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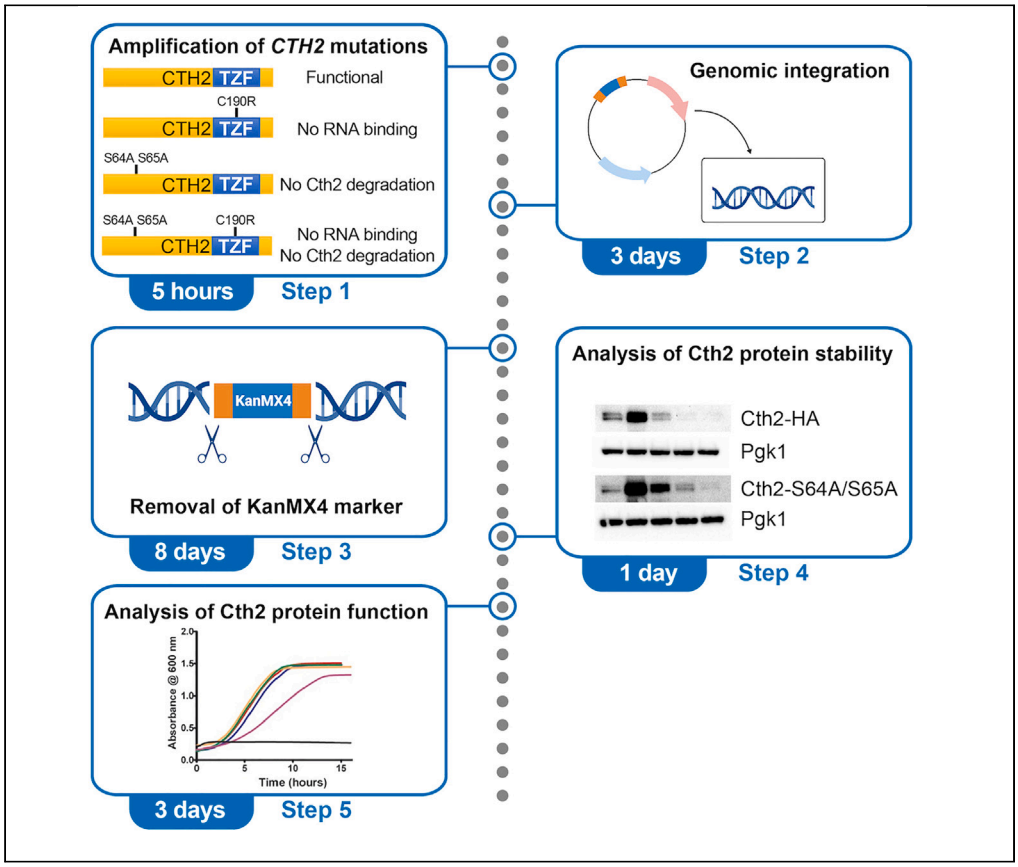
Protocol

Manipulating mRNA-binding protein Cth2 function in budding yeast *Saccharomyces cerevisiae*



Here, we present a protocol for modulating the function of the Cth2 mRNA-binding protein (RBP) in *Saccharomyces cerevisiae*. We describe steps to amplify and integrate mutations in Cth2 that affect its stability and function. Next, we detail the functional assay to verify the activity of the wild-type and mutant versions of Cth2 in yeast cells. This protocol can be adopted to modify the function of other RBPs with their respective functional mutations.

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

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Highlights

A protocol for manipulating the function of the Cth2 mRNA-binding protein in yeast

Integrates mutations in Cth2 that affect its stability and function

Analysis of Cth2 protein stability using western blotting

Quantitative assessment of the RNA-binding protein functionality

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Manipulating mRNA-binding protein Cth2 function in budding yeast *Saccharomyces cerevisiae*

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SUMMARY

Here, we present a protocol for modulating the function of the Cth2 mRNA-binding protein (RBP) in *Saccharomyces cerevisiae*. We describe steps to amplify and integrate mutations in Cth2 that affect its stability and function. Next, we detail the functional assay to verify the activity of the wild-type and mutant versions of Cth2 in yeast cells. This protocol can be adopted to modify the function of other RBPs with their respective functional mutations.

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

BEFORE YOU BEGIN

The regulation of gene expression is a complex and tightly controlled process in eukaryotic organisms, with mRNA-binding proteins playing a pivotal role in post-transcriptional gene regulation.[2] The budding yeast *Saccharomyces cerevisiae* has proven to be a valuable model system for studying these regulatory mechanisms.[3–6] The protocol described in this paper outlines detailed steps for manipulating the function of mRNA-binding proteins in yeast, focusing on the Cth2 protein as a specific example. The protocol details two distinct approaches for manipulating Cth2 protein function in yeast. First, we describe a mutagenesis strategy to disrupt the mRNA-binding function of Cth2 by specifically targeting a conserved cysteine residue (C290R) in its TZF RNA-binding domain.[7] This mutation abrogates mRNA-binding by Cth2 allowing to investigate the downstream effects of impaired RNA binding and its role in post-transcriptional regulation. In addition, activity of Cth2 may be modulated by introducing serine to alanine mutations (S64A/S65A) in the N-terminal domain of Cth2 that are known to increase its protein stability.[8] Importantly, the techniques outlined in this protocol are not limited to Cth2 and can be readily adopted to manipulate the function of other RNA-binding proteins in *S. cerevisiae*. This flexibility makes the protocol a valuable resource for researchers interested in dissecting the post-transcriptional regulatory network in yeast, thereby advancing our understanding of gene expression control in eukaryotic cells.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HA Tag monoclonal antibody (2–2.2.14), HRP (1 mg/mL)	Thermo Fisher Scientific	Cat#26183-HRP
Bacterial and virus strains		
DH5-alpha competent *E. coli* (high efficiency)	New England Biolabs	Cat#C2987H

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Yeast extract	Research Products International	SKU: Y20020
Peptone	Research Products International	SKU: P20250
D-(+)-glucose	Research Products International	SKU: G32040
Glycerol	Research Products International	SKU: G22025-0.5; CAS: 56-81-5
Agar	Sunrise Science Products	Cat#1910-500
CSM-Ura powder	Sunrise Science Products	Cat#1004-010
Yeast nitrogen base	Research Products International	SKU: Y20040
G418 sulfate	Corning	Cat#30-234-CR
Nourseothricin sulfate (NAT)	GoldBio	Cat#N500
Ampicillin, sodium salt	Research Products International	SKU: A40040-5.0
D-(+)-galactose	Research Products International	SKU: G33000
Phusion high-fidelity DNA polymerase	New England Biolabs	Cat#M0530S
10 mM dNTP mix	Thermo Fisher Scientific	Cat#R0191
E.Z.N.A. Gel Extraction Kit	Omega Bio-tek	SKU: D2500-01
NotI-HF restriction enzyme	New England Biolabs	Cat#R3189S
SalI-HF restriction enzyme	New England Biolabs	Cat#R3138S
T4 DNA ligase	New England Biolabs	Cat#M0202S
EZ-10 Spin Column Plasmid DNA Miniprep Kit	Bio Basic	Cat#BS413
Lithium acetate	Thermo Fisher Scientific	Cat#AC297110250
Polyethylene glycol 3000 (PEG 3000)	Sigma-Aldrich	SKU: 89510
Carrier single-stranded DNA (ssDNA) from salmon testes	Sigma-Aldrich	SKU: D7656
Phosphate buffered saline (PBS)	Thermo Fisher Scientific	Cat#10010023
Zymolyase	Zymo Research	Cat#E1006
Bathophenanthrolinedisulfonic acid disodium salt hydrate	Sigma-Aldrich	SKU: B1375
Cycloheximide	Research Products International	SKU: C81040-5.0
10× TBS	Thermo Fisher Scientific	Cat#J62938.K7
Tween 20	Research Products International	SKU: P20370
HEPES (1 M)	Thermo Fisher Scientific	Cat#15630130
KCl	Research Products International	SKU: P41000-500.0; CAS: 7447-40-7
EDTA (0.5 M), pH 8.0	Thermo Fisher Scientific	Cat#AM9261
Nonidet P-40 (NP-40)	Research Products International	SKU: N59000
Glass beads	Sigma-Aldrich	SKU: G8772
Pierce BCA Protein Assay Kits	Thermo Fisher Scientific	Cat#23227
NuPAGE 10%, Bis-Tris Protein gels	Thermo Fisher Scientific	Cat#NP0301
MES buffer	Thermo Fisher Scientific	Cat#NP0060
PVDF transfer membranes, 0.45 µm	Thermo Fisher Scientific	Cat#88518
10× Tris glycine buffer	Bio-Rad	Cat#1610732
Methanol	Thermo Fisher Scientific	Cat#268280025
Pierce ECL western blotting substrate	Thermo Fisher Scientific	Cat#32106
Tris-HCl buffer, 1 M, pH 7.5	Thermo Fisher Scientific	Cat#15567027
$MgCl_2$, 2 M	Sigma-Aldrich	SKU:68475-100ML-F
DTT, 1 M	Thermo Fisher Scientific	Cat#P2325
NAD+, 50 mM	New England Biolabs	CatB9007S
Polyethylene glycol 8,000 (PEG-8000)	Thermo Fisher Scientific	Cat#043443.36
Taq DNA ligase	New England Biolabs	Cat#M0208S
T5 exonuclease	New England Biolabs	Cat#M0663S
BsaI-HFv2	New England Biolabs	Cat#R3733S
Experimental models: Organisms/strains		
BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0)	Horizon Discovery	Cat#YSC1050

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
cth2Δ (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cth2Δ0)	This study	N/A
CTH2 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cth2Δ::CTH2-loxP)	This study	N/A
CTH2-S64A/S65A (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cth2Δ::CTH2-S64A/S65A-loxP)	This study	N/A
CTH2-C190R (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cth2Δ::CTH2-C190R-loxP)	This study	N/A
CTH2-S64A/S65A/C190R (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cth2Δ::CTH2-S64A/S65A/C190R-loxP)	This study	N/A
CTH2-HA (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cth2Δ::CTH2-HA-KanMX4)	This study	N/A
CTH2-S64/AS65A-HA (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 cth2Δ::CTH2-S64A/S65A-HA-KanMX4)	This study	N/A
Oligonucleotides		
oPP017_CTH2_ORF_F	Azenta Life Sciences	TTGCGGCCGCATGTGGGCTCAATTATCATATAC
oPP018_CTH2_ORF_R	Azenta Life Sciences	GCGTCGACTCACCAGGTCATTCTCTGC
oPP019_CTH2_GI_F	Azenta Life Sciences	GCAAACTCAATACGTAAAAAATAACGCAATA TGTGGGCTCAATTATCATATAC
oPP020_CTH2_GI_R	Azenta Life Sciences	AATAATTGAGTTTAGTTGAGACGCCGGTCTTCGCCAG GCCAGGAATTGTTGCATAGGCCACTAGTGGATCTG
oPP378_KanMX_chk_R	Azenta Life Sciences	CTGCAGCGAGGAGCCGTAAT
oPP325_CTH2_chk_R	Azenta Life Sciences	GTAGTGCCGAATGATTGAG
oHB69_CRISPR_gRNA_CTH2 (double-stranded DNA)	Azenta Life Sciences	TTTCGAATAAACACACATAAACAAACAAAGAAGCACTGA TGAGTCCGTGAGGACGAAACGAGTAAGCTCGTCTGCTT CTTTTTCATCAGAGAGTTTTAGAGCTAGAAATAGCAAGTT AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCA CCGAGTCGGTGCTTTTGGCCGGCATGGTCCCAGCCTCC TCGCTGGCGCCGGCTGGGCAACATGCTTCGGCATGGCG AATGGGACACAGGCCCCTTTTCCTTTGTCGATA
oHB70_Repair_cassette_oligo1	Azenta Life Sciences	AGAGAAAGCGAAACAGTGCTGCAAACTCAATAC GTAAAAAATAACGCAATAACAATTCCTGGCCTGG CGAAGACCGGCGTCTCAACTAAACTCAATTATT
oHB71_Repair_cassette_oligo2	Azenta Life Sciences	AATAATTGAGTTTAGTTGAGACGCCGGTCTTCGCC AGGCCAGGAATTGTTATTGCGTTATTTTTTACGTATT GAGTTTGCAGCACTGTTTCGCTTTCTCT
oHB64_GuideRNA_Check_F	Azenta Life Sciences	CGGTAGGTATTGATTGTAATTCTG
oHB65_GuideRNA_Check_R	Azenta Life Sciences	GCGTGAATGTAAGCGTGAC
Recombinant DNA		
Plasmid: pDZ415	Hocine et al.[9]	Addgene; Cat#45162
Plasmid: pSP414	Sergi Puig (Romero et al.[8])	N/A
Plasmid: pSP429	Sergi Puig (Romero et al.[8])	N/A
Plasmid: pSP853	Sergi Puig (Romero et al.[8])	N/A
Plasmid: pSP898	Sergi Puig (Romero et al.[8])	N/A
Plasmid: pSH47	Guldener et al.[10]	Euroscarf; Cat#P30119
Plasmid: pL59-Nat	Barre et al.[11]	Addgene; Cat# 140465
Software and algorithms		
Yeast outgrowth data analyzer (YODA)	Olsen et al.[12]	http://yoda.sageweb.org/
Other		
NanoDrop 2000 spectrophotometer	Thermo Fisher Scientific	ND-2000
ImageQuant LAS 4000 mini biomolecular imager	GE Healthcare	28-9558-10
Epoch2 microplate spectrophotometer	BioTek	EPOCH2TC

MATERIALS AND EQUIPMENT

Cell culture media and solutions

YPD		
Reagent	Final concentration	Amount
Yeast Extract	1% (w/v)	1 g
Peptone	2% (w/v)	2 g
Glucose (20% (w/v))	2% (v/v)	10 mL
ddH_2O	N/A	to 90 mL
Total	**N/A**	**100 mL**

Mix everything except the glucose, bring to a volume of 90 mL with ddH_2O, and autoclave. Separately autoclave the 20% glucose. Add the glucose and store the media at room temperature (20°C–25°C) for up to 6 weeks.

YPG		
Reagent	Final concentration	Amount
Yeast Extract	1% (w/v)	1 g
Peptone	2% (w/v)	2 g
Glycerol (50% (v/v))	3% (v/v)	6 mL
ddH_2O	N/A	to 94 mL
Total	**N/A**	**100 mL**

Mix everything except the glycerol, bring to a volume of 94 mL with ddH_2O, and autoclave. Separately autoclave the 50% glycerol. Add the glycerol and store the media at room temperature (20°C–25°C) for up to 6 weeks.

YPD plate		
Reagent	Final concentration	Amount
Yeast Extract	1% (w/v)	1 g
Peptone	2% (w/v)	2 g
Agar	2% (w/v)	2 g
Glucose (20% (w/v))	2% (v/v)	10 mL
ddH_2O	N/A	to 90 mL
Total	**N/A**	**100 mL**

Mix everything except the glucose, bring to a volume of 90 mL with ddH_2O, and autoclave. Separately autoclave the 20% glucose. Mix everything and pour 25 mL per plate. For G418 selection add G418 at 200 µg/mL final concentration. Store the plates at 4°C for up to 6 weeks.

SC-URA agar plates		
Reagent	Final concentration	Amount
CSM-Ura Powder	N/A	0.072 g
Yeast Nitrogen Base	N/A	0.167 g
Agar	2% (w/v)	2 g
Glucose (20% (w/v))	2% (v/v)	10 mL
ddH_2O	N/A	to 90 mL
Total	**N/A**	**100 mL**

Mix everything except the glucose, bring to a volume of 90 mL with ddH_2O, and autoclave. Separately autoclave the 20% glucose. Mix everything and pour 25 mL per plate. Store the plates at 4°C for up to 6 weeks.

SC-URA 2% galactose media		
Reagent	Final concentration	Amount
CSM-Ura Powder	N/A	0.072 g
Yeast Nitrogen Base	N/A	0.167 g

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Galactose (20% (w/v))	2% (v/v)	10 mL
ddH_2O	N/A	to 90 mL
Total	**N/A**	**100 mL**

Mix everything except the galactose, bring to a volume of 90 mL with ddH_2O, and autoclave. Separately autoclave the 20% galactose. Add the galactose and store the media at room temperature (20°C–25°C) for up to 6 weeks.

1 M lithium acetate (LiAc)		
Reagent	Final concentration	Amount
Lithium Acetate	1 M	659.9 mg
ddH_2O	N/A	to 10 mL
Total	**N/A**	**10 mL**

Dissolve 659.9 mg LiAc in ~8 mL ddH_2O, bring to a volume of 10 mL with ddH_2O. Filter the solution with a 0.22 μm syringe filter, store at room temperature (20°C–25°C).

10× TE buffer		
Reagent	Final concentration	Amount
Tris-HCl (1 M), pH 7.5	100 mM	1 mL
EDTA (0.5 M), pH 8.0	10 mM	0.2 mL
ddH_2O	N/A	to 10 mL
Total	**N/A**	**10 mL**

Filter the solution with a 0.22 μm syringe filter, store at room temperature (20°C–25°C).

TBST buffer		
Reagent	Final concentration	Amount
10× TBS	1 X	50 mL
Tween 20	0.1% (v/v)	500 μL
ddH_2O	N/A	449.5 mL
Total	**N/A**	**500 mL**

Store the solution at room temperature (20°C–25°C) for up to 2 months.

Tris glycine transfer buffer		
Reagent	Final concentration	Amount
10× Tris Glycine buffer	1 X	50 mL
Methanol	20% (v/v)	100 mL
ddH_2O	N/A	350 mL
Total	**N/A**	**500 mL**

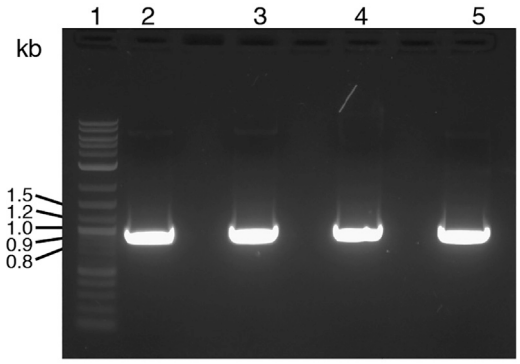
Store the solution at room temperature (20°C–25°C) for up to 2 months.

STEP-BY-STEP METHOD DETAILS

Amplification of *CTH2-C190R*, *CTH2-S64A/S65A*, and *CTH2-S64A/S65A/C190R* sequences

Timing: 5 h

In this section, the process of generating plasmids containing *CTH2*, *CTH2-C190R*, *CTH2-S64A/S65A*, and *CTH2-S64A/S65A/C190R* sequences is described. PCR amplified *CTH2* and *CTH2* with



1. Ladder
2. CTH2
3. CTH2-C190R
4. CTH2-S64A/S65A
5. CTH2-S64A/S65A/C190R

Figure 1. Representative agarose gel of PCR products containing *CTH2* and *CTH2* with mutations, which were digested with NotI and SalI restriction enzymes

mutations are then cloned into pDZ415 [9] by using NotI and SalI restriction sites upstream of loxP-KanMX-loxP.

1. Amplify sequences for *CTH2*, *CTH2-C190R*, *CTH2-S64A/S65A*, and *CTH2-S64A/S65A/C190R* from plasmids pSP414, pSP429, PSP853, and pSP898, respectively.
   a. Setup the reaction mixture for PCR amplification of the target gene fragments as indicated below.

Reagent	Amount
Template DNA (up to 10 ng)	1 μL
Phusion DNA Polymerase	0.5 μL
10 μM Forward Primer oPP017	2.5 μL
10 μM Reverse Primer oPP018	2.5 μL
5× Phusion HF Buffer	10 μL
10 mM dNTPs	1 μL
ddH_2O	32.5 μL

   b. Perform the PCR amplification using the following thermocycler parameters:

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	25–35 cycles
Annealing	59°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	10 min	1
Hold	4°C	forever	

Note: The extension time is dependent on the length of DNA fragments. An extension time of 30 seconds per kb is recommended.

   c. Load 10 μL of each PCR sample into 1 well of the 1% TAE agarose gel.
   d. Run the gel at 200 V for 15 min.

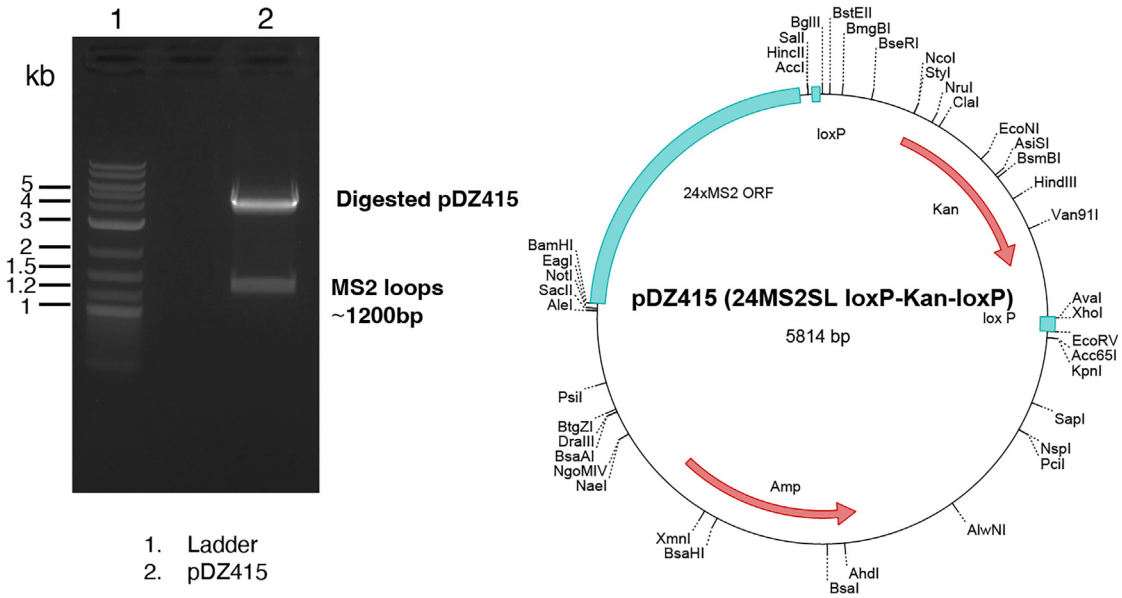


Figure 2. Digestion of the pDZ415 vector with NotI and SalI restriction enzymes releases the MS2 loops insert
The map of the pDZ415 vector is shown on the right.

e. Purify DNA fragments using E.Z.N.A. Gel Extraction Kit (Omega Bio-tek) according to the manufacturer's protocol.
f. Quantify the concentration of DNA fragments using Nanodrop by measuring the absorbance at 260 nm.

2. Perform restriction enzyme digestion of the PCR product and pDZ415 vector.
   a. Set up restriction enzyme digestion reactions separately for the PCR product and the pDZ415 vector with NotI and SalI restriction enzymes as indicated below.

Reagent	Amount
PCR amplified product or pDZ415 vector (1 μg)	X μL
10× NEB CutSmart Buffer	2 μL
NotI Restriction Enzyme	1 μL
SalI Restriction Enzyme	1 μL
ddH_2O	(16 μL–X μL)

Note: The total volume of each restriction enzyme digestion reaction is 20 μL.

b. Incubate at 37°C for 1 h.
c. Load 10 μL of each PCR sample into 1 well of the 1% TAE agarose gel.
d. Run the gel at 200 V for 15 min (Figure 1).
e. Purify the digested DNA fragments and pDZ415 vector using E.Z.N.A. Gel Extraction Kit (Omega Bio-tek) according to the manufacturer's protocol.
f. Quantify the concentration of the digested DNA fragments and pDZ415 vector using Nanodrop by measuring the absorbance at 260 nm.

Note: The pDZ415 plasmid contains an insert (24× MS2 stem-loops) between NotI and SalI cleavage sites, which will be released upon the restriction enzyme digestion (Figure 2).

3. Set up the DNA ligation reaction for the gel-purified digested DNA fragments and pDZ415 vector as indicated below.

Reagent	Amount
Gel-purified digested pDZ415 vector	25 ng
Gel-purified digested PCR product	18.75 ng
10× T4 DNA Ligase Buffer	1 μL
T4 DNA ligase	0.5 μL
ddH_2O	to 10 μL

a. Incubate at room temperature (20°C–25°C) for 1 h.
b. Heat inactivate at 65°C for 10 min.

4. Use 1 μL of the ligation reaction mixture for transformation of the DH5α *E. coli* competent cells.
   a. Thaw 20 μL of the DH5α *E. coli* competent cells on ice.
   b. Add 1 μL of the ligation reaction mixture.

   Note: Do not use pipetting to mix the ligation product with competent cells, carefully tap the tube several times to mix.

   c. Incubate on ice for 30 min.
   d. Heat-shock the tube at 42°C for 30 s.
   e. Place the tube on ice for 5 min.
   f. Add 1 mL SOC outgrowth medium (supplied with competent cells) to the tube.
   g. Incubate the cells at 37°C in a shaker incubator for 1 h with shaking at 220 rpm.
   h. Spread 50–100 μL of cells on the LB-agar plate supplemented with 100 μg/mL ampicillin.
   i. Incubate the plates at 37°C overnight (16–24 h).
   j. Pick a single colony with a sterile pipette tip and place the tip into 4 mL LB medium supplemented with 100 μg/mL ampicillin.
   k. Incubate at 37°C with shaking at 220 rpm overnight (16–24 h).
   l. Extract the plasmid from the cells using an EZ-10 Spin Column Plasmid DNA Miniprep Kit according to the manufacturer's protocol.
   m. Verify the sequence of the plasmid by Sanger sequencing using M13 Forward primer.
   n. Store the plasmid at −20°C for the subsequent experiments.

⚠ CRITICAL: Verify the correct DNA sequence of the target gene and that it contains the desired mutations. The sequencing reaction using M13 Forward primer will cover the whole *CTH2* gene containing the mutated regions.

Pause point: The plasmid DNA may be kept long-term at −20°C.

Genomic integration of Cth2 mutations

Timing: 3 days

In this step, *CTH2* and *CTH2* with mutations along with loxP-KanMX-loxP are amplified using the primers flanked on the 5′ side by sequence homologous to the *CTH2* ORF and on the 3′ side by sequence homologous to the *CTH2* 3′-UTR and integrated into the genome using homologous recombination.

5. Amplify sequences for *CTH2*, *CTH2-C190R*, *CTH2-S64A/S65A*, and *CTH2-S64A/S65A/C190R* along with loxP-KanMX-loxP using the primers flanked on the 5′ side by sequence homologous to *CTH2* ORF (oPP019) and on the 3′ side by sequence homologous to *CTH2* 3′-UTR (oPP020) (Figure 3).
   a. Setup the reaction mixture for PCR amplification of the target gene fragments as indicated below.

Reagent	Amount
Template DNA (up to 10 ng)	1 μL
Phusion DNA Polymerase	0.5 μL
10 μM Forward Primer oPP019	2.5 μL
10 μM Reverse Primer oPP020	2.5 μL
5× Phusion HF Buffer	10 μL
10 mM dNTPs	1 μL
ddH_2O	32.5 μL

b. Perform the PCR amplification using the following thermocycler parameters:

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	25–35 cycles
Annealing	59°C	30 s	
Extension	72°C	1 min 30 s	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

c. Run 10 μL of the PCR sample on a 1% TAE agarose gel to verify the correct size of the PCR product.

6. Transform the PCR product into BY4741 yeast cells.
   a. Inoculate a single colony from freshly streaked BY4741 strain into 4 mL of YPD media.
   b. Incubate overnight (16–24 h) at 30°C with shaking at 220 rpm.
   c. Next day dilute cells to 0.1 OD_{600} units/mL in 10 mL of fresh YPD.
   d. Incubate at 30°C for 5–6 h.
   e. Spin down cells by centrifuging at 3,000 × *g* for 5 min at room temperature (20°C–25°C).
   f. Remove the media and wash the cell pellet with 10 mL of autoclaved water.
   g. Resuspend the cells in 1 mL of autoclaved water and transfer the cells to a 1.6 mL Eppendorf tube.
   h. Spin down cells by centrifuging at 3,000 × *g* for 5 min at room temperature (20°C–25°C).
   i. Remove the supernatant and wash the cells in 1 mL of 0.1 M Lithium Acetate (LiAc).
   j. Spin down cells by centrifuging at 3,000 × *g* for 5 min at room temperature (20°C–25°C).
   k. Remove as much liquid as possible and resuspend the cells in 200 μL of 0.1 M LiAc.
   l. Aliquot 50 μL of cells for each transformation.
   m. Add the transformation mixture along with 15 μL of the PCR product.

Reagent	Amount
Cells	50 μL
PCR product	15 μL
50% PEG 3000 (w/v)	300 μL
10 mg/mL ssDNA	7 μL
1 M LiAc	40 μL
10× TE buffer	40 μL

Note: For the negative control avoid adding the PCR product.

   n. Vortex 2 min.
   o. Incubate the cells at 30°C for 30 min with shaking at 220 rpm.
   p. Heat-shock the tube at 42°C for 15 min.
   q. Centrifuge the transformation mixture at 5,000 × *g* for 10 s.
   r. Remove the supernatant.
   s. Resuspend the cells in 1 mL of YPD medium.
   t. Incubate for 3–4 h in the 30°C incubator with shaking at 220 rpm.

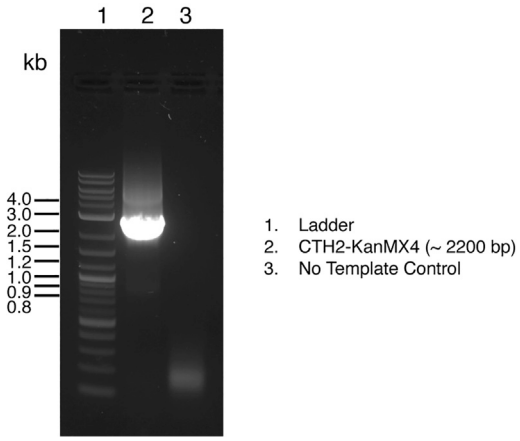


Figure 3. CTH2-KanMX4 is PCR amplified using Phusion polymerase to genome-integrate the mutated versions of *CTH2*

u. Centrifuge the cells at 5,000 × *g* for 1 min at room temperature (20°C–25°C).
v. Remove the supernatant.
w. Resuspend cells in 100 μL of autoclaved water and plate them on YPD agar plates supplemented with 500 μg/mL G418 sulfate.
x. Incubate the plates at 30°C for 2–3 days.

7. Verify the proper integration of the *CTH2* and *CTH2* with mutations into the yeast genome.
   a. Patch out about 10 individual yeast colonies using a sterile tip or a toothpick on to a fresh YPD plate supplemented with 200 μg/mL G418 sulfate.
   b. Incubate at 30°C for 1 day.

Note: The patches can be kept at 4°C until the sequences are confirmed.

   c. Swirl yeast cells from a patch using sterile tip into the solution containing 5 μL 1× PBS and 0.2 μL of zymolyase (5 U/μL)
   d. Incubate the cells at 37°C for 20 min.
   e. Heat the lysate at 95°C for 5 min.
   f. Dilute the lysate with 50 μL water (mix the solution by pipetting).
   g. Analyze 1 μL of the lysate by PCR.
   h. Amplify *CTH2*-KanMX4 by PCR with Phusion DNA polymerase using the primers specific to *CTH2* ORF (oPP017) and KanMX4 (oPP378).

Reagent	Amount
Template DNA	1 μL
Phusion DNA Polymerase	0.5 μL
10 μM Forward Primer oPP017	2.5 μL
10 μM Reverse Primer oPP378	2.5 μL
5× Phusion HF Buffer	10 μL
10 mM dNTPs	1 μL
ddH_2O	32.6 μL

   i. Perform the PCR amplification using the following thermocycler parameters:

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	25–35 cycles
Annealing	59°C	30 s	
Extension	72°C	1 min 30 s	

(*Continued on next page*)

Continued

Steps	Temperature	Time	Cycles
Final extension	72°C	10 min	1
Hold	4°C	forever	

j. Check for positive colonies by running the PCR products on a 1% TAE agarose gel.
k. Inoculate the confirmed colony into 4 mL of YPD media containing 200 μg/mL G418 sulfate.
l. Incubate the cells at 30°C overnight (16–24 h) with shaking at 220 rpm.
m. Prepare glycerol stocks for the verified strains by adding 700 μL of overnight (16–24 h) culture to 300 μL of 50% glycerol.
n. Transfer the tubes to −80°C.

⚠ CRITICAL: Verify the DNA sequences using Sanger sequencing.

Pause point: Verified strains may be stored long-term at −80°C.

Removal of KanMX4 marker

Timing: 8 days

In this step, the KanMX4 cassette flanked with loxP sites is removed by transforming yeast cells with the pSH47 plasmid encoding galactose-inducible Cre recombinase.[10]

8. Remove the KanMX4 cassette via Cre-mediated recombination.
   a. Perform transformation of the yeast cells containing genome-integrated *CTH2*, *CTH2-C190R*, *CTH2-S64A/S65A*, and *CTH2-S64A/S65A/C190R* along with loxP-KanMX-loxP with the pSH47 plasmid as described in Steps (6.a-6.p)
   b. Centrifuge the cells at 5,000 × *g* for 1 min at room temperature (20°C–25°C).
   c. Remove the supernatant.
   d. Resuspend cells in 100 μL of autoclaved water and plate them on SC-URA agar plates for the selection of colonies transformed with the pSH47 plasmid harboring Cre recombinase.
   e. Incubate the plates at 30°C for 2–3 days.
   f. Inoculate single yeast colonies in SC-URA media containing 2% galactose.
   g. Grow the cells at 30°C overnight (16–24 h) to induce the expression of Cre recombinase, which is under the control of the *GAL1* promoter.
   h. Mix 10 μL of the overnight (16–24 h) culture with 90 μL of autoclaved water and plate the cells onto a YPD plate.
   i. Incubate the plate at 30°C for 2 days.
   j. Patch out about 5 individual yeast colonies onto a fresh YPD plate.
   k. Incubate at 30°C for 1 day.
   l. Replica plate each colony onto a YPD plate and a YPD plate supplemented with 200 μg/mL of G418 sulfate.
   m. Incubate at 30°C for 2 days (Figure 4).
   n. Select the colonies that did not grow on YPD + G418 sulfate plates and prepare glycerol stocks as described in Step (7.m).

Note: Removal of KanMX4 cassette can be further confirmed by PCR using oPP017 and oPP325 primers. Colonies lacking KanMX4 cassette produce a PCR product of a smaller size (~1.3 kb smaller) compared to cells harboring KanMX4 cassette (Figure 5).

Pause point: Verified strains may be stored long-term at −80°C.

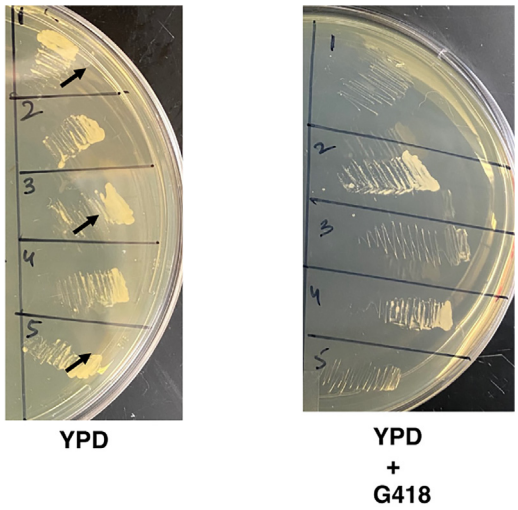


Figure 4. After inducing Cre recombinase by growing cells in SC-URA media containing 2% galactose, cells are plated on YPD plates

Subsequently, 5 individual colonies are replica plated onto a YPD plate and a YPD plate supplemented with 200 μg/mL of G418 sulfate to identify colonies lacking the KanMX4 marker. Positive colonies that have lost the KanMX4 cassette are unable to grow on G418 selection plates (indicated with an arrow).

Optional: The pSH47 plasmid possessing Cre recombinase can be cured from yeast strains containing genome-integrated *CTH2* and *CTH2* with mutations.

9. Remove pSH47 plasmid.
   a. Inoculate yeast cells that lost KanMX4 cassette into 3 mL of YPD.
   b. Incubate at 30°C for 1 day with shaking at 220 rpm.
   c. Dilute 5 μL of overnight (16–24 h) culture into 3 mL of YPD media.
   d. Incubate at 30°C for 1 day with shaking at 220 rpm.
   e. Dilute 5 μL of overnight (16–24 h) culture into 3 mL of YPD media.
   f. Incubate at 30°C for 1 day with shaking at 220 rpm.
   g. Dilute 5 μL of overnight (16–24 h) culture into 100 μL autoclaved water and plate 10 μL onto a YPD plate.
   h. Incubate at 30°C for 2–3 days.
   i. Patch out about 5 single colonies onto a fresh YPD plate and incubate for 1 day at 30°C.
   j. Replica plate several colonies from the patches onto YPD and SC-URA plates and incubate at 30°C for 1 day.
   k. Pick yeast colonies that do not grow on SC-URA plates from YPD plate and grow overnight (16–24 h) in 4 mL YPD media.
   l. Prepare glycerol stocks as described in Step (7.m).

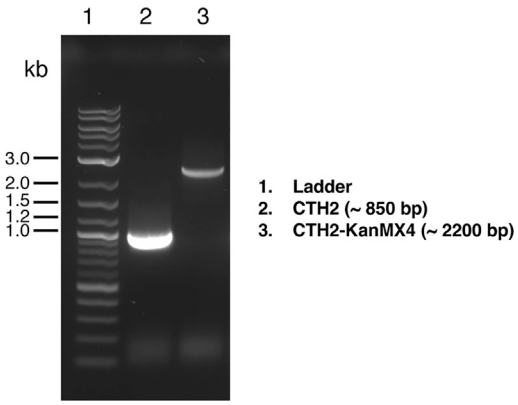


Figure 5. Representative agarose gel of PCR products amplified with CTH2 ORF Forward (oPP017) and CTH2 3′UTR Reverse (oPP325) primers

Colonies lacking KanMX4 cassette produce a PCR product of a smaller size (~1.3 kb smaller) compared to cells harboring KanMX4 cassette.

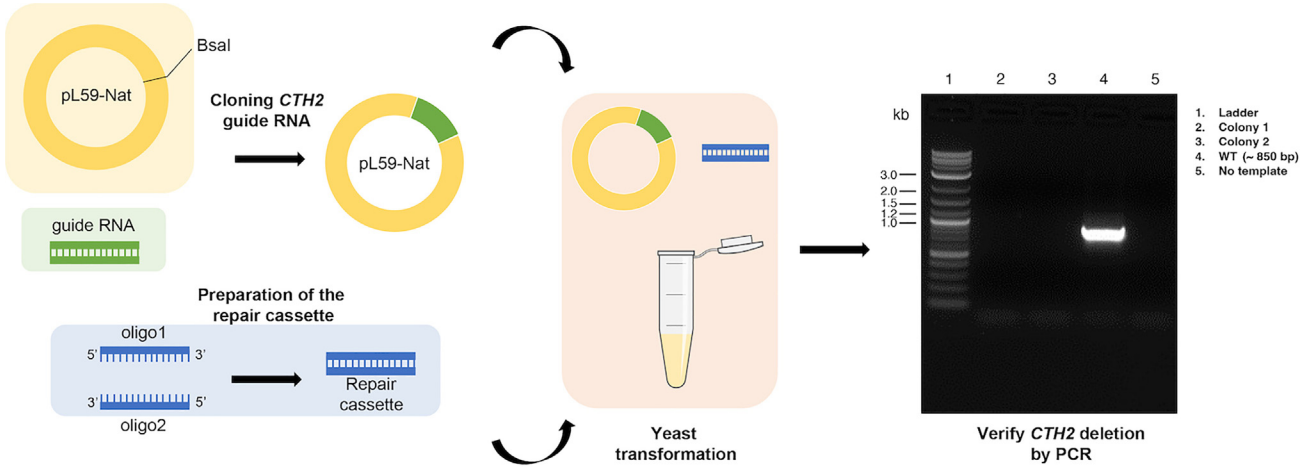


Figure 6. Scheme illustrating the markerless deletion of *CTH2* using CRISPR-Cas9

Note: Following the removal of the pSH47 plasmid, KanMX4 selection marker can be re-used for subsequent integration of the C-terminal HA-tag into the yeast strains containing genome-integrated *CTH2* and *CTH2* with mutations for analysis of the Cth2 protein stability. For this, sequences encoding HA-tag can be amplified and integrated into the yeast genome using the same protocol described in Steps (1–8).

Optional: As an alternative to integrating *CHT2* mutations using the loxP protocol described above, CRISPR-Cas9 system can be used to introduce genetic alterations into the yeast genome, including point mutations, markerless deletions, integration of the HA-tag or other tags into endogenous genes. As an example, here we describe detailed steps for markerless deletion of *CTH2* using CRISPR-Cas9 protocol originally described in Barre et al.[11] The same strategy can be applied to introduce *CTH2-C190R*, *CTH2-S64A/S65A*, and *CTH2-S64A/S65A/C190R* mutations for manipulating the function of Cth2 in *S. cerevisiae*.

10. Markerless deletion of *CTH2* using CRISPR-Cas9 (Figure 6).
    a. Clone *CTH2* guide RNA into the pL59-Nat plasmid containing CRISPR-Cas9 using Gibson Assembly Cloning.
        i. Perform digestion of the pL59-Nat vector using the BsaI-HF restriction as indicated below.

Reagent	Amount
BsaI-HF	1 μL
rCutSmart Buffer	2 μL
Vector (1 μg of DNA)	X μL
ddH_2O	(17 μL – X μL)

Note: The total volume of the restriction enzyme digestion reaction is 20 μL.

        ii. Incubate the reaction at 37°C for 1 h.
        iii. Load 20 μL of the digested pL59-Nat vector into 1 well of the 1% TAE agarose gel.
        iv. Run the gel at 200 V for 15 min.
        v. Purify the digested pL59-Nat vector using E.Z.N.A. Gel Extraction Kit (Omega Bio-tek) according to the manufacturer's protocol.
        vi. Prepare 2.5 x Isothermal (ISO) Reaction Buffer.

Reagent	Final conc.
Tris-HCl 7.5	250 mM
$MgCl_2$	25 mM
DTT	25 mM
NAD+	2.5 mM
dNTPs	2 mM
PEG-8000	12.5% (w/v)

vii. Prepare 1.33 x Gibson Master Mix.

Reagent	Amount
Taq DNA Ligase (40 u/μL)	50 μL
T5 exonuclease (10 u/μL)	0.2 μL
Phusion polymerase (2 u/μL)	6.25 μL
2.5 x ISO buffer	200 μL
ddH_2O	118.55 μL

viii. Set up the Gibson Assembly Cloning reaction using the linearized vector and *CTH2* guide RNA (oHB69).

Note: For optimized cloning efficiency use 50 ng of vector with 3-fold molar excess of the guide RNA. To calculate X (vector) and Y (guide RNA) use the following formulas: X μL = 50 ng / [vector] ng/μL; Y μL = 4.2 ng / [guide RNA] ng/μL.

Reagent	Amount	Final concentration
1.33 x Gibson Master Mix	15 μL	1×
Vector	X μL	50 ng
Guide RNA	Y μL	4.2 ng (1:3 M ratio)
ddH_2O	(5 μL – (X + Y) μL)	
Total volume	20 μL	

ix. Incubate at 50°C for 1 h.
x. Use 1 μL of the Gibson Assembly reaction mixture to perform transformation of the DH5α *E. coli* competent cells as described in Steps (4.a-4.k).
xi. Extract the plasmid from the cells using an EZ-10 Spin Column Plasmid DNA Miniprep Kit according to the manufacturer's protocol.
xii. Confirm the insertion of the sequence encoding guide RNA into the pL59-Nat vector by performing PCR using oHB64_GuideRNA_Check_F and oHB65_GuideRNA_Check_R primers and analyzing the size of the PCR products on 1% TAE agarose gel.

Note: The expected PCR product size: Empty plasmid = 211 bp; guide RNA insert = 401 bp.

xiii. Verify the sequence of the plasmid by Sanger sequencing using oHB64_GuideRNA_Check_F primer.
xiv. Store the plasmid at −20°C.

Pause point: The plasmid DNA may be kept long-term at −20°C.

b. Prepare the Repair Cassette.
i. Dilute oHB70 and oHB71 oligos in nuclease free water to the final concentration of 100 μM.
ii. Add 5 μL of each oligo and 90 μL of ddH_2O in a PCR tube and mix.

Reagent	Amount
oHB70_Repair_cassette_oligo1 (100 μM)	5 μL
oHB71_Repair_cassette_oligo2 (100 μM)	5 μL
ddH_2O	90 μL

iii. Incubate the tube 10 min at 95°C and cool down at room temperature (20°C–25°C) for 10 min (at a rate ~0.1 °C/s) to anneal the Repair Cassette oligos using a heat block or PCR thermocycler.

c. Transform the Repair Cassette and pL59-Nat plasmid containing CTH2 guide RNA into BY4741 yeast cells.
   i. Inoculate a single colony from freshly streaked BY4741 strain into 4 mL of YPD media.
   ii. Incubate overnight (16–24 h) at 30°C with shaking at 220 rpm.
   iii. Next day dilute cells to 0.1 OD_{600} units/mL in 10 mL of fresh YPD.
   iv. Incubate at 30°C for 5–6 h.
   v. Spin down cells by centrifuging at 3,000 × *g* for 5 min at room temperature (20°C–25°C).
   vi. Remove the media and wash the cell pellet with 10 mL of autoclaved water.
   vii. Resuspend the cells in 1 mL of autoclaved water and transfer the cells to a 1.6 mL Eppendorf tube.
   viii. Spin down cells at 3,000 × *g* for 5 min at room temperature (20°C–25°C).
   ix. Remove the supernatant.
   x. Wash the cells in 1 mL of 0.1 M LiAc.
   xi. Spin down cells at 3,000 × *g* for 5 min at room temperature (20°C–25°C), remove as much liquid as possible.
   xii. Add the transformation mixture along with the Repair Cassette and pL59-Nat plasmid containing CTH2 guide RNA as follows.

Reagent	Amount
50% PEG 3000 (w/v)	240 μL
1 M LiAc	36 μL
Repair Cassette	50 μL
pL59NAT-CTH2 gRNA (500 ng-1 μg)	15 μL
10 mg/mL ssDNA	5 μL

   xiii. Vortex 2 min.
   xiv. Incubate the cells at 30°C for 30 min with shaking at 220 rpm.
   xv. Heat-shock the tube at 42°C for 15 min.
   xvi. Centrifuge the transformation mixture at 5,000 × *g* for 10 s.
   xvii. Remove the supernatant.
   xviii. Resuspend the cells in 1 mL of YPD medium.
   xix. Incubate for 3–4 h in the 30°C incubator with shaking at 220 rpm.
   xx. Centrifuge the cells at 5,000 × *g* for 1 min at room temperature (20°C–25°C).
   xxi. Remove the supernatant.
   xxii. Resuspend cells in 100 μL of autoclaved water and plate them on YPD agar plates supplemented with 100 μg/mL nourseothricin (NAT).
   xxiii. Incubate the plates at 30°C for 2–3 days.
   xxiv. Verify the *CTH2* deletion by PCR with oPP017_CTH2_ORF_F and oPP018_CTH2_ORF_R primers.
   xxv. Prepare glycerol stocks for the verified strains as described in Step (7.m).
   xxvi. Transfer the tubes to −80°C.

Pause point: Verified strains may be stored long-term at −80°C.

Analysis of Cth2 protein stability

Timing: 1 day

In this step, the effect of Cth2 mutations on protein stability is analyzed using Western blotting. To analyze Cth2 protein stability, yeast cells are grown in Fe-replete and Fe-deficient conditions for 6 h followed by inhibition of protein translation using cycloheximide (CHX).

11. Cell harvesting.
    a. Streak out yeast strains expressing *CTH2-HA* and *CTH2-S64A/S65A-HA* from glycerol stocks stored at −80°C freezer onto a fresh YPD plate.
    b. Incubate at 30°C for 2–3 days.
    c. Inoculate a single colony of *CTH2-HA* and *CTH2-S64A/S65A-HA* expressing yeast cells into YPD liquid media.
    d. Grow overnight (16–24 h) at 30°C in a shaker incubator with 220 rpm.
    e. Measure the absorbance of the overnight (16–24 h) culture and dilute cells to 0.1 OD_{600} units/mL in 50 mL fresh YPD media.
    f. Grow at 30°C in a shaker incubator with 220 rpm.

    Note: Harvest 5 mL of cells before adding the Fe^{2+} chelator bathophenanthrolinedisulfonic acid (BPS) and flash freeze the pellet, which serves as Fe-replete sample.

    g. When the cells reach 0.3–0.4 O.D units/mL density, add BPS to a final concentration of 100 μM.
    h. Incubate at 30°C in a shaker incubator with 220 rpm for 6 h.
    i. Following incubation with BPS, stop protein translation by adding cycloheximide (CHX) to a final concentration of 50 μg/mL.
    j. Harvest cells at 10 min, 30 min and 60 min by centrifuging the tubes at 3,000 × *g* for 5 min at 4°C.
    k. Flash freeze the pellet.
12. Preparation of protein lysates and Western blotting.
    a. Add 200 μL of lysis buffer (50 mM HEPES, 150 mM KCl, 1 mM EDTA pH 8.0, 10% glycerol, 0.1% NP-40) to the cell pellets.
    b. Mix by pipetting and transfer to a new 1.6 mL Eppendorf tube containing 0.2 g of glass beads.
    c. Lyse cells by vortexing the tubes for seven 30 s cycles.

    Note: Keep the tubes on ice for 30 sec after each cycle.

    d. Spin down the tubes at 10,000 × *g* for 5 min at 4°C.
    e. Transfer the supernatant to a new 1.6 mL Eppendorf tube.
    f. Measure the protein concentration using the BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer's protocol.
    g. Load 10 μg of each protein sample into 1 well of the 10% NuPAGE Bis-Tris gel.
    h. Run the gel at 200 V for 35 min using MES buffer.

    Note: Run a pre-stained molecular weight marker for assessing the size off the proteins in a separate well.

    i. Transfer the proteins onto a PVDF membrane at 50 V for 2 h at room temperature (20°C–25°C) using Tris Glycine Transfer Buffer containing 20% (v/v) methanol according to the manufacturer's instructions.
    j. Block the membrane for 1 h using 5% w/v nonfat dry milk in TBST buffer.

k. Incubate the membrane with horseradish peroxidase (HRP)-conjugated anti HA antibody (1:1,000 dilution) in TBST buffer containing 5% w/v nonfat dry milk for 1–2 h at room temperature (20°C–25°C).
l. Wash the membrane three times (15 min each wash) with TBST buffer.
m. Develop the blot using Pierce ECL Western Blotting Substrate according to the manufacturer's protocol.
n. Take images with an ImageQuant LAS 4000 Mini Biomolecular Imager (GE Healthcare).

Analysis of the Cth2 protein function

Timing: 3 days

Because many of the Cth2 targets include mRNA encoding for mitochondrial proteins and components of the mitochondrial electron transport chain, increased activity of Cth2 during Fe deficiency leads to inability to grow on non-fermentable carbon sources that require respiration.[13] In this step, activity of the Cth2 and Cth2 with mutations is quantified using analyses of their growth rate on YPG media containing glycerol as a carbon source.

13. Growth rate analyses.
    a. Streak out *CTH2*, *CTH2-S64A/S65A*, *CTH2-C190R*, and *CTH2-S64A/S65A/C190R* expressing yeast cells from glycerol stocks stored at −80°C freezer onto a fresh YPD plate.
    b. Incubate at 30°C for 2–3 days.
    c. Inoculate a single colony into 3 mL YPD liquid media.
    d. Grow overnight (16–24 h) at 30°C in a shaker incubator with 220 rpm.
    e. Measure the absorbance and dilute cells to 1 OD_{600} units/mL in 1 mL fresh YP media without any carbon source.
    f. Grow at 30°C in a shaker incubator with 220 rpm.
    g. Take a 96 well plate and add 175 μL of YPD or YPG in triplicates for each strain.
    h. Transfer 1.25 μL of the diluted cells into each well.
    i. Measure the absorbance at 600 nm every 30 min for 72 h using Epoch2 Microplate Spectrophotometer at 30°C with shaking.
    j. Use the obtained OD_{600} values to calculate the doubling time using the Yeast Outgrowth Data Analyzer (YODA) software.[12]

EXPECTED OUTCOMES

The successful outcome of this protocol is the ability to manipulate the Cth2 mRNA-binding protein function in yeast. Characterization of the yeast cells expressing *CTH2-C190R*, *CTH2-S64A/S65A*, and *CTH2-S64A/S65A/C190R* mutants using analysis of Cth2 protein stability and function, and representative results are shown in Figures 7 and 8. First, Western blot analysis of the Cth2 protein levels (Figure 7) demonstrated that mutation of the conserved serine residues within the N-terminal domain of Cth2 (S64A/S65A) results in significant increase of the Cth2 protein levels compared to wild-type Cth2, consistent with a previously published study.[8] We have further confirmed the functionality of the serine mutants of Cth2 by quantifying the growth of cells expressing *CTH2-C190R*, *CTH2-S64A/S65A*, and *CTH2-S64A/S65A/C190R* mutants in YPG medium containing glycerol as a carbon source, which requires respiration (Figure 8). We found that the population doubling time of cells expressing the *CTH2-S64A/S65A* (gain of function) mutant was significantly increased ($p < 0.001$) in YPG medium compared with *cth2Δ* and *CTH2-C190R* mutants. Furthermore, combining the C190R and S64A/S65A mutations completely prevented this effect.

LIMITATIONS

In this protocol, *CHT2* mutations are genomically integrated using the loxP system.[10] After curing the selection marker cassette, Cre recombinase leaves a loxP site downstream of the stop codon.

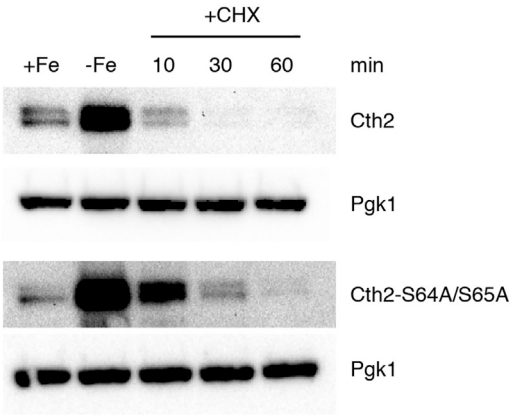


Figure 7. Mutation of S64 and S65 residues increases Cth2 protein stability

Yeast cells expressing HA-tagged Cth2 and Cth2-S64A/S65A were grown in SC media containing 100 μM BPS (-Fe) for 6 h. Following incubation with BPS, 50 μg/mL cycloheximide (CHX) was added to cease translation and aliquots were collected at the indicated times. Total proteins were extracted and Cth2 protein levels were determined by Western blot analysis with HA-tag antibody. Pgk1 was used as an internal control for protein loading.

Even though it is less than 50 bp in length, genes which have signal sequences within close distance to the stop codon cannot be mutated using the loxP system. As an alternative, we provide a detailed protocol for using the CRISPR-Cas9 strategy that allows seamless integration of genetic alterations into the yeast genome.

TROUBLESHOOTING

Problem 1

No PCR product observed (steps 1, 5, 7, and 10).

Potential solution

Annealing temperatures may vary for different primer combinations. Run a gradient PCR by setting up different annealing temperatures to determine the optimum one.

Problem 2

Restriction sites used for cloning are present within the target gene (step 2).

Potential solution

Here we used NotI and SalI restriction sites to digest the Cth2 PCR product for cloning. If these restriction sites are present within the gene of interest, alternate restriction sites present in the pDZ415 plasmid can be used for cloning.

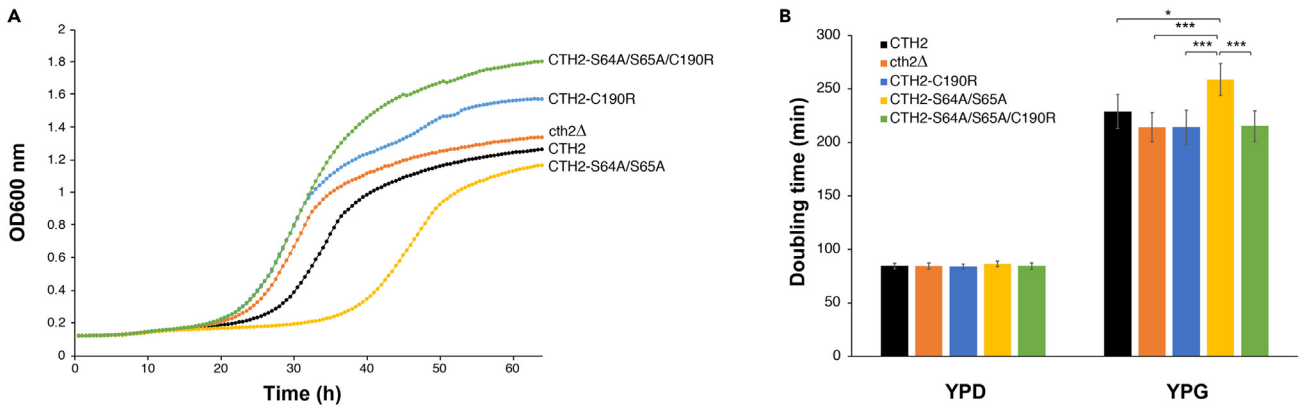


Figure 8. Mutation of the conserved Cys residue within the TZF domain of Cth2 to Arg (C190R) is sufficient to inhibit Cth2 protein function

(A) Growth of yeast cells expressing mutated versions of Cth2 on YPG media containing glycerol. (B) Doubling times in YPD and non-fermentable YPG media were calculated using Yeast Outgrowth Data Analysis (YODA) software. Error bars represent SEM of three biological replicates. *$p < 0.05$ and ***$p < 0.001$ (two-way ANOVA).

Problem 3
No yeast colonies obtained after transformation (steps 6, 8, and 10).

Potential solution
This could be because yeast cells entered stationary phase before transformation. Utilize yeast cells in log phase cultivated to no more than OD_{600} of 1 per mL. Use at least 10 OD units of yeast cells per each transformation.

Problem 4
No yeast growth in SC-URA media containing 2% galactose during overnight (16–24 h) incubation (step 8.g).

Potential solution
Yeast cells may exhibit a slower growth rate in a medium containing galactose. Incubate yeast cells for a longer period (36–48 h).

Problem 5
No signal detected for the protein after Western blot (step 12).

Potential solution
N-terminal and C-terminal tags have the potential to impact protein stability. Determining the optimal tagging position in the protein is typically explored through empirical testing. It is recommended to use a commercial antibody if available for the protein of interest.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Vyacheslav M. Labunskyy (vlabuns@bu.edu).

Technical contact
Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Praveen K. Patnaik (patnaik@bu.edu).

Materials availability
The yeast strains generated by this study can be requested by contacting the lead contact.

Data and code availability
This study did not generate new unique datasets or code.

ACKNOWLEDGMENTS
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AUTHOR CONTRIBUTIONS
P.K.P., H.B., and V.M.L. drafted the manuscript. P.K.P. and H.B. performed the experiments. P.K.P., H.B., and V.M.L. revised the manuscript. V.M.L. supervised the study.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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