Protocol

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Highlights

- Generation and validation of IRE1 and IRE1ΔLD query strains with a UPR reporter
- Detailed protocol of query strains mated to the yeast deletion library using SGA
- High-throughput measurement of reporter fluorescence levels by flow cytometry
- Data analysis to identify gene deletions activating the UPR by lipid bilayer stress

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Protocol

A high-throughput genetic screening protocol to measure lipid bilayer stress-induced unfolded protein response in *Saccharomyces cerevisiae*

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SUMMARY

The endoplasmic reticulum (ER) stress is defined by the accumulation of unfolded proteins at the ER and perturbation at the ER membrane, known as lipid bilayer stress (LBS). In turn, ER stress triggers the unfolded protein response (UPR) to restore ER homeostasis. Here, we provide a modified protocol based on the synthetic genetic array analysis in *Saccharomyces cerevisiae* to identify genetic perturbations that induce the UPR by LBS. This method is adaptable to other canonical stress pathways. For complete details on the use and execution of this protocol, please refer to Ho et al. (2020), Jonikas et al. (2009) and Baryshnikova et al. (2010).

BEFORE YOU BEGIN

To identify genes that are required to maintain endoplasmic reticulum (ER) membrane homeostasis, we employed a genome-wide high-throughput screen using an engineered *Saccharomyces cerevisiae* Ire1 sensor that activates the unfolded protein response (UPR) exclusively by lipid bilayer stress (LBS). The engineered sensor lacks the proteotoxic stress-sensing luminal domain (Ire1\textsubscript{LD}). This query strain was mated to the yeast deletion library using synthetic genetic array methodology. A parallel screen was carried out using a query strain expressing full-length Ire1. Both query strains contain an integrated reporter system in which GFP is driven by the UPR element (UPRE)-containing promoter and mCherry is driven by a constitutive promoter.

Generating Ire1 and Ire1\textsubscript{LD} query strains and library preparation

© Timing: 4 days

Both the *IRE1* and *IRE1\textsubscript{LD}* genes are genomically integrated in YMS612 strain with the dominant hygromycin resistance marker (Hygr\textsuperscript{R}) (Figure 1A and key resources table).

1. Genomic integration of *IRE1* and *IRE1\textsubscript{LD}* with Hygr\textsuperscript{R}
   a. PCR-mediated amplification of *IRE-Hygr\textsuperscript{R}* and *IRE1\textsubscript{LD-Hygr\textsuperscript{R}}* from plasmid pGT0453 and pGT0454
   b. Amplify *IRE1-Hygr\textsuperscript{R}* and *IRE1\textsubscript{LD-Hygr\textsuperscript{R}}* cassettes with primer pair HN148 and HN149 using *Phusion* polymerase following manufacturer’s protocol.
c. Inoculate a single colony of YMS612 strain in 10 mL of YPD media and grow 16 h at 30 °C to an absorbance at 600 nm of 0.6–0.8 OD/mL.

d. Pellet at 1,000 × g, 5 min, wash with 20 mL LiOAc mix and resuspend in 200 μL LiOAc mix.

e. Add 150 μL of washed cells to 700 μL PLATE mix, 10 μL of denatured ssDNA and 2 μg of purified PCR product and incubate 1 h at 30 °C.

f. Add 118.6 μL DMSO to the transformation mixture and heat shock 22 min at 42 °C.

g. Pellet the cells for 15 s at maximum speed.

h. Resuspend the pellet in 200 μL of YPD and incubate for 1.5 h at 30 °C.

i. Pellet the cells for 15 s at maximum speed and remove most of the supernatant.

j. Plate onto YPD plates containing 200 μg/mL Hyg.

k. Positive clones will appear after 48–72 h incubation at 30 °C.

l. Successful integration of IRE1 (YGT1228) and IRE1ΔLD (YGT1202) will yield PCR fragments of 6054 bp and 4285 bp, respectively, by amplifying the genomic DNA with primer pair HN148 and HN149.

Pause point: The selected strains can be stored at −80 °C as glycerol stocks until ready to proceed to the next steps.

Note: The genotyping strategy at step 1l is only insert-specific. Therefore, the subsequent functional validation steps are important. Otherwise, a flanking primer can be used to replace either HN148 or HN149 for orientation-specific genotyping.

2. Validate the functionality of Ire1 and Ire1ΔLD in the query strains by measuring the UPR activation using confocal fluorescence microscopy:

a. Grow YGT1228 and YGT1202 strains each in two tubes containing 500 μL of YPD media at 30 °C to mid-log phase.

CRITICAL: The absorbance at 600 nm should not exceed 0.4 OD/mL to prevent UPR activation in these cells.

b. Add 0.5 μL of 1 M DTT (final concentration of 1 mM) into each tube and incubate for 1 h to induce the UPR.

c. Coat coverslips evenly with 1 mg/mL of ConA and incubate at ~22 °C for 15 min.

d. Rinse the coverslips three times with 500 μL of sterile ddH2O.
e. Place cells on coated coverslips mounted onto Attofluor cell chambers.
f. Set up three acquisition channels to acquire:
   (1) Brightfield
   (2) GFP (excitation 488 nm, emission 507 nm)
   (3) mCherry (excitation 532 nm, emission 600 nm)
g. Image cells using a Zeiss LSM 710 confocal microscope with a 100× 1.4 NA oil plan-Apochromat objective (Figures 1B and 1C).

3. Sterilizing the pin tools

    Note: All pinning steps are performed using a 96 Floating E-Clip style Pin Multi-Blot Replicator (V&P Scientific). Alternatively, a ROTOR HDA (Singer Instruments), a BioMatrix Colony Processing Robot, or any other desired pinning tools can be used.

   a. Set up five sterile reservoirs containing the following: (1) 30 mL of sterile ddH₂O, (2) 40 mL of 10% bleach, (3) 50 mL of sterile ddH₂O, (4) 70 mL of sterile ddH₂O, and (5) 90 mL of 95% ethanol.
   b. Soak the pin replicator for 1 min in the reservoir containing 30 mL of sterile ddH₂O to remove the cells from the pins.
   c. Immerse the pin replicator in 10% bleach for approximately 20 s.
   d. Rinse off the bleach from the pins by transferring the replicator to the reservoir containing 50 mL of sterile ddH₂O and then to reservoir containing 70 mL of ddH₂O.
   e. Immerse the replicator pins in 95% ethanol for 30 s.
   f. Shake off the excess ethanol and flame the pin replicator.
   g. Allow the pin replicator to cool for 3 min before use.

    Note: Ensure thorough removal of cells after each pinning step to avoid cross contamination.
    The hand pinner must be sterilized after each use.

4. Constructing the deletion mutant array in 384-density array format

   a. Take out the desired microplates containing glycerol stocks of the deletion array from −80°C.
   b. Immediately place the plate on dry ice to ensure gentle thawing of the glycerol stocks. Do not allow the glycerol stocks to thaw out completely.
   c. Carefully peel off the aluminum sealer from each 96-well plate.

   △ CRITICAL: Be cautious not to cross-contaminate wells.

   d. Using the pin replicator, copy the array on a single-well YPD agar plate containing 200 μg/mL G418. Use the library copier VP 381 as a guide for printing the library (Figure 2).
   e. Reseal the master plates with sterile aluminum seals and immediately return to −80°C.
   f. Incubate the plates at ~22°C for 2 days.
   g. Use the 96 Floating E-Clip style Pin Multi-Blot Replicator to array the 96-density format colonies into 384-density array format with the help of the library copier VP 381. Incubate the colonies at ~22°C for 1 day.

    Note: The potent UPR inducer gene deletion opi3Δ strain and wild-type (WT) strains are included in each 96 well plate. Typically, each library microplates contain at least 2 empty wells that were used to include positive (opi3Δ) and negative (WT) controls. OPI3 deletion was selected because it causes high UPR by lipid bilayer stress in both query strains IRE1 and IRE1ΔLD (Ho et al., 2020).

    Note: Freshly grown colonies of the 384-density deletion array should be generated for each batch of the genetic screen.
Yeast deletion library colonies grown on the source plates (YPD agar plate containing G418) were created by inoculation from frozen glycerol stock in 96-format arrays. Before the subsequent pinning steps, fit the destination plate (YPD agar plate containing G418) into the middle of the library copier VP381. To condense four 96-format arrays into a single 384-format array, pin the colonies on the first source plate using a sterile 96-pin replicator and replicate onto the destination plate aligned to the ‘A’ alignment holes. Repeat the pinning step with the next three source plates aligned to the ‘B’, ‘C’, and ‘D’ alignment holes and transfer to the same destination plate. The resulting 384-format mutant array will then be used for subsequent SGA steps to generate the final mutant array expressing the UPR sensors and reporters. Each 384-format array can be expanded to four 96-format liquid culture for flow cytometry acquisition.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** | | |
| Bacto agar | Becton Dickinson | 214010 |
| Bacto peptone | Becton Dickinson | 211677 |
| Bacto yeast extract | Becton Dickinson | 212750 |
| Adenine sulfate | Sigma-Aldrich | A2786 |
| Tyrosine | Sigma-Aldrich | T8566 |
| Isoleucine | Sigma-Aldrich | I7403 |
| Phenylalanine | Sigma-Aldrich | P5482 |
| Glutamic acid | Sigma-Aldrich | G8415 |
| Aspartic acid | Sigma-Aldrich | A7219 |
| Valine | Sigma-Aldrich | V0513 |
| Threonine | Sigma-Aldrich | T8441 |
| Serine | Sigma-Aldrich | S4311 |
| Tryptophan | Sigma-Aldrich | T8941 |
| Leucine | Sigma-Aldrich | L8912 |

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## REAGENT or RESOURCE SOURCE IDENTIFIER

| Item | Source | Identifier |
|------|--------|------------|
| Inositol | Sigma-Aldrich | I5125 |
| p-Aminobenzoic acid | Sigma-Aldrich | A9878 |
| Alanine | Sigma-Aldrich | A7469 |
| Asparagine | Sigma-Aldrich | A4159 |
| Cysteine | Sigma-Aldrich | C7352 |
| Glutamine | Sigma-Aldrich | 49419 |
| Glycine | Sigma-Aldrich | G8790 |
| Proline | Sigma-Aldrich | P5607 |
| Difco Yeast Nitrogen Base without Amino Acids (YNB w/o AA) | Becton Dickinson | 291930 |
| Difco Yeast Nitrogen Base without Amino Acids and Ammonium Sulfate (YNB w/o AA, AS) | Becton Dickinson | 233520 |
| L-Glutamic acid monosodium salt hydrate (MSG) | Sigma-Aldrich | G1626 |
| D-Glucose monohydrate (Glucose) | Duchefa Biochemie | G0802-1000 |
| Glycerol | Promega | H5433 |
| DMSO | Sigma-Aldrich | D8418 |
| Hygromycin B (Hyg) | Nacalai Tesque | 07296-11-E |
| Geneticin (G418 sulfate) (50 mg/mL) | Gibco | 10131035 |
| L-canavanine sulfate crystalline (canavanine) | Sigma Aldrich | C9758 |
| S-(2-Aminoethyl)-L-cysteine hydrochloride (thialysine) | Sigma-Aldrich | A2636 |
| Taq DNA polymerase | New England Biolabs | M0273 |
| Standard Taq reaction buffer | New England Biolabs | B9014 |
| Phusion high-fidelity DNA polymerase | New England Biolabs | M0530 |
| Phusion HF buffer | New England Biolabs | B0518 |
| 10 mM Deoxynucleotide (dNTP) Solution Mix | New England Biolabs | N0447 |
| Poly(ethylene glycol), average MW 3350 | Sigma-Aldrich | P4338 |
| Lithium acetate | Sigma-Aldrich | S17992 |
| EDTA | Bio-Rad | 1610729 |
| Tris | Bio-Rad | 1610719 |
| Commercial bleach | N/A | N/A |
| Salmon sperm DNA (ssDNA) | Sigma-Aldrich | 31149 |
| Dithiothreitol (DTT) | Gold Biotechnology | DTT50 |
| Concanavalin A Type IV (ConA) | Sigma-Aldrich | C2010 |

### Experimental models: Organisms/strains

| Name | Source |
|------|--------|
| MATa, URE2::URA TEF2pr-mCherry::MET15 his3Δ1 leu2Δ0 lys2 met15Δ0 ura3Δ0 can1Δ::STE2pr-LEU2 cyh2, BY4741 background | (Haass et al., 2007) |
| ire1Δ::IRE1-HygR, YMS612 background | YMS612 |
| ire1Δ::IRE1-LD-HygR, YMS612 background | YGT1228 |
| Yeast Mat-A Haploid deletion clones | (Ho et al., 2020) |
| 95401.H2P | YGT1202 |

### Oligonucleotides

| Name | Source |
|------|--------|
| Forward primer for IRE1-HygR/IRE1-LD-HygR cassettes: ACAAAAGAAGTAAATGACATTTAAATGCTATTATACAG | IDT |
| Reverse primer for IRE1-HygR/IRE1-LD-HygR cassettes: CGGTCGGAAAAATGTCGATAGTAC | IDT |
| Recombinant DNA | |
| pRS313-IRE1-HygR | This study |
| pRS313-IRE1-LD-HygR | This study |

(Continued on next page)
## MATERIALS AND EQUIPMENT

### Stock solutions

| Reagent                                      | Final concentration |
|----------------------------------------------|---------------------|
| 50% Glucose                                  | 50% (w/v)           |
| 100 mg/mL Canavanine, store at −20°C         | 100 mg/mL           |
| 100 mg/mL Thialysine, store at −20°C         | 100 mg/mL           |
| 100 mg/mL Hyg, store at 4°C                  | 100 mg/mL           |
| 1 M Lithium acetate                          | 1 M                 |
| 50% PEG                                       | 50% (w/v)           |
| 10 mg/mL ssDNA, store at −20°C               | 10 mg/mL            |
| 10× Tris-EDTA buffer, pH 7.4                 | 100 mM Tris, 10 mM EDTA |
| 1 M DTT, store at −20°C                      | 1 M                 |
| 10% bleach                                   | 10% (v/v)           |
| 1 mg/mL ConA, store at −20°C                 | 1 mg/mL             |

### PLATE mix

| Reagent                                      | Final concentration | Amount |
|----------------------------------------------|---------------------|--------|
| 1 M Lithium acetate                          | 100 mM              | 5 mL   |
| 10× Tris-EDTA, pH 7.4                        | 1×                  | 5 mL   |
| 50% PEG                                       | 40%                 | 40 mL  |

Sterile filter before use. Prepare fresh before use.
### LiOAc Mix

| Reagent                | Final concentration | Amount   |
|------------------------|---------------------|----------|
| 1 M Lithium acetate    | 100 mM              | 5 mL     |
| 10x Tris-EDTA          | 1x                  | 5 mL     |
| Sterile ddH₂O          | 40%                 | 40 mL    |

Sterile filter before use. Prepare fresh before use.

### 10× Amino acid supplement drop-out mixture (drop out His, Arg, Lys, Ura, Met)

| Reagent               | Final concentration | Amount   |
|-----------------------|---------------------|----------|
| Adenine sulfate       | 1 g/L               | 1 g      |
| Tyrosine              | 0.7 g/L             | 0.7 g    |
| Isoleucine            | 0.7 g/L             | 0.7 g    |
| Phenylalanine         | 0.7 g/L             | 0.7 g    |
| Glutamic acid         | 0.7 g/L             | 0.7 g    |
| Aspartic acid         | 0.7 g/L             | 0.7 g    |
| Valine                | 0.7 g/L             | 0.7 g    |
| Threonine             | 0.7 g/L             | 0.7 g    |
| Serine                | 0.7 g/L             | 0.7 g    |
| Tryptophan            | 0.7 g/L             | 0.7 g    |
| Leucine               | 3.6 g/L             | 3.6 g    |
| Inositol              | 0.7 g/L             | 0.7 g    |
| p-Aminobenzoic acid   | 0.1 g/L             | 0.1 g    |
| Alanine               | 0.7 g/L             | 0.7 g    |
| Asparagine            | 0.7 g/L             | 0.7 g    |
| Cysteine              | 0.7 g/L             | 0.7 g    |
| Glutamine             | 0.7 g/L             | 0.7 g    |
| Glycine               | 0.7 g/L             | 0.7 g    |
| Proline               | 0.7 g/L             | 0.7 g    |
| ddH₂O                 | –                   | Up to 1 L|

Sterile filter before use. Store at 4°C, use within a month.

### YPD medium with or without G418 and/or Hyg

| Reagent               | Final concentration | Amount   |
|-----------------------|---------------------|----------|
| Yeast extract         | 1% (w/v)            | 10 g     |
| Peptone               | 2% (w/v)            | 20 g     |
| ddH₂O                 | –                   | Up to 960 mL |
| Autoclave             | –                   | –        |
| 50% D-Glucose monohydrate | 2% (w/v)     | 40 mL |
| 50 mg/mL G418 (optional) | 200 µg/mL      | 4 mL*   |
| 100 mg/mL Hyg (optional) | 200 µg/mL     | 2 mL*   |

*Adjust ddH₂O accordingly before autoclaving. Store at 20°C or 4°C with antibiotics, use within a month.

### YPD agar with or without G418 and/or Hyg

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| Yeast extract | 1% (w/v) | 10 g |
| Peptone | 2% (w/v) | 20 g |
| Agar | 2% (w/v) | 20 g |

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**Continued**

| Reagent                        | Final concentration | Amount          |
|--------------------------------|---------------------|-----------------|
| ddH2O                          | –                   | Up to 954 mL    |
| Autoclave                      | –                   | –               |
| 50% Glucose                    | 2% (w/v)            | 40 mL           |
| 50 mg/mL G418 (optional)       | 200 µg/mL           | 4 mL*           |
| 100 mg/mL Hyg (optional)       | 200 µg/mL           | 2 mL*           |
| Pour plates                    | –                   | –               |

*Adjust ddH2O accordingly before autoclaving. Store at 20°C or 4°C with antibiotics, use within a month.

**Sporulation amino acids supplement powder**

| Reagent    | Amount |
|------------|--------|
| Histidine  | 2 g    |
| Leucine    | 10 g   |
| Uracil     | 2 g    |

Store at 4°C protected from light, use within a month.

**Enriched sporulation agar**

| Reagent                              | Final concentration | Amount |
|--------------------------------------|---------------------|--------|
| Potassium acetate                    | 1% (w/v)            | 10 g   |
| Yeast extract                        | 0.1% (w/v)          | 1 g    |
| D-Glucose monohydrate                | 0.05% (w/v)         | 0.5 g  |
| Sporulation amino acids supplement powder | 0.01% (w/v)     | 0.1 g  |
| Agar                                 | 2% (w/v)            | 20 g   |
| ddH2O                                | –                   | Up to 996 mL |
| Autoclave                            | –                   | –      |
| 50 mg/mL G418                        | 200 µg/mL           | 4 mL   |
| Pour plates                          | –                   | –      |

Store at 4°C, use within a month.

**SD-His/Arg/Lys/Ura/Met containing canavanine/thialysine**

| Reagent                                | Final concentration | Amount |
|----------------------------------------|---------------------|--------|
| YNB (w/o AA)                           | 6.7 g/L             | 6.7 g  |
| Agar                                   | 2% (w/v)            | 20 g   |
| ddH2O                                  | –                   | Up to 859 mL |
| Autoclave                              | –                   | –      |
| 50% Glucose                            | 2% (w/v)            | 40 mL  |
| 10X Amino acid supplement drop-out mixture | 1 x               | 100 mL |
| 100 mg/mL Canavanine                   | 50 µg/mL            | 0.5 mL |
| 100 mg/mL Thialysine                   | 50 µg/mL            | 0.5 mL |
| Pour plates                            | –                   | –      |

Store at 4°C, use within a month.

**SDMSG-His/Arg/Lys/Ura/Met containing canavanine/thialysine/G418**

| Reagent                                | Final concentration | Amount |
|----------------------------------------|---------------------|--------|
| YNB (w/o AA, AS)                       | 1.7 g/L             | 1.7 g  |
| MSG                                    | 1 g/L               | 1 g    |
| Agar                                   | 2% (w/v)            | 20 g   |
| ddH2O                                  | –                   | Up to 855 mL |
| Autoclave                              | –                   | –      |

(Continued on next page)
Selection of query strains mated to the yeast deletion library

© Timing: 3 weeks

Note: Keeping the environment as sterile as possible at the bench is critical. Pinning steps leave plates vulnerable to contaminations.

1. Freshly grow the query strains \textit{IRE1} (YGT1228) and \textit{IRE1}ΔLD (YGT1202) from frozen glycerol stocks.
   a. Streak out the query strains on YPD agar plates containing 200 μg/mL Hyg and incubate at 30°C for 2–3 days.
   b. Inoculate a single colony into 5 mL of YPD liquid medium. Incubate at 30°C for 2 days using a culture rotator (40 rpm).
   c. Prepare the query strain lawns to start the screen.
      i. Using a spreader, evenly spread 800 μL of each saturated liquid culture onto a single-well YPD agar plate.
      ii. Repeat and prepare a total of thirteen query strain lawns for a genome-wide screen with thirteen condensed 384-density format deletion arrays.
      iii. Allow the lawns to dry and incubate at 30°C for 2 days.

Note: Perform both \textit{IRE1} and \textit{IRE1}ΔLD query strain screens in parallel for fair comparison of downstream data.

2. Mate query strains with the gene deletion arrays (Figures 2 and 3).

### Reagent Final concentration Amount
| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| 50% Glucose | 2% (w/v) | 40 mL |
| 10X Amino acid supplement drop-out mixture | 1X | 100 mL |
| 100 mg/mL Canavanine | 50 μg/mL | 0.5 mL |
| 100 mg/mL Thialysine | 50 μg/mL | 0.5 mL |
| 50 mg/mL G418 | 200 μg/mL | 4 mL |
| Pour plates | – | – |

**Store at 4°C, use within a month.**

### SD\textsubscript{MSG}-His/Arg/Lys/Ura/Met containing canavanine/thialysine/G418/Hyg

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| YNB (w/o AA, AS) | 1.7 g/L | 1.7 g |
| MSG | 1 g/L | 1 g |
| Agar | 2% (w/v) | 20 g |
| ddH2O | – | Up to 853 mL |
| Autoclave | – | – |
| 50% Glucose | 2% (w/v) | 40 mL |
| 10X Amino acid supplement drop-out mixture | 1X | 100 mL |
| 100 mg/mL Canavanine | 50 μg/mL | 0.5 mL |
| 100 mg/mL Thialysine | 50 μg/mL | 0.5 mL |
| 50 mg/mL G418 | 200 μg/mL | 4 mL |
| 100 mg/mL Hyg | 200 μg/mL | 2 mL |
| Pour plates | – | – |

**Store at 4°C, use within a month.**
a. Pin the query strain (MATa, HygR) from the lawn onto fresh single-well YPD agar plates using the 384 Solid Pin Multi-Blot Replicator.

b. Pin the 384-density deletion library (MATa, KanR) on top of the query strain.

c. Incubate at ~22°C for 1 day.

3. Select for diploids by pinning the colonies onto single-well YPD agar plates containing G418/Hyg. Incubate the plates 2 days at 30°C.

4. Sporulate the selected diploids by pinning the colonies onto single-well enriched sporulation agar plates. Incubate 7 days at 22°C.

5. Select for MATa meiotic haploid progenies by pinning the spores onto single-well SD-His/Arg/Lys/Ura/Met agar plates containing canavanine/thialysine. Incubate at 2 days at 30°C.

6. Select for MATa, KanR meiotic haploid progenies by pinning the spores onto single-well SDMSG-His/Arg/Lys/Ura/Met agar plates containing canavanine/thialysine/G418 solid medium. Incubate 2 days at 30°C.

Figure 3. Reporter-synthetic genetic array functional genomic screen for UPR activation

(A) A MATa query strain containing either one of the two integrated recombinant UPR sensors selectable by Hygromycin (Hyg) due to the dominant HygR selectable marker. The query strain also possesses a MATa-specific reporter (can1::STE2pr-Sp_his5), a recessive marker (lyp1::STE3pr-LEU2) to counter-select diploid population, and the UPR reporter with auxotrophic markers MET15 and URA3. Each yeast strain of the MATa array inoculated from the yeast deletion library contains a single gene deletion replaced by the dominant KanR selectable marker. The query strain is mated to the array strains on YPD agar plates.

(B) Successfully mated strains yield heterozygous diploid with HygR and KanR phenotype that grow on YPD agar plates containing Hyg and G418.

(C) Sporulation of the diploid strains occur upon transfer onto the enriched sporulation agar plates, resulting in the formation of haploid meiotic progeny.

(D) Selection of the MATa meiotic haploid progeny on SD-His/Arg/Lys/Ura/Met agar plates containing canavanine/thialysine.

(E) KanR meiotic haploid progeny selection on SDMSG-His/Arg/Lys/Ura/Met agar plates containing canavanine/thialysine/G418.

(F) KanR (xxxx) and HygR (IRE1 or IRE1ΔLD) haploids are selected with the same media as in (E) with the additional supplementation of Hyg (SDMSG-His/Arg/Lys/Ura/Met containing canavanine/thialysine/G418/Hyg).
7. Select for MATa, KanR, HygR meiotic haploid progenies by pinning the haploids onto single-well SDMSG-His/Arg/Lys/Ura/Met agar plates containing canavanine/thialysine/G418/Hyg. Incubate at 2 days at 30°C.

Note: The resulting selected progenies will contain either IRE1 or IRE1ΔLD, marked by HygR, and a KanR cassette that replace a non-essential gene.

Note: Ammonium sulfate interferes with G418 selection. It is replaced with monosodium glutamic acid (MSG) at steps 6 and 7.

Measurement of reporter fluorescence levels by flow cytometry

 Timing: 2 days

8. Preparation of cell cultures for flow cytometry
   a. Inoculate the selected haploid progenies from the 384-density format arrays into four 96 well plates using the 96 Floating E-Clip style Pin Multi-Blot Replicator. Each well should be prefilled with 200 µL of YPD liquid media (Figure 2).
   b. Grow 12–16 h at 30°C to allow cell cultures to reach saturation.
   c. Using a multi-channel pipette, dilute the cell cultures to a density of 0.05 OD/mL while maintaining the 200 µL volume in each well.
   d. Incubate the diluted cells 4.5 h at 30°C.

   CRITICAL: The absorbance at 600 nm should not exceed 0.4 OD/mL to prevent UPR activation in these cells.

9. Measure the fluorescence levels of GFP and mCherry reporters from each well with an automated high-throughput sampler (HTS) connected to the LSRFortessa X-20.
   a. It is important to resuspend the cells with a multi-channel pipette before reading with the flow cytometer to prevent clogging of the instrument.
   b. Acquire cells at low speed.
   c. Excite GFP and mCherry at 488 and 561 nm and collect through a 505 and 595 nm long-pass filter and a 530/30 and 610/20 band pass filter, respectively.
   d. One 96-well plate is read within 5 min, with a read of 10,000 cells per well.
   e. Use the software FACSDiVA v 8.0 to acquire data in .fcs file format. Read files with the software FlowJo 10.8.0 (Figure 4).

Note: The potent UPR inducer gene deletion opi3Δ strain and wild-type (WT) strains are included in each 96 well plate. Typically, each library microplates contain at least 2 empty wells that were used to include positive (opi3Δ) and negative (WT) controls. OPI3 deletion was selected because it causes high UPR by lipid bilayer stress in both query strains IRE1 and IRE1ΔLD (Ho et al., 2020).

Data analysis

 Timing: 1 day

10. Normalizing the GFP fluorescence levels using Microsoft Excel.
   a. Calculate the GFP/mCherry ratio signal for each mutant (m) strain (GFPm/mCherrym).
   b. Calculate the median of GFP/mCherry ratio signal for each plate (GFPmedian/mCherrymedian).
   c. Calculate the UPR reporter level of each mutant strain (Equation 1)
**UPRreporterlevels** = \( \log_2 \left( \frac{GFP_{m}}{mCherry_{m}} \right) \times \left( \frac{GFP_{median}}{mCherry_{median}} \right) \)  

**(Equation 1)**

**Figure 4. Typical flow cytometry gating workflow to quantify UPR activity**

(A) Density plot of the recorded events was gated (gate 1, left panel) to include most intact or viable cells but not ruptured or dead cells. This population was then replotted in another density plot (right panel) where the sequential gate 2 was defined to isolate single cells. Only the gate 2 subpopulation was included in the subsequent fluorescence quantification.

(B) Overlay histograms displaying the fluorescent activity of the constitutive reporter mCherry (left panel) and the inducible UPR reporter GFP (right panel) in the gate 2 subpopulation of IRE1 query strain. In this example, the blue population was treated with DMSO (carrier) and red population with the UPR inducer, DTT, respectively. The median of the GFP was normalized to the median of mCherry to determine the relative UPR activity.

**11. Validation of selected positive hits**

a. Grow each selected strain in 500 μL of YPD media to a cell density of up to an absorbance of 0.4 OD/mL.

**Pause point:** The selected strains can be stored at −80°C as glycerol stocks until ready to proceed to next steps.

i. Measure the relative GFP fluorescence using the LSRFortessa X-20 flow cytometer by exciting GFP and mCherry at 488 and 561 nm and collect through a 505 and 595 nm long-pass filter and a 530/30 and 610/20 band pass filter, respectively (Figures 5A and 5B).
ii. Place cells on slides coated with 1 mg/mL ConA mounted onto Attofluor cell chambers. Image cells using a Zeiss LSM 710 confocal microscope with a 100 x 1.4 NA oil plan-Apochromat objective (Figures 5C and 5D).

EXPECTED OUTCOMES

Disruption of ER membrane integrity activates the UPR in an Ire1 luminal domain independent manner. This protocol is designed to identify gene deletions that activate the UPR to identify cellular processes necessary for ER membrane integrity by comparing the $\text{IRE1}^\text{LD}$ query strains.

Out of the 4,847 mutants screened, we identified 629 and 958 gene deletions that activate the UPR in an Ire1 LD-dependent and -independent manner, respectively (Figure 6).

We expect most deleted genes identified in $\text{IRE1}^\text{LD}$ query strains to exhibit functions related to protein folding and protein quality control at the ER. For instance, functions should include protein N- and
O-glycosylation, ER-associated protein degradation (ERAD). Additionally, genes with functions related to protein trafficking and lipid biosynthesis should be identified.

Out of the 181 genes that specifically activated the UPR by lipid bilayer stress, we found that loss of ARV1, GET1, PMT2, OP13, SCJ1, SPC2, and STE24 activated the UPR independently of Ire1 LD as previously reported (Promlek et al., 2011). The deletion of other genes such as vacuolar protein sorting VPS8, VPS29, VPS61, VPS63, and VPS72, highly activated the UPR, confirming their requirement for ER membrane integrity (Figure 7) (Markgraf et al., 2009). Another important process is the ERAD machinery. The ERAD component Hrd1 forms a ubiquitin-gated protein conducting channel for the retro-translocation of misfolded ER luminal protein across the ER lipid bilayer. Given that Hrd1-Hrd3 is part of an ER membrane integrated complex (Schoebel et al., 2017; Wu et al., 2020), this complex might regulate ER membrane integrity. In conclusion, our findings suggest that components of vesicular trafficking and the ERAD are necessary to maintain ER membrane integrity.

LIMITATIONS

This screen was performed manually which made it technically challenging to complete in one iteration, considering both query strains had to be screened in parallel. As a result, we first screened deletion library plates 1 to 20, followed by deletion library plates 21 to 40 and lastly, deletion library...
plates 41 to 51 and 70 to 71. Therefore, it is important to include a negative and positive control to each screened deletion library plate. A researcher would benefit from having access to a robotic pinner and accompanying software to accelerate and ease this part of the protocol.

Genes that are essential in the absence of Ire1 LD will be missing from the screen of IRE1ΔLD query strain. As the UPR will not be activated in IRE1ΔLD query strain during proteotoxic stress, some diploids might not yield any selected progenies due to synthetic lethality or the growth of the resulting progeny might be beyond optimal for the screen.

**TROUBLESHOOTING**

**Problem 1**
Difficulty in generating transformed query strains (before you begin section, step 1).

**Potential solution**
Ensure that YMS612 cells are freshly activated from glycerol stock and then re-inoculated and grown to an absorbance of 0.6–0.8 OD/mL at 600 nm before transformation. Use freshly made reagents for all transformation steps.

**Problem 2**
Cell density is too low for fluorescence confocal acquisition (before you begin section, step 2; step-by-step method details section, step 11).

**Potential solution**
The absorbance at 600 nm of the cell culture should never exceed 0.4 OD/mL to avoid the activation of the UPR in unstressed cells. For slow growing mutants, concentrate the cells by spinning down a large volume of the cell suspension at low speed and resuspend in a smaller volume before adhering cells to the coated slides.

**Problem 3**
Uneven transfer of yeast cells during the pinning steps (before you begin section, step 4; step-by-step method details section, steps 2–7).

**Potential solution**
Avoid using excessive agar media when making the agar plate as it may lead to uneven surface along the edges, causing the pins to puncture the agar. Otherwise, a floating-pin replicator can be used to facilitate the pinning steps.

**Problem 4**
Contamination during the SGA steps (step-by-step method details section, steps 1–7).

**Potential solution**
Even with applied aseptic techniques, contamination may still occur due to the relatively long period of time required for the completion of SGA. The antibiotics used for selections throughout the SGA procedure can be optimized by the user. However, it is important to avoid using antibiotics in excess during SGA as some mutants may exhibit sensitivity to certain antibiotics.

**Problem 5**
High level of basal UPR activity causes false positive hits (step-by-step method details section, step 8).

**Potential solution**
Cell density should not exceed 0.4 OD/mL (absorbance at 600 nm) for flow cytometry. Subculture the cells if they have grown beyond 0.4 OD/mL or re-inoculate liquid media from the mutant arrays.
Overgrowing cells will induce the UPR even in wild-type cells due to nutrient depletion. Overgrown cells should be diluted at lower density and grown for at least 8 h without exceeding 0.4 OD/mL to allow the UPR to be deactivated to the basal level. It should also be noted that the query strains induce high expression of free GFP proteins which in turn are very stable regardless of UPR deactivation. Therefore, we highly recommend to re-inoculate the media from the mutant arrays.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Guillaume Thibault (thibault@ntu.edu.sg).

Materials availability
Strains and plasmids described in this study are available upon request.

Data and code availability
This study did not generate nor analyze datasets.

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AUTHOR CONTRIBUTIONS

Conceptualization, G.T.; methodology, N.H. and W.S.Y.; investigation, N.H. and W.S.Y.; writing – original draft, N.H., W.S.Y., and G.T.; writing – review & editing, W.S.Y. and G.T.; funding acquisition, N.H. and G.T.; supervision, G.T.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Baryshnikova, A., Costanzo, M., Dixon, S., Vizeacoumar, F.J., Myers, C.L., Andrews, B., and Boone, C. (2010). Synthetic genetic array (SGA) analysis in Saccharomyces cerevisiae and Schizosaccharomyces pombe. Methods Enzymol. 470, 145–179.

Haass, F.A., Jonikas, M., Walter, P., Weissman, J.S., Jan, Y.N., Jan, L.Y., and Schuldiner, M. (2007). Identification of yeast proteins necessary for cell-surface function of a potassium channel. Proc. Natl. Acad. Sci. U S A. 104, 18079–18084.

Ho, N., Yap, W.S., Xu, J., Wu, H., Koh, J.H., Goh, W.W.B., George, B., Chong, S.C., Taubert, S., and Thibault, G. (2020). Stress sensor Ire1 deploys a divergent transcriptional program in response to lipid bilayer stress. J. Cell Biol. 219, e201909165.

Jonikas, M.C., Collins, S.R., Denic, V., Oh, E., Quan, E.M., Schmid, V., Weibezahn, J., Schwappach, B., Walter, P., Weissman, J.S., and Schuldiner, M. (2009). Comprehensive characterization of genes required for protein folding in the endoplasmic reticulum. Science 323, 1693–1697.

Markgraf, D.F., Ahnert, F., Arlt, H., Mari, M., Peplowska, K., Epp, N., Griffith, J., Reggiori, F., and Ungermann, C. (2009). The CORVET subunit Vps8 cooperates with the Rab5 homolog Vps21 to induce clustering of late endosomal compartments. Mol. Biol. Cell 20, 5276–5289.

Promlek, T., Ishiwata-Kimata, Y., Shido, M., Sakuramoto, M., Kohno, K., and Kimata, Y. (2011). Membrane aberrancy and unfolded proteins activate the endoplasmic reticulum stress sensor Ire1 in different ways. Mol. Biol. Cell. 22, 3520–3532.

Schoebel, S., Mi, W., Stein, A., Ovchinnikov, S., Pavlovic, R., Dimaio, F., Baker, D., Chambers, M.G., Su, H., Li, D., et al. (2017). Cryo-EM structure of the protein-conducting ERAD channel Hrd1 in complex with Hrd3. Nature 548, 352–355.

Wu, X., Siggel, M., Ovchinnikov, S., Mi, W., Svetlov, V., Nuddler, E., Liao, M., Hummer, G., and Rapoport, T.A. (2020). Structural basis of ER-associated protein degradation mediated by the Hrd1 ubiquitin ligase complex. Science 368, eaaa2449.