2-16-2012

A Co-Opted DEAD-Box RNA helicase enhances tombusvirus plus-strand synthesis

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Kovalev, Nikolay; Pogany, Judit; and Nagy, Peter D., "A Co-Opted DEAD-Box RNA helicase enhances tombusvirus plus-strand synthesis" (2012). *Plant Pathology Faculty Publications*. 12.  
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Digital Object Identifier (DOI)
http://dx.doi.org/10.1371/journal.ppat.1002537

Notes/Citation Information
Published in PLoS Pathogens, v. 8, no. 2, e1002537.

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A Co-Opted DEAD-Box RNA Helicase Enhances Tombusvirus Plus-Strand Synthesis

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Abstract

Replication of plus-strand RNA viruses depends on recruited host factors that aid several critical steps during replication. In this paper, we show that an essential translation factor, Ded1p DEAD-box RNA helicase of yeast, directly affects replication of Tomato bushy stunt virus (TBSV). To separate the role of Ded1p in viral protein translation from its putative replication function, we utilized a cell-free TBSV replication assay and recombinant Ded1p. The in vitro data show that Ded1p plays a role in enhancing plus-strand synthesis by the viral replicase. We also find that Ded1p is a component of the tombusvirus replicase complex and Ded1p binds to the 3'-end of the viral minus-stranded RNA. The data obtained with wt and ATPase-deficient Ded1p mutants support the model that Ded1p unwinds local structures at the 3’-end of the TBSV (−)RNA, rendering the RNA compatible for initiation of (+)strand synthesis. Interestingly, we find that Ded1p and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is another host factor for TBSV, play non-overlapping functions to enhance (+)-strand synthesis. Altogether, the two host factors enhance TBSV replication synergistically by interacting with the viral (−)RNA and the replication proteins. In addition, we have developed an in vitro assay for Flock house virus (FHV), a small RNA virus of insects, that also demonstrated positive effect on FHV replicase activity by the added Ded1p helicase. Thus, two small RNA viruses, which do not code for their own helicases, seems to recruit a host RNA helicase to aid their replication in infected cells.

Introduction

All eukaryotic plus-stranded (+)RNA viruses have similar replication cycles in infected cells. After translation of their mRNA-sense genomic RNAs, the viral RNA and the viral replication proteins are recruited to the site of viral replication in membranous compartments. After the assembly of the membrane-bound viral replicase complexes (VRC), the viral replicase uses the viral RNA as a template to produce complementary (−)RNA. This is then followed by (+)-strand synthesis in an asymmetric manner, producing excess amounts of (+)-strand progeny, which is released from replication for other viral processes. For efficient replication, (+)RNA viruses recruit numerous host proteins [1–5]. Among the identified host proteins are RNA-binding proteins, such as translation factors, ribosomal proteins and RNA-modifying enzymes [1]. The co-opted host proteins likely affect several steps in viral RNA replication, including the assembly of the replicase complex and/or viral RNA synthesis. However, the functions of host factors in (+)RNA virus replication are known only for a small number of host factors [1–9].

Tomato bushy stunt virus (TBSV) is a model plant RNA virus that is used intensively for identification and characterization of host factors [1,10–12]. The single genomic RNA codes for two replication proteins, p33 and p92pol, which are sufficient to support TBSV replication [rep]/RNA replication in yeast (Saccharomyces cerevisiae) model host [13,14]. p33 and p92pol are components of the membrane-bound VRC that also requires the tombusviral (+)repRNA as a platform during its assembly and activation [11,15–17]. Recent genome-wide screens and global proteomics approaches with TBSV based on yeast revealed a large number of host factors interacting with viral components or affecting TBSV replication. A large fraction of the identified host proteins are RNA binding proteins, which might affect viral RNA synthesis [10,18–20].

The highly purified tombusvirus VRC is known to contain at least six permanent resident host proteins, including the heat shock protein 70 chaperones (Hsp70, Ssa1/2p in yeast) [21–24], glyceraldehyde-3-phosphate dehydrogenase (GAPDH, encoded by TDH2 and TDH3 in yeast) [25], pyruvate decarboxylase (Pdc1p) [24], Cdc34p E2 ubiquitin conjugating enzyme [24–26], eukaryotic translation elongation factor 1A (eEF1A) [27,28], eukaryotic translation elongation factor 1Bgamma (eEF1By) [29] and two temporary resident proteins, Pex19p shuttle protein [30] and the Vps23p adaptor ESCRT protein [28,31,32]. Although the functions of several of these proteins have been studied in some details, additional host proteins might be also present in tombusvirus VRC [5,10,21–23,25]. One of the intriguing questions is if tombusviruses use host-coded helicases for their replication. This is because, unlike larger RNA viruses, the tombusvirus genome does not code for a protein with helicase function. Thus, can tombusviruses and other small RNA viruses replicate without the use of a helicase or they subvert a cellular helicase(s) to assist their replication?
DEAD-box proteins constitute the largest family of RNA helicases that are involved in all aspects of cellular metabolism and perform RNA duplex unwinding and remodeling of RNA-protein complexes in cells [33–35]. Ded1p, which is essential for yeast growth, is among the best-characterized DEAD-box helicases [36]. It is involved in initiation of translation of every yeast mRNA [37–39] and down-regulation of Ded1p level also reduced p33 and p92 levels in yeast [20]. Therefore, not surprisingly, TBSV replication also decreased significantly in yeast with reduced level of Ded1p. Due to its essential role in translation, it is critical to separate the possible role of Ded1p in viral replication from its effect on viral translation.

In this paper, a yeast-based cell-free TBSV replication assay and recombinant Ded1p was used to test the role of Ded1p in TBSV replication. We define that Ded1p plays a role in enhancing plus-strand (+)RNA synthesis by the viral replicase. We also find that Ded1p is a component of the tombusvirus VRC and binds to the 3'-end of the viral minus-stranded (−)RNA. The obtained data support the model that Ded1p unwinds the 3'-end of the TBSV (−)RNA, rendering the RNA compatible for initiation of (+)-strand synthesis. Interestingly, we find that Ded1p and GAPDH play non-overlapping functions to enhance (+)-strand synthesis. Altogether, the two host factors enhance TBSV replication synergistically by interacting with the viral (−)RNA and the replication proteins.

Results

Reduced level of TBSV RNA replication in yeast cell-free extract depleted for Ded1p DEAD-box RNA helicase

Since Ded1p is an essential translation factor for all yeast mRNAs [38,39], it is difficult to separate its direct versus indirect effect on TBSV replication in yeast model host. To circumvent this problem, we used whole cell extracts (CFE) prepared from yeast containing reduced level of Ded1p (Figure S1) to support cell-free TBSV replication. As we have shown previously, TBSV (+)RNA can perform one complete cycle of replication in the CFE-based replication assay when purified recombinant p33 and p92 are replication proteins are provided [23,40]. The CFE-based replication assay showed that both (+) and (−)RNA synthesis decreased by over 4-fold when Ded1p was down-regulated as compared with the control CFE prepared from yeast with high level of Ded1p expression (Figure S1, lanes 4–6 versus 1–3). Thus, these data confirm that Ded1p is important for TBSV replication. However, the observed decrease in the CFE-based TBSV replication could be due to either reduced replication when Ded1p is limiting (direct effect) or lesser amounts of other critical host factors needed for TBSV replication in the CFE with reduced level of Ded1p (indirect effect due to Ded1p’s role in host translation). To address these points, we first studied if Ded1p is present within the tombusvirus replicase and then, what is the mechanistic role of Ded1p during TBSV RNA synthesis.

Ded1p is co-purified with the tombusvirus replicase

To examine if Ded1p is present within the tombusvirus replicase complex, we FLAG affinity-purified the tombusvirus replicase from yeast cells actively replicating TBSV repRNA [14,28]. This yeast also expressed HA-tagged Ded1p from yeast chromosome based on the native promoter. We found that the tombusvirus replicase preparation, which is highly active on added templates in vitro (not shown), contained Ded1p (Figure 1, lane 4), while Ded1p was undetectable in the control yeast sample obtained using the same affinity purification (Figure 1, lane 3). The control yeast also expressed the tombusvirus replication proteins (including the 6×His-tagged p33, but lacking FLAG-tag) and the HA-tagged Ded1p from yeast chromosome based on the native promoter and supported TBSV repRNA replication (not shown).

We also FLAG-affinity-purified 6×His/FLAG-tagged Ded1p from the membrane-fraction of yeast co-expressing HA-p33 and HA-p92 and found that the purified Ded1p preparation contained HA-tagged p33 (Figure S2A, lane 1). The affinity-purified Ded1p preparation also showed TBSV replicase activity in vitro on added TBSV template (Figure S2B, lane 2), suggesting that the membrane-bound Ded1p is associated with the active tombusvirus replicase. Altogether, these data support the model that Ded1p is part of the tombusvirus replicase.

![Figure 1. Co-purification of Ded1p with the p33 replication protein from yeast.](image)

The FLAG/6×His-tagged p33HF or 6×His-tagged p33 was purified from yeast extracts using a FLAG-affinity column (lanes 3–4). Top panel: Western blot analysis of Ded1p tagged with 6×HA, expressed from the native promoter in the chromosome, with anti-HA antibody in the purified p33 preparations. Bottom panel: Western blot analysis of HF-tagged p33 or 6×His-tagged p33 with anti-His antibody. Note that “total” represents the total protein extract from yeast expressing the shown proteins. Each experiment was repeated three times.

doi:10.1371/journal.ppat.1002537.g001
Recombinant Ded1p DEAD-box helicase enhances TBSV RNA replication in yeast cell-free extract

To address if Ded1p could play a direct role in TBSV replication, we added purified recombinant Ded1p to yeast CFE with reduced level of Ded1p (Figure 2A). We observed a ~2-fold increased level of TBSV RNA replication (based on total single-stranded repRNA level) in the presence of recombinant Ded1p when compared with the MBP control (Figure 2B, lane 2 versus 4). Interestingly, the amount of double-stranded RNA, which correlates with (~) strand levels [23], did not increase in the presence of Ded1p in the CFE assay (Figure 2B, lanes 1 versus 3). This finding suggests that (~) strand RNA did not contribute to the 2-fold increase in total TBSV RNA levels when recombinant Ded1p was added to the CFE-based replication assay. Therefore, we conclude that the added recombinant Ded1p selectively increased TBSV (~) strand RNA synthesis in vivo.

To demonstrate if the ATPase/helicase activity of Ded1p is important for the enhancement of TBSV RNA replication in the CFE-based replication assay, we tested an ATPase defective mutant of Ded1p (D1 mutant) [41]. This mutant could not enhance TBSV replication in vitro (Figure 2C, lanes 1–2 versus 3–4), suggesting that the ATPase activity of Ded1p is important for TBSV replication.

Ded1p selectively promotes TBSV (~) RNA synthesis in yeast cell-free extract

To further test what step(s) Ded1p promotes during TBSV replication, we performed a two-step replication assay based on yeast CFE. In this assay, the first step includes the assembly of the replicase complex on the endogenous membranes present in CFE in the presence of the viral (~) repRNA, the p33/p92 replication proteins and ATP/GTP [23] (Figure 3A, step 1). Under these conditions, the viral replication proteins recruit the repRNA to the membrane and the replicase becomes partially RNase and protease insensitive, but it cannot initiate minus-strand synthesis yet, due to the absence of CTP/UTP [23]. Then, centrifugation and washing the membranes will remove all the proteins and molecules not bound to the membrane. This is followed by addition of ATP/CTP/GTP/UTP to initiate RNA synthesis during the second step.

Addition of purified Ded1p to the CFE during the first step did not increase TBSV replication (Figure 3B, lane 4 versus 5). This suggests that Ded1p did not facilitate the assembly of the replicase complex, unlike other host factors, such as Hsp70 and eEF1A [23,27]. Moreover, Ded1p was likely lost during the centrifugation/washing step in this assay due to its low association with the membrane. However, addition of Ded1p exclusively during the second step of the CFE assay resulted in ~2-fold increase in TBSV RNA replication (Figure 3B, lane 14 versus 15), similar to the stimulatory effect of Ded1p during standard CFE replication assay (Figure 2). Interestingly, a Ded1p mutant (D1) deficient in ATPase activity could not stimulate TBSV RNA synthesis in this assay (Figure 3B, lane 13), while a mutant (D11, Figure S3C) with increased ATPase activity [41] promoted TBSV RNA synthesis by ~2-fold (Figure 3B, lane 11). Altogether, these findings indicate that Ded1p has a direct stimulatory function during TBSV RNA synthesis, while Ded1p is unlikely to affect viral RNA recruitment for replication or VRC assembly.

Ded1p enhances TBSV (~) RNA synthesis by the purified tombusvirus replicase

To further test the direct effect of Ded1p on RNA synthesis, we utilized detergent-solubilized and affinity-purified tombusvirus...
replicase from Ded1p-depleted yeast (Figure 4A). This purified replicase can only synthesize complementary RNA products on added TBSV templates, but, unlike the above membrane-bound replicase in the CFE-based assay, it cannot perform a complete cycle of RNA synthesis [14,15].

We found that addition of purified recombinant Ded1p to the purified tombusvirus replicase programmed with the (−)repRNA stimulated (+)-strand synthesis by ~2.5–3-fold (Figure 4B, lanes 3–4 versus 1–2; and Figure S3D, lane 3 versus 2). Interestingly, Ded1p stimulated the production of the full-length (+)-strand RNA product, while the amount of 3′-terminal extension product (3′TEX; due to self-priming by the 3′ end of the template [42–44], Figure S3B) decreased in the presence of added recombinant Ded1p. Therefore, we suggest that Ded1p facilitates the de novo initiation on the (−)RNA template by the tombusvirus replicase.

The ATPase inactive mutant D1 (Figure 4B, lanes 5–6 and S3D, lane 4), D5 and D10 (Figure S3D, lanes 6 and 8) could not promote (+)-strand synthesis, suggesting that the ATPase activity of Ded1p is required for the above stimulatory effect on TBSV RNA synthesis. Also, two active ATPase mutants, D3 and D11 (Figure S3C), did facilitate (+)-strand synthesis (Figure S3D, lanes 5 and 9), confirming that the ATPase activity of Ded1p is required during TBSV (+)-strand RNA synthesis. Interestingly, Ded1p mutant D11, albeit has increased ATPase activity, has wt-like strand displacement activity in vitro [41]. We found that D11 behaved similarly in TBSV replicase assay (Figure S3D) to wt Ded1p, suggesting that the unwinding activity of Ded1p is important during TBSV replication.

To test if Ded1p can also stimulate the activity of the tombusvirus replicase on (+)-stranded RNA templates, we used DI-72(+) RNA in the purified tombusvirus replicase assay (Figure S4A). The TBSV (+)RNA is known to carry a replication silencer element (RSE) at the 3′ end that inhibits (−)RNA synthesis in vitro [45]. Addition of Ded1p to the tombusvirus replicase assay did not enhance (−)RNA synthesis (Figure S4B, lane 3 versus 1–2). Also, D1 mutant deficient in ATPase activity had no much effect on (−)RNA synthesis (Figure S4B, lane 4 versus 1–2). Based on these data, we suggest that Ded1p does not affect (−)RNA synthesis by the tombusvirus replicase in vitro.

Ded1p binds to the 3′ end of the TBSV (−)RNA in vitro

To identify the region(s) of the TBSV RNA bound by the recombinant Ded1p, we performed electrophoresis mobility shift assay (EMSA) with purified components. Comparison of 32P-labeled TBSV (+) and (−)RNAs in binding to purified recombinant Ded1p revealed that (−)RNA bound more readily to Ded1p in vitro than (+)RNA (Figure 5B, lanes 2–6 versus 8–12). Additional EMSA experiments using the four regions in DI-72 (−)repRNA (Figure 5A) as unlabeled competitors revealed that RI(−) was the most efficient in outcompeting the 32P-labeled TBSV (−)repRNA in binding to Ded1p (Figure 5C, lanes 8–9). This is important since RI(−) is the 3′ end of (−)repRNA and contains important cis-acting elements, such as the promoter and a short enhancer sequence for (+)RNA synthesis [46,47].

Further testing of Ded1p binding to (−)repRNA regions revealed that RIV(+), representing the 3′ noncoding region in TBSV RNA, was bound more efficiently by Ded1p (Figure S5,
To test if Ded1p can interact with the TBSV p33 replication protein, we used the membrane-based split-ubiquitin assay [31]. We found that Ded1p interacted with p33 in yeast (Figure S6A). The pull-down experiments with MBP-p33 and MBP-p92 also showed that Ded1p bound to the tombusvirus replication proteins in vitro (Figure S6B). This was further supported by the reverse pull-down experiments with GST-tagged Ded1p, which resulted in the co-purification of p33 and p92 (Figure S6C).

RH20, a DEAD-box helicase from Arabidopsis stimulates TBSV (+)repRNA synthesis by the purified tombusvirus replicase

To test if a DEAD-box helicase from plants might have similar stimulatory function on the activity of tombusvirus replicase, we have cloned and purified RH20 cytosolic DEAD-box helicase from Arabidopsis thaliana, which shows high degree of similarity to yeast Ded1p (Figure S7). Addition of the recombinant AtRH20 to the CFE prepared from Ded1p-depleted yeast strain increased TBSV repRNA replication by ~2-fold (Figure 6A, lane 3). Also, adding the recombinant RH20 to the replicase assay led to almost

Figure 4. Ded1p promotes plus-strand synthesis by the affinity-purified tombusvirus and FHV replicases. (A) Scheme of the tombusvirus replicase assay. Yeast with depleted Ded1p co-expressing p33 and p92pol replication proteins and DI-72 (+)repRNA were used to affinity-purify the RNA-free tombusvirus replicase. The in vitro assays were programmed with DI-72 (−)repRNA, and they also contained purified recombinant Ded1p, mutants or MBP in addition to ATP/CTP/GTP and 32P-UTP. (B) Representative denaturing gel of 32P-labeled RNA products synthesized by the purified tombusvirus replicase in vitro in the presence of 0.5 μg or 1.0 μg of purified recombinant Ded1p or its mutants is shown. The level of complementary RNA synthesis producing “repRNA” (marked as “FL”, the full-length product, made via de novo initiation from the 3′-terminal promoter) in each sample was compared to that of the replicase activity obtained in the absence of added recombinant protein (lane 1). Note that this replicase preparation also synthesizes de novo internal initiation products (“ii”) and 3′-terminal extension products (“3′TEX”). Each experiment was repeated three times. (C) Representative denaturing gel of 32P-labeled RNA products synthesized by the purified FHV replicase in vitro in the presence of 1.0 μg of purified recombinant Ded1p or its mutants is shown. Note that the FHV replicase can use TBSV (−)repRNA as a template in vitro. Each experiment was repeated three times. (D) Representative denaturing gel of 32P-labeled RNA products synthesized by the purified FHV replicase in vitro in the presence of 1.0 μg of purified recombinant Ded1p or its mutants is shown. Note that the FHV DI-634 (−)repRNA was used as a template in the FHV replicase assay.

doi:10.1371/journal.ppat.1002537.g004

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PLoS Pathogens | www.plospathogens.org 5 February 2012 | Volume 8 | Issue 2 | e1002537

lane 11) than the other regions of DI-72(+)-RNA were (lanes 2–9). However, the binding of Ded1p to RIV(+)-RNA was readily outcompeted by the unlabeled DI-72 (+)repRNA (Figure S5, lane 12).
3-fold increase of the activity of the purified tombusvirus replicase on TBSV (−)RNA template (Figure 6B, lanes 2–3 versus 1). To test if AtRH20 can interact with the TBSV p33 replication protein, we used the membrane-based split-ubiquitin assay [31]. We found that AtRH20, similar to Ded1p, interacted with p33 protein in yeast (Figure S6A). Altogether, these data strongly suggest that plants also have DEAD-box helicases that could play similar role to the yeast Ded1p DEAD-box helicase in tombusvirus (−)RNA synthesis.

Ded1p enhances Flock house virus (+)-RNA synthesis by the purified replicase

To test if Flock house virus (FHV) replicase is affected by Ded1p, first we have developed an in vitro FHV replication assay based on affinity-purified FHV replicase preparation that can be programmed with exogenously added RNAs (Figure 4C, lanes 1 versus 10). The in vitro FHV replication assay revealed that the purified recombinant Ded1p increased (+)-strand RNA synthesis on the (−)-stranded FHV template by ∼3-fold (Figure 4D, lanes 2 versus 1) and by ∼2-fold on the heterologous TBSV (−)repRNA template (Figure 4C, lane 2 versus lane 1). Addition of D1 ATPase inactive mutant of Ded1p decreased RNA synthesis by the FHV replicase by 20–40% (Figure 4C, lane 3; Figure 4D, lane 3), confirming that the ATPase activity of Ded1p is important for the stimulatory function on (+)RNA synthesis. Ded1p did not stimulate (−)-RNA synthesis by the FHV replicase on (−)stranded TBSV RNA (Figure S4C, lane 3 versus 1–2) or FHV (+)RNA (Figure S4D, lane 2 versus 1). It is likely that the observed stimulation of FHV replicase activity by Ded1p is direct, since we found that Ded1p bound to the FHV repRNA (Figure S8A) and protein A RdRp protein in vitro (Figure S8B). Altogether, these data show that, similar to the tombusvirus replicase, the activity of FHV replicase is stimulated by Ded1p only on the (−)RNA templates, but not when using the (+)RNA templates.

Ded1p facilitates initiation by the tombusvirus replicase on RNA/DNA duplex

To gain further insights into the mechanism of Ded1p-driven stimulation of TBSV and FHV RNA synthesis, we exploited template structures, such as an RNA/DNA duplex, that are known to hinder RdRp-driven RNA synthesis [48,49]. Since Ded1p is an RNA helicase [39,41] and the ATPase/helicase...
function of Ded1p is needed for stimulation of (+)RNA synthesis by the tombusvirus and FHV replicases, we wanted to examine if Ded1p might facilitate RNA synthesis on a partial DNA/RNA duplex. We chose partial duplex for this assay, since Ded1p and Tdh2p might facilitate RNA synthesis on a partial DNA/RNA duplex by the tombusvirus and FHV replicases, we wanted to examine if p92pol replication proteins and DI-72 (AtRH20 is shown. Yeast with depleted Ded1p co-expressing p33 and p92pol replication proteins and DI-72 (+)repRNA were used to affinity-purify the RNA-free tombusvirus replicase. The in vitro assays were programmed with DI-72 (+)repRNA, and they also contained purified recombinant AtrH20 (Lanes 2–4) in addition to ATP/CTP/GTP and 32P-UTP. Lane 4 represents the sample with no tombusvirus replicase added. The experiment was repeated three times.

doi:10.1371/journal.ppat.1002537.g006

Non-overlapping functions of Ded1p and GAPDH in promoting initiation by the tombusvirus replicase

GAPDH (Tdh2p in yeast) RNA binding protein is also a host factor stimulating (+)RNA synthesis by the tombusvirus replicase [25,50]. To test if Ded1p and GAPDH could play a complementary role during (+)RNA synthesis, we added the purified recombinant Ded1p and Tdh2p to the in vitro tombusvirus replicase assay based on the purified preparation (Figure 8A). While Ded1p mostly stimulated de novo (+)RNA synthesis initiated from the 3′ end of the (+)repRNA up to ~5-fold (Figure 8B, lane 6), Tdh2p enhanced both de novo (+)RNA synthesis and 3′TEX by ~2- and ~3-fold, respectively (Figure 8B, lane 7). Interestingly, the two host proteins together had the largest (4.5-fold) effect on (+)RNA synthesis, while their effect on 3′TEX was only ~2-fold. Based on these data, we suggest that Ded1p and Tdh2p have a

Figure 7. Ded1p facilitates the RNA synthesis by the tombusvirus and FHV replicases on short partial DNA/RNA duplex. (A) Schematic representation of the DNA/RNA duplex used in the replicase assays. The template consists of DI-72 (+)repRNA, whose 3′ end forms a 21 nt duplex with a complementary DNA oligo as shown. The following cis-acting sequences involved in (+)-strand synthesis are shown: cPR is an 11 nt long sequence used as a (+)-strand initiation promoter; PPE REN is a promoter proximal replication enhancer; while RIII(−)REN is a strong replication enhancer within RIII(−) sequence. (B) Representative denaturing gel of 32P-labeled RNA products synthesized by the purified tombusvirus replicase in vitro in the presence of 1.0 µg of purified recombinant Ded1p or D1 mutant. The level of complementary RNA synthesis using the DNA/RNA duplex (panel A) in each sample was compared to that of the replicase activity obtained in the absence of added recombinant protein (lane 1). See further details in Figure 4. (C) Representative denaturing gel of 32P-labeled RNA products synthesized by the purified FHV replicase in vitro in the presence of 1.0 µg of purified recombinant Ded1p or D1 mutant on the DNA/RNA duplex (panel A). Each experiment was repeated three times.

doi:10.1371/journal.ppat.1002537.g007
synergistic effect on (+)RNA synthesis by promoting de novo initiation from the 3’ end of the (-)RNA template.

We also performed similar experiments with a short partial RNA/DNA duplex as a template. Both Ded1p and Tdh2p alone could enhance (+)RNA synthesis on the short RNA/DNA duplex (Figure 8C, lanes 2–3 versus 1) but the largest stimulatory effect (i.e., close to 5-fold increase) was seen when both host factors were included in the assay (Figure 8C, lane 4). Altogether, these data...
further support that Ded1p and GAPDH play synergistic roles in (+)-RNA synthesis by the tombusvirus replicase.

**Discussion**

RNA viruses form several different ribonucleic acid-protein complexes (RNP) in infected cells during various steps of infection [9]. Since the viral RNA plays multiple roles during infection, it is likely that remodelling of the viral RNAs and RNP complexes during the switch from one step to another requires RNA helicases or RNA chaperones. Accordingly, the larger RNA viruses all code for RNA helicases [51,52]. However, RNA viruses with shorter than 6 kbp genomes usually do not code for RNA helicases. They could still use RNA helicases during infections if they can subvert selected host helicases for viral purposes. Indeed, we show here that Ded1p helicase is recruited for TBSV replication to aid (+)-strand RNA synthesis.

It is not yet known if Ded1p is the only host helicase needed for TBSV replication, since Genome-wide screens and global proteomics approaches with TBSV have identified additional host helicases as well [9,10,20]. However, Ded1p helicase seems to be an ideal host factor to be recruited for viral replication because it is involved in mRNA and viral RNA translation, thus it is co-localized with the viral RNA prior to replication. Also, by recruiting Ded1p, viruses could affect RNA degradation (P-body formation; [53]) and initiation of translation of new RNAs, likely affecting subsequent host translation (including virus-induced mRNAs coding for anti-viral proteins or required for anti-viral signaling).

The essential nature of Ded1p for host mRNA translation, however, makes characterization of Ded1p function as a host factor difficult. Therefore, the combined use of *in vivo* and *in vitro* approaches might be necessary to dissect the function of Ded1p in RNA virus replication as shown in this paper. By using a Ded1p-depleted yeast CFE in combination with recombinant Ded1p, we allowed us to define that the ATPase (helicase) activity of Ded1p is required for efficient TBSV (+)-strand synthesis (see below). Ded1p also increased the RdRp activity of the FHV replicase, suggesting that recruitment of DEAD-box helicases might also be useful for additional small RNA viruses (see below).

**Ded1p helicase is a component of the tombusvirus replicase complex**

Co-purification experiments revealed that Ded1p is present in the tombusviral VRC (Figure 1). In addition, Ded1p affected (+)-strand synthesis, but not the assembly of the VRC *in vitro* (Figures 2–4), indicating that Ded1p is likely present in the VRC.

Surprisingly, Ded1p, unlike other previously tested host factors Hsp70, GAPDH, or eEF1A [21–23,27,54], was able to affect TBSV repRNA replication even after the replicase assembly step took place *in vitro* (Figure 3). This suggests that Ded1p can enter the membrane-bound VRC, possibly due to its interaction with p33 (Figure S6) and the helicase activity of Ded1p might lead to some remodeling of VRC.

It is not yet known if additional members of the large helicase family could perform similar function to Ded1p during TBSV replication. Our *in vitro* data with AtRH20 plant helicase protein, which is very similar to Ded1p (Figure S7), suggests that this protein can likely perform similar function in plant infections. For example, AtRH20 has been shown to boost TBSV (+)-RNA synthesis *in vitro* (Figure 6) and bind to p33 replication protein (Figure S6A), which can facilitate its recruitment into the VRC. Since more than 50 RNA helicases are present in plants, further experiments will be needed to define if additional host helicases might also be involved in TBSV replication.

**Role of Ded1p helicase in stimulation of TBSV RNA synthesis**

The *in vitro* data, based on the CFE assay containing the membrane-bound VRC as well as the solubilized/purified tombusvirus replicase, showed the Ded1p can mainly stimulate TBSV (+)-strand synthesis, while its effect on (−)-RNA synthesis is less pronounced. The ATPase activity of Ded1p is required for this stimulatory effect, suggesting that the helicase function of Ded1p is likely important for unwinding the secondary structure of (−)-RNA template with the purified tombusvirus replicase or destabilizing the replication intermediate with the membrane-bound VRC, which might contain dsRNA structure. This function of Ded1p can explain why yeast with down-regulated Ded1p level produced small amount of (−)-stranded RNA progeny, albeit it has also shown that depleted Ded1p reduced the level of p33/p92 in yeast [20]. However, the decreased p33/p92 levels are expected to reduce both (+) and (−)-RNA levels [53] (Figure S1). Since the recombinant Ded1p enhanced (+)-strand synthesis by the purified recombinant tombusvirus replicase, we propose that Ded1p directly affect TBSV RNA synthesis via affecting the structure of the RNA templates. However, we cannot exclude that Ded1p could also affect the activity of the VRC due to its interaction with p33, albeit the assembly of the VRC was not affected by Ded1p in the CFE-based assay (Figure 3). Overall, the recruitment of a host DEAD-box helicase for replication of a small RNA virus is remarkable, since RNA viruses with less than 6 kbp genomes are usually do not code for their own helicases [51]. These viruses are thought to replicate without needing a helicase by using RNA chaperones or possibly recruiting host helicases. The emerging picture with TBSV is that this virus utilizes both the viral-coded p33 RNA chaperone [48] and the host Ded1p helicase for replication by promoting (+)-strand synthesis. Both p33 and Ded1p have been shown to open up short DNA/RNA duplexes, although the activity of Ded1p was more robust than that of p33 *in vitro* [48]. Why would TBSV utilize both an RNA chaperone and an RNA helicase? It is possible that both proteins are needed for robust (+)-RNA synthesis to make excess amount of progeny RNA. Also, Ded1p helicase could be involved in remodeling the viral RNA bound by the viral RdRp or host proteins prior or during RNA synthesis. Since Ded1p can work in both 5′-to-3′ and 3′-to-5′ directions [56], it could be used for multiple purposes during RNA synthesis.

**A model on the synergistic roles of host factors in TBSV (+)-RNA synthesis**

Based on the available data, it seems that TBSV (+)-RNA synthesis is not only affected by the p33/p92 replication proteins, but by GAPDH and Ded1p host proteins as well. Since the viral replication proteins were shown to bind to TBSV (−)-RNA nonspecifically [57], we propose that the above host proteins, which bind strongly to the TBSV (−)repRNA, are involved in facilitating the proper and efficient recruitment of the p92 RdRp protein to the (−)RNA template (or alternatively to the dsRNA intermediate) within the VRC as shown in Figure 9. For example, Ded1p might unwind either local secondary structure in (−)repRNA or dsRNA region and that, in turn, could favor binding of GAPDH or p92 to the (−)RNA template. This is followed by binding of GAPDH to an AU-rich internal site and proper positioning of the p92 RdRp (bound to GAPDH, [50]) over the (+)-strand initiation promoter, leading to (+)-RNA synthesis.
The synergistic effect of these host proteins could promote efficient recycling of the viral RdRp resulting in multiple rounds of (+)RNA synthesis (Figure 9). Thus, the roles of these host proteins are to serve as “matchmakers” between the viral RNA template and the viral RdRp. It is intriguing that two host proteins, eEF1A and eEF1Bγ, are proposed to serve somewhat similar functions during (−)RNA initiation for TBSV [27,29]. Therefore, the emerging picture is that TBSV utilizes different host proteins for promoting (−) versus (+)RNA synthesis. This strategy could be beneficial for the virus by allowing asymmetric RNA synthesis, thus leading to excess amount of progeny (+)RNA.

Overall, host DEAD-box or related RNA helicases have been shown to affect many different aspects of virus infections, including translation of viral proteins [58–60]; viral RNA replication [61–64]; reverse transcription [65]; the activity of anti-viral proteins [66,67], and virus-mediated regulation of host gene transcription [68]. Interestingly, Ded1p is known to affect minus-strand synthesis during replication of the L-A dsRNA virus of yeast [69], suggesting that the use of DEAD-box helicases is widely spread among RNA viruses.

Materials and Methods

Yeast strains and expression plasmids

Saccharomyces cerevisiae strain BY4741 (MATα his3D1 leu2D0 met15D0 ura3D0) and TET::DED1 yeast strain (yTHC library, Figure 9. A model describing the functions of Ded1p and GAPDH during tombusvirus replication. The (−)repRNA, which is shown with a “closed” 3′ end due to either a secondary structure within the promoter (cPR) in the (−)repRNA or part of a dsRNA structure, could be unwound by Ded1p around the 3′ end, making the (−)repRNA “open”. The open ssRNA structure would facilitate (+)RNA synthesis by the tombusvirus P92 RdRp protein. GAPDH could facilitate (+)RNA synthesis via binding to a 3′ AU-rich sequence (indicated as a box) and also bringing in the P92 protein and positioning it over the cPR promoter as shown. Thus, Ded1p and GAPDH might play a complementary role, leading to synergistic effect on (+)RNA synthesis. In the absence of Ded1p, GAPDH could promote both 3′-terminal extension and (+)RNA synthesis by facilitating the positioning of P92 over the 3′ end.

doi:10.1371/journal.ppat.1002537.g009
Recombinant protein purification from E. coli

Expression and purification of the recombinant MBP-tagged host proteins and the MBP-tagged TBSV p33 and p92 replication proteins and Ded1p helicase from E. coli were carried out as described earlier with modifications [71]. Briefly, the expression plasmids were separately introduced into E. coli strain BL21 (DE3) CodonPlus. Protein expression was induced using isopropyl β-D-thiogalactopyranoside (IPTG) for 8 h at 23°C in the case of host proteins and at 16°C in the case of p33 and p92, then the cells were collected by centrifugation (5,000 rpm for 5 min). The cells were suspended and sonicated in MBP column buffer containing 30 mM HEPES-KOH pH 7.4, 25 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol. The extract was then centrifuged at 16,000 g for 10 min, followed by incubation with amylose resin (NEB) for 15 min at 4°C. After washing the resin 2 times with the column buffer, the proteins were eluted with column buffer containing 0.1% (N/2) maltose. Purification of GST-tagged TDH2 (pGEX-TDH2) [50] was carried out using glutathione resin and eluted with 10 mM glutathione, 10 mM β-mercaptoethanol in the column buffer following the same protocol as MBP-proteins. Eluted proteins were aliquoted for storage at −80°C. Protein fractions used for the replication assays were at least 95% pure, as determined by SDS-PAGE (not shown). We have previously shown that our Ded1p preparation has helicase activity on short RNA/DNA duplexes in vitro [48].

RNA probes and RNA competitors used for RNA–protein interactions

The 32P-labeled or unlabeled full-length DI-72 (+) and (−) RNAs and the four separate regions (RI-IV), were generated as described [57]. Transcripts for replicate or CFE replication assays were purified as described earlier [57]. The amounts of transcripts were quantified by UV spectrophotometer (Beckman). To obtain full-length FHV-derived DI-634 RNA [72], we used primers #3042 (GGATAATGACTCACTATAGTAACGAGGAATGTCG- AAAATTG) and #3509 (ACCTTGCTGTCGTAATTGAACCTG) or #3519 (GAACAGATATCCAGGTTCC) and #3527 (GTAATGACTCAGTCATAGGAAGTTACCCATAGGTAATTAGCAATGG) or (3516) for (+) or (−)DI-634 RNAs, respectively, and pDI634 [72] as a template in PCR reactions. The RNA transcripts were synthesized on the PCR templates using T7-based transcription [47].

In vitro TBSV replication assay in cell-free yeast extract

CFEs from BY4741 or TET::DED1 strains capable of supporting TBSV replication in vitro were prepared as described earlier [23,40]. Briefly, the in vitro TBSV replication assays were performed in 20-μl total volume containing 2 μl of CFE, 0.15 μg DI-72 (+)repRNA transcript, 200 ng purified MBP-p33, 200 ng purified MBP-p92 (both recombinant proteins were purified from E. coli), 30 mM HEPES-KOH, pH 7.4, 150 mM potassium acetate, 5 mM magnesium acetate, 0.13 M sorbitol, 0.4 μl actinomycin D (5 mg/ml), 2 μl of 150 mM creatine phosphate, 0.2 μl of 10 mg/ml creatine kinase, 0.2 μl of RNase inhibitor, 0.2 μl of 1 M dithiothreitol (DTT), 2 μl of 10 mM ATP, GTP, and TTP and 0.25 mM UTP and 0.1 μl of [32P]UTP [23,40]. Host proteins were added in different amounts as indicated in the Figure legends. The reaction was performed as described [23,40]. The newly synthesized 32P-labeled RNA products were separated by electrophoresis in a 5% polyacrylamide gel (PAGE) containing 0.5 × Tris-borate-EDTA (TBE) buffer with 8 M urea. To detect the double-stranded RNA (dsRNA) in the cell-free replication assay, the 32P-labeled RNA samples were divided into two aliquotes: one half was loaded onto the gel without heat treatment in the presence of 25% formamide, while the other half was heat denatured at 85°C for 5 min in the presence of 50% formamide [27]. Fractionation of the whole cell extract was done according to [23,40]. The total extract was centrifuged at 21,000 × g at 4°C for 10 min to separate the “soluble” (supernatant) and “membrane” (pellet) fraction. The pellet was re-suspended and washed with buffer A (30 mM HEPES-KOH pH 7.4, 150 mM potassium acetate, and 5 mM magnesium acetate) followed by centrifugation at 21,000 × g at 4°C for 10 min and re-suspension of the pellet in buffer A. In vitro TBSV replication in the fractions was performed as described [23,40].

Tombusvirus replication assay from yeast and in vitro RdRp assay

Yeast strains (BY4741 and TET::DED1) were transformed with plasmids pGBK-HisFlagp33 (or pGBKHisFlag33 as a control), pGAD-HisFlagp92 (or pGAD-Hisp92 as a control) and pYC-DI72. The 6×His-Flag double-tagged HF-p33 and p92 were expressed under the ADH1 promoter and DI-72 repRNA was used under the Gal1 promoter. Transformed yeast were pre-grown on SC-ULH− media containing 2% glucose at 29°C. After centrifugation at 2,000 rpm for 3 min and washing pellet with selective media containing 2% galactose and 1 mg/ml Doxycycline, yeast were grown for 24 hours in SC-ULH− media containing 2% galactose at 23°C. The replication reaction was done according to a previously described procedure [24] with the following modification. Briefly, 200 mg of yeast cells were re-suspended and homogenized in TG buffer [50 mM Tris-HCl [pH 7.5], 10% glycerol, 15 mM MgCl2, 10 mM KCl, 0.5 M
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NaCl, and 1% [V/V] yeast protease inhibitor cocktail (Ypic) by glass beads using FastPrep Homogenizer (MP Biomedicals). The membrane fraction containing the viral replicase complex was solubilized with 1 ml TG buffer containing 1% Triton X-100, 1% [V/V] Ypic as described [14,15]. After affinity purification of HF-p33 on anti-FLAG M2-agarose affinity resin (Sigma), the resin-bound replicase complex was eluted in 100 μl elution buffer [50 mM Tris–HCl [pH 8.0], 10 mM KCl, 10 mM NaCl, 1% Triton X-100, and 0.15 mg/ml Flag peptide (Sigma)].

In vitro RdRp activity assay was performed by using DI-72(−) or (+) RNA template transcribed in vitro by T7 transcription [14]. To measure the effect of host proteins in the replicase assay with DI-72(−) RNA containing short double-stranded region at the 3′-end, a heat denatured RNA transcript (94°C for 2 min) was annealed with a 21-nt oligodeoxynucleotide (nt) complementary to the 3′ end of DI-72(−)RNA in STE buffer (10 mM TRIS pH 8.0, 1 mM EDTA, and 100 mM NaCl) and then slowly (in 30 min) cooled them down to 25°C (Panavis and Nagy, 2005). RNase ONE digestion to remove single-stranded 32P-labeled RNA was performed at 37°C for 30 min in a 1× RNase ONE buffer containing 0.1 μl of RNase ONE (Promega).

FHV replicase purification from yeast and in vitro replicase assay

To obtain FHV replicase preparation, BY4741 yeast strain was transformed with plasmid pGAD/Cup/FHV/propionateC-termin/HA/FLAG and pESC-His-GAL1::FHVRNA1framshift. After selection of transformed yeast on SC-ULH media containing 2% galactose, yeast were grown for 36 hours in SC-LH medium. Pre-grown overnight in selective media containing 2% glucose at 29°C. After centrifugation at 2,000 rpm for 3 min and washing the pellet with selective media containing 2% galactose, yeast were grown 36 hours at 29°C in SC-LH media containing 2% galactose and 50 μM CuSO4 to induce FHV RNA replication. Affinity-purification was done similarly as for tombusvirus, except for using different buffers: homogenization buffer consisted of 50 mM Tris–HCl [pH 8.0], 0.4 M sorbitol, 5 mM MgCl2, 50 mM KCl, 0.03% β-mercaptoethanol and 1% [V/V] yeast protease inhibitor cocktail (Ypic). Elution buffer also contained 0.15 mg/ml Flag peptide (Sigma).

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 CVN p33 replication protein has been described earlier [26]. The prey constructs were made by PCR amplification of individual genes using gene specific primers: #3957 (CCAGGCTG-CAGTCCACCCAGGCCAGGATCTG) / #4602 (CCAGGCGATG-GGCGACAAAGAGAAGTTG) followed by digestion with EcoRI and NotI for DED1 and #4312/CCAGGAGATCCGAGATGC-TTAGGCTTGGTAGAAG / #4603 (CCAGGCGATGGAAGTTTGGAAACGACCTG) / #3418 (CCAGGAGATCCGAGATGCTGC-TAGATGACCG) / #4604 (CCAGGCGATGGAAGTTTGGAAACGACCTG-TAGATGACCG) followed by digestion with BamHI and NotI in the case of RH20 gene, respectively. Digested PCR products were fused to NubG at either the 5′- or 3′- termini [NubG-x and x-NubG] by cloning into pPRN-N-RE or pPRN-G-RE vectors [26], respectively, using the same enzymes. Yeast strain NMY51 was co-transformed with pGAD-BT2-N-His33 and pPR-N-RE or one of the prey constructs carrying the cDNA for a given helicase 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–helicase protein interactions as described [26].

Protein co-purification with the viral replicase

S. cerevisiae strain DED1::6×HAprNT1 was generated by homologous recombination using strain BY4741. PCR was performed using plasmid pYM-16 (EUROSCARF) [73] as template and primers #2493 (GCCAGAACCAGAAATCTC-TACCCTAGTTTGCTGAAATCGATGATTCGAGCTC) / #2494 (GGCTGGGATACAGGCCTGTTTCACAAACACCTTCTTTGCGGTAGTACGCGAGTCGAC). The PCR products were transformed to BY4741 and recombinant yeast colonies were selected in YPD plates supplemented with hygromycin. Recombinant yeast strains were transformed with plasmids pGBK-HisFlapp33, pGAD-HisFlag92 and pYC-DI72 [24]. 6×His/Flag-tagged HF-p33 and HF-p92 were expressed from ADH1 promoter and DI-72 transcript was under GAL1 promoter. After selection of transformed yeast on SC-ULH− plates, yeast were pre-grown overnight in selective media containing 2% glucose at 29°C. After centrifugation at 2,000 rpm for 3 min and washing the pellet with selective media containing 2% galactose, yeast were grown for 36 hours in SC-ULH− media containing 2% galactose at 23°C. 0.2 ml of pelleted yeast were used to affinity-purify HF-p33 and HF-p92 with anti-FLAG M2 agaroase as described previously (see also Text S1) [14,15]. HF-p33 and HF-p92 were detected with anti-His, antibody (1/5,000 dilution) and AP-conjugated anti-mouse antibody (1/5,000). DED1-6×HA protein was detected with anti-HA antibody from rabbit (Bethyl; 1/10,000 dilution) and AP-conjugated anti-rabbit (1/10,000) followed by NBT-BCIP detection.

Supporting Information

Figure S1 Reduced TBSV replication in CFE prepared from Ded1-depleted yeast. CFEs were prepared from TET::DED1 yeast strain cultured in the absence of doxycycline (i.e., Ded1p is not containing 6×His/FLAG -Ded1p (lane 1) or not containing 6×His/FLAG -Ded1p (lanes 2–4) or not containing 6×His/FLAG -Ded1p (lanes 5). The CFEs contained comparable amounts of host proteins (not shown). (EPS)

Figure S2 Co-purification of the p33 replication protein with Ded1p from yeast. (A) 6×His/FLAG-tagged Ded1p was purified from yeast extracts containing 6×His/FLAG -Ded1p (lane 1) or not containing 6×His/FLAG-tagged Ded1p (lane 2) using a FLAG-affinity column Top panel: Western blot analysis of p33 tagged with 6×HA with anti-HA antibody in the purified Ded1p preparations. Bottom panel: Western blot analysis of 6×His/FLAG-tagged Ded1p with anti-His antibody. Each experiment was repeated three times. (B) Co-purification of tombusvirus replicase activity with Ded1p from yeast. FLAG-tagged Ded1p was purified from yeast expressing FLAG-Ded1p (lanes 2–4) or yeast not expressing FLAG-Ded1p (lane 5) using a FLAG-affinity column. Yeast also co-expressed p33/p92/DI-72 repRNA as indicated by “+”. External DI-72−repRNA template was given to samples as shown. Representative denaturing gel of 32P-labeled

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RNA products synthesized in vitro by the purified FLAG-Ded1p preparation is shown. Lane 1 is a size-control [621 nt DI-72(+)] RNA. (EPS)

**Figure S3** Ded1p mutants with ATPase activity promote plus-strand synthesis by the affinity-purified tombusvirus replicase. (A) Scheme of the tombusvirus replicase assay. Yeast with depleted Ded1p co-expressing p33 and p92 and replication proteins and DI-72 (+)repRNA were used to affinity-purify the RNA-free tombusvirus replicase. The in vitro assays were programmed with DI-72 (--repRNA), and they also contained purified recombinant Ded1p. (B) Representative denaturing gel of 32P-labeled RNA products synthesized by the purified tombusvirus replicase in vitro in the presence of 1.0 μg of purified recombinant Ded1p. The level of complementary RNA synthesis producing “repRNA” (marked as “FL”, the full-length product, made via initiation from the 3′-terminal promoter) in each sample was compared to that of the replicase activity obtained in the absence of added recombinant protein. Note that this replicase preparation also synthesizes internal initiation products (“ii”) and 3′-terminal extension products (“3′TEX”). RNase One digestion was used to confirm the replicase products: the de novo products are insensitive, while the 3′TEX product changes migration after RNase treatment (marked with an open arrowhead in lanes 2 and 4). Each experiment was repeated three times. (C) The ATPase activities of the Ded1p mutants based on REF 38. (D) Representative denaturing gel of 32P-labeled RNA products synthesized by the purified tombusvirus replicase in vitro in the presence of 1.0 μg of purified recombinant Ded1p mutants. See Panel B for further details. (EPS)

**Figure S4** Ded1p helicase does not promote minus-strand synthesis by the affinity-purified tombusvirus and FHV replicases. (A) Scheme of the procedure. Yeast with depleted Ded1p co-expressing p33 and p92 and replication proteins and DI-72 (+)repRNA were used to affinity-purify the RNA-free tombusvirus replicase. (B) Representative denaturing gel of 32P-labeled RNA products synthesized by the purified tombusvirus replicase in vitro in the presence of 0.9 μg of purified recombinant Ded1p or the D1 mutant is shown. The in vitro assays were programmed with DI-72 (+)repRNA, and they also contained purified recombinant Ded1p (lane 3) or the D1 mutant (lane 4) in addition to ATP/CTP/GTP and 32P-UTP. (C) Representative denaturing gel of 32P-labeled RNA products synthesized by the purified FHV replicase in vitro in the presence of 1.0 μg of purified recombinant Ded1p or D1 mutant on the TBSV DI-72 (+)RNA template. (D) Representative denaturing gel of 32P-labeled RNA products synthesized by the purified FHV replicase in vitro in the presence of 1.0 μg of purified recombinant Ded1p or D1 mutant on the FHV DI-634 (+)RNA template. (EPS)

**Figure S5** Ded1p binds to various regions of TBSV (+)RNA in vitro. (A) In vitro binding assay with purified Ded1p using 32P-labeled ssRNA templates representing the four regions of DI-72(+)-RNA. The assay contained ~0.1 pmol labeled RNA plus 1.0 μg of purified recombinant Ded1p. The unlabeled competitor was DI-72 (+) RNA (3 pmol). The free or Ded1p-bound ssRNA was separated on nondenaturing 5% acrylamide gels. (EPS)

**Figure S6** Interaction between Ded1p and the TBSV replication proteins. (A) Split ubiquitin assay was used to test binding between p33 and the shown full-length proteins. The bait p33 was co-expressed with the prey proteins in yeast. Ssa1p (HSP70 chaperone), and the empty prey vector (NubG) were used as positive and negative controls, respectively. The image shows 10-fold dilutional doses of yeast cultures. (B) In vitro pull-down assay of GST-His-tagged Ded1p with MBP-p33 or MBP-p92 using amylene resin. Top panel: Western blot analysis with anti-His antibody of GST-His-tagged Ded1p co-purified with MBP-p33 and MBP-p92. MBP was used as a negative control. Bottom panel: Coomassie stained SDS-PAGE gel, showing quality and quantity of purified MBP, MBP-p33 and MBP-p92 proteins. (C) In vitro reverse pull-down assay of MBP-p33 or MBP-p92 with GST-His-tagged Ded1p using glutathione resin. Western blot analysis with anti-MBP antibody of MBP-p33 and MBP-p92 co-purified with GST-His-tagged Ded1p. GST-His was used as a negative control. Bottom panel: Coomassie stained SDS-PAGE gel, showing quality and quantity of purified proteins GST-HisDed1 and GST-His. (EPS)

**Figure S7** Comparison of the amino acid sequence of the yeast Ded1p (top) and the Arabidopsis RH20 helicases. (PDF)

**Figure S8** Ded1p binds to FHV RNA in vitro. (A) In vitro binding assay with purified Ded1p using 32P-labeled FHV and TBSV RNA templates representing (+) and (−)RNAs. The assay contained ~0.1 pmol labeled RNA plus 0.4 μg (lanes 3, 6, 8) or 1.0 μg (lanes 2, 5, 7) of purified recombinant Ded1p. The free or Ded1p-bound ssRNAs were separated on nondenaturing 5% acrylamide gels. (B) In vitro pull-down of GST-His-tagged Ded1p or GST-His with MBP-protA of FHV using amylene resin. Top panel: Western blot analysis with anti-His antibody of GST-His-tagged Ded1p co-purified with MBP-protA. MBP and GST-His were used as negative controls. Bottom panel: Coomassie stained SDS-PAGE gel, showing quality and quantity of purified MBP, and MBP-protA proteins. (EPS)

**Text S1** Materials and Methods used in the supplementary experiments. (DOC)

**Acknowledgments**

The authors thank Dr. Daniel Barajas for valuable comments. Ded1p mutants are the generous gift of Dr. N.K. Tanner (Centre Medical U., Geneva, Switzerland). Dr. Shou-Wei Ding (UC Riverside) is thanked for the FHV clones. The *Eichneria coli* expression plasmids GST-Tdh2 was provided by Dr. Tyng-Shyan Huang (Nagy lab).

**Author Contributions**

Conceived and designed the experiments: NK JP PDN. Performed the experiments: NK JP. Analyzed the data: NK JP PDN. Contributed reagents/materials/analysis tools: JP. Wrote the paper: NK JP PDN.

**References**

1. Nagy PD (2000) Yeast as a model host to explore plant virus-host interactions. Annu Rev Phytopathol 48: 217-242.
2. den Boon JA, Ahlquist P (2010) Cytoplasmic viral replication complexes. Cell Host Microbe 8: 77–85.
3. den Boon JA, Díaz A, Ahlquist P (2010) Cytoplasmic viral replication complexes. Cell Host Microbe 8: 77–85.
4. Nagy PD, Wang RY, Pogany J, Hafren A, Makinen K (2011) Emerging picture of host chaperone and cyclophilin roles in RNA virus replication. Virology 411: 374–382.
32. Barajas D, Jiang Y, Nagy PD (2009) A Unique Role for the Host ESCRT
31. Barajas D, Nagy PD (2010) Ubiquitination of tombusvirus p33 replication protein. RNA Biol 8: 1055–105
30. Pathak KB, Sasvari Z, Nagy PD (2008) The host Pex19p plays a role in tombusvirus replication. Ann N Y Acad Sci 1151: 207–219.
29. Shi ST, Lai MM (2005) Viral and cellular proteins involved in coronavirus replication. Curr Top Microbiol Immunol 297: 95–131.
28. Li Z, Pogany J, Panavas T, Xu K, Esposito AM, et al. (2009) Translation elongation factor 1A is a component of the tombusvirus replicase complex and Stimulates Minus-Strand Synthesis. PLoS Pathog 6: e1001175.
27. Pathak KB, Pogany J, Xu K, White KA, Nagy PD (2012) Defining the Roles of cis-Acting RNA Elements in Tombusvirus Replicase Assembly In Vitro. J Virol 86: 156–171.
26. Pogany J, Stork J, Li Z, Nagy PD (2008) In vitro assembly of the Tomato bushy stunt virus replicase requires the host Heat shock protein 70. Proc Natl Acad Sci U S A 105: 7326–7331.
25. Wang RY, Stork J, Nagy PD (2008) A temperature sensitive mutant of a positive-strand RNA virus. Proc Natl Acad Sci U S A 105: 13764–13769.
24. Panavas T, Serviene E, Brasher J, Nagy PD (2005) Yeast genome-wide screen for factors affecting tombusvirus replication. Virology 385: 245–260.
23. Pogany J, Stork J, Li Z, Nagy PD (2008) Host transcription factor Rpb11p affects and accumulates in Saccharomyces cerevisiae. Virology 368: 388–404.
22. Wang RY, Stork J, Pogany J, Nagy PD (2009) A key role for heat shock protein 70 in the localization and insertion of tombusvirus replication proteins to intracellular membranes. J Virol 83: 3276–3287.
21. Wang RY, Stork J, Nagy PD (2009) Heat shock protein 70 associates with the virus accumulation complex, and is essential for infection. J Virol 83: 1768–1777.
20. Jiang Y, Serviene E, Gal J, Panavas T, Nagy PD (2006) Identification of essential host factors affecting tombusvirus RNA replication based on the yeast Tet promoters Hughes Collection. J Virol 80: 7394–7405.
19. Linder P (2003) Yeast RNA helicases of the DEAD-box family of RNA helicases. Gene 367: 17–37.
18. Kushner DB, Lindenbach BD, Grdzelishvili VZ, Noueiry AO, Paul SM, et al. (2003) Systematic, genome-wide identification of host genes affecting replication of a positive-strand RNA virus. Proc Natl Acad Sci U S A 100: 15864–15869.
17. Pathak KB, Pogany J, Xu K, White KA, Nagy PD (2012) RNA chaperone activity of the Tomato bushy stunt virus Minus-Strand Synthesis. PLoS Pathog 7: e1002438.
16. Panavas T, Serviene E, Brasher J, Nagy PD (2005) Yeast genome-wide screen reveals dissimilar sets of host genes affecting replication of RNA viruses. Proc Natl Acad Sci U S A 102: 7326–7331.
15. Jiang Y, Serviene E, Gal J, Panavas T, Nagy PD (2006) Temperature of an essential host factor affects tombusvirus RNA replication based on the yeast Tet promoters Hughes Collection. J Virol 80: 7394–7405.
14. Panafiez, Z, Savari, N, Nagy, P.D. (2011) RNA chaperone activity of the tombusviral p33 replication protein, which facilitates initiation of RNA synthesis by the viral RdRp in vitro. Virology 409: 338–347.
13. Panavas T, Nagy PD (2003) Internal initiation by the cucumber mosaic virus. Virology 314: 315–325.
12. Nagy PD (2011) The roles of host factors in tombusvirus RNA replication. RNA Biol 8: 1055–105.
11. Nagy PD, Pogany J (2006) Yeast as a model host to dissect functions of viral and cellular RNA helicases. Cell 125: 219–221.
10. Nagy PD, Pogany J (2006) Yeast as a model host to study replication and transcription of replicative activity. J Virol 70: 8224–8236.
9. Nagy PD, Pogany J (2005) Role of an internal and two 3′-terminal RNA elements in assembly of tombusvirus replicase. J Virol 79: 10068–10016.
8. Pogany J, White KA, Nagy PD (2005) Specific Binding of Tombusvirus Replication Protein p33 to an Internal Replication Element in the Viral RNA is Essential for Replication. J Virol 79: 4859–490.
7. Pathak KB, Pogany J, Xu K, White KA, Nagy PD (2012) Defining the Roles of cis-Acting RNA Elements in Tombusvirus Replicase Assembly In Vitro. J Virol 86: 156–171.
6. Nagy PD, Pogany J (2003) Systematic, genome-wide identification of host genes affecting replication of a positive-strand RNA virus. Proc Natl Acad Sci U S A 100: 15764–15769.
5. Panavas T, Serviene E, Brasher J, Nagy PD (2005) Yeast genome-wide screen reveals dissimilar sets of host genes affecting replication of RNA viruses. Proc Natl Acad Sci U S A 102: 7326–7331.
4. Wang RY, Stork J, Pogany J, Nagy PD (2009) A temperature sensitive mutant of heat shock protein 70 reveals an essential role during the early steps of tombusvirus replication. Virology 394: 28–39.
3. Wang RY, Stork J, Nagy PD (2009) A key role for heat shock protein 70 in the localization and insertion of tombusvirus replication proteins to intracellular membranes. J Virol 83: 3276–3287.
2. Pogany J, Stork J, Li Z, Nagy PD (2008) In vitro assembly of the Tomato bushy stunt virus replicase requires the host Heat shock protein 70. Proc Natl Acad Sci U S A 105: 19956–19961.
1. Serva S, Nagy PD (2006) Proteomics analysis of the tombusvirus replicase: Hsp70 molecular chaperone is associated with the replicase and enhances viral RNA replication. J Virol 80: 2162–2165.
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protein and the potential role of p68 in HCV RNA replication. J Virol 78: 5288–5298.

65. Wang H, Kim S, Ryu WS (2009) DDX3 DEAD-Box RNA helicase inhibits hepatitis B virus reverse transcription by incorporation into nucleocapsids. J Virol 83: 5815–5824.

66. Ye P, Liu S, Zhu Y, Chen G, Gao G (2010) DEXH-Box protein DHX30 is required for optimal function of the zinc-finger antiviral protein. Protein Cell 1: 956–964.

67. Sumpter R, Jr., Loo YM, Foy E, Li K, Yoneyama M, et al. (2005) Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J Virol 79: 2689–2699.

68. Jong JE, Park J, Kim S, Seo T (2010) Kaposi’s sarcoma-associated herpesvirus viral protein kinase interacts with RNA helicase a and regulates host gene expression. J Microbiol 48: 206–212.

69. Chong JL, Chuang RY, Tung L, Chang TH (2004) Ded1p, a conserved DEaD/H-box translation factor, can promote yeast L-A virus negative-strand RNA synthesis in vitro. Nucleic Acids Res 32: 2031–2038.

70. Serviene E, Jiang Y, Cheng CP, Baker J, Nagy PD (2006) Screening of the yeast yTHC collection identifies essential host factors affecting tombusvirus RNA recombination. J Virol 80: 1231–1241.

71. Rajendran KS, Pogany J, Nagy PD (2002) Comparison of turnip crinkle virus RNA-dependent RNA polymerase preparations expressed in Escherichia coli or derived from infected plants. J Virol 76: 1707–1717.

72. Dasgupta R, Cheng LL, Bartholomay LG, Christensen BM (2003) Flock house virus replicates and expresses green fluorescent protein in mosquitoes. J Gen Virol 84: 1789–1797.

73. Jauk C, Magiera MM, Rathfelder N, Taxis C, Reber S, et al. (2004) A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes. Yeast 21: 947–962.