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TRAPPII Complex Assembly Requires Trs33 or Trs65

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Abstract

TRAPP is a multi-subunit complex that acts as a Ypt/Rab activator at the Golgi apparatus. TRAPP exists in two forms: TRAPP I is comprised of five essential and conserved subunits and TRAPP II contains two additional essential and conserved subunits, Trs120 and Trs130. Previously, we have shown that Trs65, a nonessential fungi-specific TRAPP subunit, plays a role in TRAPP II assembly. *TRS33* encodes another nonessential but conserved TRAPP subunit whose function is not known. Here, we show that one of these two subunits, nonessential individually, is required for TRAPP II assembly. Trs33 and Trs65 share sequence, intra-cellular localization and interaction similarities. Specifically, Trs33 interacts genetically with both Trs120 and Trs130 and physically with Trs120. In addition, *trs33* mutant cells contain lower levels of TRAPP II and exhibit aberrant localization of the Golgi Ypts. Together, our results indicate that in yeast, TRAPP II assembly is an essential process that can be accomplished by either of two related TRAPP subunits. Moreover, because humans express two Trs33 homologues, we propose that the requirement of Trs33 for TRAPP II assembly is conserved from yeast to humans.

Introduction

TRAPP is a multi-subunit complex required for trafficking through the Golgi apparatus. TRAPP exists in at least two forms. TRAPP I consists of five essential and conserved subunits and functions at the cis-Golgi. TRAPP II contains two additional essential and conserved subunits, Trs120 and Trs130, and functions at the trans-Golgi (Table 1). In addition, three other TRAPP subunits are not essential for cell viability: Trs33, Trs65 and Trs85 (1). Sequence and structure analyses showed that the TRAPP I/II subunits, which are shared between the two complexes, fall into two families: the Bet3-like family includes Bet3, Trs31, Trs33, and the Bet5-like family includes Bet5, Trs20 and Trs23 (1). Here, we show that Trs65 is also related to the Bet3 family.

Two different activities were assigned to the TRAPP complexes: activation of Ypt/Rab GTPases and vesicle tethering. TRAPP I acts as a guanine-nucleotide exchange factor (GEF) for Ypt1, the Ypt/Rab GTPase required for entry into the Golgi (2–5). Four of the five essential TRAPP I/II subunits are required and sufficient for this activity: Bet3, Bet5, Trs23 and Trs31 (6). TRAPP II acts as a GEF for Ypt31/32, which are a functional pair required

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for exit from the trans-Golgi (3,7,8). We have previously shown that the two TRAPP II-specific subunits, Trs120 and Trs130, are required for switching the GEF activity of TRAPP from Ypt1 to Ypt31/32 (8). We proposed that conversion of TRAPP I to TRAPP II, via the addition of Trs120 and Trs130, is required for coordination between Ypt1-mediated Golgi entry and Ypt31/32-mediated Golgi exit (8). Therefore, we expect that assembly of TRAPP II is an essential process.

Trs65, a non-essential TRAPP subunit, is conserved only among fungi (9). We have recently shown that this subunit plays a role in TRAPP II assembly (10). The question arises: if TRAPP II assembly is an essential process, how does it happen in the absence of Trs65? Trs33, another non-essential TRAPP I/II subunit, is not required for TRAPP I assembly or for the Ypt1-GEF activity of TRAPP I (6). Unlike Trs65, Trs33 is conserved from yeast to humans, and humans have two Trs33 homologues: TRAPPC6A and TRAPPC6B. However, to date, the role of this subunit is not clear. Here, we show that Trs33 plays a role in the assembly of the TRAPP II complex.

TRS33 exhibits synthetic lethality with *TRS65*, and this lethality can be suppressed by over-expression of a Ypt31 mutant protein restricted to its GTP-bound form (Ypt31-GTP, (11)). In addition, the two genes share similar genetic interactions with other genes whose products function in the late Golgi, e.g. *PIK1*, *SEC14* and *DRS2* ((12), summarized in Figure 1A). These genetic interactions suggest that Trs33 and Trs65 share a function in the late Golgi related to the proper function of Ypt31/32. Here, we show that Trs33 and Trs65 share sequence similarity and that, like Trs65, Trs33 functions in the assembly of TRAPP II complex and the proper localization of Ypt31/32. Moreover, because either Trs33 or Trs65 is required for cell viability, and because Trs33 is conserved from yeast to humans, we propose that assembly of TRAPP II is an essential and conserved process.

Results

Trs33 and Trs65 share sequence and localization similarities

Based on a synthetic lethal interaction that can be suppressed by over-expression of Ypt31-GTP ((11) and Figure 1A), we tested whether the two non-essential TRAPP subunits Trs33 and Trs65 share similarities in their intra-cellular localization and sequence. Endogenous Trs33 was tagged with YFP at its C-terminus. Like Trs65 (10), Trs33 co-localizes best with the trans-Golgi marker Sec7 and to a lesser extent with the cis-Golgi marker Cop1, but not with the endosomal marker FYVE domain (Figure 1B). Thus, the intra-cellular localization of Trs33 is similar to that of Trs65.

Multiple alignment analysis of Trs33 proteins from yeast to humans shows a number of conserved domains ((13,14) and Figure 2, domains I–III). In contrast, Trs65 is conserved only amongst some fungi (9). When Trs65 from proteomes of twelve different fungi, including *S. cerevisiae*, are also included in the multiple alignments analysis with the Trs33-related proteins, the same conserved domains can be seen at the N-terminal half of the Trs65 proteins (Figure 2). Domain I is important for the interaction of Trs33 with Bet3 ((13,14), and Figure 2C). The roles of the two other conserved domains, II and III, are not known. Based on results presented here, we propose that these domains are important for the role of the two proteins in the assembly of the TRAPP II complex.

The Trs33-related domain of Trs65 is sufficient for its role in TRAPP II assembly

To determine whether the N-terminal half of Trs65, which shares sequence similarity with Trs33, is sufficient for its known interactions and function, we constructed yeast two-hybrid plasmids that express residues 1–375 (Trs65N) and 376–560 (Trs65C). Both constructs express polypeptides of the expected size. However, only the Trs65N, and not Trs65C,

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interacts with the TRAPP II specific subunits Trs120 and Trs130 (Figure 3A). Moreover, like the full-length Trs65, expression of the Trs65N domain can complement the growth defect of *trs65ts* mutant cells (Figure 3B). Most importantly, like the full-length Trs65, Trs65N also complements the lower level of Trs130 protein in *trs65ts* mutant cells (Figure 3C). Together, these results show that the Trs33-related domain of Trs65 is sufficient for its interactions with the TRAPP II-specific subunits and for its role in the assembly of TRAPP II.

Physical and genetic interactions of Trs33 with TRAPP II subunits

To assess a possible role for Trs33 in the assembly of the TRAPP II complex, we first used the yeast two-hybrid assay to determine whether Trs33 exhibits physical interactions with TRAPP II-specific subunits. In this assay, Trs33 interacts with Trs120, but not with Trs130 or Trs65 (Figure 4A). To determine whether Trs33 interacts directly with Trs120 or Trs130, tagged versions of the three proteins were expressed in bacteria and used in a co-precipitation analysis. Trs120-His6 can be pulled down with GST-Trs33, but not with GST (~2–5% Trs120 co-precipitates with ~50% of the GST-Bet5; Figure 4B) while calmodulin-HA, as a negative control, cannot. A smaller proportion of Trs130-HA can also be pulled down with GST-Trs33 but not with GST (~0.5% Trs130 co-precipitates with ~25% of the GST-Bet5, Figure S1). Because the Trs33-Trs120 interaction was shown by two independent ways, yeast-two hybrid and co-precipitation, these results indicate that Trs33 can interact directly with at least one of the TRAPP II-specific subunits, Trs120. A possible Trs33-Trs130 interaction requires further study.

Previously, we have shown that deletion of *TRS65* from cells in which endogenous Trs120 and Trs130 are both tagged at the C-terminus results in temperature sensitivity for growth (10). To determine whether *TRS33* also interacts genetically with the TRAPP II-specific subunits, *TRS33* was deleted in cells expressing tagged versions of Trs120 or Trs130. Deletion of *TRS33* in cells expressing either Trs120-myc or Trs130-HA results in a temperature sensitive growth phenotype (Figure 5A). Deletion of *TRS33* in cells expressing tagged versions of both Trs120 and Trs130 results in lethality (Figure 5B). In addition, as in the case of *trs65ts*, over-expression of the TRAPP I/II subunit Bet5 in *trs33ts* mutant cells results in a more severe growth phenotype (Figure S2). These results show that deletion of *TRS33* and *TRS65* exhibit similar genetic interactions with genes expressing TRAPP I and TRAPP II subunits, except that deletion of *TRS33* results in more severe growth phenotypes than those caused by deletion of *TRS65*.

We have previously shown that *trs65ts* mutant cells exhibit a general secretory defect and accumulate secretory vesicles (10). Like *trs65ts* mutant cells, *trs33ts* (trs33Δ Trs130-HA) mutant cells at their restrictive temperature exhibit a general secretory defect (Figure 5C), and accumulate secretory vesicles (Figure 5D). Together, these results indicate that Trs33 plays a joint role in the late Golgi with the other TRAPP II-specific subunits.

Assembly of TRAPP II is defective in *trs33ts* mutant cells

We have previously shown that the level of TRAPP II is lower in *trs65ts* mutant cells when compared with wild-type cells (10). The level of the TRAPP II-specific subunits was tested in cell lysates made from *trs33ts* mutant cells. The level of Trs120-myc remained unchanged in *trs33ts* mutant cells even when Bet5 was over-expressed (Figure S3A, left). In contrast, the level of Trs130-HA is lower in *trs33ts* mutant cells even at their permissive temperature (Figure S3B) and gets even lower in mutant cells over-expressing Bet5 (Figure 6A). These results suggest that in *trs33ts* mutant cells, TRAPP II contains low levels of Trs130.

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Moreover, when TRAPP complexes were purified from *trs33ts* mutant cells expressing tagged Trs130-HA, the level of Trs130 was lower than that seen in wild-type cells (Figure 6B). Consistent with these findings, Trs130-GFP localization is diffuse in *trs33ts* mutant cells even at their permissive temperature (Figure 6C). No such effect of *trs33*Δ was seen on the level of Trs120 in purified TRAPP complexes (Figure S3A, right). These results suggest that in the absence of Trs33, Trs120 can join the TRAPP complex, whereas Trs130 does not join the complex efficiently. Importantly, because Trs130 is required for TRAPP II function as a GEF for Ypt31/32 (8), assembly of a functional TRAPP II complex in *trs33ts* mutant cells is defective. In agreement, Ypt32 GEF activity of GST-Bet5-associated complexes purified from *trs33ts* mutant cells is defective, while the complexes possess Ypt1 GEF activity (Figure 6D).

Trs33 and Ypt31/32 interactions

TRAAP II acts as a GEF for Ypt31/32 but not for Ypt1 (3,8). In agreement with this, the growth defect of mutant cells defective in TRAPP II assembly, *trs120*, *trs130* and *trs65ts*, can be suppressed by over-expression of Ypt31/32 but not Ypt1 (8,10). Because assembly of functional TRAPP II is defective in *trs33ts* mutant cells, we expected these mutant cells to show similar genetic interactions with the Golgi Ypts. Indeed, over-expression of Ypt31, and not Ypt1, can suppress the growth defect of *trs33ts* mutant cells in which either Trs120 or Trs130 is tagged (Figure 7A). Thus, *TRS33* exhibits genetic interaction with genes encoding Golgi Ypts similar to those of the other three TRAPP II-specific subunits.

Moreover, we have previously shown that the localization of the Golgi Ypts is affected in opposite ways in cells carrying mutations in the TRAPP II-specific subunits, Trs130 or Trs65 (8,10). The localization of Ypt31/32 and Ypt1 is affected in opposite ways also in *trs33ts* mutant cells. Specifically, whereas Ypt31/32 localization is diffuse in *trs33ts* mutant cells especially at their restrictive temperature, the Ypt1 staining in *trs33ts* mutant cells at the restrictive temperature is more intense than in wild type cells (Figure 7B). Importantly, there is no change in the localization of the trans-Golgi marker Sec7 in *trs33ts* mutant cells at any temperature (Figure S3C), suggesting that the structure of the Golgi is not affected. Thus, like Trs130 and Trs65, the absence of Trs33 affects the localization of the substrates of the TRAPP II GEF, Ypt31/32, to the trans-Golgi. The enhancement of Ypt1 staining in *trs130ts*, *trs65ts* and *trs33ts* mutant cells can be attributed to the uncoupling of the coordination between Ypt1 and Ypt31/32 resulting in accumulation of Ypt1 on the Golgi.

Trs65 and Trs33 can substitute for each other

Because in wild type cells either Trs33 or Trs65 is required for viability, we tested whether over-expression of these proteins can substitute for each other in mutant cells with tagged Trs120 or Trs130. Over-expression of Trs65 from a 2μ plasmid suppresses the temperature sensitivity of *trs33ts* mutant cells in which either Trs130 is tagged with HA or Trs120 is tagged with myc. In addition, over-expression of Trs33 from a 2μ plasmid suppresses the temperature sensitivity of *trs65ts* mutant cells (Figure 8A). Importantly, the level of Trs130 in *trs33ts* and *trs65ts* mutant cells over-expressing Trs65 or Trs33, respectively, is also significantly restored (Figure 8B). This last result indicates that Trs65 and Trs33 can substitute for each other in restoring the level of TRAPP II in cells deleted for the other gene, *trs33*Δ and *trs65*Δ, respectively.

Trs33 and Trs65 can be on the same TRAPP complex

One question that arises is whether Trs33 and Trs65 can reside on the same TRAPP complex. We first determined their interactions with TRAPP I/II subunits using the yeast two-hybrid assay. Structural studies have shown that Trs33 interacts with Bet3 and Bet5, and that these interactions are conserved also between the mammalian subunits (6,13,14).

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Our yeast two-hybrid analysis shows that both Trs33 and Trs65 interact with Bet3 and Trs31, and to a lesser extent with Trs20. However, whereas Trs33 interacts with Bet5, Trs65 interacts with Trs23; the latter interaction is mediated through its N-terminal half (Figure S4). Based on the proposed architecture of the TRAPP I complex (6,15), the interactions of Trs33 and Trs65 with TRAPP I subunits do not completely overlap (summarized in Figure 9A).

The ability of Trs33 and Trs65 to reside on the same TRAPP complex was determined directly using a pull-down assay. GST-Trs65 or GST-Trs33 was pulled down from yeast cell lysates that also express Trs33-YFP or YFP-Trs65, respectively. Trs33-YFP co-precipitated with both GST-Bet5 and GST-Trs65, but not with the negative controls, GST and GST-Sec2 (Figure 9B, left). Similarly, YFP-Trs65 co-precipitates with GST-Bet5 and GST-Trs33, but not with GST or GST-Sec2 (Figure 9B, right). Together, the incomplete overlap of Trs33 and Trs65 interactions with TRAPP I subunits, along with the ability of Trs33 and Trs65 to pull each other down, suggest that these two TRAPP subunits do not compete for interaction with the TRAPP I complex and can reside on the same TRAPP complex.

Discussion

The conserved TRAPP II complex plays an essential function in protein trafficking. Here, we show that two parallel mechanisms exist in yeast cells to ensure efficient assembly of TRAPP II, one mediated by Trs33 and the other by Trs65. While each mechanism by itself is not essential, at least one is required for cell viability. Improper TRAPP II assembly caused by mutations in the essential TRAPP II subunits, Trs120 or Trs130, or deletion of one of the non-essential subunits, Trs33 or Trs65, combined with tagging of the essential subunits, results in mis-localization of the Golgi Ypts, Ypt1 and Ypt31/32.

We also show here that Trs65 and Trs33 share sequence similarity, and define Trs65 as a new distant member of the Bet3 family of TRAPP subunits (Figure 2). Trs33 was previously defined as a TRAPP I subunit solely based on its co-migration with TRAPP I subunits on a sizing column (16). However, to date there is no evidence that Trs33 plays a role in TRAPP I assembly or function. In contrast, here we assign a function for Trs33 in TRAPP II complex assembly together with Trs65. Therefore, we propose that both Trs33 and Trs65 are important for TRAPP II assembly and function.

The architecture of TRAPP I has been resolved (6,15). However, very little is currently known about the architecture or mechanism of assembly of TRAPP II. This study provides new insights into both of these questions. First, we show that the interactions of Trs33 and Trs65 with TRAPP I/II subunits overlap only partially, and that both subunits can reside on the same TRAPP complex (Figure 9A and B). Multiple alignment analyses (Figure 2) and yeast two-hybrid interactions (Figure 9A) show that the N-terminal domain of Trs65 is similar to Trs33. Based on this similarity, we propose that this domain is involved in the interaction of Trs65 with Bet3 or another Bet3 family member, Trs31. Our results suggest that when both Trs33 and Trs65 are present, they interact with opposite sides of the TRAPP I complex. But, in the absence of Trs65, Trs33 can also interact with the other side (Figure 9C).

Second, we propose a model for TRAPP II architecture and mechanism of assembly, based on results from our previous papers and this study (Figure 9C). We propose that Trs120 joins the TRAPP complex due to its interactions, assisted by Trs65, with TRAPP I subunits. This idea is based on the finding that Trs120 does not assemble with TRAPP in *trs65ts* mutant cells, but that it does so in *trs130* and *trs33ts* mutant cells ((8, 10) and this study). On the other hand, Trs130 joins the complex due to its interaction with Trs120 and the

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assistance of Trs33, and perhaps Trs65. This idea is based on the finding that Trs130 does not join the TRAPP complex in *trs120*, *trs65ts* and *trs33ts* mutant cells ((8, 10) and this study). The finding that Trs33 interacts directly with Trs120, but facilitates bringing only Trs130 to the complex, suggests that Trs33 induces structural changes in Trs120 that increase the latter's interaction with Trs130. Together, our results indicate that even though Trs120 and Trs130 interact with each other and with TRAPP I/II subunits, the assistance of Trs33 or Trs65 is required for TRAPP II assembly.

Importantly, one of the mechanisms of TRAPP II assembly is conserved. Mammalian cells do not have a homologue for Trs65, but they do have two Trs33 homologues: TRAPPC6A and TRAPPC6B. TRAPPC6A is expressed ubiquitously, and a TRAPPC6A mutation in mice implicates it in melanosome biogenesis. The mosaic nature of the TRAPPC6A mutation suggests that there is a stage during development in which the function of TRAPPC6A is redundant, perhaps due to the presence of TRAPPC6B (17). Melanosome biogenesis is a process that requires transport beyond the ER-to-Golgi transport step. The finding that a mutation in TRAPPC6A affects melanosome biogenesis is consistent with our idea that Trs33 functions in transport from the late Golgi and that this role is conserved.

In summary, we propose that assembly of TRAPP II is a conserved and essential process in yeast and in human cells. In yeast, either Trs33 or Trs65 is required for this process. We expect that the Trs33 homologues TRAPPC6A and TRAPPC6B are required for the assembly of the mammalian TRAPP II complex.

Materials and Methods

Strains, plasmids and reagents

Strains and plasmids used in this study are summarized in Table S1. Trs33 was tagged with YFP at the C-terminus on the chromosome in cells, containing Trs120-myc, Cop1-RFP or Sec7-DsRed, to create NSY1415, 1428 and 1416, respectively, using PCR and homologous recombination in yeast (18). To create *trs33ts* strain, *TRS33* was deleted with KAN in Trs130-HA cells (NSY991), Trs120-myc cells (NSY1040), to create NSY1429 and 1430, respectively, or in Trs120-myc Trs130-HA cells, harboring GST-*TRS130* (*URA3*) plasmid, to create NSY1431. To create Trs130-GFP Sec7-DsRed strain, cells expressing Trs130-GFP (a gift from Scott Emr) were mated with cells expressing Sec7-DsRed (a gift from Benjamin Glick), diploids were sporulated and dissected to select for haploids expressing tagged versions of both proteins.

For yeast two-hybrid assay, TRAPP subunits' constructs were cloned into pACT2 and pGBDU-C2 using PCR followed by restriction enzyme digestion and ligation at SmaI and XhoI, or SmaI and SalI sites, respectively. For expression in *E. Coli TRS33* was cloned into pETDuet-1 (EMD Chemicals, Gibbstown, NJ), modified to contain GST-tag from vector pET-41a(+) (EMD Chemicals) in its multiple cloning site (MCS) 1, using SpeI and SalI restriction sites. *TRS120* and *TRS130-HA* were cloned into pCDFDuet-1 (EMD Chemicals) at MCS1 using Sac1/Sal1, and MCS2 using BglII/AvrII sites, respectively. For genetic interaction experiments, *TRS33* and *TRS65* constructs were cloned into yeast 2μ plasmid (pRS327). For *TRS33*, open reading frame including 1380 bp upstream and 571 bp downstream was introduced at SmaI and HindIII restriction sites. For *TRS65* open reading frame including 500 bp upstream and 300 bp downstream was introduced at SmaI and EcoRI sites. All yeast transformations were done using lithium acetate method combined with heat-shock. *E. Coli* transformation was done using electroporation.

Antibodies used in this study are: mouse monoclonal anti-HA (Covance, WI, USA); rabbit anti-glucose-6-phosphate dehydrogenase (G-6-PDH, Sigma-Aldrich, MO, USA), rabbit anti-

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GST (Molecular probes, OR, USA), mouse monoclonal anti-HIS (R&D systems, MN, USA), mouse monoclonal anti-myc (Santa-Cruz Biotechnology, Santa-Cruz, CA); affinity-purified rabbit anti-Ypt31 (7); affinity-purified rabbit anti-Ypt1 (4); mouse monoclonal anti-GFP (Roche Diagnostics, Indianapolis, IN); Texas Red-conjugated anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, West Grove, PA); anti-rabbit and anti-mouse IgG-Horseradish Peroxidase linked (GE Healthcare, Chalfont St. Giles, United Kingdom).

All chemical reagents were purchased from Thermo Fisher Scientific (Fair Lawn, NJ), unless otherwise noted. Media components, other than amino acids, as well as 5-Fluoroortic Acid (5-FOA) were purchased from US Biological (Swampscott, MA). ProtoGel for western blots was purchased from National Diagnostics (Atlanta, GA). Amino Acids and protease inhibitors were purchased from Sigma-Aldrich. EDTA-free protease inhibitor cocktail (PIC) was purchased from Roche Diagnostics. Restriction enzymes and buffers were purchased from New England Biolabs (Ispwich, MA). Isopropil-beta-D-thiogalactopyranoside (IPTG) was purchased from ACROS Organics (Fair Lawn, NJ). Dithiothreitol (DTT) was purchased from Invitrogen. Quantification of the bands on western blots was done using the Spot Denso option of the Alpha Imager (Alpha Innotech, San-Leonardo, CA).

Multiple sequence alignment

Multiple sequence alignment was done using the ClustalW2 (19) program with default values (gap opening penalty 10, and gap extension penalty 0.2, using the Gonnet250 matrix, no end gaps, and gap separation penalty 4). The GI Accession numbers of the sequences used for multiple alignments are given in Table S2. The structure of the Bet3-Trs33 heterodimer (PDB id-2c0j; (13)) was edited using the Rasmol Version 2.7.4.2 (20); (21)

Yeast culture conditions

For yeast two-hybrid and genetic interaction assays yeast cultures were grown overnight at 26°C in minimal (SC) media, normalized to the same density by OD_{600} and spotted onto agar plates in serial dilutions of 1-to-5 or 1-to-10. For live and immuno-fluorescence microscopy yeast cultures were grown at permissive temperature (26°C) in rich (YPD) or selective (when plasmid is used) media to log phase, and switched to a restrictive temperature (37°C) for two hours, when needed. For live microscopy using DsRed-FYVE construct, cells were grown in SC media lacking leucine and methionine overnight to allow for expression of DsRed-FYVE from *MET3* promoter, with consequent growth to mid-log in YPD to boost the expression of Trs33-YFP that appears poorly expressed in SC media.

Protein expression and western blotting

To determine the expression level of yeast two-hybrid constructs and level of endogenous Trs130-HA, 5 OD_{600}s of overnight or mid-log cell cultures, respectively, were spun down, re-suspended in 100 μl of Laemmli buffer, supplemented with PIC (Roche Diagnostics), 1 mM Pefabloc SC and 1 mM Benzamidine (Sigma-Aldrich, St. Lois, MO), boiled, vortexed with equal volume of glass beads (BioSpec Products, Bartlesville, OK) and subjected to Western blot analysis with anti-HA antibody.

Expression and co-precipitation of recombinant proteins

Plasmids encoding *HISX6-TRS120* or *Trs130-HA*, together with either empty GST or GST-Trs33 were transformed into Rosetta™ *E. coli* (EMD Chemicals). Transformants were grown in Terrific broth (http://www.bd.com/ds/technicalCenter/inserts/Terrific_Broth.pdf), at 37oC to OD_{600} 0.5. Cells were induced with IPTG to a final concentration of 0.1 mM for 4 hours at 21oC.

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Co-precipitation was done by resuspending cells in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) supplemented with 1.5 mM DTT, 1 mM PMSF, and 2X complete protease inhibitor cocktail (Roche Diagnostics). Cells were lysed by sonication, and lysates cleared at 10,000 g for 10 minutes at 4oC. Cleared lysates were then added to glutathione sepharose 4B (GE Healthcare). After 90 minutes of agitation at 4oC, the glutathione sepharose was washed 5 times in wash buffer (PBS supplemented with 1 mM PMSF and 1.5 mM DTT). Proteins were removed from sepharose beads by boiling 5 minutes in Laemmli buffer. Results were obtained by Western blotting using anti-His, anti-HA, and anti-GST antibodies.

General secretion

General secretion assay was performed as described previously (10), except media proteins' amounts were quantified using phosphor screen (GE Healthcare) and phosphor imager STORM860 (Molecular Dynamics, Sunnyvale, CA) and were normalized to the amounts of labeled intracellular proteins.

Precipitation of GST complexes from yeast lysates

GST-tagged proteins were expressed in yeast and precipitated as described previously (22), except the extracts were made in buffer containing 1M NaCl, PIC, 1 mM Pefabloc SC and 1 mM benzamidine, and further diluted with buffer containing no NaCl to create a final NaCl concentration of 200 mM. 0.1% Triton × 100 was added to lysates with the following incubation at room temperature for 10 min to break membranes, and centrifugation at 600g for 10 min at 4°C to clear lysates. Lysates were then incubated with glutathione-agarose beads for 2 hours and washed twice with wash buffer (100 mM Tris HCl pH 7.5, 4 mM $MgCl_2$, 10% glycerol, 200 mM NaCl, 5 mM DTT, 0.2% Triton ×100). Precipitated proteins were eluted with Laemmli buffer and subjected to Western blot analysis using anti-GST, anti-myc and anti-HA antibody. GDP release assays were done as previously described (10).

Microscopy

Immuno-fluorescence microscopy was performed as previously described (23) using anti-Ypt1 in 1:500 dilution, or anti-Ypt31 in 1:250 dilution, as a primary antibody. Slides were visualized using deconvolution Axioscope microscope (Carl Zeiss, Thornwood, NY) as described (10), except a series of 5–9 Z stacks, 500 nm each, were collected for each field. Electron microscopy was done as previously described (10).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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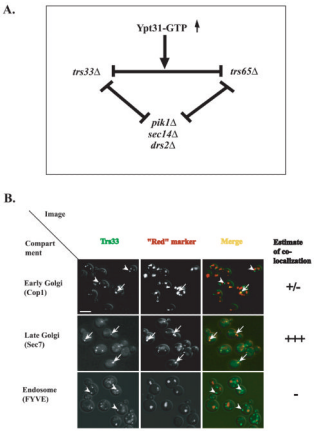

Figure 1.

Trs33 and Trs65 share genetic interactions and localization similarities. **(A)** Either *TRS33* or *TRS65* is required for cell viability and the two genes share genetic interactions. TRS33 and TRS65 exhibit synthetic lethality that can be suppressed by over-expression of Ypt31/32 (top, (17)). In addition, both genes are synthetic lethal with genes that function in the late Golgi: *PIK1*, *SEC14*, and *DRS2* (bottom, (12)). Flat arrows depict lethality of the double-deletion mutant, whereas the arrow represents suppression of the lethality. **(B)** Trs33 localizes mainly to the trans-Golgi. Trs33-YFP co-localizes with the trans-Golgi marker Sec7-DsRed (middle), and to a lesser extent with the cis-Golgi marker Cop1-RFP (top), but not with the endosomal marker DsRed FYVE domain (bottom). Endogenous Trs33 was tagged at its C-terminus with YFP in cells expressing either Sec7-DsRed or Cop1-RFP from the chromosome, or DsRed FYVE domain from a plasmid. Cells were grown to mid-log phase and examined by de-convolution microscopy. Panels show from left to right: YFP-Trs33, the red-labeled compartmental marker, a merge, and merge pus differential interference contrast (DIC). Arrows point to regions of co-localization, and arrowheads point to regions in which Trs33-YFP does not co-localize with the compartmental markers. Bar, 5 μm. Results shown are representative of two independent experiments.

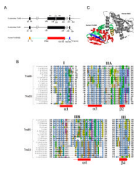

Figure 2.

Trs33 and Trs65 share sequence similarity. **(A)** Domain diagram of Trs65 (amino acids 1–350/560; top) and Trs33 (middle) from *S. cerevisiae*, and TRAPPCB, one of the human homologues of Trs33 (bottom). Domain number is shown at the top and amino-acid number is shown at the bottom of each diagram. The conserved domains I, II and III, are shown in orange, red and blue, respectively, and domains not conserved between the three proteins are shown in green. **(B)** Multiple alignments in domains I, II and III of Trs65 proteins from twelve fungi genomes and Trs33 proteins from nine genomes from yeast to humans. Multiple alignment analysis was done using ClustalW2. Structural elements, α-helices and β-sheets, from the published structure of TRAPPC6B are marked at the bottom. **(C)** The structure of the human Trs33-Bet3 hetero-dimer is shown, with domains I, II, and III, conserved between Trs33 and Trs65, shown in orange, red and blue (as in panel A). The α-helix-1 in domain I (orange) of TRAPP6B is inserted between two helices of Bet3. The other conserved domains are free for other interactions.

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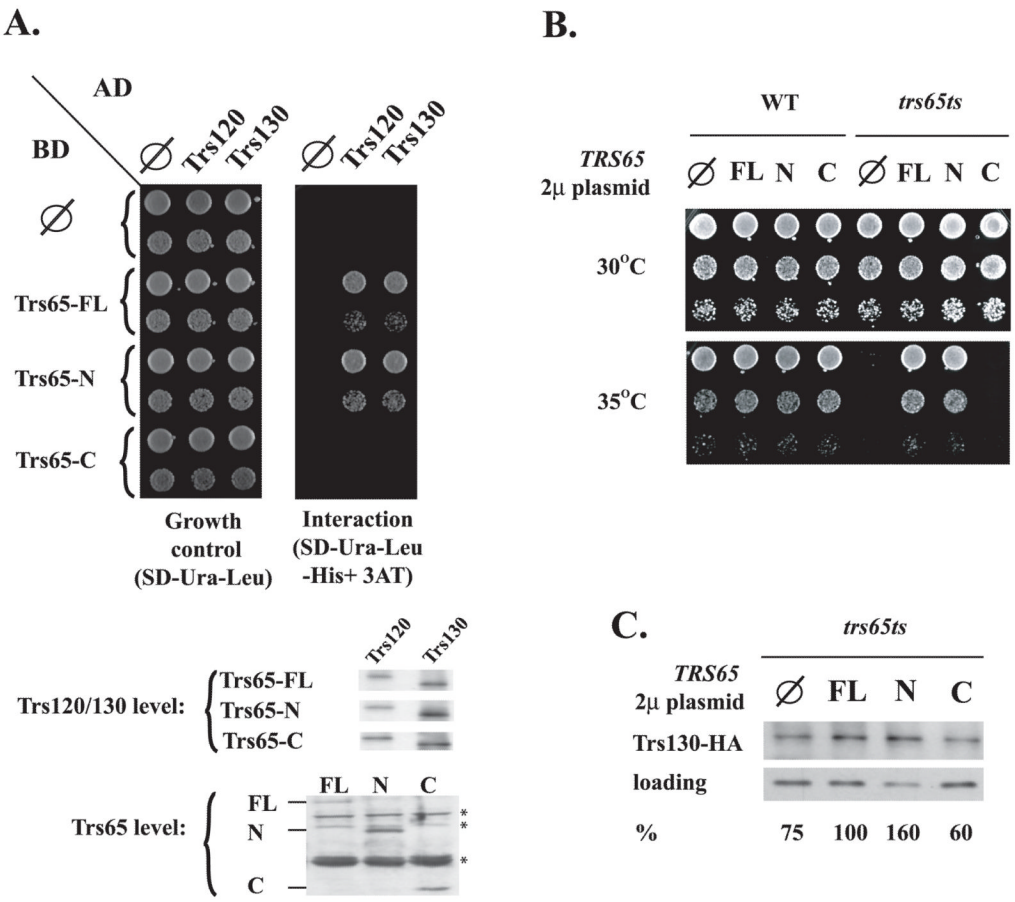

Figure 3.

The Trs33-related domain of Trs65, Trs65N, is sufficient for its interactions and function. **(A)** Like Trs65 full-length (FL), Trs65N, but not Trs65C, interacts with Trs120 and Trs130 in the yeast two-hybrid assay. Cells were transformed with two plasmids: pGBDU (*GAL4-BD, URA3*) binding domain-tagged Trs65-FL, Trs65N or Trs65C, and pACT2 (*GAL4-AD, LEU2*) activation domain-tagged TRAPP II subunits: Trs120 or Trs130. Cells were plated in 10-fold serial dilutions from top to bottom and incubated at 30°C. The growth control of the double transformants is shown on the left (SD-Ura-Leu), and interaction is shown on the right (SD-Ura-Leu-His+3 mM 3AT). Expression, as determined by immuno-blot analysis, is shown at the bottom (* denote non-specific bands). **(B)** The Trs65N domain is sufficient for the complementation of the *trs65ts* growth phenotype. *Trs65ts* mutant cells were transformed with 2μ plasmids expressing Trs65 (full-length, FL), Trs65N or Trs65C. Cells were plated in 10-fold serial dilutions from top to bottom on SD-Ura plates and incubated at 30°C for growth control (top) or 35°C (bottom), a restrictive temperature for the *trs65ts* mutant strain (see empty vector control, ∅). *Trs65ts* mutant cells expressing Trs65 FL or Trs65N, but not Trs65C, can grow at 35°C. **(C)** The Trs65N domain is sufficient to complement Trs65's function in maintaining Trs130 protein level. Transformants from panel B were grown to mid-log phase and cell lysates were subjected to immuno-blot analysis using anti-HA (for Trs130), and anti-G-6-PDH (as a loading control). Bands were quantified and the percentage of Trs130-HA is indicated at the bottom (100% corresponds to mutant cells complemented by FL-Trs65). Empty vector (∅) for all plasmids is shown as a negative control. Results presented in this figure are representative of at least two independent experiments.

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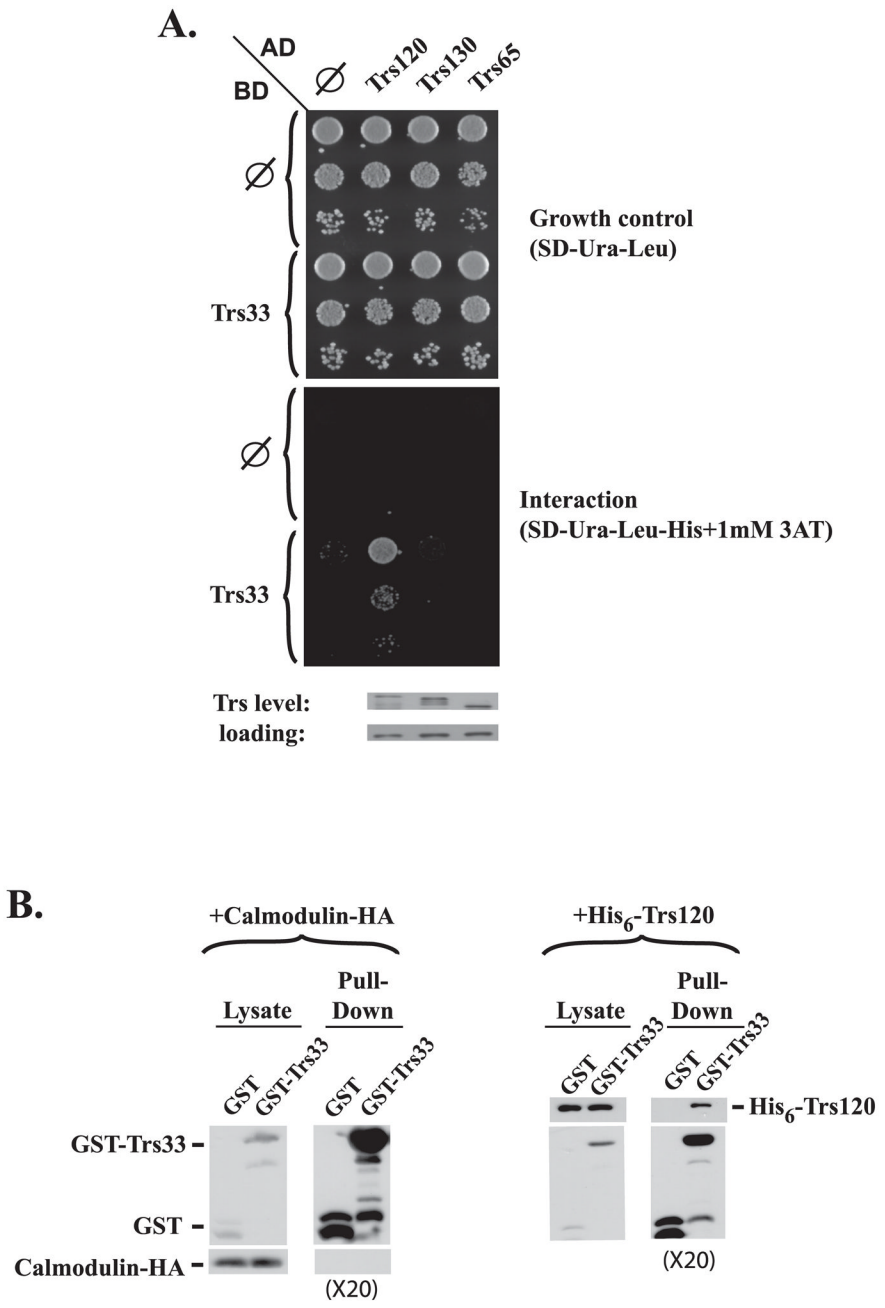

Figure 4.

Trs33 interacts directly with the TRAPP II-specific subunit Trs120. **(A)** Trs33 interacts with the TRAPP II-specific subunit Trs120 in the yeast-two hybrid assay. The experiment was done as described for Figure 3A, except that Trs33 was expressed from the BD vector and Trs120, Trs130 and Trs65 were expressed from the AD vectors. **(B)** Direct interaction of bacterially expressed recombinant Trs33 protein with Trs120. GST-Trs33, or GST as a negative control for precipitation, was co-expressed with His6-Trs120 or calmodulin-HA as a negative control for co-precipitation. GST was pulled down using glutathione sepharose beads and the co-precipitation of His6-Trs120 and calmodulin-HA was detected using immuno-blot analysis with anti-His6 or anti-HA antibodies. Trs120 and calmodulin were present in all lysates (left), but only Trs120 co-precipitated with GST-Trs33 and not with

GST (right, the concentration of the pull-downs is 20-fold higher than the concentration of the lysates). Results shown in this figure are representative of at least two independent experiments.

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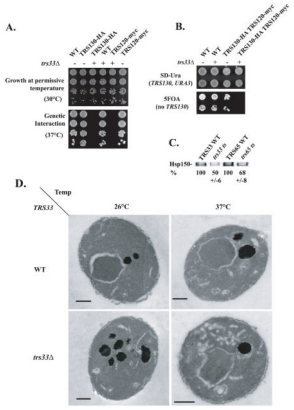

Figure 5.

TRS33 interacts genetically with TRAPP II-specific subunits: Characterization of the *trs33ts* mutants. **(A)** Deletion of *TRS33* in cells expressing either Trs120-myc or Trs130-HA results in a temperature-sensitive growth phenotype. Cells containing the different combinations of *TRS33, TRS120* and *TRS130* (as detailed at the top), were plated on YPD plates (in 10– fold serial dilutions from top to bottom) and incubated at the indicated temperatures. All the strains can grow at 30°C (top), but *trs33*Δ *TRS120*-myc and *trs33*Δ *TRS130*-HA cannot grow at 37°C (bottom). **(B)** Deletion of *TRS33* in cells expressing the tagged versions of both *TRS120* and *TRS130* results in lethality. *TRS33* was deleted in wild type cells or in cells expressing Trs120-myc and Trs130-HA on the chromosome and Trs130 from a 2μ *URA3* plasmid. All strains can grow on SD-Ura plates. However, only the triple mutant, *trs33*Δ *TRS120*-myc *TRS130*-HA, cannot grow on 5FOA plates, which select against the presence of the Trs130-expressing *URA3* plasmid. **(C)** General secretion is defective in *trs33ts* mutant cells. Wild type and *trs33ts* mutant cells (*trs33*Δ *TRS130*-HA) were tested for their ability to secrete Hsp150 to the medium. The 150 kDa band was quantified and the percentage of Hsp150 secreted by mutant versus wild type cells (+/− represents the SEM) is indicated at the bottom. For comparison, we included *trs65ts* mutant cells and their corresponding wild type in the assay. **(D)** Aberrant membranous structures accumulate in *trs33ts* mutant cells at their permissive temperature. Wild type and *trs33ts* mutant cells (*trs33*Δ *TRS130*-HA) were incubated at the indicated temperature for 90 minutes, and then were fixed and processed for electron microscopy analysis. Representative cells are shown. Bar, 1 μm. Results shown in this figure are representative of at least two independent experiments.

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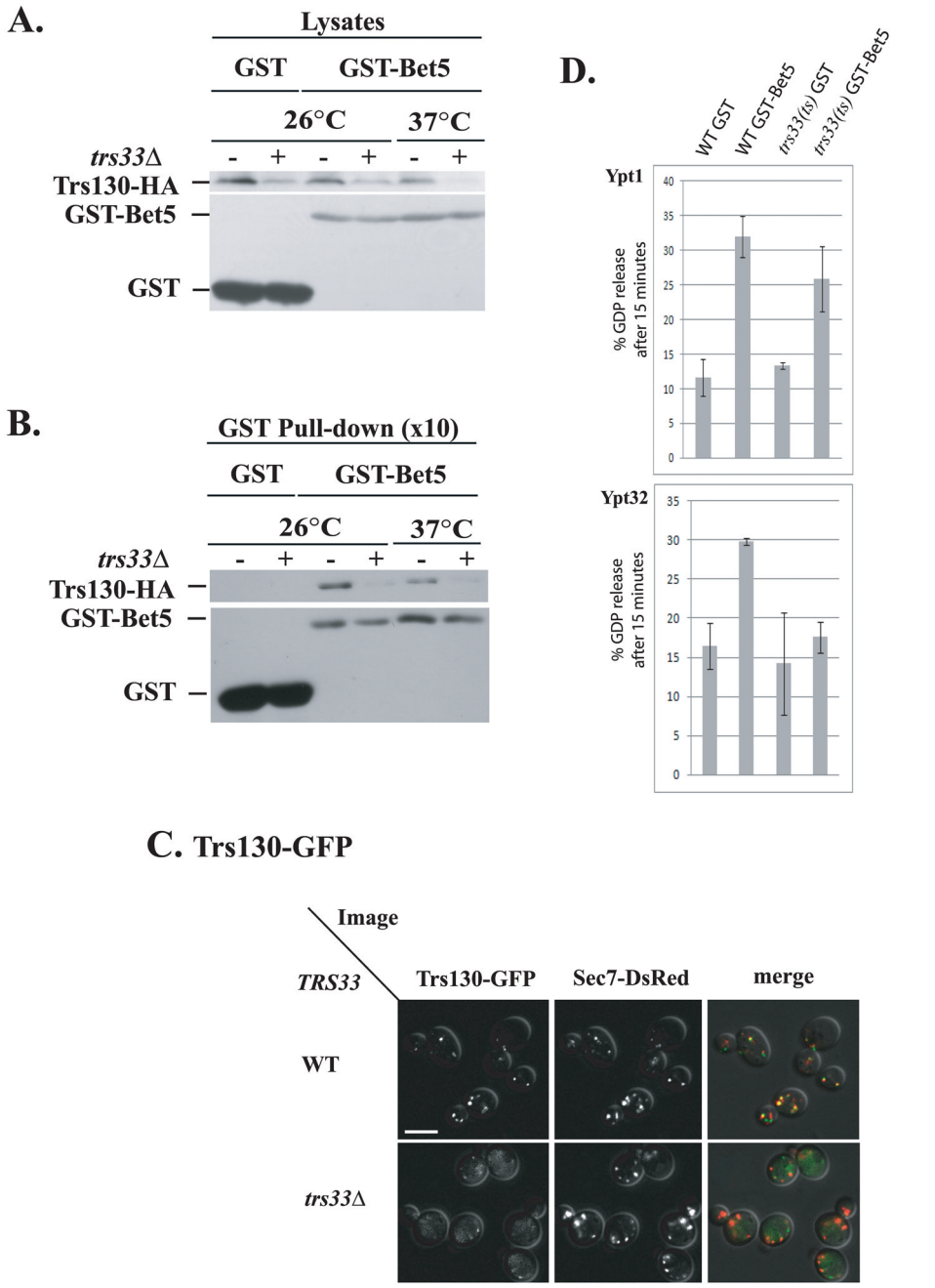

Figure 6.

Effect of *TRS33* deletion on the TRAPP II-specific subunit Trs130. **(A)** Trs130 protein level is lower in *trs33*Δ cell lysates when compared to wild-type cells even at the permissive temperature (26°C). Cells expressing GST or GST-Bet5 were grown to mid-log phase at 26°C or shifted to 37°C for 1.5 h. Cell lysates were subjected to immuno-blot analysis using anti-HA (for Trs130), anti-GST (for GST and GST-Bet5) or anti-G-6-PDH (as a loading control). **(B)** Trs130 protein level is lower in GST-Bet5 associated complexes purified from *trs33*Δ mutant cell lysates. Cells were grown as described in panel A, and GST-Bet5- or GST-associated complexes were purified from wild type and mutant cell lysates and subjected to immuno-blot analysis, using anti-GST (for GST and GST-Bet5) and anti-HA

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(for Trs130-HA) antibodies. **(C)** Trs130-GFP localization is diffuse in *trs33*Δ cells. Wild type and mutant cells expressing Sec7-DsRed as a Golgi marker were grown at 26°C and examined by live-cell microscopy as described for Figure 1B. Trs130-GFP is shown on the left, Sec7-DsRed in the middle, and the merge on the right. Bar, 5 μm. **(D)** TRAPP purified from *trs33ts* mutant cells is defective in Ypt32, but not Ypt1, GEF activity. Bet5-associated complexes purified from wild type or mutant cells, as described in panel B, were used in a GDP-release assay for Ypt1 or Ypt32. Percent GDP release after 15 minutes is shown. Results shown in this figure are representative of at least three independent experiments.

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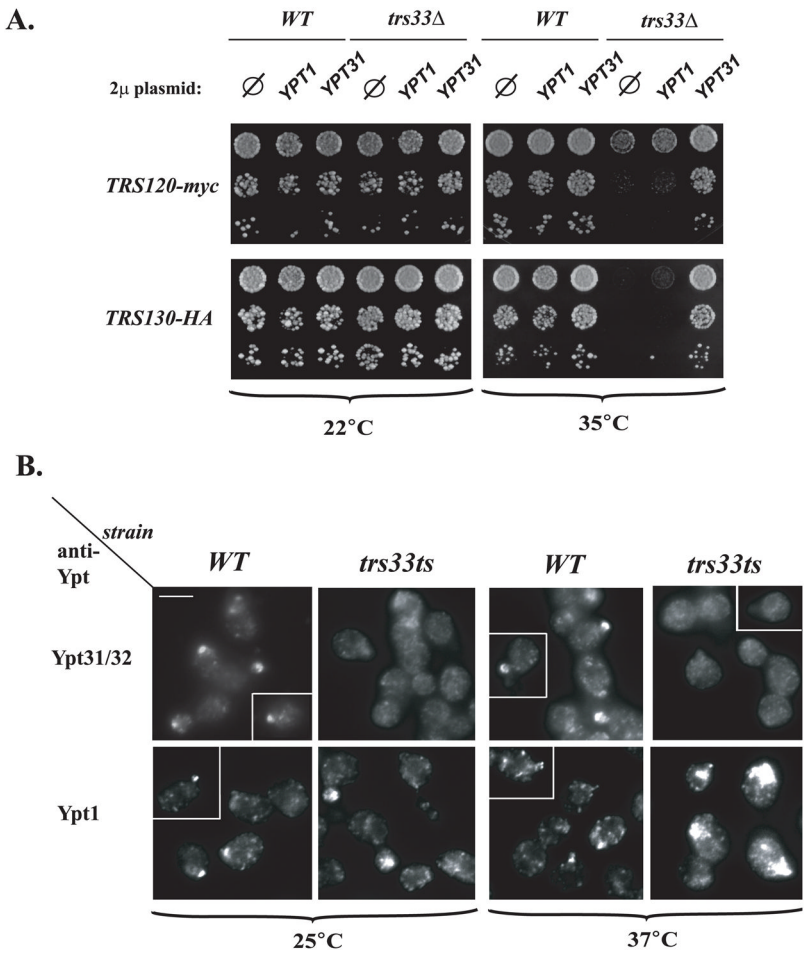

Figure 7.

Interactions between *trs33ts* on Ypt1/Ypt31/32. **(A)** Over-expression of Ypt31, but not Ypt1, rescues the temperature-sensitive growth phenotype of *trs33ts* mutant cells. Wild type and *trs33*Δ mutant cells (*TRS120*-myc, top; *TRS130*-HA, bottom) were transformed with 2μ plasmids expressing Ypt1, Ypt31 or empty vector (∅) as a negative control. Transformants were plated on SD-Ura as described for Figure 5A and incubated at 22°C for growth control (left) and at 35°C for testing the ability of the plasmid to rescue the mutant. **(B)** Ypt31/32 and Ypt1 intra-cellular localization is affected in opposite ways by the *trs33ts* mutation. Wild type and *trs33ts* mutant cells (both expressing Trs120-myc) were grown to mid-log phase at 25°C (left), or shifted to 37°C for 1.5 h (right). Cells were fixed and processed for immuno-fluorescence microscopy using anti-Ypt31/32 (top) or anti-Ypt1 (bottom) antibodies. Inset panels show additional small-budded cells taken from separate fields. The Ypt31/32 staining pattern is more diffuse in *trs33ts* mutant cells, especially at 37°C, as compared with wild type cells. In contrast, the Ypt1 staining pattern at 37°C is more intense than that seen in wild type cells. Bar, 5 μm. Results shown in this figure are representative of at least two independent experiments.

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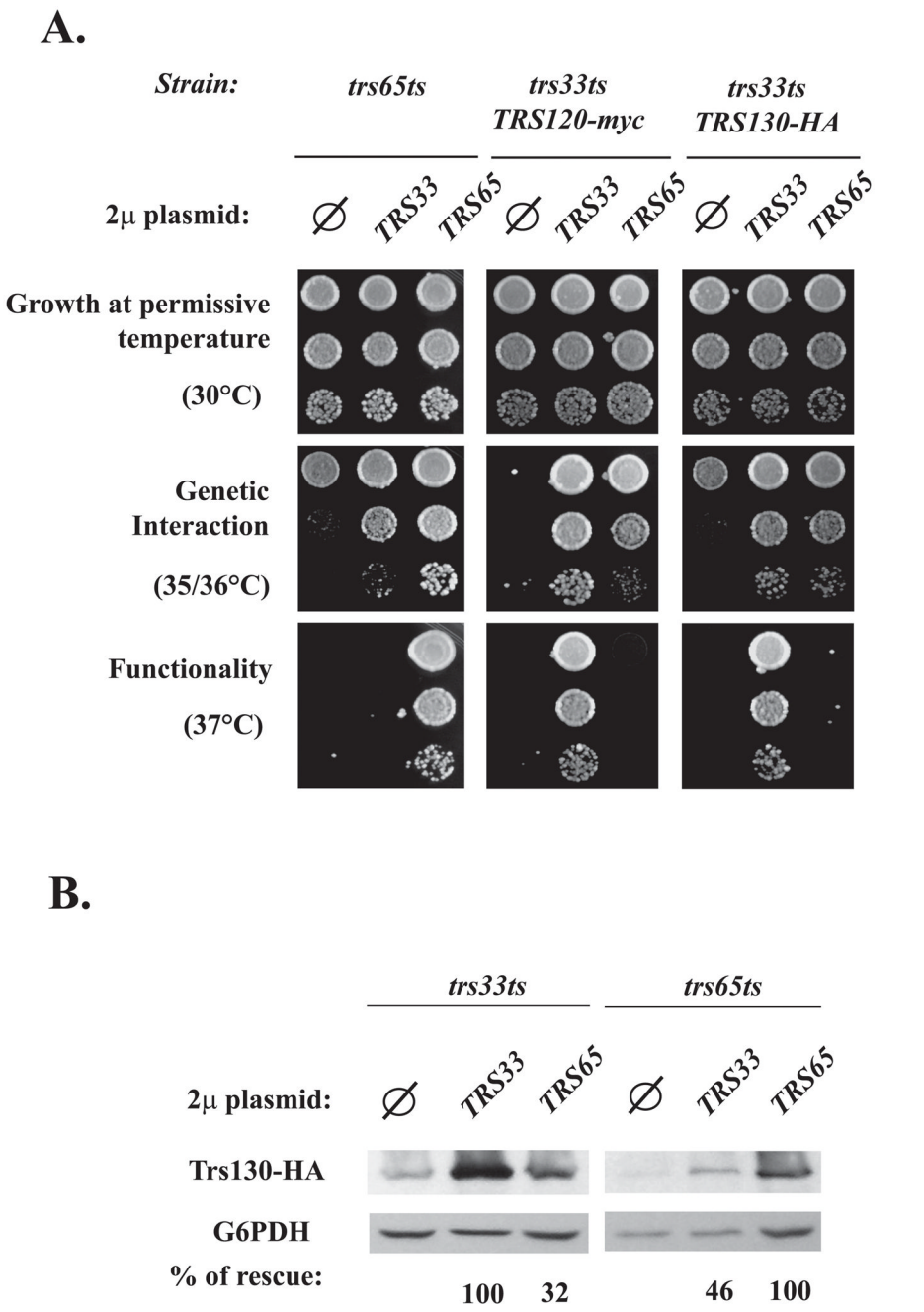

Figure 8.

Trs33 and Trs65 can rescue each other's mutant phenotypes. **(A)** Over-expression of Trs33 and Trs65 can rescue the growth defect of *trs65ts* and *trs33ts*, respectively. Mutant cells, *trs65ts* (left), *trs33*Δ *TRS120*-myc (middle) and *trs33*Δ *TRS130*-HA (right), were transformed with the following 2μ plasmids: empty vector (∅) as a negative control, and plasmids expressing Trs33 or Trs65. Transformants were plated as described for Figure 7 at the following temperatures: permissive temperature to show cell growth (top), restrictive temperature to show mutant phenotype (bottom), and an intermediate temperature to show rescue (middle, 35°C for *trs65ts* and *trs33ts-TRS130-HA* and 36°C for *trs33ts-TRS130-HA*). All three mutant strains do not grow at 37°C unless they are complemented by their

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corresponding gene. Importantly, over-expression of Trs33 can rescue the growth phenotype of *trs65ts* mutant cells, and over-expression of Trs65 can rescue the growth phenotype of *trs33ts* mutant cells up to one degree below the temperature in which they can be rescued by their corresponding gene. **(B)** Over-expression of Trs65 can rescue the low Trs130 protein level phenotype of *trs33ts* mutant cells and vise versa. *Trs33ts* (*trs33*Δ *TRS130*-HA; left panel) or *trs65ts* (*trs65*Δ *Trs130-HA Trs120-myc*; right panel) mutant cells were transformed with 2μ plasmids as described for panel A. The level of Trs130-HA was determined by immuno-blot analysis as described for Figure 3C and is indicated at the bottom as percentage of rescue (100% corresponds to *trs33ts* mutant cells complemented by Trs33 or *trs65ts* mutant cells complemented by Trs65). Results shown in this figure are representative of at least two independent experiments.

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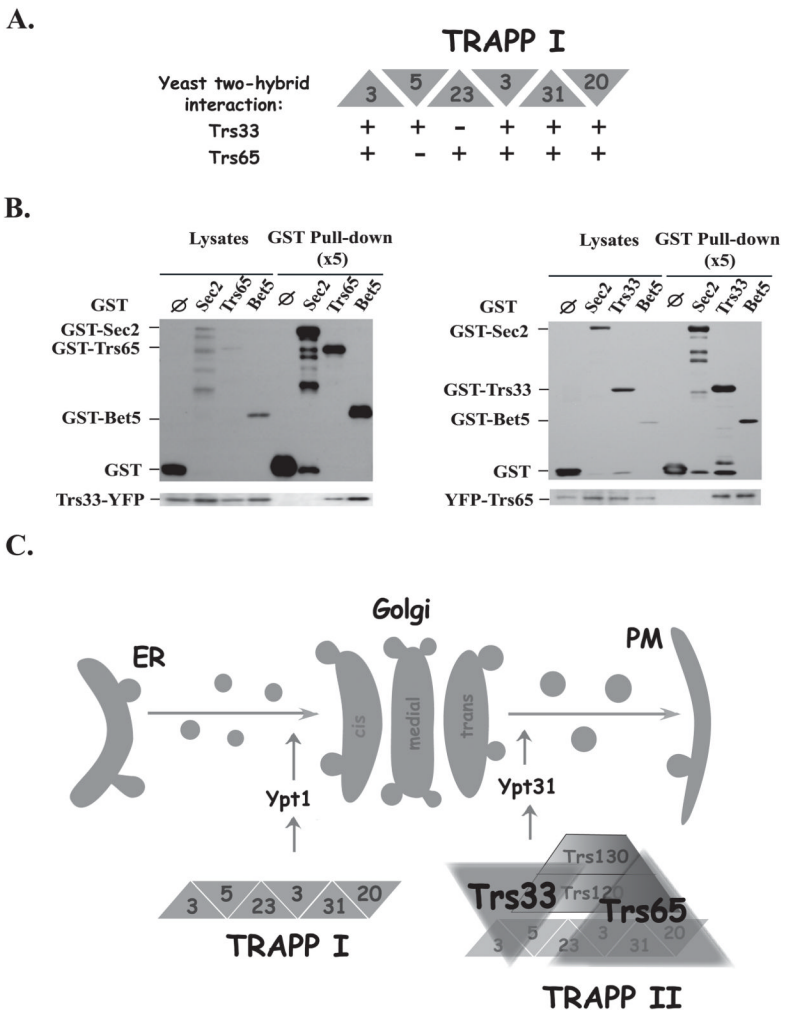

Figure 9.

Trs33 and Trs65 can reside on the same TRAPP complex. **(A)** The interactions of Trs33 and Trs65 with TRAPP I/II subunits do not completely overlap. The diagram summarizes yeast-two hybrid interactions of Trs33 and Trs65 with TRAPP I/II subunits shown in Figure S3. Whereas Trs33 interacts with Bet5 and not Trs23, Trs65 interacts with Trs23 and not Bet5. The proposed architecture of the TRAPP I complex is shown at the top (6). **(B)** Trs33 co-purifies with GST-Trs65 and vise versa. Yeast cells expressing Trs33-YFP (left panel) or YFP-Trs65 (right panel) were transformed with plasmids over-expressing GST (∅), GST-Sec2, GST-Trs65, GST-Trs33 or GST-Bet5. GST-associated complexes were purified and subjected to immuno-blot analysis as described for Figure 6A and B. Whereas all the tested strains expressed Trs33-YFP or YFP-Trs65 (left lanes), these proteins co-purified with Bet5 (as a positive control), and Trs65 or Trs33, respectively, but not with GST or GST-Sec2 (right lanes). Results shown are representative of two independent experiments. **(C)** Model for assembly of the TRAPP II complex. The five TRAPP I/II subunits are assembled on the cis-Golgi, where the complex acts as a GEF for Ypt1. On the trans-Golgi, Trs33 and Trs65 assist in bringing Trs120 and Trs130 to TRAPP I to form the TRAPP II complex that can act as a GEF for Ypt31/32. When both Trs33 and Trs65 are present, they can each reside one side of the complex: Trs33 on the Bet3/Bet5 side and Trs65 on the Trs23/Bet3/Trs31/Trs20 side. Because Trs33 can also interact with Trs31, we propose that in *trs65*Δ cells, Trs33 can reside on both sides of the TRAPP complex.

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Table 1

TRAPP subunits

Subunit	Essentiality[b]
TRAPP I/II [a]:	
Bet3	+
Trs31	+
Bet5	+
Trs23	+
Trs20	+
Trs33	−
Trs85/GSG1	−
TRAPP II-specific [a]:	
Trs120	+
Trs130	+
Trs65/Kre11	−

[a] Based on co-migration on a sizing column (16).

[b] Data from SGD (12).