Title: Tri-SUS: A Yeast Split-Ubiquitin assay to examine protein interactions governed by a third binding partner

Short Title: Tri-SUS System for Tripartite Protein Interaction analysis

Author names and affiliations:

Ben Zhang[1*], Lingfeng Xia[2], Yaxian Zhang[1], Hui Wang[1], Rucha Karnik[2]

1. School of Life Science, Shanxi University, Taiyuan, Shanxi, China 030006
2. Plant Science Group, Laboratory of Plant Physiology and Biophysics, Institute of Molecular, Cell, and Systems Biology, University of Glasgow, Glasgow G12 8QQ, United Kingdom

* Correspondence: Email: benzhang@sxu.edu.cn Tel:+0086-0351-7010599

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Author contributions

RK and BZ conceived the project, designed vectors and wrote the manuscript, BZ and LX prepared constructs, BZ, LX and HW carried out Tri-SUS assays, BZ and YZ did immunoblot.

One-Sentence Summary:

The yeast Tri-SUS system can be used to study tripartite protein-protein interactions between bait and prey protein pairs by modulation of expression of their binding partner

Dear Editor:

Study of protein-protein interactions is an essential first step for understanding the biochemical networks in living cells. Several technologies have emerged to support protein-protein interaction research, including *in vitro* pull-down assays, *in vivo* live-cell fluorophore-based imaging assays, and yeast growth assays (Xing et al., 2016). These technologies are well suited to study binary interactions between so-called 'bait' and 'prey' proteins. However, cellular processes are often driven by higher-order protein complexes. For analysis of multiprotein interactions, researchers can use pull-down and yeast growth assays (Bolle, 2009; Horaruang and Zhang, 2017), but such technologies come with several limitations (Louche et al., 2017; Vikis and Guan, 2015). For example, pull-downs require purified proteins, which make experiments technically challenging and expensive (Louche et al., 2017). The Yeast-3-Hybrid assay (Y3H) (Tirode et al., 1997; Zhang and Lautar, 1996) has limited efficacy because there is no control over expression of proteins, and the modified mating-based split-ubiquitin system (mbSUS) assay (Grefen, 2014) has reduced transformation and hybrid yeast selection efficiencies because it requires transformation of yeast cells with multiple plasmids. Most importantly in the yeast growth assays, unless the expression of the third protein can be modulated, it is not possible to resolve how the physical connection with the third protein influences binding between the bait and prey proteins.

We developed a yeast growth-based Tri-SUS system to study bait and prey interactions under the influence of a third binding partner in a complex. The Tri-SUS system is designed to allow chemical-induced modulation of partner protein expression to determine whether the partner protein facilitates or outcompetes trimeric complex formation between the bait and prey proteins. Preparation of hybrid yeast expressing three proteins in the Tri-SUS system is mating based and the assay can be used for analysis of both membrane-anchored and soluble bait proteins. Thus, the Tri-SUS system marries the benefits of the yeast mating-based SUS assays, overcomes limitations for efficacy of existing multiprotein interaction assays, and has the added advantage of the ability to probe dynamics of tripartite protein interactions.

Tri-SUS vector sets are Gateway compatible for ease of cloning and use a two-vector system for mating based preparation of hybrid yeast expressing three proteins (Figure 1 A-B). We used the unique restriction sites KpnI and XhoI to engineer artificially synthesized *ptdh3*

promotor for stabilized and uniform bait and prey protein expression. The bait vector sets were developed from the vectors of pExg2Met-Dest of the GPS system (Zhang et al., 2018) to give the option of expressing soluble bait proteins with a membrane-anchoring N-terminal glycosyl-phosphatidylinositol (GPI) hydrophobic signal sequence. GPI membrane anchoring of soluble baits reduces false positive results when soluble bait proteins transit to the nucleus and autoactivate reporter gene expression (Zhang et al., 2018). The Tri-SUS prey-partner vector was developed from the pNX35-Dest mbSUS vector (Grefen et al., 2009) and it uses the multisite Gateway cloning cassettes (Grefen and Blatt, 2012; Hecker et al., 2015) to allow recombination of two genes of interest in a one-step recombination reaction. The advantage is that transformation of yeast cells with separate plasmids for expression of prey and partner proteins is circumvented, thus increasing efficiency of transformation and hybrid yeast selection. We engineered the *pmet25* promotor into the cassette for partner protein expression, which makes it possible to modulate expression of the partner protein with addition of methionine (Met) in the growth media and to effectively probe the role of the partner in bait-prey tripartite interactions (Figure 1A).

To check the quality of the bait in the SUS assay, negative and positive controls are needed (Grefen et al., 2009; Horaruang and Zhang, 2017). Unlike the classic mbSUS assay, in the Tri-SUS assay, the negative control should express both the soluble NubG and partner proteins. So, transfer of the partner gene in the prey vector (leaving the prey Gateway cassette empty or transferring an unrelated gene as prey) was used as the negative control. Similarly, after changing NubG to NubWt (NubI) by site-directed mutagenesis (Karnik et al., 2013a), transfer of ‘partner’ gene alone into the new vector pPrey-PartnerSUS-PC-Dest was used as the positive control.

In *Arabidopsis thaliana*, SEC11 (=KEULE) outcompetes SNARE protein SYP121 for Kv channel KC1 binding, and these interactions were characterized using *in vitro* pull-down assays (Waghmare et al., 2019; Karnik et al., 2017; Zhang et al., 2020). To test the Tri-SUS system with a partner protein that outcompetes bait-prey interactions, we took advantage of the SYP121-KC1-SEC11 tripartite interactions. KC1 was used as bait, SYP121 as 2in1-prey, and SEC11 as 2in1-partner in the assay. Diploid yeast was obtained and grown as before using standard mbSUS protocols (Supplemental Figure S1). Yeast growth and supporting

immunoblot data (Figure 2A) from one of three independent experiments showed similar results. As expected, we observed no yeast growth in yeast containing KC1 and negative prey control, and the yeast growth was recovered when there were KC1 and positive prey control. When SYP121 was used as prey and the partner site was left empty, the diploid yeast grew well, and the addition of Met had little or no effect. The growth of diploid yeast was suppressed in the $CSM_{-LTUMAH}$ media without Met when carrying KC1-SYP121-SEC11. In the $CSM_{-LTUMAH}$ media with 500 μM Met, which suppressed the expression of SEC11, the yeast growth recovered. These results suggest that SEC11 competes with SYP121 for KC1 binding, consistent with published pull-down studies (Karnik et al., 2013b, 2015; Waghmare et al., 2019). Immunoblot analysis of each bait, prey, and partner protein in haploid yeast (Figure 2B) confirmed that bait or prey expression was not affected in the Tri-SUS yeast. We tested the suppression of partner protein (SEC11) expression by growing mated yeast on CSM_{-TUM} media containing Met at increasing concentrations (0, 50, and 500 μM). Immunoblots with α-Myc antibody were carried out to detect SEC11 expression alongside immunoblots for α-HA to detect the prey SYP121. Gels were loaded with ~ 5 μg/lane total protein (Figure 2B) and band intensity for SEC11 and SYP121 was measured using ImageJ and normalized to total protein. We found that the expression of SEC11 (partner) was suppressed in the presence of Met in a dose-dependent manner, but there was no effect on SYP121 (prey) expression. Thus, suppression of expression of partner proteins in the Tri-SUS system is Met dose-dependent. Together these data demonstrate that Tri-SUS works efficiently to detect bait-prey interactions that are outcompeted by the partner protein.

To test the Tri-SUS assay with a partner protein that facilitates bait-prey interactions, we used the KC1-Arabidopsis K^+ Transporter 1 (AKT1)-Calcineurin B-like protein-interacting protein kinase 23 (CIPK23) tripartite complex. In Arabidopsis, the AKT1 and KC1 subunits form the functional K^+ channel (Geiger et al., 2009). A Ser/Thr protein kinase, CIPK23, phosphorylates AKT1 but not KC1, suggesting that the AKT1 protein bridges tripartite interactions between KC1 and CIPK23 (Xu et al., 2006). In the Tri-SUS, we cloned KC1 as bait, CIPK23 as prey, and AKT1 as partner (Figure 2C). On the $CSM_{-LTUMAH}$ selective media, the growth of diploid yeast expressing KC1-AKT1-CIPK23 was better than that of yeast carrying KC1 and CIPK23. The addition of Met only suppressed the growth of yeast containing KC1-AKT1-CIPK23. Yeast

growth indicated that AKT1 facilitates the interaction between KC1 and CIPK23, as expected. Thus, Tri-SUS can be used to detect bait-prey interactions that are facilitated by the partner protein. We expect that if the partner does not participate in the bait-prey interactions, yeast growth will remain unchanged when added Met suppresses partner protein expression.

A major advantage of the Tri-SUS system that yeast cells are transformed more efficiently with a single bait or prey+partner plasmid (Supplemental Figure S1). A comparison of yeast transformation efficiencies of Tri-SUS vectors alongside systems using three separate vectors to express bait, prey and partner/bridge proteins (Supplemental Figure S2) showed that relative to transformation with a single plasmid (Relative transformation efficiency ~100%), transformation with three plasmids reduces efficiency by almost five-fold (Relative transformation efficiency ~20%) (Supplemental Figure S3).

In summary, the Tri-SUS system is a promising tool that overcomes the inherent limitations of existing yeast-based multi-protein interaction assays and allows for investigation of interactions between bait and prey proteins that are governed by a third 'partner' protein. The Tri-SUS system is efficient in yeast transformations, suitable for soluble baits, and allows the study of tripartite interactions that facilitate or outcompete bait-prey binding.

Resources

Vector maps and information for vector requests are available from the corresponding author or through requests at www.psrg.org.uk or www.rdrp.sxu.edu.cn.

Supplemental Data

Supplemental Figure S1. Experimental flowchart for the Tri-SUS System

Supplemental Figure S2. Experimental flowchart for the three-plasmid system.

Supplemental Figure 3. Comparative analysis of yeast transformation efficiencies for Tri-SUS and the three-plasmid systems

Figure legends

Figure 1. The Tri-SUS system schematic. (A) Tri-SUS vector maps. The Tri-SUS vector sets are developed from traditional mbSUS and GPS vectors (Grefen et al., 2009; Horaruang and Zhang, 2017; Zhang et al., 2018) by DNA synthesis (Sangon Biotech (Shanghai) Co., Ltd.), overlap-PCR, and restriction-ligation reactions. The genes for bait, prey, and partner proteins are be cloned into vectors using Gateway recombination. The vector ptdh3YC-Dest allows the expression of membrane protein as bait under control of *ptdh3* promotor. The vector ptdh3Exg2YC-Dest allows the expression of non-membrane protein as bait under control of *ptdh3* promotor. And an N-terminal GPI hydrophobic signal peptide from yeast glucan-1,3-β-glucosidase 2 (Exg2) is anchored to the bait to prevent its release from the membrane. Expression of prey protein with 2x Haemagglutinin (HA) tag is under control of *ptdh3* promotor in and the expression of bridge protein with c-Myc tag is under control of *pmet25* promotor. **(B) Schematic representation of Tri-SUS assay.** The bait and prey proteins are fused to mutated N-terminal ubiquitin (NubG) and C-terminal ubiquitin (Cub) linked with transcription reporter complex Protein A-LexA-VP16 (PLV), respectively. If the bait and prey have binary interactions, the NubG and Cub assemble as a functional ubiquitin to release the PLV, which activates expression of reporter genes (ADE2, HIS3) for the synthesis of adenine (ADE) and histidine (HIS). Yeast that growth on nutrient selective media is identified as interactors. The expression of the ‘partner’ protein is under control of *pmet25* promotor is suppressed with the addition of methionine (Met). If the ‘partner’ protein competes with the bait or prey proteins for binding, its expression out-competes the bait -prey interactions, and addition of Met to suppress ‘partner’ expression recovers bait-prey binding and yeast growth. If the ‘partner’ forms a tripartite complex, it will facilitate bait and prey interactions. The addition of Met to suppress ‘partner’ expression would reduce the tripartite interactions and suppress yeast growth. If the ‘partner’ does not participate in the interactions, yeast growth will remain unchanged in the presence of Met (not shown).

Figure 2. Protein-protein interaction analysis using the Tri-SUS assay. (A) Tri-SUS system tested with 'partner' that out-competes bait-prey interactions. Diploid yeast expressing bait: KC1-CubPLV, 2in1-prey: NubG-SYP121 and 2in1-partner: SEC11 or controls (prey negative: NubG; prey positive: NubI) were dropped on different selective media as indicated. The yeast growth on CSM(-LTUM) was used to verify the presence of both bait and 2in1 vectors in the diploid yeast. The yeast growth on CSM(-LTUMAH) was used to verify interaction and additions of different concentrations of Met were used to suppress the expression of 'partner' protein. Diploid yeast was dropped at 1.0, 0.1, and 0.01 (OD600) in each case. Incubation time was 24 h on CSM(-LTUM) and 72 h on CSM(-LTUMAH). Immunoblot analysis (5 μg total protein/lane) of the haploid yeast used for mating was performed using commercial α-VP16 antibody for bait, α-HA antibody for prey, and α-Myc antibody for the partner. **(B) Suppression of 'partner' protein expression is Methionine dose dependent.** Diploid yeast expressing prey SYP121 and partner SEC11 proteins were grown in the media CSM(-TUM) supplemented with different concentrations of Met. To test expression of SEC11 protein following the addition of Met, immunoblot analysis (5 μg total protein/lane) of harvested yeast was performed using commercial α-HA antibody for 'prey' and α-Myc antibody for the partner. Protein expression was quantified using ImageJ and normalized against Ponceau S stain of the membrane for total protein detection. The bar graph shows Mean ± SE of relative protein levels for partner SEC11 (bottom left) and prey SYP121 (bottom, right). n= 3, alphabets represent statistical difference by ANOVA ($p<0.05$). **(C) Tri-SUS system tested with 'partner' protein that facilitates bait-prey interactions.** Diploid yeast expressing bait: KC1-CubPLV, 2in1-prey: NubG-CIPK23, 2in1-partner: AKT1, and controls (prey negative: NubG; prey positive: NubI) were dropped on different selective media as indicated. The yeast growth on CSM(-LTUM) was used to verify the presence of both bait and 2in1 vectors in the diploid yeast. The yeast growth on CSM(-LTUMAH) was used to verify interaction and additions of different concentrations of methionine (Met) were used to suppress the expression of 'partner' protein. Diploid yeast was dropped at 1.0, 0.1, and 0.01 (OD600) in each case. Incubation time was 24 h on CSM(-LTUM) and 72 h on $CSM_{-LTUMAH}$. To verify protein expression, immunoblot analysis (5 μg total protein/lane) of the haploid yeast used for mating was performed using commercial α-VP16 antibody for bait, α-HA antibody for prey, and α-Myc antibody for the partner.

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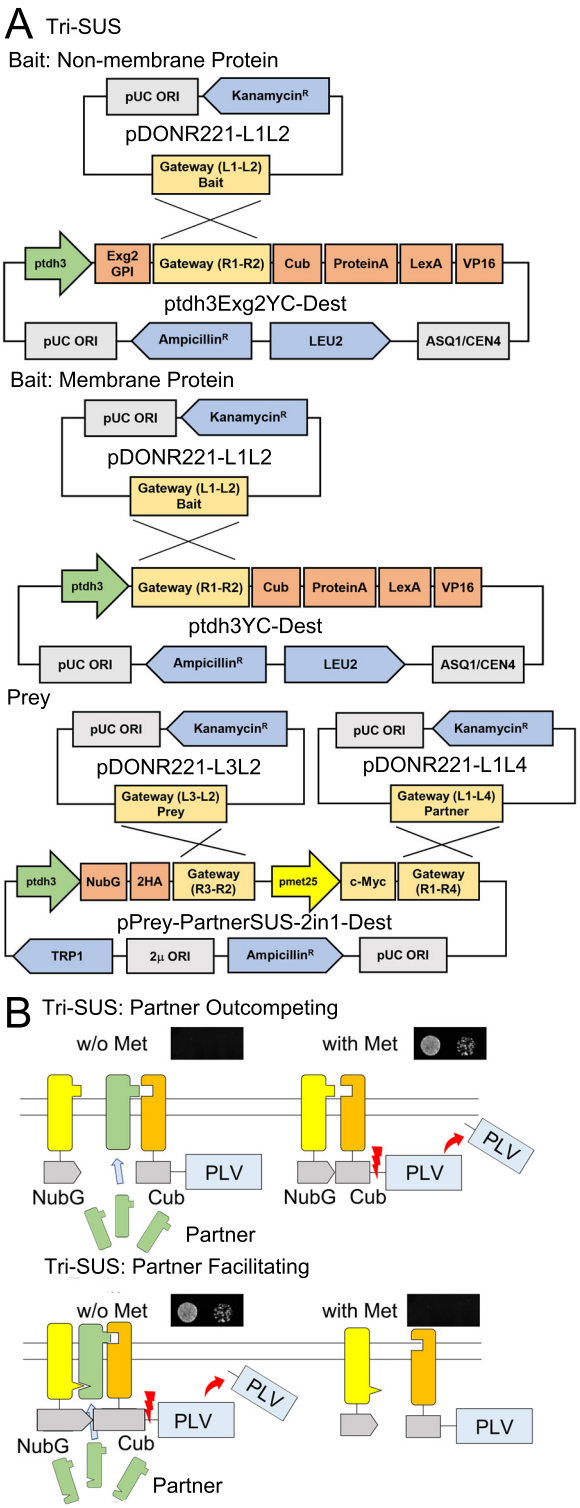
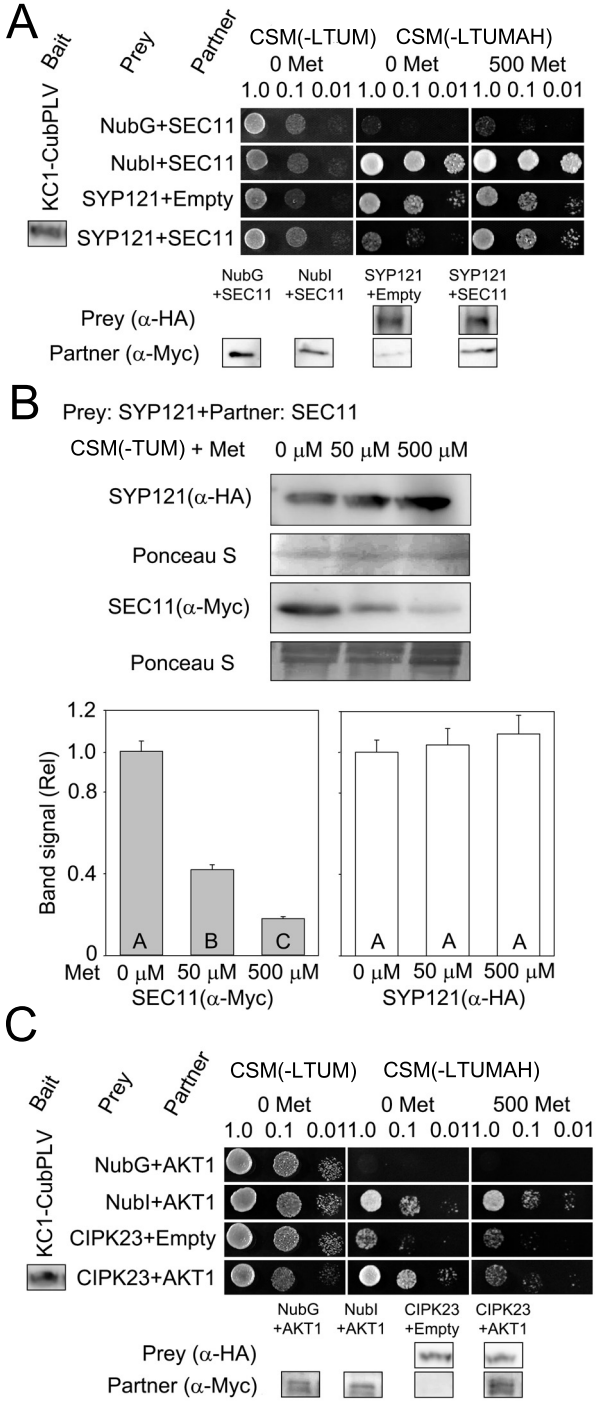

A Tri-SUS
Bait: Non-membrane Protein
pUC ORI
KanamycinR
pDONR221-L1L2
Gateway (L1-L2)
Bait
ptdh3
Exg2
GPI
Gateway (R1-R2)
Cub
ProteinA
LexA
VP16
ptdh3Exg2YC-Dest
pUC ORI
AmpicillinR
LEU2
ASQ1/CEN4
Bait: Membrane Protein
pUC ORI
KanamycinR
pDONR221-L1L2
Gateway (L1-L2)
Bait
ptdh3
Gateway (R1-R2)
Cub
ProteinA
LexA
VP16
ptdh3YC-Dest
pUC ORI
AmpicillinR
LEU2
ASQ1/CEN4
Prey
pUC ORI
KanamycinR
pDONR221-L3L2
Gateway (L3-L2)
Prey
pUC ORI
KanamycinR
pDONR221-L1L4
Gateway (L1-L4)
Partner
ptdh3
NubG
2HA
Gateway
(R3-R2)
pmet25
c-Myc
Gateway
(R1-R4)
pPrey-PartnerSUS-2in1-Dest
TRP1
2μ ORI
AmpicillinR
pUC ORI
B Tri-SUS: Partner Outcompeting
w/o Met
with Met
PLV
PLV
PLV
NubG
Cub
NubG
Cub
Partner
Tri-SUS: Partner Facilitating
w/o Met
with Met
PLV
PLV
PLV
NubG
Cub
Partner


A
Bait
Prey
Partner
CSM(-LTUM)
CSM(-LTUMAH)
0 Met
0 Met
500 Met
1.0 0.1 0.01 1.0 0.1 0.01 1.0 0.1 0.01
KC1-CubPLV
NubG+SEC11
NubI+SEC11
SYP121+Empty
SYP121+SEC11
NubG +SEC11
NubI +SEC11
SYP121 +Empty
SYP121 +SEC11
Prey (α-HA)
Partner (α-Myc)
B
Prey: SYP121+Partner: SEC11
CSM(-TUM) + Met
0 μM 50 μM 500 μM
SYP121(α-HA)
Ponceau S
SEC11(α-Myc)
Ponceau S
Band signal (Rel)
1.2
1.0
0.8
0.4
0
A
B
C
A
A
A
Met 0 μM 50 μM 500 μM
SEC11(α-Myc)
0 μM 50 μM 500 μM
SYP121(α-HA)
C
Bait
Prey
Partner
CSM(-LTUM)
CSM(-LTUMAH)
0 Met
0 Met
500 Met
1.0 0.1 0.01 1.0 0.1 0.01 1.0 0.1 0.01
KC1-CubPLV
NubG+AKT1
NubI+AKT1
CIPK23+Empty
CIPK23+AKT1
NubG +AKT1
NubI +AKT1
CIPK23 +Empty
CIPK23 +AKT1
Prey (α-HA)
Partner (α-Myc)

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