step 38
c
Primary optimization: metabolic labelling conditions
I. Probe concentration
II. Labelling time
III. Required material
[Lipid probe]
Cells
18 h
Analyse
Optimum
Lipid probe incorporation
[Lipid probe]
Lipid probe
t
Time (t)
Scaling up sample size
T175 flask(s)
10 cm dish
6-well plate
d
Secondary optimization: competition at optimized conditions
IV. Determine inhibitor IC50 + achieve full inhibition
[Inhibitor]
30 min
Optimized t
IC50
10× IC50
[Inhibitor]

Figure 4

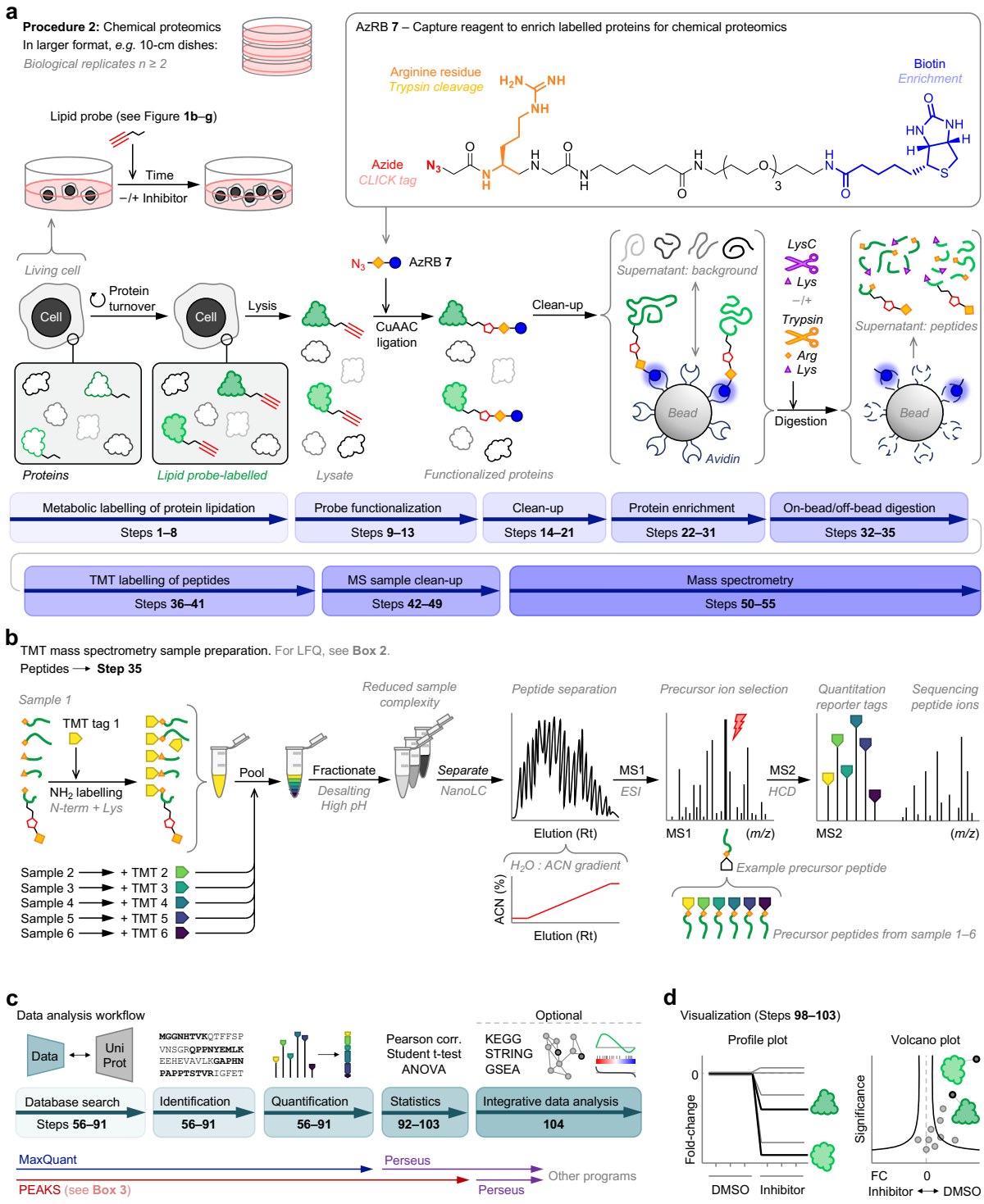

a
Procedure 2: Chemical proteomics
In larger format, e.g. 10-cm dishes:
Biological replicates n ≥ 2
Lipid probe (see Figure 1b–g)
Time
−/+ Inhibitor
Living cell
AzRB 7 – Capture reagent to enrich labelled proteins for chemical proteomics
Arginine residue
Trypsin cleavage
Azide
CLICK tag
Biotin
Enrichment
N3
AzRB 7
Protein turnover
Cell
Lysis
CuAAC ligation
Clean-up
Proteins
Lipid probe-labelled
Lysate
Functionalized proteins
Supernatant: background
Bead
Avidin
LysC
Lys
−/+
Trypsin
Arg
Lys
Digestion
Supernatant: peptides
Metabolic labelling of protein lipidation
Steps 1–8
Probe functionalization
Steps 9–13
Clean-up
Steps 14–21
Protein enrichment
Steps 22–31
On-bead/off-bead digestion
Steps 32–35
TMT labelling of peptides
Steps 36–41
MS sample clean-up
Steps 42–49
Mass spectrometry
Steps 50–55
b
TMT mass spectrometry sample preparation. For LFQ, see Box 2.
Peptides → Step 35
Sample 1
TMT tag 1
NH2 labelling
N-term + Lys
Pool
Fractionate
Desalting
High pH
Reduced sample complexity
Separate
NanoLC
Peptide separation
Elution (Rt)
MS1
ESI
Precursor ion selection
MS1 (m/z)
MS2
HCD
Quantitation reporter tags
Sequencing peptide ions
MS2 (m/z)
H2O : ACN gradient
ACN (%)
Elution (Rt)
Example precursor peptide
Precursor peptides from sample 1–6
Sample 2 → + TMT 2
Sample 3 → + TMT 3
Sample 4 → + TMT 4
Sample 5 → + TMT 5
Sample 6 → + TMT 6
c
Data analysis workflow
Data
Uni Prot
MGGNHTVKQTFFSP
VNSGRQPPNYEMLK
EEHEVAVLKGAPHN
PAPPTSTVRIGFET
Optional
Pearson corr.
Student t-test
ANOVA
KEGG
STRING
GSEA
Database search
Steps 56–91
Identification
56–91
Quantification
56–91
Statistics
92–103
Integrative data analysis
104
MaxQuant
PEAKS (see Box 3)
Perseus
Perseus
Other programs
d
Visualization (Steps 98–103)
Profile plot
Fold-change
0
DMSO
Inhibitor
Volcano plot
Significance
FC
0
Inhibitor
DMSO

Figure 5

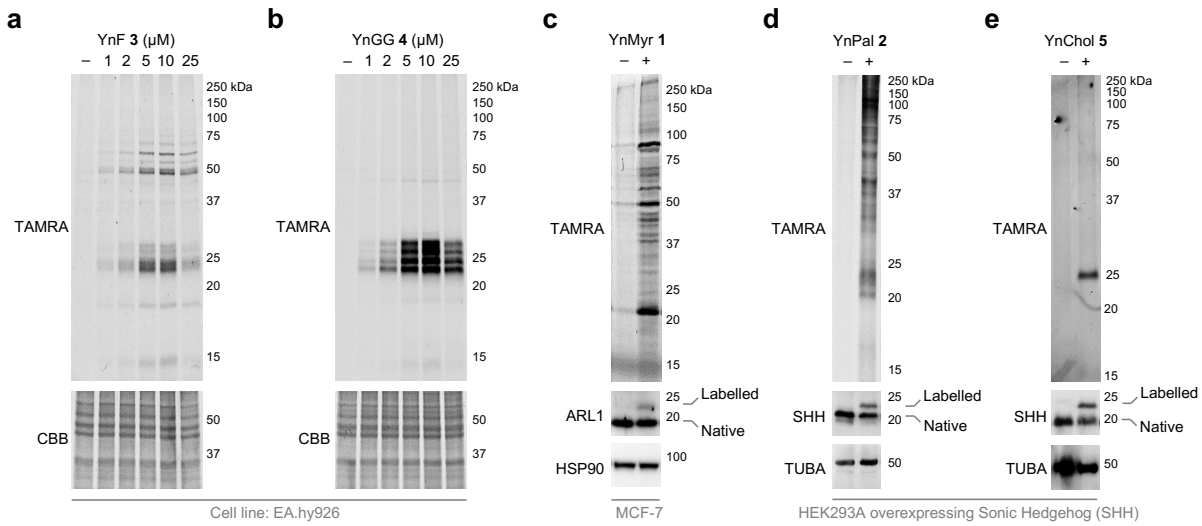

a
YnF 3 (μM)
– 1 2 5 10 25
TAMRA
CBB
250 kDa
150
100
75
50
37
25
20
15
b
YnGG 4 (μM)
– 1 2 5 10 25
TAMRA
CBB
c
YnMyr 1
– +
TAMRA
ARL1
HSP90
Labelled
Native
MCF-7
d
YnPal 2
– +
TAMRA
SHH
TUBA
e
YnChol 5
– +
TAMRA
SHH
TUBA
Cell line: EA.hy926
HEK293A overexpressing Sonic Hedgehog (SHH)

Figure 6

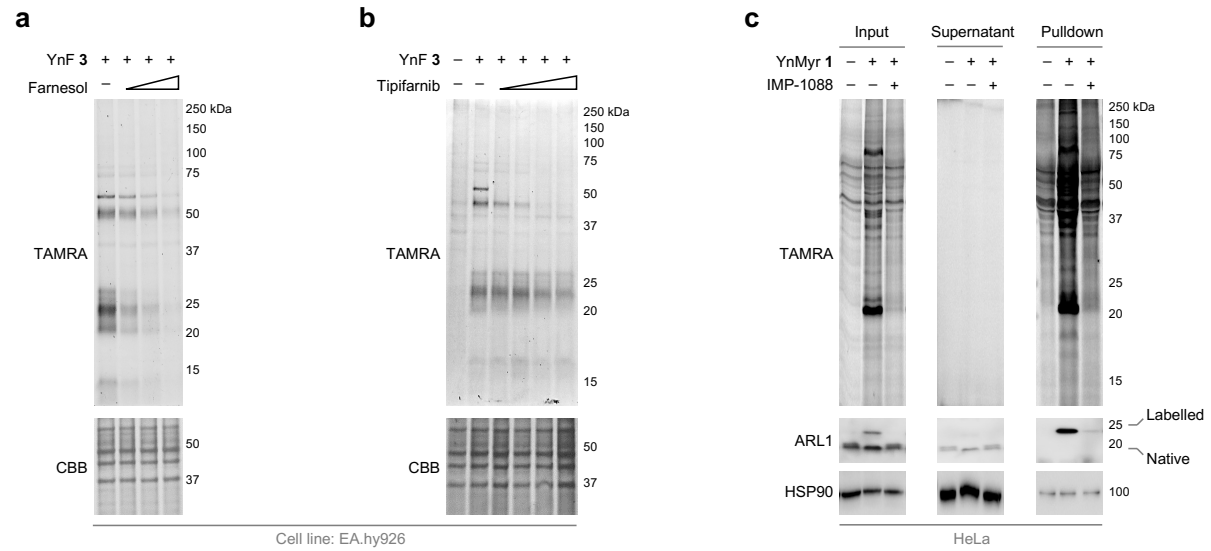
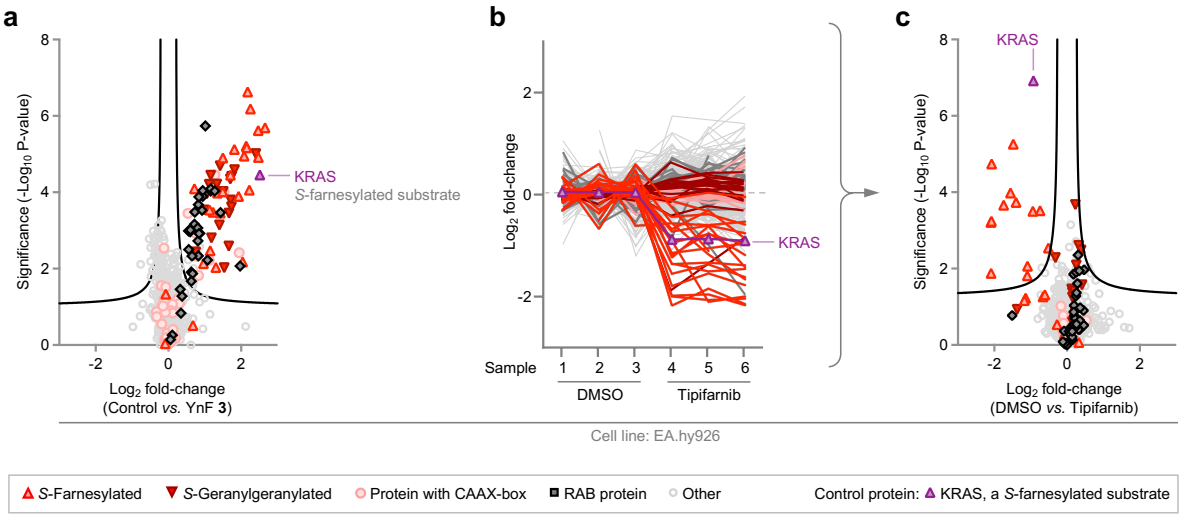
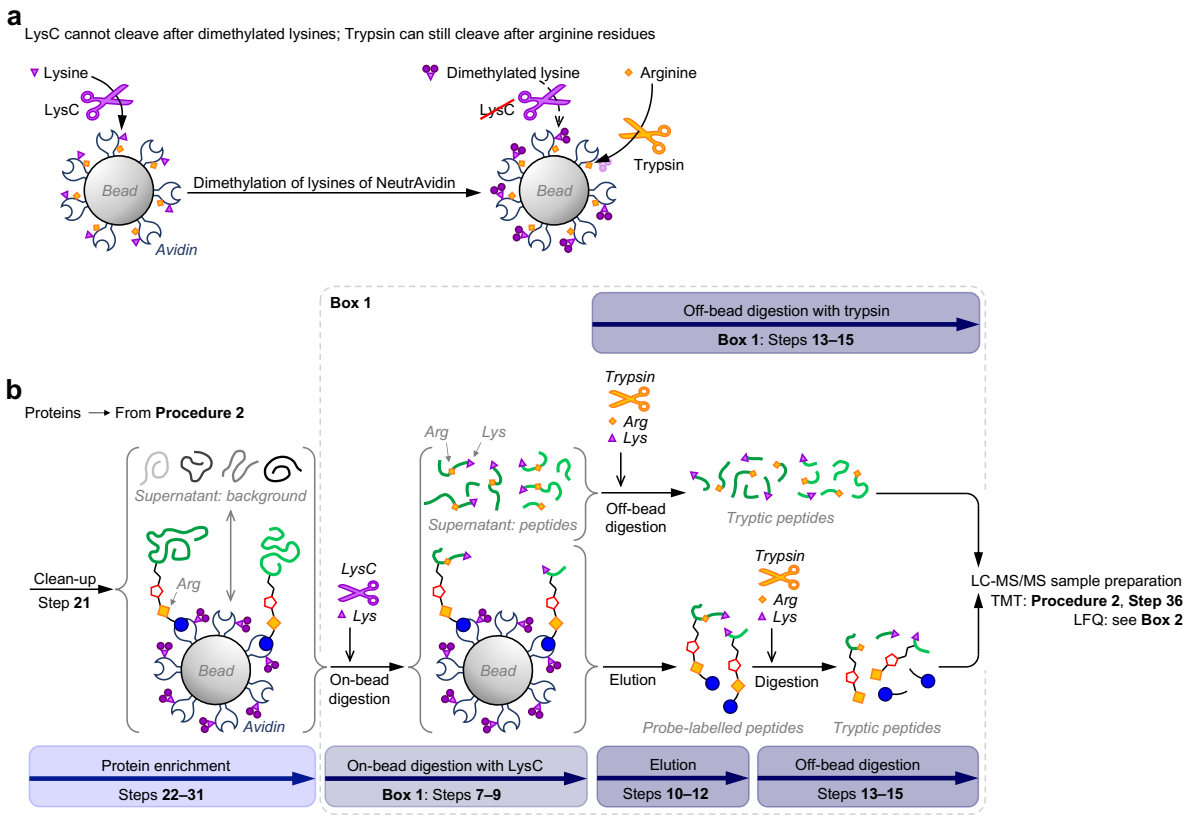

a
YnF 3 + + + +
Farnesol – ▲
250 kDa
150
100
75
50
37
25
20
15
TAMRA
50
37
CBB
Cell line: EA.hy926
b
YnF 3 – + + + + +
Tipifarnib – – ▲
250 kDa
150
100
75
50
37
25
20
15
TAMRA
50
37
CBB
c
Input
Supernatant
Pulldown
YnMyr 1 – + + – + + – + +
IMP-1088 – – + – – + – – +
250 kDa
150
100
75
50
37
25
20
15
TAMRA
25
20
Labelled
Native
ARL1
HSP90
100
HeLa

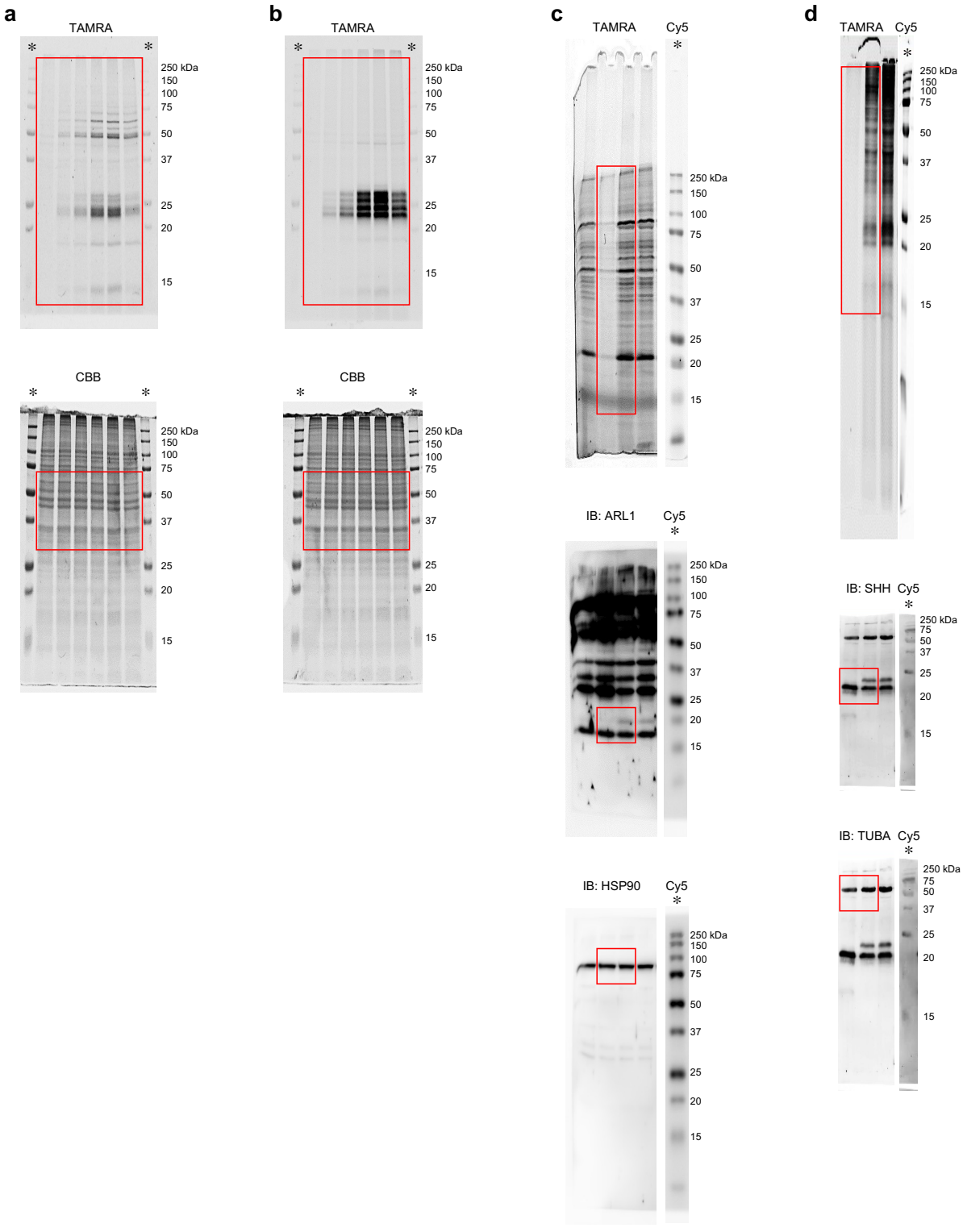
Figure 7


a
b
c
Significance (-Log10 P-value)
Log2 fold-change
(Control vs. YnF 3)
KRAS
S-farnesylated substrate
Log2 fold-change
Sample
1 2 3 4 5 6
DMSO
Tipifarnib
KRAS
KRAS
Significance (-Log10 P-value)
Log2 fold-change
(DMSO vs. Tipifarnib)
Cell line: EA.hy926
S-Farnesylated
S-Geranylgeranylated
Protein with CAAX-box
RAB protein
Other
Control protein: KRAS, a S-farnesylated substrate

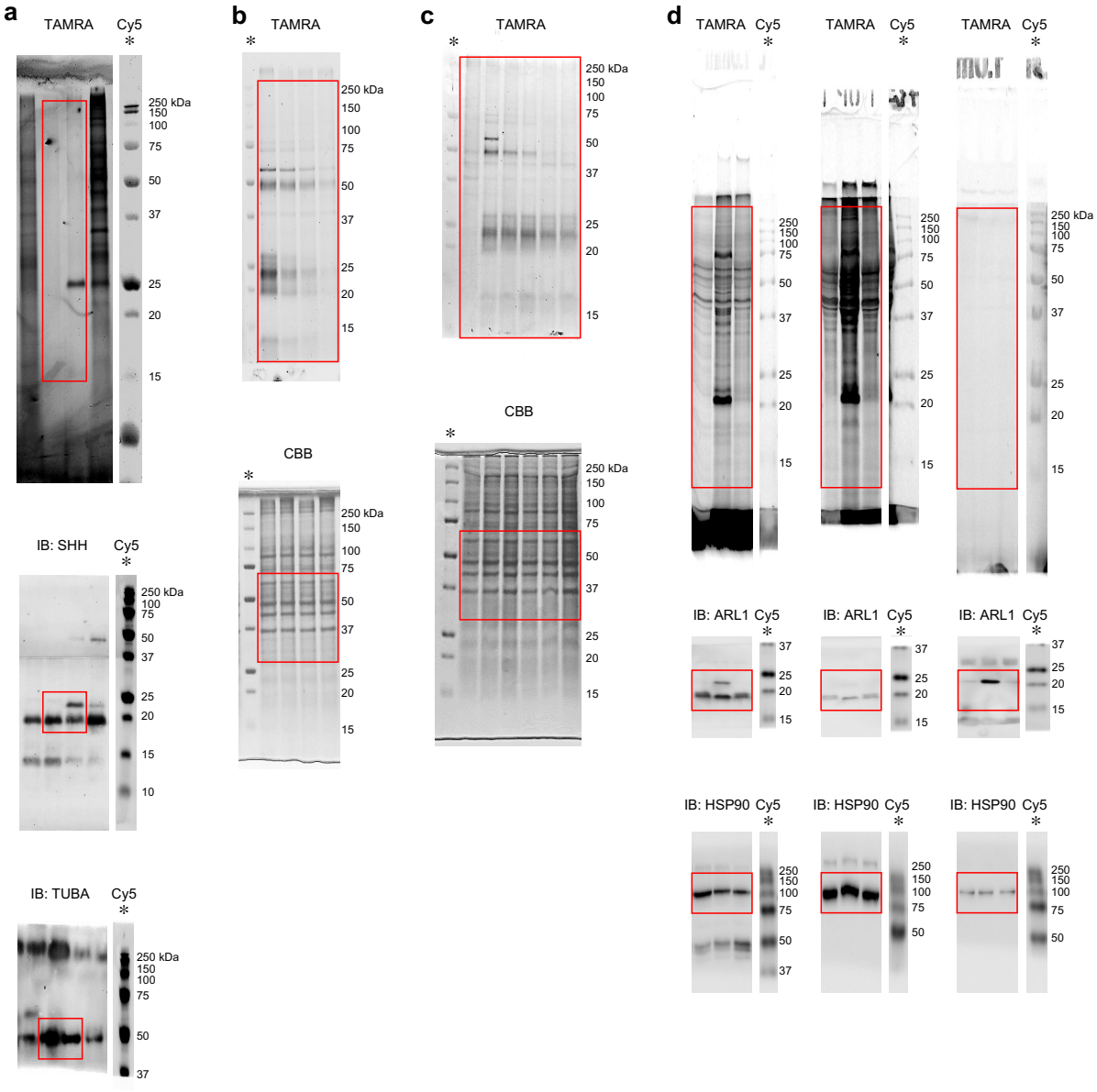
Box 1. Figure 1



SI Figure 1


a
TAMRA
CBB
b
TAMRA
CBB
c
TAMRA
Cy5
IB: ARL1
Cy5
IB: HSP90
Cy5
d
TAMRA
Cy5
IB: SHH
Cy5
IB: TUBA
Cy5
250 kDa
150
100
75
50
37
25
20
15

SI Figure 2


a
TAMRA
Cy5
*
250 kDa
150
100
75
50
37
25
20
15
IB: SHH
Cy5
*
250 kDa
100
75
50
37
25
20
15
10
IB: TUBA
Cy5
*
250 kDa
150
100
75
50
37
b
TAMRA
*
250 kDa
150
100
75
50
37
25
20
15
CBB
*
250 kDa
150
100
75
50
37
25
20
15
c
TAMRA
*
250 kDa
150
100
75
50
37
25
20
15
CBB
*
250 kDa
150
100
75
50
37
25
20
15
d
TAMRA
Cy5
*
250 kDa
150
100
75
50
37
25
20
15
IB: ARL1
Cy5
*
37
25
20
15
IB: HSP90
Cy5
*
250 kDa
150
100
75
50
37