Co-translational Localization of an LTR-Retrotransposon RNA to the Endoplasmic Reticulum Nucleates Virus-Like Particle Assembly Sites

Jung H. Doh¹, Sheila Lutz¹, M. Joan Curcio¹,²*  
1 Laboratory of Molecular Genetics, Wadsworth Center, New York State Department of Health, Albany, New York, United States of America, 2 Department of Biomedical Sciences, School of Public Health, University at Albany, Albany, New York, United States of America

Abstract  
The transcript of retrovirus-like transposons functions as an mRNA for synthesis of capsid and replication proteins and as the genomic RNA of virus-like particles (VLPs), wherein the genome is replicated. Retrotransposon RNA and proteins coalesce in a cytoplasmic focus, or retrosome, to initiate VLP assembly, but it is not known how the retrosome is nucleated. We determined how the RNA and Gag protein of the Saccharomyces cerevisiae Ty1 retrotransposon are directed to the retrosome. We found that Ty1 RNA is translated in association with signal recognition particle (SRP), a universally conserved chaperone that binds specific ribosome-nascent chain (RNC) complexes and targets the nascent peptide to the endoplasmic reticulum (ER). Gag is translocated to the ER lumen; yet, it is also found in the cytoplasm, associated with SRP-RNC complexes. In the absence of ER translocation, Gag is synthesized but rapidly degraded, and Ty1 RNA does not coalesce in retrosomes. These findings suggest that Gag adopts a stable conformation in the ER lumen, is retrotranslocated to the cytoplasm, binds to Ty1 RNA on SRP-RNC complexes and multimerizes to nucleate retrosomes. Consistent with this model, we show that slowing the rate of co-translational ER translocation by limiting SRP increases the prevalence of retrosomes, while suppressing the translocation defect of srp hypomorphs by slowing translational elongation rapidly decreases retrosome formation. Thus, retrosomes are dynamic foci of Ty1 RNA-RNC complexes whose formation is modulated by the rate of co-translational ER translocation. Together, these findings suggest that translating Ty1 mRNA and the genomic RNA of VLPs originate in a single pool and moreover, that co-translational localization of Ty1 RNA nucleates the presumptive VLP assembly site. The separation of nascent Gag from its RNA template by transit through the ER allows Gag to bind translating Ty1 RNA without displaying a cis-preference for its encoding RNA.

Introduction  
Long terminal repeat (LTR)-retrotransposons are ubiquitous molecular symbionts of eukaryotic genomes whose mobility is responsive to environmental and developmental cues and can result in host cell genome remodeling. These retroelements are the evolutionary progenitors of retroviruses, which have acquired env genes, and with them, the ability of their nucleocapsids to undergo exocytosis and infection of a naïve cell [1]. In contrast, LTR-retrotransposons lack env genes and replicate intracellularly. Because of their streamlined genomes and complex life cycles, both retroviruses and LTR-retrotransposons rely extensively on host cell factors to proliferate, yet much remains to be learned about the role of host cell pathways in retroelement replication.

Our understanding of the mechanism ofLTR-retrotransposon replication is derived largely from the study of Ty elements in Saccharomyces cerevisiae. Ty1 elements comprise the most abundant and most active family of LTR-retrotransposons in budding yeast. Ty1 contains a gag ORF that encodes a single structural protein with capsid and nucleocapsid functions, and a pol ORF, which encodes protease (PR), integrase (IN) and reverse transcriptase (RT) activities. A 5.7 kb sense-strand RNA expressed from genomic Ty1 elements functions both as an mRNA and as the genomic RNA of nucleocapsids, or VLPs. Ty1 RNA is reverse transcribed in cytoplasmic VLPs to form a DNA copy (cDNA). The Ty1 cDNA is transported to the nucleus and inserted into the host cell genome by integration or more rarely, homologous recombination [2].

Ty1 RNA is translated into two precursor proteins, p49-Gag and p199-Gag-Pol, the latter a result of programmed translational frameshifting from gag to pol. The p49-Gag and p199-Gag-Pol proteins multimerize to assemble into VLPs. Gag binds Ty1 RNA and encapsidates it as a dimer into the VLP during assembly [3,4]. Initiated by autocatalytic processing of p20-PR from the p199-Gag-Pol precursor, proteolytic processing of p49-Gag and p199-Gag-Pol to mature p45-Gag, p20-PR, p71-IN and p63-RT is thought to occur within the assembled VLP [5,6,7,8].

Ty1 RNA and Gag co-localize in microscopically distinct cytoplasmic RNA foci known as T bodies [9] or retrosomes [10]. Ty1 retrosomes partially co-localize with P bodies, and many factors involved in translational repression and P-body formation are also activators of Ty1 retrosome formation and

[111x354]2014 Doh et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.
[117x366]Copyright: © 2014 Doh et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

* E-mail: curcio@wadsworth.org

Citation: Doh JH, Lutz S, Curcio MJ (2014) Co-translational Localization of an LTR-Retrotransposon RNA to the Endoplasmic Reticulum Nucleates Virus-Like Particle Assembly Sites. PLoS Genet 10(3): e1004219. doi:10.1371/journal.pgen.1004219

Editor: Harmit S. Malik, Fred Hutchinson Cancer Research Center, United States of America

Received November 14, 2013; Accepted January 18, 2014; Published March 6, 2014

Abstract

The transcript of retrovirus-like transposons functions as an mRNA for synthesis of capsid and replication proteins and as the genomic RNA of virus-like particles (VLPs), wherein the genome is replicated. Retrotransposon RNA and proteins coalesce in a cytoplasmic focus, or retrosome, to initiate VLP assembly, but it is not known how the retrosome is nucleated. We determined how the RNA and Gag protein of the Saccharomyces cerevisiae Ty1 retrotransposon are directed to the retrosome. We found that Ty1 RNA is translated in association with signal recognition particle (SRP), a universally conserved chaperone that binds specific ribosome-nascent chain (RNC) complexes and targets the nascent peptide to the endoplasmic reticulum (ER). Gag is translocated to the ER lumen; yet, it is also found in the cytoplasm, associated with SRP-RNC complexes. In the absence of ER translocation, Gag is synthesized but rapidly degraded, and Ty1 RNA does not coalesce in retrosomes. These findings suggest that Gag adopts a stable conformation in the ER lumen, is retrotranslocated to the cytoplasm, binds to Ty1 RNA on SRP-RNC complexes and multimerizes to nucleate retrosomes. Consistent with this model, we show that slowing the rate of co-translational ER translocation by limiting SRP increases the prevalence of retrosomes, while suppressing the translocation defect of srp hypomorphs by slowing translational elongation rapidly decreases retrosome formation. Thus, retrosomes are dynamic foci of Ty1 RNA-RNC complexes whose formation is modulated by the rate of co-translational ER translocation. Together, these findings suggest that translating Ty1 mRNA and the genomic RNA of VLPs originate in a single pool and moreover, that co-translational localization of Ty1 RNA nucleates the presumptive VLP assembly site. The separation of nascent Gag from its RNA template by transit through the ER allows Gag to bind translating Ty1 RNA without displaying a cis-preference for its encoding RNA.

Introduction

Long terminal repeat (LTR)-retrotransposons are ubiquitous molecular symbionts of eukaryotic genomes whose mobility is responsive to environmental and developmental cues and can result in host cell genome remodeling. These retroelements are the evolutionary progenitors of retroviruses, which have acquired env genes, and with them, the ability of their nucleocapsids to undergo exocytosis and infection of a naïve cell [1]. In contrast, LTR-retrotransposons lack env genes and replicate intracellularly. Because of their streamlined genomes and complex life cycles, both retroviruses and LTR-retrotransposons rely extensively on host cell factors to proliferate, yet much remains to be learned about the role of host cell pathways in retroelement replication.

Our understanding of the mechanism of LTR-retrotransposon replication is derived largely from the study of Ty elements in Saccharomyces cerevisiae. Ty1 elements comprise the most abundant and most active family of LTR-retrotransposons in budding yeast. Ty1 contains a gag ORF that encodes a single structural protein with capsid and nucleocapsid functions, and a pol ORF, which encodes protease (PR), integrase (IN) and reverse transcriptase (RT) activities. A 5.7 kb sense-strand RNA expressed from genomic Ty1 elements functions both as an mRNA and as the genomic RNA of nucleocapsids, or VLPs. Ty1 RNA is reverse transcribed in cytoplasmic VLPs to form a DNA copy (cDNA). The Ty1 cDNA is transported to the nucleus and inserted into the host cell genome by integration or more rarely, homologous recombination [2].

Ty1 RNA is translated into two precursor proteins, p49-Gag and p199-Gag-Pol, the latter a result of programmed translational frameshifting from gag to pol. The p49-Gag and p199-Gag-Pol proteins multimerize to assemble into VLPs. Gag binds Ty1 RNA and encapsidates it as a dimer into the VLP during assembly [3,4]. Initiated by autocatalytic processing of p20-PR from the p199-Gag-Pol precursor, proteolytic processing of p49-Gag and p199-Gag-Pol to mature p45-Gag, p20-PR, p71-IN and p63-RT is thought to occur within the assembled VLP [5,6,7,8].

Ty1 RNA and Gag co-localize in microscopically distinct cytoplasmic RNA foci known as T bodies [9] or retrosomes [10]. Ty1 retrosomes partially co-localize with P bodies, and many factors involved in translational repression and P-body formation are also activators of Ty1 retrosome formation and
Retrotransposons are mobile elements that have invaded the genomes of organisms from bacteria to humans. Facilitated by host co-factors, retrotransposon proteins copy their RNA genomes into DNA that integrates into the host genome, causing mutations and genome instability. The yeast Ty1 element belongs to a family of retrotransposons that are related to infectious retroviruses. Ty1 RNA and its coat protein, Gag, assemble into virus-like particles, wherein the RNA is copied into DNA. It was not previously known how Ty1 RNA and Gag are concentrated in a specific cellular location to initiate the assembly of virus-like particles. In this study, we show that Ty1 RNA is brought to the presumptive assembly site during translation by the protein chaperone, signal recognition particle. As Ty1 RNA is translated, the nascent Gag polypeptide enters the lumen of the endoplasmic reticulum, where Gag adopts a stable conformation before returning to the cytoplasm to bind to translating Ty1 RNA. An interaction between Gag molecules bound to translating Ty1 RNA results in the nucleation of the virus-like particle assembly site. Our findings identify new host co-factors in retrotransposon mobility and suggest potential approaches to controlling retrotransposon-associated genome instability in aging and cancer.

Results

A defect in N-glycosylation results in Ty1 Gag instability

Deletion of DFG10, a gene encoding polyenol reductase, was identified in a screen for Ty1 retrotransposition-defective mutants [23]. Dfg10 catalyzes the synthesis of dolichol, the precursor for N-linked protein glycosylation in the ER. Western blot analysis of Gag protein expressed from the ~30 endogenous Ty1 elements demonstrated that the steady-state level of Gag is substantially decreased in the dfg10A mutant (Figure 2A). A C-terminal fusion of GFP to p45-Gag (Gag-GFP), expressed from the LTR promoter on a plasmid, was also present at a reduced level in the dfg10A mutant. Ty1 RNA foci were visualized by performing fluorescent in situ hybridization (FISH) with a Cy3-labeled antisense primer in the gag ORF and detecting the hybrid by fluorescent microscopy. Ty1 RNA failed to efficiently localize to foci, or retosomes, in the dfg10A mutant (Figure 2B). Similarly, deletion of the ribosome biogenesis factor gene, BUD21, resulted in decreased Gag, Gag-GFP and Ty1 RNA foci. To determine whether the lack of
Gag accumulation in these mutants results from a reduced level of Ty1 RNA, inefficient translation or protein instability, we performed northern blot analysis of Ty1 RNA and pulse-chase labeling followed by immunoprecipitation of Gag. The steady-state level of Ty1 RNA in a dfg10Δ mutant was very low relative to the congenic wild-type strain or the bud21Δ mutant (Figure 2C). Surprisingly though, the amount of labeled Gag that was immunoprecipitated immediately after pulse-labeling of proteins in a dfg10 mutant was 89% of that immunoprecipitated from the wild-type strain (Figure 2D, 0 min chase). In contrast, pulse-labeled Gag in the bud21Δ mutant was reduced to 26% of that in the wild-type strain, consistent with the proposed role for Bud21 in translation of Ty1 RNA [23]. These data indicate that synthesis of Gag is not significantly reduced by deletion of DFg10Δ, despite the reduced steady-state level of Ty1 RNA. However, Gag is degraded rapidly after synthesis in the dfg10Δ mutant, as evidenced by the significant reduction in pulse-labeled Gag after a 30 or 60 min chase relative to the level in the wild-type strain at the same time points (Figure 2D). Thus, accumulation of Ty1 RNA and Gag is substantially reduced by a post-translational mechanism in the dfg10Δ mutant. The increased turnover of Gag in a mutant with a defect in N-linked glycosylation suggests that Ty1 Gag is degraded as a result of induction of the ER stress response. However, Ty1 Gag has not previously been shown to be associated with the ER.

Gag is translocated to the ER lumen

Several experiments were performed to determine whether Ty1 Gag is associated with the ER membrane or is a soluble protein in the ER lumen. To investigate the possible association of Gag with the ER membrane, equilibrium density gradient centrifugation was performed to investigate the flotation behavior of Gag (Figure 3A). To provide a membrane-associated control protein for this analysis, we used a strain in which the KAR2 ORF, which encodes the ER chaperone, Kar2/BiP, was fused at the C-terminal end to the tandem affinity purification (TAP) tag. Kar2 is a soluble lumen protein; however, it is tethered to the membrane via interactions with the Sec63 complex and the Hrd1/Hrd3-Yos9 complex [18,24], so Kar2 co-purifies primarily with membrane fractions [24,25]. Cell lysate adjusted to 2 M sucrose was loaded between higher and lower sucrose cushions in a step gradient (Figure 3A). After centrifugation of the gradient, nine fractions of equal volume were collected and analyzed by western blotting. Ty1 Gag was concentrated in high-density fractions 7 to 9. As expected, Kar2-TAP was found primarily in low-density fractions 3 to 5, but was also present in high-density fractions 7 to 9. In an identical analysis of a congenic ADH5-TAP strain, the cytosolic Adh5-TAP protein was found only in high-density fractions 7 to 9. The presence of Gag exclusively in high-density fractions suggests that little if any Gag associates with the ER membrane.

To determine whether Gag is present as a soluble protein in the lumen of the ER, we prepared ER microsomes from the KAR2-TAP strain. Microsomes are cell fractions enriched for closed vesicles of fragmented ER. Following disruption of microsomes with sodium carbonate, the membrane fraction was separated from the soluble lumen fraction by centrifugation. Western blot analysis was performed on total microsomes (T), as well as the membrane-enriched pellet (P) and lumen-enriched supernatant (S) fractions of sodium carbonate-extracted microsomes (Figure 3B). Ty1 Gag co-purified with total microsomes, as did Kar2-TAP. As expected, Kar2-TAP co-fractionated with both the pellet and the supernatant fractions of sodium carbonate-treated microsomes. Gag segregated primarily with the supernatant, which is indicative of its presence in the lumen of the ER. A low level of Gag was also present in the pellet fraction, which was unexpected because Gag was not apparent in low-density membrane fractions in the flotation assay (Figure 3A). The presence of Gag in the microsome pellet could result from aggregation of Gag during the incubation of microsomes with sodium carbonate. Alternatively, if a low level of Gag is transiently associated with the ER membrane, it might be detectable in these concentrated microsomal fractions as compared to the membrane fractions of the density gradient in the flotation assay.
Figure 2. Ty1 RNA and Gag are destabilized subsequent to Gag synthesis in the N-glycosylation mutant, dfg10Δ. (A) Steady state levels of Ty1 Gag and Gag:GFP relative to alpha-tubulin, a loading control, determined by western blot analysis of strain BY4741 (WT) and congenic mutant derivatives, each harboring plasmid pLTRp:Gag1–401:GFP:ADH1TER. The spt3Δ strain, which lacks Ty1 transcription, is a negative control. In this strain background, Gag migrates as two or more bands with faster migration than unprocessed p49-Gag (Figure S1). Multiple Gag bands may result from post-translational modification of p45-Gag. (B) FISH analysis of Ty1 RNA (red) detected using a Cy3-labeled antisense oligomer hybridizing in the Ty1 gag ORF. DNA (blue) was stained with DAPI, and representative merged images are presented. Cells were visualized by DIC (differential interference contrast) microscopy. The percentage of DAPI-stained cells that have one or more Ty1 RNA foci is indicated for each strain. (C) Northern blot analysis of Ty1 RNA in total RNA isolated from strains indicated. The ratio of Ty1 RNA to 25S rRNA in each strain relative to that in the wild-type strain is indicated. (D) Immunoprecipitation of Gag from cells pulse-labeled for 15 min with the methionine analog, HPG and chased with excess methionine for 0, 30 or 60 min, as indicated. HPG-labeled Gag was immunoprecipitated with anti-VLP antibody and detected by conjugation with TAMRA. TAMRA-conjugated Gag was analyzed on a 10% SDS-PAGE gel and the fluorescent signal was quantified. The values presented in the graphs are the average fluorescent signal in each strain relative to the WT strain at the 0 min chase time point. The average value from two experiments is shown. Error bars represent standard deviation.

doi:10.1371/journal.pgen.1004219.g002
As expected, Gag was present in microsomes prepared from the ADH5-TAP strain as well, but Adh5-TAP was not detected, suggesting that there was minimal contamination of microsome preparations with cytosolic proteins (unpublished result). To directly determine whether Gag association with ER microsomes is due to its presence in the ER lumen or to contamination of microsomes with cytosolic Gag, we treated the microsome preparation with trypsin (Figure 3C). If Gag is present on the outer surface of microsomes because of cytosolic contamination, it should be digested by trypsin; however, the level of Gag was not reduced by treatment with trypsin. Kar2-TAP protein was also resistant to trypsin digestion, which demonstrates that ER lumen proteins were protected in the microsome preparation. In contrast, trypsin efficiently digested small non-specific proteins, indicating that trypsin digested contaminating proteins under these conditions. When microsomes were treated with trypsin and Triton X-100, which disrupts the microsomal membrane, both Gag and Kar2-TAP were markedly reduced. Together, these findings demonstrate that some fraction of Gag is present in the lumen of the ER.

Ty1 RNA and Gag are associated with SRP-RNC complexes

A major pathway by which proteins enter the ER is co-translational translocation mediated by SRP (Figure 1). SRP binds a hydrophobic domain of nascent peptides, including N-terminal signal sequences and transmembrane domains; however, not all peptide domains that are recognized by SRP have been defined [26]. Therefore, it is conceivable that the Ty1 Gag polypeptide, although lacking a discernible N-terminal signal sequence or transmembrane domain, could be co-translationally targeted to the ER lumen by SRP. To explore the possibility, we examined the results of a genome-wide study of SRP targets by del Alamo et al. [26]. These authors identified a collection of yeast ORFs corresponding to mRNAs that associate with SRP-RNC complexes despite the absence of a signal sequence or transmembrane domain in the corresponding nascent polypeptide. Three ORFs that encode Ty1 Gag were present in this collection: YJR027W, YBL005W-A and YGR109W-A. These findings suggest that Ty1 RNA-ribosome-nascent Gag complexes are targets of the SRP chaperone.

As an independent test of the hypothesis that Ty1 RNA is translated in association with SRP, we treated cells of an SRP54-TAP strain with cycloheximide to stabilize SRP-RNC complexes, affinity-purified Srp54-TAP complexes and analyzed the co-purifying RNA by RT-PCR using gene-specific primers (Figure 4A). The enrichment of SRP subunit 7SL RNA and 18S rRNA in the Srp54-TAP purification confirmed that SRP-RNC complexes were specifically enriched in the Srp54-TAP purification. As controls for non-specific binding, cells expressing TAP-tagged Lsm1, an activator of mRNA decapping, or no TAP tag were purified under identical conditions. The lack of enrichment of 7SL RNA and 18S rRNA in the control purifications indicates that SRP-RNC complexes were specifically enriched in the Srp54-TAP purification. Next, we assayed for the association of Ty1 RNA with SRP-RNC complexes. We found that Ty1 RNA was substantially enriched in the Srp54-TAP purification, whereas its enrichment was minimal or absent in the Lsm1-TAP and no-TAP controls, respectively. Furthermore, a Ty1 PCR product was not detected in a reaction lacking reverse transcriptase, demonstrating that amplification of Ty1 sequences was not due to the presence of contaminating DNA.
reaction as a negative control (No RT). 7SL RNA, the RNA subunit of SRP, 18S rRNA and cDNA was amplified for 29 and 32 cycles (indicated by wedge) with gene-specific primers. Reverse transcriptase was omitted from the cDNA synthesis.

IgG Sepharose and released by TEV protease. RNA isolated from TAP-purified complexes or the untagged control was reversed transcribed, and the

cycloheximide to stabilize RNC complexes were immediately frozen and pulverized. TAP-tagged proteins and associated complexes were bound to

affinity purified SRP-RNC complexes by western blotting. Gag co-

VLPs [10]. Therefore, we assayed for the presence of Gag in

RNA represses its translation and promotes its packaging into

Three uncharacterized ORFs, YEL076C, YLR463C and YHL049C, corresponding to the mRNA of the subtelomeric repetitive element, Y’ were also identified as interacting with SRP-RNC complexes in the genome-wide study [26]. Notably, Y’ RNA is enriched in Ty1 VLPs [27]. We found that Y’ RNA was substantially enriched in the Srp54-TAP purification but not in the Lsm1-TAP or mock purification. Similar results were obtained with EAR2 mRNA, which is also known to interact with SRP during translation. On the other hand, mRNAs encoding the transmembrane protein Hrd1 and the ER-lumen protein Yos9, which undergo SRP-independent translation [26], were not enriched in Srp54-TAP complexes (Figure 3A). Together, these data confirm that SRP-associated RNC complexes were selectively purified in the Srp54-TAP affinity purification. Thus, we conclude that Ty1 RNA is likely translated in association with SRP and therefore, that Gag is translocated to the ER lumen during translation.

It has been proposed that binding of Ty1 Gag to translating Ty1 RNA represses its translation and promotes its packaging into VLPs [10]. Therefore, we assayed for the presence of Gag in affinity purified SRP-RNC complexes by western blotting. Gag co-purified with Srp54-TAP, but was not detected in the Lsm1-TAP or no TAP-tag purification (Figure 4B). The co-purification of Gag with SRP-associated translation complexes indicates that Gag is present not only in the ER lumen but also in the cytoplasm. Moreover, the results suggest that Gag binds Ty1 RNA that is being translated on SRP-RNC complexes. However, it is also possible that Gag bridges an interaction between non-translationing Ty1 RNA and SRP-RNC complexes. To rule out this possibility and support the conclusion that Ty1 RNA is translated in association with SRP, we determined whether Ty1 RNA is associated with SRP-RNC complexes in the retrotransposition-defective ypl7aΔ mutant [23]. In this mutant, Ty1 RNA and Gag fail to co-localize in retrosomes, even though Ty1 RNA and Gag are present at wild type or modestly reduced levels, respectively (Figure S2 and unpublished data). These observations suggested to us that the interaction between Ty1 RNA and Gag is disrupted in the ypl7aΔ mutant. (The retrotransposition defect of the ypl7aΔ mutant will be described in detail elsewhere). We found that Ty1 RNA co-purified with 7SL RNA, 18S rRNA and Y’ RNA in Srp54-TAP complexes from the ypl7aΔ mutant (Figure S2A); however, no Gag was detected in the Srp54-TAP complexes (Figure S2B). In contrast, Gag co-purified with Srp54-TAP in the bud21Δ mutant, despite the low level of Gag in this mutant (Figure S2B). Thus, Ty1 RNA is present in SRP-RNC complexes even when Gag is not detectably associated, and therefore, Gag does not bridge the interaction between Ty1 RNA and SRP. Together, these results are consistent with a model in which Gag binds Ty1 RNA that is translated in association with SRP. Notably, the lack of Gag association with Ty1 RNA on SRP-RNC complexes in the ypl7aΔ mutant is correlated with an absence of Ty1 RNA and Gag localization in retrosomes (Figure S2C), raising the possibility that Gag binding to Ty1 RNA translation complexes is required for the nucleation of retrosomes.

Depletion of co-translational ER translocation factors reduces Gag stability and Ty1 retrotransposition

The association of Ty1 RNA with SRP-RNC complexes and the presence of Gag in the ER lumen suggest that Gag is co-translationaly translocated to the ER lumen. SRP directs nascent polypeptides to the ER lumen by binding to the SRP receptor and delivering the nascent peptides to the ER translocon, (Figure 1). To further explore the role of the SRP-mediated translocation pathway in the synthesis of Gag, we examined the effect of mutations in genes encoding subunits of SRP, SRP receptor, ER translocon and translocon-associated complexes on Ty1 RNA and
Gag levels. Because deletion of these genes results in severe growth defects, and because Ty1 retrotransposition is heat-sensitive, we used Decreased Abundance by mRNA Perturbation (DAmP) mutations. DAmP alleles contain a selectable marker, *KimMX*, inserted into the 3' UTR region of the gene, which results in variable levels of mRNA destabilization and reduced gene expression [29]. To determine whether SRP-dependent translocation was compromised in the DAmP mutants, we used a phenotypic assay for translocation of a Pho8-Ura3 reporter protein to the ER [29] (Figure S3 and data not shown). The efficiency of Pho8-Ura3 translocation varied among strains carrying DAmP alleles of different co-translational translocation genes. Most mutants had partial (srp54-DAmP, srp72-DAmP and srp101-DAmP) or severe (srp68-DAmP, sec61-DAmP and sec63-DAmP) defects in Pho8-Ura3 translocation, and sec61-DAmP and srp68-DAmP mutants grew more slowly than the wild-type strain. Only the srp21-DAmP and srp102-DAmP mutants had no detectable deficiency in Pho8-Ura3 translocation relative to the wild-type strain. The relative steady-state level of Gag in each mutant was determined by western blot analysis (Figure 5A). Gag levels were reduced to varying degrees in eight mutants harboring DAmP alleles of genes encoding subunits of SRP (Srp21, Srp54, Srp68 and Srp72), the SRP receptor (Srp101 and Srp102), the ER translocon (Sec61) or the ER chaperone, Kar2. However, Gag levels were not decreased in the sec63-DAmP strain (Figure 5A), even though Pho8-Ura3 translocation was strongly compromised in this mutant (Figure S3). In contrast, Gag levels were markedly decreased in srp21-DAmP and srp102-DAmP mutants (Figure 5A), which lacked a detectable defect in Pho8-Ura3 translocation (Figure S3 and data not shown). Together, the data indicate that each of the nine DAmP mutants analyzed are hypomorphic; however, some of the mutations affect Gag accumulation differently than they do Pho8-Ura3 translocation. Overall, we find that Gag accumulation is reduced when co-translational ER translocation is compromised.

The results above suggest that the level of SRP could directly determine how much Ty1 Gag accumulates. To test this interpretation, we depleted functional SRP complexes by shutting off the expression of Srp54, which is required for SRP to translocate proteins to the ER [30,31], and measuring the level of Ty1 Gag as a function of declining Srp54 levels (Figure 5B). An *srp54A* strain harboring plasmid pGAL1-SRP54-HA was shifted from galactose medium to glucose medium to halt transcription of the *GAL1*::*SRP54-HA* cassette. Srp54-HA decreased from 0 to 28 hours after the carbon source shift, and Gag levels decreased concomitantly, whereas the level of the control protein, GDPDH was unaffected. These data indicate that the level of Gag is directly correlated with the level of functional SRP, suggesting that SRP-mediated ER translocation is necessary for the accumulation of Gag.

Depletion of Gag in mutants with hypomorphic alleles of SRP, SRP receptor and ER translocon subunit genes is correlated with a defect in retrotransposition of a chromosomal Ty1his3AT element (Figure 5C). The mobility of Ty1his3AT was measured quantitatively by determining the frequency of His* prototroph formation in each strain [32]. Mutants with a small reduction in Gag accumulation, such as *srp54-DAmP* and *srp72-DAmP*, displayed an insignificant reduction in retrotransposition, while those with very low levels of Gag, such as *srp68-DAmP* and *srp102-DAmP* mutants, had a strong reduction in retrotransposition to ~2% to 7% of that in a wild-type strain. The reduction in Gag levels and retrotransposition were not due to a decrease in Ty1 RNA levels, as the amount of Ty1 RNA in the hypomorphic *srp54, srp68, srp72, sec61* or *kar2* mutant was equivalent to that in the wild-type strain (Figure 5D). To determine whether these ER translocation mutants have defects in Gag synthesis or stability, we performed pulse-chase labeling and immunoprecipitation of Gag using two strains, *kar2-DAmP* and *srp68-DAmP*, which have low or undetectable steady-state levels of Gag (Figure 5E). After a 15-min pulse-label, the amount of labeled Gag detected in the *kar2-DAmP* and *srp68-DAmP* mutants was 96% or 83% of that in the wild-type strain, respectively. However, the level of pulse-labeled Gag was two or four-fold lower, respectively, after a 60 min chase in the *kar2-DAmP* or *srp68-DAmP* mutant relative to the wild-type strain (Figure 5E). The data demonstrate that Gag is synthesized efficiently but degrades more rapidly when ER translocation is compromised. We conclude that translocation to the ER is necessary for the stability of Gag. Possibly the oxidizing environment of the ER lumen enables Gag to adopt a stable conformation, or Gag may undergo post-translational modification in the ER. Since Gag also associates with SRP-RNC complexes in the cytoplasm (Figure 4B), the most plausible scenario to explain these findings is that Gag, once it folds into a stable conformation in the ER lumen, is retrotranslocated to the cytoplasm, where it associates with Ty1 RNA on SRP-RNC complexes.

**Retrosomes accumulate when ER translocation is blocked by treatment with tunicamycin.**

A key question raised by the findings above is how Gag-associated Ty1 RNA-SRP-RNC complexes are temporally and spatially related to retrosomes, in which Ty1 RNA and Gag accumulate in VLPs. We considered the possibility that co-translational localization of Ty1 RNA to the ER membrane and binding of Gag to Ty1 RNA-SRP-RNC complexes nucleate retrosomes. To test this hypothesis, we treated cells with tunicamycin and monitored the effect on retrotransome abundance. Tunicamycin is an inhibitor of N-glycosylation that induces the unfolded protein response, which, in *S. cerevisiae*, does not attenuate translation initiation but impedes ER translocation [33,34,35]. Our expectation, based on the phenotype of the N-glycosylation defective mutant, *dfg10A* (Figure 2), is that degradation of Gag would be enhanced. In accordance with this expectation, steady-state levels of Gag decreased gradually with increasing time of exposure to tunicamycin from 1 to 18 hours (Figure 6A). To determine the effect of blocking ER translocation on the formation of retrosomes, Ty1 RNA foci were visualized in cells treated with tunicamycin for 8 hours, when Gag was decreased but not completely depleted, because Ty1 RNA foci do not form in the absence of Gag (Figure 2B). FISH and fluorescent microscopy were performed to detect Ty1 RNA. The number of Ty1 RNA foci increased substantially from 0.46 foci/cell in mock-treated cells to 1.28 foci/cell in tunicamycin-treated cells (Figure 6B). Elevated accumulation of Ty1 RNA foci comprise an increase in the percentage of cells that contained at least one retrograde from 34% to 90% and an increase in the percentage of cells with two or more retrosomes from 7% to 26%. Direct visualization of Gag-GFP expressed from plasmid pLTR:Gag1–401:GFP:ADH1TER showed that Gag-GFP and Ty1 RNA co-localize in virtually all cells with detectable Gag-GFP foci, consistent with the idea that the tunicamycin-induced foci are retrosomes. These findings are consistent with the hypothesis that coalescence of translating Ty1 RNA gives rise to retrosomes. However, we also considered two alternative models that are formally consistent with these results: First, Ty1 RNA bound by Gag could be recruited to stress granules during ER stress, resulting in the formation of abnormal Ty1 RNA-Gag foci; or second, treatment with tunicamycin could
induce the coalescence of non-translating Ty1 RNA-Gag complexes, or VLPs, to form retrosomes.

To determine whether Ty1 RNA and Gag accumulate in stress granules when cells are treated with tunicamycin, we measured the induction of Ty1 RNA-Gag foci by tunicamycin in strains lacking stress-granule components Pub1 and Pbp1 (Figure 6B). Pub1, an ortholog of mammalian TIA-1, and Pbp1, an ortholog of mammalian ataxin-2, are both required for stress granule assembly in yeast and mammalian cells [36,37,38]. The absence of Pbp1 had no effect on the prevalence of Ty1 RNA foci or the co-localization of Gag in mock-treated cells or tunicamycin-treated cells. In the pub1Δ mutant, Ty1 RNA-Gag foci were absent from

Figure 5. Co-translational ER translocation machinery is required for Ty1 Gag stability. (A) Western blot analysis of Ty1 Gag in strain BY4741 and the hypomorphic mutant derivatives indicated, using anti-VLP polyclonal antibody to detect Gag and anti-GAPDH monoclonal antibody to detect GAPDH as a loading control. (B) Western blot analysis of an srp54Δ strain expressing GAL1p:SRP54-HA after shutting off the GAL1 promoter by addition of glucose to the medium. Lysate from cells removed from 0 to 28 hr after glucose addition was analyzed using anti-HA monoclonal antibody to detect Srp54-HA, anti-VLP polyclonal antibody to detect Gag, and anti-GAPDH monoclonal antibody to detect GAPDH. (C) Frequency of retrotransposition of a chromosomal Ty1his3AI element in strain JC3212 and hypomorphic mutant derivatives, as indicated. When the Ty1his3AI element undergoes retrotransposition, the intron in the his3AI indicator gene is spliced from the Ty1his3AI transcript, resulting in integration of a Ty1HIS3 cDNA that confers a His⁺ phenotype. The frequency of retrotransposition is the frequency of His⁺ prototroph formation in each strain following growth in rich media at 20 °C, the permissive temperature for retrotransposition. (D) Bar graph of the relative level of Ty1 RNA in three biological replicates of strain BY4741 and mutant derivatives, as determined from three independent Northern blots. The ratio of Ty1 RNA to 25S rRNA in each RNA sample was determined, and the mean ratio in each strain relative to the wild-type strain is expressed as a percentage. The average relative Ty1 RNA level determined from three experiments is shown. Error bars represent standard deviation. (E) Immunoprecipitation of HPG-labeled Gag from cells pulse-labeled for 15 min with HPG and chased with excess methionine for 0, 30 or 60 min, as indicated. HPG-Gag was immunoprecipitated with anti-VLP antibody and detected by conjugation to TAMRA. TAMRA-conjugated Gag was analyzed on a 10% SDS-PAGE gel and the fluorescent signal was quantified using a Typhoon scanner. The values presented in the graphs are the average fluorescent signal in each strain relative to the WT strain at the 0 min chase time point. The average value from two experiments is shown for the WT and srp68ΔAmP strains; values presented for the kar2ΔAmP strain are from one experiment. Error bars represent standard deviation.

doi:10.1371/journal.pgen.1004219.g005
Figure 6. The prevalence of Ty1 retroelements increases when ER translocation is blocked with tunicamycin. (A) Western blot analysis of strain BY4741 harboring plasmid pLTRp:Gag1–401:GFP:ADH1TER after addition of tunicamycin to a final concentration of 5 μg/ml and incubation for the times indicated above each lane. Gag:GFP was detected with anti-GFP antibody, endogenous Gag was detected using anti-VLP polyclonal antibody, and alpha-tubulin was detected as a loading control using anti-alpha tubulin monoclonal antibody. (B) FISH analysis of Ty1 RNA and direct visualization of Gag:GFP in strain BY4741 (WT) and congenic pBP1Δ and pub1Δ derivatives expressing plasmid pLTRp:Gag1–401:GFP:ADH1TER after addition of tunicamycin (TUNI) to a final concentration of 5 μg/ml or an equal volume of DMSO (mock-treatment) for 8 hours. Cells were visualized by DIC (differential interference contrast) microscopy and fluorescence microscopy. DAPI (blue) stained nuclei. A Cy3-labeled gag anti-sense probe detected Ty1 RNA (red) by FISH. Gag:GFP (green) in fixed cells prepared for FISH analysis was visualized directly. \( f/c \) is the Ty1 RNA foci per DAPI-stained cell and \( n \) is the total number of DAPI stained cells counted. The graphs to the right of each image indicate the percentage of cells that have 0, 1, 2, or 3 or more RNA foci per cell.

doi:10.1371/journal.pgen.1004219.g006
mock-treated cells, which is likely a consequence of the reduced Ty1 RNA stability and Gag levels in this mutant [39,40]. However, treatment with tunicamycin for 8 hours completely suppressed the Ty1 RNA localization defect of the pub1A mutant, and remarkably, restored Ty1 RNA foci to levels approaching those in wild-type cells treated with tunicamycin. Moreover, Gag co-localized with Ty1 RNA foci in tunicamycin-treated pub1A cells. These data demonstrate that neither Pph1 nor Pub1 is necessary for the accumulation of Ty1 RNA-Gag foci in tunicamycin-treated cells, and therefore the observed Ty1 RNA-Gag foci are not stress granules. Furthermore, the tunicamycin-induced formation of retrosomes in a pub1 mutant, which is normally depleted of Gag and therefore VLPs, strongly suggests that retrosomes are not nucleated by a coalescence of VLPs. Thus, stalled Ty1 RNA translation complexes that accumulate when ER translocation is blocked by inhibiting N-linked glycosylation likely nucleate retrosomes.

Retrosome abundance is a function of the rate of ER translocation and Gag accumulation

As an independent test of the model that retrosomes are nucleated by an accumulation of Ty1 RNA-RNC complexes, we determined whether retrosome formation increases when ER translocation is stalled by a limited depletion of SRP (Figure 7). To examine the effect of reducing the level of SRP, we used hypomorphic alleles of SRP54, SRP60 and SRP72, which encode components of the S domain of SRP. Depletion of these SRP subunits reduces the rate of SRP-dependent protein translocation [41]. In the srp54-DAmP and srp72-DAmP strains, which have a slight reduction in the steady-state level of Gag (Figure 7A) and a modest Pho8-Ura3 translocation defect (Figure S3 and data not shown), Ty1 RNA foci increased to 0.70 and 0.91 foci/cell, respectively, compared to 0.48 foci/cell in the wild-type strain (Figure 7B). Gag-GFP foci that were detected co-localized with Ty1 RNA foci, indicating that the foci are retrosomes. These data are consistent with the accumulation of Ty1 RNA-RNC complexes in foci when SRP is limiting, and thus translocation of Gag to the ER lumen is stalled. In contrast, the srp60-DAmP mutant, which has a very low level of Gag (Figure 7A) and a severe Pho8-Ura3 translocation defect (Figure S3), lacked Ty1 RNA foci. This phenotype is similar to that of the bud21A mutant, which also has a wild-type level of Ty1 RNA and a very low level of Gag (Figure 2). Together, the data suggest that a minimum level of Gag is needed for Ty1 RNA to coalesce in foci and support the idea that multimerization of Gag molecules bound to Ty1 RNA on SRP-RNC complexes nucleates the formation of retrosomes.

A further prediction of the model that retrosomes form by accumulation of Ty1 RNA-RNC complexes is that suppressing the translocation deficiency of the srp54-DAmP or srp72-DAmP mutant should reverse the elevated formation of retrosomes in each mutant. Treatment of srp hypomorphs with a very low concentration of cycloheximide suppresses their translocation deficiency by providing more time for SRP to sample RNC complexes for a cognate nascent peptide [31]. Therefore, we treated srp54-DAmP and srp72-DAmP mutants and the wild-type strain with 0.44 μg/ml cycloheximide for 30 min. Exposure to this low concentration of cycloheximide did not decrease the level of Gag in the srp54-DAmP or srp72-DAmP mutant (Figure 7A). In fact, Gag levels were slightly increased, consistent with the suppression of stalled translocation of Gag. In contrast, the number of Ty1 RNA foci decreased sharply, from 0.7 to 0.36 foci/cell in the srp54-DAmP strain and from 0.91 to 0.45 foci/cell in the srp72-DAmP strain. This effect of cycloheximide on the accumulation of retrosomes was specific for the srp hypomorphs, as there was no change in the number of Ty1 RNA foci/cell as a result of cycloheximide treatment of the wild-type strain. Thus, slowing down translational elongation to complement the limiting levels of SRP in the srp54 or srp72 hypomorph rapidly reverses the elevated formation of retrosomes. Together, the results demonstrate that retrosomes are dynamic foci formed by the coalescence of Ty1 RNA translation complexes at the ER.

Discussion

Translocation of Gag to the ER lumen by SRP-mediated translation of Ty1 RNA

This study revealed an unanticipated association of Ty1 RNA and Gag with the ER. Ty1 Gag co-purifies with the lumen fraction of ER microsomes and is protected from trypsin digestion by the microsomal membrane (Figure 3), providing direct biochemical evidence that Gag is translocated to the ER lumen. Although Gag is not a secreted protein and lacks a recognizable signal sequence, two lines of evidence suggest that SRP mediates the cotranslational translocation of Gag across the ER membrane. First, Ty1 RNA is enriched in affinity-purified SRP-RNC complexes (Figure 4A) [26]. The enrichment of Ty1 RNA is observed even in a mutant that lacks a detectable association of Gag with SRP-RNC complexes, indicating that Gag does not bridge the association of Ty1 RNA with SRP (Figure S2). Second, disruption of ER translocation by depleting subunits of SRP, the SP receptor, the ER translocon or the ER chaperone, Kar2/BiP, results in decreased accumulation of Gag (Figure 5A). In the translocation-deficient srp60-DAmP strain, which has a very low steady-state level of Gag, newly synthesized Gag is present at 85% of that in a wild-type strain but is rapidly degraded (Figure 5E). Therefore, most if not all of the nascent Gag is co-translationally localized to the ER lumen, or at least, nascent Gag that is synthesized in the cytoplasm does not contribute significantly to the cellular pool.

A model for the nucleation of presumptive VLP assembly sites

Our findings support a model in which the coalescence of Ty1 RNA-ribosome-nascent Gag complexes nucleates the formation of cytoplasmic Ty1 retrosomes, where the concentration of Ty1 RNA and proteins enables the assembly of VLPs (Figure 8). In this model, Ty1 RNA-ribosome-nascent Gag complexes are specifically recognized and bound by SRP, which docks the Ty1 RNA translation complex to the SRP receptor on the ER membrane. Nascent Gag is threaded through the ER translocon into the ER lumen. Gag adopts a stable conformation in the ER lumen and is subsequently retrotranslocated to the cytoplasm. When Gag enters the cytoplasm, it binds Ty1 RNA that is being translated on SRP-RNC complexes, perhaps aided by the proximity of Ty1 RNC complexes that are docked onto the ER membrane. Multimerization of Gag bound to Ty1 RNA-SRP-RNC complexes results in the coalescence of Ty1 RNA and Gag to form the presumptive VLP assembly site. When sufficient Gag is synthesized and bound to translating Ty1 RNA, Gag likely sequesters Ty1 RNA from translation and promotes its dimerization and packaging into assembling VLPs, although the details of this transition have not yet been elucidated.

In wild-type cells, VLP assembly is inefficient, as Ty1 VLPs are rarely detected, and only about 20% Ty1 RNA is protected from nuclease digestion by encapsidation in VLPs [11,12,15]. Nonetheless, retrosomes are visualized in 40% to 50% of wild-type cells, suggesting that there is sufficient Gag to drive the coalescence of Ty1 RNA-RNC complexes into foci in many cells, but insufficient
Localization of Ty1 RNA to VLP Assembly Sites
Gag enables the efficient transition of Ty1 RNA from translation to packaging in VLPs. Thus, retrosum formation might represent a bottleneck in the retrotransposition cycle in which Ty1 RNA-SRP-RNC complexes accumulate because a component of the ER translocation machinery is limiting. This model explains why retrosums increase in number and size when Ty1 RNA is overexpressed [11], since increasing the level of Ty1 RNA would create a larger bottleneck of Ty1 RNA-SRP-RNC complexes awaiting recognition by the SRP receptor or ER translocon.

Several lines of evidence indicate that multimerization of Gag bound to Ty1 RNA on SRP-RNC complexes results in the nucleation of retrosums. First, Gag co-purifies with SRP-RNC complexes (Figure 4B). In an spf7aA mutant, in which SRP-RNC complexes are associated with Ty1 RNA but not Gag, Ty1 RNA-RNC complexes fail to coalesce in retrosums (Figure S2). Second, both the bud21A mutant, which has inefficient Gag synthesis (Figure 2D), and the spf68-DAmP mutant, in which Gag is rapidly degraded (Figure 5E), lack retrosums. Thus, the level of Gag in these mutants may be too low to enable coalescence of Ty1 RNA-SRP-RNC complexes into foci. Third, modestly reducing the rate of co-translational ER translocation increases the prevalence of retrosums. Treatment of cells with tunicamycin dramatically increased Ty1 RNA foci co-localize with Ty1 Gag. This tunicamycin-induced accumulation of Ty1 RNA-Gag foci occurs in the absence of Pbp1 and Pub1, which are required for stress granule formation. Ty1 VLP formation is likely to be severely impaired by the paucity of Ty1 RNA and Gag in the pub1A mutant [39,40]; therefore, the tunicamycin-induced formation of retrosums in the pub1A mutant further supports the idea that Ty1 RNA-RNC complexes, rather than VLPs, nucleate retrosums. The accumulation of Ty1 retrosums that results from limiting the amount of SRP in the spf54-DAmP and spf72-DAmP hypomorphs is completely reversed by exposure of cells to a very low concentration of cycloheximide. The fact that altering the efficiency of co-translational translocation rapidly alters the prevalence of retrosums strongly suggests that the Ty1 RNA that coalesces in retrosums is being translated. Overall, these findings support the idea that Ty1 RNA is localized to the retrosum during translation.

We previously showed that nearly all Ty1 RNA in the cell resides in very high molecular weight complexes whose migration in sucrose gradients is not significantly altered by treatment with EDTA, which causes the dissociation of ribosomes [12]. Thus, we concluded that Ty1 RNA was translationally repressed in complexes that appeared to be devoid of translating ribosomes. However, the data presented here clearly indicate that Ty1 RNA is translated in association with SRP. Moreover, translation of Ty1 RNA within these high molecular weight complexes is consistent with the broader molecular weight distribution of Ty1 ribonucleoprotein complexes in a mutant with a defect in 40S ribosomal subunit biogenesis [39]. The fact that very little Ty1 RNA is released into low molecular weight complexes when ribosomes are dissociated by EDTA suggests that nearly all Ty1 RNA, including that being translated, is in a complex with Gag dimers.

The co-purification of Gag with SRP-RNC complexes in which Ty1 RNA is translated suggests that Gag binding to translating Ty1 RNA promotes the transition of Ty1 RNA from translation to packaging in VLPs. Thus, our findings provide indirect evidence that Ty1 RNA is not partitioned into separate mRNA and...
genomic RNA pools. Binding of a retroelement RNA chaperone protein to its RNA during translation is thought to enable the preferential mobilization of the encoding RNA in cis, which results in mobilization of only those elements that encode functional proteins. For example, human L1 retrotransposon proteins show a strong cis-preference for mobilizing their own RNA template, presumably because they bind to L1 RNA co-translationally. As a consequence, only functional L1 elements transpose efficiently [42,43]. However, Ty1 proteins efficiently package Ty1 RNA in trans, and defective elements are mobilized as efficiently as competent elements [17,44], raising the question of how Gag associates with translating RNA without interacting preferentially with its own RNA template. A similar paradox is manifest by studies on the nucleation of HIV-1 assembly sites: while HIV-1 mRNA and genomic RNA reside in a single pool, and Gag binds HIV-1 RNA in the cytoplasm before transport to the plasma membrane assembly site, HIV-1 Gag does not display a cis-preference for packaging its encoding RNA [45,46]. Our data reveal a novel mechanism by which Gag is temporally separated from its RNA template by translocation to the ER and retro-translocation to the cytoplasm before binding Ty1 RNA translation complexes. This model may have implications for other retrotransposons or retroviruses that display efficient trans-packaging of RNA.

The role of Gag in Ty1 RNA stability

The observation that Ty1 RNA levels are not reduced in mutants with very low levels of Gag, including the bud21A mutant (Figure 2C) and the spg68-DAmP mutant (Figure 5D) indicates that Gag binding to Ty1 RNA is probably not required for the exceptionally long half-life of Ty1 RNA [47]. Our conclusion that Gag is dispensable for Ty1 RNA stability differs from the conclusion reached in a recent study in which the authors analyzed a Ty1 transcript with a premature stop codon placed directly after the start codon [16]. The difference between our results may indicate that a minimal level of Ty1 RNA translation, rather than the presence of stable Gag, is required for Ty1 RNA stability. In the dfg10A mutant, Ty1 RNA is unstable (Figure 2B), although its degradation occurs subsequent to Gag synthesis (Figure 2D). Perhaps Ty1 RNA instability in the dfg10A strain is an indirect result of N-linked glycosylation, which is expected to induce unfolded protein accumulation in the ER, whereas Ty1 RNA is not destabilized in the spg68-DAmP strain because the cellular stress response to blocking ER translocation is distinct from that of the unfolded protein response.

SRP-mediated translocation of Gag to the ER and retrotranslocation to the cytoplasm

The essential role of SRP in the nucleation of presumptive Ty1 VLP assembly sites is particularly interesting in light of the association of 7SL RNA with nucleocapsids of several retroviruses [46,48,49]. It is not known whether the 7SL RNA has a function in retroviral replication, but it has been implicated in the incorporation of the human antiviral restriction factor APO-BEC3G into HIV-1 particles [50]. In addition, SRP interacts co-translational with the Gag polypeptide of the murine endogenous retrovirus, IAP, and brings Gag to the ER membrane, although Gag was not translocated to the ER lumen in an in vitro system [51]. Our data raise the possibility that the association of 7SL RNA with retroviral particles evolved from an ancient functional role of SRP in retrotransposition.

The SRP-mediated translocation of Ty1 Gag to the ER lumen and the presence of Gag in the cytoplasm raise many questions about the mechanism and purpose of Gag transit into and out of the ER lumen. For example, the target sequence in the nascent Gag peptide that is recognized by SRP is not known. A systematic identification of nascent peptides that interact with SRP demonstrated that approximately 20% of SRP targets lack a predicted N-terminal signal sequence or transmembrane domain [26]. Many of these nascent peptides, including Ty1 Gag, are encoded by mRNAs that are membrane-associated and therefore are validated targets of SRP. Binding of SRP to a hydrophobic domain of the nascent peptide is required for the high affinity association of SRP with RNPs complexes and for translocation [22], so it is likely that Gag has a specific target sequence that is bound by SRP. Analysis of the Ty1-H3 Gag sequence with the Kyte-Doolittle algorithm reveals that the longest hydrophobic region of Gag resides in the C-terminal end of p45-Gag from amino acid 334 to 345 (NTVAELFLDIHA). This region is predicted to form an alpha-helical domain, which could promote recognition by SRP [52,53]. Interestingly, amino acids 341 through 346 (LDIHAI) have been shown to be critical for the formation of VLPs [54,55].

The findings reported here suggest that Gag that is not co-translationally translocated to the ER is retained in the cytoplasm and unstable, perhaps because it cannot fold properly. What aspect of the ER lumen environment promotes the stability of Gag? One possibility is that Gag is post-translationally modified in the lumen. Although the C-terminal domain of Gag harbors five to eight potential N-glycosylation sites revealed by NetNglyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and N-GlycoSite (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html) algorithms, digestion of Gag with Endo H did not alter its mobility on SDS-PAGE gel (unpublished result). Thus, there is no evidence that Gag is subject to N-glycosylation. The migration of mature Gag in two bands on SDS-PAGE gels indicates that processed Gag may be subject to post-translational modification (Figure S1). Indeed, phosphorylation of Ty1 Gag in cells treated with maphorin has been observed previously [56]; however, there is no evidence that these modifications are linked to transit through the ER. We suggest that a more likely possibility is that the oxidizing environment of the ER supports the folding of the Gag and Gag-Pol precursors into conformations that promote their stability, enabling them to return to the cytoplasm to bind Ty1 RNA and initiate VLP assembly. Notably, maturation of the Moloney murine leukemia virus (MMLV) Gag and Gag-Pol proteins is regulated by the redox environment. Proteolytic processing of MMLV Gag and Gag-Pol proteins is constrained in infected cells exposed to a mild oxidizing agent and induced by treatment of the immature viral particles with a reducing agent [57,58]. Perhaps folding of Ty1 Gag and Gag-Pol in the ER lumen prevents premature maturation of Gag and Gag-Pol until they are retrotranslocated to the cytoplasm, where Gag can bind Ty1 RNA and multimerize to initiate the formation of Ty1 VLPs.

How is Gag retrotranslocated from the ER lumen to the cytoplasm? Misfolded ER proteins are retrotranslocated to the cytoplasm via the ER-associated degradation (ERAD) pathway, in which transport from the ER to the cytoplasm is coupled to polyubiquitination and proteosomal degradation [reviewed in [59]]. Some proteins that are known to be retrotranslocated from the ER to the cytoplasm, such as A/B toxins that enter the ER following endocytosis [60], may utilize the ERAD pathway but escape proteasome degradation. One example of a cellular protein that is retrotranslocated from the ER is the mammalian and Trypanosoma cruzi calcium-binding chaperone, calreticulin [61,62]. Calreticulin is targeted to the ER lumen via the canonical SRP-mediated pathway by recognition and cleavage of its N-terminal signal sequence. A fraction of luminal calreticulin is subsequently retrotranslocated to the cytoplasm by a process that is regulated by
the concentration of calcium in the ER. Several viruses are also known to hijack the retrotranslocation pathway to promote viral assembly. For example, the N-glycosylated ORF2 capsid protein of Hepatitis E virus is retrotranslocated to the cytoplasm. In this instance, retrotranslocation is dependent on glycosylation of the ORF2 protein in the ER [63]. Further studies aimed at understanding how Ty1 Gag is recognized as an ER substrate, what conformational changes or modifications are required for Gag stability and how Gag is retrotranslocated to the cytoplasm will likely provide significant insights into the host-retrotransposon relationship as well as illuminate poorly understood aspects of SRP target specificity and protein retrotranslocation from the ER to the cytoplasm.

**Materials and Methods**

**Plasmids and yeast strains**

The plasmid pLTR\_Gag1\_401\_GFP\_ADH1\_TER is a LEU2-marked, CEN-based plasmid containing a Ty1 U3 promoter, 5’ UTR and GAG ORF from amino acid 1 to 401 (corresponding to the processed p45-Gag protein) fused to the GFP (S65T) ORF and followed by the Adh1 terminator. The plasmid pGAL1-SRP54-HA, carrying a SRP54-HA expression cassette on a 2-micron vector, pRS315, was obtained from Martin Pool (University of Manchester), strain JC6159 is a haploid p54\_LEU2\_ura2\_0 leu2\_0 his3\_1 strain carrying plasmid pGAL1-SRP54-HA [65]. The strain was constructed by PCR-mediated gene disruption of a typl\_hisG/tp1\_hisG derivative of strain BY4743 with a p54\_LEU2/ SRP54 diploid strain. Plasmid pGal-SRP4-HA was transformed into the p54\_LEU2/2SRP54 diploid strain, and tetrads were dissected to obtain the segregant JC6159.

**Western blot analyses**

Strains were grown at 20°C, a temperature that is permissive for Ty1 retrotransposition, to mid-log phase (OD\_600 of 0.4–0.6) unless otherwise noted. Total cell lysates were prepared as described by Yarrington et al. [70] and proteins were separated on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated in phosphate-buffered saline (PBS) plus 0.05% Tween 20 and 1% nonfat milk containing a 1:50,000 dilution of affinity-purified anti-HA monoclonal antibody (Abcam) to detect actin, or a 1:500 dilution of a polyclonal antibody to detect Srp54-TAP, or a 1:5,000 dilution of an anti-calmodulin binding protein (CBP) antibody and SuperSignal West Pico chemiluminescent substrate (Pierce), and then the blots were exposed to film. The PVDF membranes were stripped of antibody in 50 mM Tris-HCl (pH 7), 2% SDS, and 50 mM DTT at 70°C for 30 min and washed in 1× PBS several times. The membrane was incubated with a 1:10,000 dilution of anti-GFP polyclonal antibody (Sigma) to detect GaggFP, a 1:7,500 dilution of peroxidase-anti-Peroxidase (PAP) complex (Sigma) to detect Kar2-TAP or Adh5-TAP, a 1:5,000 dilution of anti-Calmodulin binding protein (CBP) polyclonal antibody (Millipore) to detect Srp45-TAP, or a 1:500 dilution of anti-HA (F-7) monoclonal antibody to detect Srp54-HA (Santa Cruz Biotechnology). Subsequently, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and SuperSignal West Pico chemiluminescent substrate (Pierce), and then the blots were exposed to film. The PVDF membranes were stripped of antibody in 50 mM Tris-HCl (pH 7), 2% SDS, and 50 mM DTT at 70°C for 30 min and washed in 1× PBS several times. The membrane was incubated with a 1:1,000,000 dilution of anti-alpha tubulin monoclonal antibody (Millipore) to detect alpha-tubulin, a 1:7,300 dilution of anti-actin monoclonal antibody (Abcam) to detect actin, or a 1:5,000 dilution of anti-GAPDH monoclonal antibody (Thermo Scientific) to detect GAPDH as a loading control, and bands were visualized as described above.

**Northern blot analyses**

Northern blot analysis of total RNA prepared from cells grown to mid-log phase in CSW-Leu 2% Ghu or YPD broth at 20°C, was performed as described previously [27]. Plasmid pJC940 DNA was used as a template to synthesize a 32P-labeled riboprobe to detect Ty1 RNA [12]. Plasmid pDG513, a gift of David Garfinkel, was used as a template to synthesize a riboprobe to detect 25S rRNA. Bands were quantified by phosphorimaging.
Pulse-chase labeling and immunoprecipitation

Proteins were pulse-labeled by incubation of cells in culture with L-homopropargylglycine, an analog of L-methionine with an alkylene side chain. Strains were grown to mid-log phase in YPD broth at 20°C. 30 OD₆₀₀ units of cells per strain were harvested and washed three times in 5 ml of CSM-Met 2% Glu media. Click-IT L-Homopropargylglycine (Life Technologies) was added to 5 ml cell suspensions in CSM-Met broth to a final concentration of 80 μM. Cultures were incubated at 20°C with vigorous shaking, and equal OD₆₀₀ units of cells were harvested at 0, 30 and 60 min time points. Cells were centrifuged at 1000 × g for 1 min, and cell pellets were washed twice with 5 ml of chase media (CSM-Met 2% Glu+50 mM methionine).

Cell pellets were resuspended in 100 μl HB buffer [25 mM Tris-Cl (pH 7.5), 125 mM NaCl, 5 mM EDTA, 0.5% IPEGAL, Complete Mini, EDTA-free protease inhibitor cocktail (Roche)] and vortexed at 4°C for 3 min with 0.15 g of glass beads. Following addition of 5 μl of 10% SDS, the cell extract was boiled for 5 min, and 500 μl ice-cold HB buffer was added. The extract was centrifuged for 1 min at 15,000 × g, and 50 μl Protein A Sepharose (GE Healthcare) was added to the supernatant, which was incubated at 4°C for 4 hr. Anti-VLP polyclonal antibody was added to pre-cleared lysate and rotated at 4°C for 18 hr. 50 μl of Protein A Sepharose was added to each sample and rotated at 4°C for 30 min. Samples were washed 3 times with 500 μl IP wash buffer [50 mM Tris (pH 7.5), 125 mM NaCl, 5 mM EDTA, 1% IPEGAL, 1× EDTA-free protease inhibitor cocktail (Roche)], and proteins were eluted from the Protein A Sepharose with 50 mM Glycine (pH 3.0) and equilibrated in 200 μl of 50 mM Tris-Cl (pH 5.0), 1% SDS.

Eluted proteins were precipitated by methanol/chloroform precipitation. Copper-catalyzed triazole formation click reactions were performed using Click-IT Cell Reaction Buffer Kit (Life Technologies) to conjugate TAMRA (Tetramethylrhodamine 5-Carboxamido-6-Azidohexanyl), 5-isomer; Life Technologies) to HPG-labeled Gag. SDS loading buffer was added to each reaction, proteins were separated on 10% SDS-PAGE gels. The fluorescent signal of TAMRA-conjugated Gag was detected using a Typhoon Scanner with a 580 BP30 filter, and bands were quantified with ImageQuant TL Software (GE Healthcare).

Membrane flotation assay

Strain BY4741 was grown to mid-log phase in YPD broth at 20°C. The cells were harvested, washed, and then resuspended in Buffer F [50 mM HEPES-NaOH pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol supplemented with Complete Mini, EDTA-free protease inhibitor cocktail (Roche)] to an OD₆₀₀ of 10. Glucose beads were added and cells were agitated on a Vortex mixer for 5 min at 4°C. The extract was removed and centrifuged at 300 × g for 2 min at 4°C. 50 μl of the supernatant was mixed with 300 μl of 2.5 M sucrose in Buffer F, to obtain lysate in 2.0 M sucrose. This lysate was layered onto a 300 μl cushion of 2.0 M sucrose in Buffer F in a centrifuge tube. 1.5 M sucrose in Buffer F (500 μl) and 0.25 M sucrose in Buffer F (350 μl) were successively layered onto the gradient and the tube was centrifuged in a Beckman SW35 rotor at 100,000 × g for 4 hr at 4°C. A 130 μl fraction was removed from the top of the gradient and discarded. Nine 130 μl fractions were collected from the top of the gradient, and protein profiles were analyzed by western blot analysis as described above.

Microsome preparation, sodium carbonate extraction, and protease protection assay

Microsomes were prepared from a culture of strain BY4741 grown to mid-log phase in YPD broth at 20°C as described in Brotsky et al. [1]. The microsomes were resuspended in B88 buffer [20 mM HEPES, pH 6.8, 250 mM sorbitol, 150 mM KOAc, 5 mM MgOAc] to an OD₆₀₀ of 10. Sodium carbonate extractions were performed by incubating 50 μl of microsomes with 1 ml extraction buffer [200 mM Na₂CO₃ (pH 11.5), 10 mM DTT, Complete Mini, EDTA-free protease inhibitor cocktail (Roche), 0.5 M sucrose, 2% glycerol] on ice for 30 min. Samples were centrifuged at 230,000 × g at 4°C for 1 hr, and the pellet was dissolved in 1× SDS loading buffer. Proteins in the supernatant were precipitated by incubation on ice for 30 min with trichloroacetic acid (TCA) added to a final concentration of 10%, followed by a 10-min centrifugation at 16,060 × g at 4°C. The pellet was solubilized in 1× SDS loading buffer, and proteins separated on 10% SDS-PAGE gels were analyzed by western blot analysis, as described above.

Protease protection assays were performed by incubating 50 μl of microsomes with and without 1% Triton X-100 with 0.2 mg/ml TPCK-treated trypsin from bovine pancreas (Sigma) for 15 min on ice. The reactions were stopped by the addition of 5× SDS loading buffer. Ty1 Gag and Kar2-TAP were detected by western blot analysis, as described above.

Affinity purification of TAP complexes, RNA and protein analysis

Srps4-TAP and control complexes were purified following the procedure of del Alamo et al [26]. Strain BY4741 and derivatives harboring a chromosomal SRP54-TAP or LSM1-TAP allele were grown to an OD₆₀₀ of 0.6–0.7 at 20°C. Cycloheximide was added to a final concentration of 0.1 mg/ml and growth was continued for 1 min at 20°C with vigorous shaking. Cells were immediately harvested by filtration onto 0.45 μm pore size nitrocellulose filters (Whatman), and cells were resuspended in 0.5 ml of ice-cold buffer A [50 mM Heps-KOH (pH 7.5), 140 mM KCl, 10 mM MgCl₂, 0.1% NP-40, 0.1 mg/ml cycloheximide, 0.5 mM DTT, Complete Mini EDTA-free protease inhibitor cocktail (Roche), 0.2 mg/ml heparin, 50 U/ml Superasin (Ambion), and 50 U/ml RNAsin (Promega)]. The cell suspension was dripped into a 50-ml conical tube containing liquid nitrogen. Frozen cells were pulverized for six cycles of 3 min at 15 Hz, using a Retsch MM301 mixer mill. Sample chambers were chilled in liquid nitrogen before each pulverization cycle. Pulverized cells were thawed and resuspended in 5 ml of buffer A. Cell debris was removed by two sequential centrifugation steps at 8,000 × g for 5 min at 4°C. A 100 μl aliquot of the supernatant was removed for total RNA isolation, and a 50 μl aliquot was removed for western blot analysis. The remaining supernatant was incubated with IgG-Sepharose 6 Fast Flow (GE Healthcare) at 4°C for 2 hr. Beads were washed once in 5 ml of buffer A for 2 min and 5 times in 1 ml buffer B [50 mM Heps-KOH (pH 7.5), 140 mM KCl, 10 mM MgCl₂, 0.01% NP-40, 10% glycerol, 0.5 mM DTT, 10 U/ml SUPERase-In (Life Technologies), 10 U/ml RNasin, 0.1 mg/ml cycloheximide] for 1 min. Beads were resuspended in 100 μl of buffer B, and 0.3 U/ml AcTEV protease (Invitrogen) was added. The samples were incubated for 2 hr at 16°C, and the eluate was recovered. A 50 μl aliquot of the eluate was mixed with 5× SDS loading buffer, and the proteins were separated on 10% SDS-PAGE gels. Srps54-TAP and Tyl1 Gag proteins were analyzed by western blot analysis, as described above. Total RNA and RNA from the eluate were isolated by sequential extraction with Phenol/Chloroform [5:1] (Ambrosc), Phenol/Chloroform/Isosamyl Alcohol [25:24:1] (Sigma), and chloroform followed by isopropanol precipitation with 15 μg of Glycoblue (Ambion) as carrier. Each RNA sample was treated with 0.04 U/μl Turbo DNase (NEB) for 30 min at 30°C and inactivated with DNase Inactivation Reagent for 5 min at
room temperature, and cDNA was synthesized using the First-Strand cDNA Synthesis Kit (Affymetrix). PCR reactions were performed using 0.5 µg of cDNA as a template with gene-specific primers. Each reaction was subject to 29 or 32 cycles of amplification. PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide. The sequence of gene-specific RT-PCR primers is provided in Table S2.

Srp54 depletion

Cells of strain J6159 were diluted to an OD_{600} of 0.01–0.02 in CSM-Ura-Leu 2% Gal 2% Raf 2% Suc and grown at 20°C for 24 hours. Glucose was added to the culture to a final volume of 2%, and 4 OD_{600} units of cells were harvested, pelleted and frozen at different time points. Equal volumes of lysate prepared from each cell pellet were separated on 10% SDS-PAGE gels and analyzed by western blot analysis as described above.

Transposition frequency assays

To measure transposition of the chromosomal Ty1his3A1/A1/-3114 element [69], strain JC3212 and mutant derivatives were grown in YPD broth at 30°C to saturation. Cultures were diluted 1:1000 in YPD broth and grown at 20°C for 3 days. A 1:1000 dilution of each culture was plated on YPD agar to determine the colony forming units (CFU). Aliquots (1–2 ml) of each culture were plated on CSM-His 2% Glu agar, and all plates were incubated at 30°C for 3 days. The frequency of Ty1his3A1 retrotransposition is the number of His+ colonies divided by the total number of CFU plated on CSM-His 2% Glu agar, which was determined from the colony count on YPD agar. The average frequency and standard error for each genotype tested were calculated from nine separate cultures.

Tunicamycin time course

A 250 ml culture of BY4741 harboring plasmid pLTR_p:Gag_{401}:GFP:ADH1_TER was grown to an OD_{600} of 0.25 at 20°C in CSM-Leu 2% Glu broth, and 250 µl of 5 mg/ml tunicamycin in DMSO [Research Products International, Corp] or DMSO only was added. Four OD_{600} units of cells were harvested 0, 1, 4, 8, and 18 hr after addition of tunicamycin. Lysates of each cell pellet were separated on 10% SDS-PAGE gels and analyzed by western blot analysis, as described above.

FISH and fluorescence microscopy

Fluorescence in situ hybridization was performed essentially as described in Amberg et al. [72], with the following modifications. Strains were grown in YPD broth or, in the case of strains harboring plasmid pLTR_p:Gag_{401}:GFP:ADH1_TER, CSM-Leu 2% Glu broth, to mid-log phase (OD_{600} of 0.4–0.6) at 20°C. For experiments with tunicamycin, a solution of tunicamycin in DMSO was added to a final concentration of 5 µg/ml or an equivalent volume of DMSO was added, and cultures were grown for an additional 8 hr at 20°C. For experiments with cycloheximide, a solution of cycloheximide in DMSO was added to a final concentration of 0.44 µg/ml cycloheximide, or an equivalent volume of DMSO was added, and cultures were incubated with gentle turning at 20°C for 30 min. Cultures were treated with formaldehyde (4% final concentration) and incubated at room temperature for 15 min on a turning platform. Cells were collected by centrifugation, resuspended in 5 ml of 0.1 M KPO_4 (pH 6.5)/4% formaldehyde, and rotated for 90 min at 23°C. Cells were washed twice with 0.1 M KPO_4 (pH 6.5) and once in 1 ml wash buffer [0.1 M KPO_4 (pH 6.5), 1.2 M sorbitol]. The cell pellet was resuspended in 1 ml wash buffer containing 500 µg of 100T Zymolyase (MP Biomedicals) and incubated for 30 min at 30°C. Spheroplasts were washed gently with wash buffer and resuspended in a volume of wash buffer approximately twice the volume of the pellet and transferred to 10-well slides (Electron Microscopy Sciences) pretreated with 0.1% poly-L-lysine (Sigma). After aspirating non-adhered cells, adhered cells were washed twice with 100 µl of 2× SSC (1× SSC = 0.15 NaCl, 0.015 M sodium citrate) per well. Cells in each well were incubated with 12 µl of prehybridization buffer (50% formamide, 10% dextran sulfate, 4× SSC, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll-400, 125 µg/ml of tRNA, 500 µg/ml of denatured salmon sperm DNA) at 37°C for 1 hr in a humid chamber. Subsequently, 0.75 pmol of a Cy3-labeled gag anti-sense oligomer, PJ798 (5’-Cy3/TCT GTT TTG GAA GCT ACG TGT AAC GGA TCT TGA TTT GTG TGG ACT TC-3’), obtained from Integrated DNA Technologies, Inc., was added to each well, and slides were incubated for 12–18 hr at 37°C in a dark humid chamber. Slides were washed in 2× SSC for 3 min, 1× SSC for 5 min, 1× SSC containing 2 mg/ml DAPI for 3 min, 1× SSC for 5 min and 0.5× SSC for 5 min. After drying, coverslips were adhered to the slides with anti-fade solution [1% Mowiol-488 (Sigma), 3% glycerol, 50 mM Tris HCl (pH 8.5)].

Cells were visualized by fluorescence microscopy using a Zeiss Axioskop 200 M inverted microscope equipped with filter set: 31 (Cy3), 34 (DAPI) and 38 EX Band Pass 470/40 (GFP), at a magnification of 63 or 100×. A Q Imagining camera (or Hamamatsu ORCA ER) was used to obtain the images, which were then colored and merged in Openlab 4.0.4 software (Improvision) and modified with Photoshop CS software.

Pho8-Ura3 translocation assay

Strains harboring plasmid pMP234 [29] were grown overnight in CSM-Leu 2% Glu broth at 20°C to an OD_{600} of 0.6 to 1.0. The OD_{600} of each culture was adjusted to 0.5 by addition of CSM-Leu 2% Glu broth. A 6 µl aliquot of each culture, and 10-fold serial dilutions of each culture in water, were spotted onto selective media containing glucose and grown at 20°C for 4 to 7 days.

Supporting Information

Figure S1 Ty1 Gag isoforms present at steady-state in strain BY4741 do not correspond to unprocessed p49-Gag. Western blot analysis of Gag in wild-type strain BY4741, or the p53 derivative expressing plasmid pGTy1-H3(pr-1682) [72], a GAL1-driven Ty1 element harboring a protease active-site mutation that blocks processing of p49-Gag. Anti-VLP polyclonal antibody was used to detect Gag. (TIF)

Figure S2 Ty1 RNA, but not Gag, is associated with SRP-RNC complexes, and Ty1 retroelements fail to form in an npl7a mutant. (A) RT-PCR analysis of RNA co-purified with affinity-purified TAP complexes from the SRP54-TAP (WT) and SRP54-TAP npl7a (npl7a) derivatives of strain BY4741 (top panel) or the SRP54-TAP (WT) and SRP54-TAP bud21A (bud21A) derivative of strain BY4741 (bottom panel). RNA isolated from cells treated with cycloheximide before TAP purification (Total RNA) or after purification of Srp54-TAP complexes (Sr5p4-TAP/TEV chateau) was analyzed by RT-PCR with gene-specific primers. Amplification was performed for 29 and 32 cycles (indicated by wedge). Reverse transcriptase was omitted from the cDNA synthesis reaction as a negative control (No RT). 38S RNA, 18S rRNA and Y′ RNA were detected as positive controls for purification of SRP-RNC complexes. (B) Western blot analysis of whole cell lysate (WCL) using anti-CBP polyclonal antibody to detect Srp54-TAP.
and anti-VLP polyclonal antibody to detect Gag, and Srp54+TAP-purified complexes (TEV chime) using anti-VLP antibody to detect Gag. (C) FISH analysis of Ty1 RNA and direct visualization of GagGFPU cells in strain BY4741 (WT) and a congenic pEF/a derivative, both harboring plasmid pLTR-Gag-GFP: ADH1-5′. Cells were visualized by DIC (differential interference contrast) microscopy and fluorescence microscopy. DAPI (blue) stained nuclei. Ty1 RNA (red) was detected using a Cy3-labeled gag anti-sense probe. GagGFPU (green) was visualized directly. E/c is the Ty1 RNA foci per DAPI-stained cell and n is the total number of DAPI stained cells counted. (TIF)

Figure S3 DmMP alleles of SRP, SRP receptor, ER translocon and Sec63 complex genes have varied effects in co-translational translocation of Pho8-Ura3 to the ER. Ten-fold serial dilutions of each strain (genotype indicated) harboring LEU2-based plasmid pMP234 expressing the Pho8-Ura3 reporter protein, were spotted onto CSM-Leu medium to monitor growth, CSM-Leu+5-FOA to detect Gag. (TIF) (DOCX)

Localization of Ty1 RNA to VLP Assembly Sites

The references cited in the document include:

1. Malik HS, Henikoff S, Eickbush TH (2000) Posed for contagion: evolutionary origins of the infectious abilities of invertebrate retroviruses. Genes Res 10: 1307–1318.

2. Voytas DF, Boeke JD (2002) Ty1 and Ty5 of Saccharomyces cerevisiae. In: Craig N, Craigie R, Gellert M, Lambowitz A, editors. Mobile DNA II. Washington, DC: ASM Press. pp. 631–662.

3. Paryszycka KJ, Legiewicz M, Matusek E, Eisenstadt LD, Laervaardi S, et al. (2013) Exploring Ty1 retrotransposon RNA structure within virus-like particles. Nucleic Acids Res 41: 463–473.

4. Yen YX, Moore SP, Garfinkel DJ, Rein A (2000) The genomic RNA in Ty1 virus-like particles is dimeric. J Virol 74: 10019–10021.

5. Al-Rhayat HB, Denis, K, Kennedy JM, Roth JF, Krugman AJ, Martin-Rendon E, et al. (1999) Yeast Ty retrotransposons assemble into virus-like particles whose T-numbers depend on the C-terminal length of the capid protein. J Mol Biol 292: 65–73.

6. Garfinkel DJ, Hodge AM, Youngren SD, Copeland TD (1991) Proteolytic processing of pol-TyB proteins from the yeast retrotransposon Ty1. J Virol 65: 4573–4581.

7. Meller J, Fulton AM, Dobson MJ, Roberts NA, Wilson W, et al. (1985) The Ty transcript of Saccharomyces cerevisiae determines the synthesis of at least three proteins. Nucleic Acids Res 13: 6249–6263.

8. Merkulov GV, Lawler JF, Jr., Eby Y, Boeke JD (2001) Ty1 proteolytic cleavage of the nuclear transcriptase and virus-like particles. Cell 105: 507–517.

9. Malagon F, Jensen TH (2008) The T body, a new cytoplasmic RNA granule in Saccharomyces cerevisiae. Mol Cell Biol 28: 6022–6032.

10. Nyathi Y, Wilkinson BM, Pool MR (2013) Co-translational targeting and translocation of proteins to the endoplasmic reticulum. Biochim Biophys Acta 1833: 2519–2525.

11. Halic M, Becker T, Pool MR, Spahn CM, Grusscui RA, et al. (2004) Structure of the signal recognition particle interacting with the elongation-attenuated ribosome. Nature 427: 801–814.

12. Sarosi I, Shao SO (2011) Molecular mechanism of co-translational protein targeting by the signal recognition particle. Traffic 12: 355–352.

13. Rider JK, Kenny AE, Palumbo RJ, Gramache ER, Curcio MJ (2012) Host cofactors of the retrovirus-like transposon Ty1. Mobile DNA 3: 12.

14. Broudy JL, Schekman R (1993) A Sec63p-BiP complex from yeast is required for protein translocation in a reconstituted proteoliposome. J Cell Biol 123: 1355–1363.

15. Zhang Y, Nijbroek G, Sullivan ML, McCracken AA, Watkins SC, et al. (2001) Hepatitis C molecular chaperone facilitates endoplasmic reticulum-associated protein degradation of cystic fibrosis transmembrane conductance regulator in yeast. Mol Biol Cell 12: 1303–1314.

16. del Amo M, Hogan DJ, Pechmann S, Albanese V, Brown PO, et al. (2011) Defining the specificity of cotranslationally acting chaperones by systematic analysis of mRNAs associated with ribosome-nascent chain complexes. PLoS Biol 9: e1001100.

17. Maxwell PH, Coombes C, Kenny AE, Lawler JF, Boeke JD, et al. (2004) Ty1 mobilizes subdominant Y′ elements in telomerase-negative Saccharomyces cerevisiae survivors. Mol Cell Biol 24: 9887–9898.

18. Breslow DK, Cameron DM, Collins SR, Schuldiner M, Stewart-Ornstein J, et al. (2000) A comprehensive strategy enabling high-resolution functional analysis of the yeast genome. Nat Methods 5: 711–718.

19. Dalley JA, Seilkirk A, Pool MR (2008) Access to ribosomal protein Rpl23p by the signal recognition particle is required for efficient cotranslational translocation. Mol Cell Biol 28: 2676–2684.

20. Haun BC, Walter P (1991) The signal recognition particle in S. cerevisiae. Cell 67: 131–144.

21. Ogg SC, Walter P (1995) SRP samples nascent chains for the presence of signal sequences by interacting with ribosomes at a discrete step during translation elongation. Cell 81: 1075–1088.

22. Curcio MJ, Garfinkel DJ (1991) Single-step selection for Ty1 element retrotransposition. Proc Natl Acad Sci U S A 88: 936–940.

23. Mori K (2009) Signalling pathways in the unfolded protein response: development from yeast to mammals. J Biochem 146: 743–750.

24. Schuck S, Prinz WA, Thorn KS, Voss C, Walter P (2009) Membrane expansion alleviates endoplasmic reticulum stress independently of the unfolded protein response. J Cell Biol 187: 525–536.

Acknowledgments

We are very grateful to Jeffrey Brodsky for his helpful advice on ER microsome analysis, to Martin Pool for providing translocation reporter plasmids, to David Garfinkel for providing riboprobe templates and to Ryan Palumbo for sharing data prior to publication. We thank Joseph Florko and Darlena Henderson for technical assistance with plasmid construction. We thank Ryan Palumbo and Richard Zitomer for critical review of the manuscript. Finally, we are grateful to the Wadsworth Center for use of the DAI Microscope Facility and to the Wadsworth Center Applied Genomic Technologies Core for DNA sequencing.

Author Contributions

Conceived and designed the experiments: JHD SL MJC. Performed the experiments: JHD SL. Analyzed the data: JHD SL MJC. Wrote the paper: JHD MJC. Edited the manuscript: JHD SL MJC.
Localization of Ty1 RNA to VLP Assembly Sites

35. Rubio C, Pincus D, Korennyakh A, Schuck S, El-Samad H, et al. (2011) Homeostatic adaptation to endoplasmic reticulum stress depends on Ire1 kinase activity. J Cell Biol 193: 171–184.

36. Buchan JR, Muhlrad D, Parker R (2008) P bodies promote stress granule mediated by the signal recognition particle. J Cell Biol 183: 441–455.

37. Gilks N, Kedersha N, Ayodele M, Shen L, Stoecklin G, et al. (2004) Stress granule assembly is mediated by prion-like aggregation of TIA-1. Mol Cell Biol 15: 5383–5398.

38. Nonhoff U, Ralser M, Welzel F, Piccini I, Balzerriev D, et al. (2007) Axin-2 interacts with the DEAD/H-box RNA helicase DDX6 and interferes with P-bodies and stress granules. Mol Biol Cell 18: 1385–1396.

39. Dombroski BA, Mathias SL, Nanthakumar E, Scott AF, Kazazian HH, Jr. (1991) Isolation of an active human transposable element. Science 254: 1805–1808.

40. Xu H, Boeke JD (1990) Localization of sequences required in cis for yeast Ty1 element transposition near the long terminal repeats: analysis of mini-Ty1 elements. Mol Cell Biol 10: 2699–2702.

41. Jouvenet N, Laine S, Pessel-Vivares L, Mougel M (2011) Cell biology of retroviral RNA packaging. RNA Biol 8: 572–580.

42. Wei W, Gilbert N, Ooi SL, Lawler JF, Ostertag EM, et al. (2001) Human L1 retrotransposition: cis preference versus trans complementation. Mol Cell Biol 21: 1429–1439.

43. Buchan JR, Muhlrad D, Parker R (2008) P bodies promote stress granule mediated by the signal recognition particle. J Cell Biol 183: 441–455.

44. Xu H, Boeke JD (1990) Localization of sequences required in cis for yeast Ty1 element transposition near the long terminal repeats: analysis of mini-Ty1 elements. Mol Cell Biol 10: 2699–2702.

45. Jouvenet N, Laine S, Pessel-Vivares L, Mougel M (2011) Cell biology of retroviral RNA packaging. RNA Biol 8: 572–580.

46. Wach A, Brachat A, Alberti-Segui C, Rebischung C, Philippe P (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. Yeast 13: 1065–1073.

47. Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, et al. (1999) Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science 285: 901–906.

48. Gharimaghami S, Huh WK, Bower K, Howson RW, Belle A, et al. (2003) Global analysis of protein expression in yeast. Nature 425: 737–741.

49. Storici F, Resnick MA (2006) The delitto perfetto approach to in vivo site-directed mutagenesis and chromosome rearrangements with synthetic oligonucleotides in yeast. Methods Enzymol 409: 329–345.

50. Storici F, Resnick MA (2006) The delitto perfetto approach to in vivo site-directed mutagenesis and chromosome rearrangements with synthetic oligonucleotides in yeast. Methods Enzymol 409: 329–345.

51. Storici F, Resnick MA (2006) The delitto perfetto approach to in vivo site-directed mutagenesis and chromosome rearrangements with synthetic oligonucleotides in yeast. Methods Enzymol 409: 329–345.

52. Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, et al. (1999) Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. Science 285: 901–906.

53. Gharimaghami S, Huh WK, Bower K, Howson RW, Belle A, et al. (2003) Global analysis of protein expression in yeast. Nature 425: 737–741.

54. Martin-Rendon E, Marfany G, Wilson S, Ferguson DJ, Kingsman SM, et al. (1996) Structural determinants within the subunit protein of Ty1 virus-like particles. Mol Microbiol 22: 667–679.

55. Monoskin GM, Blaxter LM, Boeke JD (1994) In-frame linker insertion mutagenesis of yeast transposon Ty1: mutations, transposition and dominance. Gene 139: 9–16.

56. Xu H, Boeke JD (1991) Inhibition of Ty1 transposition by mating pheromones in Saccharomyces cerevisiae. Mol Cell Biol 11: 2736–2745.

57. Surjit M, Jameel S, Lal SK (2007) Cytoplasmic localization of the ORF2 protein of hepatitis E virus is dependent on its ability to undergo retrotranslocation from the endoplasmic reticulum. J Virol 81: 3339–3345.

58. Wach A, Brachat A, Alberti-Segui C, Rebischung C, Philippe P (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. Yeast 13: 1065–1073.

59. Wach A, Brachat A, Alberti-Segui C, Rebischung C, Philippe P (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. Yeast 13: 1065–1073.

60. Wach A, Brachat A, Alberti-Segui C, Rebischung C, Philippe P (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. Yeast 13: 1065–1073.

61. Wach A, Brachat A, Alberti-Segui C, Rebischung C, Philippe P (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. Yeast 13: 1065–1073.

62. Wach A, Brachat A, Alberti-Segui C, Rebischung C, Philippe P (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. Yeast 13: 1065–1073.

63. Wach A, Brachat A, Alberti-Segui C, Rebischung C, Philippe P (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. Yeast 13: 1065–1073.

64. Wach A, Brachat A, Alberti-Segui C, Rebischung C, Philippe P (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. Yeast 13: 1065–1073.

65. Wach A, Brachat A, Alberti-Segui C, Rebischung C, Philippe P (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. Yeast 13: 1065–1073.

66. Wach A, Brachat A, Alberti-Segui C, Rebischung C, Philippe P (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. Yeast 13: 1065–1073.