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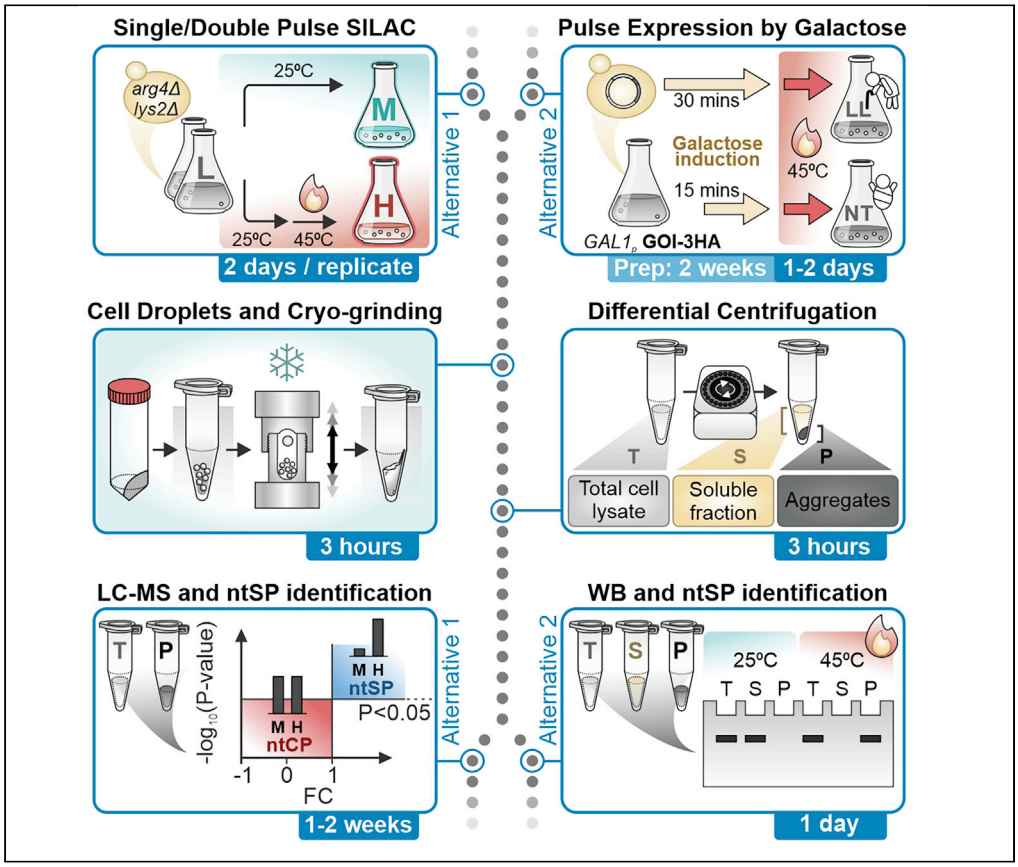
Identification of newly translated thermo-sensitive proteins using pulse SILAC mass spectrometry and the GAL promoter system



Some newly translated proteins are more susceptible to misfolding and aggregation upon heat shock in comparison to other proteins. To study these newly translated thermo-sensitive proteins on a proteomic scale, we present here a protocol that combines pulse-SILAC with biochemical fractionation for mass spectrometry analysis, followed by an orthogonal validation protocol for selected candidates using the *GAL* promoter system in *Saccharomyces cerevisiae*. This approach can be further developed to study other stresses and specific post-translational modifications or adapted to mammalian cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Pulse-SILAC labeling to identify newly translated proteins by mass spectrometry

Detailed steps of yeast cryo-lysis and pellet fraction collection

Galactose induction for temporally defined protein expression in yeast

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Identification of newly translated thermo-sensitive proteins using pulse SILAC mass spectrometry and the GAL promoter system

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SUMMARY

Some newly translated proteins are more susceptible to misfolding and aggregation upon heat shock in comparison to other proteins. To study these newly translated thermo-sensitive proteins on a proteomic scale, we present here a protocol that combines pulse-SILAC with biochemical fractionation for mass spectrometry analysis, followed by an orthogonal validation protocol for selected candidates using the *GAL* promoter system in *Saccharomyces cerevisiae*. This approach can be further developed to study other stresses and specific post-translational modifications or adapted to mammalian cells.

For complete details on the use and execution of this protocol, please refer to Zhu et al. (2022).[1]

BEFORE YOU BEGIN

This protocol was developed to identify by mass spectrometry (MS) a subset of newly translated proteins in order to then characterize their associated chemicophysical properties. In our case, we sought to identify which proteins are more thermo-sensitive following synthesis in comparison to other proteins in the cell. We presumed that proteins that have not yet completed their maturation into their stable native state would become insoluble following heat stress. Therefore, we used centrifugation to isolate proteins that become enriched in the pellet fraction after heat shock in *Saccharomyces cerevisiae*. Other stress conditions of interest may be used instead. In addition, the protocol could be adapted to other biochemical fractionation methods to identify specific post-translation modifications for instance.

In the first part of the protocol, pulse SILAC (Stable Isotope Labelling by Amino acids in Cell culture), where medium or heavy labeled amino acids are added for a short period of time, is used to identify by MS newly translated proteins (SILAC labeled) versus long-lived proteins (unlabeled). The use of pulse SILAC allows for a relatively easy workflow in comparison to other approaches such as click chemistry. For SILAC in yeast cells, a strain lacking the ability to synthesize their own lysine and arginine residues must be used in order to get good labeling of proteins with heavy labeled SILAC amino acids. The common laboratory strain BY4742 (*MATα*) is *lys2Δ0* and *MATa* cells with *lys2Δ0* can be obtained through mating and sporulation if desired. Further deletion of the *ARG4* gene can be achieved through homologous recombination.[2,3]

SILAC quantitation is limited to three channels (*light*, *medium* and *heavy*) in most mass spectrometry instruments. Therefore, the identification of newly translated thermo-sensitive proteins required two

set of experiments to first determine which newly translated proteins are enriched in the pellet fraction, and then to resolve which of these newly translated proteins are specifically enriched in the pellet fraction after the stress. These two experiments are respectively labeled Exp 1 and Exp 3 in our previously published study.[1] In this protocol, we first describe a single pulse-SILAC labeling experiment (step 1 is similar to Exp 1) and then how to repeat the protocol to execute a double pulse-SILAC labeling experiment (step 2 is similar to Exp 3).

In the second part of the protocol, we describe how to validate selected hits by Western blotting using the yeast *GAL* promoter system. For practical reasons, this protocol is limited to a small subset of candidate proteins, as each of them needs to be individually evaluated, which is time consuming. The second section of the protocol could be used independently to specifically evaluate properties of newly translated proteins using Western Blots. It first requires each candidate to be sub-cloned before they can be assessed, which can affect the proposed timeline depending on each laboratory set up. For instance, if the candidate contains an intron, which is relatively rare is yeast, a different strategy is required by either using a cDNA library instead of genomic DNA, or Gibson assembly to fuse the exons together.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-HA	ABLab	Cat# 12CA5
Rabbit polyclonal anti-Pgk1	Acris	Cat# AP21371AF-N
IRDye® 800CW goat anti-mouse IgG	LI-COR	Cat# 926-32210
IRDye® 800CW goat anti-rabbit IgG	LI-COR	Cat# 926-32211
Bacterial and virus strains		
NEB5α competent *E. coli*	NEB	Cat# C2987I
Chemicals, peptides, and recombinant proteins		
Adenine hemisulfate salt	Sigma-Aldrich	Cat# A9126-100G
$^{13}C_6$ Arg-6	Silantes	Cat# 201204102
13C615N4 Arg-10	Silantes	Cat# 201604102
13C615N2 Lys-8	Silantes	Cat# 211604102
D_4 Lys-4	Silantes	Cat# 211104113
Dextrose/D-Glucose	Fisher Chemical	Cat# D16-10
Difco™ Yeast Nitrogen Base without Amino Acids (YNB)	BD Life Sciences	Cat# 291940
D-(+)-Galactose	Bio Basic	Cat# GB0215
IGEPAL® CA-630 (NP-40)	Sigma-Aldrich	Cat# I3021-100ML
L-Histidine monohydrochloride monohydrate	Sigma-Aldrich	Cat# H8125-100G
L-Leucine	Sigma-Aldrich	Cat# L8000-100G
L-Lysine monohydrochloride	Sigma-Aldrich	Cat# L5626-100G
L-Methionine	Sigma-Aldrich	Cat# M9625-100G
L-Tryptophan	Sigma-Aldrich	Cat# T0254-100G
Phenylmethylsulfonyl fluoride (PMSF)	Roche	Cat# 1359061
cOmplete™, EDTA-free Protease Inhibitor Cocktail (PIC)	Roche	Cat# 5056489001
Raffinose, pentahydrate, high purity	Bio Basic	Cat# RJ392
Sequencing grade modified Trypsin, frozen	Promega	Cat# V5113
Sera-Mag SpeedBeads A (GE Healthcare Sera-Mag SpeedBeads™ carboxyl magnetic beads, hydrophilic)	Thermo Scientific	Cat# 09-981-121
Sera-Mag SpeedBeads B (GE Healthcare Sera-Mag SpeedBeads™ carboxyl magnetic beads, hydrophobic)	Thermo Scientific	Cat# 09-981-123
Sodium carbonate ReagentPlus®, ≥99.5%	Sigma-Aldrich	Cat# S2127-500G
Synthetic Dropout medium for yeast without Lysine	Sigma-Aldrich	Cat# Y1896-20G
Uracil	Sigma-Aldrich	Cat# U0750-100G

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Gibson Assembly Master mix	NEB	Cat# E2611L
Q5® high-fidelity DNA polymerase	NEB	Cat # M0491L
Deposited data		
Mass spectrometry results of pellet and total cell lysate fractions from single and double pulse SILAC (Exp1 and Exp3)	[1] PRoteomics IDEntification (PRIDE) repository	PXD024336
Experimental models: Organisms/strains		
BY4741 strains (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*)	Hieter Lab	YTM408
SILAC strain (*MATa, his3Δ1, leu2Δ0, ura3Δ0, MET15, arg4Δ::KanMX6, lys2Δ0*)	Measday Lab	YTM1173
Recombinant DNA		
pRS426-*GAL1*$_p$-3HA-*CYC1*$_t$	Mayor Lab	BPM1809
Software and algorithms		
Bruker Compass HyStar 5.0 SR1	Bruker Daltonics	Ver. 5.0.37.0
MaxQuant	Computational Systems Biochemistry under Prof. Jürgen Cox	1.6.14
Other		
Agarose gel electrophoresis system (BioRad Mini-Sub Cell GT)	Bio-Rad	1664288EDU
Blue light box	Clare Chemical Research Dark Reader	DR89X
Mass spectrometry (MS) instrument (Bruker Impact II)	Bruker	Impact II
Thermo Scientific Nanodrop One	Thermo Scientific	ND-ONE-W
LI-COR Odyssey CLx	LI-COR	Odyssey CLx
Retsch MM400 mixer mill	Retsch	MM400
Thermocycler	Bio-Rad	S1000
Trans-Blot Turbo	Bio-Rad	1704150

MATERIALS AND EQUIPMENT

The following is a list of materials and equipment we used, however, you may also use alternatives/different models.

- Heating block for 1.5 mL tubes (set at 95°C).
- HPLC (Agilent 1200 series with Zorbax extend column: 1.0 × 50 mm, 3.5 μm particles).
- Liquid nitrogen.
- Magnetic rack capable of holding 1.5 mL microcentrifugation tubes.
- Nano-liquid chromatography (LC) coupled to MS instrument.
- PCR plate (Axygen PCR-96-FS-C-S).
- Refrigerated microcentrifuge.
- Shaker incubator.
- Shaking water bath.
- Sonicating water bath.
- Spectrophotometer.
- SpeedVac.
- Tabletop refrigerated clinical centrifuge.
- Two 1.5 mL grinding jars (Retsch #014620230).
- Two 2 mm stainless steel grinding balls (Retsch #224550010).

100× SD drop-out mix (leave out desired nutrient for specific drop-out)

Reagent	Final concentration	Amount
Adenine hemisulfate	2 g/L	0.2 g
L-Histidine	2 g/L	0.2 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
L-Leucine	10 g/L	1 g
L-Lysine	3 g/L	0.3 g
L-Methionine	2 g/L	0.2 g
L-Tryptophan	2 g/L	0.2 g
Uracil	2 g/L	0.2 g
ddH_2O	N/A	Up to 100 mL
Total	**100×**	**100 mL**

Filter sterilize with a 0.2 μm filter before use. Store at 20°C–22°C in the dark. Shelf life is 1 year.

Pre-SILAC Mix		
Reagent	Final concentration	Amount
10× YNB	1×	100 mL
50% dextrose	2%	40 mL
100× SD drop-out mix without lysine	1×	10 mL
ddH_2O	N/A	Up to 1,000 mL
Total	**1×**	**1,000 mL**

Lysine is omitted from the 100× SD drop out mix. All stock reagents must be sterile. Prepare fresh or, if needed, store at 4°C. Shelf life is 1 week.

***Light* SILAC medium**		
Reagent	Final concentration	Amount
Pre-SILAC Mix	1×	300 mL
150 mg/mL Lys-0 Stock	0.03 mg/mL	60 μL
85 mg/mL Arg-0 Stock	0.02 mg/mL	70 μL
Total	**1×**	**300 mL**

Make fresh.

***Medium* SILAC medium**		
Reagent	Final concentration	Amount
Pre-SILAC Mix	1×	300 mL
150 mg/mL Lys-4 Stock	0.03 mg/mL	60 μL
85 mg/mL Arg-6 Stock	0.02 mg/mL	70 μL
Total	**1×**	**300 mL**

Make fresh.

***Heavy* SILAC medium**		
Reagent	Final concentration	Amount
Pre-SILAC Mix	1×	300 mL
150 mg/mL Lys-8 Stock	0.03 mg/mL	60 μL
85 mg/mL Arg-10 Stock	0.02 mg/mL	70 μL
Total	**1×**	**300 mL**

Make fresh.

Single dropout synthetic defined medium without uracil (SD-Ura) with appropriate sugars		
Reagent	Final concentration	Amount
10× YNB	1×	50 mL
100× SD drop-out mix without uracil	1×	5 mL
50% dextrose or 40% raffinose	2%	20 mL (dextrose) 25 mL (raffinose)
ddH_2O	N/A	Up to 500 mL
Total	**1×**	**500 mL**

Store at 20°C–22°C. Shelf life is 1 year.

Uracil is omitted from the 100× SD drop out mix to ensure the plasmid with the GOI to be assessed is maintained in the cells. Store at room temperature.

Tris-buffered saline (TBS)		
Reagent	Final concentration	Amount
Tris pH 7.5	50 mM	6.05 g
NaCl	150 mM	8.76 g
1 M HCl	To adjust to pH 7.5	N/A
ddH_2O	N/A	Up to 1,000 mL
Total	**1×**	**1,000 mL**

Filter sterilize and store at 4°C. Shelf life is 6 months.

2× Native Lysis buffer	
Reagent	Final concentration
Tris pH 7.5	100 mM
NaCl	150 mM
PMSF	2 mM
PIC	2×

Prepare fresh and maintain ice-cold.

1× Native Lysis buffer with 1.5% NP-40	
Reagent	Final concentration
Tris pH 7.5	50 mM
NaCl	150 mM
PMSF	1 mM
PIC	1×
IGEPAL® CA-630 (NP-40)	1.5% v/v

Prepare fresh and maintain ice-cold.

1× Native Lysis buffer with 1% NP-40	
Reagent	Final concentration
Tris pH 7.5	50 mM
NaCl	150 mM
PMSF	1 mM
PIC	1×
IGEPAL® CA-630 (NP-40)	1% v/v

Prepare fresh and maintain ice-cold.

3× Modified Laemmli buffer		
Reagent	Final concentration	Amount
Tris Base	0.1875 M	1.1205 g
SDS	6%	3 g
Glycerol	30%	15 mL
ddH_2O	N/A	Up to 50 mL
Total	**3×**	**50 mL**
Store at 20°C–22°C. Shelf life is 1 year.		

⚠ CRITICAL: SDS is a potentially harmful/toxic chemical, check MSDS for proper PPE before handling.

1× Modified Laemmli buffer		
Reagent	Final concentration	Amount
Tris Base	0.0625 M	0.3735 g
SDS	2%	1 g
Glycerol	10%	5 mL
ddH_2O	N/A	Up to 50 mL
Total	**1×**	**50 mL**
Store at 20°C–22°C. Shelf life is 1 year.		

⚠ CRITICAL: SDS is a potentially harmful/toxic chemical, check MSDS for proper PPE before handling.

Buffer A		
Reagent	Final concentration	Amount
Formic acid	0.1%	1 mL
Acetonitrile	5%	50 mL
ddH_2O	N/A	Up to 1,000 mL
Total	**1×**	**1,000 mL**
Store in amber bottle at 20°C–22°C. Shelf life is 6 months.		

⚠ CRITICAL: Organic Acid and solvent are potentially harmful/toxic chemicals, check MSDS for proper PPE before handling.

Buffer B		
Reagent	Final concentration	Amount
Formic acid	0.1%	1 mL
Acetonitrile	90%	900 mL
ddH_2O	N/A	Up to 1,000 mL
Total	**1×**	**1,000 mL**
Store at 20°C–22°C. Shelf life is 6 months.		

⚠ CRITICAL: Organic Acid and solvent are potentially harmful/toxic chemicals, check MSDS for proper PPE before handling.

200 mM Ammonium formate pH10		
Reagent	Final concentration	Amount
Ammonium formate	200 mM	6.30559 g
Ammonium hydroxide	N/A	Adjust pH to 10
ddH_2O	N/A	Up to 500 mL
Total	**1×**	**500 mL**
Store at 20°C–22°C. Shelf life is 6 months.		

⚠ CRITICAL: Ammonium hydroxide is a corrosive/hazardous chemical, check MSDS for proper PPE before handling.

High pH Buffer A		
Reagent	Final concentration	Amount
200 mM Ammonium formate pH10	5 mM	25 mL
Acetonitrile	2%	20 mL
ddH_2O	N/A	Up to 1,000 mL
Total	**1×**	**1,000 mL**
Store in amber bottle at 20°C–22°C. Shelf life is 6 months.		

⚠ CRITICAL: Organic solvents are potentially harmful/toxic chemicals, check MSDS for proper PPE before handling.

High pH Buffer B		
Reagent	Final concentration	Amount
200 mM Ammonium formate pH10	5 mM	25 mL
Acetonitrile	90%	900 mL
ddH_2O	N/A	Up to 1,000 mL
Total	**1×**	**1,000 mL**
Store at 20°C–22°C. Shelf life is 6 months.		

⚠ CRITICAL: Organic solvents are potentially harmful/toxic chemicals, check MSDS for proper PPE before handling.

STEP-BY-STEP METHOD DETAILS

Preparation of pulse SILAC labeled cells

Timing: 2 days per replicate

The following steps describe the procedures for labelling newly translated proteins in yeast using a single pulse SILAC with lysine and arginine residues labeled in *light* (Lys-0, Arg-0) then medium (Lys-4, Arg-6) stable isotopes prior to harvesting cells (step 1; Figure 1). Instead of *medium* SILAC, *heavy* SILAC (Lys-8, Arg-10) could also be used, as the pulse labeled proteins will be distinct from *light* SILAC in both cases. Step 2 describes how the double pulse-SILAC experiment is performed. Both experiments must be performed in parallel to identify newly translated thermo-sensitive proteins. We recommend collecting at least 3 replicates for each experiment.

1. Prepare and collect single pulse SILAC culture.

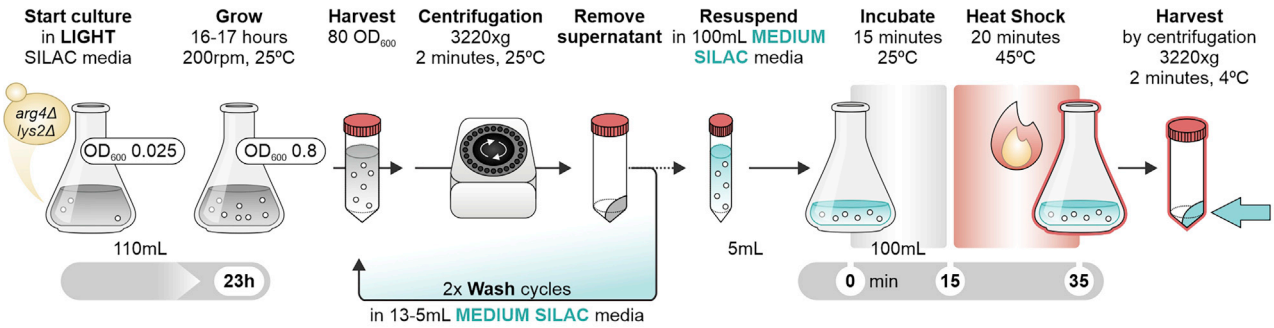

Figure 1. Schematic of the preparation of single pulse SILAC labeled cells (step 1) and cell harvesting (step 3a)

a. Resuspend 3–4 yeast colonies (*arg4Δ*, *lys2Δ*) from a fresh plate (less than two weeks old and stored at 4°C) in a 1.5 mL microcentrifuge tube with 100 µL of sterile ddH_2O or Pre-SILAC mix for a final OD_{600} ~35.
b. Measure the OD_{600} by diluting the cell suspension within the linear range of the spectrophotometer (typically between 0.1 and 0.8).
c. Add the proper amount of the cell suspension to 110 mL of *light* SILAC medium to obtain an overnight exponential culture. For instance, dilute to a final 0.025 OD_{600} for an overnight culture (5 cell doublings).
d. Incubate the culture at 25°C with shaking at 200 RPM overnight. Depending on the strain, media and laboratory conditions, cells will need 16–17 h of growth. Troubleshooting 1.
e. Collect 80 OD_{600} of cells from an ~0.8 OD_{600} culture.
    i. Collect cells in two 50 mL centrifuge tubes by centrifugation at 3,220 × *g* and at room temperature (RT) in a clinical centrifuge for 2 min.
    ii. Discard supernatant and wash cells by resuspending them with 13 mL of *medium* SILAC medium at room temperature and transfer to a 15 mL tube.
    iii. Pellet cells again by centrifugation at 3,220 × *g* at RT for 2 min.
    iv. Wash cells again by resuspending them with 5 mL of *medium* SILAC medium at room temperature.
    v. Pellet cells again by centrifugation at 3,220 × *g* at RT for 2 min.
    vi. Carefully aspirate all supernatant.
f. Resuspend cells in 100 mL *medium* SILAC medium and incubate at 25°C for 15 min.
g. At the end of the pulse-SILAC, transfer the culture flask to a 45°C shaking water bath and incubate for 20 min.

Note: within a couple of minutes, translation is fully inhibited in these conditions.

h. At the end of incubation, collect cells and snap-freeze in liquid nitrogen as cell pellets as described below in step 3 and 4 in cell harvesting, preparation of cell droplets and cell lysis.
i. Repeat step a-h for desired number of replicates. Replicate cultures are recommended to be setup and collected on different dates.

2. Prepare and collect double pulse SILAC culture (Figure 2).
    a. Resuspend 3–4 yeast colonies (*arg4Δ*, *lys2Δ*) from fresh plate (stored at 4°C for less than 2 weeks) into a 1.5 mL microcentrifugation tube with 100 µL of sterile ddH_2O or Pre-SILAC Mix for a final OD_{600} ~35.
    b. Measure the OD_{600} by dilution of the cell suspension within the linear range of the spectrophotometer.
    c. Set up one flask containing 110 mL of *light* SILAC medium. Add the proper amount of the cell suspension for a final OD_{600} of 0.025.

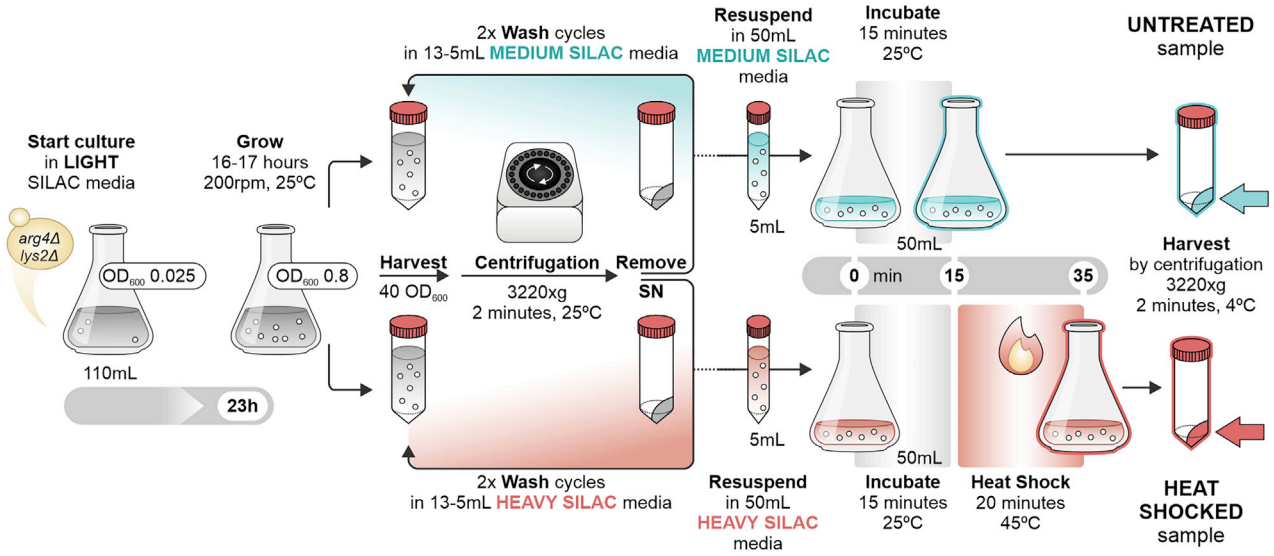

Figure 2. Schematic of the preparation of double pulse SILAC labeled cells (step 2) and cell harvesting (step 3a)
SN, supernatant.

d. Incubate the yeast culture at 25°C with shaking at 200 RPM overnight (16–17 h). Troubleshooting 1.
e. Once the culture reaches ~0.8 OD_{600}, collect two aliquots (one for *medium* and another for *heavy* pulse SILAC, each ~40 OD_{600}).
   i. For each aliquot, collect 50 mL of culture in a 50 mL centrifuge tubes and spin the cells in a clinical centrifuge at 3,220 × *g* at RT for 2 min (two 50 mL tubes in total).
   ii. Discard supernatant and resuspending each cell population in 13 mL of the corresponding SILAC medium (*medium* SILAC or *heavy* SILAC media) at room temperature and transfer to a 15 mL tube without mixing *medium* and *heavy* SILAC cells (each 15 mL tube should contain ~40 OD_{600} of cells).
   iii. Pellet cells by centrifugation at 3,220 × *g* at RT for 2 min.
   iv. Wash cells by resuspending in 5 mL of the corresponding SILAC medium at RT.
   v. Pellet cells by centrifugation at 3,220 × *g* at RT for 2 min.
   vi. Carefully aspirate all supernatant.
f. Resuspend each cell population in 100 mL of corresponding SILAC medium and incubate at 25°C for 15 min.
g. Collect cells in the *medium* pulse labeled cells right away as described in step 3.
h. At the same time, transfer the *heavy* pulse labeled culture to a 45°C shaking water bath and incubate for another 20 min, then proceed to step 3.

Note: The two cultures will be mixed after first wash before freezing and lysis in step 3.

Cell harvesting, preparation of cell droplets and cell lysis

Timing: 3 h

This section describes how to harvest cells (step 3), generate frozen cell droplets (step 4) and lyse the cells via cryogrinding (step 5). Cell harvesting may span several days, each time the process of harvesting and freezing cells into droplets takes ~2–2.5 h. We recommend that lysis of all samples and further processing in step 6 occur on the same day. Lysis is carried out in batches of 2 samples (due to the capacity limits of the mixer mill) taking ~0.5–0.75 h/batch.

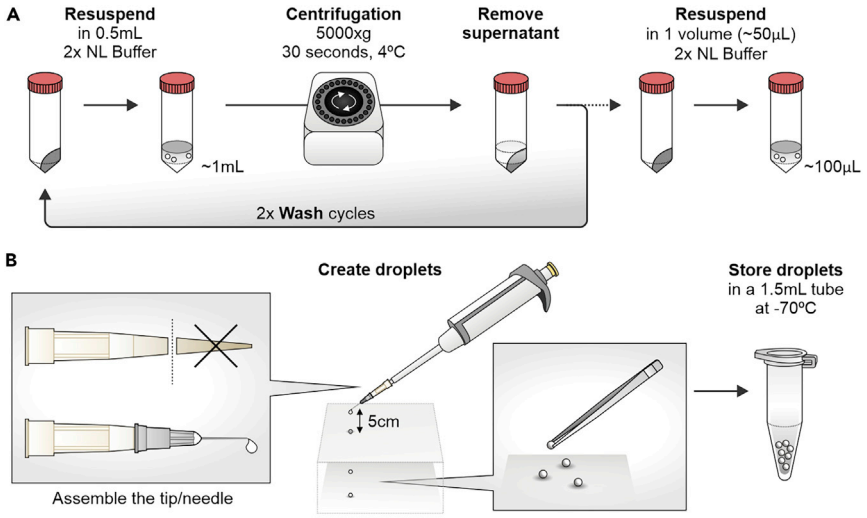

Figure 3. Cell harvest and freezing

Schematic of (A) cell washes (step 3) and (B) the preparation of cell droplets (step 4). NL, Native Lysis buffer.

3. Harvest and wash cells (Figure 3A).

⚠ CRITICAL: Perform all steps on ice and pre-chill all equipment and solutions.

   a. Collect cells from steps 1, 2 or 21–24 in 50 mL centrifuge tubes and immediately harvest them by centrifugation at 3,220 × *g* at 4°C for 5 min.
   b. Discard supernatant, resuspend the cell pellet in 13 mL of pre-chilled TBS and transfer to a 15 mL tube.
   c. Centrifuge at 3,220 × *g* at 4°C for 5 min and discard supernatant.
   d. Repeat steps b-c one more time with 5 mL of pre-chilled TBS, for a total of two washes. For double pulse SILAC samples, use the same 5 mL TBS to resuspend both *medium* and *heavy* pulse labeled cells to mix them into one sample.
   e. Resuspend the final cell pellet in an equivalent volume of pre-chilled 2× Native Lysis buffer. Use about 100 µL for the pellet of 100 mL of OD_{600} 0.8 culture.
   f. Proceed to step 4 with snap freezing.

4. Snap-freeze cell droplets (Figure 3B).
   a. Cut off about 2 cm from a P200 tip so that a 27-gauge needle can be tightly fitted and assembled as illustrated in Figure 3B.
   b. Place a disposable sterile pipette reservoir filled with liquid nitrogen in a large insulated container with liquid nitrogen up to ~ half the height of the reservoir. The pipette reservoir serves to limit distribution of the cell droplets.
   c. Using a P200 fitted with the modified tip, draw up the cells previously suspended in 2× Native Lysis buffer from step 3.
   d. Slowly drip the cell suspension into the liquid nitrogen forming small 1–2 mm droplets (alternatively use a repeater pipette set at 5 µL intervals), which will freeze and accumulate at the bottom.

Note: Hold the needle about 5 cm from the liquid nitrogen surface when forming droplets. If the needle does not allow droplets to form, it is likely frozen due to the cold vapor from the nitrogen. In that case, placing a finger (gloved) on the side of the needle will melt the frozen contents and re-enable the flow of droplets.

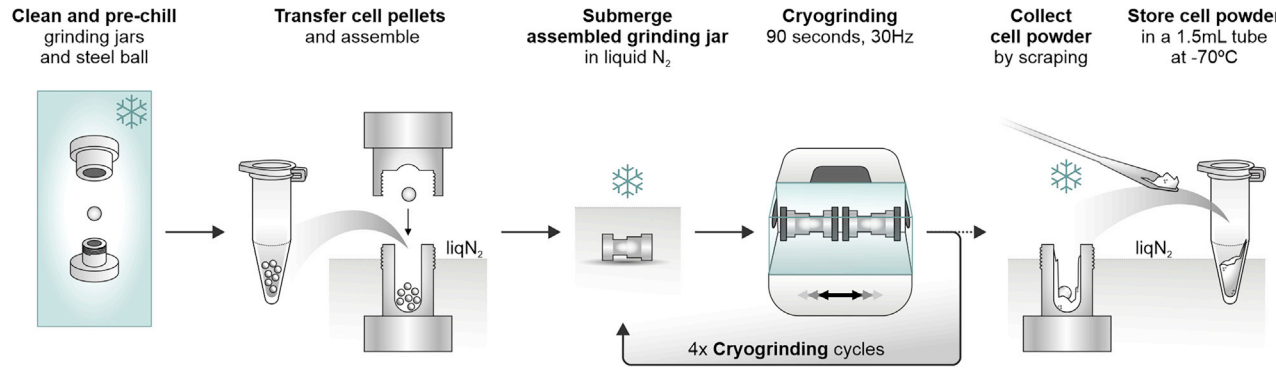

Figure 4. Schematic describing the cell lysis of the cell pellets with cryo-grinding (step 5)

e. Using tweezers with the tip previously chilled in liquid nitrogen, collect the droplets and transfer them to a pre-chilled 2 mL cryogenic vial.

Pause point: After snap freezing the cells in droplets, the samples can be stored in the vapor phase of liquid nitrogen indefinitely before generating the lysates by cryo-grinding.

5. Generate cell lysates by cryo-grinding (Figure 4).

CRITICAL: Keep the grinding jars, grinding balls, tubes (empty or with samples) and the contact surfaces of all tools sitting in a pool of liquid nitrogen. Do not let either the cell droplets or the cell powder thaw!

a. Clean the 1.5 mL grinding jars and the 2 mm stainless steel grinding ball by soaking them in a solution of 1% (w/v) sodium carbonate, rinse 3 times in distilled water and finally thoroughly rinse with 100% Ethanol and air dry in a fume or laminar flow hood.
b. Pre-chill the grinding jars (cup and lid separated) and one grinding ball per jar in liquid nitrogen, until the bubbling/boiling of the liquid nitrogen stops. Avoid getting liquid nitrogen into the cup or lid of the grinding jars. Use tongs and tweezers with tips pre-chilled in liquid nitrogen to manipulate cooled grinding jars.
c. Transfer the frozen cell droplets generated in step 4 to the cup of the grinding jar, place a stainless-steel grinding ball on top of the droplets and cap the jar tightly with the lid (use insulated gloves to screw on lid).
d. Keep the entire grinding jar submerged in liquid nitrogen until the bubbling stops and you are ready to proceed with the next step.
e. Mount the grinding jars on the Retsch MM400 mixer mill. The mixer mill can accommodate a maximum of 2 grinding jars at a time. If processing only one sample, a second empty jar must be mounted to balance the mill.
f. Cryo-grind at 30 Hz for 90 s.
g. Detach the jars from the mixer and submerge them in liquid nitrogen until the bubbling stops.
h. Repeat steps d-g for 3 additional cycles, for a total of 4 cryo-grinding cycles.
i. Submerge the grinding jars in liquid nitrogen to transport them from the mixer mill to your workbench.
j. At your workbench, remove the jars from the nitrogen and open carefully (using insulated gloves) identifying where the cell powder has settled (i.e., lid or cup).
k. Scrape the cell powder from the inner walls of the grinding jar using a spatula (pre-chilled in liquid nitrogen) to transfer the powder to a pre-chilled 1.5 mL microcentrifuge tube. Proceed to step 6.

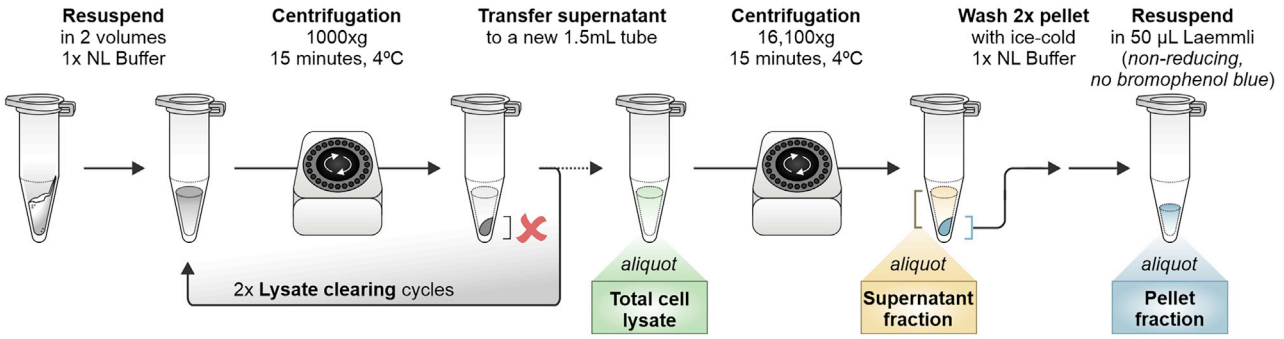

Figure 5. Schematic describing the differential centrifugation required to obtain pellet and supernatant fractions (step 6)

Differential centrifugation to obtain pellet and supernatant fractions

Timing: 3 h

This section describes how to generate the insoluble protein pellet and supernatant fractions for MS and Western blot analyses. For the MS experiment, both an aliquot of the total cell lysate (TCL) and pellet fractions are analyzed. For the Western Blot analysis an aliquot of the supernatant fraction is also analyzed. We recommend that up to 12 samples are prepared in parallel.

6. Collect pellet and supernatant protein fractions (Figure 5).
   a. Thaw cell lysate from step 5 on ice and add 2 volumes of 1× Native Lysis buffer with 1.5% NP-40 (i.e., add 400 µL–100 µL of cells that were lysed in 100 µL of 2× Native Lysis buffer for a final volume of 600 µL). This gives a final concentration of 1% NP-40.
   b. Pre-clear the lysate twice by centrifugation in a microcentrifuge at 1,000 × *g* at 4°C for 15 min. Save an aliquot of this as the total cell lysate (TCL) control (50 µL for MS in step 8 and ~20 µL for western blots in step 25). Fully solubilize proteins in the aliquot by adding 3× modified Laemmli buffer to 1× final concentration.
   c. Pellet insoluble proteins by centrifugation at 16,100 × *g* at 4°C for 15 min.
   d. Transfer the supernatant to a new microcentrifuge tube. This is the supernatant fraction. Collect desired amount of supernatant (in most cases 50 µL is sufficient) and add 3× modified Laemmli buffer to 1× final concentration to fully solubilize proteins.
   e. Wash the insoluble protein pellet twice with ice-cold 1× Native Lysis buffer with 1% NP-40.
   f. Resuspend the pellet in 50 µL of 1× modified Laemmli buffer and incubate at 37°C with shaking at 1,400 RPM for an hour, incubate at 95°C for 5 min to fully solubilize proteins. This is the pellet fraction.

⚠ CRITICAL: For samples prepared for MS, test the tubes by incubating them at 95°C for 5 min with the 1× modified Laemmli sample buffer only and test for potential polymer contamination. See troubleshooting 2.

   g. Determine protein concentration of the fractions.
      i. Identify a suitable protein assay that is compatible with SDS such as the bicinchoninic acid (BCA) or detergent compatible (DC) protein assays that are widely used colorimetric assays based on the protein-induced reduction of copper.
      ii. Prepare a suitable dilution of TCL, Supernatant and Pellet fractions in a compatible buffer. ~20–50-fold dilutions recommended. Check the assay manufacturer's protocols (BCA or DC) for reagent compatibility and follow their protocols to determine protein concentrations.

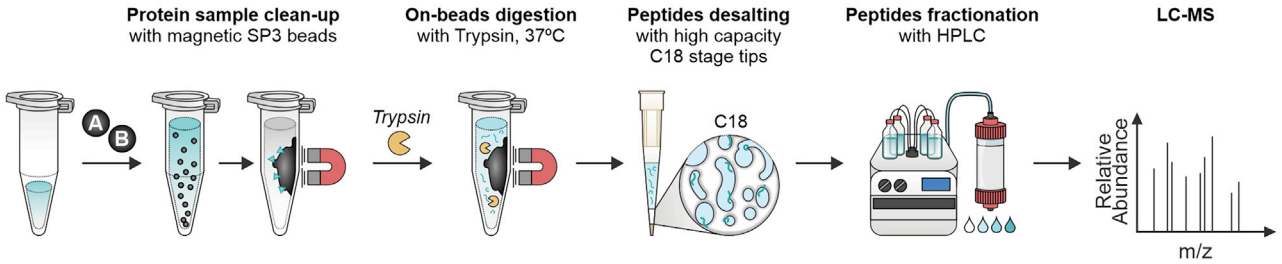

Figure 6. Schematic of the steps summarizing protein sample clean up, further digestion on SP3 beads, peptides desalting with high capacity C18 stage tips and further off-line high pH reverse phase fractionation (steps 7–14)

Pause point: All protein samples in 1× modified Laemmli buffer can be stored at −20°C indefinitely after snap-freezing in liquid nitrogen.

Protein sample clean up and digestion with SP3 beads

Timing: 2 days

These steps remove detergent used to solubilize proteins in the lysis process and digest proteins for downstream off-line peptide fractionation (Figure 6). This protocol is based on the Single-Pot Solid-Phase-enhanced Sample Preparation (SP3).[4] To increase the analysis depth, we performed an offline high pH fractionation (see steps 11 and 12) and 200 µg per sample is required to have enough material. These steps could be omitted and less material may be required when using more sensitive mass spectrometry instrument.

7. Beads preparation.
   a. Equilibrate Sera-Mag SpeedBeads A and B at room temperature. Sera-Mag SpeedBeads are provided at a stock concentration of 50 mg/mL.
   b. Prepare a 1:1 working solution of Sera-Mag SpeedBeads A and B (SP3 beads) mixture with a concentration of 20 mg/mL (the following example is for a single sample of 200 µg of proteins, scale up or down as needed).
      i. Take 20 µL of each bead stock and combine in a 1.5 mL microcentrifuge tube.
      ii. Place on a magnetic rack for 1 min to collect the beads and remove the supernatant.
      iii. Remove the tube from the rack and reconstitute the beads in 1 mL of water and mix well with pipette.
      iv. Place on a magnetic rack for 1 min to collect the beads and remove the supernatant.
      v. Remove the tube from the rack and reconstitute the beads in 100 µL of water.
8. SP3 cleanup and on beads digestion. For each sample, TCL and pellet fraction should be analyzed. The supernatant fraction can be saved for later or processed together if deemed necessary.
   a. Reduce 200 µg of the protein sample with TCEP at 3 mM final concentration. Incubate in the dark at 25°C for 20 min using a thermomixer with 500 RPM shaking.
   b. Alkylate the lysate with 2-chloroacetamide at 55 mM final concentration. 2-chloroacetamide must be freshly prepared. Incubate in the dark at 25°C for 30 min using a thermomixer with 500 RPM shaking.
   c. SP3 beads are used at a minimum ratio of 10:1 (w/w) beads:protein. For a single sample, add 100 µL of 20 mg/mL SP3 beads. Pipette to mix beads and lysate.
   d. Add 100% ethanol to a final concentration of 50%.

Note: Depending on the amount of protein present, the beads may clump and become very sticky immediately after the addition of ethanol. Minimize the amount of pipetting to avoid material loss due to beads sticking to the pipette tip.

e. Place tubes in the thermomixer and incubate at 25°C for 10 min with shaking at 1,000 RPM.
f. Place tubes in a magnetic rack for 2 min, or until the beads have settled on the tube wall, then remove and discard the supernatant taking care not to disrupt the beads.
g. Remove the tubes from the magnetic rack and add 200 µL of 80% ethanol. Gently tap or vortex to reconstitute and rinse the beads.
h. Place the tubes in the magnetic rack and incubate for 2 min, or until the beads have settled on the tube wall, then remove and discard the supernatant.
i. Repeat steps g-h two more times.
j. Use a P200 tip to remove as much ethanol from the tubes as possible.

Note: Try to remove as much ethanol as possible with pipetting prior to digestion. It is not necessary to air-dry the beads prior to the addition of the digestion solution.

k. Add 100 µL of 50 mM HEPES pH 8.0 containing trypsin (1:25 (w/w) enzyme: protein) to each tube. Do not pipette to mix, use a pipette tip to gently push the beads from the tube wall into the liquid.

Note: The beads may be very sticky, and material can be lost on pipette tips. It is recommended to add the digestion solution and simply push the beads into it using a pipette tip. Sonicating the mixture in a water bath will break up the beads clump.

l. Sonicate the tubes in a room temperature water bath 20 kHz and 320 W for 15 s, or scrape the tube along a tube rack, to reconstitute the beads.
m. Incubate tubes overnight (14 h) at 37°C with interval mixing (5 s on, 25 s off) at 1,000 RPM in a Thermomixer.
n. Centrifuge at 16,000 × *g* for 1 min to pellet the beads.
o. Place in a magnetic rack for 2 min, or until the beads have settled on the tube wall, and recover the supernatant in a new 1.5 mL tube.

Pause point: The peptides can be frozen and stored indefinitely at ≤ −70°C.

Peptides desalting with high capacity C18 stage tip

Timing: 4 h

This step describes the process of making high capacity C18 stage tips and using them for peptide desalting. Lower capacity stage tips should be used if steps 11 and 12 are omitted and less material required.

9. Packing high capacity C18 stage tip.
   a. Prepare regular stage tip based on previously published protocol.[5]
   b. Resuspend C18 powder in 50% acetonitrile.
   c. Add C18 suspension to stage tip and spin down at 1,000 × *g* to pack it. Adjust the volume of C18, so the resulting C18 column is ~3 mm tall from the C18 disc at the end of the tip.
10. Desalt peptides with high capacity C18 stage tip.
   a. Wash stage tip with 100 µL 100% methanol by centrifugation at 1,000 × *g* for 2 min.
   b. Wash stage tip 2× with 100 µL Buffer A by centrifugation at 1,000 × *g* for 2 min.
   c. Make sure the pH of the sample is < 2. If needed, use acetic acid to lower the pH.

d. Centrifuge the sample at maximum speed (>10,000 × *g*) for 2 min.
e. Load the sample, 100 μL at a time, into stage tip by centrifugation at 1.000 × *g* for 5 min (or longer if the liquid does not completely pass through C18).
f. Wash stage tip twice with 100 μL Buffer A by centrifugation at 1,000 × *g* for 2 min or until all liquid passes through the C18.

Pause point: Peptides on stage tip may be stored at 4°C for long period of time (months to a year) until they are ready to be eluted for downstream procedures.

g. Elute the peptides twice with 20 μL 40% Acetonitrile with 0.1% formic acid by centrifugation at 1,000 × *g* for 2 min.
h. Dry the peptides by vacuum centrifugation. Avoid over-drying to decrease peptide loss to adhesion to tube wall.
i. Resuspend dried peptides in 20 μL of High pH Buffer A.

Pause point: Samples can be stored at −20°C until they are ready for off-line fractionation.

Off-line high pH reverse phase fractionation of peptides

Timing: 2 h per sample excluding column conditioning (1–2 h to overnight)

The off-line high pH reverse phase fractionation protocol provides an orthogonal method to separate peptides and decrease sample complexity, while maintaining the ability to utilize the full low pH LC gradient on the mass spectrometer, leading to deeper coverage of the proteome.[6] The column used in this protocol is Agilent Zorbax Extend column: 1.0 × 50 mm, 3.5 μm particles.

11. Preparation of C18 Column.
    a. Change the lines from the storage buffers (50% methanol) to High pH buffers and purge each line at a flow rate of 50 μL/min for 6–7 min.
    b. Run 100% High pH Buffer B through the column for 10 min. This washes the column.
    c. Switch to 100% High pH Buffer A to equilibrate the column until a stable UV signal is observed. If pressure is higher than normal (a 50 μL/min flow rate should give around 50 bar column pressure), proceed to step d as the column is dirty. Otherwise, directly proceed to step 12.
    d. To wash the column, run a gradient as in step 12a (or a similar gradient with half of the time) 10–12 times. This step is recommended regardless of column cleanness as it helps to condition the column. Repeat step c before proceeding to step 12.

Optional: run a standard of choice to ensure the column is performing properly. i.e: 100pmol of BSA digest. If the chromatogram of BSA is satisfactory, proceed to step 12.

12. High pH reverse phase fractionation.
    a. Setup a HPLC method with following gradient at a flow rate of 50 μL/min:
        i. Minute 0 – 6% High pH Buffer B (against High pH Buffer A).
        ii. Minute 5 – 6% High pH Buffer B.
        iii. Minute 7 – 8% High pH Buffer B.
        iv. Minute 45 – 27% High pH Buffer B.
        v. Minute 49 – 31% High pH Buffer B.
        vi. Minute 53 – 39% High pH Buffer B.
        vii. Minute 60 – 60% High pH Buffer B.
        viii. Minute 69.5 – 60% High pH Buffer B.
        ix. Minute 70 – 0% High pH Buffer B.

x. Minute 80 – 0% High pH Buffer B.

b. During the gradient, collect the samples at 2 min intervals in a 96-well PCR plate (e.g., Axygen PCR-96-FS-C-S). The resulting 40 fractions are then pooled in a non-contiguous manner into 12 fractions per sample (i.e., samples from well A1, B1, C1 and D1 are pooled if the samples are collected from A1 to A12 then B1 to B12 and so on).

c. Dry the fractions after pooling by vacuum centrifugation.

Pause point: Dried samples can be stored in −20°C until they are ready for MS analysis.

d. Re-solubilize peptides with 20 μL 1% trifluoroacetic acid. This sample is MS-ready with no need for further desalting *via* stage tip. The use of trifluoroacetic acid helps to lower sample's high pH due to the basic buffer used in fractionation. This will extend the life of the column used in LC-MS.

LC-MS analysis

Timing: 2 h per sample

The off-line fractionated samples are analyzed on mass spectrometer in these steps. In our case, we used an Impact II (Bruker) mass spectrometer that was coupled to an EASY-nLC 1000 (Thermo Scientific) or a nanoElute (Bruker) liquid chromatography (LC). Other instruments can be used and the protocol should be accordingly adapted. For this analysis, we analyzed peptides from both the pellet fraction and the TCL, which corresponded to a total of 96 samples from the four replicates. The amount of peptides to be loaded on the instrument is typically optimized on each platform. In our case, we loaded 100 ng of peptides for each sample.

13. HPLC separation of peptides in samples.
    a. The LC is equipped with a 2 cm long, 100 μm inner diameter trap column packed with 5 μm-diameter Aqua C-18 beads (Phenomenex) and a 40-cm long, 50-μm-inner diameter fused silica analytical column packed with 1.9 μm-diameter Reprosil-Pur C-18-AQ beads (Dr. Maisch). The column is heated to 50°C using tape heater (SRMU020124, Omega.com and in-house build temperature controller).
    b. Peptides are separated at 0.4 μL/min flow rate with following 120-min gradient followed by a 15 min wash:
        i. Minute 0 – 5% Buffer B (against Buffer A).
        ii. Minute 60 – 13% Buffer B.
        iii. Minute 120 – 35% Buffer B.
        iv. Minute 122 – 90% Buffer B.
        v. Minute 135 – 90% Buffer B.
14. Peptides eluted from the LC column are analyzed by mass spectrometry with the following parameters.
    a. The nano ESI source was operated at 1,900 V capillary voltage, 0.25 Bar nanoBuster pressure with methanol in the nanoBooster, 3 L/min drying gas and 150°C drying temperature.
    b. The Impact II was set to acquire in a data-dependent auto-MS/MS mode with inactive focus fragmenting the 20 most abundant ions (at 18 Hz rate) after each full-range scan from 200 to 2000 Th (at 5 Hz rate).
    c. The isolation window for MS/MS was 2 to 3 Th.
    d. Depending on parent ion mass to charge ratio and the collision energy ranged from 23 to 65 eV depending on ion mass and charge.
    e. Parent ions were then excluded from MS/MS for the next 0.3 min and reconsidered if their intensity increased more than 5 times.
    f. Singly-charged ions were excluded since, in ESI mode, peptides usually carry multiple charges.

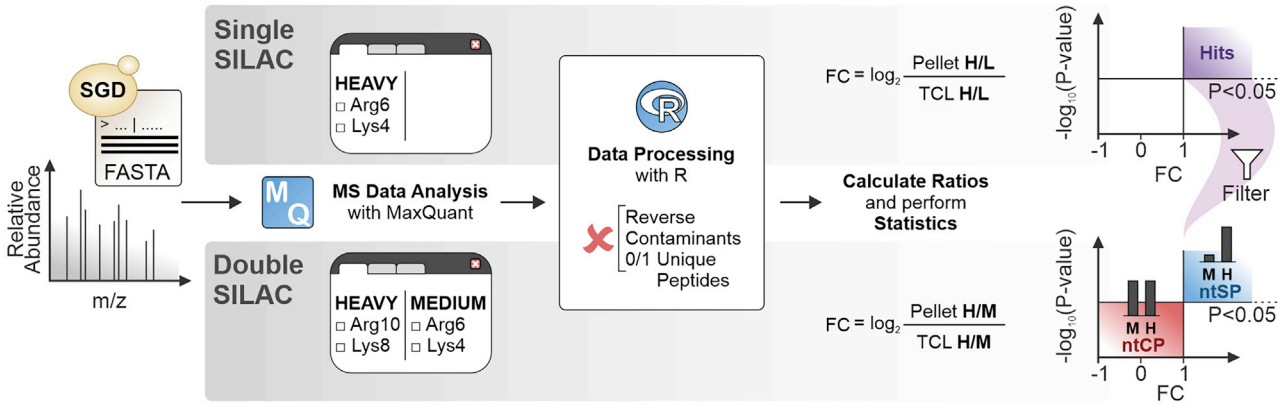

Figure 7. Schematic of the steps necessary for LC-MS analysis and final determination of newly translated thermo-sensitive proteins (ntSP) enriched in the pellet fraction (steps 15 and 16)

g. Strict active exclusion was applied. Mass accuracy: error of mass measurement is typically within 5 ppm and cannot exceed 10 ppm.
h. Following the analysis, collect the mass spectrometry raw file and proceed to step 15 for data analysis.

MS data analysis and determination of newly translated thermos-sensitive proteins (ntSP) enriched in the pellet fraction

Timing: Days to weeks (depending on how large the data is and how powerful the computer used for search is)

This step describes how to use MaxQuant to perform a proteomic search on the raw MS files and used the results from both single and double pulse SILAC experiments to determine which newly translated proteins are thermos-sensitive (ntSP) and which newly translated proteins accumulate constitutively in the pellet fraction (ntCP) (Figure 7). The most recent version of MaxQuant is recommended as it includes the newest features and updates to the search algorithm. Here, we used version 1.6.14. Other software can also be used and parameters should be adjusted accordingly. A key element of this analysis is that, following a pulse experiment, the SILAC ratio for each protein will be distinct in the TCL analysis (according to its translation and turnover rates) and therefore needs to be taken in account in the analysis.

15. Proteomic search using MaxQuant.
    a. Search raw files in MaxQuant against the latest released yeast proteome database provided by the Saccharomyces Genome Database (we used SGD_orf_trans_all_20150113) with built-in common contaminants from MaxQuant.
    b. Perform separate searches for single and double pulse-SILAC samples.
    c. In the *Raw data* tab, set all fractions from the same sample as a single experiment and assign with the corresponding fraction numbers for MaxQuant to properly determine the relationship between different raw files.
    d. In the *Group-specific parameters* and submenu *Type*, set *Multiplicity* to 2 for single pulse SILAC search; 3 for double pulse SILAC search. No specific selection is required for *light* labels. For single pulse SILAC search, set *heavy* labels to Arg6 and Lys4; for double pulse SILAC search, set *medium* labels to Arg6 and Lys4 and *heavy* labels to Arg10 and Lys8.
    e. In the *Global parameters* tab and submenu *Identification*, turn on match-between-runs and in the *Group-specific parameters*. In the submenu *Misc.*, turn on re-quantification to increase the number of peptides that are identified and quantified.

f. All other settings can be left at default as MaxQuant has those setting optimized for the selected instrument in most cases.

Note: The MaxQuant website provides detailed documentation for MaxQuant analysis workflow (http://coxdocs.org/doku.php?id=maxquant:manual:beginner) and training videos from previous MaxQuant summer school (https://www.youtube.com/c/MaxQuantChannel).

16. Analysis of MaxQuant search results.
    a. Open the proteinGroups.txt file from MaxQuant output with R or Excel. R is highly recommended as Excel struggles with large text dataset and may convert certain gene name to dates due to software flaw.
    b. Remove protein groups that are flagged as *Reverse* or *Potential contaminant*.
    c. Remove protein groups that are identified with only 1 peptide or 0 unique peptide. The resulting data is saved as `proteinGroups.csv`.

Note: For original code used for published MS data analysis,[1] please see https://doi.org/10.5281/zenodo.6640632.

    d. For single pulse SILAC results, divide the *Ratio H/L* of the pellet sample (H_P/L_P) by the *Ratio H/L* of the TCL (H_T/L_T) to calculate the $\log_2$ [(H_P/L_P) /(H_T/L_T)] for each protein in each replicate. Determine the averaged $\log_2$ ratio for each protein and calculate the *p*-value using Student's *t*-test against 0. The *t*-test can be easily performed using R's built-in *t*-test function. `YOUR_DATA` is the list containing you calculated $\log_2$ [(H_P/L_P) /(H_T/L_T)] for each protein you need perform *t*-test with, then call.

```
> t.test(YOUR_DATA, mu=0)$p.value
```

and the resulting p-value will be returned. Select proteins with at least a two-fold enrichment and p-value < 0.05. This will be the Hit set from single pulse SILAC. This is the group of newly translated proteins enriched in the pellet fraction. A sample script is shown below:

```
>library(dplyr)

>#import the proteinGroups.csv file

>MS_Data <- read.csv(proteinGroups.csv, header=TRUE)

>#T is total cell lysate(TCL), P is pellet, Ratio.H.L.MS_1_P et al are the experiment name you
set for MaxQuant search

>df <- select(MS_Data, Protein.IDs, Majority.protein.IDs, Fasta.headers, Ratio.H.L.MS_
1_P, Ratio.H.L.MS_1_T, Ratio.H.L.MS_2_P, Ratio.H.L.MS_2_T, Ratio.H.L.MS_3_P, Ra-
tio.H.L.MS_
3_T, Ratio.H.L.MS_4_P, Ratio.H.L.MS_4_T)

#Calculate TCL normalized Ratio

>df$Ratio.MS_P_T_1 <- df$Ratio.H.L.MS_1_P/df$Ratio.H.L.MS_1_T

>df$Ratio.MS_P_T_2 <- df$Ratio.H.L.MS_2_P/df$Ratio.H.L.MS_2_T

>df$Ratio.MS_P_T_3 <- df$Ratio.H.L.MS_3_P/df$Ratio.H.L.MS_3_T

>df$Ratio.MS_P_T_4 <- df$Ratio.H.L.MS_4_P/df$Ratio.H.L.MS_4_T


>#Log2 of TCL normalized Ratio

>df$Log2.MS_P_T_1 <- log2(df$Ratio.MS_P_T_1)
```

```
>df$Log2.MS_P_T_2 <- log2(df$Ratio.MS_P_T_2)

>df$Log2.MS_P_T_3 <- log2(df$Ratio.MS_P_T_3)

>df$Log2.MS_P_T_4 <- log2(df$Ratio.MS_P_T_4)

>#mean of Log2 ratio not counting NA using 'na.rm = T'

>df$Log2.MS_P_T_mean <- apply(select(df, Log2.MS_P_T_1, Log2.MS_P_T_2, Log2.MS_P_T_3,
Log2.MS_P_T_4), MARGIN = 1, mean, na.rm = T)

>#number of replicates quantified

>df$MS_P_T_count <- apply(select(df, Log2.MS_P_T_1, Log2.MS_P_T_2, Log2.MS_P_T_3,
Log2.MS_P_T_4), MARGIN = 1, function(x) length(which(!is.na(x))))

>#selected proteins that are quantified at least 3 times

>df_tri <- df[which(df$MS_P_T_count>=3),]

>#one sample t-test against 0 (mu=0)

>for(i in 1:nrow(df_tri))

> df_tri$MS_P_T_p.val[i] <- t.test(select(df_tri, Log2.MS_P_T_1, Log2.MS_P_T_2,
Log2.MS_P_T_3, Log2.MS_P_T_4)[i,], mu=0)$p.value

>#calculate -log10 p-value

>df_tri$MS_P_T_log10p.val <- -log10(df_tri$MS_P_T_p.val)

>#Select proteins enriched in pellet as newly translated proteins after HS with at least a two-
fold enrichment and p-value < 0.05

>hit <- df_tri[which(df_tri$Log2.MS_P_T_mean>=1),]

>hit <- hit[which(df_hits$MS_P_T_p.val<=0.05),]

>hit$Hits <- "Hits"
```

e. For double pulse SILAC results, divide the *Ratio H/M* in the pellet fraction (H_P/M_P) by TCL (H_T/M_T) to calculate the $\log_2$ [(H_P/M_P) /(H_T/M_T)] for each protein in each sample. Determine the average $\log_2$ ratio for each protein and calculate the *p*-value using Student's *t*-test against 0 same as what is described in step 16-d. For this analysis, only consider proteins that are in the *Hit* set from the single pulse SILAC experiment (step 16-d). The double pulse SILAC data alone cannot confidently determine which newly translated proteins are enriched in the pellet fraction after heat-shock, due to the fact the *light* channel proteins are a composite of proteins from both heat-shocked and non-heat-shocked samples. The *heavy* over *medium* ratio differentiates between newly translated proteins that are affected by heat-shock, which correspond to newly translated and thermo-sensitive proteins (*ntSP*) and newly translated proteins that are always enriched in pellet fraction (*ntCP*). The *ntSP* set is composed of proteins with at least a two-fold enrichment and *p*-value < 0.05; the *ntCP* set consists of proteins with less than two-fold enrichment and *p*-value > 0.05. A few additional proteins will not be considered further due to ambiguity.

f. Proteins of interest from *ntSP* and *ntCP* sets can now be selected for further validation by *Pulse Expression by Galactose Induction*. Troubleshooting 2.

Generation of 3HA-tagged constructs under GAL inducible promoter

Timing: 1 week

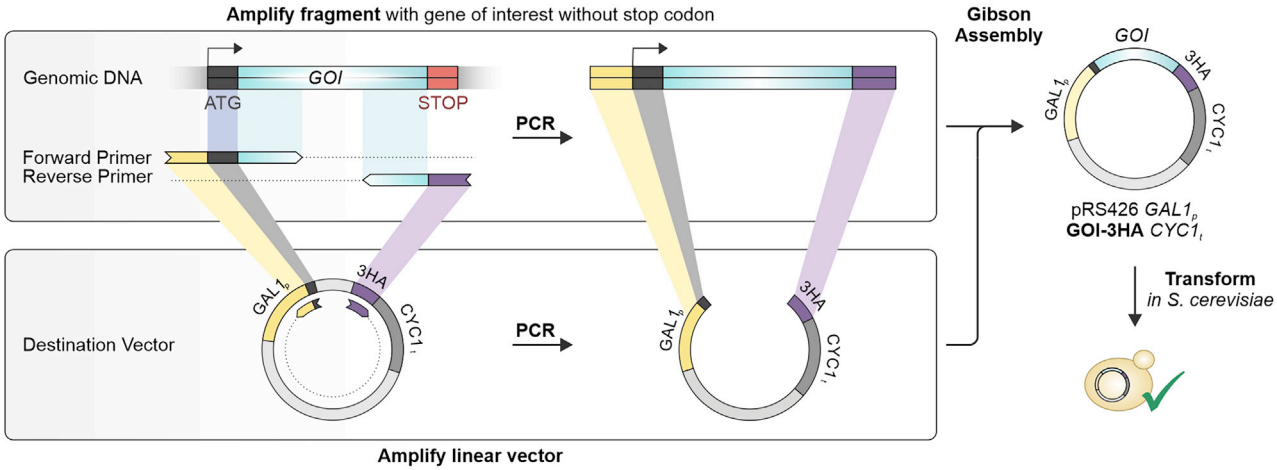

Figure 8. Schematic of the strategy for the generation of 3HA-tagged constructs under GAL-inducible promoter (steps 17–20)

This section describes how to generate *GAL* inducible plasmids to validate the proteins identified as hits in *MS data analysis and determination of newly translated and thermo-sensitive proteins (ntSP) enriched in the pellet* (steps 15 and 16) following the pulse-SILAC experiments (Figure 8).

17. Primers are designed to amplify the gene of interest (GOI) from genomic DNA and insert it into BPM1809 (pRS426-*GAL1*$_p$-3HA-*CYC1*$_t$) between the promoter and 3HA-tag by Gibson assembly (Figure 8). If the GOI contains an intron, an alternate strategy is needed (e.g., RT-PCR).
    a. The forward primers amplifying the GOI must contain the ATG start codon (in bold) and the reverse primer must omit the stop codon. Both primers should have overhangs which anneal to the insertion region of BPM1809 (Forward primer: 5′ ACGTCAAGGAGAAAAAACC **ATG**$n_{15\text{-}25}$; Reverse primer: 5′ GAACATCGTATGGGTAAGATCCTCTn$_{15\text{-}25}$; $n_{15\text{-}25}$ corresponds to the GOI sequence).
        i. The primers should have a minimum Tm of 58°C in the annealing region (sequence shown) and 55°C in the region that anneals to the GOI ($n_{15\text{-}25}$) in the first round of amplification.
        ii. Ensure the GOI is inserted in-frame with the 3HA-tag (i.e., the last codon of the GOI before the stop codon is in frame with the following sequence: AGA GGA TCT TAC CCA TAC GAT GTT CCT GAC TAT GCG GGC…).
    b. The primers for amplifying BPM1809 must be complementary to the overhangs on the insert primers.
        i. Forward primer: 5′ AGAGGATCTTACCCATACGATGTTCCTG.
        The forward primer anneals directly to the start of the linker and HA tag sequences.
        ii. Reverse primer: 5′ **CAT**GGTTTTTTCTCCTTGACGTTAAAG.
        The reverse primer may include a CAT overhang (bold) corresponding to the ATG codon of the GOI.
18. Backbone and inserts are amplified with Q5® High-Fidelity DNA Polymerase (NEB), but other high-fidelity DNA polymerases can also be used.
    a. If using Q5®, calculate annealing temperatures using https://tmcalculator.neb.com using the primer regions that would anneal on the first cycle (i.e., the $n_{15\text{-}25}$ of the GOI or the entire primer sequences listed in step 17 b).
    b. 50 μL PCR reactions were prepared and run according to the polymerase manufacturer guidelines, using High GC Enhancer (Q5 polymerase) where needed. 5 ng of plasmid DNA or 200 ng of genomic DNA was used as template for the reactions. All PCRs were run for a total of 30 cycles with extension times calculated according to manufacturer guidelines.

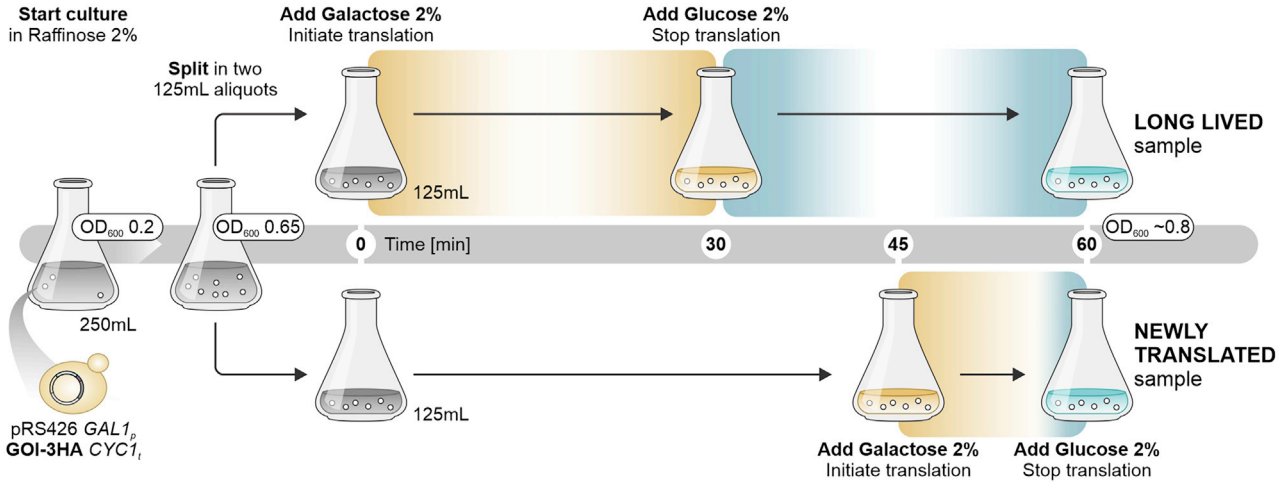

Figure 9. Schematic of the steps for the protein pulse expression experiment by galactose induction illustrating the timings of galactose and glucose addition (steps 21–23)

c. Gel purify PCR products before using for Gibson assembly following manufacturer's protocol (e.g., Epoch GenCatch™ Gel Extraction Kit).
d. Measure DNA concentration using a Nanodrop device.

19. Ligate insert and backbone using Gibson Assembly kit (NEB). Other Gibson assembly kits can also be used.
    a. Gibson Assembly Master-Mix was used to make a 5 μL reaction following manufacturer protocol (3:1 insert to backbone ratio; final DNA concentration varies).
    b. Transform 2 μL of the ligation mix into competent DH5α (NEB5α) cells and spread onto LB-Amp plates (100 μg/mL). Incubate at 37°C overnight until colonies appear.
    c. Screen colonies for inserts by colony PCR.[7] To facilitate this step, we typically use a reverse 15–25 nucleotide long primer with a Tm of ~55°C located 100–300 nucleotides downstream of the ATG of the GOI and the universal T3 primer located upstream of the *GAL1* promoter to amplify a ~500–800 bp product indicating a positive hit.
    d. Verify correct insertion of the GOI without mutations by sequencing (check for local companies that offer whole plasmid sequencing at low costs).
20. Transform *GAL* inducible constructs into yeast cells using the LiAc/ssDNA/PEG method,[8] and plate with appropriate selection.

Protein pulse expression by galactose induction

Timing: 1–2 days

This section describes how to perform a pulse expression experiment to confirm if a protein is more susceptible to aggregation upon heat shock when newly translated (Figures 9 and 10).

21. Culture the yeast transformed with the *GAL* inducible constructs using 2% raffinose as the sole-carbon source.
    a. Inoculate 250 mL of SD-Ura raffinose media to a maximum of 0.2 OD_{600} to grow to 0.65 OD_{600} at 25°C. This can be done by overnight or same-day growth. Troubleshooting 1.

⚠ CRITICAL: Do not culture the cells in media containing glucose, as glucose actively represses the *GAL* induction system leading to a large delay from addition of galactose

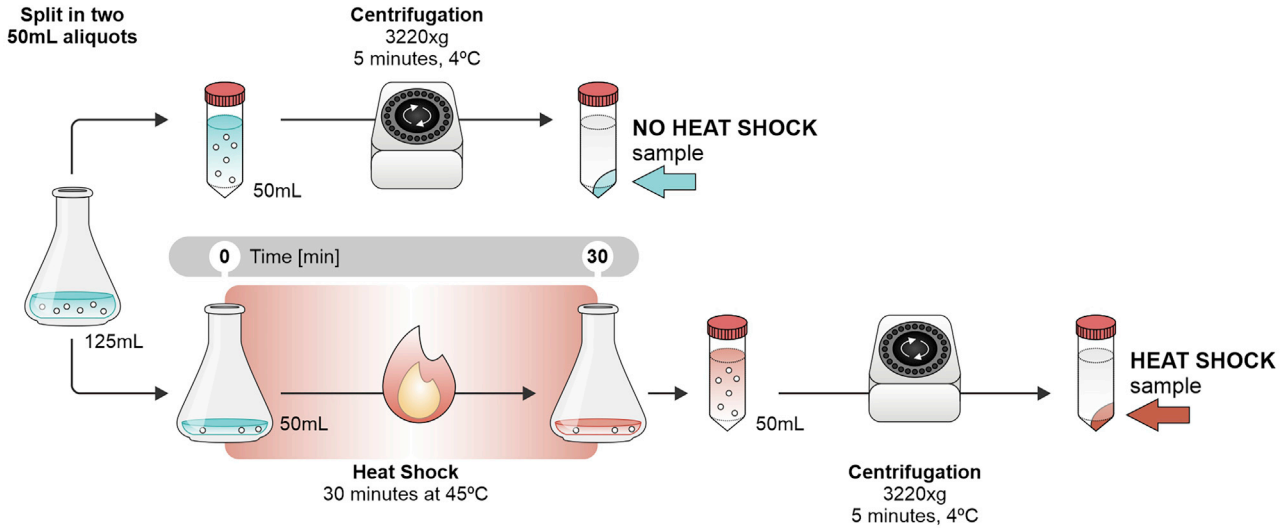

Figure 10. Schematic of the steps for the protein pulse expression experiment by galactose induction illustrating the heat shock treatment (step 24)

to the start of transcription from *GAL1* promoter. Raffinose and galactose must be high purity as low-quality reagents often contain glucose, inhibiting the *GAL* induction system.

Note: Cells grow considerably slower in raffinose compared to glucose. Allow extra time to determine doubling times or inoculate several cultures a few hours apart to ensure you get log phase cells. Some strains grow really poorly in raffinose if too diluted. In that case, include a low amount of glucose (0.1%) in the starting culture and verify that *GAL* induction is effective in these conditions.

22. Prepare for heat-shock.
    a. Pre-heat your shaking water bath, along with two 125 mL Erlenmeyer flasks (per protein of interest) to 45°C.
    b. Allow adequate time for all components to get to temperature.
23. Galactose induction of "long-lived" and "newly translated" proteins.
    a. When the pre-cultures hit 0.65 OD_{600}, split them into two 125 mL aliquots. One aliquot will have galactose added and the other will continue to grow.
    b. To one aliquot, add galactose to 2% final concentration and allow to grow for 30 min, before adding glucose to 2% to repress *GAL* driven expression. Let it grow for another 30 min (Figure 9). This is the *long-lived* sample.
    c. 15 min after adding glucose to the first aliquot, add galactose to 2% in the second aliquot that was left to grow. Let it grow for 15 min (Figure 9). This is the *newly translated* sample.
    d. Expected cell density at the end of these steps is about 0.8 OD_{600}.
24. Heat-shock and controls (Figure 10).
    a. Collect a 50 mL aliquot of the *long-lived* and *newly translated* samples in centrifuge tubes and immediately harvest by centrifugation at 3,220 × *g* at 4°C. These are the "no heat-shock" controls.
    b. Collect another 50 mL aliquot of the long-lived and newly translated samples in centrifuge tubes and transfer by pouring into the pre-heated Erlenmeyer flasks. Heat-shock for 30 min in the water bath with shaking at 200 RPM.
    c. While the heat shock is occurring. Collect the cell pellets from the *no heat-shock* controls and process as described in steps 3–6, to generate TCL, supernatant and pellet fractions.
    d. Collect the "heat-shocked" samples by decanting back into the 50 mL centrifuge tubes and harvest and process cells as in the *no heat-shock* controls.

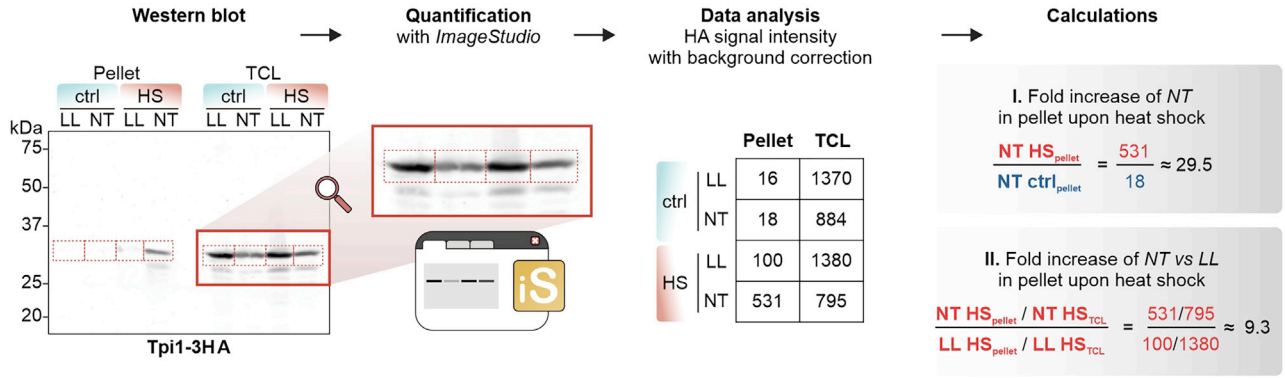

Figure 11. Schematic demonstrating quantification of Western blots by image analysis software to obtain band intensities (step 25) for Tpi1

The original Western blot was previously published and the results were reprinted with permission from Zhu et al.[1]

25. Perform Western blots using the TCL and pellet fractions. The supernatant can also be assessed if desired for additional controls. In our case, we used a precast 10% SDS-PAGE gel (BioRad Mini-Protean TGX), the Trans-Blot Turbo transfer system (BioRad) and the Odyssey® Imaging system (LI-COR), but other similar systems can be used. For blotting, we used the mouse anti-HA antibodies (AbLab; 1:3000) and rabbit anti-PGK1 for loading controls (Acris; 1:10,000; there should be no significant PGK1 signal in the pellet fractions), and IRDye® secondary antibodies (LI-COR; 1:20,000). We recommend using the IRDye® 800CW Goat Anti-Mouse IgG (800 nm channel) for visualizing the HA signal, as the 800 nm channel has less interference from membrane autofluorescence. IRDye® 680RD secondaries (700 nm channel) can be used to multiplex (i.e., for the loading control), however you should test the strength of the signal from the 700 nm channel for your target before proceeding. Alternatively, the antibodies can be stripped with 2% (w/v) NaOH for 15 min after imaging for HA signal and then re-probed for loading control, to utilize the 800 nm channel for both HA and loading control. Different secondary antibodies can be used depending on the imaging platform.
    a. Equilibrate the TCL samples from step 6 to 1 μg/μL protein concentration with a final concentration of 5% 2-mercaptoethanol and 0.002% bromophenol blue. Complement the 50 μL pellet samples from step 6 adding 2-mercaptoethanol and bromophenol blue as above.
    b. Heat the samples at 95°C for 3 min. Load 25 μg of TCL (for us ~5% of the total volume of TCL from step 6a.) on a 10% SDS-PAGE gel.
    c. Since the pellet contains a smaller portion of the total protein, a larger proportion of this sample should be loaded, in this case ~10 μL or ~20%, to make a 4× concentrated pellet fraction. Keep this ratio consistent across all samples.
    d. Transfer the gel to a nitrocellulose or PVDF membrane for Western blotting using standard procedures (Figure 11).[9]
    e. Band intensities were determined in LI-COR Image Studio (other image analysis software may be used) by defining all lanes then selecting a box centered around the HA signal bands and duplicating that box and placing the duplicate at the same molecular weight region for all other lanes. Duplicating the box ensures the same area is quantified. To control for background, select the "lane" background subtraction method (Figure 11). We recommend two different analyses:
        i. To determine whether the *newly translated* GOI is thermo-sensitive, compare the HA signal in the pellet fractions of the *heat-shocked* vs. *no heat shock* samples. A thermo-sensitive GOI should display a high *heat-shocked/ no heat shock* ratio (e.g., >2). In contrast, HA signal levels in the corresponding supernatant fractions, if assessed, should not increase.

ii. To determine whether only the *newly translated* GOI is thermo-sensitive or if it is more thermo-sensitive than the corresponding *long-lived* GOI, compare the HA signal levels in the *heat shocked* samples. First, normalize the signal of the pellet fractions with the signal in the corresponding TCL, then compare the normalized signals between the *newly synthesized* and *long-lived* samples of a given GOI. Troubleshooting 3.

EXPECTED OUTCOMES

With a modern high resolution mass spectrometer, one typically expects 3,000–5,000 peptides identified in a single off-line fractionated sample. The total number of protein groups identified should be ~4,000 with all fractions searched together. In this study, we identified 3,410 proteins in at least one of the four replicates of the single pulse-SILAC experiment; 2,610 of them were quantified in at least three experiments. 574 *Hit* proteins with at least a 2-fold enrichment and *p*-value < 0.05 were identified in the single pulse SILAC experiment. Similarly, we quantified 2,520 proteins in the double pulse SILAC experiment in at least three replicates with large overlap of proteins quantified in the single pulse SILAC experiment. We identified 368 ntSP proteins and 72 ntCP proteins from the combined single and double pulse SILAC experiments.

For the validation using the *GAL* induction system (steps 17–24, steps 3–6 and step 25), we observed an increased signal in the pellet fraction of the *newly translated* proteins in comparison to the *no heat-shock* conditions (Analysis i) and to *long-lived* proteins (Analysis ii) for the four selected candidates to validate by Western blots (see Figure 11 for Tpi1 and Figure 4D in published study).[1] We also observed a lower signal in the supernatant fraction, as a portion of the target protein has gone to the insoluble pellet.

Expected outcomes table

Experiment	Expected outcomes
Single Pulse SILAC with heat shock	Identification of hit proteins significantly enriched in the pellet fraction upon exposure to heat stress (p-value $\leq$ 0.05).
Double Pulse SILAC with heat shock and time-dependent labelling	Further refinement of hit proteins from the *Single Pulse SILAC with heat shock* experiment to identify which newly translated proteins become significantly enriched in the pellet fraction upon exposure to heat stress because they are thermo-sensitive (ntSP).
Protein Pulse Expression by galactose induction	Further validation by orthogonal approach of newly translated thermo-sensitive (ntSP) hit proteins from *Double Pulse SILAC with heat shock and time-dependent labelling* experiment. The expression is regulated by galactose induction and samples are analyzed by SDS-PAGE and Western blot. The signal of ntSP proteins should increase in the pellet fraction upon exposure to heat stress.

LIMITATIONS

The temporal resolution of pulse-SILAC labeling is restricted by the rate at which SILAC amino acids can be incorporated into nascent peptides as well as the translation and protein turnover rates. We recommend a minimum of 5%–15% pulse signal for good SILAC quantitation. Thus, the application of the pulse SILAC labeling is optimum for organisms with short doubling times, like yeast. To circumvolve this issue in mammalian cells, a booster signal can be generated using tandem mass tags.[10]

While using the *GAL* promoter system provides the advantage of the inducible expression of our gene of interest, the expression level will differ from that of the endogenous protein. Overexpression of the protein may overwhelm the protein homeostasis network and cause artificial aggregation of proteins. In addition, small as the 3HA tag (used for Western blotting) may be, it could possibly hinder proper protein folding, possibly leading to unwanted effects. As the *GAL* system control transcription and not translation, some newly translated proteins could still be generated following the 30 min dextrose chase, especially if the mRNA is very stable. As the *GAL* system only works in yeast, a

different promoter system is needed in other model organisms, such as the well-established tetracycline transcriptional activator in mammalian cells.[11]

TROUBLESHOOTING

Problem 1

No cells grow in the overnight culture.

Potential solution

Make sure additional amino acids such as lysine and arginine, and a proper carbon source is added as this protocol uses a variety of uncommon yeast culture medium.

Problem 2

Low number of protein groups identified in MS or low percentage of MS2 identified.

Potential solution

Check the mass accuracy in meta data output from MaxQuant. If mass accuracy is large, re-calibrate the instrument and re-run the samples. Check the chromatogram for late eluting polymer contaminations. Polymers are characteristic for their 1 Th interval pattern of +1 charged ions. Polymers suppress ion signals. Identify the source of polymer contamination (e.g., possible change in brand or batch of tubes or tips, avoid the use of low-binding/siliconized tubes and avoid extend period of incubation of tube at high temperature). All MS buffers should be stored in glass bottles and not come in contact with caps. None of the glassware should be washed with detergent.

Problem 3

No target signal observed in Western blots.

Potential solution

First, verify reactivity of your antibodies. Then check for expression of your target protein by growing transformed yeast directly in galactose media overnight or perform a time series to verify that sufficient signal can be detected after 15–30 min galactose induction. Lyse the cells directly in 1× Laemmli buffer and do a Western or dot blots to check for target signal.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Thibault Mayor (mayor@msl.ubc.ca).

Materials availability

All unique reagents generated in this study (e.g., yeast strains and plasmids) are listed in the key resources table and available upon reasonable request from the lead contact.

Data and code availability

All Raw proteomic data are deposited in the PRIDE repository from the ProteomeXchange Consortium (PXD024336).

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AUTHOR CONTRIBUTIONS

Experimental design, M.Z., R.W.K.W., G.C., T.M.; Experimental work, M.Z., R.W.K.W., G.C.; Computational analyses, M.Z.; Figure design and illustrations, G.C.; Resources, T.M.; Writing & editing, M.Z., R.W.K.W., G.C., T.M.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Zhu, M., Kuechler, E.R., Wong, R.W.K., Calabrese, G., Sitarik, I.M., Rana, V., Stoynov, N., O'Brien, E.P., Gsponer, J., and Mayor, T. (2022). Pulse labeling reveals the tail end of protein folding by proteome profiling. Cell Rep. *40*, 111096. https://doi.org/10.1016/j.celrep.2022.111096.

2. Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P., and Boeke, J.D. (1998). Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast *14*, 115–132. https://doi.org/10.1002/(SICI)1097-0061(19980130)14:2<115::AID-YEA204>3.0.CO;2-2.

3. Gardner, J.M., and Jaspersen, S.L. (2014). Manipulating the yeast genome: deletion, mutation, and tagging by PCR. Methods Mol. Biol. *1205*, 45–78. https://doi.org/10.1007/978-1-4939-1363-3_5.

4. Hughes, C.S., Foehr, S., Garfield, D.A., Furlong, E.E., Steinmetz, L.M., and Krijgsveld, J. (2014). Ultrasensitive proteome analysis using paramagnetic bead technology. Mol. Syst. Biol. *10*, 757. https://doi.org/10.15252/msb.20145625.

5. Rappsilber, J., Mann, M., and Ishihama, Y. (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat. Protoc. *2*, 1896–1906. https://doi.org/10.1038/nprot.2007.261.

6. Udeshi, N.D., Mertins, P., Svinkina, T., and Carr, S.A. (2013). Large-scale identification of ubiquitination sites by mass spectrometry. Nat. Protoc. *8*, 1950–1960. https://doi.org/10.1038/nprot.2013.120.

7. Bergkessel, M., and Guthrie, C. (2013). Colony PCR. Methods Enzymol. *529*, 299–309. https://doi.org/10.1016/B978-0-12-418687-3.00025-2.

8. Gietz, R.D., and Schiestl, R.H. (2007). High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat. Protoc. *2*, 31–34. https://doi.org/10.1038/nprot.2007.13.

9. Burnette, W.N. (1981). "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. *112*, 195–203. https://doi.org/10.1016/0003-2697(81)90281-5.

10. Klann, K., and Münch, C. (2022). Quantitative translation proteomics using mePROD. Methods Mol. Biol. *2428*, 75–87. https://doi.org/10.1007/978-1-0716-1975-9_5.

11. Gossen, M., and Bujard, H. (1992). Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. USA *89*, 5547–5551. https://doi.org/10.1073/pnas.89.12.5547.