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Tombusviruses upregulate phospholipid biosynthesis via interaction between p33 replication protein and yeast lipid sensor proteins during virus replication in yeast

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Abstract

Positive-stranded RNA viruses induce new membranous structures and promote membrane proliferation in infected cells to facilitate viral replication. In this paper, the authors show that a plant-infecting tombusvirus upregulates transcription of phospholipid biosynthesis genes, such as *INO1*, *OPI3* and *CHO1*, and increases phospholipid levels in yeast model host. This is accomplished by the viral p33 replication protein, which interacts with Opi1p FFAT domain protein and Scs2p VAP protein. Opi1p and Scs2p are phospholipid sensor proteins and they repress the expression of phospholipid genes. Accordingly, deletion of *OPI1* transcription repressor in yeast has a stimulatory effect on TBSV RNA accumulation and enhanced tombusvirus replicase activity in an *in vitro* assay. Altogether, the presented data convincingly demonstrate that *de novo* lipid biosynthesis is required for optimal TBSV replication. Overall, this work demonstrates that a (+)RNA virus reprograms the phospholipid biosynthesis pathway in a unique way to facilitate its replication in yeast cells.

INTRODUCTION

Positive-strand (+)RNA viruses usurp various intracellular and organellar membranes for their replication. These cellular membranes concentrate membrane-bound viral replication proteins, being used as a platform to facilitate the formation of viral replicase complexes (VRCs), and provide protection against cellular nucleases and proteases (Belov and van Kuppeveld, 2012; Castorena, Stapleford, and Miller, 2010; Hsu et al., 2010; Mine and Okuno, 2012; Nagy and Pogany, 2012; Pogany et al., 2008; Stapleford, Rapaport, and Miller, 2009; Xu, Huang, and Nagy, 2012). Also, lipids and cellular proteins in the subverted membranes may serve as scaffolds for targeting the viral replication proteins or for the assembly of VRCs. Moreover, membranes may also provide key lipids or protein cofactors for activation of the viral replicase. Accordingly, special membrane invaginations, called spherules, consisting of lipid membranes bended inward that contain viral replication proteins and recruited host proteins, have been documented for several (+)RNA viruses

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(Barajas, Jiang, and Nagy, 2009; Barajas et al., 2014; de Castro, Volonte, and Risco, 2013; Kopek et al., 2007; McCartney et al., 2005; Schwartz et al., 2002). These viral-induced spherules are the sites of viral replication. Since several (+)RNA viruses also induce membrane proliferation that requires new lipid biosynthesis, it is not surprising that genome-wide screens for identification of host factors affecting (+)RNA virus replication identified lipid biosynthesis and metabolism genes (Cherry et al., 2005; Kushner et al., 2003; Panavas et al., 2005; Serviène et al., 2006).

Alteration of cellular lipid metabolism by viruses is exemplified by picornaviruses, which recruit the host PI4PKIII β for phosphatidylinositol-4-phosphate (PI4P) synthesis in order to modify the lipid composition of membranes during replication (Belov and Ehrenfeld, 2007; Belov et al., 2007; Hsu et al., 2010; Sasvari and Nagy, 2010). Hepatitis C virus (HCV) recruits PI4PKIII α , which is also involved in PI4P synthesis, to modulate phospholipid biosynthesis and facilitate the formation of the “membranous-web”, which serves as the site of HCV RNA replication (Berger et al., 2009). A related flavivirus (Dengue virus, DENV) retargets FASN (fatty acid synthase, a major rate-limiting enzyme in fatty acid biosynthesis) to the ER membrane, the site of DENV replication (Heaton et al., 2010). Additional examples include the effects of host fatty acid synthase on Drosophila C virus (Cherry et al., 2006) and Ole1p, which affects the amount of unsaturated fatty acids, and acyl coenzyme A on *Brome mosaic virus* replication (Lee and Ahlquist, 2003; Zhang et al., 2012). DENV was also shown to promote autophagy and beta-oxidation of lipids to generate extra ATP needed for virus replication (Heaton and Randall, 2010). These examples illustrate the various use of lipids by different (+)RNA viruses during their infections.

Tomato bushy stunt virus (TBSV), a small plant (+)RNA virus, is a model virus to study virus replication, recombination, and virus - host interactions based on yeast (*Saccharomyces cerevisiae*) model host (Nagy and Pogany, 2006; Panavas and Nagy, 2003). In addition to the viral-coded p33 RNA chaperone and p92^{pol} RNA-dependent RNA polymerase (RdRp), which are essential components of the TBSV VRC (Stork et al., 2011; White and Nagy, 2004), TBSV also recruits a number of host proteins based on extensive proteomics screens (Li et al., 2008b; Li et al., 2009; Mendu et al., 2010; Nagy and Pogany, 2010; Serva and Nagy, 2006). Interestingly, genome-wide and global proteomics screens in *S. cerevisiae* identified ~20 host genes involved in lipid biosynthesis and metabolism affecting tombusvirus replication and recombination, suggesting that tombusvirus replication depends on active lipid biosynthesis and extensive remodeling of membranes (Jiang et al., 2006; Li et al., 2008b; Li et al., 2009; Mendu et al., 2010; Nagy and Pogany, 2010; Panavas et al., 2005; Rochon et al., 2014; Serva and Nagy, 2006; Serviène et al., 2006; Serviène et al., 2005).

Additional studies with one of the identified genes, *INO2*, which is a transcription activator involved in regulation of phospholipid biosynthesis revealed that deletion of *INO2*, reduced TBSV replication and inhibited the activity of the tombusvirus replicase (Sharma, Sasvari, and Nagy, 2011). In addition, the stability of the viral replication protein is decreased and the pattern of viral protein localization changed dramatically in *ino2 ino4* yeast. In contrast, over-expression of Ino2p stimulated TBSV RNA accumulation (Sharma, Sasvari,

and Nagy, 2011). Thus, phospholipid biosynthesis is required for efficient replication of TBSV.

In this paper, we explored the role of FFAT-motif (two phenylalanines with acidic tail)-containing protein, namely Opi1p, in tombusvirus replication in yeast. Opi1p, similar to other FFAT-motif containing proteins present in mammals, interacts with phospholipids (namely with phosphatidic acid, PA) and VAP proteins [(VAMP-associated protein, named Scs2p in yeast) in the ER (Carman and Han, 2011). Opi1p is a repressor of phospholipid biosynthesis and it also serves as sensor to detect the amount of PA in the ER. The release of Opi1p from the ER leads to relocalization to the nucleus, where Opi1p strongly represses the transcriptional activity of Ino2p, which is complexed with Ino4p, and regulates the transcription of phospholipid and inositol synthesis genes from the UAS_{INO} transcriptional regulatory sequences (Chen, Hancock, and Lopes, 2007; Nohturfft and Zhang, 2009a). When Opi1p binds to PA and Scs2p, then Opi1p is retained in the ER, thus preventing the repressor activity of Opi1p, which depends on nuclear localization and binding to the nuclear Ino2p (Carman and Han, 2011). Therefore, the ER localization of Opi1p is important for regulation of transcription of the phospholipid biosynthesis genes in the cell.

Based on the ability of Opi1p to act as phospholipid PA sensor in the ER, where most of the phospholipid synthesis takes place, Opi1p tightly controls phospholipid biosynthesis in the cell (Chen, Hancock, and Lopes, 2007; Daum, 2004; Loewen et al., 2004; Nohturfft and Zhang, 2009a). We hypothesized that interfering with the function of Opi1p host protein by the virus could lead to increased phospholipid synthesis needed for virus replication. Accordingly, we show that transcription of phospholipid biosynthesis genes is greatly upregulated by the p33 replication protein in yeast, leading to increased level of phospholipids. We also find that p33 interacts with Opi1p, which leads to induction of phospholipid biosynthesis genes in *OPI1* and *SCS2*-dependent way. Deletion of *OPI1* leads to increased TBSV accumulation and high tombusvirus replicase activity *in vitro*. Altogether, the obtained evidence suggests that a (+)RNA virus can actively reprogram host cell lipid and membrane biogenesis to promote virus replication.

RESULTS

TBSV up-regulates transcription of phospholipid biosynthesis genes and increases phospholipid levels in yeast

To test if TBSV replication could affect the expression of phospholipid biosynthesis genes in yeast model host, we chose three well-characterized phospholipid biosynthesis genes, namely *INO1*, *OPI3* and *CHO1* (Carman and Han, 2011). Induction of TBSV replication in BY4741 (wt) yeast cells led to ~4-to-9-fold induction of transcription of *INO1*, *OPI3* and *CHO1* genes when compared with BY4741 yeast carrying the empty expression plasmids (Fig. 1A, lanes 3–4 versus 1–2). Thus, transcription of several key phospholipid biosynthesis genes is induced by TBSV replication in yeast. Since phospholipid biosynthesis is mainly regulated at the transcription level (Carman and Han, 2011), the increased mRNA levels for these genes indicate robust phospholipid biosynthesis in the presence of TBSV replication.

To see if TBSV replication or only the expression of the p33 replication protein is needed for the induction of yeast phospholipid biosynthesis genes, we compared *INO1* mRNA level in BY4741 yeast expressing only the tombusvirus p33 or supporting full TBSV repRNA replication. These experiments showed that p33 expression in yeast is sufficient to induce transcription of yeast phospholipid genes (Fig. 1B, lane 3 versus 2).

To demonstrate that the upregulated transcription of phospholipid biosynthesis genes leads to altered phospholipid levels in yeast replicating TBSV repRNA, we measured the total phospholipid content of yeast cells with or without TBSV using mass-spectrometry. These experiments revealed that the phospholipid levels of the yeast cells increased to ~270% of those in yeast cells lacking the virus (Fig. 1C). Thus, the presence of TBSV proteins contributes to the increased synthesis of phospholipids in yeast.

The TBSV-driven upregulation of transcription of phospholipid biosynthesis genes depends on the Opi1p and Scs2p lipid sensor proteins

Expression of many phospholipid biosynthesis genes, including *INO1*, *OPI3* and *CHO1*, depends on Ino2p/Ino4p transcription regulators, which are under the control of Opi1p PA-sensor protein (Henry, Kohlwein, and Carman, 2012). Opi1p represses Ino2p function when low amount of PA is present in the ER (Carman and Han, 2011). In *opi1* yeast (in the absence of Opi1p repression), the Ino2p-Ino4p activator complex induces the constitutive expression of *INO1*, *OPI3* and *CHO1* by 5-to-16-fold (Fig. 1A, control lanes 9–10).

We found that TBSV replication in *opi1* yeast only led to less than two-fold induction of transcription of *INO1*, *OPI3* and *CHO1* genes when compared with the *opi1* yeast lacking tombusvirus (Fig. 1A, lanes 11–12 versus 9–10). Similarly, in *scs2* yeast, which results in constitutive inhibition of Ino2p and repression of phospholipid biosynthesis genes (Carman and Han, 2011), TBSV replication induced the transcription of *INO1*, *OPI3* and *CHO1* genes only by ~two-fold (Fig. 1A, lanes 7–8 versus 5–6). Thus, TBSV-driven induction of the transcription of yeast phospholipid biosynthesis genes depends on functional Opi1p and Scs2p in yeast.

Deletion of the yeast Opi1p repressor stimulates TBSV replication in yeast

Due to its repressor function during transcription of phospholipid biosynthesis genes, Opi1p might be a negative regulator of TBSV replication. To test this possibility, we measured TBSV repRNA accumulation in *opi1* yeast, in which many phospholipid biosynthesis genes are constitutively expressed (Henry, Kohlwein, and Carman, 2012; Kumme et al., 2008; Nohturfft and Zhang, 2009b). Interestingly, TBSV replication increased by ~5-fold in *opi1* yeast (Fig. 2, lanes 5–6 versus 1–2), indicating that up-regulation of transcription of phospholipid biosynthesis genes in the absence of Opi1p repressor is beneficial for TBSV replication.

To test if the increased accumulation of TBSV repRNA in *opi1* yeast is due to enhanced tombusvirus replicase activity, we isolated the membrane-bound tombusvirus replicase carrying the endogenous repRNA (Fig. 3A) (Panaviene et al., 2004). The activity of replicase preparations obtained from *opi1* yeast were up by ~3-fold of those preparations

obtained from wt yeast (Fig. 3B, lanes 7–9 versus lanes 1–3). Thus, the increased phospholipid levels in yeast in the absence of Opi1p repressor enhance tombusvirus replicase activity *in vitro*.

Over-expression of Opi1p repressor decreases TBSV replication in yeast

To further test the inhibitory function of Opi1p repressor on TBSV RNA replication, we over-expressed the wt Opi1p from the galactose-inducible *GALI* promoter (Fig. 4A) in BY4741 yeast strain carrying the tombusvirus expression plasmids. We found that the over-expression of Opi1p inhibited TBSV repRNA accumulation by ~50% (Fig. 4B, lanes 3–4 versus 1–2). This level of inhibition is significant, because over-expression of most yeast proteins reduces TBSV repRNA replication in yeast only by ~20% (based on over-expression of 5,500 yeast proteins) (Shah Nawaz-Ul-Rehman et al., 2012). Over-expression of Opi1p also led to reduced accumulation of p92^{Pol} replication protein.

To test if the ability of Opi1p to interact with Ino2p transcription factor is important for inhibition of TBSV repRNA accumulation in yeast, we expressed a C-terminally truncated version of Opi1p lacking the Ino2p interaction domain (Fig. 4A). Interestingly, unlike in case of wt Opi1p, the expression of Opi1- C did not inhibit TBSV repRNA accumulation (Fig. 4B, lanes 5–6), suggesting that it is critical for Opi1p to interact with Ino2p transcription factor to suppress TBSV replication.

TBSV replication reduces the level of Opi1p repressor in yeast

The robust induction of transcription of phospholipid biosynthesis genes during TBSV replication could be due to inhibition of the repressor function of Opi1p. Therefore, we tested Opi1p levels in wt and *scs2* yeasts. In this experiment, Opi1p was expressed from a plasmid using *GALI* promoter, so it could not be auto-regulated by Opi1p level via transcriptional control. Interestingly, TBSV replication in wt yeast led to markedly reduced level of Opi1p (by ~8-fold, Fig. 5A, lanes 3–4 versus 1–2). The reduction of Opi1p level was also observed in *scs2* yeast by ~2-fold (Fig. 5A, lanes 7–8 versus 5–6). Based on these data, we propose that a tombusvirus affects the steady-state level of Opi1p repressor in yeast.

To test if Scs2p, the major interactor of Opi1p in yeast ER membrane, could stabilize Opi1p in yeast replicating TBSV, we over-expressed Scs2p in BY4741 yeast. We observed that the accumulation of Opi1p was still dramatically reduced (Fig. 5B, lanes 4–6 versus 1–3), suggesting that tombusvirus can efficiently reduce the amount of Opi1p in yeast cells.

Tombusvirus p33 replication protein interacts with the yeast Opi1p repressor

To test if p33 could interact with Opi1p, we performed co-purification experiments from detergent-solubilized membrane fraction from yeast co-expressing HA-tagged Opi1p and FLAG-tagged p33. These experiments revealed selective co-purification of Opi1p with the tombusvirus p33 (Fig. 6A, lane 4). The interaction between p33 : Opi1p, however, was weaker than interaction between p33 : Scs2p based on similar co-purification experiments (Fig. 6A, lane 2).

To test if the interaction between p33 : Opi1p was dependent on Scs2p, we also performed co-purification experiments from *scs2* yeast or *scs2 scs22* yeast [also lacking the related Scs22p, a minor protein with similar role to Scs2p) (Loewen and Levine, 2005)] co-expressing HA-Opi1p and FLAG-p33. We found that Opi1p was co-purified with p33 at the same extent (Fig. 6B, lanes 4 and 6 versus 2), excluding that Scs2p/Scs22p could serve as a “bridge” between p33 and Opi1p during co-purification.

DISCUSSION

(+)RNA viruses depend on cellular membranes during their replication (Belov and van Kuppeveld, 2012; de Castro, Volonte, and Risco, 2013; den Boon, Diaz, and Ahlquist, 2010; Miller and Krijnse-Locker, 2008). In order to assemble multiple VRCs or form viral replication organelles in infected cells, these viruses likely have to reshape/deform membranes to new structures, such as spherules and vesicles. In addition, they likely have to induce membrane proliferation or stimulate the synthesis of new lipids, which can be hijacked for the assembly of new VRCs. Based on these needs for (+)RNA virus replication, it is an emerging theme that (+)RNA viruses modify the lipid biosynthesis machinery in infected cells (Heaton et al., 2010; Heaton and Randall, 2010; Hsu et al., 2010; Reiss et al., 2011). Among the targeted lipids are phospholipids, which are major components of cellular membranes, affecting the size, shape and rigidity of membranes and intracellular organelles (Carman and Han, 2011; Nohturfft and Zhang, 2009a).

In this paper, we show biochemical and genetic evidence that TBSV reprograms the cellular phospholipid biosynthesis pathway leading to upregulation of transcription of several phospholipid synthesis genes and ~2.5-fold increase in phospholipids levels in yeast model host. These events likely occur due to interaction of p33 : Opi1p repressor and reduction of Opi1p level, which then leads to increased expression of phospholipids biosynthesis genes. The interaction between p33 and the yeast Scs2p protein in regulation of transcription of phospholipid synthesis genes might also be important, but not explored in much detail here. Ultimately, these processes are predicted to lead to membrane proliferation in cells replicating TBSV, a phenomenon that have been shown by using ultrastructural analyses in plants and yeast (Navarro et al., 2006; Rubino, Di Franco, and Russo, 2000; Russo, Di Franco, and Martelli, 1983). Indeed, a large number of deformed peroxisomes and other membranes has previously been documented in tombusvirus-replicating cells (Barajas, Jiang, and Nagy, 2009; Barajas et al., 2014; McCartney et al., 2005; Rochon et al., 2014; Rubino, Di Franco, and Russo, 2000; Russo, Di Franco, and Martelli, 1983). Also, blocking the phospholipid biosynthesis pathway via deletion of *INO2/INO4* transcription activator genes has resulted in reduction of TBSV replication, altered the subcellular distribution and stability of the viral replication proteins and decreased the activity of the viral replicase (Sharma, Sasvari, and Nagy, 2011).

The current work indicates that the tombusvirus p33 replication protein binds to the FFAT-motif containing Opi1p, and this results in reduced level of Opi1p accumulation (Fig. 5). It is possible that p33 binding to both Opi1p and Scs2p inhibits the interaction between Scs2p and Opi1p. All these activities by p33 might cause lower activity and/or reduced stability of Opi1p repressor, which ultimately leads to increased transcription of phospholipid

biosynthesis genes (Fig. 1). We show genetic evidence that the TBSV-induced reprogramming of the host phospholipid biosynthesis pathway depends on the *OPI1* and *SCS2* genes (Fig. 2).

The increased phospholipid biosynthesis is beneficial for the activity of the tombusvirus replicase based on our *in vitro* replication experiments with tombusvirus replicase preparations from *opi1* yeast (Fig. 3). It seems that the tombusvirus-induced phospholipid biosynthesis is important during replication possibly due to the limited availability of membrane surfaces or particular lipids for the rapidly replicating TBSV. Accordingly, deletion of the yeast lipin, called *PAH1* (phosphatidic acid phosphohydrolase), which leads to proliferation and expansion of the endoplasmic reticulum (ER) membrane, resulted in the subversion of the vastly expanded ER membranes by tombusviruses to build VRCs and support increased level of viral replication (Chuang et al., 2014). Thus, tombusviruses could efficiently exploit expanded membrane surfaces in cells. Altogether, the presented data convincingly demonstrate that *de novo* lipid biosynthesis is required for optimal TBSV replication.

Based on the available data, we propose the following model to describe the mechanism of TBSV-driven reprogramming of the host cellular phospholipid biosynthesis machinery in yeast cells (Fig. 7): The viral p33 replication protein binds to Opi1p repressor and the Scs2p VAP protein in the ER, which might inhibit the dynamic interaction between Opi1p repressor and Scs2p that depends on PA levels in the ER. Under this condition, Opi1p will not get activated/translocated to the nucleus and it likely becomes degraded. Second, in the presence of only low amount of activated and nucleus-bound Opi1p repressor, Ino2p/Ino4p complex activates efficient transcription of numerous phospholipid biosynthesis genes, which will lead to new phospholipid synthesis (Fig. 7A–B). Then, the newly made phospholipids participate in membrane proliferation and formation of new structures (spherules/replication organelles) guided by viral and host proteins (such as the host ESCRT proteins) (Barajas, Jiang, and Nagy, 2009; Barajas et al., 2014; Barajas and Nagy, 2010) (Fig. 7B). Altogether, the TBSV-induced reprogramming of the host phospholipid biosynthesis pathway depends on targeting the Opi1p repressor and Scs2p VAP protein by the tombusviral p33 protein, which might occur via “blinding the lipid sensors” to force the host to produce abundant phospholipids to be hijacked by the virus for replication.

In summary, we have identified a new pathway exploited by a tombusvirus to reprogram the host phospholipid biosynthesis to benefit virus replication in yeast cells. This pathway is based on conserved FFAT-motif and VAP-containing host proteins. This is beneficial for TBSV since virus replication requires new cellular membranes for replication, similar to many other (+)RNA viruses (Castorena, Stapleford, and Miller, 2010; Netherton et al., 2007).

MATERIALS AND METHODS

Yeast strains and expression plasmids

Saccharomyces cerevisiae strain BY4741 (*MATa his3 1 leu2 0 met15 0 ura3 0*) and the Yeast Knock Out (YKO) strains *scs2* and *opi1* were obtained from Open Biosystems.

Strain Sc1 was obtained from Invitrogen. Other yeast strains were generated by homologous recombination. To create the double deletion strain *scs2* Δ *scs22*, the nourseotricin resistance gene *natNT2* was amplified from plasmid pFA6-natNT2 (Euroscarf) with primers #3668 and #3669 (Table 1) and the product transformed into *scs2* strain. To introduce C-terminal 6xHA tags, the 6xHA-hphNT1 cassette was amplified from plasmid pYM16 (Janke et al., 2004) with primers #3263 / #3264 or #3260 / #3261 (Table 1), and the products were transformed into BY4741 or deletion strains to generate SCS2-6xHA and OPI1-6xHA strains, respectively.

Plasmids pGBK-His33-CUP1/DI72-GAL1, pGAD-His92-CUP1 and pGAD-FLAG-92-CUP1 used for TBSV replication assays in yeast have been described before (Barajas, Li, and Nagy, 2009). Plasmid pYC-HF, expressing a 6xHis/FLAG peptide from the *GALI* promoter, has been described (Serva and Nagy, 2006). Plasmid pGBK-His33-CUP1 expressing a 6xHis-tagged tombusvirus p33 protein from the *CUP1* promoter has been described (Jaag, Stork, and Nagy, 2007). To create plasmid pGBK-FLAG-p33-CUP1, expressing a FLAG-tagged CNV p33 from the *CUP1* promoter, the FLAG-p33 ORF was excised from plasmid pGBK-FLAG-p33 (Li et al., 2008b) by *NcoI/PstI* digestion and inserted into pGBK-His33-CUP1, in which the original 6xHis-p33 had been removed by *NcoI/PstI* digestion.

To create plasmid pYES-CT-Opi1, which expresses 6xHis-tagged Opi1 protein (the tag is placed at the C-terminus) from the *GALI* promoter, *OPI1* ORF was PCR amplified using yeast DNA with primers #3975 and #3976, digested with *BamHI* and *NheI* and inserted into *BamHI/XbaI*-digested pYES2/CT (Invitrogen). For the C-terminally truncated version, *OPI1* sequence coding amino acids 1-321 was amplified with primers #3975 and #4351, digested with *BamHI* and *NheI* and inserted into *BamHI/XbaI*-digested pYES2/CT. To create plasmid pPR-N-Opi1 which over-expresses and N-terminal HA-tagged Opi1p, *OPI1* ORF was amplified with primers # 3203 and #3204, digested with *BamHI* and *NheI* and inserted into *BamHI/NheI*-digested pPR-N-RE. Additionally, to create pPR-FLAG-opi1 expressing a FLAG-tagged Opi1p, *OPI1* ORF was amplified with primers #4167 and #3976, digested with *SpeI* and *XhoI* and inserted into pPR-N-RE, in which the NubG-HA tag had been removed by *SpeI/SalI* digestion.

Co-purification of host proteins with the tombusvirus p33 or with SCS2p

Yeast strains expressing 6xHA-tagged host proteins from their chromosomal locations were transformed with plasmids pGBK-His33-CUP1 (expressing a 6xHis-tagged CNV p33 protein) or pGBK-FLAG-p33-CUP1 (expressing a FLAG-tagged CNV p33). Transformed yeasts were pre-grown in SC media containing 2% glucose for 16 hours at 29°C and then, in SC media with 2% galactose and reduced inositol (10 μ M) for 24 hours at 29°C. Then 50 μ M CuSO₄ was added and the yeast cultures were incubated for 2 hours at 29°C. The yeast cultures were then centrifuged, washed with phosphate buffer saline (PBS) and incubated in PBS plus 1% formaldehyde for 1 hour on ice to crosslink proteins. Then, the formaldehyde was quenched by adding glycine (to 0.1 M) and the yeast was recovered by centrifugation (Pathak, Sasvari, and Nagy, 2008). Yeast was broken with glass beads and p33 protein was purified using anti-FLAG M2 agarose as described before (Li et al., 2008b). Affinity-

purified p33 was analyzed by Western blot using anti-FLAG antibody, followed by anti-mouse antibody conjugated to alkaline phosphatase. Co-purified 6xHA-tagged host proteins were analyzed with anti-HA antibody, followed by alkaline phosphatase- conjugated anti-rabbit and detection with NBT-BCIP as described (Barajas, Jiang, and Nagy, 2009; Panaviene et al., 2004).

Analysis of TBSV replication in yeast

Yeast strain BY4741 or the gene deletion strains were transformed with plasmids pGBK-His33-CUP1/DI72-GAL1 and pGAD-His92-CUP1 or pGAD-FLAG-92-CUP1 to launch TBSV replication. Transformed yeasts were grown in SC media supplemented with 2% galactose for 16 hours at 23°C. Then CuSO₄ was added to a final concentration of 50 µM and the cultures incubated for 24 hours at 23°C. RNA was extracted and the accumulation of DI-72 repRNA was analyzed by Northern blot, using the 18S rRNA for normalization, as previously described (Barajas, Jiang, and Nagy, 2009; Panaviene et al., 2004). Accumulation of the viral proteins p33 and p92 was analyzed by Western blot. Proteins were extracted from the same cultures used to analyze repRNA accumulation, using NaOH and SDS-PAGE loading buffer as described (Panaviene et al., 2004). Proteins were detected with anti-6xHis or anti-FLAG antibodies followed by alkaline phosphatase-conjugated anti-mouse antibody and NBT-BCIP detection (Panaviene et al., 2004).

For the analysis of Opi1p effect on TBSV replication, the yeast strain BY4741 was transformed with plasmids pGBK-His33-CUP1/DI72-GAL1, pGAD-His92-CUP1 plus pYES2/CT, pYES-CT-Opi1 or pYES-CT-Opi1(1-321). The transformed yeasts were cultured and process as above to analyze the accumulation of repRNA and viral proteins.

In vitro replicase assay using yeast membrane-enriched fractions

The BY4741 or deletion strains carrying plasmids pGBK-His33-CUP1/DI72-GAL1 and pGAD-His92-CUP1 were pre-grown in SC media with 2% glucose at 23°C for 16 hours. Then, the yeast cultures were transferred to SC media with 2% galactose and 50 µM CuSO₄ and incubated at 23°C for 36 hours. Yeast cultures were collected by centrifugation and broken with glass beads as described (Barajas, Li, and Nagy, 2009) to obtain membrane-enriched fractions containing the active TBSV replication complexes including the repRNA. The amount of p33 protein in each preparation was analyzed by Western blot and the volumes adjusted in order to use comparable amounts of replicase from each preparation. The activity of the TBSV replicase in each preparation was analyzed as previously described (Barajas, Li, and Nagy, 2009). The reactions were performed in 100 µl containing 25 µl of the normalized membrane-enriched fraction preparations, 50 mM Tris-Cl pH 8.0, 10 mM MgCl₂, 10 mM DTT, 0.2 µl RNase inhibitor, 1 mM ATP, 1 mM CTP, 1 mM GTP and 0.1 µl of α³²P UTP (3000 Ci/mmol). Reaction mixtures were incubated for 2 hours at 25°C and then the RNA was obtained by phenol/chloroform extraction and isopropanol/amonium acetate (10:1) precipitation. The α³²P-UTP-labeled repRNA products were separated in 8% acrylamide/8M urea gels and detected using a phosphorimager.

Analysis of the expression of phospholipids biosynthesis genes by Northern blot

The BY4741 or deletion strains containing plasmids pGBK-His33-CUP1/DI72-GAL1 and pGAD-FLAG-92-CUP1, or yeast containing empty plasmids were grown as described in the analysis of TBSV replication, with the exception that the media contained reduced amounts of inositol (10 μ M instead of 400 μ M in the regular SC media). RNA was extracted as described previously (Panaviene et al., 2004). The mRNAs of *INO1*, *OPI3* and *CHO2* phospholipids biosynthesis genes were detected using specific α^{32} P-UTP-labeled RNA probes complementary to the 5' region of each gene.

Analysis of the accumulation of Opi1 protein

Yeast strains were transformed with pYES-CT-opi1, which expresses the C-terminal 6xHis-tagged Opi1 protein, plus pGBK-His33-CUP1/DI72-GAL1 and pGAD-His92-CUP1 or empty plasmids. Additionally, Sc1 yeasts were transformed with plasmids pYC-NT-scs2 expressing a 6xHis-tagged Scs2p, pPR-FLAG-opi1 and either pGBK-His33-CUP1/DI72-GAL1, pGAD-His92-CUP1 or the cognate empty plasmids. Yeast was grown essentially as described for analysis of TBSV replication. Total proteins were extracted using NaOH and SDS-PAGE loading buffer as described (Panaviene et al., 2004). Accumulation of the Opi1 protein was analyzed by Western blot using anti-6xHis antibody or anti-FLAG antibody followed by alkaline phosphatase-conjugated anti-mouse antibody followed by NBT-BCIP detection as described (Panaviene et al., 2004).

Phospholipid analysis in yeast

Yeast were pre-cultured in glucose containing media overnight, washed and diluted to 0.3 OD₆₀₀ units/ml in galactose containing media and cultured to 2 OD₆₀₀ units/ml. In a 15 ml glass tube, 0.4 g yeast cells, 1.2 ml water, 2 ml chloroform, 4 ml methanol, 0.8 g glass beads were mixed and vortexed vigorously. Then 2 ml chloroform and 2 ml water were added and mixed into the tube and centrifuged at low speed. Lower organic phase were collected. 2 ml chloroform was mixed with the remaining inorganic phase; lower organic phase was withdrawn. Chloroform extraction step was repeated. All the organic phases from previous steps were combined and washed with 0.5 ml 1M KCl and water, respectively. Washed organic phase was analyzed on a triple quadrupole MS/MS equipped for electrospray ionization (ESI).

The total biomass of yeasts was measured before extraction of phospholipids. Spiked lipid standards di 10:0 PE/PC/PC (Avanti Polar Lipids, Inc) were added to the cells before breaking the cells by glass beads. These spiked lipid standards do not exist in the yeast, so they are used as control for normalization for the loss of different phospholipids classes during the extraction step. In addition, two internal lipids standards of each phospholipid class were mixed with each sample before ESI-MS/MS analysis for quantification (Li et al., 2008a). Altogether, we measured 5 major phospholipids: PC, PE, PG, PS and PI, which were used to represent the total phospholipids. The final total amount (in moles) of these 5 major phospholipids from each sample was normalized against the yeast biomass value, which is measured before extraction, and then, the normalized data were compared between different treatments. Three biological repeats were performed for different treatment.

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REFERENCES

- Barajas D, Jiang Y, Nagy PD. A Unique Role for the Host ESCRT Proteins in Replication of Tomato bushy stunt virus. *PLoS Pathog.* 2009; 5(12):e1000705. [PubMed: 20041173]
- Barajas D, Li Z, Nagy PD. The Nedd4-Type Rsp5p Ubiquitin Ligase Inhibits Tombusvirus Replication by Regulating Degradation of the p92 Replication Protein and Decreasing the Activity of the Tombusvirus Replicase. *J Virol.* 2009; 83(22):11751–64. [PubMed: 19759160]
- Barajas D, Martin IF, Pogany J, Risco C, Nagy PD. Noncanonical Role for the Host Vps4 AAA+ ATPase ESCRT Protein in the Formation of Tomato Bushy Stunt Virus Replicase. *PLoS Pathog.* 2014; 10(4):e1004087. [PubMed: 24763736]
- Barajas D, Nagy PD. Ubiquitination of tombusvirus p33 replication protein plays a role in virus replication and binding to the host Vps23p ESCRT protein. *Virology.* 2010; 397(2):358–368. [PubMed: 20004458]
- Belov GA, Ehrenfeld E. Involvement of cellular membrane traffic proteins in poliovirus replication. *Cell Cycle.* 2007; 6(1):36–8. [PubMed: 17245115]
- Belov GA, Habbersett C, Franco D, Ehrenfeld E. Activation of cellular Arf GTPases by poliovirus protein 3CD correlates with virus replication. *J Virol.* 2007; 81(17):9259–67. [PubMed: 17567696]
- Belov GA, van Kuppeveld FJ. (+)RNA viruses rewire cellular pathways to build replication organelles. *Curr Opin Virol.* 2012; 2(6):740–7. [PubMed: 23036609]
- Berger KL, Cooper JD, Heaton NS, Yoon R, Oakland TE, Jordan TX, Mateu G, Grakoui A, Randall G. Roles for endocytic trafficking and phosphatidylinositol 4-kinase III alpha in hepatitis C virus replication. *Proc Natl Acad Sci U S A.* 2009; 106(18):7577–82. [PubMed: 19376974]
- Carman GM, Han GS. Regulation of phospholipid synthesis in the yeast *Saccharomyces cerevisiae*. *Annu Rev Biochem.* 2011; 80:859–83. [PubMed: 21275641]
- Castorena KM, Stapleford KA, Miller DJ. Complementary transcriptomic, lipidomic, and targeted functional genetic analyses in cultured *Drosophila* cells highlight the role of glycerophospholipid metabolism in Flock House virus RNA replication. *BMC Genomics.* 2010; 11:183. [PubMed: 20236518]
- Chen M, Hancock LC, Lopes JM. Transcriptional regulation of yeast phospholipid biosynthetic genes. *Biochim Biophys Acta.* 2007; 1771(3):310–21. [PubMed: 16854618]
- Cherry S, Doukas T, Armknecht S, Whelan S, Wang H, Sarnow P, Perrimon N. Genome-wide RNAi screen reveals a specific sensitivity of IRES-containing RNA viruses to host translation inhibition. *Genes Dev.* 2005; 19(4):445–52. [PubMed: 15713840]
- Cherry S, Kunte A, Wang H, Coyne C, Rawson RB, Perrimon N. COPI activity coupled with fatty acid biosynthesis is required for viral replication. *PLoS Pathog.* 2006; 2(10):e102. [PubMed: 17040126]
- Chuang C, Barajas D, Qin J, Nagy PD. Inactivation of the Host Lipin Gene Accelerates RNA Virus Replication through Viral Exploitation of the Expanded Endoplasmic Reticulum Membrane. *PLoS Pathog.* 2014; 10(2):e1003944. [PubMed: 24586157]
- Daum G. Membrane targeting: glued by a lipid to the ER. *Curr Biol.* 2004; 14(17):R711–3. [PubMed: 15341762]
- de Castro IF, Volonte L, Risco C. Virus factories: biogenesis and structural design. *Cell Microbiol.* 2013; 15(1):24–34. [PubMed: 22978691]
- den Boon JA, Diaz A, Ahlquist P. Cytoplasmic viral replication complexes. *Cell Host Microbe.* 2010; 8(1):77–85. [PubMed: 20638644]

- Heaton NS, Perera R, Berger KL, Khadka S, Lacount DJ, Kuhn RJ, Randall G. Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. *Proc Natl Acad Sci U S A*. 2010; 107(40):17345–50. [PubMed: 20855599]
- Heaton NS, Randall G. Dengue virus-induced autophagy regulates lipid metabolism. *Cell Host Microbe*. 2010; 8(5):422–32. [PubMed: 21075353]
- Henry SA, Kohlwein SD, Carman GM. Metabolism and regulation of glycerolipids in the yeast *Saccharomyces cerevisiae*. *Genetics*. 2012; 190(2):317–49. [PubMed: 22345606]
- Hsu NY, Ilnytska O, Belov G, Santiana M, Chen YH, Takvorian PM, Pau C, van der Schaar H, Kaushik-Basu N, Balla T, Cameron CE, Ehrenfeld E, van Kuppeveld FJ, Altan-Bonnet N. Viral reorganization of the secretory pathway generates distinct organelles for RNA replication. *Cell*. 2010; 141(5):799–811. [PubMed: 20510927]
- Jaag HM, Stork J, Nagy PD. Host transcription factor Rpb1 1p affects tombusvirus replication and recombination via regulating the accumulation of viral replication proteins. *Virology*. 2007; 368(2):388–404. [PubMed: 17689583]
- Janke C, Magiera MM, Rathfelder N, Taxis C, Reber S, Maekawa H, Moreno-Borchart A, Doenges G, Schwob E, Schiebel E, Knop M. A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. *Yeast*. 2004; 21(11):947–62. [PubMed: 15334558]
- Jiang Y, Serviène E, Gal J, Panavas T, Nagy PD. Identification of essential host factors affecting tombusvirus RNA replication based on the yeast Tet promoters Hughes Collection. *J Virol*. 2006; 80(15):7394–404. [PubMed: 16840320]
- Kopek BG, Perkins G, Miller DJ, Ellisman MH, Ahlquist P. Three-dimensional analysis of a viral RNA replication complex reveals a virus-induced mini-organelle. *PLoS Biol*. 2007; 5(9):e220. [PubMed: 17696647]
- Kumme J, Dietz M, Wagner C, Schuller HJ. Dimerization of yeast transcription factors Ino2 and Ino4 is regulated by precursors of phospholipid biosynthesis mediated by Opi1 repressor. *Curr Genet*. 2008; 54(1):35–45. [PubMed: 18542964]
- Kushner DB, Lindenbach BD, Grdzlishvili VZ, Noueiry AO, Paul SM, Ahlquist P. Systematic, genome-wide identification of host genes affecting replication of a positive-strand RNA virus. *Proc Natl Acad Sci U S A*. 2003; 100(26):15764–9. [PubMed: 14671320]
- Lee WM, Ahlquist P. Membrane synthesis, specific lipid requirements, and localized lipid composition changes associated with a positive-strand RNA virus RNA replication protein. *J Virol*. 2003; 77(23):12819–28. [PubMed: 14610203]
- Li W, Wang R, Li M, Li L, Wang C, Welti R, Wang X. Differential degradation of extraplastidic and plastidic lipids during freezing and post-freezing recovery in *Arabidopsis thaliana*. *J Biol Chem*. 2008a; 283(1):461–8. [PubMed: 17962199]
- Li Z, Barajas D, Panavas T, Herbst DA, Nagy PD. Cdc34p Ubiquitin-Conjugating Enzyme Is a Component of the Tombusvirus Replicase Complex and Ubiquitinates p33 Replication Protein. *J Virol*. 2008b; 82(14):6911–26. [PubMed: 18463149]
- Li Z, Pogany J, Panavas T, Xu K, Esposito AM, Kinzy TG, Nagy PD. Translation elongation factor 1A is a component of the tombusvirus replicase complex and affects the stability of the p33 replication co-factor. *Virology*. 2009; 385(1):245–60. [PubMed: 19131084]
- Loewen CJ, Gaspar ML, Jesch SA, Delon C, Ktistakis NT, Henry SA, Levine TP. Phospholipid metabolism regulated by a transcription factor sensing phosphatidic acid. *Science*. 2004; 304(5677):1644–7. [PubMed: 15192221]
- Loewen CJ, Levine TP. A highly conserved binding site in vesicle-associated membrane protein-associated protein (VAP) for the FFAT motif of lipid-binding proteins. *J Biol Chem*. 2005; 280(14):14097–104. [PubMed: 15668246]
- McCartney AW, Greenwood JS, Fabian MR, White KA, Mullen RT. Localization of the tomato bushy stunt virus replication protein p33 reveals a peroxisome-to-endoplasmic reticulum sorting pathway. *Plant Cell*. 2005; 17(12):3513–31. [PubMed: 16284309]

- Mendu V, Chiu M, Barajas D, Li Z, Nagy PD. Cpr1 cyclophilin and Ess1 parvulin prolyl isomerases interact with the tombusvirus replication protein and inhibit viral replication in yeast model host. *Virology*. 2010; 406(2):342–51. [PubMed: 20709345]
- Miller S, Krijnse-Locker J. Modification of intracellular membrane structures for virus replication. *Nat Rev Microbiol*. 2008; 6(5):363–74. [PubMed: 18414501]
- Mine A, Okuno T. Composition of plant virus RNA replicase complexes. *Curr Opin Virol*. 2012; 2(6):669–75. [PubMed: 23083891]
- Nagy PD, Pogany J. Yeast as a model host to dissect functions of viral and host factors in tombusvirus replication. *Virology*. 2006; 344(1):211–20. [PubMed: 16364751]
- Nagy PD, Pogany J. Global genomics and proteomics approaches to identify host factors as targets to induce resistance against tomato bushy stunt virus. *Adv Virus Res*. 2010; 76:123–77. [PubMed: 20965073]
- Nagy PD, Pogany J. The dependence of viral RNA replication on co-opted host factors. *Nature Reviews Microbiology*. 2012; 10(2):137–149. [PubMed: 22183253]
- Navarro B, Russo M, Pantaleo V, Rubino L. Cytological analysis of *Saccharomyces cerevisiae* cells supporting cymbidium ringspot virus defective interfering RNA replication. *J Gen Virol*. 2006; 87(Pt 3):705–14. [PubMed: 16476994]
- Netherton C, Moffat K, Brooks E, Wileman T. A guide to viral inclusions, membrane rearrangements, factories, and viroplasm produced during virus replication. *Adv Virus Res*. 2007; 70:101–82. [PubMed: 17765705]
- Nohturfft A, Zhang SC. Coordination of lipid metabolism in membrane biogenesis. *Annu Rev Cell Dev Biol*. 2009a; 25:539–66. [PubMed: 19575637]
- Nohturfft A, Zhang SC. Coordination of Lipid Metabolism in Membrane Biogenesis. *Annual Review of Cell and Developmental Biology*. 2009b; 25:539–566.
- Panavas T, Nagy PD. Yeast as a model host to study replication and recombination of defective interfering RNA of Tomato bushy stunt virus. *Virology*. 2003; 314(1):315–25. [PubMed: 14517084]
- Panavas T, Serviène E, Brasher J, Nagy PD. Yeast genome-wide screen reveals dissimilar sets of host genes affecting replication of RNA viruses. *Proc Natl Acad Sci U S A*. 2005; 102(20):7326–31. [PubMed: 15883361]
- Panaviene Z, Panavas T, Serva S, Nagy PD. Purification of the cucumber necrosis virus replicase from yeast cells: role of coexpressed viral RNA in stimulation of replicase activity. *J Virol*. 2004; 78(15):8254–63. [PubMed: 15254197]
- Pathak KB, Sasvari Z, Nagy PD. The host Pex19p plays a role in peroxisomal localization of tombusvirus replication proteins. *Virology*. 2008; 379(2):294–305. [PubMed: 18684480]
- Pogany J, Stork J, Li Z, Nagy PD. In vitro assembly of the Tomato bushy stunt virus replicase requires the host Heat shock protein 70. *Proc Natl Acad Sci U S A*. 2008; 105(50):19956–61. [PubMed: 19060219]
- Reiss S, Rebhan I, Backes P, Romero-Brey I, Erfle H, Matula P, Kaderali L, Poenisch M, Blankenburg H, Hiet MS, Longerich T, Diehl S, Ramirez F, Balla T, Rohr K, Kaul A, Buhler S, Pepperkok R, Lengauer T, Albrecht M, Eils R, Schirmacher P, Lohmann V, Bartenschlager R. Recruitment and activation of a lipid kinase by hepatitis C virus NS5A is essential for integrity of the membranous replication compartment. *Cell Host Microbe*. 2011; 9(1):32–45. [PubMed: 21238945]
- Rochon D, Singh B, Reade R, Theilmann J, Ghoshal K, Alam SB, Maghodia A. The p33 auxiliary replicase protein of Cucumber necrosis virus targets peroxisomes and infection induces de novo peroxisome formation from the endoplasmic reticulum. *Virology*. 2014; 452–453:133–42.
- Rubino L, Di Franco A, Russo M. Expression of a plant virus non-structural protein in *Saccharomyces cerevisiae* causes membrane proliferation and altered mitochondrial morphology. *J Gen Virol*. 2000; 81(Pt 1):279–86. [PubMed: 10640568]
- Russo M, Di Franco A, Martelli GP. The fine structure of Cymbidium ringspot virus infections in host tissues. III. Role of peroxisomes in the genesis of multivesicular bodies. *J Ultrastruct Res*. 1983; 82(1):52–63. [PubMed: 6848773]
- Sasvari Z, Nagy PD. Making of Viral Replication Organelles by Remodeling Interior Membranes. *Viruses-Basel*. 2010; 2(11):2436–2442.

- Schwartz M, Chen J, Janda M, Sullivan M, den Boon J, Ahlquist P. A positive-strand RNA virus replication complex parallels form and function of retrovirus capsids. *Mol Cell*. 2002; 9(3):505–14. [PubMed: 11931759]
- Serva S, Nagy PD. Proteomics analysis of the tombusvirus replicase: Hsp70 molecular chaperone is associated with the replicase and enhances viral RNA replication. *J Virol*. 2006; 80(5):2162–9. [PubMed: 16474124]
- Serviene E, Jiang Y, Cheng CP, Baker J, Nagy PD. Screening of the yeast yTHC collection identifies essential host factors affecting tombusvirus RNA recombination. *J Virol*. 2006; 80(3):1231–41. [PubMed: 16415000]
- Serviene E, Shapka N, Cheng CP, Panavas T, Phuangrat B, Baker J, Nagy PD. Genome-wide screen identifies host genes affecting viral RNA recombination. *Proc Natl Acad Sci U S A*. 2005; 102(30):10545–50. [PubMed: 16027361]
- Shah Nawaz-Ul-Rehman M, Martinez-Ochoa N, Pascal H, Sasvari Z, Herbst C, Xu K, Baker J, Sharma M, Herbst A, Nagy PD. Proteome-wide overexpression of host proteins for identification of factors affecting tombusvirus RNA replication: an inhibitory role of protein kinase C. *J Virol*. 2012; 86(17):9384–95. [PubMed: 22718827]
- Sharma M, Sasvari Z, Nagy PD. Inhibition of phospholipid biosynthesis decreases the activity of the tombusvirus replicase and alters the subcellular localization of replication proteins. *Virology*. 2011; 415(2):141–52. [PubMed: 21561636]
- Stapleford KA, Rapaport D, Miller DJ. Mitochondrion-enriched anionic phospholipids facilitate flock house virus RNA polymerase membrane association. *J Virol*. 2009; 83(9):4498–507. [PubMed: 19244330]
- Stork J, Kovalev N, Sasvari Z, Nagy PD. RNA chaperone activity of the tombusviral p33 replication protein facilitates initiation of RNA synthesis by the viral RdRp in vitro. *Virology*. 2011; 409(2):338–47. [PubMed: 21071052]
- White KA, Nagy PD. Advances in the molecular biology of tombusviruses: gene expression, genome replication, and recombination. *Prog Nucleic Acid Res Mol Biol*. 2004; 78:187–226. [PubMed: 15210331]
- Xu K, Huang TS, Nagy PD. Authentic in vitro replication of two tombusviruses in isolated mitochondrial and endoplasmic reticulum membranes. *J Virol*. 2012; 86(23):12779–94. [PubMed: 22973028]
- Zhang J, Diaz A, Mao L, Ahlquist P, Wang X. Host acyl coenzyme A binding protein regulates replication complex assembly and activity of a positive-strand RNA virus. *J Virol*. 2012; 86(9):5110–21. [PubMed: 22345450]

tombusvirus p33 replication protein interacts with FFAT-domain host protein;
tombusvirus replication leads to upregulation of phospholipids;
tombusvirus replication depends on de novo lipid synthesis;
Deletion of FFAT-domain host protein enhances TBSV replication
TBSV rewires host phospholipid synthesis

Fig. 1.

TBSV replication induces transcription of phospholipid biosynthesis genes in yeast. (A) Northern blot analysis of *INO1*, *OPI3* and *CHO1* mRNA levels in wt, *scs2* and *opi1* yeasts at 24 h time point after induction of replication by addition of 50 μ M CuSO₄. To launch TBSV repRNA replication, we expressed His₆-p33 and FLAG-tagged p92 from the copper-inducible *CUP1* promoter and DI-72(+) replicon RNA from the galactose-inducible *GAL1* promoter in the parental (BY4741) and in *scs2* and *opi1* yeast strains. The yeast cells were cultured for 16 hours at 23 °C on 2% galactose SC minimal media with reduced amounts of inositol (10 μ M), and then for 24 h at 23 °C on 2% galactose SC minimal media (10 μ M inositol) supplemented with 50 μ M CuSO₄. The bottom panel shows the total RNA extract in the ethidium-bromide stained agarose gel as a loading control. The accumulation of TBSV repRNA is pointed by a black arrowhead, while rRNAs are yeast ribosomal RNAs. Standard deviation from all the experimental repeats (3 repeats) is shown. (B) p33 replication protein alone can induce the expression of *INO1* gene in yeast. The Northern blot analysis shows *INO1* mRNA level in wt yeast at 24 h time point after expression of viral proteins/repRNA as shown. (C) Increased levels of phospholipids in yeast replicating TBSV repRNA. Total phospholipid level of yeast cells was measured by mass-spectrometry. BY4741 yeast without TBSV repRNA (also lacking the CNV p33 and p92^{pol}) was used as control (100%). Standard deviation from all the experimental repeats (3 repeats) is shown.

Fig. 2.

Increased TBSV repRNA accumulation in *opi1* yeast. Yeast cells were pre-grown and TBSV repRNA replication was initiated as described in the legend to Fig. 1A. Northern blot analysis was used to detect DI-72(+) repRNA accumulation after yeasts were cultured for 24 h at 23 °C on 2% galactose SC minimal media supplemented with 50 μ M CuSO₄. The accumulation level of DI-72(+) repRNA was normalized based on 18S rRNA levels. Bottom panels: Western blot analysis of the accumulation level of His₆-tagged p33 and FLAG-tagged p92^{pol} proteins using anti-6xHis or anti-FLAG antibodies. Each experiment was performed three times.

Fig. 3.

Increased activity of the tombusvirus replicase assembled in *opi1* yeast. (A) Scheme of the experimental design. (B) Top image: Denaturing PAGE analysis of *in vitro* replicase activity in the membrane-enriched fraction from *scs2* and *opi1* yeasts using the co-purified repRNA. The yeast cells were harvested for analysis at 36 h time point after launching TBSV replication. Note that this phosphorimager image shows the ³²P-UTP-labeled repRNAs made by the replicase *in vitro*. The quantified data indicate the relative activity of the tombusvirus replicase in these samples, due to the adjustment of the p33 level to comparable level in each sample. Lower image: Western blot analysis of the level of His₆-tagged p33 replication protein using anti-6xHis antibodies. Each experiment was performed three times.

Fig. 4.

Inhibition of TBSV replication by Opi1p over-expression requires Opi1p : Ino2p interaction. (A) Domain structure of the yeast Opi1p with the interacting partners of the known domains. Sin3p is part of a histone deacetylase complex involved in transcription regulation; PA represents the phosphatidic acid binding region; Scs2p is a VAP protein involved in regulation of phospholipid biosynthesis; Ino2p is a transcriptional activator for expression of phospholipid biosynthesis genes. (B) Replication of TBSV repRNA was launched by expressing 6xHis-p33 and 6xHis-p92 from the *CUP1* promoter and DI-72 (+)repRNA from the *GALI* promoter. 6xHis-tagged Opi1p or the C-terminal truncated Opi1p (containing aa 1-321 out of 404 aa), which lacked the Ino2p interaction domain, were expressed from the *GALI* promoter. The yeast cells were cultured for 16 hours at 23 °C in 2% galactose SC minimal media, and then for 24 hours at 23 °C in 2% galactose SC minimal media supplemented with 50 μM CuSO₄. Accumulation of DI-72 (+)repRNA was analyzed by Northern blot and normalized based on 18S rRNA levels. Bottom panel: Western blot analysis of the accumulation of 6xHis-p33 and 6xHis-p92 viral proteins as well as Opi1p, using anti-6xHis antibody.

Fig. 5.

Reduced accumulation of Opi1p in yeast replicating TBSV repRNA. (A) Western blot analysis of 6xHis-tagged Opi1p and 6xHis-tagged p33 proteins using anti-His antibody. BY4741 or *scs2*^Δ yeasts expressing Opi1p in the absence or presence of TBSV replication were studied. Quantification of the steady-state levels of Opi1p was done using Western-blots and a Bio-Rad imager. Loading was normalized based on total proteins (not shown). Each experiment was repeated three times. (B) Decreased accumulation of Opi1p induced by TBSV replication in yeast over-expressing Scs2p. The 6xHis-p33 and 6xHis-p92 were expressed from *CUP1* promoter, while DI-72 (+)repRNA and 6xHis-Scs2p were expressed from *GALI* promoters. The FLAG-tagged Opi1p was expressed from a high copy number plasmid from a *CYCI* promoter. Yeast cells were cultured as above. Accumulation of FLAG-Opi1p was analyzed by Western blot using anti-FLAG antibody.

Fig. 6.

Co-purification of the Opi1p protein with the tombusvirus p33 replication protein. (A) The FLAG-tagged p33 was purified from detergent-solubilized membrane fractions of yeast extracts using a FLAG-affinity column. Top panel: Western blot analysis of co-purified 6xHA-tagged Opi1p using anti-HA antibody. Middle panel: Western blot of purified FLAG-p33 detected with anti-FLAG antibody. Bottom panel: Western blot of 6xHA-tagged Opi1p in the total yeast extract using anti-HA antibody. Note that yeast expressing 6xHis-tagged p33 was used as a negative control to detect nonspecific binding of Opi1p. 6xHA-tagged Scs2p expressed in yeast was used as a positive control. 6xHA-tagged Opi1p and 6xHA-Scs2p were expressed from their natural chromosomal locations. Each experiment was repeated three times. (B) Co-purification of Opi1p with the tombusvirus p33 protein does not require Scs2p or Scs22p. The FLAG-tagged p33 was purified from yeast extracts solubilized with a detergent using a FLAG affinity column. 6xHA-tagged Opi1p was expressed from its natural chromosomal locations. Top panel: Western blot analysis of 6xHA-tagged Opi1p using anti-HA antibody in FLAG-affinity purified p33 samples. The panel shows the amount of 6xHA-Opi1p co-purified with FLAG-p33 from BY4741, *scs2* or *scs2* *scs22* yeast strains. Bottom panel: Western blot of purified FLAG-p33 using anti-FLAG antibody. See details as in panel A.

Fig. 7.

Model on reprogramming the phospholipid biosynthesis pathway by the p33 replication protein. (A) In the absence of p33 and when low amount of PA is present in the ER membrane, then Opi1p repressor is released from the ER and relocated to the nucleus to inhibit transcription by Ino2p/Ino4p. This leads to repression of transcription of phospholipid biosynthesis genes and inhibition of phospholipid biosynthesis. (B) The tombusvirus p33 replication protein binds to Opi1p repressor and the Scs2p VAP protein. These interactions prevent the activation and possibly the nuclear relocation of Opi1p repressor and might lead to degradation of Opi1p. As a consequence, Ino2p/Ino4p transcription activators induce the constitutive transcription of the phospholipid biosynthesis genes, leading to the production of the corresponding mRNAs, followed by new phospholipid synthesis and membrane formation. We propose that these new lipids and membranes are hijacked by the virus for replication in yeast cells.

List of primers used.

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4251	CAATGAAGTAAAGAGAAAATG
4252	GCCGGATCCATGAGTAACGAGCTTCTCAC
4253	CGGCTCGAGTCATGTCCTTTCATAATG
4254	ATTTTGTTGAAGGGATCTG
4255	GCCGGATCCATGACCGGCGTTGGCGAG
4256	CGGCTCGAGTTATGTGGGAGAAGCTAAG
4257	GTGTTGAAGCTCAAGAG
4258	GCCGGATCCATGACGACCGGAGATCTCG
4259	CGGCTCGAGTTATATCCGGTTCAATAAGTAG
4260	TGAAGCTAACCCCATGGC
4351	CCGGCTCGAGTTAGCTAGCCGAGGCTGAGTCGTCGC