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# Cpr1 cyclophilin and Ess1 parvulin prolyl isomerases interact with the tombusvirus replication protein and inhibit viral replication in yeast model host

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ABSTRACT

To identify host proteins interacting with the membrane-bound replication proteins of tombusviruses, we performed membrane yeast two-hybrid (MYTH) screens based on yeast cDNA libraries. The screens led to the identification of 57 yeast proteins interacting with replication proteins of two tombusviruses. Results from a split ubiquitin assay with 12 full-length yeast proteins and the viral replication proteins suggested that the replication proteins of two tombusviruses interact with a similar set of host proteins. Follow-up experiments with the yeast Cpr1p cyclophilin, which has prolyl isomerase activity that catalyzes *cis–trans* isomerization of peptidyl–prolyl bonds, confirmed that Cpr1p interacted with the viral p33 replication protein in yeast and *in vitro*. Replication of *Tomato bushy stunt virus* replicon RNA increased in *cpr1*Δ yeast, while over-expression of Cpr1p decreased viral replication. We also show that the Ess1p parvulin prolyl isomerase partly complements Cpr1p function as an inhibitor of tombusvirus replication.



## Introduction

Viruses are intracellular parasites that rely on the vast resources of the host cells for their replication. The viral replication process requires co-opting of an unknown number of host proteins and the reprogramming of cellular pathways. Another level of complexity in virus–host interactions is the activation of host antiviral responses that target different players and steps in the viral replication process. Not surprisingly, all these processes depend on protein–protein interactions. Therefore, there is a major on-going effort to identify all the host proteins interacting with viral replication proteins.

*Tomato bushy stunt virus* (TBSV) and the closely related *Cucumber necrosis virus* (CNV) and *Carnation Italian ringspot virus* (CIRV) are tombusviruses with small (+)RNA genomes. Due to the development of yeast (*Saccharomyces cerevisiae*) as a model host, tombusviruses have recently emerged as model viruses to study virus replication, recombination, and virus–host interactions (Jaag et al., 2010; Nagy and Pogany, 2006; Panavas and Nagy, 2003b; Panaviene et al., 2004c; Pogany and Nagy, 2008; White and Nagy, 2004). TBSV codes for two essential replication proteins, p33 and $p92^{pol}$. The auxiliary p33 replication protein is involved in the recruitment of the TBSV (+)RNA to the site of replication, which is the cytosolic surface of peroxisomal membranes (Jonczyk et al., 2007; McCartney et al., 2005; Panavas et al., 2005a; Pogany et al., 2005). The $p92^{pol}$ RNA-dependent RNA polymerase (RdRp) protein, which is the translational readthrough product of the p33 open reading frame, binds to p33 replication protein leading to the assembly of the functional membrane-bound replicase complex (Panavas et al., 2005a; Panaviene et al., 2004c, 2005; Pogany and Nagy, 2008).

Our current knowledge on tombusvirus–host interactions is based on recent genome-wide screens covering 95% of yeast genes that have identified more than 150 host genes affecting TBSV replication or recombination (Jiang et al., 2006; Panavas et al., 2005b; Serviene et al., 2006; Serviene et al., 2005). In addition, proteomics analysis of the highly purified tombusvirus replicase complex identified 6 host proteins in the replicase, in addition to the viral p33 and $p92^{pol}$ replication proteins (Li et al., 2008a, 2009; Serva and Nagy, 2006). These host proteins, such as heat shock protein 70 (hsp70), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Cdc34p Ub-conjugating enzyme, and eEF1A translation elongation factor affect the assembly of the viral replicase complex or regulate viral RNA replication (Li et al., 2008a, 2009; Pogany et al., 2008; Wang and Nagy, 2008; Wang et al., 2009a).

The tombusvirus replication proteins are bound to the peroxisomal membrane or in its absence to the ER membrane in infected cells (Cheng et al., 2005; Jonczyk et al., 2007; McCartney et al., 2005; Pathak et al., 2008). Therefore, many host protein–viral replication protein interactions are expected to occur on membrane surfaces. However, the previous global proteomics approach using the yeast

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**Table 1**
The name and functions of yeast proteins bound to CNV p33, p92 and CIRV p36.

| Gene[1] | FR[2] | Ver[3] | Gene function[4] |
|---|---|---|---|
| ANB1 | 6/1/0 | + | Translation elongation factor eIF-5A, previously thought to function in translation initiation; similar to and functionally redundant with Hyp2p |
| AQY1 | 3/0/0 | + | Spore-specific water channel that mediates the transport of water across cell membranes |
| ARC15 | 0/0/1 | nd | Subunit of the ARP2/3 complex, which is required for the motility and integrity of cortical actin patches |
| ARF1 | 1/0/0 | + | ADP-ribosylation factor, GTPase of the Ras superfamily involved in regulation of coated vesicle formation in intracellular trafficking within the Golgi; functionally interchangeable with Arf2p |
| BGL2 | 0/0/2 | nd | Endo-beta-1,3-glucanase, major protein of the cell wall, involved in cell wall maintenance |
| CCW12 | 2/0/0 | – | Cell wall mannoprotein |
| COF1 | 1/0/0 | + | Cofilin, promotes actin filament depolarization in a pH-dependent manner; binds both actin monomers and filaments and severs filaments; thought to be regulated by phosphorylation; ubiquitous and essential in eukaryotes |
| CPR1 | 2/0/0 | + | Cytoplasmic peptidyl-prolyl cis-trans isomerase (cyclophilin), catalyzes the cis-trans isomerization of peptide bonds N-terminal to proline residues |
| EGD2 | 13/3/0 | + | Alpha subunit of the heteromeric nascent polypeptide-associated complex (NAC) involved in protein sorting and translocation, associated with cytoplasmic ribosomes |
| FAA3 | 0/1/0 | nd | Long chain fatty acyl-CoA synthetase, has a preference for C16 and C18 fatty acids |
| FBA1 | 1/0/0 | – | Fructose 1,6-bisphosphate aldolase, required for glycolysis and gluconeogenesis; catalyzes conversion of fructose 1,6 bisphosphate to glyceraldehyde-3-P and dihydroxyacetone-P |
| FIP1 | 1/0/0 | + | Subunit of cleavage polyadenylation factor (CPF), interacts directly with poly(A) polymerase (Pap1p) to regulate its activity |
| FRE8 | 1/0/0 | nd | Protein with sequence similarity to iron/copper reductases, involved in iron homeostasis |
| GND1 | 1/0/0 | – | 6-phosphogluconate dehydrogenase, catalyzes an NADPH regenerating reaction in the pentose phosphate pathway |
| HST1 | 0/1/0 | nd | NAD(+)-dependent histone deacetylase |
| HTA2 | 2/0/0 | + | Histone H2A, core histone protein required for chromatin assembly and chromosome function; one of two nearly identical (see also HTA1) subtypes |
| ICY2 | 1/0/0 | + | Protein of unknown function; mobilized into polysomes upon a shift from a fermentable to nonfermentable carbon source |
| KIN4 | 0/1/0 | nd | Serine/threonine protein kinase that inhibits the mitotic exit network (MEN) when the spindle position checkpoint is activated |
| MFalpha1 | 3/0/0 | nd | Mating pheromone alpha-factor, pheromone-dependent signal transduction |
| PGK1 | 1/0/0 | nd | 3-phosphoglycerate kinase, catalyzes transfer of high-energy phosphoryl groups from the acyl phosphate of 1,3-bisphosphoglycerate to ADP to produce ATP |
| PKH2 | 0/0/1 | nd | Serine/threonine protein kinase involved in sphingolipid-mediated signaling pathway that controls endocytosis; redundant with Pkh1p |
| RHR2 | 1/0/1 | + | Constitutively expressed isoform of DL-glycerol-3-phosphatase; involved in glycerol biosynthesis, induced in response to both anaerobic and osmotic stress |
| RNR4 | 1/0/0 | + | Ribonucleotide-diphosphate reductase (RNR), small subunit; the RNR complex catalyzes the rate-limiting step in dNTP synthesis |
| RPL4A | 0/1/0 | nd | N-terminally acetylated protein component of the large (60S) ribosomal subunit, nearly identical to Rpl4Bp and has similarity to E. coli L4 and rat L4 ribosomal proteins |
| RPL9A | 1/0/0 | + | Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl9Bp and has similarity to E. coli L6 and rat L9 ribosomal proteins |
| RPL11A | 0/0/1 | nd | Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl11Bp; involved in ribosomal assembly |
| RPL13A | 1/0/0 | + | Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl13Bp; not essential for viability; has similarity to rat L13 ribosomal protein |
| RPL14A | 0/1/0 | nd | N-terminally acetylated protein component of the large (60S) ribosomal subunit, nearly identical to Rpl14Bp and has similarity to rat L14 ribosomal protein |
| RPL30 | 1/0/0 | + | Protein component of the large (60S) ribosomal subunit, has similarity to rat L30 ribosomal protein |
| RPL32 | 1/0/0 | – | Protein component of the large (60S) ribosomal subunit, has similarity to rat L32 ribosomal protein |
| RPL35A | 1/0/0 | + | Protein component of the large (60S) ribosomal subunit, identical to Rpl35Bp and has similarity to rat L35 ribosomal protein |
| RPP2B | 2/0/0 | nd | Ribosomal protein P2 beta, a component of the ribosomal stalk, which is involved in the interaction between translational elongation factors and the ribosome; regulates the accumulation of Rpp1Ap and Rpp1Bp in the cytoplasm |
| RPS2 | 1/0/0 | nd | Protein component of the small (40S) subunit, essential for control of translational accuracy; phosphorylation by C-terminal domain kinase I (CTDK-I) enhances translational accuracy; similar to E. coli S5 and rat S2 ribosomal proteins |
| RPS3 | 0/1/0 | nd | Protein component of the small (40S) ribosomal subunit, has apurinic/apyrimidinic (AP) endonuclease activity |
| RPS4A | 0/1/0 | nd | Protein component of the small (40S) ribosomal subunit; mutation affects 20S pre-rRNA processing; identical to Rps4Bp and has similarity to rat S4 ribosomal protein |
| RPS12 | 2/0/0 | + | Protein component of the small (40S) ribosomal subunit; has similarity to rat ribosomal protein S12 |
| RPS15 | 0/1/0 | nd | Protein component of the small (40S) ribosomal subunit; has similarity to E. coli S19 and rat S15 ribosomal proteins |
| RPS30B | 2/0/1 | + | Protein component of the small (40S) ribosomal subunit; nearly identical to Rps30Ap and has similarity to rat S30 ribosomal protein |
| SAM37 | 1/0/0 | + | Component of the Sorting and Assembly Machinery (SAM or TOB complex) of the mitochondrial outer membrane, which binds precursors of beta-barrel proteins and facilitates their outer membrane insertion; contributes to SAM complex stability |
| SCS2 | 1/0/0 | + | Integral ER membrane protein that regulates phospholipid metabolism, disruption causes inositol tauxotrophy above 34 degrees C, VAP homolog |
| SGT2 | 1/0/0 | – | Glutamine-rich cytoplasmic protein that contains tetratricopeptide (TPR) repeats, which often mediate protein-protein interactions; has similarity to human SGT, which is a cochaperone that negatively regulates Hsp70 |
| SHO1 | 1/0/0 | + | Transmembrane osmosensor, participates in activation of both the Cdc42p- and MAP kinase-dependent filamentous growth pathway and the high-osmolarity glycerol response pathway |
| SPC2 | 2/0/0 | + | Subunit of signal peptidase complex, which catalyzes cleavage of N-terminal signal sequences of proteins targeted to the secretory pathway; homologous to mammalian SPC25 |
| SSN8 | 1/0/0 | nd | Cyclin-like component of the RNA polymerase II holoenzyme, involved in phosphorylation of the RNA polymerase II C-terminal domain |
| SSS1 | 3/0/0 | + | Subunit of the Sec61p translocation complex that forms a channel for passage of secretory proteins through the endoplasmic reticulum membrane |
| STM1 | 1/0/0 | – | Protein required for optimal translation under nutrient stress; perturbs association of Yef3p with ribosome's; involved in TOR signaling; binds G4 quadruplex and purine motif triplex nucleic acid |
| SUR7 | 1/0/0 | nd | Plasma membrane protein that localizes to furrow-like invaginations, membrane sphingolipid content are altered in mutants |
| TDH2 | 1/0/0 | + | Glyceraldehyde-3-phosphate dehydrogenase, involved in glycolysis and gluconeogenesis |
| TDH3 | 1/0/0 | nd | Glyceraldehyde-3-phosphate dehydrogenase, involved in glycolysis and gluconeogenesis |
| TEF1* | 6/0/2 | + | Translational elongation factor EF-1 alpha; also encoded by TEF2; functions in the binding reaction of aminoacyl-tRNA (AA-tRNA) to ribosomes |
| TMA19 | 1/0/0 | + | Protein that associates with ribosome; homolog of translationally controlled tumor protein; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and relocates to the mitochondrial outer surface upon oxidative stress |

(*continued on next page*)

**Table 1** (*continued*)

| Gene[1] | FR[2] | Ver[3] | Gene function[4] |
|---|---|---|---|
| **TSA1** | **1/0/0** | **nd** | Thioredoxin peroxidase, acts as both a ribosome-associated and free cytoplasmic antioxidant; self-associates to form a high-molecular weight chaperone complex under oxidative stress |
| **YAL044W** | **2/0/0** | **+** | Putative protein of unknown function; similar to S. pombe uvi31 which is a putative DNA repair protein; overlaps with SSA1 |
| **YDR210W** | **1/0/0** | **–** | Protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the cell periphery |
| **YNL190W** | **1/0/0** | **–** | Cell wall protein of unknown function; proposed role as a hydrophilin induced by osmotic stress; contains a putative GPI-attachment site |
| **YOP1(YIP2)** | **1/0/0** | **+** | Membrane protein that interacts with Yip1p to mediate membrane traffic; interacts with Sey1p to maintain ER morphology |
| **YSY6** | **2/0/0** | **+** | Nuclear actin-related protein involved in chromatin remodeling |

[1]Proteins binding specifically to various regions of TBSV p33 and p92 and CIRV p36.
[2]Frequency of independent identification (first number for TBSV p33, second number for CIRV p36 and third number in case of TBSV p92).
[3]Interaction of the full-length host protein with TBSV p33 was confirmed in the split-ubiquitination assay.
[4]Documented functions of the yeast proteins are based on the *Saccharomyces* Genome Database at http://www.yeastgenome.org/
NOTE: Host proteins that have been identified in other screens with tombusviruses are highlighted with gray.

protein array is underrepresented for membrane proteins (Li et al., 2008a). In addition, lipids within the membranes could affect membrane-bound protein–protein interactions as well. Thus, to complement the previous protein array approach that targets mostly soluble host proteins, we now performed a membrane-based global protein–protein approach. The assay is based on MYTH (membrane yeast two-hybrid) split-ubiquitin assay. Briefly, the split-ubiquitin assay is based on the ability of N-terminal (NubG) and C-terminal (Cub) halves of ubiquitin to reconstitute a functional protein (Fetchko et al., 2003; Fetchko and Stagljar, 2004). NubG and Cub (fused separately to interacting proteins) are brought to close proximity by the interacting proteins, resulting in reconstitution of a functional ubiquitin protein. This leads to the cleavage of the reconstituted ubiquitin by endogenous ubiquitin specific proteases, resulting in the release of the transcription factor, which allows growth on selective media. Altogether, we identified 57 host proteins interacting with the tombusvirus replication proteins using the MYTH screens.

To determine the role of the identified host proteins interacting with p33 replication protein in tombusvirus replication, in this work, we further analyzed Cpr1p (Cyclosporin A-sensitive proline rotamase 1), which is a member of the cyclophilin family of proteins. Cyclophilins are ubiquitous, highly conserved proteins having prolyl isomerase (PPIase or rotamase) activity. Cyclophilins, together with the structurally unrelated FKB proteins and parvulins, constitute a family of 13 prolyl isomerases in yeast involved in catalyzing *cis–trans* isomerization of the peptidyl–prolyl bonds that could alter the structure, function or localization of the client proteins (Wang and Heitman, 2005) (Arevalo-Rodriguez et al., 2004). Overall, isomerization of the peptidyl–prolyl bonds is frequently required for protein refolding following traffic through cellular membranes and PPIases play a global role in facilitating correct protein folding and conformational changes (Arevalo-Rodriguez et al., 2004). The best-known members of prolyl isomerases are the bacterial trigger factor (TF) and cyclophilin A (CypA in mammals and Cpr1p in yeast). TF associates with the nascent polypeptide chain and plays a general role in protein folding (Craig et al., 2003). Cpr1p protects cells during stress, affects cellular transcription and protein localization. For example, Cpr1p is involved in import of fructose-1,6-biphosphatase into Vid vesicles (vacuole import and degradation) in the absence of glucose (Arevalo-Rodriguez et al., 2004). Because of their roles in immunosuppression, cyclophilins and FKBs are also called immunophilins.

In this paper, Cpr1p is shown to bind to the viral p33 protein and inhibits TBSV replication in yeast based on deletion and over-expression analysis. We also show interaction between p33 and Ess1p, which is a parvulin prolyl isomerase. Deletion of *CPR1* in combination with inactivation of a temperature-sensitive *ess1* mutant led to a 5-fold increase in TBSV replication in yeast, demonstrating that prolyl isomerases are potent inhibitors of TBSV and they might be part of the innate response of the host against viruses.

## Results

### *Screening MYTH libraries for yeast proteins interacting with tombusvirus replication proteins*

To identify yeast proteins interacting with the tombusvirus p33 replication protein in the intracellular membranes, we screened two cDNA libraries prepared from yeast, in which the yeast cDNAs were fused to the NubG prey construct either at the 5′- or 3′-termini (NubG-x and x-NubG libraries) (Iyer et al., 2005; Kittanakom et al., 2009). The plasmids were recovered from yeast colonies growing on selective media and the cDNA inserts were sequenced. The above screens led to the identification of 45 host proteins that interacted with the CNV p33 replication protein in the MYTH assay (Table 1).

Among the host proteins identified in the MYTH screens, the most frequent proteins were those involved in translation/ribosome biogenesis (14 proteins, 32% of the identified proteins) and protein/vesicle-mediated transport (7 proteins). The remaining host proteins are involved in RNA or lipid metabolism (1 and 2 proteins, respectively), general metabolism (6 proteins), RNA transcription (1 protein), DNA remodeling (3 proteins) or they function as chaperones/stress-related proteins (6 proteins), while 4 proteins have other functions and the function of 1 protein is not yet identified (Table 1).

Since sequencing of the cDNAs for the interactors showed mostly partial clones in the above cDNA libraries (not shown), we cloned the full-length ORFs for 35 of the identified interactors into the NubG split-ubiquitin construct (Table 1). The split-ubiquitin assay confirmed that 28 of the 35 full-length yeast proteins interacted with the CNV p33 (Fig. 1 and Table 1). Among these, Tef1p translation elongation factor 1A has previously been shown to be a permanent resident in the tombusvirus replicase complex and it interacts with p33 and $p92^{pol}$ replication proteins as well as with the viral RNA (Li et al., 2009). The identification of Tef1p in the MYTH screens suggests that this approach is useful to find relevant interactors of membrane-bound viral replication proteins.

We have also tested the MYTH libraries against the CIRV p36 replication protein, which unlike the peroxisomal CNV p33, is localized to the outside mitochondrial membrane (Hwang et al., 2008; Weber-Lotfi et al., 2002). The screen with CIRV p36 was less sensitive, but we still identified 10 host proteins interacting with p36 (Table 1). Interestingly, two of those, Anb1p and Egd2p also interacted with the CNV p33 (Table 1).

Screening the MYTH libraries against the CNV $p92^{pol}$ replication protein led to the identification of 7 host proteins (Table 1), three of which also interacted with the CNV p33 (Table 1). These yeast proteins included Tef2p (eEF1A), Rhr2p and Rps30B. Overall, the MYTH screens led to the identification of 57 new yeast proteins that interacted with the replication proteins of tombusviruses.

Since library screens depend on several factors for successful identification of host protein interactors of the tombusvirus replication

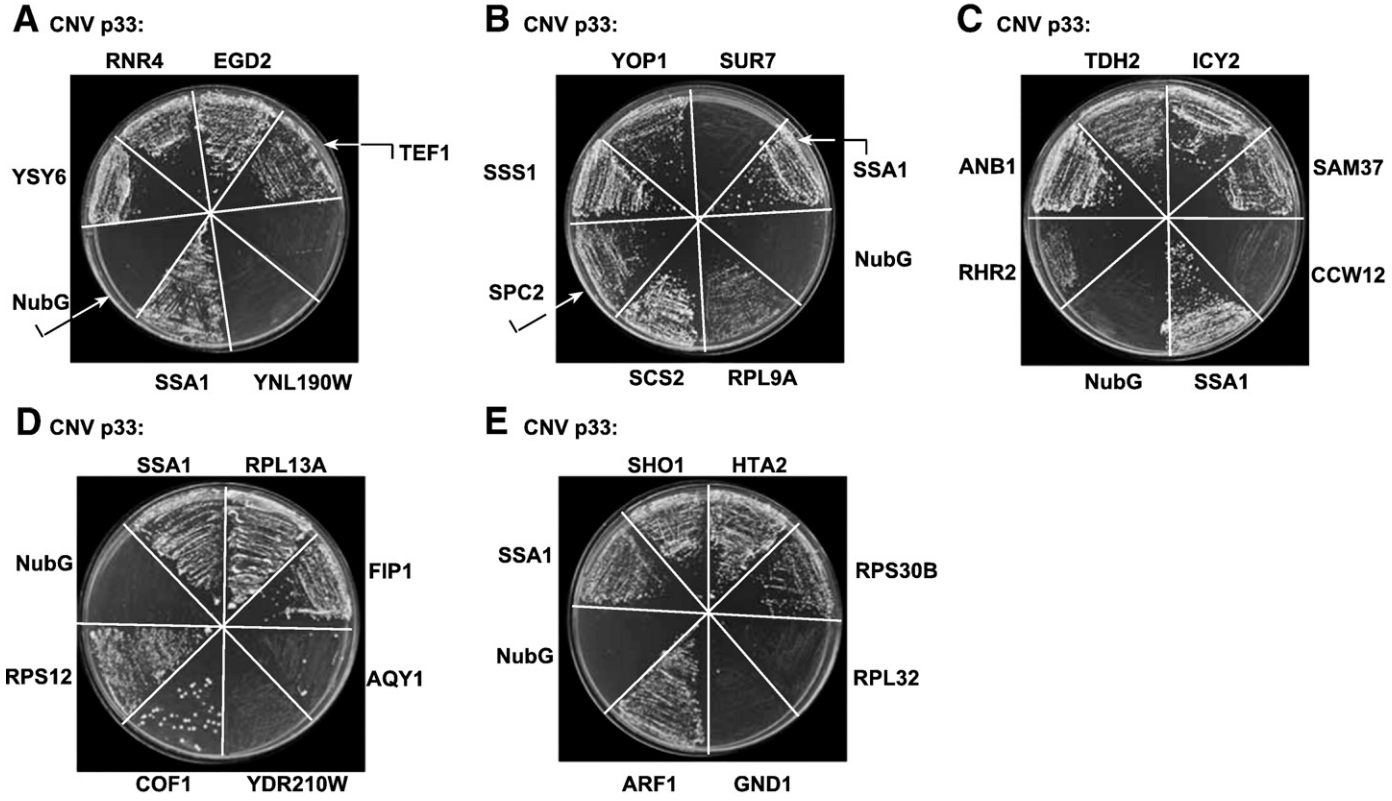


**Fig. 1.** Confirmation of interaction between selected yeast proteins and the p33 replication protein. (A–E) Split ubiquitin assay was used to test binding between p33 and the shown full-length yeast proteins chosen from the MYTH screens (Table 1). The bait p33 was co-expressed with the prey proteins in yeast. SSA1 (HSP70 chaperone), and the empty prey vector (NubG) were used as positive and negative controls, respectively.

proteins, we compared whether 12 yeast proteins, which were identified in the MYTH screens with CNV p33, could also interact with CNV $p92^{pol}$ and CIRV p36 replication proteins in the split-ubiquitin assay. These experiments were based on the full-length ORF clones. Interestingly, we found that 9 of the 12 yeast proteins tested also interacted with CNV $p92^{pol}$ and all 12 tested interacted with the CIRV p36 replication protein (Fig. 2). These data indicate that in spite of the different subcellular localization, the replication proteins of CNV and CIRV likely interact with a similar set of host proteins. This observation might not be surprising since the cytosolic C-terminal portion of CNV p33 and CIRV p36 is highly homologous. Also, the N-terminal portion of $p92^{pol}$ overlaps with p33 due to the expression strategy of tombusvirus genome (White and Nagy, 2004).

*Cpr1p cyclophilin binds to p33 replication protein in yeast and in vitro*

To test the functional relevance of the interaction between the identified yeast proteins and the CNV p33 replication protein, we have chosen *CPR1* for the follow-up experiments. Cpr1p is a cyclophilin localized in the cytosol and the nucleus. It has prolyl isomerase

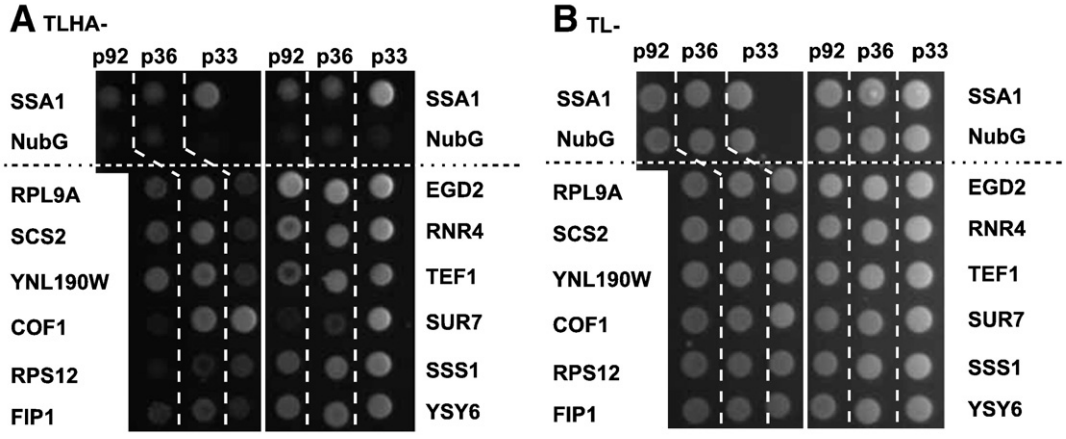


**Fig. 2.** Testing the interaction between full-length yeast proteins and CNV p33, CNV p92 and CIRV p36 replication proteins. (A) Split ubiquitin assay was used to test binding between the replication proteins (used as bait proteins) and the shown yeast proteins selected from the MYTH screens (Table 1). The yeast was grown on $TLHA^{-}$ plates. SSA1 (HSP70 chaperone), and the empty prey vector (NubG) were used as positive and negative controls, respectively. (B) A control plate showing growth of the yeast transformants on nonselective $TL^{-}$ media.

activity that catalyzes *cis–trans* isomerization of peptidyl–prolyl bonds (Arevalo-Rodriguez et al., 2004; Wang and Heitman, 2005).

To confirm the interaction between Cpr1p and p33, we performed protein pull-down experiments with purified recombinant p33. For the protein pull-down experiments, yeast extract containing soluble 6xHis-tagged Cpr1p was applied to a column containing immobilized MBP-p33 fusion. After elution of the bound proteins from the column, we analyzed if 6xHis-Cpr1p host protein was present in the eluted fraction by using Western blotting (Fig. 3A). Similarly prepared recombinant MBP immobilized to beads was the negative control to exclude non-specific binding by Cpr1p. As expected, Cpr1p bound efficiently to MBP-p33 (Fig. 3A, lane 3), while it did not bind to MBP (lane 2), confirming that Cpr1p can interact with p33 *in vitro*.

To further test the interaction between the full-length Cpr1p and p33, we performed a split-ubiquitin assay, which confirmed the interaction (Fig. 3B). Interestingly, it seems that Cpr1p interaction with p33 is as robust as the interaction of p33 with Ssa1p (Hsp70 chaperone), which is an important component of the tombusvirus replicase complex (Pogany et al., 2008; Serva and Nagy, 2006; Wang et al., 2009a,b). Altogether, the pull-down experiments and the split-ubiquitin assay confirmed that Cpr1p interacts strongly with the membrane-bound p33.

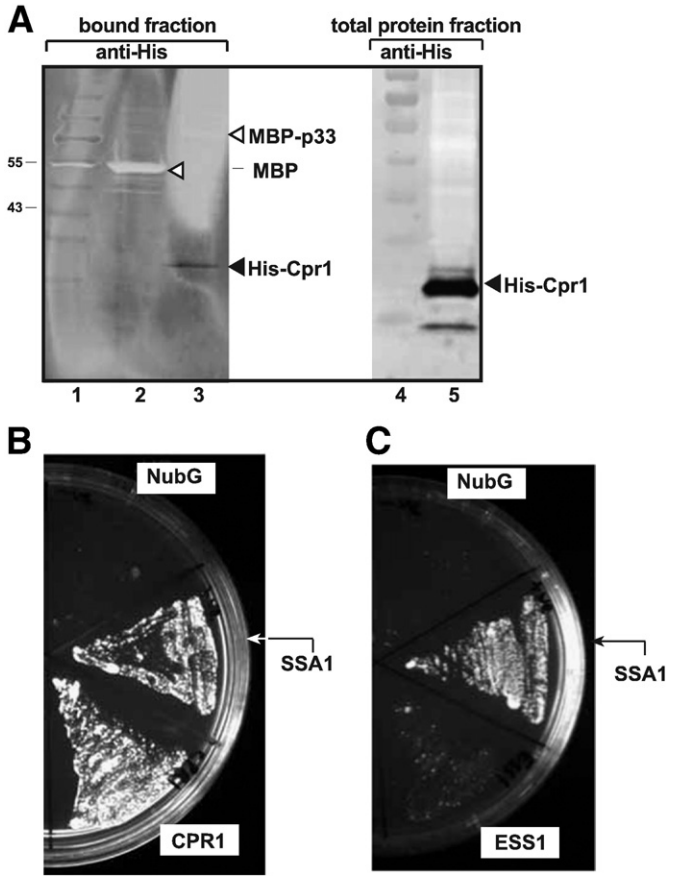


**Fig. 3.** Binding of Cpr1p and Ess1p to p33 replication protein. (A) Affinity binding (pull-down) assay to detect interaction between 6xHis-Cpr1p and the MBP-tagged p33 of TBSV. The MBP-p33 and MBP produced in *E. coli* were immobilized on amylose-affinity columns. Then, yeast extract containing 6xHis-Cpr1p was passed through the amylose-affinity columns with immobilized MBP (lane 2) or MBP-p33 (lane 3) proteins. The affinity-bound proteins were specifically eluted with maltose from the columns. The eluted proteins were analyzed by Western blotting with anti-His-antibody to detect the amount of 6xHis-Cpr1p specifically bound to MBP-p33. The presence of 6xHis-Cpr1p in total yeast lysate was confirmed with Western blotting (lane 5). (B) Confirmation of interaction between the full-length Cpr1p yeast protein and the p33 replication protein using the split ubiquitin assay. The bait p33 protein was co-expressed with the Cpr1p prey protein in yeast grown on TLHA$^-$ plates. SSA1 and the empty prey vector (NubG) were used as positive and negative controls, respectively. (C) Interaction between the full-length Ess1p yeast protein and the p33 replication protein using the split ubiquitin assay. The bait p33 was co-expressed with the Ess1p prey protein in yeast grown on TLHA$^-$ plates. See further details in panel B.

*Deletion of CPR1 increases TBSV RNA accumulation in yeast*

To test if *CPR1* could affect tombusvirus RNA replication, we took advantage of the previously developed efficient tombusvirus replication system in yeast (Panavas and Nagy, 2003b; Panavas et al., 2005b). We launched TBSV replicon (rep)RNA replication from the *GAL1* promoter in *cpr1Δ* yeast cells. Comparable amounts of yeast cells were harvested 40 h later, followed by Northern blotting to measure the level of TBSV repRNA produced. We used ribosomal RNA as a loading control for normalization of data on repRNA accumulation in yeast. These experiments revealed that TBSV repRNA accumulation increased in *cpr1Δ* yeast cells by 60% when compared with the wt BY4741 control (Fig. 4A, lanes 7–12 versus 1–6). Western blotting revealed that the amounts of p33 and p92$^{pol}$ replication proteins did not change in *cpr1Δ* yeast cells (Fig. 4B, top panel, lanes 3–4 versus 1–2), suggesting that Cpr1p is an inhibitor of tombusvirus replication.

*Over-expression of Cpr1p reduces TBSV repRNA replication in yeast*

To test if over-expression of Cpr1p could affect tombusvirus RNA replication, we first over-expressed Cpr1p-zz (carrying a C-terminal zz-tag) from the galactose inducible *GAL1* promoter (Gelperin et al., 2005) in yeast cells for 16 h. Subsequently, we launched TBSV repRNA replication from the *CUP1* promoter in the same cells. Comparable amounts of yeast cells were harvested 24 h later. The accumulation level of repRNA in yeast carrying pYES plasmid, which expresses

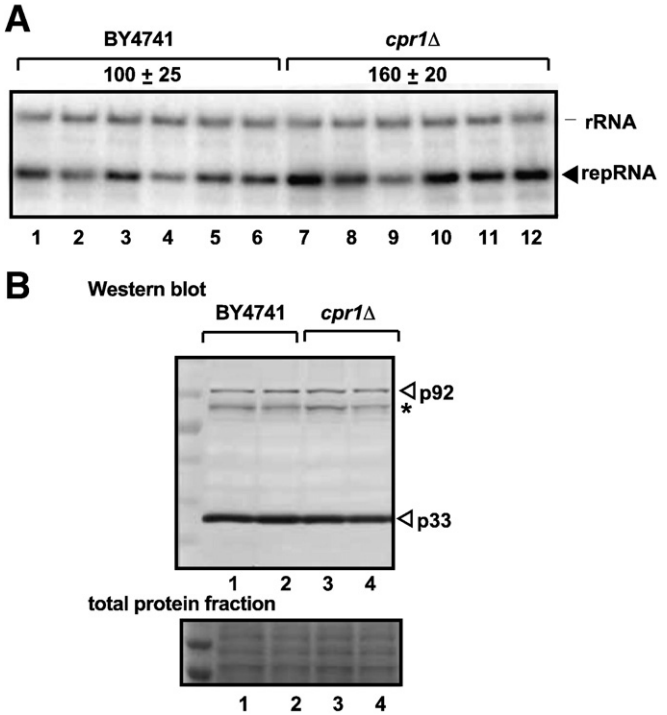


**Fig. 4.** Increased TBSV repRNA accumulation in *cpr1Δ* yeast. (A) To launch TBSV repRNA replication, we expressed 6xHis-p33 and 6xHis-p92 from the constitutive *ADH1* promoter and DI-72(+) repRNA from the galactose-inducible *GAL1* promoter in the parental (BY4741) and in *cpr1Δ* yeast strains. The yeast cells were cultured for 40 h at 23 °C on 2% galactose SC minimal media. Northern blot analysis was used to detect DI-72(+) repRNA accumulation. The accumulation level of DI-72(+) repRNA was normalized based on 18S rRNA. (B) Western blot analysis of the accumulation level of 6xHis-tagged p33 and 6xHis-tagged p92 proteins using anti-His antibody. Asterisk marks the SDS-resistant p33 homodimer. The coomassie-stained SDS-PAGE image at the bottom shows the total protein in the yeast extract as a loading control.

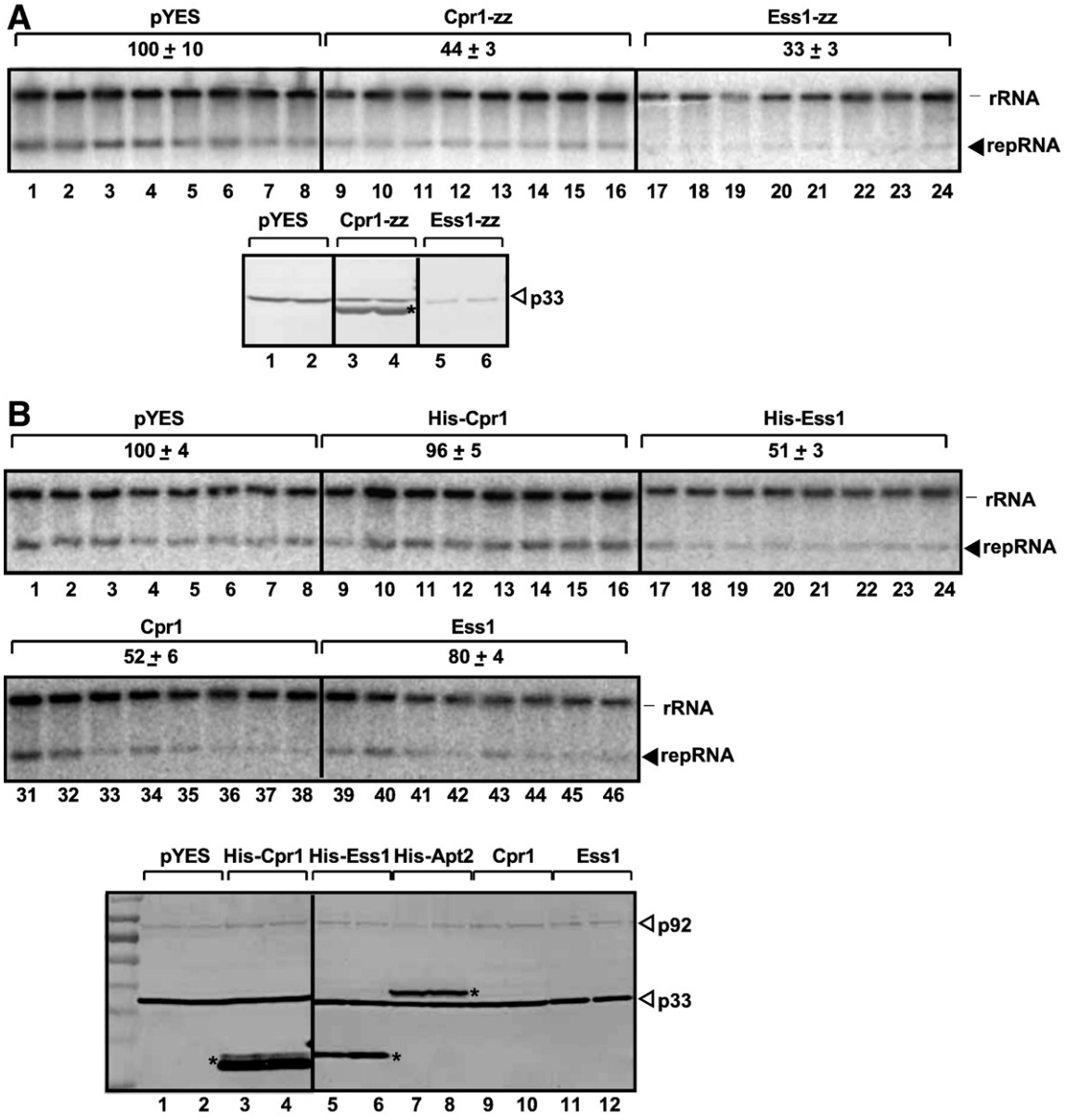


**Fig. 5.** Inhibition of TBSV repRNA accumulation by over-expression of Cpr1p and Ess1p in yeast. (A) Top panel: Northern blot analysis was used to detect DI-72(+) repRNA accumulation in BY4741 yeast strain over-expressing the C-terminal zz-tagged Cpr1-zz, Ess1-zz or a small peptide (as a control) from the high copy number pYES plasmid as shown. The continuous expression of Cpr1-zz and Ess1-zz from the *GAL1* promoter started 16 h prior to launching repRNA replication at 29 °C by inducing with 50 μM $CuSO_4$. Note that samples were obtained after 24 h of repRNA replication. See further details in the legend to Fig. 4. Bottom panel: Western blot analysis of the accumulation level of 6xHis-tagged p33, 6xHis-tagged p92 and the zz-tagged Cpr1-zz, Ess1-zz proteins using anti-6xHis antibody. Note that the zz-tag includes a His-tag. The asterisk mark (*) indicates Cpr1-zz, while Ess1-zz was under detectable level. Also, expression of Ess1-zz slowed down yeast growth as indicated by the reduced level of rRNA (lanes 17–24). (B) Top and middle panels: Northern blot analysis to measure the accumulation of DI-72(+) repRNA in BY4741 yeast strain over-expressing the N-terminal 6xHis-tagged His-Cpr1, His-Ess1 or native Cpr1p and Ess1p from the high copy number pYES plasmid as shown. See further details in panel A. Bottom panel: Western blot analysis of the accumulation level of 6xHis-tagged p33, 6xHis-tagged p92 and the 6xHis-tagged His-Cpr1, His-Ess1 proteins using anti-6xHis antibody. Note that the native Cpr1p and Ess1p are not detected with this antibody. The asterisk mark (*) indicates His-Cpr1 and His-Ess1. Note that the 6xHis-tagged Apt2 was used as a control to show that over-expression of a noninteracting protein with p33 does not affect the accumulation of p33 in yeast cells.

only a short peptide, was taken as 100% (Fig. 5). Over-expression of Cpr1p-zz led to 44% repRNA accumulation when compared with yeast carrying the pYES control (Fig. 5A, lanes 9–16 versus 1–8). Similarly, over-expression of the native (nontagged) Cpr1p in yeast reduced TBSV repRNA accumulation by ~50% (Fig. 5B, lanes 31–38), while over-expression of the His-tagged Cpr1p (6xHis-tag at the N-terminus) decreased TBSV repRNA accumulation by ~4% (lanes 9–16). This suggests that the N-terminally tagged Cpr1p is likely non-functional in yeast, in contrast with the C-terminally tagged or native Cpr1p. Western blotting revealed that over-expression of Cpr1p did not decrease p33 levels (see Cpr1 and His-Cpr1, Fig. 5B, bottom panel) or decreased it by 20% (Cpr1-zz, Fig. 5A, bottom panel). Altogether, these data further support that Cpr1p is an inhibitor of TBSV repRNA replication in yeast.

*Over-expression of Ess1p parvulin reduces TBSV repRNA replication in yeast*

There are 8 known yeast cyclophilins, which are part of the large prolyl isomerase family (13 members in yeast, 54 in *Arabidopsis*). These genes might have overlapping functions and could complement each other. Indeed, it has been documented that over-expression of

*CPR1* in yeast leads to complementation of the function of the structurally unrelated *ESS1* parvulin, which is an essential prolyl isomerase (Arevalo-Rodriguez et al., 2004). Therefore, Ess1p and Cpr1p might complement each other's function in yeast. To test if Ess1p might have a similar role in TBSV replication to Cpr1p, first we tested if Ess1p could interact with p33 replication protein. The spilt-ubiquitin assay revealed interaction between Ess1p and CNV p33 (Fig. 3C). The weak interaction between Ess1p and CNV p33 is likely due to the low expression level of Ess1p in yeast (not shown). Nevertheless, the data are consistent with Ess1p playing a role in TBSV replication.

To further test the possible role of Ess1p in TBSV replication, we over-expressed Ess1p in yeast as described above for Cpr1p. Interestingly, over-expression of Ess1-zz and His-Ess1 from plasmids led to 50–70% decrease in TBSV repRNA accumulation, while over-expression of the nontagged Ess1p resulted in a 20% decrease (Fig. 5B). Western blotting was not sensitive enough to detect the accumulation of Ess1p in these experiments in yeast, except when it was 6xHis-tagged (Fig. 5A–B). Overall, the reduced replication of TBSV repRNA in yeast containing the Ess1p expression plasmids suggests that, similar to Cpr1p cyclophilin, Ess1p is also an inhibitor of TBSV replication in yeast.

*Expression of a temperature sensitive mutant of Ess1p in cpr1Δ yeast greatly increases TBSV repRNA*

Since it is likely that Ess1p in *cpr1Δ* yeast partly complements *CPR1* function in inhibition of TBSV replication (Fig. 4A), and *ESS1* is an essential gene for yeast growth (thus the double deletion *cpr1Δ ess1Δ* yeast is nonviable), we decided to use a temperature sensitive mutant of *ESS1* in *cpr1Δ* yeast background to test TBSV replication near or at the nonpermissive temperature. To keep the yeast alive, we only used the temperature treatment for a shorter period as shown in Fig. 6. Interestingly, TBSV replication increased ~5-fold after a short treatment of *ess1*$^{ts}$ *cpr1Δ* yeast at 38 °C, which is nonpermissive temperature (Fig. 6D, lanes 7–12 versus 1–6). Similar ~5-fold increase in TBSV repRNA accumulation was observed after a short treatment of *ess1*$^{ts}$ *cpr1Δ* yeast at 35 °C (Fig. 6C, lanes 7–12 versus 1–6). These treatments did not affect p33 accumulation level in *ess1*$^{ts}$ *cpr1Δ* yeast when compared with the parental BY4741 yeast (Fig. 6C, bottom panel). In contrast, TBSV repRNA accumulation was only 40–50% above the control when *ess1*$^{ts}$ *cpr1Δ* yeast was grown at 23 °C or 30 °C, both of which are permissive temperatures (Fig. 6A–B, lanes 7–12 versus 1–6). This increase in TBSV repRNA accumulation at the permissive temperatures is likely due to deletion of cpr1 (see also Fig. 4). Based on these experiments, we suggest that Cpr1p and Ess1p play a partly complementary role in inhibiting TBSV replication in yeast. Thus, prolyl isomerases, including cyclophilins and parvulins, might be components of the host antiviral defense.

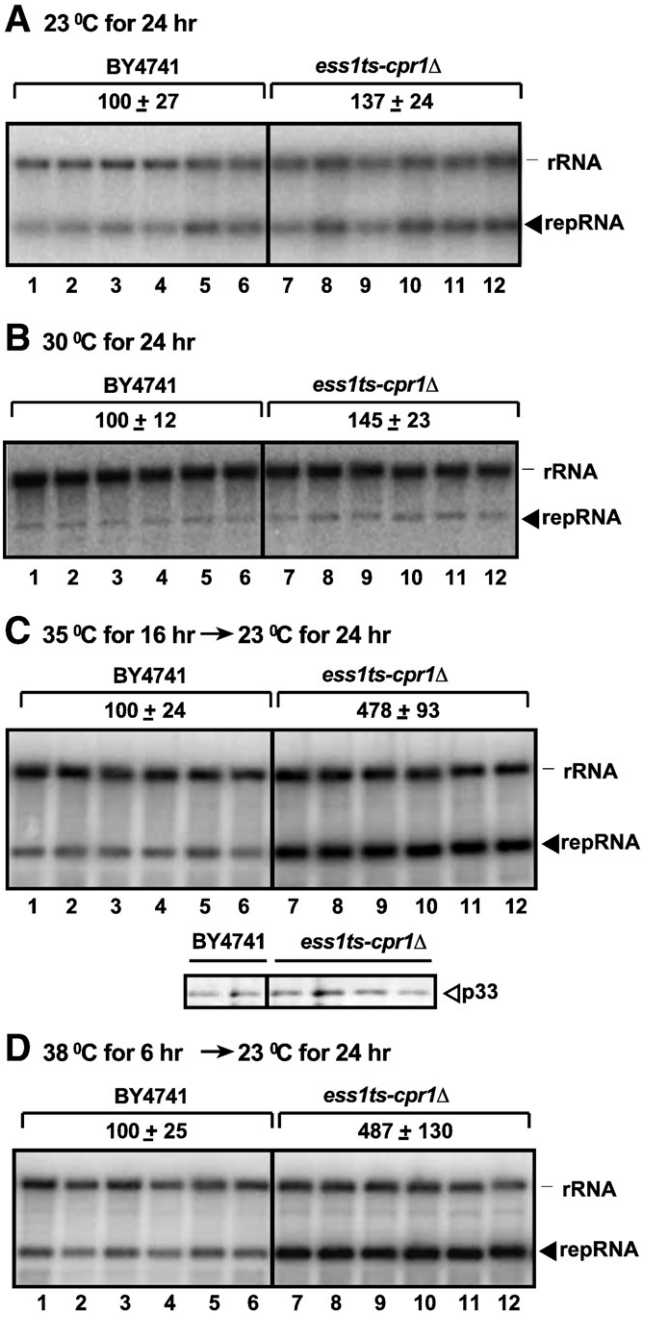


**Fig. 6.** Increased TBSV repRNA accumulation in *ess1*$^{ts}$*cpr1Δ* yeast. (A) To launch TBSV repRNA replication, we expressed 6xHis-p33 and 6xHis-p92 from *CUP1* promoter and DI-72(+) repRNA from the *ADH1* promoter in the parental (BY4741) and in *ess1*$^{ts}$*cpr1Δ* yeast strains. The yeast cells were cultured for 24 h at 23 °C on 2% glucose SC minimal media plus 50 μM $CuSO_4$. Northern blot analysis was used to detect DI-72(+) repRNA accumulation. The accumulation level of DI-72(+) repRNA was normalized based on 18S rRNA. (B) The yeast cells were cultured for 24 h at 30 °C on 2% glucose SC minimal media plus 50 μM $CuSO_4$. (C) The yeast cells were cultured for 16 h at 35 °C in 2% glucose media in the absence of $CuSO_4$. Then, the yeast cells were shifted for 24 h at 23 °C on 2% glucose SC minimal media plus 50 μM $CuSO_4$. Top image: Northern blot analysis was used to detect DI-72(+) repRNA accumulation. Bottom image: Western blot analysis was used to detect 6xHis-tagged p33 accumulation. (D) The yeast cells were cultured for 6 h at 38 °C in 2% glucose media in the absence of $CuSO_4$. Then, the yeast cells were shifted for 24 h at 23 °C on 2% glucose SC minimal media plus 50 μM $CuSO_4$. Northern blot analysis was used to detect DI-72(+) repRNA accumulation.

## Discussion

Genome-wide genomics and global proteomics screens are powerful approaches to identify numerous host factors affecting virus replication as demonstrated for TBSV based on yeast and *in vitro* approaches (Jiang et al., 2006; Li et al., 2008a, 2009; Nagy, 2008; Panavas et al., 2005b; Serva and Nagy, 2006; Serviene et al., 2005, 2006). Yet, in the current screens to identify interactors of the tombusvirus replication proteins based on the MYTH approach and yeast cDNA libraries, we found many new, previously not identified host proteins (Table 1). This is probably due to the differences in approaches, such as the current screens focused on interactions taking place in the intracellular membranes, where the TBSV RNA is replicated by the viral replication proteins in association with cellular proteins and lipids (Nagy, 2008; Sharma et al., 2010). In contrast, the previous global proteomics approaches were based on protein arrays containing mostly soluble (cytoplasmic and nuclear) yeast proteins and the membrane-bound proteins were under-represented (Li et al., 2008a, 2009). Overall, the identification of many new host proteins in the current MYTH screens suggests that we still have not reached the saturation level with our screens and justify additional screens for identification of all the host factors involved in tombusvirus replication.

The current MYTH screens together with the previous protein array and proteomic approaches led to the identification of over 100 yeast proteins interacting with the tombusvirus replication proteins (Table 1) (Li et al., 2008a, 2009; Serva and Nagy, 2006). The identified host proteins have numerous cellular functions, which might also be utilized during TBSV replication. The emerging picture is that several of the identified host proteins do play important roles in TBSV replication. For example, the MYTH screens identified Tef1p (eEF1A) and Tdh2p / Tdh3p (GAPDH), which are present in the tombusvirus replicase complex (Li et al., 2009; Serva and Nagy, 2006; Wang and Nagy, 2008) and affect the stability of the viral replication proteins or the activity of the viral replicase. In addition, Hsp70 chaperones, several ESCRT proteins, Cdc34p ubiquitin-conjugating enzyme, Rsp5p ubiquitin-ligase, and Pex19p peroxisomal shuttle protein, all of which have been identified in previous screens, affected TBSV RNA replication in yeast or in plants (Barajas et al., 2009a,b; Barajas and Nagy, 2010; Li et al., 2008a; Pathak et al., 2008; Pogany et al., 2008; Wang et al., 2009a,b). Although, the roles of the other host proteins identified in the MYTH screens or in previous screens are not yet known, based on the above examples, it seems likely that the tombusvirus replication proteins are involved in a large number of protein–protein interactions in the host cells.

In addition to identification of the known host factors involved in TBSV replication, such as Tef1p /Tef2p (eEF1A) and Tdh2p / Tdh3p (GAPDH), we also validated the results from the MYTH screens for Cpr1p cyclophilin (Table 1). We found that Cpr1p interacts with the tombusvirus replication proteins in yeast and *in vitro* and the interaction results in inhibition of TBSV replication. This is supported by deletion of *CPR1* from yeast that led to 60% increase, while over-expression of Cpr1p resulted in 50–60% decrease in TBSV RNA accumulation. Albeit the mechanism of inhibition by Cpr1p is currently unknown, it does not seem to be the results of degradation of p33/p92 replication proteins. It is possible that direct binding between Cpr1p results in inhibition of p33/p92 functions or the structural changes in p33/p92 caused by the prolyl isomerase activity of Cpr1p might be responsible for the inhibitory effect of Cpr1p. Future *in vitro* approaches will be needed to address these points.

Another observation in this paper is that Cpr1p does not seem to be the only prolyl isomerase acting as the inhibitor of TBSV replication. We found that Ess1p parvulin, whose function can be complemented by over-expression of *CPR1* (Arevalo-Rodriguez et al., 2004), could also inhibit TBSV replication (Fig. 5). Indeed, deletion of *CPR1* in combination with inactivation of a temperature-sensitive *ess1* mutant resulted in a 5-fold increase in TBSV replication in yeast (Fig. 6). These data indicate that prolyl isomerases are potent inhibitors of TBSV and they might be part of the innate response of the host against some viruses. Since prolyl isomerases are conserved and ubiquitous proteins, their role could be common against viruses.

Accordingly, CypA, the mammalian homolog of Cpr1p, was shown to bind to the matrix protein (M1) of influenza A virus and this binding interfered with the nuclear localization of M1 (Liu et al., 2009). Over-expression of CypA increased the self-association of M1 and led to decreased viral replication.

Another example for the antiviral activity of cyclophilins is shown for HIV-1 (human immunodeficiency virus-1). CypA was found to affect the formation and infectivity of HIV-1 virions. This is likely due to the incorporation of CypA into HIV-1 virions and direct binding between CypA and Gag, the polyprotein precursor of virion structural proteins (Franke et al., 1994; Luban et al., 1993; Strebel et al., 2009). The role of CypA as an anti-HIV protein is reduced by the retroviral Vif protein, which inhibited the incorporation of CypA into the viral particles (Takeuchi et al., 2007).

In contrast to the above examples on the potent antiviral activities of cyclophilins, prolyl isomerases might also facilitate virus replication as demonstrated in case of hepatitis C virus (HCV). Knockdown of CypA level or inhibition of CypA activity by cyclosporine or DEBIO-025 (a cyclosporine analogue) resulted in greatly reduced HCV replication in hepatoma cell lines (Kaul et al., 2009). The authors propose that CypA affects the processing of HCV polyprotein as well as binding of CypA to the HCV NS5B RdRp is required for replicase activity due to the induced conformation change in NS5B. Additional cyclophilins might also be involved in HCV replication (Gaither et al., 2010). Also, the effect of cyclophilins could be complex during HCV replication, since depletion of cyclophilin G even stimulated HCV accumulation (Gaither et al., 2010).

Another example of co-opting cyclophilins for facilitating virus infections was documented in case of human papillomavirus type 16 (HPV16). CypB, localized on the cell surface, has been shown to promote structural changes in the L2 capsid protein of HPV16. The resulting exposure of the N-terminus of the L2 capsid protein of HPV16 is required for functional internalization of the virions and HPV16 infections (Bienkowska-Haba et al., 2009). Altogether, these observations suggest that prolyl isomerases play complex roles during many viral infections.

## Materials and methods

### *Yeast strains and expression plasmids*

Yeast (*S. cerevisiae*) strains BY4741 (*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and *cpr1Δ* were obtained from Open Biosystems (Huntsville, AL, USA). Yeast strain NMY51 [*MAT***a** *his3Δ 200 trp1-901 leu2-3, 112 ade2 LYS2*::(*lexAop*)$_4$-*HIS3 ura3*::(*lexAop*)$_8$-*lacZ ade2*::(*lexAop*)$_8$-*ADE2 GAL4*] was obtained from Dualsystems. The plasmids for over expression of zz-tagged Cpr1 and Ess1 were obtained from the collection of ZZ-ORF library from Open Biosystems. The construction of pGBK-ADH::His33 and pGAD-ADH::His92 (Panaviene et al., 2004b) as well as pYC-GAL::DI72sat have been described previously (Panavas and Nagy, 2003a).

To express 6xHis-Cpr1p and 6xHis-Ess1p in yeast, we PCR-amplified *CPR1* and *ESS1* from yeast genomic DNA with primers #3028/#3029 and #3033/#3034, respectively. The obtained PCR products were cloned into pYES-NT using *Bam*HI and *Xho*I sites. We used similar strategy to make the expression vectors for the native Cpr1p and Ess1p expression by using PCR with primers 3068/#3029 and #3069/#3034 and *Hin*dIII and *Xho*I sites to clone into pYES-NT.

To generate plasmid pHisGBK-CUP1::His33-ADH::DI72, the ADH1 promoter was amplified from pGBK-His33 (Panaviene et al., 2005) with primers #953 and #954 and digested with *Bsp*1407I and *Xho*I. The DI72sat sequence was amplified from pYC-DI72sat (Panaviene et al., 2005) with primers #471 and #1069# and digested with *Xho*I and *Sac*I. The two digested PCR products were ligated into pHisGBK-CUP1::His33-GAL::DI72 (Barajas et al., 2009b), where the GAL1::DI72 had been excised by *Bsp*1407I/*Sac*I digestion.

### *MYTH screening of yeast cDNA libraries*

The MYTH screens were performed as described (Iyer et al., 2005; Kittanakom et al., 2009). The bait construct pGAD-BT3-N-His33, carrying CNV p33 ORF was made from pGAD-BT2-N-His33 (Li et al., 2008a) by digesting with *Pvu*I and *Sda*I to remove the ampicillin resistance gene and replacing it with a *Pvu*I/*Sda*I portion from pGBK-His33 (Panaviene et al., 2005) containing a kanamycin resistance gene. The two yeast cDNA libraries, in which the yeast cDNAs were fused to NubG at either at the 5′- or 3′-termini (NubG-x and x-NubG) were obtained from Dr. Stagljar (Iyer et al., 2005; Kittanakom et al., 2009).

The stock cDNA libraries were transformed into TOP10 *E. coli* competent cells via electroporation for propagation, then the plasmid DNA representing the cDNA libraries was extracted from *E. coli* and transformed into NMY51 yeast already containing the plasmid pGAD-BT3-N-His33. The transformed yeasts were plated onto Trp-/Leu-/His-/Ade- (TLHA-) media to select for colonies that contain a library cDNA expressing a protein interacting with p33. Plasmid DNA was

extracted from the yeast colonies, transformed into *E. coli* and plated on ampicillin plates to recover the "prey" plasmids containing host cDNAs. The resulting plasmids were sequenced to identify the host proteins interacting with p33.

The same procedure was followed to identify host proteins interacting with CNV p92 and CIRV p36 proteins. To make plasmid pGAD-BT3-N-His92, pGAD-BT3-N-His33 was digested with *Spe*I and *Nco*I to remove the His33, then the 6xHis tagged CNV p92 was amplified from pGAD-His92 (Panaviene et al., 2005) with primers #2261 and #2945, digested with *Spe*I and *Nco*I and inserted into the SpeI/NcoI digested pGAD-BT3-N-vector. To make pGAD-BT3-N-p36 the CIRV p36 was amplified from a full length CIRV clone (American Type Culture Collection, ATCC) with primers #2944 and #972, digested with *Spe*I and *Nco*I and inserted into the *Spe*I/*Nco*I digested pGAD-BT3-N vector.

*Analysis of protein–protein interactions using the split-ubiquitin assay*

The split-ubiquitin assay was based on the Dualmembrane kit3 (Dualsystems). The bait construct, pGAD-BT2-N-His33, expressing the CNV p33 replication protein has been described earlier (Li et al., 2008b). The prey constructs were made by PCR amplification of individual genes using gene specific primers (available upon request). PCR products were digested with *Bam*HI and *Nhe*I and cloned into pPRN-N-RE vector using the same enzymes. Yeast strain NMY51 was co-transformed with pGAD-BT2-N-His33 and pPR-N-RE (NubG) or one of the prey constructs carrying a given host gene and plated onto $Trp^-/Leu^-$ synthetic minimal medium plates. Transformed colonies were picked with a loop, re-suspended in water, and streaked onto $TLHA^-$ ($Trp^-/Leu^-/His^-/Ade^-$) plates to test for p33/p36/p92–host protein interactions as described (Li et al., 2008b).

*Analysis of protein–protein interactions in vitro*

The MBP-tagged p33 was purified from *E. coli* as described previously (Rajendran and Nagy, 2003). Briefly, expression of the MBP-p33 and MBP as a control was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) in Epicurion BL21-codon-plus (DE3)-RIL cells (Stratagene). Cells were resuspended in the extraction buffer and were broken by sonication, and then the cell lysate was passed through amylose columns to bind MBP-p33 or MBP. After passing the cell lysate, the columns were washed three times with ice-cold column buffer (10 mM Tris–HCl [pH 7.4], 1 mM EDTA, 25 mM NaCl, 10 mM β-mercaptoethanol). For the pull-down assay of 6xHis-Cpr1p, the cleared yeast lysate (6xHis-Cpr1 expressed from pYES2-NT vector) containing 6xHis-Cpr1 through the amylose columns carrying the pre-bound MBP-p33 or MBP. The columns were washed five times with cold column buffer, and then the bound protein complexes were eluted with column buffer supplemented with 10 mM maltose. The presence of 6xHis-Cpr1 protein in the eluate was analyzed by SDS-PAGE and Western blotting using anti-His antibody followed by an alkaline phosphatase-conjugated anti-mouse secondary antibody (Panaviene et al., 2004a).

*TBSV replication assay*

Yeast strains BY4741 and *cpr1Δ* were transformed with pGBK-ADH::His33 and pGAD-ADH::His92 (Panaviene et al., 2004b) as well as with pYC-GAL::DI72sat (Panavas and Nagy, 2003a). Replication assay was performed by measuring the accumulation of DI-72(+) repRNA relative to the 18S rRNA (Panavas and Nagy, 2003a).

The Ess1 temperature sensitive strain (*ess1*-H164R, or $ess1^{ts}$) was obtained from Prof. C. Boone (U. Toronto, Canada). To generate $ess1^{ts}cpr1\Delta$ strain, we used homologous recombination in $ess1^{ts}$ strain to delete *cpr1Δ* using Hygromycin antibiotic selection. The Hygromycin marker (hphNT1) was PCR amplified from pFA6a-HPH (Euroscarf) using the primers #3070/3071 and transformed into the $ess1^{ts}$ strain. Deletion of the *cpr1* gene in $ess1^{ts}cpr1\Delta$ strain (GS #275) was confirmed with primers #3141 and #2501. The $ess1^{ts}cpr1\Delta$ strain was transformed with pGAD-CUP1::His92 and pHisGBK-CUP1::His33-ADH::DI72. Replication was induced by adding 50 μM $CuSO_4$ to the media and then yeast was cultivated for 24 h at different temperatures (Fig. 6).

*Northern blotting*

For the analysis of TBSV repRNA accumulation, total RNA was extracted from yeast and Northern blot analysis was performed as described earlier (Panaviene et al., 2004b). Briefly, the yeast cells were centrifuged and re-suspended in the RNA extraction buffer (50 mM sodium acetate, pH 5.2, 10 mM EDTA, 1% SDS) and water-saturated phenol, followed by incubation at 65 °C for 5 min and then on ice for 5 min. After centrifugation, the upper aqueous phase was removed and mixed with ethanol for precipitation, followed by recovering the RNA by centrifugation. The denatured total RNA was subjected to agarose (1.5%) gel electrophoresis and transferred to a Hybond-XL membrane (Amersham). For the replication assay, RNA hybridization was done with a mixture of two probes to detect DI-72 (+)RNA and 18S yeast ribosomal RNA (rRNA) as described previously (Cheng et al., 2005). Hybridization signals were detected using a Typhoon 9400 imaging scanner (Amersham) and quantified by ImageQuant software.

*Over-expression of host proteins*

To evaluate the accumulation of viral RNA during the over-expression of Cpr1p and Ess1p, viral replication proteins were expressed using the *CUP1* promoter, while the host proteins were expressed under *GAL1* promoter (Li et al., 2008a). Yeast strain BY4741 was transformed with CPR1-zz and ESS1-zz along with pGAD-CUP1::His92 and pHisGBK-CUP1::His33-*GAL1*::DI72 (Li et al., 2008a). The transformants were selected on synthetic complete medium lacking Ura, Leu, and His (SC-$ULH^-$ medium). Transformants were cultured at 30 °C for 16 h and the expression of virus replication proteins was launched by adding 5 μM $CuSO_4$ to the media, followed by additional 24 h culturing at 30 °C. Western and Northern blot was performed as described above.

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