Electronic Supplementary Material (ESI) for RSC Chemical Biology.

Supporting information for:

A peptide-crosslinking approach identifies HSPA8 and PFKL as selective interactors of an actin-derived peptide containing reduced and oxidized methionine

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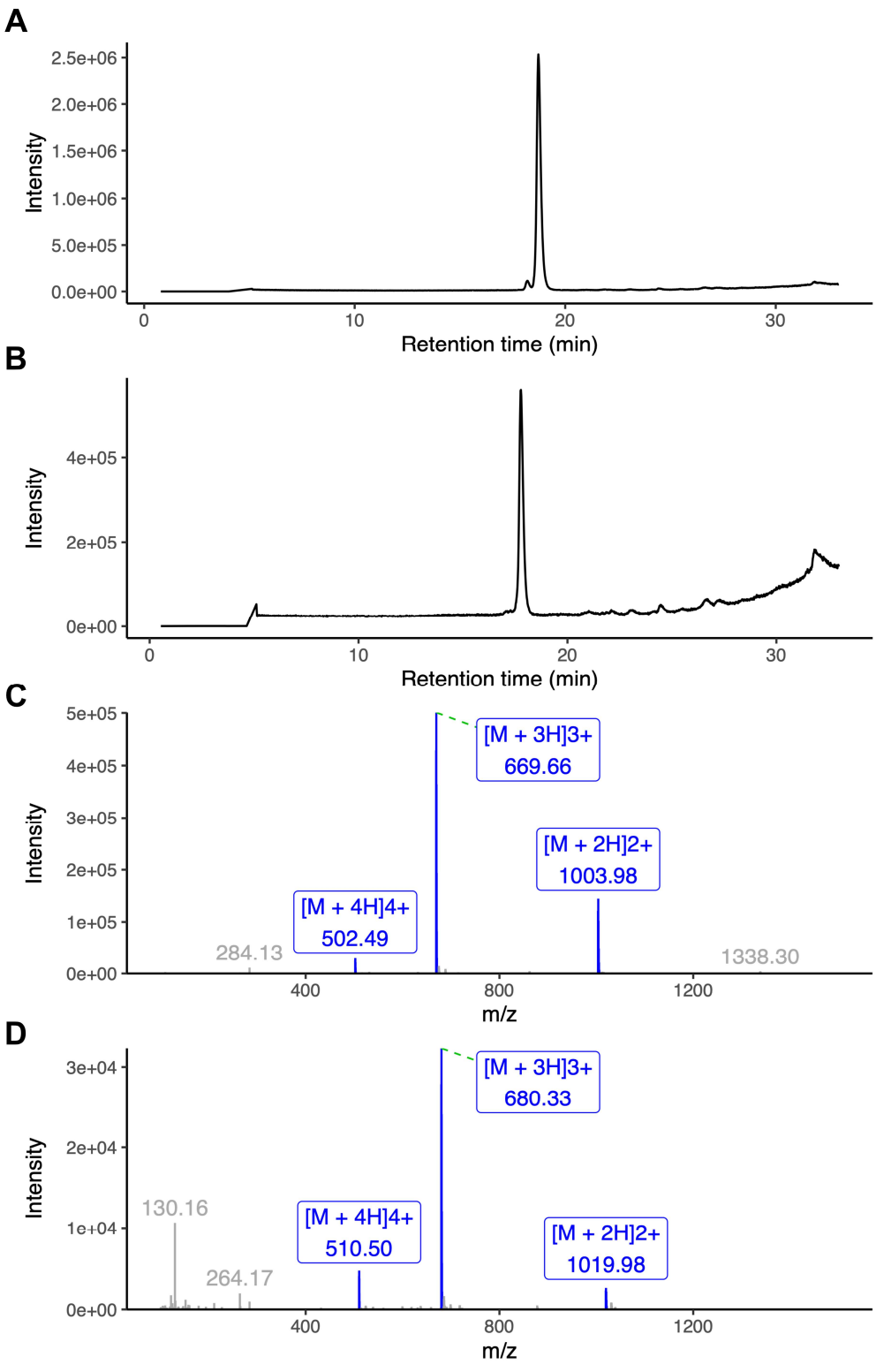

Figure S1: LC/MS characterization of probe **1** (reduced Met) (panels A and C) and probe **2** (oxidized Met) (panels B and D).

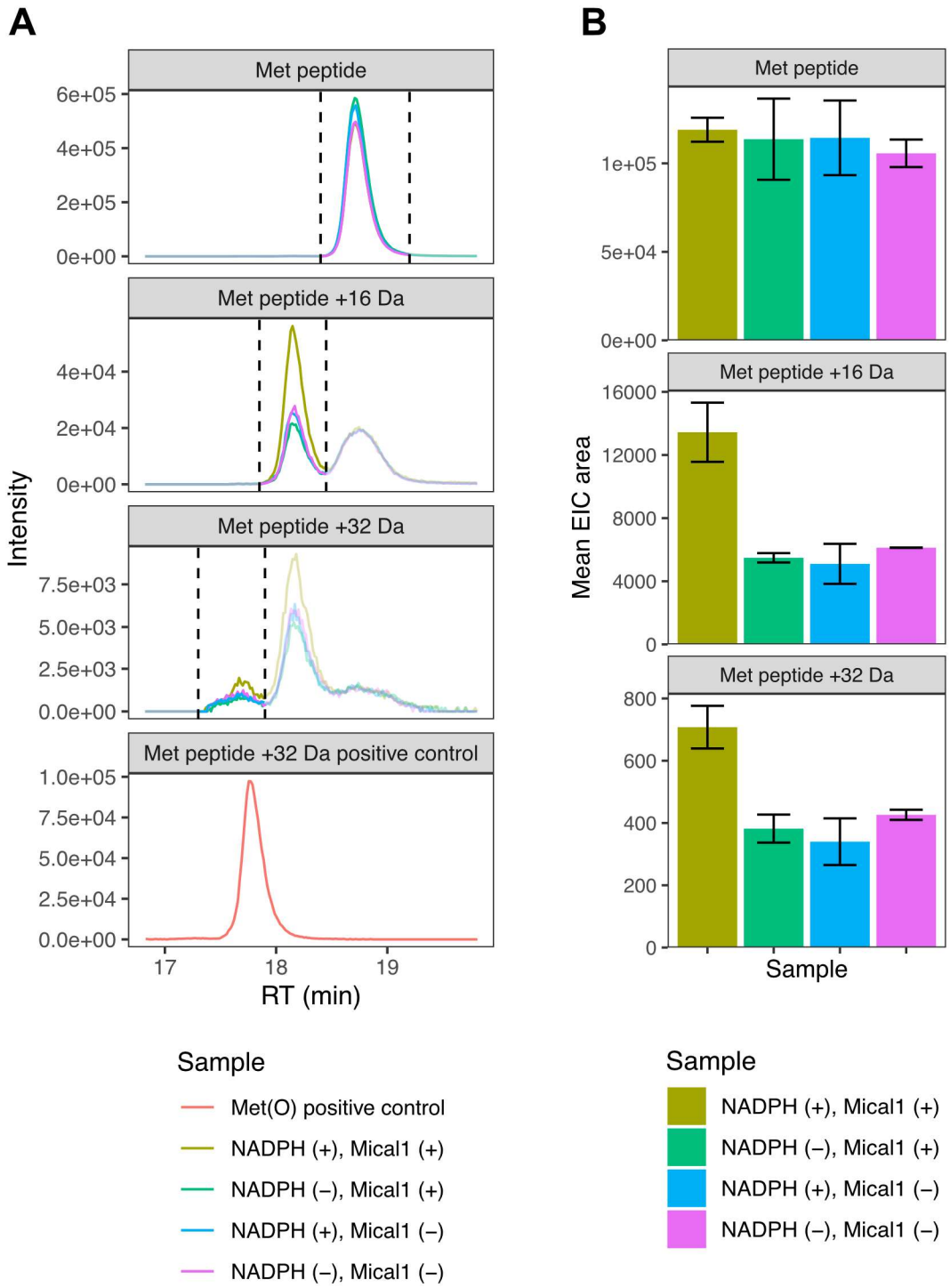

Figure S2: Mical1 oxidizes probe **1**. 100 µM probe **1** was treated with 1 µM Mical1 in the presence or absence of 200 µM NADPH. The doubly oxidized probe **2** was run as a positive control. **(A)** Representative extracted ion chromatograms (EICs) of the mass of fully reduced, singly oxidized, and double oxidized probe **1**. Dotted lines represent peak areas that were integrated to quantify each species. **(B)** Mean EIC area of 3 technical replicates. Errorbars represent the standard deviation of the 3 measurements.

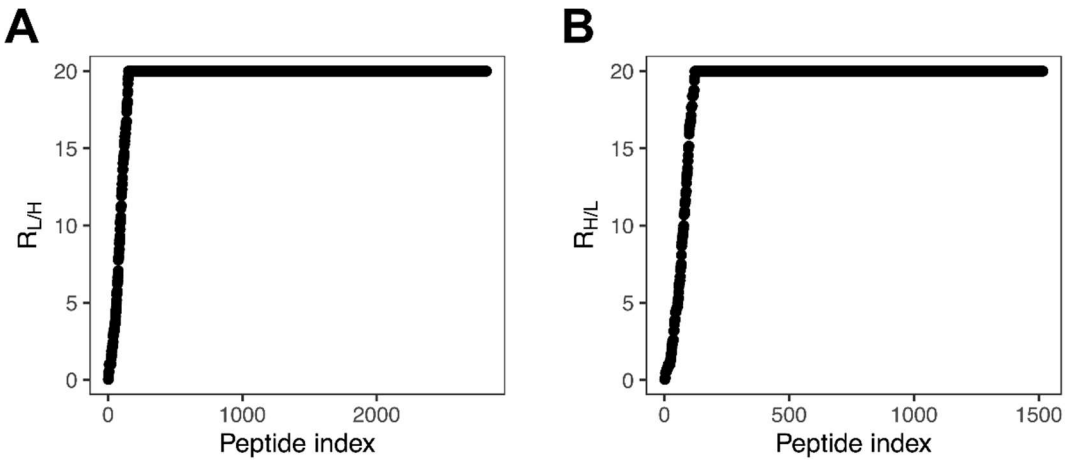

Figure S3: Verification of SILAC labeling in MCF7 cells. **(A)** Light to heavy peptide ratios in light-labeled cell line. **(B)** Heavy to light peptide ratios in heavy-labeled cell line.

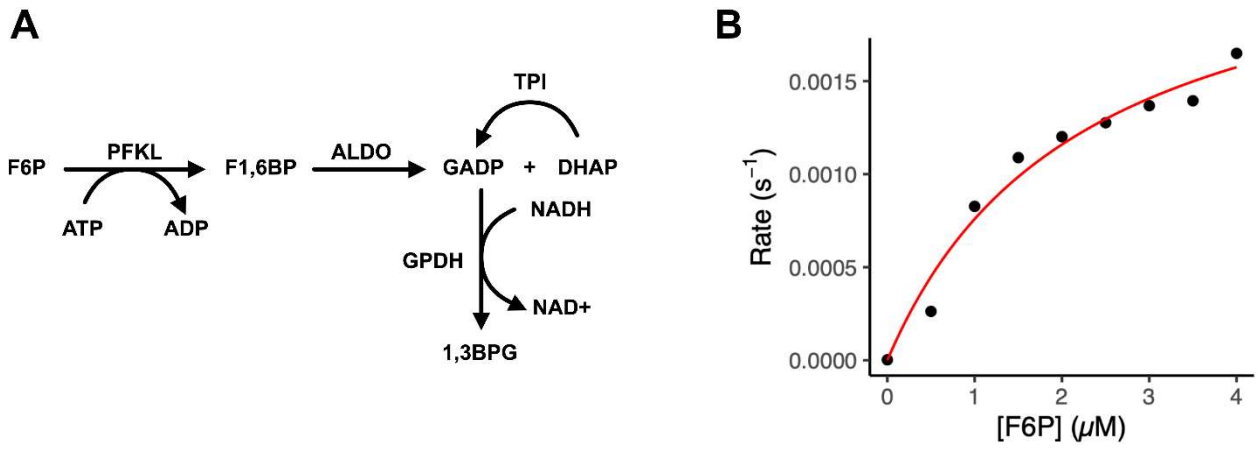

Figure S4: PFKL activity assay. **(A)** Coupled enzyme assay used to measure PFKL activity. **(B)** Michaelis–Menten plot of recombinant PFKL activity at varying concentrations of fructose 6-phosphate (F6P).

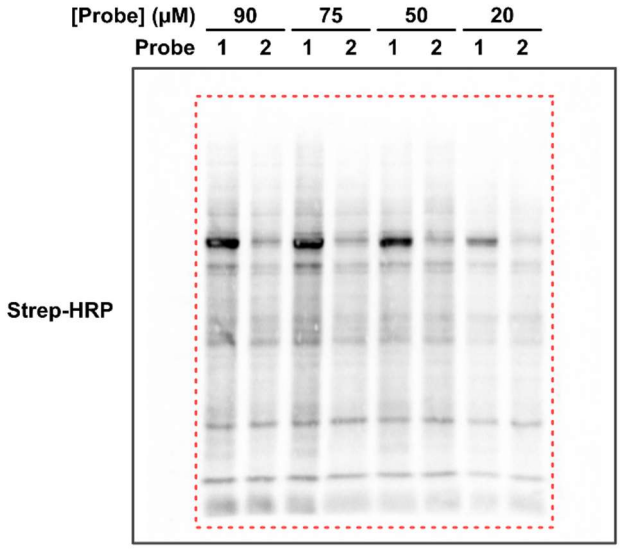

Figure S5: Uncropped gel image corresponding to Figure 2B.

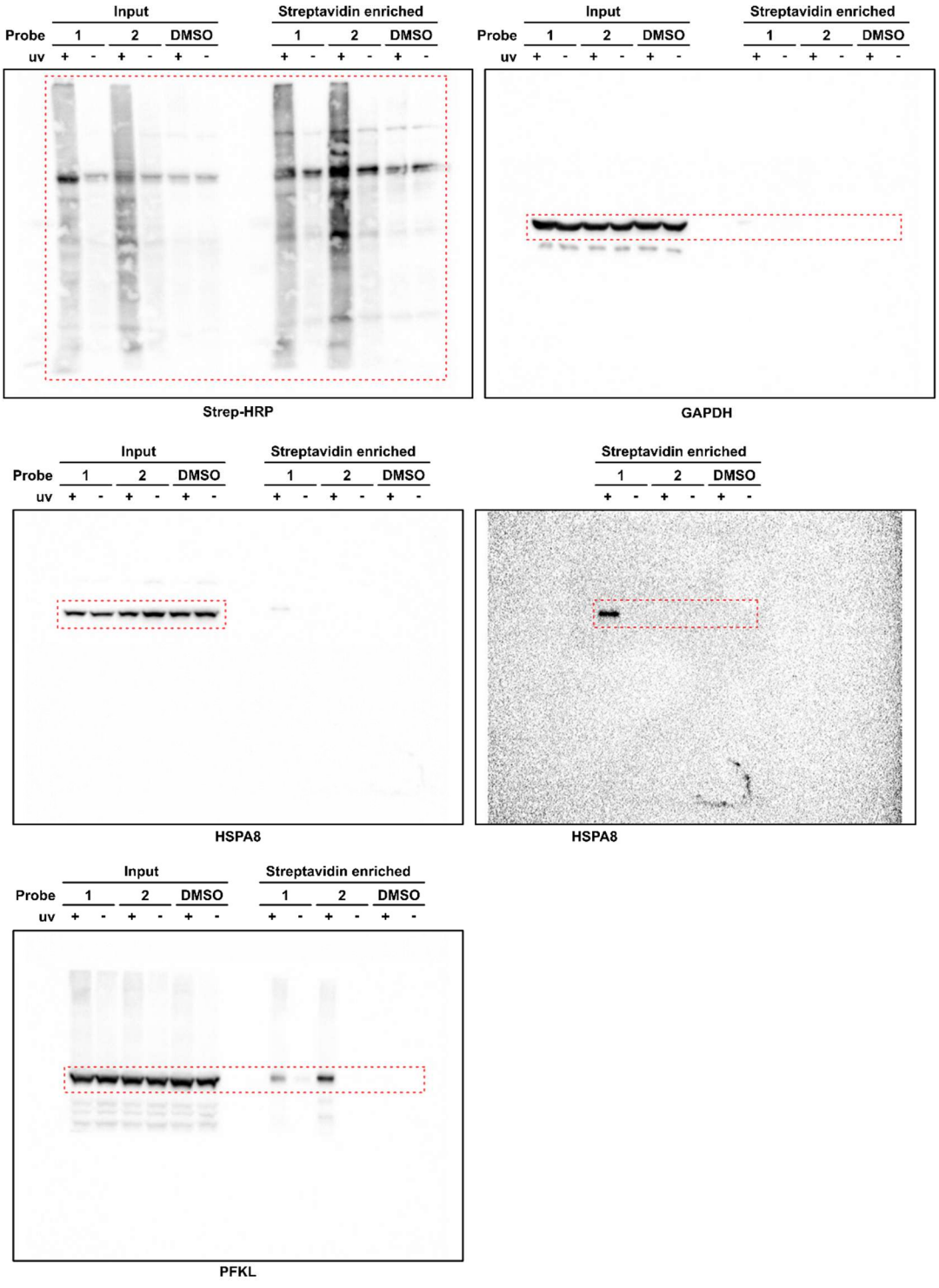

Figure S6: Uncropped gel images corresponding to Figure 2A.

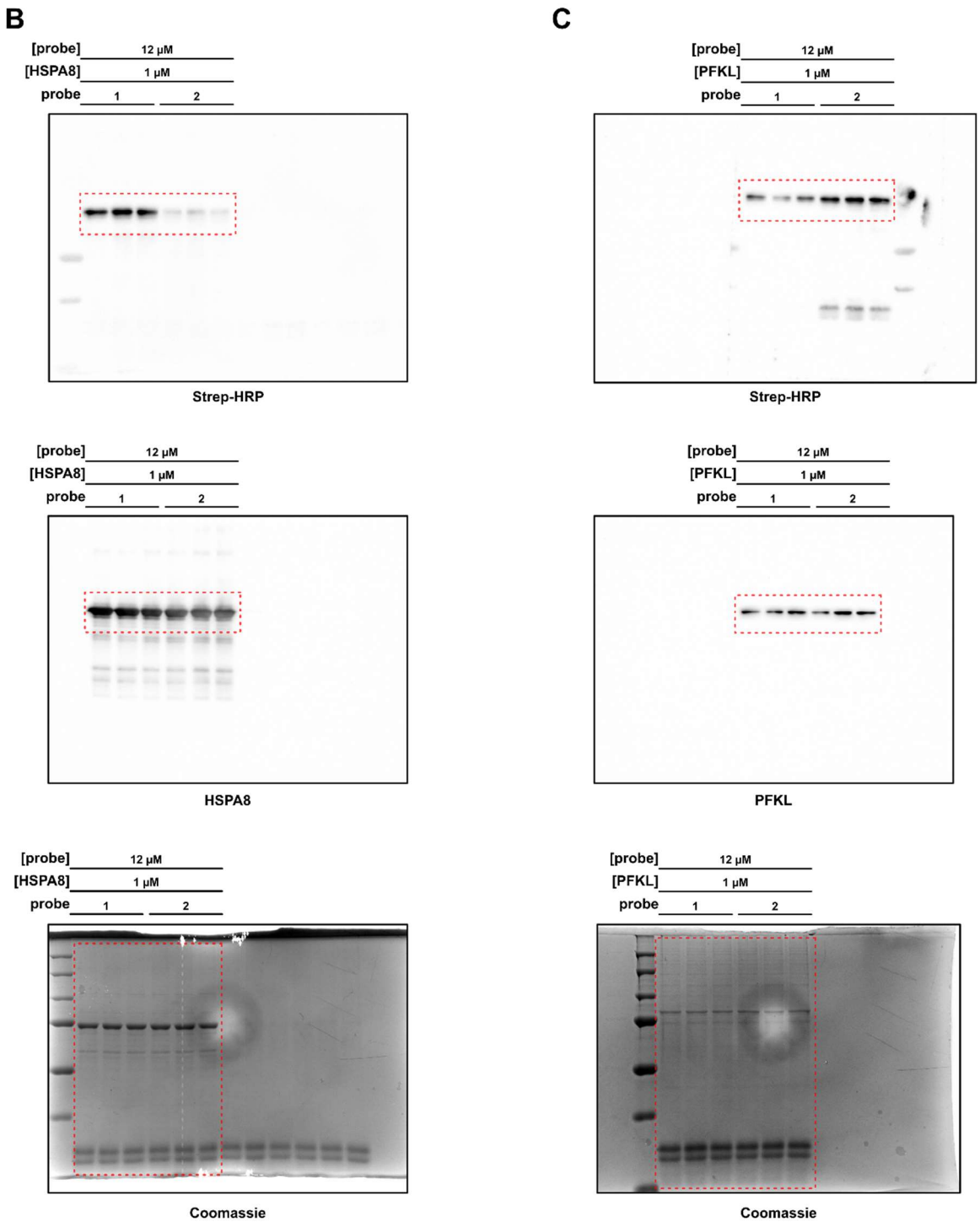

Figure S7: Uncropped gel images corresponding to Figures 2B and C.

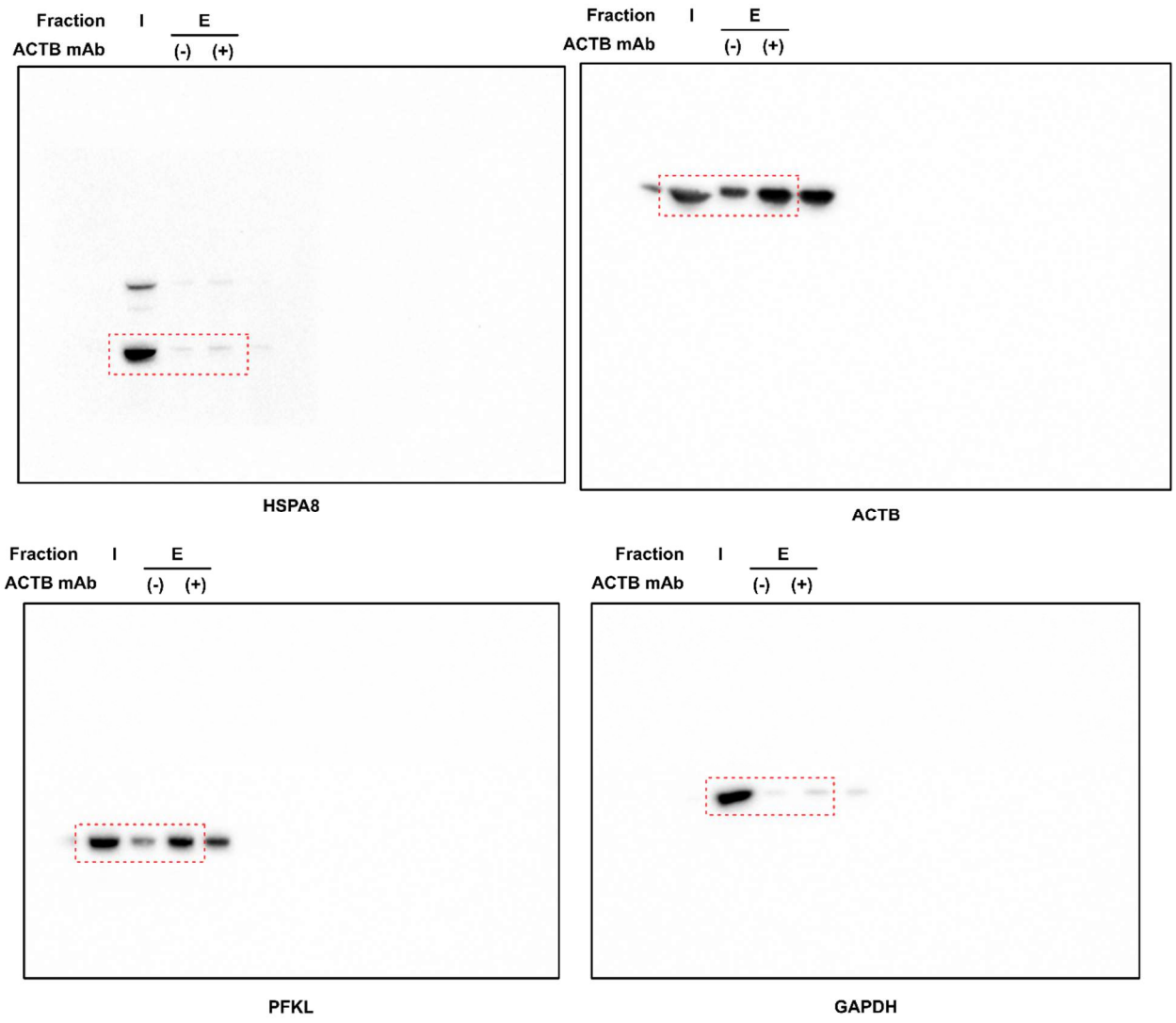

Figure S8: Uncropped gel images corresponding to Figure 3A.

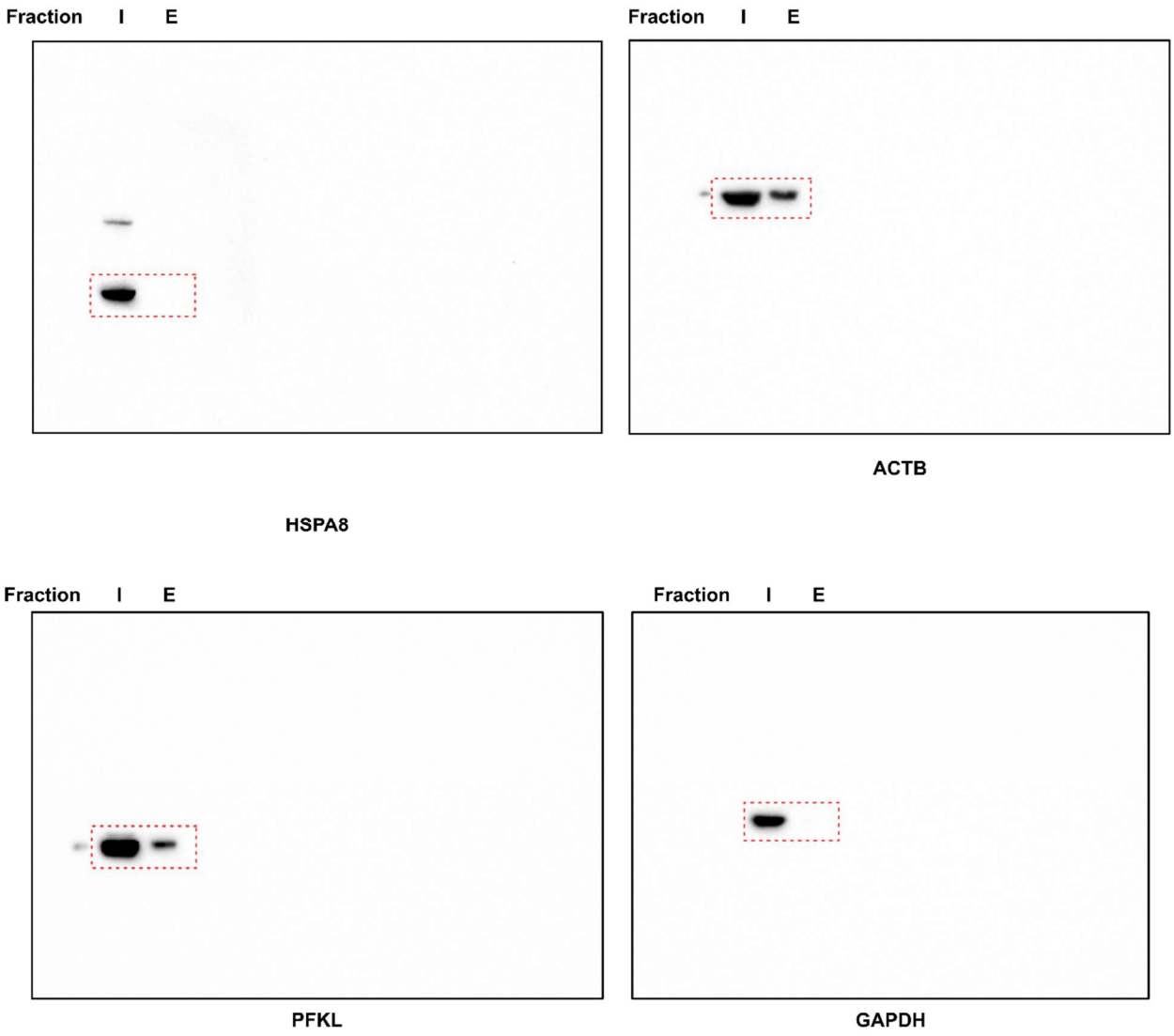

Figure S9: Uncropped gel images corresponding to Figure 3B.

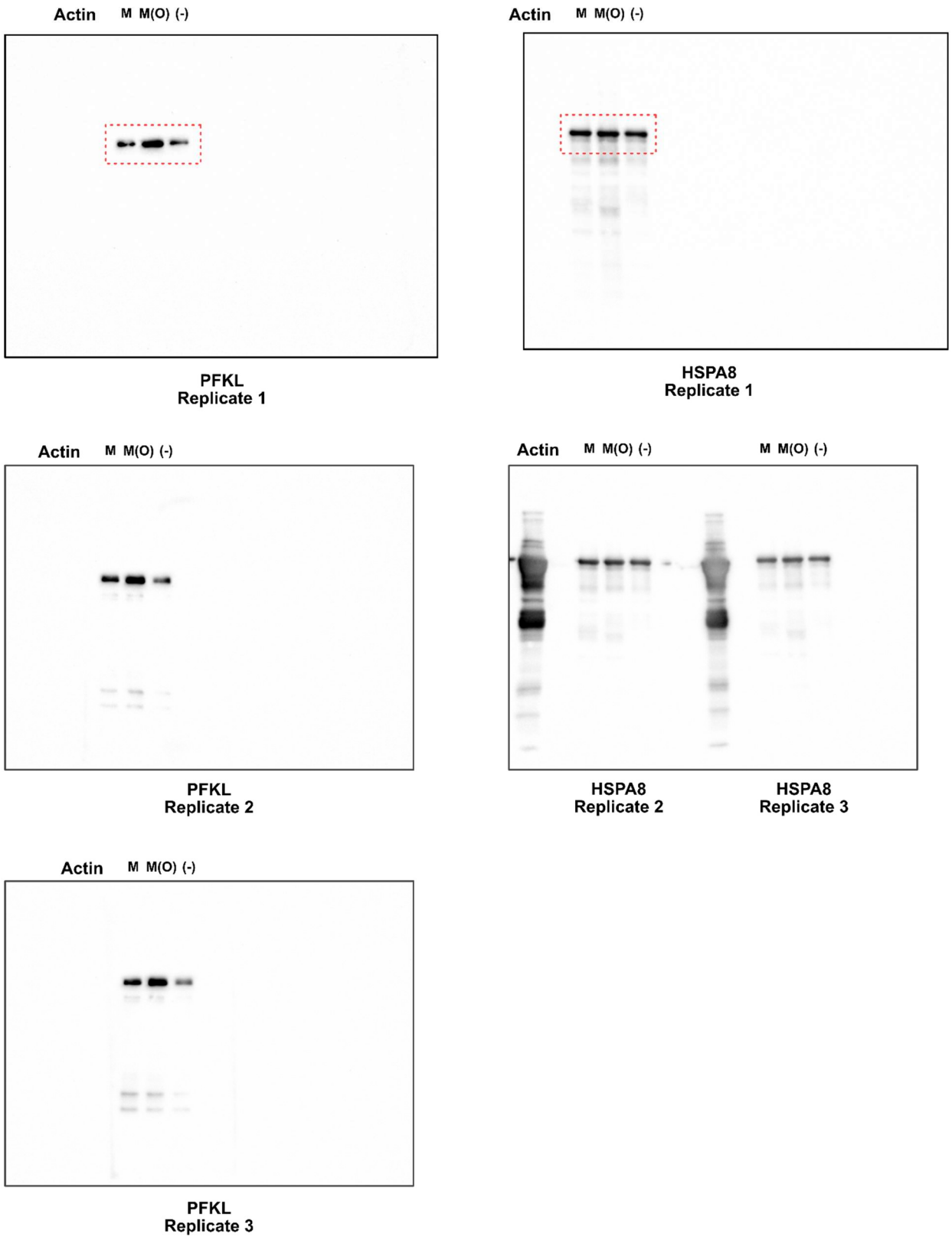

Figure S10: Uncropped gel images corresponding to Figure 3C.

Materials and methods

Synthesis of Met/Met(O) peptide probes

Ac-F(p-Bz)-H-Q-G-V-**M**-V-G-**M**-G-Q-Ahx-PEG3-Biotin

Met probe (1)

Ac-F(p-Bz)-H-Q-G-V-**M(O)**-V-G-**M(O)**-G-Q-Ahx-PEG3-Biotin

Met(O) probe (2)

Peptides were synthesized by manual solid-phase synthesis on Fmoc-PEG Biotin NovaTag™ resin (Millipore Sigma) using Fmoc as the protecting group for α-amino groups. The success of each deprotection and coupling was confirmed by Kaiser test following the standard protocol. 6-(Fmoc-amino) hexanoic acid, Fmoc-Lys(Boc)-OH, Fmoc-Gln(trt)-OH, Fmoc-Gly-OH, Fmoc-Met-OH, Fmoc-Gly-OH, Fmoc-Val-OH, Fmoc-Met-OH, Fmoc-Val-OH, Fmoc-Gly-OH, Fmoc-Gln(trt)-OH, Fmoc-His(trt)-OH, Fmoc-p-Bz-Phe-OH residues were added under standard coupling conditions. The same order of residues was used for the Met(O) probe with the substitution of Fmoc- Met(O)-OH for Fmoc-Met-OH. After the final Fmoc deprotection, dry DCM was added to the resin. Acetic anhydride (10 eq) and pyridine (10 eq) were added drop- wise and the vessel was shaken for 3 hours. After acetyl capping the peptide was cleaved from the resin in 90 % TFA, 5 % DCM, 2.5 % TIS, 2.5 % water for 2 hours. The peptide was purified by preparative HPLC with a gradient of increasing ACN 0.1 % TFA (solvent) in water- 0.1 % TFA (solvent A) and analyzed on an Agilent triple TOF mass spectrometer coupled to an Agilent 1200 series LC (Figure S1). HRMS for Met probe ($C_{92}H_{144}N_{22}O_{22}S_3 + 3H^+$): 669.6667 *m/z* calcd.; obsd. [M + $3H^+$] 669.6755, HRMS for Met(O) probe ($C_{92}H_{144}N_{22}O_{24}S_3 + 3H^+$): 680.3333 *m/z* calcd.; obsd. [M + $3H^+$] 680.3427

Cell culture

MCF7 cells were grown in RPMI media minus l-lysine and l-arginine (Thermo Fisher) supplemented with 10 % dialyzed FBS (Gibco), 1 % Penicillin-Streptomycin- Amphotericin B (Gibco), and either 84 μg mL^{-1} [$^{13}C/^{15}N$]-l-arginine (R10) and 146 μg mL^{-1} [^{13}C]-l-lysine (K6) or 84 μg mL^{-1} l-arginine (R0) and 146 μg mL−1 l-lysine (K0) at 37 °C and 5 % CO2 for a minimum of 6 passages.

Verification of Heavy Amino Acid Incorporation for SILAC

Soluble cell lysates were diluted to 1 mg mL^{-1} and precipitated with the addition of 10 % TCA followed by incubation at −80 °C for 1 hour. Proteins were pelleted by centrifugation (17, 000 × g for 10 min) at 4 °C and pellets washed twice with ice-cold acetone. Pellets were air dried at room temperature then resuspended in 8 M urea in PBS (30 μL) and solubilized by heating (65 °C for 5 min) and sonication. Once the pellet had dissolved, 70 μM NH_3HCO_3 (70 μL) was added, and the sample was treated with 15 mM DTT (15 min 65 °C) and 20 mM iodoacetamide (room temperature, 30 min). The urea concentration was diluted to 2 M with PBS and 1 mM $CaCl_2$ and 2 μg trypsin (Promega) were added and the sample was incubated overnight at 37 °C. The trypsin was quenched with the addition of formic acid (15 μL) and the samples were centrifuged at 15000 rpm for 20 min. Our observation of light/heavy intensity ratios ($R_{L/H}$) for most peptides conformed complete metabolic labeling with heavy amino acids (Figure S3).

MS sample prep

Probes 1 or 2 (50 μM) were added to soluble, isotopically heavy or light, MCF7 lysates at 2 mg mL^{-1} (500 μL). The mixture was subjected to UV irradiation for 1 hour on ice. The heavy and light samples were combined and precipitated with the addition of 10 % TCA and incubated at −80 °C for 1 hour. Samples were centrifuged at 17, 000 × g for 10 min and the pellets were washed twice with ice cold acetone. After allowing the protein pellets to air dry, pellets were resuspended in 1.2 % SDS by repeated rounds of heating at 65 °C and sonication. The SDS was diluted to 2.5 % with the addition of 5 mL PBS and the solution was incubated with 170 μL streptavidin agarose beads (Thermo Fisher) overnight at 4 °C then 3 hours at room temperature. The beads were washed with PBS (3×5 mL), and water (3×5 mL). The beads were pelleted by centrifugation (1400 ×g, 3 min) between washes. The washed beads were resuspended in 500 μL 6 M urea / PBS and 10 mM DTT and incubated at 65 °C for 15 min. 20 mM iodoacetamide was then added and the samples were incubated at 37 °C for 30 min. After reduction and alkylation, the beads were pelleted by centrifugation (1400 × g, 3 min), and resuspended in 200 μL 2 M urea / PBS, 1 mM $CaCl_2$, and 2 μg trypsin (Promega) and incubated overnight at 37 °C. The tryptic digest was collected, and the beads were washed 2 × 75 μL with water and the washes combined with the tryptic digest for a final sample volume of ∼350 μL. The trypsin was quenched with the addition of 15 μL formic acid and stored at −20 °C until MS analysis.

Liquid chromatography mass spectrometry

Mass spectrometry was performed on an LTQ Orbitrap Discovery (Thermo Fisher) coupled to an Agilent 1200 series HPLC. Protein digests were pressure loaded onto a 250 μm fused silica desalting column, packed with 4 cm Aqua C18 reverse phase resin (Phenomenex). Peptides were

eluted onto a 100 μm fused silica biphasic column, packed with 4 cm strong cation exchange resin (Watman) and 10 cm C18 resin using a using a five-step multidimensional LC/LC-MS/MS protocol (MudPIT)[1]. Each of the five runs begins with a salt push (0 %, 25 %, 50 %, 80 %, and 100 % buffer C), followed by a gradient 0–100 % buffer B in buffer A (buffer A: 95 % water, 5 % ACN, 0.1 % formic acid; buffer B: 20 % water, 80 % ACN, 0.1 % formic acid; buffer C 95 % water, 5 % ACN, 500 mM ammonium acetate). The flowrate was ~0.25 μL min^{-1} and the spray voltage was set to 2.75 kV. One survey MS1 scan (400-1800 *m/z*) was followed by 8 data dependent scans of the n^{th} most intense ion.

Peptide identification

Peptides were assigned to MS2 spectra using the SEQUEST[2] algorithm, searching against a human reverse-concatenated non-redundant protein database. Data sets were searched independently for peptides containing isotopically light and heavy amino acids. For the light search the default masses of lysine and arginine were used, for the heavy search a static modification was specified for lysine (+6.020 13 Da) and arginine (+10.008 26 Da). A static modification was specified for cysteine residues (+57.0215 Da, iodoacetamide alkylation) in both heavy and light samples. MS2 matches were assembled by protein and filtered to achieve a false discovery rate of 5 % using DTASelect 2.0[3].

Ratio quantification

Light/heavy intensity ratios ($R_{L/H}$) were calculated using the CIMAGE quantification package as described previously[1, 4]. CIMAGE reports peptide $R_{L/H}$ for co-eluting light and heavy peptides.

The peptides are grouped by protein and a representative $R_{L/H}$ for each protein is calculated from the median $R_{L/H}$ for each peptide identified for that protein.

PFKL expression and activity assay

Recombinant PFKL was prepared by baculovirus expression as described previously[5, 6]. Briefly, complementary DNA (cDNA) encoding isoform b of Homo sapiens PFKL (NP_002617) was inserted into pFastBac HTa vectors. Baculovirus was used to infect sf9 cells at a density of 2 × 106 cells mL^{-1} at a multiplicity of infection (MOI) of 2 for 48 hours in a shaking flask at 26 °C. The activity of recombinant PFKL was confirmed with an auxiliary enzyme assay[6]. Reactions were performed at a final volume of 200 µL containing 50 mM HEPES at pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 0.15 mM NADH, 0.675 U mL^{-1} aldolase, 5 U mL^{-1} triosephosphate isomerase, 2 U mL^{-1} glycerol phosphate dehydrogenase, 0.25 mM ATP, and 0.25 mM ADP. Auxiliary enzymes were de-salted using an Amicon Ultracel-10K Centrifugal Filter Unit prior to use. The consumption of NADH was monitored by the measuring the absorbance at 340 nm using a SpectraMax M5 plate reader (Molecular Devices). The initial reaction rates were measured at various concentrations of F6P. The rate and the concentration of F6P were fit the Michaelis-Menten equation having the form:

$$r = \frac{V_{max} C}{K_m + C}$$

where r is the reaction rate, C is the substrate concentration V_{max} is the maximum rate of the system, and K_m is the Michaelis–Menten constant.

Constructs and antibodies

Mouse Mical1 (NP_612188.1) (residues 1-611)[7], and transcript variant 1 of human HSPA8 (NP_006588.1), were cloned into pET28b and expressed in BL21 E. Coli. Streptavidin-HRP Conjugate (EMD Millipore); PFKL, and HSPA8 (1F2-H5) (Novus Biologicals); β-actin mouse mAb (15G5A11/E2) (Invitrogen); β-actin Rabbit Ab, HA-Tag (C29F4), GAPDH (Cell Signaling) antibodies were used for western blotting and/or immunoprecipitation assays. Purified reduced and Mical oxidized rabbit skeletal muscle actin were purchased from Cytoskeleton Inc.

Protein purification

Cell pellets were thawed on ice and resuspended in PBS (5 mL). Cells were lysed by sonication and centrifuged at 10,000 × g for 20 minutes at 4 °C. The supernatant was loaded onto a Ni-NTA column equilibrated with PBS, 10 mM imidazole (pH 7.4). The column was washed with 10 column volumes 25 mM imidazole in PBS, and the purified protein was eluted with 4 × 1 column volume fractions with 250 mM imidazole in PBS. Elution fractions were analyzed for purity by SDS-PAGE and the protein containing fractions were combined. Protein fractions were desalted to remove imidazole using a NAP-5 column (Thermo Fisher).

Immunoprecipitation

200 µg MCF7 lysate at 1 mg mL^{-1} was incubated with 10 µg of a mouse α-ACTB mAb overnight at 4 °C. The mixture was then incubated with 50 µL protein-G agarose beads (Thermo Fisher) for 2 hours at room temperature. The bound proteins were eluted from the beads by heating to 85 °C in SDS-PAGE loading buffer for 30 minutes. The input and eluting fractions were then analyzed by western blot.

F-actin pulldown

200 µg MCF7 lysate at 1 mg mL^{-1} was incubated with 25 µg biotin-phalloidin (Thermo Fisher) incubated at room temperature for 1 hour. The mixture was incubated with 50 µL streptavidin-agarose beads overnight at 4 °C with constant rotation. The bound proteins were eluted from the beads by heating to 85 °C in SDS-PAGE loading buffer for 30 minutes. The input and eluting fractions were then analyzed by western blot.

Actin pulldown

10 µg reduced or Mical oxidized rabbit skeletal muscle actin was solubilized in G-actin buffer as described previously[8] and diluted to 6 µM, then labeled with 20 molar excess biotin-LC-LC-NHS ester (Thermo Fisher) according to the manufacturer's protocol. The biotinylated actin was desalted on a NAP-5 column to remove excess linker then immobilized on 50 µL streptavidin-agarose beads by incubation at room temperature for 2 hours with constant rotation (200 µL 6 µM actin per sample). Recombinant PFKL and HSPA8 were combined and diluted to a final concentration of 6 µM with actin G-buffer and added to the immobilized actin (200 µL per sample), and incubated overnight at 4 °C overnight with constant rotation. The beads were washed with PBS (3 × 1 mL) and pelleted by centrifugation (1400 × g, 3 min) between washes. The bound proteins were eluted from the beads by heating to 85 °C in SDS-PAGE loading buffer for 10 minutes. The input and eluting fractions were then analyzed by western blot.

Western blotting

Samples were separated on a 10 % SDS-PAGE gel then transferred to nitrocellulose membranes at 75 V for 100 minutes. The membranes were blocked with 5 % non-fat powdered milk in TBS-T for 1 hour at room temperature. The membrane was probed with primary antibody (1:1000) in 5 % powdered milk / TBS-T overnight at 4 ◦C. The blot was washed (3 × 5 min) with TBS-T then incubated with a α-rabbit-HRP conjugated secondary antibody (1:2000) for 2 hours at room temperature. For biotin- blots, after electrotransfer, membranes were blocked overnight with 5 % non-fat dry milk in TBS-T at room temperature. The membrane was then washed (3 × 5 min) and probed with streptavidin-HRP (1:1000) in TBS-T for 1 hour at room temperature. After probing with secondary antibody or streptavidin-HRP, membranes were washed 3 × 5 min with TBS-T and treated with chemiluminescence reagents (Thermo Fisher) and developed on a Bio-Rad ChemDoc MP imaging system.

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