Published in final edited form as:

Methods Mol Biol. 2018 ; 1787: 115–127. doi:10.1007/978-1-4939-7847-2_9.

Discovery of Modulators of Adipocyte Physiology Using Fully Functionalized Fragments

Andrea Galmozzi^{*,#}, Christopher G. Parker[#], Bernard P. Kok, Benjamin F. Cravatt, and Enrique Saez

Department of Molecular Medicine, The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, CA 92037, USA

Summary

Defects in adipocyte function associated with obesity drive the development of systemic insulin resistance and type 2 diabetes. Agents that correct obesity-linked adipocyte dysfunction serve as useful insulin sensitizers in humans, as is exemplified by the thiazolidinediones (TZDs). We have developed a new platform that integrates advanced chemoproteomics with phenotypic screening to identify small molecules that promote differentiation and lipid storage in adipocytes, and, in tandem, their molecular target(s). These molecules mimic the activity of TZDs in culture and thus may also serve as insulin sensitizers *in vivo*. Central to this platform is the use of fully functionalized fragment (FFF) probes that consist of a variable, fragment-like recognition element linked to an alkyne-diazirine group that enables the photo-activated capture of probe-bound proteins directly in living cells and subsequent copper-catalyzed azide-alkyne cycloaddition to reporter tags for enrichment and identification of these probe-bound proteins by mass spectrometry. This platform, which can be adapted to diverse screens and cell types beyond adipocytes, has the potential to uncover new biological pathways amenable to pharmacological modulation that may impact human disease.

Keywords

Phenotypic screening; adipose tissue; adipogenesis; photoreactive small molecules; chemical proteomics; target identification

1. Introduction

As the incidence of obesity and obesity-associated type 2 diabetes continues to increase, there is a critical need to develop new diabetes drugs with a mechanism of action distinct from that of existing treatments. Advances in our understanding of adipose tissue highlight the central role that adipocytes play in systemic glucose homeostasis and in the development of obesity-linked diabetes [1,2]. The ability of obese, hypertrophied adipocytes to dispose of glucose, store lipids, and secrete insulin-sensitizing adipokines is severely compromised, and this contributes to insulin resistance, hyperglycemia, and deposition of lipids in other

*Correspondence: galmozzi@scripps.edu.

#Co-first author

tissues (liver, muscle) that impairs whole-body insulin action [3]. Agents such as TZDs that can revert these defects and restore adipocyte function (e.g., lipid retention, secretion of beneficial adipokines) and normal lipid partitioning amongst tissues are used as diabetes medications in humans.

Phenotypic screens in adipocytes are an attractive strategy to discover molecular pathways that may be pharmacologically modulated to correct obesity-linked adipocyte dysfunction. It has been shown that molecules that mimic the effects of TZDs in cultured preadipocytes can have therapeutic effects in diabetic mice [4–6]. However, technical hurdles intrinsic to phenotypic screens have restricted wider application of this approach. Primary among these is the fact that identification of the molecular target(s) of bioactive compounds is often an arduous task [7,8]. Without a target, understanding the mode of action of hits and optimizing leads can be difficult.

To address this limitation of cell-based screens, we recently described an integrated platform for phenotypic screening and chemoproteomics-driven target identification based on the use of libraries of Fully Functionalized Fragment (FFF) probes [9] that enable broad exploration of the proteome and direct progression from phenotypic screen to target identification in living cells. Each member of the FFF library consists of a variable drug-like low-molecular weight ($MW < 300$ Da) small-molecule that is affixed to a conserved element comprised of a photoreactive diazirine group to enable UV light-induced crosslinking of probe to interacting proteins, and a “clickable” alkyne handle for reporter tag conjugation to visualize, enrich, and identify crosslinked proteins (Figure 1). The use of FFF libraries in phenotypic screens offers important advantages over classical approaches, such as affinity-based chromatography, where a hit compound is immobilized on a solid support for target enrichment from cell lysates. First, unlike affinity chromatography, FFFs enable direct identification of phenotype-relevant target proteins without requiring chemical modification of the screening hit(s). Second, treatment of cells with FFFs, followed by UV light-induced covalent trapping of small molecule-protein interactions provides a way to enrich and identify FFF-interacting proteins directly from intact cells. Finally, because the identification of FFF probe-interacting proteins is performed in living cells, lower-affinity or labile interactions prone to disruption by cell lysis/processing protocols used in biochemical affinity enrichment strategies are preserved, thus facilitating the discovery of context-dependent and/or short-lived probe-protein interactions.

These notable strengths of FFF-based screens are balanced by the need to perform careful target deconvolution studies to discern phenotypically-relevant target(s) of FFF hits from proteins that may interact with these compounds, but not contribute to the phenotypic effect. Accordingly, both inactive control compounds and paired competitors lacking the photoreactive/clickable element serve as critical components of the workflow for target deconvolution, and we outline below how these reagents can be used in quantitative mass spectrometry (MS)-based competitive assays to facilitate target identification of FFF probes that promote adipocyte differentiation [9].

In a typical workflow, a phenotypic screen is performed in preadipocytes or adipocytes and hit FFFs are validated and prioritized for target identification. Protein target(s) of prioritized

bioactive FFFs are identified using quantitative MS-based chemical proteomics methods, such as Stable Isotope Labeling with Amino acids in Cell culture (SILAC) [10] or isotopic reductive dimethylation (ReDiMe) [11] coupled to Multidimensional Protein Identification Technology (MudPIT) [12]. The relevant protein target of the prioritized FFF for the elicited biological response is then confirmed using genetic tools (e.g. RNAi). Here, we describe the general procedure to identify positive regulators of adipocyte differentiation and lipid storage using the preadipocyte line 3T3-L1, although other cell lines or primary preadipocytes can also be used. The sections on FFF hit selection and protein target identification also apply to screens in adipocytes, or in any other cell type, in which a different phenotype is desired (e.g., glucose-stimulated insulin secretion).

2 Materials

2.1 Phenotypic Screen

1. 3T3-L1 preadipocyte cell line (ATCC, CL-173).
2. Culture medium: DMEM supplemented with 10% bovine calf serum (BCS), 1% penicillin and streptomycin.
3. Adipogenesis induction medium: DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin and streptomycin, 1 µg/mL insulin, 0.4 µg/mL dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX).
4. Maintenance medium: DMEM supplemented with 10% FBS and 1 µg/mL insulin.
5. Rosiglitazone (a thiazolidinedione that is a direct synthetic ligand of the proadipogenic transcription factor PPAR γ).
6. Library of Fully Functionalized Fragment probes (FFFs) [9].
7. Nile Red stain (AdipoRed). Working solution is 1:250 dilution of AdipoRed reagent in DPBS. BODIPY 493/503 can also be used for neutral lipid staining instead of Nile Red.
8. Hoechst 33342. Working concentration: 0.1 µg/mL.
9. 4% Paraformaldehyde (PFA) solution in DPBS.
10. DPBS, no calcium, no magnesium.
11. High Content Imager (e.g., Celigo from Nexcelom, or CellInsight CX-5 from Thermo Fisher)
12. 0.05% trypsin-EDTA.
13. 96-well black-walled, clear bottom plates.

2.2 Gel- and Mass Spectrometry-based Studies

All solutions are prepared with Mass Spectrometry (MS)-grade water/DPBS.

1. DMEM medium for SILAC.

2. Fetal Bovine Serum, dialyzed, heat-inactivated.
3. [^{12}C , ^{14}N]-L-Lysine, [^{12}C , ^{14}N]-L-Arginine (“light”) and [^{13}C , ^{15}N]-L-Lysine, [^{13}C , ^{15}N]-L-Arginine (“heavy”) amino acids.
4. UV crosslinker for 365 nm UV light exposure.
5. Probe sonicator.
6. Click-chemistry reaction mix (10X): 10 mM tris-(2-carboxyethyl)-phosphine HCl (TCEP, freshly made), 1 mM tris-(benzyltriazolylmethyl)-amine (TBTA), 10 mM CuSO_4 , and 250 μM tetramethylrhodamine (TAMRA) azide (for gel-based visualization) or 1 mM biotin-PEG-azide (for MS analysis).
7. 4–12% Bis-Tris Protein gels.
8. Methanol/chloroform 4:1 solution.
9. 6M urea (proteomics grade) in DPBS.
10. 10% SDS solution in DPBS.
11. 600 mM potassium carbonate solution in DPBS.
12. 400 mM iodoacetamide solution in DPBS.
13. Streptavidin agarose resin.
14. Calcium chloride.
15. Sequencing grade modified trypsin.
16. Formic acid.

3 Methods

3.1 Phenotypic Screen

1. Seed 10,000 3T3-L1 cells in culture medium on gelatin-coated 96-well plates (final volume 100 μL /well). Replace medium every two days until preadipocytes reach confluence. (See Note 1)
2. After cells reach confluence (day-2), wait 2 days before starting differentiation regimen.
3. At day 0, gently aspirate/remove medium and replace with adipogenesis induction medium supplemented with vehicle (DMSO), rosiglitazone 2 μM (positive control), or FFFs (10–50 μM). Make sure to include multiple control wells per 96-well plate at different spots. The screen should be performed at least in duplicate.

¹Edge effects due to uneven evaporation and humidity amid wells can be common in 96-well plates, especially when cells are cultured for several days. This usually results in reduced adipocyte differentiation in wells along the edges and at corners. To avoid these problems and minimize variability, external wells should not be used and should instead be filled with 200 μL of medium or DPBS only. In addition, to verify that differentiation is comparable between plates, several wells of vehicle- and rosiglitazone-treated cells should be present in each plate and located in different positions.

4. On day 2, carefully aspirate/remove adipogenesis induction medium and replace with maintenance medium supplemented with vehicle, rosiglitazone, or FFFs.
5. Refresh maintenance medium every two days with minimal agitation (i.e. on day 4 and day 6).
6. On day 8, gently aspirate medium, wash cells with DPBS and add 80 μ L of staining solution containing the fluorescent neutral lipid dye Nile red and Hoechst 33342 (for nuclei counterstain). Incubate cells for 10 min at 37°C. Wash with DPBS and add 50 μ L of DPBS to each well.
7. Measure fluorescence and image all wells for lipid (red channel) and nuclei (blue channel) staining using a high content imager. (See Note 2) A general workflow scheme is shown in Figure 2.

(Optional)

Alternatively, cells can be fixed without staining and plates stored at 4°C for a short time (up to 2 weeks) before staining and imaging:

8. On day 8, wash cells with DPBS, add 80 μ L of 4% paraformaldehyde solution. Incubate cells for 10 min at room temperature, remove fixative, wash with DPBS, and add 80 μ L of DPBS to each well.
9. Seal plates with parafilm and aluminum foil. Store at 4°C. Perform lipid and nuclei staining right before imaging as described in step 6 of section 3.1.

3.2 Hit Selection and Prioritization for Target Identification

In this screen, FFFs that induce lipid accumulation >3-fold relative to vehicle are selected as positive hits. Hit FFF probes are then re-tested at different concentrations (at least 2, the screening concentration and a lower dose) in larger formats (e.g., 24-well) and with additional biological replicates. Lipid staining, as well as gene expression analysis of adipocyte markers evaluated using real-time quantitative PCR (e.g., PPAR γ , Adiponectin, FABP4, GLUT4, CD36), are used to rank order FFF hits based on their potency. (See Note 3) To prioritize FFF probes that work via novel mechanisms of action, validated hits are also tested for their ability to directly activate PPAR γ in a luciferase reporter assay [4,9]. Compounds that act as direct PPAR γ agonists, an established mechanism for their proadipogenic activity, are eliminated.

²The adipogenic potential of 3T3-L1 cells can vary significantly from batch to batch and must be considered carefully before setting up the phenotypic screen. If the chief goal is to find positive modulators of adipogenesis, vehicle-treated cells should display a low-medium level of differentiation, such that the assay window is optimal to identify compounds that promote differentiation. On the other hand, if negative modulators of adipogenesis are sought, a batch of cells showing a medium-high level of basal differentiation is preferred.

³As described, this phenotypic screen constitutes an intrinsic filter of toxicity, given that cells are treated for several days with compounds and hits are identified using a positive readout. Therefore, hit FFF probes are likely to be well tolerated at the screening concentration. If the screen is run to identify compounds that block differentiation, hits must be carefully filtered in secondary cell viability assays (e.g., CellTiter-Glo) to exclude compounds that show significant toxicity and can thus be considered false positive hits.

3.3 Design and Identification of Control Compounds

The availability of control molecules is critical for successful target identification. FFF probes that are structurally similar to active FFFs may share many, but not all, interactions with protein targets. However, if these structurally-related compounds exhibit little or no ability to promote differentiation and lipid accumulation, it is likely that the protein(s) responsible for these effects are exclusive to the active FFF probe. As such, comparisons of interacting proteins of active versus inactive control FFF probes allow for the exclusion of proteins shared between related molecules but unrelated to the observed bioactivity, as well as the identification of phenotypically-relevant target(s) specific to the active FFF probe (Figure 3). Further, to identify high-occupancy, saturable targets specific to the active FFF probe, it is also essential to perform competitive assays using paired non-functionalized versions of both the phenotypically active and inactive FFF probes (i.e. molecules lacking the photoreactive/clickable element). Analysis of primary screening data should ideally furnish a set of control compounds that includes:

- Inactive probes: FFF probes that structurally resemble active hits, but show no effect in the phenotypic screen. Inactive probes can assist in the sorting of targets that are more selective for the active FFF probe. They are expected to share some, but not all, protein targets of active FFF probes.
- Active competitors: non-functionalized derivatives of the hit FFF probe. Active competitors show similar performance in the phenotypic assay. When incubated in excess with an active FFF probe, active competitors allow the identification of high-occupancy targets of the active FFF probe and therefore potentially relevant to the observed activity.
- Inactive competitors: non-functionalized derivatives of inactive FFF probes. Similar to their functionalized counterparts, they have no effect in the phenotypic screen. Inactive competitors are used to identify high-occupancy targets of the active FFF probe that are unrelated to the observed phenotype. (See Note 4)
- Background FFF probe: This probe has a methyl group substituted at the recognition element and is designed to identify non-specific interactions of the cross-linking enrichment tag that is shared by all FFF probes. Proteins enriched with this control FFF can be considered as the “background noise” of the method.

3.4 Preparation of Probe-labeled SILAC Samples for MS- and Gel-based Protein Analysis

1. Grow cells in which target identification is to be performed (e.g., 3T3-L1 preadipocytes) for 5 passages in SILAC DMEM - 10% dialyzed FBS supplemented with isotopically “light” and “heavy” amino acids, to allow their complete incorporation into proteomes. For gel-based analyses, cells can be grown in standard label-free medium.

⁴Control FFF probes can be sought within the library of FFFs that has been screened. A set of structurally similar compounds that do not affect differentiation/lipid accumulation can be selected for initial validation. Small, second generation libraries can also be synthetically generated around hit FFF probes to identify additional active and inactive analogs.

2. Seed approximately 2 million heavy and light 3T3-L1 cells in separate 10 cm dishes. Grow cells to confluence.
3. Wash cells with DPBS.
4. Add serum-free DMEM containing FFF hits to be tested. For active-versus-inactive probe experiments, treat light and heavy cells with active and inactive FFF probes, respectively. For competition experiments, treat light cells with serum-free DMEM containing the active FFF probe and vehicle (DMSO), and heavy cells with the active FFF probe in the presence of a 10X excess of active or inactive competitor. Incubate at 37°C for 30 min. (See Note 5)
5. Remove medium and expose cells to 365 nm UV light for 10 min at 4°C. Include a no UV condition in which cells are incubated at 4°C for 10 min under ambient light. (See Note 6)
6. Wash cells once with ice-cold DPBS (5 mL).
7. Harvest cells with cold DPBS and spin down to pellet cells. Cell pellets can be stored at –80°C until analysis.
8. Resuspend cell pellets in 500 µL of ice-cold DPBS.
9. Sonicate with a probe sonicator (if using a Branson Analog Sonifier 250, 5 pulses, 30% duty cycle, output setting = 4).
10. Adjust cell lysates to a concentration of 1.5 mg/mL.

3.5 Proteome Processing for Mass Spectrometry Analysis

1. Combine light and heavy proteomes in equal proportions to obtain 1 mL of 1.5 mg/mL (50% light, 50% heavy).
2. Add 110 µL of freshly prepared 10X click chemistry reaction to each sample to conjugate biotin to FFF probe-labeled proteins.
3. Rotate at room temperature for 1 hr.
4. Transfer to a 15 mL tube. Incubate on ice and add 2.5 mL of cold 4:1 methanol (MeOH)/chloroform (CHCl₃) mixture, and 1 mL of cold DPBS.
5. Vortex until solution appears homogeneously cloudy.

⁵Prior to SILAC MS analysis, it is important to define the temporal window of action of active FFF probes (the time at which the relevant protein target is present in the cells), as well as the minimal concentration at which active FFF probes exert their effects (to reduce the number of non-specific interactions). In initial MS-based target deconvolution studies, concentrations between the apparent EC₅₀/IC₅₀ and EC₉₀/IC₉₀ should be used. Using lower concentrations may run the risk of failure to detect lower abundance targets, while using significantly higher concentrations may result in the enrichment of low-affinity off-targets that are unlikely to be relevant for the observed bioactivity. Cells can be induced to differentiate and be exposed to different concentrations of active FFFs at different times for varying duration. This will help to characterize the effects of hit FFF probes and select optimal experimental conditions for successful target identification.

⁶Proteome profiling of FFF probes and target deconvolution can be challenging to interpret. FFF probes can potentially interact with a large number of proteins with varying affinity and selectivity. Similarly, some proteins can be very promiscuous and may interact with many different FFF probes. Having several structurally similar, but inactive analogs greatly assists in the identification of the FFF target responsible for the phenotype observed. Further, it is important to confirm that FFF probe targets are enriched in a UV-dependent manner. This can be accomplished by comparing FFF probe-treated cells that are exposed to UV irradiation to those treated with probe but not exposed to UV irradiation.

6. Centrifuge samples at $5,000 \times g$ for 10 min at 4°C to fractionate the protein interphase from the organic and aqueous solvent layers.
7. Wash the protein disc carefully 3 times with 1 mL cold 1:1 MeOH:CHCl₃.
8. Add 3 mL of cold 4:1 MeOH:CHCl₃ and sonicate samples to ensure that unreacted click chemistry reagents are efficiently removed.
9. Pellet the remaining precipitate by centrifugation ($5,000 \times g$, 10 min, 4°C).
10. Discard supernatant and add 500 μL of 6M MS-grade urea solution (prepared fresh in DPBS) containing 10 μL of 10% SDS.
11. Resuspend pellet by sonication.
12. Add 50 μL of a 1:1 mixture containing TCEP (200 mM in DPBS) and potassium carbonate (600 mM in DPBS) to reduce disulfide bonds. Incubate for 30 min at 37°C .
13. Alkylate reduced thiols by adding 70 μL of 400 mM iodoacetamide. Incubate for 30 min at room temperature, protected from light.
14. Add 130 μL of 10% SDS (in DPBS) and 5.5 mL DPBS and incubate with pre-equilibrated streptavidin agarose resin (100 μL 1:1 slurry) for 1.5 hr at ambient temperature on a rotator.
15. Pellet streptavidin beads by centrifugation ($1,400 \times g$, 2 min).
16. Wash with 5 mL of 0.2% SDS in DPBS. Pellet beads by centrifugation.
17. Wash 2 times with 5 mL of detergent-free DPBS. Pellet beads by centrifugation.
18. Wash 2 times with 5 mL of dH₂O. Pellet beads by centrifugation.
19. Transfer streptavidin beads to a Protein LoBind tube (Eppendorf).
20. Digest bound proteins on-bead overnight at 37°C under constant shaking in 200 μL of DPBS containing 2 μg sequencing grade modified trypsin, 2 M urea, 1mM CaCl₂.
21. Transfer proteolyzed supernatant to a new Protein LoBind tube and acidify with formic acid (5% final).
22. Analyze samples by liquid chromatography-tandem mass spectrometry (LC/LC-MS/MS) or store samples at -20°C until analysis. See references [9,14] for examples of instrument settings, quantitation methods, and data analysis.

3.6 Identification of Relevant Active FFF Probe Protein Target(s)

To be classified as active FFF probe targets, proteins must be: 1) labeled in a UV-dependent manner (>5-fold enrichment in UV versus no UV experiments, see Note 6), 2) selectively enriched by the active FFF probe over the inactive FFF probe (>3-fold enrichment, see Note 7), and 3) competed by active non-functionalized competitor (>3-fold), but not competed by inactive non-functionalized competitors (<2-fold).

3.7 Gel-based Analysis

Direct hit FFF probe labeling of proteins that respect the above criteria is confirmed by overexpression of putative targets in HEK293T cells in 6-well plates. Mock-transfected and target-overexpressing cells are exposed to active FFF probe and labeling is competed with increasing concentrations of active or inactive non-functionalized competitors (Figure 4). Treatments are performed as described above (see section 3.4.4), but cells do not need to be metabolically labeled unless subsequent MS analysis is desired. The following steps are performed after UV light exposure:

1. Wash cells with ice-cold DPBS (1 mL).
2. Harvest cells with 200 μ L of ice-cold DPBS and keep on ice.
3. Lyse cells by sonication.
4. Quantify protein concentration.
5. Aliquot 50 μ g of proteomes into new tubes and adjust volume to 50 μ L with DPBS.
6. Add 6 μ L of 10X click chemistry reaction mix to each sample to conjugate rhodamine to FFF probe-labeled proteins.
7. Mix vigorously and incubate for 1 hr at room temperature.
8. Quench click chemistry reaction by adding 17 μ L of 4X SDS loading buffer.
9. Load 25 μ L on a 4–12% Bis-Tris acrylamide gel.
10. Run SDS-PAGE for 45 minutes at 165V.
11. Visualize in-gel fluorescence using a fluorescence scanner.
12. Transfer proteins to PVDF or nitrocellulose membrane and perform western blot analysis to confirm overexpression and normalize fluorescent signals.

3.8 Genetic Validation of Relevant Active FFF Probe Target Protein

This phenotypic screening strategy can identify compounds that either inhibit or act as gain-of-function ligands on their targets. To discern the relevant protein target for a compound's effects, lentiviruses (e.g., pLKO.1 and/or regulated versions if needed) can be used to express shRNA against each putative target identified in 3.6 (4 constructs/target) in the cells in which the compound exhibits its effects. The infected cells are then phenotyped in the presence and absence of active FFF probe. For FFFs behaving as inhibitors, knockdown of the relevant protein target will mimic the effect of the FFF. For gain-of-function ligands, knockdown of the relevant protein target will abolish the effect of the FFF. In this case, complementation studies can be used to further establish the identity of the relevant target.

⁷Active and inactive FFF probes may bind the same interacting proteins but with notably different affinity. By directly comparing relative target enrichment between active and inactive probes, differential affinity towards those targets can be directly assessed and higher-affinity targets of the active probe identified.

Acknowledgments

This work was supported by NIH DK099810, DK114785, CA132630, 1S10OD16357.

References

1. Rosen ED, Spiegelman BM. What we talk about when we talk about fat. *Cell*. 2014; 156(1–2):20–44. [PubMed: 24439368]
2. Kusminski CM, Bickel PE, Scherer PE. Targeting adipose tissue in the treatment of obesity-associated diabetes. *Nat Rev Drug Discov*. 2016; 15(9):639–660. [PubMed: 27256476]
3. Samuel VT, Petersen KF, Shulman GI. Lipid-induced insulin resistance: unravelling the mechanism. *Lancet*. 2010; 375(9733):2267–2277. [PubMed: 20609972]
4. Dominguez E, Galmozzi A, Chang JW, Hsu KL, Pawlak J, Li W, Godio C, Thomas J, Partida D, Niessen S, O'Brien PE, Russell AP, Watt MJ, Nomura DK, Cravatt BF, Saez E. Integrated phenotypic and activity-based profiling links *Ces3* to obesity and diabetes. *Nat Chem Biol*. 2014; 10(2):113–121. [PubMed: 24362705]
5. Waki H, Park KW, Mitro N, Pei L, Damoiseaux R, Wilpitz DC, Reue K, Saez E, Tontonoz P. The small molecule harmine is an antidiabetic cell-type-specific regulator of PPAR γ expression. *Cell Metab*. 2007; 5(5):357–370. [PubMed: 17488638]
6. Galmozzi A, Sonne SB, Altshuler-Keylin S, Hasegawa Y, Shinoda K, Luijten IH, Chang JW, Sharp LZ, Cravatt BF, Saez E, Kajimura S. ThermoMouse: an in vivo model to identify modulators of UCP1 expression in brown adipose tissue. *Cell Rep*. 2014; 9(5):1584–1593. [PubMed: 25466254]
7. Schenone M, Dancik V, Wagner BK, Clemons PA. Target identification and mechanism of action in chemical biology and drug discovery. *Nature Chemical Biology*. 2013; 9(4):232–240. [PubMed: 23508189]
8. Swinney DC, Anthony J. How were new medicines discovered? *Nature Reviews Drug Discovery*. 2011; 10(7):507–519. [PubMed: 21701501]
9. Parker CG, Galmozzi A, Wang Y, Correia BE, Sasaki K, Joslyn CM, Kim AS, Cavallaro CL, Lawrence RM, Johnson SR, Narvaiza I, Saez E, Cravatt BF. Ligand and Target Discovery by Fragment-Based Screening in Human Cells. *Cell*. 2017; 168(3):527–541. [PubMed: 28111073]
10. Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics*. 2002; 1(5):376–386. [PubMed: 12118079]
11. Hsu JL, Huang SY, Chow NH, Chen SH. Stable-isotope dimethyl labeling for quantitative proteomics. *Anal Chem*. 2003; 75(24):6843–6852. [PubMed: 14670044]
12. Washburn MP, Wolters D, Yates JR 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. *Nat Biotechnol*. 2001; 19(3):242–247. [PubMed: 11231557]
13. Bembenek SD, Tounge BA, Reynolds CH. Ligand efficiency and fragment-based drug discovery. *Drug Discov Today*. 2009; 14(5–6):278–283. [PubMed: 19073276]
14. Cisar JS, Cravatt BF. Fully functionalized small-molecule probes for integrated phenotypic screening and target identification. *J Am Chem Soc*. 2012; 134(25):10385–10388. [PubMed: 22667687]

Figure 1. Basic Structure of Fully Functionalized Fragment Probes (FFFs)

(a) Schematic of FFF probes showing their basic features: 1) a variable head group that confers specificity towards target proteins, 2) a photoreactive diazirine group for UV light-induced crosslinking of probes to their interacting proteins, and 3) an alkyne handle for copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) of reporter tags (e.g., fluorophores or biotin) for downstream applications. (b) Examples of FFF probes are shown, along with their paired non-functionalized competitors used in target identification studies.

Figure 2. Phenotypic Screen to Isolate Preadipogenic FFFs

(a) Preadipocytes are seeded and induced to differentiate into adipocytes in the presence of FFFs. On day 8, cells are stained with the lipid dye Nile Red and imaged and quantified using a high content imager. FFFs are ranked based on their ability to promote differentiation and lipid accumulation. (b) Following validation of hit FFF probes, control FFF probes and non-functionalized active and inactive competitors are selected to facilitate target deconvolution.

Figure 3. SILAC-MudPIT Analysis to Identify Relevant Protein Targets of FFF Probes

Experimental workflow used to identify protein target(s) of active FFF probes. (a) Light and heavy SILAC cells are treated with the indicated FFF probes in the presence or absence of excess active or inactive competitor. After 30 min, cells are irradiated with UV light for 10 min to crosslink probe-interacting proteins. (b) Heavy and light proteomes are mixed in equal proportions, and click chemistry is used to add a biotin tag to the FFF probe to enable enrichment of target proteins. After streptavidin pull down, FFF targets are digested on-bead with trypsin and the resulting peptides analyzed by liquid chromatography and tandem mass spectrometry. (c) Relevant targets are defined as proteins with >3-fold enrichment by the active FFF probe over the inactive FFF probe, competed >3-fold with the active non-functionalized competitor, *and* competed <2-fold by any inactive non-functionalized competitor.

Figure 4. Visualization of FFF Probe Target Proteins by SDS-PAGE

Cells overexpressing the putative relevant protein target of an active FFF are exposed to the FFF probe in the presence and absence of non-functionalized competitors for 30 min, followed by UV light-induced photocrosslinking, cell lysis, click chemistry conjugation of a fluorescent tag, SDS-PAGE separation, and visualization by in-gel fluorescence scanning and Western blot.