Protocol

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

Engineering yeast to induce the synthesis of GPI-APs with a permanent phosphoethanolamine on mannose 2 of the glycan moiety

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SUMMARY

GPI-APs are a family of proteins attached to the plasma membrane by a glycolipid that undergoes remodeling of the glycan and lipid structure during transport to the cell surface. We describe a protocol to induce the synthesis of a GPI-anchored protein whereby EtNP is added to Man2 but not removed. By temporally manipulating the expression of Gpi7p, the enzyme that adds EtNP to Man2, in tet1Δ dcr2Δ cells prior to the expression of a canonical GPI-AP (mNeon-Gas1p), EtNP is attached to Man2 of de novo synthesized mNeon-Gas1p and cannot be removed. This strategy provides a means to temporally and spatially track the transport of remodeling-defective GPI-APs in yeast cells.

For complete details on the use and execution of this protocol, please refer to Chen et al. (2021).

BEFORE YOU BEGIN

Glycosylphosphatidylinositol (GPI)-anchored proteins (GPI-APs) represent an evolutionary conserved group of post-translationally modified plasma membrane (PM) localized proteins in eukaryotic cells with diverse functions. During their synthesis in the lumen of the ER, GPI-APs undergo a succession of remodeling events that exclusively impact the structure of the GPI moiety. These modifications / remodeling events have generally been considered to function in quality assurance whereby the preceding GPI modification / remodeling event is a prerequisite for and ensures the fidelity and robustness of the subsequent one. One such remodeling event – the addition of phosphoethanolamine (EtNP) to mannose 2 (Man2) of the GPI moiety, and its subsequent removal, functions as an ER transport warrant. Indeed, GPI-APs in which EtNP has not been removed from Man2 show a kinetic delay in their export from the ER. Nevertheless, GPI-APs deficient in remodeling are still able to reach the plasma membrane and the phenotypic consequences of this are diverse – at least in budding yeast cells were this has been most thoroughly studied. We have established that GPI-APs in which EtNP remains attached to Man2 are still delivered to the PM where they elicit a unique stress response culminating in activation of the spindle assembly checkpoint (SAC). The methods described herein provide a genetic basis by which to engineer yeast strains that conditionally express GPI-APs in which EtNP is added to Man2 but not removed. These reagents provide a tool kit with which to dissect the spatial and temporal delivery of incompletely remodeled GPI-APs to the PM and in so doing a means by which to elicit stress-induced ectopic activation of the SAC. Such strains will permit interrogation of the signaling pathway leading to SAC activation in GPI-AP Man2 remodeling deficient mutants.
The following preparation steps guide you through the generation of yeast strains with a variety of genetic backgrounds for use in the temporal expression, spatial tracking of un-remodeled GPI-APs, and the ectopic activation of the SAC that arises from the presence of such proteins at the PM. The yeast strains described in Chen et al., are available from the lead contact should you not wish to generate yourself.

**Amplification of the knock-in/knock-out cassettes for integration of exogenous DNA sequences to target regions on chromosomes**

© Timing: 4 h

1. Use high-fidelity DNA polymerase (e.g., Phusion™) to amplify the knock-out cassette (named “dcr2-KO LEU”, fragment size ~2550 bp) for deleting the promoter and parts of the open reading frame (ORF) of DCR2 (i.e., from -255 to 1490 bp) a deletion larger than this will likely impact the expression of VPS38 as the DNA sequence of the DCR2 ORF after 1701 bp overlaps with the ORF of VPS38).
   a. Template: pUG73 (Gueldener et al., 2002).
   b. Forward primer: DCR2-KO-LoxP-F.
   c. Reverse primer: DCR2-KO-LoxP-R.

2. Use high-fidelity DNA polymerase (e.g., Phusion™) to amplify the knock-in cassette (named “GPI7-9myc-KI”, fragment size ~2921 bp) for appending 9 tandem copies of the myc epitope to the C-terminus of the Gpi7p.
   a. Template: C-9myc-Leu-pRS416.
   b. Forward primer: GPI7-9myc-KI-F.
   c. Reverse primer: GPI7 KO-CL-R.

3. Use high-fidelity DNA polymerase (e.g., Phusion™) to amplify the knock-in cassette (named “Kl LEU-PGAL-GPI7”, fragment size ~2979 bp) for replacing the endogenous promoter of GPI7 with the inducible GAL1 promoter.
   a. Template: pRS415-prom-Leu-pGAL-Sed5.
   b. Forward primer: KlLEU-pGAL-KI-GPI7-F.
   c. Reverse primer: KlLEU-pGAL-KI-GPI7-R.

4. Use high-fidelity DNA polymerase (e.g., Phusion™) to amplify the knock-out cassette (named “Kl LEU-KO URA3”, fragment size ~1692 bp) for deleting the first half of Kluyveromyces lactis (K.l.) LEU2 selectable marker including the first LoxP sequence.
   a. Template: pUG72 (Gueldener et al., 2002).
   b. Forward primer: KLURA-deKL LEU-F.
   c. Reverse primer: KLURA-deKL LEU-R.

5. Use high-fidelity DNA polymerase (e.g., Phusion™) to amplify the knock-out cassette (named “PGAS1-KO LEU”, fragment size ~2550 bp) for replacing the promoter and the start codon (ATG) of GAS1 with the LEU2 gene from Kluyveromyces lactis.
   a. Template: pUG73.
   b. Forward primer: GAS1prom-LoxP-KO-F.
   c. Reverse primer: GAS1prom-LoxP-KO-R.

**Note:** Plasmid pUG73 contains two LoxP sequences, between which there is a coding sequence of K.l. LEU2 which can be used as a selection marker to knockout the gene of interest. Similarly, pUG72 includes LoxP-K.l. URA3-LoxP.

**Alternatives:** For amplification of knock-out cassettes, using rTaq polymerase is an option to reduce the cost. However, a high-fidelity DNA polymerase is a better choice to amplify knock-in cassettes for introducing exogenous DNA sequences into chromosomes.
Note: Verification of amplified cassettes on 1% agarose is required to ensure that the amplification conditions are optimal. Purification of the PCR products for knock-in or knockout experiments is necessary.

Generation of yeast strains in which GPI7 expression is regulatable

© Timing: 4 weeks

6. Generation of a ted1 Δ dcr2Δ strain containing a counter-selectable TED1-containing plasmid (gTED1-pRS416) (i.e., SARY4602) see Figure 1A.

   a. Culture the ted1 Δ cells overnight (~16 h) in YEPD medium at 25°C with shaking at 250 RPM, take a portion of the overnight culture and resuspend cells at 0.3 x 10^7 cells/mL in YEPD and culture cells for a further 4–5 h at 25°C.

   b. When the culture has reached exponential growth phase (i.e., an OD660 of ~0.6–0.8) transform ~10^7 cells with the plasmid gTED1-pRS416 as described below (Hill et al., 1991).

      i. Centrifuge cells for 15 s at room temperature (RT) in a 1.5 mL Eppendorf tube at the top speed using a benchtop microcentrifuge and discard the supernatant.

      ii. Resuspend the cells with 1 mL 0.1 M lithium acetate pH8.0.

      iii. Repeat centrifugation procedure described in step 6-b-i, and discard the supernatant.

      iv. Resuspend the cells with 100 μL 0.1 M lithium acetate pH8.0.

      v. Mix the plasmid gTED1-pRS416 (~1 μg) with the cell suspension and incubate the cells at room temperature for 5 min.

      vi. Mix 280 μL 50% (w/v) PEG4000 (dissolved in 0.1 M lithium acetate, TE pH8.0) with the cell suspension and incubate the cells at room temperature for 60 min.

      vii. Mix 43 μL DMSO with the mixture and heat shock the cells at 42°C for 5 min.

      viii. Centrifuge cells for 30 s at room temperature (RT) in a 1.5 mL Eppendorf tube at the top speed using a benchtop microcentrifuge and discard the supernatant.

      ix. Resuspend the cells with 1 mL ddH2O.

      x. Centrifuge cells for 15 s at room temperature (RT) in a 1.5 mL Eppendorf tube at the top speed using a benchtop microcentrifuge and discard the supernatant.

      xi. Resuspend the cells with 100 μL ddH2O and spread the suspension onto an appropriate selection plate (i.e., -ura).
xii. Incubate the plate at 25°C for 3 days.

Note: All reagents should be sterile.

Note: gTED1-pRS416 contains the open reading frame of TED1 flanked by 200 base pairs of its 5’UTR region (from position -200 to -1) i.e., the promoter region, and 125 base pairs of its 3’UTR region (125 bp after stop codon) i.e., the terminator region. The pRS416 vector includes the URA3 gene as a selection marker. Hence the positive transformants can be isolated by being incubated on a -ura plate at 25°C after 3 days.

c. Culture single colonies that arise on -ura plates in -ura medium at 25°C by shaking at 250 rpm overnight, resuspend the cells at ~0.3 × 10^7 cells/mL in -ura medium and culture them for 4–5 h at 25°C by shaking at 250 rpm.

d. Transform ~10^7 cells with ~3 μg of the knock-out cassette “dcr2-KO LEU”.
   i. Spin down the cells in an Eppendorf tube at the top speed in a benchtop microcentrifuge at room temperature for 15 s and discard the supernatant.
   ii. Resuspend the cells with 1 mL 100 mM lithium acetate.
   iii. Spin down the cells at top speed in a benchtop microcentrifuge at room temperature for 15 s and discard the supernatant.
   iv. Resuspend the cells with PEG3350 master mix (refer to “materials and equipment”) and incubate the mixture at room temperature for 45 min.
   v. Heat-shock the cells at 42°C for 30 min.
   vi. Spin down the cells at the top speed in a benchtop microcentrifuge at room temperature for 30 s and discard the supernatant.
   vii. Resuspend the cells with 1 mL ddH₂O.
   viii. Spin down the cells at top speed in a benchtop microcentrifuge at room temperature for 15 s and discard the supernatant.
   ix. Resuspend the cells with 100 μL ddH₂O and spread the suspension onto an appropriate selection plate (i.e., -leu).
   x. Incubate the transformants at 25°C for 3 days.

Note: This method (Gietz and Schiestl, 2007) provides very high transformation efficiency to increase the success rate of integration of the exogenous DNA to the target DNA region. But it might introduce unwanted integration of the exogenous DNA from the ssDNA into the yeast genome. All reagents should be sterile.

e. Select LEU2-positive cells by incubating the transformants on a -leu plate and verify the candidates by yeast colony PCR using the primers listed below:
   i. Forward primer: KL-LEU2-R.
   ii. Reverse primer: DCR2intR/MfeI.
   This PCR results in the amplification of ~650 bp DNA fragment from integration-positive cells (Figure 2A).
**Yeast colony PCR master mix**

| Reagent                          | Amount |
|---------------------------------|--------|
| DNA template                    | 0.5 μL |
| DNA Polymerase (TaKaRa Taq™)    | 0.125 μL |
| Forward Primer (10 μM)          | 2 μL   |
| Reverse Primer (10 μM)          | 2 μL   |
| 10× PCR Buffer (Mg2+ plus)      | 2.5 μL |
| dNTP Mixture (2.5 mM)           | 2 μL   |
| ddH2O                            | up to 25 μL |

**Note:** The template DNA is derived from yeast cell lysates generated in 20 mM NaOH. To make the yeast lysate, resuspend cells from a colony (the diameter is about 1–2 mm) or a patch (~0.3 × 10^7 cells) with 20 μL 20 mM NaOH in a PCR tube. Heat the suspension in a PCR machine at 95°C for 10 min. Remove cell debris and unlysed cells by centrifugation in a microcentrifuge at top speed for 5 min. Use the resulting supernatant as the DNA template.

**TaKaRa Taq™ PCR cycling conditions**

| Steps          | Temperature | Time       | Cycles |
|----------------|-------------|------------|--------|
| Initial Denaturation | 98°C        | 30 s       | 1      |
| Denaturation    | 98°C        | 10 s       | 34 cycles |
| Annealing      | 50°C        | 30 s       |        |
| Extension      | 72°C        | 1 min per 1 Kb |      |
| Final extension| 72°C        | 10 min     | 1      |
| Hold           | 4°C         | unlimited  |        |

f. Patch the integration positive strains identified by PCR on plates containing 5-fluoro- orotic acid (5-FOA).

g. Identify those strains that cannot grow on 5-FOA plates and transform cells from these strains with pSH62 to remove *K.l. LEU2* marker (Gueldener et al., 2002).

i. Transform the cells with pSH62 (method used refers to step 6-b) and spread the transformants on -his plates.

ii. Incubate the transformants at 25°C for 3 days.

iii. Inoculate HIS3-positive colonies into 1 mL -his medium and culture the cells at 25°C for 6 h.

iv. Spin down the cells from 1 mL of culture in an Eppendorf tube at top speed in a benchtop microcentrifuge at room temperature for 15 s and discard the supernatant.

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**Figure 2. Schematic diagram indicating the position of the primers used for verifying the success of genetic manipulation**

(A) Diagram indicating the position of the verification primers used for *dcr2* knockout.

(B) Diagram indicating the position of the primers used for verifying the replacement of the *GPI7* promoter with the *GAL1* promoter.

(C) Diagram indicating the position of the primers used for verifying the deletion of the *GAS1* promoter.
v. Resuspend the cells with 1 mL YEPGal medium.
vi. Spin down the cells at the top speed of a benchtop microcentrifuge at room temperature for 15 s and discard the supernatant.

vii. Resuspend the cells with 2 mL YEPGal medium and culture them overnight to allow expression of Cre recombinase.

viii. Spread ~500 cells from the culture onto a YEPD plate and incubate the plate at 25°C for 3 days.

ix. Patch the colonies that arise on the YEPD plate in replica onto a -leu plate and a -his plate.

x. Identify colonies from the YEPD plate that cannot grow on either -leu or -his plates and store them at –80°C.

Note: The HIS3-containing plasmid pSH62 includes the coding sequence of the Cre recombinase under the control of GAL promoter. Cre recombinase removes the DNA between two LoxP sequences.

△ CRITICAL: In the following experiments the LoxP-containing selection marker is going to be re-applied for gene editing, it is therefore necessary to make sure that plasmid pSH62 has been lost from the yeast cells after removing the previous LoxP-containing marker. If the pSH62 plasmid remains, culture the cells in YEPD medium again and repeat steps 6-g–viii–6-g-x.

7. Generation of the yeast strain expressing Gpi7p-9myc [refers to SARY5529 (derived from BY4741) and SARY5533 (derived from SARY4602)] (Figure 1B).

a. Culture BY4741 and SARY4602 cells in YEPD medium overnight.
b. Take 10⁷ cells of each strain in exponential growth and transform the cells with the knock in cassette “GPI7-9myc-KI” (DNA amount: 3 μg) (transformation method refers to step 6-d).
c. Select LEU2-positive cells by incubating the transformants on -leu plates.
d. Examine Gpi7p-9myc expression in the LEU2-positive cells by immunoblotting using an anti-myc antibody. Gpi7p-9myc-positive migration at ~115 KDa on an SDS-PGE gel.
e. Transform a Gpi7p-9myc-positive strain with pSH62 to remove the K.l. LEU2 marker and store the LEU2-negative cells at –80°C (the method refers to step 6-g).

8. Generation of strains containing GAL1 promoter-driven Gpi7p-9myc [refers to SARY5598 (derived from SARY5529) and SARY5600 (derived from SARY5533)] (Figure 1C).

a. Culture SARY5529 and SARY5533 cells in YEPD medium until the culture reaches an OD660 of ~0.6–0.8 (exponential growth phase).
b. Take ~10⁷ cells of each strain and transform the cells with the knock-in cassette “KI LEU-PGAL-GPI7” (DNA amount: 3 μg) (transformation method refers to step 6-d).

Note: This cassette is inserted between the promoter and the ORF of GPI7. As the distance between GPI7 and the adjacent gene (COA3) is only 303 bp, the GPI7 promoter may partially overlap with the ORF and the 3’UTR of COA3. To minimize the influence on COA3 expression, an insertion of GAL1 promoter upstream of GPI7 ORF is utilized rather than a replacement of GPI7 promoter with the GAL1 promoter.

c. Select LEU2-positive cells by incubating the transformants on -leu plates and identify Candidates with the insert by yeast colony PCR (the method refers to step 6-e) using primers listed below:
   i. Forward primer: KL-LEU2-R.
   ii. Reverse primer: GPI7-int1-R.

   This PCR results in an amplification of a ~947 bp DNA fragment from positive cells (Figure 2B).
d. Patch cells from the PCR verified strains onto 5-FOA plates to remove the balancing plasmid gTED1-pRS416.
e. Induce the expression of GPI7-9myc from cells in exponential growth phase by culturing the cells in YEPGal (containing 2% galactose) for 7 h and examine the expression by immunoblotting using an anti-myc antibody. After induction for 2 h the Gpi7p-9myc-positive strains will reveal an immuno-positive band of ~115 K.Da.

f. Store the positive strains at −80 °C.

9. Disable K.l. LEU2 function by removing the 5' half of K.l. LEU2 marker (including one of the LoxP sequences). These strains are the same as SARY5768 (derived from SARY5598) and SARY5769 (derived from SARY5600) (Figure 1D).

**Note:** The reason for leaving the second half of the K.l. LEU2 marker on the chromosome is to maintain enough space between the GPI7 promoter and the coding region of GPI7-9myc. As such, the expression of GPI7-9myc is only controlled by the GAL1 promoter.

a. Culture SARY5598 and SARY5600 cells in YEPD medium.
b. Take 10^7 cells of each strain in exponential growth and transform the cells with the knock in cassette “Kl LEU-KO URA3” (DNA amount: 3 μg) (transformation method refers to step 6-d).
c. Select URA3-positive cells by incubating the transformants on -ura plates for 3 days and patch the candidates on a -leu plate.
d. Identify transformants that cannot grow on -leu plates.
e. Transform cells with pSH62 to remove the K.l. URA3 marker (the method refers to step 6-g).
f. Induce the expression of GPI7-9myc from cells growing in exponential phase by culturing them in YEPGal (containing 2% galactose) for 7 h and examining the expression of GPI7-9myc by immunoblotting using an anti-myc antibody. After induction for 2 h the Gpi7p-9myc-positive samples will reveal an immuno-positive band of ~115 K.Da.
g. Store the positive strains at −80 °C.

**Generation of strains in which mNeon-GAS1 is inducible**

⏱ Timing: 10 days

10. Knock -in mNeon-GAS1 into strains carrying an inducible GPI7-9myc gene (Figure 1E).
   a. Use the restriction enzyme MscI to linearize the plasmid P_{GAL1}-mNeon-GAS1-pRS406 (DNA amount: 2 μg).
   b. Purify the linearized DNA for yeast transformation using an agarose gel purification kit.
   c. Culture SARY5529, SARY5768 and SARY5769 cells in YEPD medium.
   d. Take 10^7 cells of each strain in exponential growth and transform the cells with the MscI-linearized P_{GAL1}-mNeon-GAS1-pRS406 (DNA amount: 0.3 μg for each strain) (transformation method refers to step 6-d).
   e. Select URA3-positive candidates by incubating the transformants on -ura plates and induce mNeon-Gas1p expression for 5–7 h from cells growing in exponential phase and cultured in YEPGal (containing 2% galactose). Examine the expression of mNeon-Gas1p by fluorescence microscopy.
   f. Store mNeon-Gas1p positive cells at −80 °C, named SARY6933 (derived from SARY5529), SARY6935 (derived from SARY5768), and SARY6937 (derived from SARY5769).

11. Eliminating the expression of endogenous GAS1 (Figure 1F).
   a. Culture SARY6933, SARY6935 and SARY6937 cells in YEPD medium.
   b. Take 10^7 cells of each strain in exponential growth and transform the cells with the knock-out cassette “PGAL1-KO LEU” (DNA amount: 3 μg for each strain) (transformation method refers to step 6-d).
   c. Select LEU2-positive cells and verify the candidates by yeast colony PCR (the method refers to step 6-e) using the primers listed below:
      i. Forward primer: gGAS1-BamHI-F.
ii. Reverse primer: KL-LEU2-F.
This PCR results in the amplification of ~522 bp DNA fragment from cells in which GAS1 promoter has been replaced with K.L. LEU2 (Figure 2C).
d. Induce the expression of GPI7-9myc and mNeon-GAS1 from exponentially growing cells cultured in YEPGal (containing 2% galactose) and examine protein expression by immunoblotting using an anti-myc and an anti-Gas1p antibody. After induction for 2 h the Gpi7p-9myc-positive samples will reveal an immuno-positive band of ~115 KDa., while mNeon-Gas1p appears as two bands (~180 KDa. and ~130 KDa.) after induction for 7 h.
e. Store immune-positive strains at –80°C, named SARY7331, SARY7333 and SARY7335.

Note: To visualize GPI-APs that retain EtNP on the Man2, we generated a mNeon-Gas1p chimera for expression in yeast cells. The coding region of mNeon was inserted into the GAS1 gene after the sequence which encodes GAS1’s signal peptide (i.e., amino acids 1–23). Gas1p is one of the most abundant GPI-APs in yeast, and as such, provides a useful means by which to visualize GPI-APs in cells. The expression of mNeon-Gas1p is driven by an inducible attenuated GAL1 promoter, as such, the expression of mNeon-Gas1p will be less robust than that of Gpi7p which is driven by the unattenuated GAL1 promoter. Asynchronous expression of GPI7 temporally prior to that of mNeon-Gas1p in cells lacking TED1 and DCR2 ensures that the vast majority of the mNeon-Gas1p synthesized will retain EtNP on Man2.

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse monoclonal anti Myc | Roche | Cat#11667149001; RRID:N/A |
| Rabbit polyclonal anti Gas1p | kindly provided by Prof. Howard Reizman (University of Geneva) | N/A |
| Mouse monoclonal anti Pgl1p | Molecular Probes | Cat#459250; RRID: AB_2532235 |
| Goat Anti-Mouse IgG | Sigma | Cat#A8924, RRID: AB_258426 |
| Donkey Anti-Rabbit IgG | GE Healthcare | Cat#NA934; RRID: AB_772206 |
| Chemicals, peptides, and recombinant proteins | | |
| Phusion High Fidelity DNA Polymerase | New England Biolabs | Cat#M0530L |
| TaKaRa Taq DNA Polymerase | TaKaRa | Cat#R10T1 |
| Polyethylene Glycol (4000) | Fluka | Cat#81240 |
| Polyethylene Glycol (3350) | Sigma | Cat#P3640 |
| Lithium Acetate, dihydrate | Nacalai | Cat#206-04 |
| DMSO | Sigma | Cat#D26505X5H |
| S-Fluoroorotic acid hydrate (5′-FOA) | Zymo Research | Cat#F9001-1 |
| Adenine hemisulfate salt | Sigma | Cat#A9126 |
| L-arginine | Sigma | Cat#A5131 |
| L-aspartic acid | Sigma | Cat#A9256 |
| L-glutamic acid monosodium salt | Sigma | Cat#G1251 |
| L-histidine | Sigma | Cat#H8125 |
| L-leucine | Sigma | Cat#C8000 |
| L-lysine mono-HCl | Sigma | Cat#L5626 |
| L-methionine | Sigma | Cat#M9625 |
| L-phenylalanine | Sigma | Cat#P2126 |
| L-serine | Sigma | Cat#S4500 |
| L-threonine | Sigma | Cat#T8625 |
| L-tryptophan | Sigma | Cat#T0254 |
| L-tyrosine | Sigma | Cat#T3754 |

(Continued on next page)
## Continued

| REAGENT or RESOURCE NAME | SOURCE | IDENTIFIER |
|--------------------------|--------|------------|
| D-(+)- Glucose anhydrous | RDH    | Cat#16325  |
| D-(+)- Galactose         | Sigma  | Cat#G0750  |
| R-(+)-Raffinose pentahydrate | Sigma | Cat#R0514  |
| Agar                     | Oxoid  | Cat#LP0011 |
| Yeast nitrogen base      | Difco  | Cat#233520 |
| Ammonium sulphate        | BDH    | Cat#A4915  |
| Deoxyribonucleic Acid, Sodium salt, from salmon testes | Sigma | Cat#D1626  |
| Trizma® base             | Sigma  | Cat#T1503  |
| Boric acid               | Sigma  | Cat#31146  |
| EDTA                     | Invitrogen | Cat#15576-028 |
| Glycerol                 | Sigma  | Cat#G7757  |
| Sodium dodecyl Sulfate   | Sigma  | Cat#14390  |
| Bromophenol blue         | BDH    | Cat#200152E|
| sodium chloride          | Sigma  | Cat#53014  |
| Potassium chloride       | Sigma  | Cat#12636  |
| Disodium Hydrogen Phosphate Dihydrate | BDH | Cat#28029-260 |
| Potassium Dihydrogen Phosphate | RDH | Cat#04243  |
| Tween-20                 | USB    | Cat#20605  |
| Pefabloc SC (AEBSF)      | Roche  | Cat#11429876001 |
| EDTA-free protease inhibitor cocktail | Roche | Cat#11873580001 |
| Trichloroacetic acid     | Sigma  | Cat#T8657  |
| Concanavalin-A           | Sigma  | Cat#C7275  |
| FM™ 4–64 Dye             | Invitrogen | Cat#T13320 |
| Dithiothreitol (DTT)     | USB    | Cat#15397  |
| Amersham™ Protran® Premium Western blotting membranes, nitrocellulose | Cytiva | Cat#GE10600003 |
| ECL™ Western Blotting Reagents | Cytiva | Cat#GERPN2106 |

### Critical commercial assays

#### FavorPrep™ GEL/PCR Purification Mini Kit
FAVORGEN | Cat#FAGCK001-1

#### Experimental models: Organisms/strains

| MATa his3Δ1 leu2Δ10 met15Δ0 ura3Δ0 ted1::KanMX4 | EUROSCARF | BY4741 |
| MATa his3Δ1 leu2Δ10 met15Δ0 ura3Δ0 ted1::KanMX4, dcr2::gTED1-pRts16 | EUROSCARF | Y01432 |
| MATa his3Δ1 leu2Δ10 met15Δ0 ura3Δ0 ted1::KanMX4, dcr2::gTED1-pRts16, gpi7::GPI7-9myc | This study | SARY4602 |
| MATa his3Δ1 leu2Δ10 met15Δ0 ura3Δ0 gpi7::GPI7-9myc | This study | SARY5529 |
| MATa his3Δ1 leu2Δ10 met15Δ0 ura3Δ0 K.I. LEU2-P<sub> GAL </sub>-GPI7-9myc | This study | SARY5533 |
| MATa his3Δ1 leu2Δ10 met15Δ0 ura3Δ0 K.I. LEU2-P<sub> GAL </sub>-GPI7-9myc | This study | SARY5598 |
| MATa his3Δ1 leu2Δ10 met15Δ0 ura3Δ0 K.I. LEU2-P<sub> GAL </sub>-GPI7-9myc | This study | SARY5600 |
| MATa his3Δ1 leu2Δ10 met15Δ0 ura3Δ0 P<sub> GAL </sub>-GPI7-9myc | This study | SARY5768 |
| MATa his3Δ1 leu2Δ10 met15Δ0 ura3Δ0 P<sub> GAL </sub>-GPI7-9myc | This study | SARY5769 |

(Continued on next page)
Note: Autoclave the Part A and Part B separately and store them at room temperature. Mix the two parts before use.

### MATERIALS AND EQUIPMENT

#### YEPD medium

| Reagent             | Final concentration | Amount          |
|---------------------|---------------------|-----------------|
| **Part A**          |                     |                 |
| Adenine hemisulfate salt | 55 µg/mL      | 0.055 g         |
| Yeast extract       | 10 g/L              | 10 g            |
| Bacto peptone       | 20 g/L              | 20 g            |
| ddH₂O               | n/a                 | up to 900 mL    |
| **Part B**          |                     |                 |
| Glucose             | 20 g/L              | 20 g            |
| ddH₂O               | n/a                 | up to 100 mL    |
| **Total**           | n/a                 | 1 L             |

**Note:** See Table S1 for primers and Table S2 for other reagents.

#### YEPGal medium

| Reagent             | Final concentration | Amount          |
|---------------------|---------------------|-----------------|
| **Part A**          |                     |                 |
| Adenine hemisulfate salt | 55 µg/mL      | 0.055 g         |
| Yeast extract       | 10 g/L              | 10 g            |
| Bacto peptone       | 20 g/L              | 20 g            |
| ddH₂O               | n/a                 | up to 900 mL    |

(Continued on next page)
**Note:** Autoclave the Part A and Part B separately and store them at room temperature. Mix the two parts before use.

### YEPD plate

| Reagent                  | Final concentration | Amount     |
|--------------------------|---------------------|------------|
| Part A                   |                      |            |
| Adenine hemisulfate salt | 55 µg/mL            | 0.055 g    |
| Yeast extract            | 10 g/L              | 10 g       |
| Bacto peptone            | 20 g/L              | 20 g       |
| Agar                     | 20 g/L              | 20 g       |
| ddH₂O                    | n/a                 | up to 900 mL |
| Part B                   |                      |            |
| Galactose                | 20 g/L              | 20 g       |
| Raffinose                | 10 g/L              | 10 g       |
| ddH₂O                    | n/a                 | up to 100 mL |
| Total                    | n/a                 | 1 L        |

**Note:** Autoclave the Part A and Part B separately. Mix the two parts when the agar-containing medium is still warm (around 60°C). Pour the mixture into 10 cm petri dishes. Following solidification plates can be stored at 4°C.

### Complete amino acid supplement

| Reagent                  | Final concentration | Amount |
|--------------------------|---------------------|--------|
| Adenine hemisulfate salt | 3.32%               | 2.5 g  |
| L-arginine               | 1.59%               | 1.2 g  |
| L-aspartic acid          | 7.96%               | 6 g    |
| L-glutamic acid          | 7.96%               | 6 g    |
| L-histidine              | 1.59%               | 1.2 g  |
| L-leucine                | 4.77%               | 3.6 g  |
| L-lysine                 | 2.39%               | 1.8 g  |
| L-methionine             | 1.59%               | 1.2 g  |
| L-phenylalanine          | 3.98%               | 3 g    |
| L-serine                 | 29.84%              | 22.5 g |
| L-threonine              | 15.92%              | 12 g   |
| L-tryptophan             | 3.18%               | 2.4 g  |
| L-tyrosine               | 2.39%               | 1.8 g  |
| L-valine                 | 11.94%              | 9 g    |
| Uracil                   | 1.59%               | 1.2 g  |
| Total                    | 100%                | 75.4 g |

**Note:** For selection purposes one or more of the reagents is omitted (i.e., -ura lacks only uracil). The supplement mixture lacking a particular constituent is termed the “dropout supplement”.  

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Filter the Part A (e.g., by using Stericup vacuum filtration system from Millipore) and autoclave the Part B. Mix the two parts before use.

| Dropout medium | Reagent                                      | Final concentration | Amount   |
|----------------|---------------------------------------------|---------------------|----------|
| Part A         | Yeast nitrogen base without amino acids and ammonium sulfate | 1.7 g/L             | 1.7 g    |
|                | Ammonium sulphate                           | 5 g/L               | 5 g      |
|                | Dropout supplement                           | 1.3 g/L             | 1.3 g    |
|                | ddH₂O                                       | n/a                 | up to 900 mL |
| Part B         | Glucose                                     | 20 g/L              | 20 g     |
|                | ddH₂O                                       | n/a                 | up to 100 mL |
| Total          |                                             | n/a                 | 1 L      |

**Note:** Filter the Part A (e.g., by using Stericup vacuum filtration system from Millipore) and autoclave the Part B. Mix the two parts before use.

| Dropout plate | Reagent               | Final concentration | Amount   |
|---------------|-----------------------|---------------------|----------|
| Part A        | Yeast nitrogen base   | 1.7 g/L             | 1.7 g    |
|               | Ammonium sulphate     | 5 g/L               | 5 g      |
|               | Dropout supplement    | 1.3 g/L             | 1.3 g    |
|               | ddH₂O                 | n/a                 | up to 450 mL |
| Part B        | Agar                  | 20 g/L              | 20 g     |
|               | ddH₂O                 | n/a                 | up to 450 mL |
| Part C        | Glucose               | 20 g/L              | 20 g     |
|               | ddH₂O                 | n/a                 | up to 100 mL |
| Total         |                       | n/a                 | 1 L      |

**Note:** Filter the Part A (e.g., by using Stericup vacuum filtration system from Millipore) and autoclave the Part B and C. Mix the three parts when the agar-containing solution remains warm (around 60°C). Pour the liquid into 10 cm petri dishes. Following solidification plates can be stored at 4°C.

| 5'-FOA plate | Reagent               | Final concentration | Amount   |
|--------------|-----------------------|---------------------|----------|
| Part A       | Yeast nitrogen base   | 1.7 g/L             | 1.7 g    |
|               | Ammonium sulphate     | 5 g/L               | 5 g      |
|               | Agar                  | 20 g/L              | 20 g     |
|               | ddH₂O                 | n/a                 | up to 500 mL |
| Part B       | 5'-FOA                | 1 g/L               | 1 g      |
|               | complete amino acid supplement | 1.3 g/L | 1.3 g    |
|               | Glucose               | 20 g/L              | 20 g     |
|               | ddH₂O                 | n/a                 | up to 500 mL |
| Total        |                       | n/a                 | 1 L      |
**Note:** Autoclave Part A. Dissolve Part B in prewarmed 500 mL ddH₂O (60°C) and sterilize it by filtration (e.g., by using Stericup vacuum filtration system from Millipore). Mix the warm filtered Part B when the agar-containing medium (Part A) remains warm (around 60°C). Pour the liquid into 10 cm petri dishes. Following solidification plates can be stored at 4°C.

### PEG3350 master mix (for high efficiency DNA transformation of yeast cells)

| Reagent                                          | Final concentration | Amount   |
|--------------------------------------------------|---------------------|----------|
| 50% (w/v) PEG 3350                              | 33.3%               | 240 µL   |
| Lithium Acetate (1 M)                            | 0.1 M               | 36 µL    |
| Single-stranded carrier DNA (2 mg/mL)            | 27.8 µg/µL          | 50 µL    |
| Exogenous DNA                                    | 8.33 ng/µL          | 3 µg     |
| Yeast cells                                      | n/a                 | 10³      |
| ddH₂O                                            | n/a                 | up to 360 µL |
| **Total**                                        | n/a                 | 360 µL   |

**Note:** All reagents should be sterile. Single-stranded carrier DNA (ssDNA) comes from salmon sperm DNA. Dissolve the ssDNA in sterile TE buffer (10 mM Tris–HCl, 1 mM Na₂EDTA, pH 8.0) and denature the DNA by heating at 100°C for 5 min followed by immediate ice-chilling.

### TBE buffer

| Reagent                          | Final concentration | Amount   |
|----------------------------------|---------------------|----------|
| Tris base                        | 10.8 g/L            | 27 g     |
| Boric acid                       | 5.5 g/L             | 13.75 g  |
| EDTA (0.5 M, pH8.0)              | 2 mM/L              | 10 mL    |
| ddH₂O                            | n/a                 | up to 2.5 L |
| **Total**                        | n/a                 | 2.5 L    |

### 2 x SDS sample buffer

| Reagent              | Final concentration | Amount   |
|----------------------|---------------------|----------|
| Tris buffer (1 M, pH6.8) | 0.1 M               | 14 mL    |
| Glycerol             | 14%                 | 14 mL    |
| 10% SDS              | 4%                  | 40 mL    |
| Bromophenol blue     | 0.01%               | 0.01 g   |
| ddH₂O                | n/a                 | up to 100 mL |
| **Total**            | n/a                 | 100 mL   |

**Note:** Add 2 mM Pefabloc SC, 2x protease inhibitor cocktail and 10 mM DTT into the 2 x SDS-sample buffer before mixing with the samples.

### 1 x SDS-running buffer

| Reagent       | Final concentration | Amount   |
|---------------|---------------------|----------|
| Glycine       | 28.8 g/L            | 144 g    |
| Trizma® base  | 6 g/L               | 30 g     |
| ddH₂O         | n/a                 | up to 5 L |
| **Total**     | n/a                 | 5 L      |

**Note:** Add 0.1% SDS before use.
STEP-BY-STEP METHOD DETAILS

Induction of mNeon-Gas1p synthesis whereby EtNP is not removed from Man2 and observation of protein expression and trafficking by epi-fluorescence microscopy

Timing: 4 days

This section describes how to induce the expression of mNeon-Gas1p in which Man2 has not been remodeled by the removal of EtNP.

1. Revive the yeast strains in which the expression of mNeon-Gas1p and Gpi7p-9myc are driven by the GAL1 promoter (i.e., SARY7331, SARY7333 and SARY7335).
   a. Streak yeast strains from their /C0 /C14 glycerol stocks for single colonies onto YEPD plates.
   b. Incubate the plates at 25°C for two days.
   c. Select well isolated colonies and patch them onto a new YEPD plate.

2. Inoculate cells from each strain into YEPD medium and culture them overnight at 25°C.

3. Dilute the overnight culture into 9 mL YEPD medium at a density of 0.3 x 10^7 cells/mL.

4. Culture the diluted culture at 25°C for 3 h.

5. Aliquot 6 mL of culture from each of the strains for induction in YEPGal.
   a. Spin down cells in Falcon tubes at 3,000 x g for 3 min at room temperature and discard the supernatant.
   b. Wash the cell pellet by resuspension with 5 mL YEPGal medium once.
   c. Spin down the cells again at 3,000 x g for 3 min at room temperature and discard the supernatant.
   d. Resuspend the cells in 6 mL YEPGal medium and continue culturing at 25°C.
△ CRITICAL: It is important to remove the glucose present in the YEPD as any residual glucose will delay induction via the GAL1 promoter.

6. For samples cultured in YEPD.
   a. Preparation of yeast lysates for immunoblotting.
      i. Measure the optical density of the culture in log phase (an OD660 of ~ 0.6–0.8) (these values will be used to adjust the amount of protein run on SDS-PAGE gels).
      ii. Mix 1 mL culture with 200 µL 100% TCA and store the mixture at −80°C for later SDS-PAGE and immunoblotting.
   b. Preparation of yeast cells for plasma membrane labeling with FM™ 4–64.
      i. Collect cells from 1 mL culture in log phase (an OD660 of ~ 0.6–0.8) using the highest speed of a benchtop microcentrifuge at room temperature for 15 s and discard the supernatant.
      ii. Resuspend the cells with 1 mL ice-chilled PBS.
      iii. Incubate the cells on ice for 5 min.
      iv. Spin down the cells again as in 6-b-i.
      v. Resuspend the cells with 50 µL ice-chilled PBS containing 30 µM FM™ 4–64.
      vi. Incubate the cells on ice for 20–30 min.
      vii. Spin down the cells at top speed in a benchtop microcentrifuge at 4°C for 15 s and discard the supernatant.
      viii. Wash the cells with 1 mL ice-chilled PBS.
      ix. Spin down the cells at top speed in a benchtop microcentrifuge at 4°C for 15 s and discard the supernatant.
   x. Resuspend the cells with ice-chilled PBS at ~ 10^7 cells/100 µL.
   xi. Apply a 0.8 µL suspension of each strain onto a multispot microscope slide coated with Concanavalin A and seal the slide with a cover slip and nail polish.
   xii. Acquire images of cells using a fluorescence microscope with the configuration optimized for mNeon and FM™ 4–64 excitation/emission spectra (mNeon: Ex λ 506/ Em λ 517; FM™ 4–64: Ex λ 515/ Em λ 640) ( Samples were visualized and photographed through a Nikon Plan Apo VC 100×/1.40 oil objective lens by using a Nikon ECLIPSE 80i microscope (Nikon, Japan) equipped with a SPOT-RT KE monochrome charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI).

7. Preparation of yeast cells for immunoblotting and FM™ 4–64 staining following 2-h induction in YEPGal.
   a. Preparation of samples for immunoblotting.
      i. Measure the optical density of the culture at 660 nm (these values will be used to adjust the amount of protein run on SDS-PAGE gels).
      ii. Mix 1 mL culture with 200 µL 100% TCA and store the mixture at −80°C for immunoblotting.
   b. Preparation of yeast cells for plasma membrane labeling with FM™ 4–64 (repeat step 6-b).

8. Preparation of yeast cells for immunoblotting and FM™ 4–64 staining following 2-h incubation in YEPGal.
   a. Preparation of samples for immunoblotting.
      i. Measure the optical density of the culture at 660 nm (these values will be used to adjust the amount of protein run on SDS-PAGE gels).
      ii. Mix 1 mL culture with 200 µL 100% TCA and store the mixture at −80°C for immunoblotting.
   b. Preparation of yeast cells for plasma membrane labeling with FM™ 4–64 (repeat step 6-b).

Alternatives: Measuring the absorption of yeast cultures at 600nm is an alternative means by which to estimate the number of cells / mL of yeast culture. Induction of mNeon-Gas1p synthesis can be observed by epifluorescence microscopy following 4 h of induction. However, by contrast a clearer indication of robust expression might require 7 h of induction. Using a preculture in raffinose-rich medium might be helpful to improve the galactose induction.
CRITICAL: Incubating the cells with ice-chilled PBS before and during the FM™ 4–64 staining is very important as this will reduce the rate at which the dye is endocytosed and reaches the vacuole.

Examination of the expression of Gpi7p-9myc and mNeon-Gas1p by immunoblotting

Timing: 2 days

This section describes how to monitor the expression of Gpi7p-9myc and mNeon-Gas1p following SDS-PAGE and immunoblotting.

9. Resolve Gpi7p-9myc and mNeon-Gas1p by SDS-PAGE.
   a. Take the TCA-containing samples from \(-80^\circ C\) and thaw them on ice.
   b. Spin down the pellets at the top speed in a benchtop centrifuge at \(4^\circ C\) for 10 min and discard the supernatant.
   c. Resuspend the pellets with 1 mL pre-chilled acetone (stored at \(-20^\circ C\)).
   d. Spin down the pellets again at the top speed in a benchtop centrifuge at \(4^\circ C\) for 10 min and discard the supernatant.
   e. Airdry or use a 65°C dry bath to volatilize acetone from the pellets.
   f. Dissolve the pellets with ddH₂O containing 1% SDS 0.1 M NaOH (dissolving buffer). The volume of dissolving buffer used will depend on the relative cell number, which was determined from the optical density measurements conducted in steps 6-a-i, 7-a-i and 8-a-i. The desired ratio is \(\sim 10^7\) cells/50 μL dissolving buffer.
   g. Add the same volume (9.f.) of 2× SDS-sample buffer to the samples.
   h. Heat the samples at 95°C for 10 min.
   i. Spin down the undissolved particles at the top speed in a benchtop microcentrifuge at room temperature for 3 min.
   j. Load 10 μL of the supernatants onto separate 8% SDS-polyacrylamide gels (two) in the identical order (one to be used for Gpi7p-9myc immunoblotting and the other for mNeon-Gas1p immunoblotting).
   k. Conduct SDS-PAGE.

10. Immunoblotting of Gpi7p-9myc and mNeon-Gas1p.
   a. Transfer the resolved proteins from each of the two polyacrylamide gels onto separate pieces of nitrocellulose membrane by electrophoretic transfer (200 mA for overnight or 300 mA for 3 h).
   b. Incubate the nitrocellulose membranes with 5% skim milk in PBST for 1 h to reduce non-specific protein-antibody binding.
   c. Cut each membrane into two parts to separate proteins larger than 64 KDa from proteins which are smaller (this results in 4 pieces of membrane in total – 2 pieces from each of the two original membranes).
   d. Incubate the two pieces of membranes containing larger molecular weight proteins separately with an anti-myc antibody (1:1000 dilution) and an anti-Gas1p (1:5000 dilution) with constant agitation. The antibodies were diluted in PBST with 5% skimmed milk. For the two pieces of membranes containing smaller molecular weight proteins use anti-Pgk1p antibody (1:10000 dilution) (Pgk1p serves as a gel loading control) with agitation. Incubate membranes and antibodies for 1 h at room temperature.
   e. Wash the membranes with PBST for 5 min three times with constant agitation.
   f. Incubate the anti-Gas1p-bound membranes with HRP-conjugated anti-rabbit antibody (1:3000 dilution) in PBST with 5% skimmed milk for 1 h with constant agitation. Incubate the remaining three pieces of nitro cellulose membrane with an HRP-conjugated anti-mouse antibody (1:3000 dilution) in PBST with 5% skimmed milk for 1 h with constant agitation.
   g. Wash the membranes with PBST for 5 min three times with constant agitation.
h. Drain the liquid from the membranes and incubate the membranes with chemiluminescent substrate for 1 min.

i. Put the membranes into imaging system (e.g., ChemiDoc Imaging System) to acquire a digital image of the immunoblot by using cumulative mode.

**EXPECTED OUTCOMES**

SARY7331 cells (gGPI7-9myc gas1::PGAL-mNeon-GAS1) constitutively express Gpi7p-9myc since the gene is driven by its own promoter. While for SARY7333 cells (gpi7::PGAL-GPI7-9myc gas1::PGAL-mNeon-GAS1) and SARY7335 cells (gpi7::PGAL-GPI7-9myc ted1Δ dcr2Δ gas1::PGAL-mNeon-GAS1), expression of the GPI7 gene is controlled by GAL1 promoter and as such no Gpi7p-9myc should be observed by immunoblotting when the cells were cultured in YEPD. However, within the 2-h induction period the expression of Gpi7p-9myc should be comparable to the levels seen in SARY7331 cells (Figure 3A).

When cultured in YEPD or YEPGal medium (for 2 h), the SARY7331, SARY7333 and SARY7335 cells will likely show no detectable expression of mNeon-Gas1p. As judged by either immunoblotting or epifluorescence microscopy mNeon-Gas1p will only become apparent in cells cultured in YEPGal for 7 h (Figures 3B and 3C).
Since mNeon-Gas1p is only detectable after the expression of Gpi7p-9myc reaches endogenous levels (i.e., that observed in SARY7331 cells) it is presumed that EtNP has been added to Man2 of Gas1p-mNeon.

**LIMITATIONS**

We do not directly determine if all Gas1p-mNeon molecules have not been remodeled, however we consider that as the de novo detectable synthesis of mNeon-Gas1p only appears to occur after that of Gpi7p-9myc we surmise that the bulk of the protein retains EtNP on Man2. Nevertheless, we cannot rule out the possibility that some quantity of mNeon-Gas1p is exported from the ER prior to the modification of Man2 by EtNP addition, in particular during early timepoints where the Gpi7p-9myc is not detectable by immunoblotting.

**TROUBLESHOOTING**

**Problem 1**

Failure to amplify the knock-in/knock-out cassettes (many non-specific or no PCR products, etc.) (before you begin, steps 1–5).

**Potential solution**

Optimize the annealing temperature (e.g., by using a temperature gradient PCR).

Reduce or titrate the amount of the template in the PCR mix.

**Problem 2**

No transformants using the DMSO-based transformation method (before you begin, steps 6-a and 6-b).

**Potential solution**

Increase the number of yeast cells in the transformation reaction up to $10^8$.

Increase the amount of DNA used in the transformation reaction.

**Problem 3**

No transformants are recovered using the ssDNA-based transformation method (before you begin, steps 6-c and 6-d).

**Potential solution**

Increase the number of yeast cells used up to $10^8$ cells / transformation.

Increase the amount of DNA used in the transformation reaction.

Re-denature and ice-chill the ssDNA.

Increase the time for heat shock. However, this might not be ideal for strains carrying mutations that confer temperature-sensitivity for growth.

If pSH62 was transformed into the cells beforehand, make sure the plasmid has been removed from cells before conducting another transformation using a LoxP-containing selection marker.

**Problem 4**

FM^TM 4–64 dye is internalized into the yeast cells (step-by-step method details, steps 6-b, 7-b and 8-b).
Potential solution
Wash the cells thoroughly with ice-chilled PBS.

Extend the time for incubating the cells with ice-chilled PBS.

Reduce the time for incubating the cells with FM™ 4–64 dye in cold PBS.

Finish the fluorescence microscopy as soon as possible.

Problem 5
No or low expression of Gpi7p-9myc/mNeon-Gas1p (step-by-step method details, step 5).

Potential solution
Wash the cells thoroughly with YEPGal.

Using a preculture in raffinose-rich medium might be helpful to improve the galactose induction.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, David K. Banfield (bodkb@ust.hk).

Materials availability
All strains, plasmids and reagents used in this study are available upon request from the lead contact.

Data and code availability
Data reported in this paper will be shared by the lead contact upon request.

This paper does not report novel code.

Any additional information required to re-analyze the data reported in this paper is available from the lead contact upon request.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101503.

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AUTHOR CONTRIBUTIONS
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DECLARATION OF INTERESTS
The authors declare no competing interests.
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