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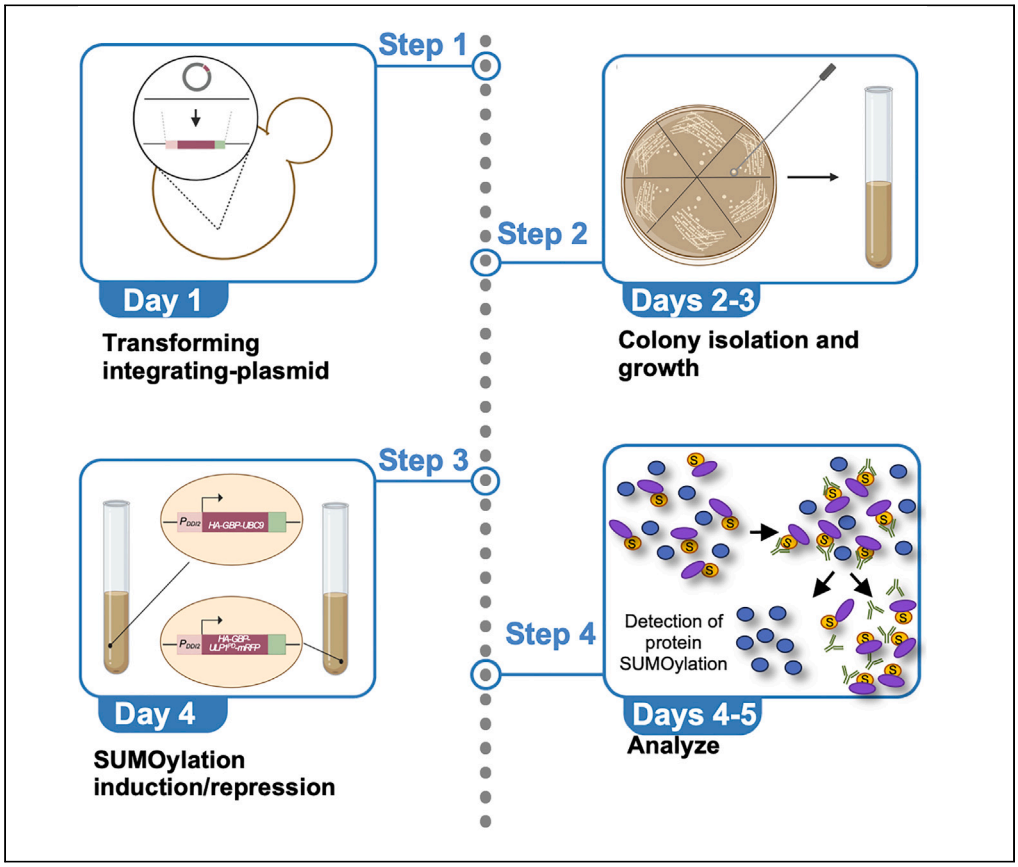
Protocol to modulate SUMOylation of a specific protein in budding yeast using chemical genetic approaches



SUMOylation (small ubiquitin-like modifier) is a ubiquitous and highly dynamic posttranslational modification. Here, we present a protocol to alter the local SUMOylation landscape of target proteins in budding yeast *Saccharomyces cerevisiae* using chemical genetic tools. We describe steps for recruiting SUMO enzymes (Ulp1^{PD} or Ubc9) to GFP-tagged proteins using GBP (GFP-binding protein)-fusion proteins. We then detail procedures for inducing SUMO conjugation/deconjugation and the subsequent SUMOylation analysis.

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

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Highlights

Complete and user-friendly protocol for altering SUMOylation of a specific protein

Steps for transforming yeast cells with plasmids expressing SUMO pathway enzymes

Procedures for the expression of SUMO enzymes from plasmids and SUMOylation analysis

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Protocol to modulate SUMOylation of a specific protein in budding yeast using chemical genetic approaches

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SUMMARY

SUMOylation (small ubiquitin-like modifier) is a ubiquitous and highly dynamic posttranslational modification. Here, we present a protocol to alter the local SUMOylation landscape of target proteins in budding yeast *Saccharomyces cerevisiae* using chemical genetic tools. We describe steps for recruiting SUMO enzymes (Ulp1^{PD} or Ubc9) to GFP-tagged proteins using GBP (GFP-binding protein)-fusion proteins. We then detail procedures for inducing SUMO conjugation/deconjugation and the subsequent SUMOylation analysis.

For complete details on the use and execution of this protocol, please refer to Gutierrez-Morton et al.[1]

BEFORE YOU BEGIN

SUMOylation is a critical mechanism for regulating protein localization, function, and stability.[2,3] Thousands of proteins have been shown to be SUMOylated in humans.[4] Targeted SUMOylation of a specific protein allows precise modulation of its function without disrupting global SUMOylation dynamics.[1] Here, we describe methods using the GBP (GFP-binding protein) system to recruit SUMO enzymes specifically to a protein of interest in yeast cells. GBP is a 13-kDa nanobody fragment with high affinity for GFP,[5,6] which allows us to recruit SUMO pathway enzymes to a GFP-tagged protein. This system is designed to modulate specific protein SUMOylation, as fusing the SUMO-conjugating enzyme Ubc9 or SUMO protease to a protein has been shown to enhance or diminish its SUMOylation, respectively (Figures 1A and 1D).[7,8] Of the two SUMO proteases in budding yeast, Ulp1 exhibits broad activity, therefore we fused the protease domain (PD) of Ulp1 to GBP. Additionally, we added a hemagglutinin (HA) tag and a red fluorescent protein (mRFP) tag, generating the plasmid pEGM4 (P_{DDI2}*HA-GBP-ULP1^{PD}-mRFP*) (Figures 1B and 1C).[9,10] The HA tag facilitates immunodetection of the expression of GBP fusion protein through western blotting, while the mRFP tag allows assessment of colocalization with GFP-tagged proteins *in vivo*. Similarly, the pEGM5 plasmid encoded the fusion protein between HA-GBP and Ubc9 (Figure 1E). Both constructs are under the control of a *DDI2* promoter, which enables inducible expression in response to cyanamide, providing temporal control over protein production. This protocol explains how to induce expression of GBP-fused SUMO enzymes in yeast cells expressing a GFP-tagged protein and subsequently monitor SUMOylation of the GFP-tagged protein through a co-immunoprecipitation approach. While we describe how to tether the SUMO machinery to a GFP-tagged protein, aspects of this approach can be adapted to tether any protein to a GFP-tagged target.

Recombinant DNA used for modulating targeted protein SUMOylation		
Reagent	Source	Identifier
pEGM4 (P_{DDI2}*HA-GBP-ULP1*^{PD}*-mRFP-LEU2*)	Gutierrez-Morton et al.[1]	N/A
pEGM5 (P_{DDI2}*HA-GBP-UBC9-LEU2*)	Gutierrez-Morton et al.[1]	N/A

Preparation of plasmids harboring GBP fused with SUMO enzymes (pEGM4 and pEGM5)

Timing: 1 h

1. Grow an overnight culture (16 h with shaking at 37°C) of *E. coli* strains harboring pEGM4 or pEGM5 plasmid in 5 mL 2×YP (yeast peptone) medium containing 100 mg/mL ampicillin.
2. Extract the plasmids with a Miniprep kit according to the manufacturer's instructions.
3. Measure the plasmid concentration with a NanoDrop.
4. Store the plasmid at −20°C until further use.

Media and buffer preparation

Timing: 2–6 h

5. Prepare YPD (yeast peptone dextrose) liquid medium.
6. Prepare leucine dropout agar plates.
7. Prepare all buffers and solutions.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-Pgk1	Invitrogen	Cat# 459250; RRID:AB_2532235
Mouse monoclonal anti-HA	BioLegend	Cat# 901515; RRID:AB_2565334
Mouse monoclonal anti-c-Myc	BioLegend	Cat# 626801; RRID:AB_2235686
Mouse monoclonal anti-FLAG	Sigma-Aldrich	Cat# F3165; RRID:AB_259529
Mouse anti-GFP antibody	Santa Cruz	Cat# Sc-9966; RRID:AB_627235
Mouse monoclonal anti-Smt3	Santa Cruz	Cat# SC-137158; RRID:AB_201891
Secondary anti-mouse IgG HRP-linked antibody	Cell Signaling Technology	Cat# 7076; RRID:AB_330924
Mouse anti-FLAG M2 (agarose beads) affinity gel	Sigma	Cat# A2220; RRID:AB_10063035
Protein A/G PLUS-agarose	Santa Cruz	Cat# SC-2003; RRID:AB_10201400
Chemicals, peptides, and recombinant proteins		
PEG (50% w/v)	Rigaku	1008054
ssDNA	Rockland	MB-103-0025
ECL	PerkinElmer	NEL 104001
Cyanamide	BeanTown Chemical	420-04-2
Ampicillin	Fisher	BP1760-25
Lithium acetate	Chem-Impex	26485
EcoRI	New England Biolabs	R0101S
EcoRV	New England Biolabs	B6004S
XcmI	New England Biolabs	R0101S
10× rCutSmart buffer	New England Biolabs	R0533S
Sodium hydroxide	Fisher Scientific	S318-500
Agar	Difco	214010
Glucose	Alfa Aesar	A16828
Yeast extract	Fisher Bioreagents	BP1422-500
Yeast nitrogen base w/o amino acids	Difco	291920
Peptone	HiMedia	RM001-500G

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Adenine sulfate	Thermo Scientific	A16964.18
Alanine	Alfa Aesar	A15804
Arginine	Alfa Aesar	A14730
Asparagine	Acros Organics	5794-13-8
Aspartic acid	Acros Organics	56-84-8
Cysteine	Alfa Aesar	A10435
Glutamine	Alfa Aesar	A14201
Glutamic acid	Acros Organics	56-86-0
Glycine	BeanTown Chemical	135880
Histidine	Acros Organics	71-00-1
Inositol	Alfa Aesar	A13586
Isoleucine	Alfa Aesar	A13699
Lysine	Alfa Aesar	A16249
Methionine	VWR	E801-500
para-aminobenzoic acid	Sigma	100536
Phenylalanine	VWR	0991-25G
Proline	Alfa Aesar	A10199
Serine	Fisher Scientific	A11179.14
Threonine	Acros Organics	72-19-5
Tryptophan	Acros Organics	73-22-3
Tyrosine	Thermo Scientific	A11141.30
Uracil	Acros Organics	66-22-8
Valine	Acros Organics	72-18-4
Glass beads	Cole-Parmer	NC0386496
Tris	VWR	0497
NaCl	Sigma	S9888
EDTA	Invitrogen	15575-038
Tween 20	ChemCruz	SC-29113
Glycerol	Fisher Bioreagents	EC200-289-5
Bromophenol blue	ICN Biomedicals	805732
MG-132	Sigma-Aldrich	474790
Rapamycin	Thermo Scientific	PHZ1235
DMSO	Sigma-Aldrich	D8418
Protease inhibitor cocktail set III	Millipore-Calbiochem	539136
N-ethylmaleimide (NEM)	Sigma-Aldrich	E3876
Critical commercial assays		
E.Z.N.A. plasmid DNA mini kit II	Omega Bio-tek	D6945-01
Experimental models: Organisms/strains		
S. cerevisiae: strain background W303; see Table 1	This paper	N/A
Other		
NanoDrop	Thermo Scientific	ND2000USCAN
Bead beater	BioSpec Products	NC0342321

MATERIALS AND EQUIPMENT

2×YP (yeast peptone)		
Reagent	Final concentration	Amount
Yeast extract	10 g/L	10 g
NaCl	5 g/L	5 g
Peptone	16 g/L	16 g
ddH_2O	–	up to 1 L
Total	–	**1 L**

Autoclave. 2×YP liquid medium can be stored at room temperature (20°C–25°C) for at least 6 months.

YPD (yeast peptone dextrose)		
Reagent	Final concentration	Amount
Yeast extract	10 g/L	10 g
(D-(+)-Glucose)	20 g/L	20 g
Peptone	20 g/L	20 g
Adenine sulfate	0.2 g/L	0.2 g
Agar (plates only)	20 g/L	20 g
ddH_2O	–	up to 1 L
Total	–	**1 L**

Autoclave. YPD liquid medium can be stored at room temperature (20°C–25°C) for at least 6 months.

Leucine dropout powder mix		
Reagent	Final concentration	Amount
Adenine	1.1%	0.5 g
Alanine	4.5%	2 g
Arginine	4.5%	2 g
Asparagine	4.5%	2 g
Aspartic acid	4.5%	2 g
Cysteine	4.5%	2 g
Glutamine	4.5%	2 g
Glutamic acid	4.5%	2 g
Glycine	4.5%	2 g
Histidine	4.5%	2 g
Inositol	4.5%	2 g
Isoleucine	4.5%	2 g
Lysine	4.5%	2 g
Methionine	4.5%	2 g
para-Aminobenzoic acid	4.5%	2 g
Phenylalanine	4.5%	2 g
Proline	4.5%	2 g
Serine	4.5%	2 g
Threonine	4.5%	2 g
Tryptophan	4.5%	2 g
Tyrosine	4.5%	2 g
Uracil	4.5%	2 g
Valine	4.5%	2 g
Total	–	**44.5 g**

Mix all the ingredients mentioned using a blender. Store mix at room temperature (20°C–25°C).

Leucine dropout media		
Reagent	Final concentration	Amount
Leucine dropout powder mix	2 g/L	2 g
(D-(+)-Glucose)	20 g/L	20 g
Agar	20 g/L	20 g
Sodium hydroxide	–	1 pellet
ddH_2O	–	up to 895 mL
10× YNB	100 mL/L	100 mL
200× Tryptophan	5 mL/L	5 mL
Total	–	**1 L**

Mix all ingredients (minus 10× YNB (yeast nitrogen base) and 200× Tryptophan solutions) and autoclave. Add 10× YNB and 200× Tryptophan right before pouring plates. Store the plates at 4°C.

200× Tryptophan solution		
Reagent	Final concentration	Amount
Tryptophan	49 g/L	49 g
ddH_2O	–	up to 1 L
Total	–	**1 L**

Filter-sterilize. Store at 4°C.

10× YNB (yeast nitrogen base) solution		
Reagent	Final concentration	Amount
Yeast nitrogen base w/o amino acids	67 g/L	67 g
ddH_2O	–	up to 1 L
Total	–	**1 L**

Filter-sterilize. Store at 4°C.

1000× MG-132		
Reagent	Final concentration	Amount
MG-132	50 mM	23.8 mg
DMSO	–	up to 1 mL
Total	–	**1 mL**

Store at −20°C.

Co-immunoprecipitation lysis buffer		
Reagent	Final concentration	Amount
Tris, pH 7.5	50 mM	2.5 mL of 1 M stock
NaCl	150 mM	1.5 mL of 5 M stock
EDTA, pH 8	5 mM	0.5 mL of 500 mM stock
Tween-20	0.05%	25 μL
ddH_2O	–	up to 50 mL
Total	–	**50 mL**

Filter-sterilize once all reagents (excluding Tween-20) have been mixed. Add Tween-20 separately. Store at 4°C. Just prior to use, add protease inhibitor cocktail (1000×), 1 mM PMSF (serine protease inhibitor), and 10 mM NEM (cysteine peptidase inhibitor).

2× loading buffer		
Reagent	Final concentration	Amount
Tris, pH 6.8	120 mM	1.2 mL of 1 M stock
Glycerol	10%	1 mL
SDS	4%	4 mL of 10% stock
BME (β-mercaptoethanol)	8%	0.8 mL
Bromophenol blue	24% w/v	2.4 mgs
ddH_2O	–	up to 10 mL
Total	–	**10 mL**

Filter-sterilize once all reagents (excluding Bromophenol blue and BME) have been mixed. Add Bromophenol blue and BME separately. Store at 4°C. Prepare 1× buffer by diluting in sterile ddH_2O.

STEP-BY-STEP METHOD DETAILS

Day 0: Preparing yeast culture for plasmid transformation

Timing: 10 min

This step enables cells to reach mid-log phase during Day 1, which improves the transformation efficiency.

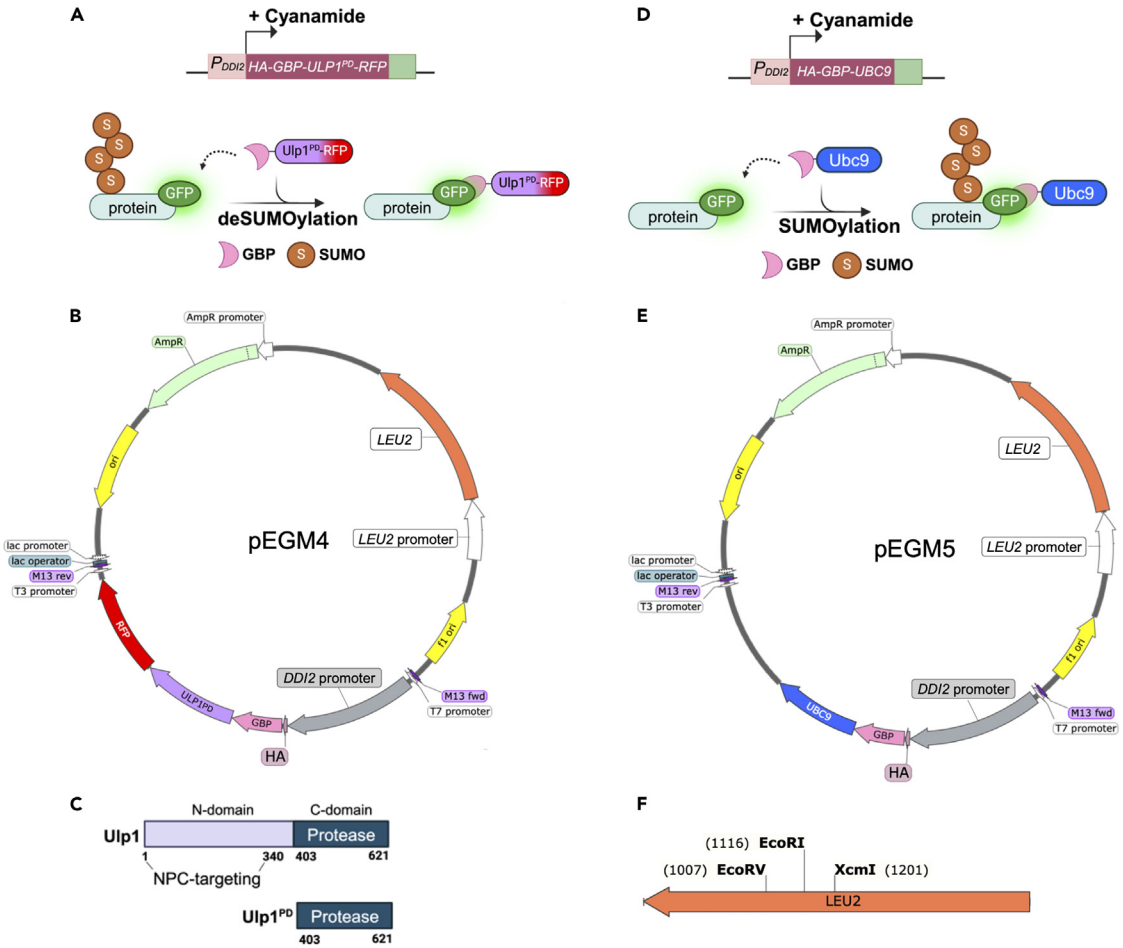

Figure 1. Annotated plasmid maps for pEGM4 and pEGM5

Figure reprinted and adapted with permission from Gutierrez-Morton et al.[1]

(A) Scheme of the inducible GBP tethering system by fusing Ulp1^{PD}-mRFP to HA-GBP, which was expressed under control of the *DDI2* promoter.

(B) pEGM4 is comprised of a *DDI2* promoter directly upstream of the *ULP1* construct (*HA-GBP-ULP1^{PD}-mRFP*).[8]

(C) Domain organization of yeast SUMO protease Ulp1. The Ulp1 SUMO protease domain (PD) localizes from residue 403 to 621.

(D) Scheme of the inducible GBP tethering system by fusing SUMO E2 enzyme Ubc9 to HA-GBP that is expressed under the control of the *DDI2* promoter.

(E) pEGM5 contains *DDI2* promoter followed by *HA-GBP-UBC9*.

(F) pEGM4 and pEGM5 plasmids can be linearized within the *LEU2* gene with an enzyme EcoRV, EcoRI, or Xcm1.

1. Inoculate a yeast strain (1:1000 dilution from a fresh saturated liquid culture) expressing a GFP-tagged protein of interest in 5 mL YPD liquid medium. Grow overnight (16 h) at 30°C with shaking until mid-log phase ($OD_{600} \approx 0.3$).

Table 1. Yeast strains used in this protocol

Strains	Relevant genotypes	Reference
Y300	Mata *ura3-1, his3-11,15 leu2-3,112 trp1-1, ade2-1, can1-100*	Allen et al.[11]
EGM2	MATa *TOF2-GFP-TRP1*	Gutierrez-Morton et al.[1]
4160-1-2	MATa *HIS-FLAG(HF)-SMT3-LEU2*	Gutierrez-Morton et al.[1]
4604-3-2	MATa P_{DDI2}*HA-GBP-ULP1^{PD}-mRFP-LEU2*	This study
4603-5-1	MATa P_{DDI2}*HA-GBP-UBC9-LEU2*	This study
4570-5-2	MATα *TOF2-GFP-TRP1 HF-SMT3-LEU2* P_{DDI2}*HA-GBP-UBC9-LEU2*	Gutierrez-Morton et al.[1]
4711-3-2	MATa *HF-SMT3 TOF2-GFP SCC1-13Myc* P_{DDI2}*HA-GBP-UBC9*	Gutierrez-Morton et al.[1]
4646-4-1	MATa *HF-SMT3 TOF2-GFP ulp2-AA* P_{DDI2}*HA-GBP-ULP1^{PD}-mRFP*	Gutierrez-Morton et al.[1]

Note: If targeting Tof2 SUMOylation/deSUMOylation, use strain EGM2 (*TOF2-GFP*). Otherwise, any yeast strain expressing a GFP-tagged protein of interest is suitable for the transformation of either pEGM4 or pEGM5.

Note: Be sure that the strain used is leucine auxotroph, because both pEGM4 and pEGM5 contain a *LEU2* marker. All the strains listed in Table 1 are isogenic to Y300, which is *leu2-3*.

Note: Fresh liquid yeast cultures should not be more than 2 weeks old as this may negatively affect cell viability.

Day 1: Digestion of plasmid pEMG4 and pEMG5 and yeast transformation

Timing: 3 h

For plasmid integration at the *LEU2* locus of the yeast genome, it is necessary to linearize plasmids pEGM4 and pEGM5 within the *LEU2* gene prior to the transformation (single digest reaction). Figure 1F shows the unique restriction enzyme sites within the *LEU2* gene that can be used for linearization.

2. Prepare for plasmid digestion.
   a. In a 1.5 mL Eppendorf tube, prepare the mix in the following order:

Component	Amount
rCutsmart	2.5 μL
Plasmid DNA (pEGM4 or pEGM5)	1–1.5 μg
Sterile ddH_2O	up to 24 μL
Restriction enzyme (EcoRI, EcoRV, or XcmI)	1 μL
Total	**25 μL**

   b. Mix gently by pipetting.
   c. Incubate the reaction at 37°C for 2 h.

Pause point: Keep reaction mix at 4°C until ready to use for yeast transformation.

Optional: Following digestion, it is optional to run 1 μL of the reaction mix on an agarose gel to verify the efficiency of plasmid digestion by the restriction enzyme.

Note: While all listed restriction enzymes can be used for plasmid digestion, EcoRI is often preferred due to its high cutting efficiency.

3. Transform yeast cells with linearized plasmids pEGM4 or pEGM5.
   a. Spin down 0.8 mL (OD600 ≈ 0.3) of overnight culture (16 h with shaking) in a 1.5 mL Eppendorf tube. The volume of yeast culture can be adjusted based on the OD.
   b. Wash with 1 mL sterile ddH2O and pellet for 10 s at 5,200 × g.
   c. Wash once with 500 μL of cold 0.1 M LiAc and spin down for 10 s at 5,200 × g.
   d. Add the transformation mix to the Eppendorf tubes with yeast cells in the following order.

Component	Amount
PEG (50% w/v)	240 μL
1.0 M LiAc	36 μL
ssDNA	25 μL
Linearized plasmid DNA	25 μL

⚠ CRITICAL: Use 1.0 M LiAc in the transformation mix.

Note: ssDNA is stored at −20°C and needs to be boiled at 100°C for 10 min prior to use. Once boiled, ssDNA is stored at 4°C.

Note: Heat inactivation (raising the temperature to 65°C for 20 min) of the plasmid digest reaction is not required as the enzyme concentration is typically too low to affect transformation efficiency.

e. Vortex the tube vigorously until the cell pellet has been completely mixed.
f. Incubate the tube for 30 min at 30°C.
g. Heat shock cells for 25 min at 42°C using either a dry bath or water bath.
h. Place tube on ice for 2 min.
i. Spin down cells at 3,800 × g for 15 s and remove the supernatant with a pipet.
j. Add 90 μL of sterile ddH_2O into the tube and carefully resuspend the pellet.
k. Spread cells onto a leucine dropout plate.
l. Incubate for 2–3 days at 30°C to allow colony formation.
m. Re-streak multiple transformed colonies onto a new selection (leucine dropout) plate.

Pause point: Plates harboring transformed colonies can be wrapped in parafilm and stored at 4°C for several weeks.

Days 4: Inoculate transformant colonies

Timing: 10 min

To ensure that colonies have successfully integrated the GBP-SUMO system at the *LEU2* locus, we grow cells overnight and verify their expression for SUMOylation induction/repression studies.

4. Grow a transformed colony in a glass tube with 5 mL YPD medium overnight (16 h with shaking) at 30°C.

Optional: Although negative colonies are uncommon, we recommend inoculating multiple colonies as a precautionary measure in case cells do not grow or do not show expression of the GBP-SUMO system.

Note: The volume of the culture will vary depending on how many time-points are required.

Day 5: Dilute saturated cultures and perform SUMOylation induction/repression experiments

Timing: 2 h

Yeast transformants with pEGM4 and pEGM5 contain P_{DDI2}-*HA-GBP-ULP1*^{PD}-*mRFP* and P_{DDI2}-*HA-GBP-UBC9*, respectively. The presence of cyanamide will induce gene expression from *DDI2* promoter in these yeast transformants,[12] and the expression of HA-GBP fused proteins will be determined by western blotting with an anti-HA antibody. In this step, dilute the fresh saturated cultures so that you will have your ideal concentration at the time of inducing the *DDI2* promoter with cyanamide.

5. Dilute the fresh saturated cultures to an OD_{600} of 0.2 and allow cells to grow for an additional 3 h.

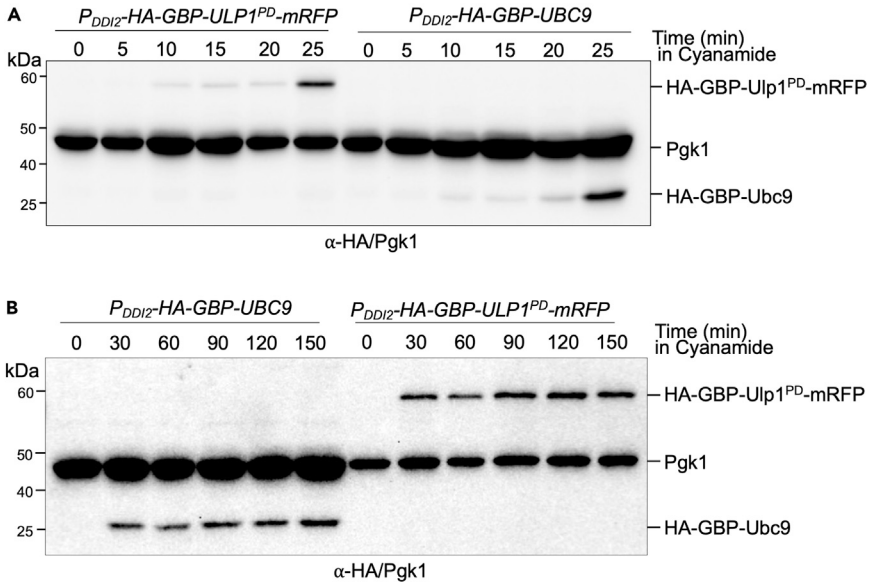

Figure 2. Cyanamide-induced expression of the SUMO-GBP system

(A) Expression induction of either HA-GBP-Ulp1^{PD}-mRFP or HA-GBP-Ubc9 from the *DDI2* promoter with cyanamide over time for 25 min. Cells were grown to log phase at 30°C and then 5 mM cyanamide was added to the cultures. Cells were collected after cyanamide addition at the indicated time points to prepare protein samples. Protein levels were detected by western blotting using an anti-HA antibody. Pgk1, loading control.

(B) Expression induction of HA-GBP-Ulp1^{PD}-mRFP and HA-GBP-Ubc9 fusion proteins with 5 mM cyanamide overtime for 150 min. The method was the same as described above.

6. Collect 1 mL of culture to be used as "pre cyanamide induction" control. Store cell pellet at −80°C until western blotting.
7. Induce expression of HA-GBP-Ubc9 or HA-GBP-Ulp1^{PD} (to either trigger SUMOylation or deSUMOylation of the GFP-tagged protein) in yeast cells by adding cyanamide to a final concentration of 5 mM. Expression of the HA-GBP-Ubc9 and HA-GBP-Ulp1^{PD} is visible after 30 min induction by cyanamide (Figures 2A and 2B).
8. Collect 1 mL samples in 30-min intervals for 2 h after cyanamide addition.

Pause point: Cell pellets used for western blotting can be stored at −80°C for several weeks.

Note: The number of time points and the duration of the time course should be tailored to each experiment and protein of interest. Given that SUMOylation is a rapid and transient modification, it's advisable to limit experiments to a maximum of 2 h to capture meaningful data.

9. Analyze protein expression by western blotting using mouse anti-HA antibody at 1:1000 dilution. Secondary anti-mouse IgG antibody should be used at 1:2500.

Note: Dilute the primary antibody at 1:1000 and the secondary antibody at 1:2500 as a starting point. According to the manufacturer's instructions, the recommended dilution range for the primary antibody is 1:1000 - 1:5000, while the secondary antibody is suggested to be used at a dilution of 1:1000 - 1:3000.

Days 5–7: Detect protein SUMOylation/deSUMOylation by co-immunoprecipitation

Timing: 2–3 days

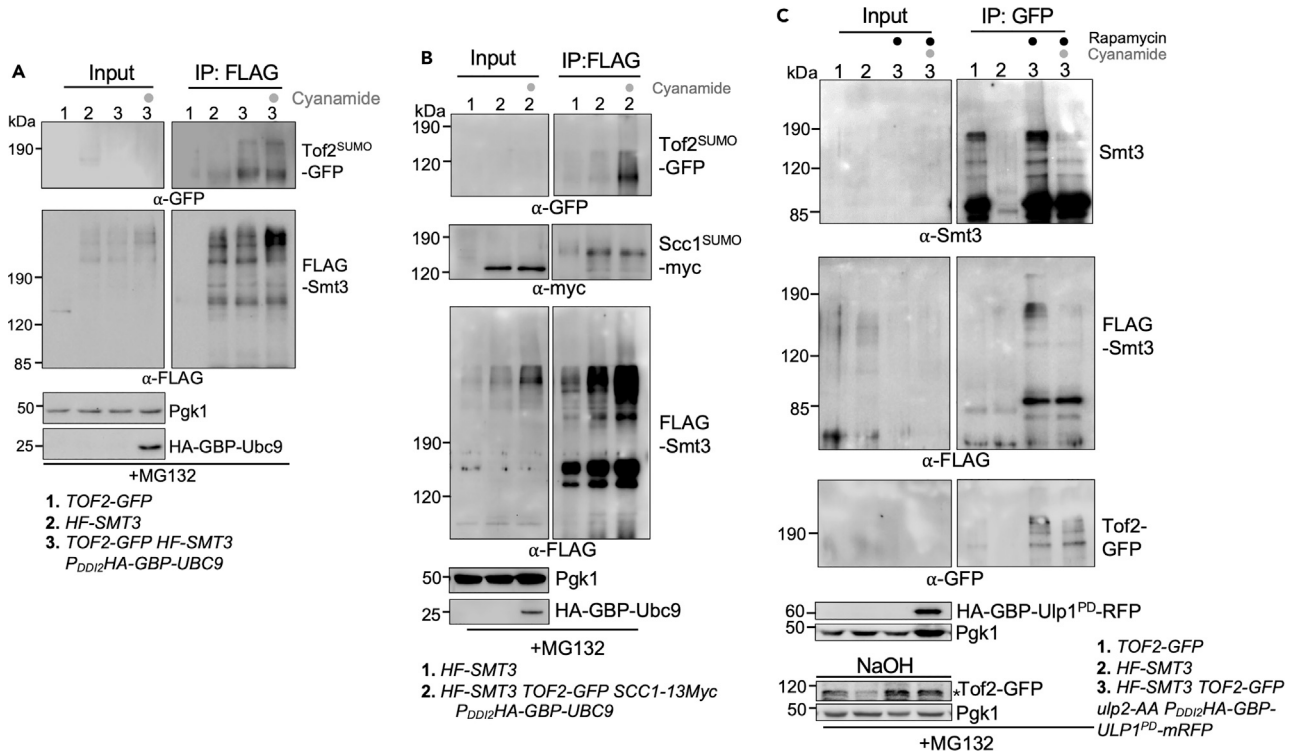

Figure 3. Expression of GBP system controls Tof2-GFP SUMOylation

Figure reprinted and adapted with permission from Gutierrez-Morton et al.[1]

(A) Expression of HA-GBP-Ubc9 triggers Tof2-GFP SUMOylation. Cells were grown asynchronously to log phase at 30°C. MG-132 (50 μM) was then added for 30 min to prevent proteasome activity. Cyanamide (5 mM) was added to induce HA-GBP-Ubc9 expression for 1 h. Cells were collected in the presence and absence of cyanamide treatment. IP was performed to precipitate His-Flag-Smt3 conjugates with anti-FLAG beads. His-Flag-Smt3, Tof2-GFP, and HA-GBP-Ubc9 protein levels in the input and IPed fractions were detected by western blotting. Cells expressing Tof2-GFP and HF-Smt3 served as controls. Pgk1, loading control.

(B) Tethering Ubc9 to Tof2-GFP does not trigger polySUMOylation of cohesin Scc1. Cells with the indicated genotypes were treated as described in panel A.

(C) Tethering the protease domain of Ulp1 to Tof2-GFP prevents Tof2 SUMOylation. Cells with the indicated genotypes were grown to log phase at 30°C. MG-132 (50 μM) was then added for 30 min to prevent proteasome activity. Cyanamide (5 mM) was added to induce HA-GBP-Ulp1^{PD}-mRFP expression for 1 h before rapamycin was added for 30 min to trigger polySUMOylation via the anchor away system.[1] Rapamycin only or rapamycin/cyanamide-treated cells were collected. Total Tof2-GFP protein levels were detected by western blotting. Cell extracts were IPed with anti-GFP antibody to isolate Tof2-GFP proteins. Protein levels were detected by western blotting using anti-Smt3, anti-FLAG, anti-GFP, and anti-HA antibodies, respectively. Cells expressing Tof2-GFP or HF-Smt3 served as controls. Pgk1, loading control. Nonspecific band denoted by *.

We monitor the changes in protein SUMOylation by performing co-immunoprecipitation (co-IP). This modular protocol allows for immunoprecipitating either SUMO/Smt3 (and probing for the GFP-tagged protein) or immunoprecipitating the GFP-tagged protein (and probing for SUMO/Smt3). In the following examples, we show both approaches. We first examined Tof2-GFP SUMOylation after inducing HA-GBP-Ubc9 expression (Figure 3A).[1] Since Tof2 localizes to the nucleolus, we also show an example experiment monitoring for off-target SUMOylation outside the nucleolus using the nuclear-localized cohesin subunit Scc1 (Figure 3B).[1] In Figures 3A and 3B, we use anti-FLAG beads to pull-down His-Flag (HF)-tagged Smt3 (the only SUMO isoform in *S. cerevisiae*) and probe for GFP-tagged protein of interest. The His-Flag-tagged Smt3 construct (N-terminal tag on Smt3) was originally generated by the Basrai Lab.[13] Tetrad dissection of the *HF-SMT3* strain generated the subsequent yeast strains used in this study (listed in Step 10) and are available for sharing.

In Figure 3C,[1] we used anti-GFP antibody-conjugated beads to immunoprecipitate Tof2-GFP and subsequently probe for SUMO modification. In the example experiment, the "anchor away" (AA) system induces global SUMOylation of nuclear proteins by depleting SUMO protease Ulp2 from the nucleus. Upon rapamycin addition, Ulp2 is sequestered to the cytoplasm via heterodimerization

with RPL13A, thereby effectively terminating its SUMO protease activity for nuclear substrates and triggering their polySUMOylation. This results in a widespread increase in polySUMOylation. The induction of global nuclear protein SUMOylation occurs simultaneously with the expression of HA-GBP-Ulp1^{PD}, which removes polySUMO moieties from Tof2-GFP. Therefore, the AA system can be used in combination with HA-GBP-Ulp1^{PD} expression to ensure the protein of interest is polySUMOylated prior to targeted deSUMOylation. For more details on the use and execution of the AA system, please refer to Gutierrez-Morton et al.[1] The yeast strains used in this study (listed in Step 10) were generated by mating strains harboring the AA system with strains harboring the GBP system, followed by tetrad dissection. All strains are available for sharing.

10. Grow the following strains (1:1000 dilution from a stock culture) in 50 mL YPD medium overnight (16 h) at 30°C with shaking until mid-log phase.
    a. *TOF2-GFP-TRP1*.
    b. *HIS-FLAG (HF)-SMT3-LEU2*.
    c. *TOF2-GFP-TRP1 HF-SMT3-LEU2 P_{DDI2}HA-GBP-UBC9-LEU2*.
    d. *HF-SMT3 TOF2-GFP SCC1-13Myc P_{DDI2}HA-GBP-UBC9*.
    e. *HF-SMT3 TOF2-GFP ulp2-AA P_{DDI2}HA-GBP-ULP1^{PD}-mRFP*.
11. Induce Tof2-GFP SUMOylation or deSUMOylation by HA-GBP-Ubc9 or HA-GBP-Ulp1^{PD} expression.
    a. The following day, add cyanamide to each culture at a final concentration of 5 mM.

    Optional: Proteasomal inhibitor MG-132 (50 μM) can be added to the culture 30 min prior to SUMOylation induction to suppress proteasomal degradation, because protein polySUMOylation may trigger its proteasomal degradation.

    ⚠ CRITICAL: For experiments using the *ulp2-AA* strains, add cyanamide to induce GBP system expression one h before the addition of rapamycin (final concentration 2 μg/mL), which triggers polySUMOylation.

12. Harvest cells and perform cell lysis.
    a. Collect cells after 1 h treatment in cyanamide.
    b. Centrifuge at 1,700 × g for 1 min and remove supernatant.
    c. Wash cells once with 20 mL sterile ddH_2O and centrifuge at 1,700 × g for 1 min.
    d. Remove supernatant.

    ⏸ **Pause point:** Cell pellets can be stored frozen at −80°C for several months.

    e. Resuspend cells in 0.7 mL of co-IP lysis buffer containing all mentioned protease inhibitors.
    f. Add 150–200 μL of glass beads and combine with lysate into a screw cap tube.
    g. Lyse cells by bead bashing for 20 s followed by 1 min on ice.
    h. Repeat for a total of 3 times.

    ⚠ CRITICAL: Following cell lysis, all samples need to be handled on ice or at 4°C to prevent protein degradation. Remove 2 μL cell lysate to a slide and examine the efficiency of cell breakage under a microscope. More than 90% of cells are expected to be broken by bead bashing.

    i. To separate the glass beads from the rest of the lysate, puncture a hole in the bottom of the tube with a needle and place it inside a fresh tube.
    j. Spin at 200 × g for 1 min at 4°C.

    Note: Due to the stacking of tubes, the centrifuge lid will not fit. At such a low speed, centrifugation can be completed without the lid.

k. Collect all supernatant, repeating spins if necessary.
l. Centrifuge cell lysate at 2,700 × g for 1 min at 4°C.
m. Transfer the supernatant into a new prechilled 1.5 mL Eppendorf tube.
n. Take 25 μL from the supernatant to be used as input.
o. Add 25 μL of 2× loading buffer to the input and boil for 5 min at 90°C.
p. Store input samples at −80°C until western blot analysis.

13. Perform co-immunoprecipitation.
    a. To immunoprecipitate the FLAG-Smt3/SUMO conjugates from the cell lysate, add 20 μL of anti-FLAG bead slurry to the leftover supernatant.

⚠ CRITICAL: For the samples prepared from *ulp2-AA* strains, immunoprecipitation (IP) is performed using anti-GFP antibody instead of anti-FLAG beads. Add 5 μL of anti-GFP antibody to the supernatant to precipitate GFP-tagged protein of interest.

    b. Allow binding for 4–16 h at 4°C while rotating.
    c. Add 30 μL of Protein A/G PLUS-Agarose bead slurry to each sample and allow binding for an additional hour at 4°C while rotating.

⚠ CRITICAL: Only add Protein A/G PLUS-Agarose beads to samples immunoprecipitated with anti-GFP antibody. The GFP-tagged protein should be bound to the anti-GFP antibody, and the addition of Protein A/G beads enables the binding of the antibody-protein complex to the beads.

    d. Centrifuge samples at 700 × g at 4°C for 30 s. Remove supernatant.
    e. Wash beads with 500 μL of co-IP lysis buffer supplemented with fresh protease inhibitors.
    f. Centrifuge the beads at 700 × g at 4°C for 30 s and remove supernatant.
    g. Repeat wash and centrifuge steps two additional times.
    h. After removing the supernatant in the final wash, add 50 μL of 1× SDS loading buffer to the beads.
    i. Gently mix buffer and beads by tapping.
    j. Heat the IP samples for 5 min at 65°C.
    k. Analyze input and IP samples by western blotting with appropriate antibodies.

⏸ **Pause point:** Samples can be stored frozen at −80°C for several weeks.

EXPECTED OUTCOMES

This protocol consists of transforming yeast cells with GBP fusion plasmids followed by the induction of either polySUMOylation or deSUMOylation of a protein tagged with GFP (27 kDa). When designing GBP/GFP constructs from scratch, there are minimal limitations. Both GFP and GBP tags can be fused to either the N- or C-terminus of a protein. However, it is generally considered best practice to place GFP at the C-terminus, as this is more likely to preserve the protein's native function and localization.[14] In some cases, including a spacer between the tag and the protein may improve protein folding and reduce any potential impact on the protein's structure or activity.[15] Here, plasmids pEGM4 and pEGM5 do not include a spacer (Figure 1B and 1D). Overall, this system is advantageous because GBP has high specificity for GFP, and there are no known endogenous targets of GBP.[5,6] While GBP primarily recognizes GFP, it can also bind the yellow variant YFP. However, GBP does not interact with CFP or any derivatives, such as mRFP, mCherry, or mOrange.

After transformation, the number of colonies on the leucine dropout plate may vary due to several factors, including the transformation efficiency, the effectiveness of plasmid linearization, the

amount of linearized plasmid used, etc. However, yeast transformation efficiency is typically high with the protocol described. In our hands, successful plasmid integrations result in detectable expression of the GBP fusion proteins within 30 min after cyanamide addition (Figure 2A).[1] HA-GBP-Ubc9 is expected to be ~27 kDa in size, meanwhile HA-GBP-Ulp1^{PD}-mRFP is ~60 kDa. The expression of GBP-fused proteins is not expected to interfere with the localization of GFP-tagged substrates. For instance, when Tof2-GFP is co-expressed with HA-GBP-Ulp1^{PD}-mRFP, both colocalize to the nucleolus, indicating that the GFP-GBP interaction does not appear to alter the localization of Tof2-GFP itself.[1]

Ensure that the GFP-tagged protein is a confirmed SUMO substrate. SUMOylation occurs on lysine residues within a SUMO consensus motif (SCM) (ΨKX(D/E)), where Ψ represents a large hydrophobic residue, and X is any amino acid.[16,17] If a protein's SUMOylation status is unknown, prediction tools such as JASSA can help to identify potential modification sites. While forced interaction with Ubc9 via GFP-GBP may enhance proximity, SUMOylation is unlikely to occur for proteins without an SCM.

An example of altered SUMOylation of Tof2-GFP by expressing GBP-fused SUMO enzymes is shown in Figure 3.[1] In Figure 3A, yeast cells expressing HA-GBP-Ubc9 showed increased Tof2-GFP SUMOylation (lane 4). Targeted SUMOylation of GFP-tagged proteins is expected, as the SUMOylation status of cohesin Scc1 is unaffected by targeting HA-GBP-Ubc9 to Tof2-GFP (Figure 3B, lane 3). In contrast, cells expressing HA-GBP-Ulp1^{PD} are expected to show reduced SUMOylation of GFP-tagged proteins following polySUMOylation induction in *ulp2-AA* cells (Figure 3C).[1]

Please consult the troubleshooting section for suggestions on how to improve SUMOylation modulation efficiency.

LIMITATIONS

While the GBP system is a useful tool for modulating the local SUMOylation landscape, we recognize the limitations of this system. First, the tag-specific limitation of using GFP may not be suitable for all experiments, especially in cases where GFP expression declines or becomes cytotoxic. In addition, the plasmids carry a *LEU2* marker, therefore yeast transformations must be carried out in a strain that is leucine auxotroph. To circumvent this issue, further cloning into a new vector with a different auxotrophic marker may be required. Another potential limitation is the specificity of the GBP system. In our previous work, we show that targeting the SUMO machinery for one protein may affect the SUMOylation status of other proteins within the same complex.[1] This is consistent with the idea of group modification seen in the DNA damage response pathway.[18] Lastly, forcing the proximity of the SUMO machinery with a GFP-tagged protein could potentially interfere with its normal function or regulation. Overall, however, we believe that the reliability, specificity, and strength of this protocol make up for any limitations.

TROUBLESHOOTING

Problem 1

The transformation efficiency is low.

Potential solution

- Increase the amount of linearized plasmid DNA in the transformation mix to 1.5 μg.
- Increase the amount of enzyme in the restriction enzyme digestion for higher efficiency.
- Following plasmid digestion, incubate the reaction at 65°C for 20 min to ensure that the restriction enzyme is heat-inactivated.
- Ensure that yeast cells used for transformation are in mid-log phase ($OD_{600} \approx 0.3$).

- Use fresh PEG solution.

Problem 2

No expression of HA-GBP-Ubc9 or HA-GBP-Ulp1^{PD} was detected.

Potential solution

- Try optimizing the cyanamide concentration to induce expression. Although we recommend using 5 mM cyanamide to induce expression of either HA-GBP-Ubc9 or HA-GBP-Ulp1^{PD}, cyanamide is not toxic to cells up to concentrations of 30 mM.[12]
- Ensure that cells are in mid log-phase and have not yet reached saturation stage.

Problem 3

The GBP construct is being expressed, but there are no differences in the GFP-tagged protein SUMOylation.

Potential solution

- SUMOylation is a transient modification, and it may be difficult to stabilize substrate SUMOylation status once cells are lysed and subject to proteolysis. To preserve protein SUMOylation, increase the concentration of the SUMO protease inhibitor NEM to 20 mM.
- Because protein polySUMOylation often causes proteasomal turnover, it may be necessary to use proteasomal inhibitors such as MG-132 in cell culture. Alternatively, perform experiments in a proteasomal-deficient mutant to mitigate protein degradation. In addition, STUBL mutant *slx5Δ* can be used to block polySUMO-mediated protein ubiquitination and the subsequent degradation.

Problem 4

The localization of the GBP-tethered SUMO machinery to GFP-tagged proteins has not been confirmed, or it is believed to be mislocalized.

Potential solution

- To verify if the GBP-fusion protein is bound to the GFP-tagged target, perform a co-IP to pull-down the GFP-tagged protein and probe for GBP-fused proteins. Alternatively, use fluorescence microscopy to verify their colocalization. The *HA-GBP-ULP1^{PD}* construct contains mRFP and has been shown to colocalize with nucleolar protein Tof2-GFP.[1] For *HA-GBP-UBC9*, further molecular cloning is required for the addition of an RFP tag.

Problem 5

Cyanamide treatment is causing cell stress or toxicity.

Potential solution

- Cyanamide is not toxic to yeast cells (in concentrations of 30 mM or less).[12] Because SUMOylation is a known cellular response to stress,[19] altered SUMOylation from driving expression of HA-GBP-Ubc9 or HA-GBP-Ulp1^{PD} may trigger a stress response. To address this, lowering the temperature, reducing the concentration of cyanamide, or reducing the amount of time spent in cyanamide may help in minimizing cellular toxicity.

Problem 6

There is non-specific targeting of the GBP system to non-GFP-tagged proteins.

Potential solution

- Inducing the GBP system may lead to off-target SUMOylation or deSUMOylation simply due to the increased concentration of SUMO machinery within the cell. The elevated SUMO enzymes can result in non-specific SUMOylation or deSUMOylation due to the higher availability of the enzymes. To minimize non-specific modification, it may help by lowering the concentration of cyanamide, which reduces the expression of the SUMO machinery.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yanchang Wang (yanchang.wang@med.fsu.edu).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Emily Gutierrez-Morton (emg19i@fsu.edu).

Materials availability

Yeast strains and plasmids generated for this study are available by request to the lead contact.

Data and code availability

This study did not generate any datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, methodology, resources, and writing – original draft, E.G.-M. and Y.W.; validation and visualization, E.G.-M. and Y.W.; writing – review and editing, E.G.-M. and Y.W.; funding acquisition, supervision, and project administration, Y.W.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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