Location Is Everything: An Educational Primer for Use with "Genetic Analysis of the Ribosome Biogenesis Factor Ltv1 of *Saccharomyces cerevisiae*"

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ABSTRACT The article by Merwin *et al.* in the November 2014 issue of *GENETICS* provides insight into ribosome biogenesis, an essential multistep process that involves myriad factors and three cellular compartments. The specific protein of interest in this study is **l**ow-**t**emperature **v**iability protein (Ltv1), which functions as a small ribosomal subunit maturation factor. The authors investigated its possible additional function in small-subunit nuclear export. This Primer provides information for students to help them analyze the paper by Merwin *et al.* (2014), including an overview of the authors' research question and methods.

Related article in *GENETICS*: Merwin, J. R., L. B. Bogar, S. B. Poggi, R. M. Fitch, A. W. Johnson, and D. E. Lycan, 2014 Genetic analysis of the ribosome biogenesis factor Ltv1 of *Saccharomyces cerevisiae*. Genetics **198:** 1071–1085

KEYWORDS ribosome biogenesis; nuclear export; education

Background

THE research described by Merwin *et al.* (2014) sheds light on the essential energy-intensive process by which cells make mature ribosomes that are ready to participate in translation. Defects in ribosomal processing are usually lethal, but some mutations in ribosomal biogenesis components are associated with diseases, including neurodevelopmental defects (Brooks *et al.* 2014) and cancer (Ruggero and Shimamura 2014).

Transcription of ribosomal RNAs and the initial steps of ribosome subunit maturation occur in the nucleolus, a darkly staining area(s) in the nucleus, with the assistant of numerous factors. The authors are investigating a specific step in the process: that of export of the small ribosomal subunit from the nucleus to the cytoplasm, where ribosome maturation is completed. They test whether the nonessential protein Ltv1 has a role in small-subunit export through mutational analysis of a putative nuclear export signal. Their results indicate that although Ltv1 is important in small ribosomal subunit biogenesis, its key role is not in transport of the subunits out of the nucleus; rather, any role in transport is redundant with other factors, and the main role of Ltv1 is likely at another step in small-subunit maturation. Information on ribosome biogenesis, yeast as a model system, and ways to study ribosome maturation are provided for readers so that they may be able to understand the context of the authors' study.

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Ribosome biogenesis

Ribosomes are the cellular machines that translate messenger RNA (mRNA) to produce protein; they are ribonuclear protein complexes containing several ribosomal RNA molecules (rRNA) and more than 75 proteins (for review, see Woolford and Baserga 2013). Ribosome biogenesis involves many additional proteins and small RNAs that are responsible for the multiple steps required to assemble the rRNA with ribosomal proteins. Ribosome assembly begins in the nucleolus, with continued maturation in the nucleoplasm, and then the final steps occur in the cytoplasm. The initial nucleolar RNA-protein complex is called the 90S complex, and it undergoes processing to become the larger 60S subunit and the smaller 40S subunit. A review of the process is described in Woolford and Baserga (2013) and is illustrated in Figure 1.

The pre-60S and pre-40S subunits are generated in the nucleus and are transported independently into the cytoplasm

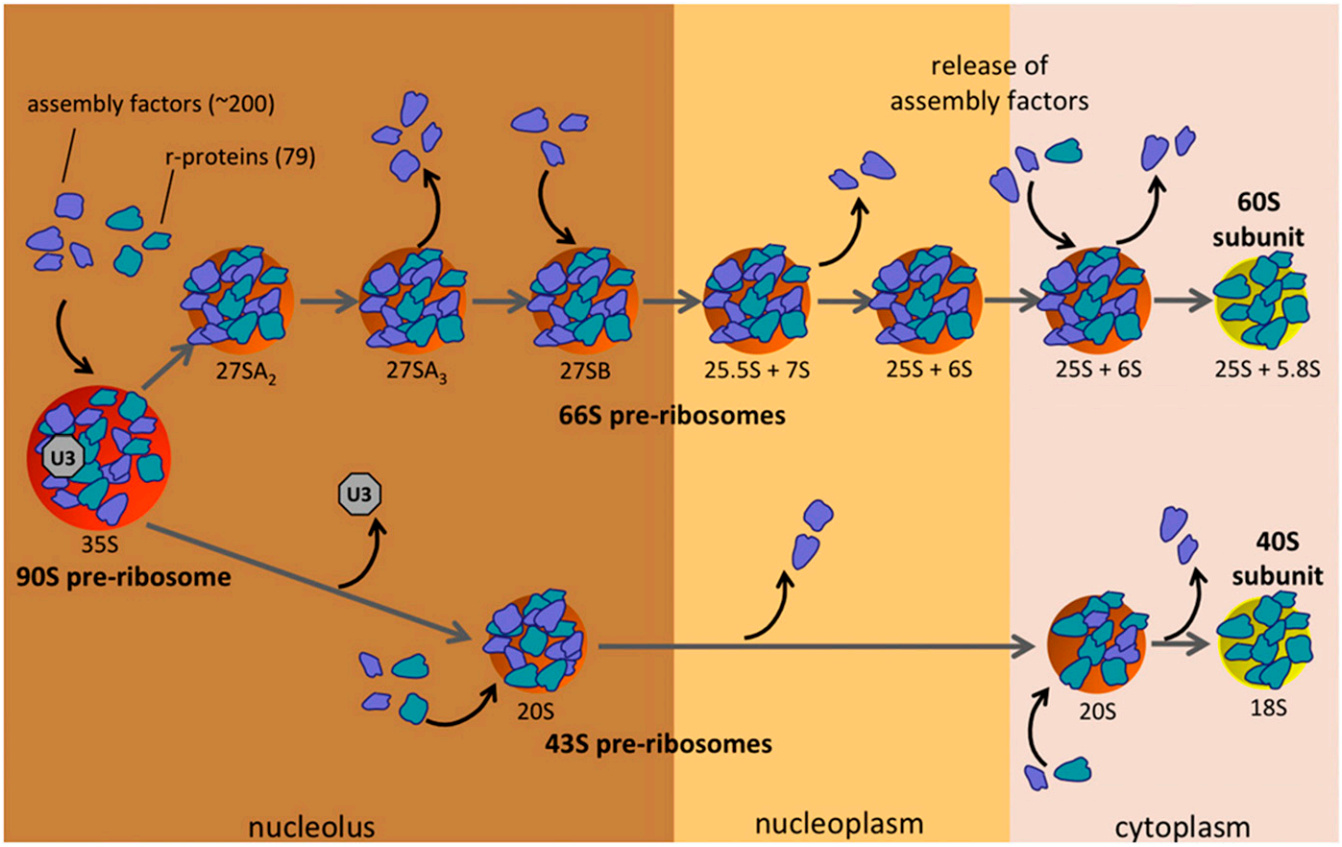

Figure 1 Pathway for maturation of pre-ribosomes to form 40S and 60S ribosomal subunits. Sequential assembly intermediates are shown, distinguished by the pre-rRNA processing intermediates contained within them. Most r-proteins (light blue) and many assembly factors (dark blue) associate with the early nucleolar/nuclear precursor particles. Some assembly factors join pre-ribosomes in midassembly or even during later steps in the cytoplasm. Release of assembly factors from pre-ribosomes occurs at early, middle, or late stages of subunit maturation (from Woolford and Baserga 2013).

for the final maturation steps. They reconnect to form an 80S ribosome during translation initiation on an mRNA. Movement of subunits to different cellular compartments is indicated in Figure 1; however, transport from the nucleus to the cytoplasm requires specific export proteins, and those proteins are the focus of the research by Merwin *et al.* (2014).

Nuclear export

The bulk of a cell's energy is dedicated to protein synthesis, including transcription of rRNAs, synthesis of ribosome building blocks, and translation (Warner 1999). In addition, ribosome biogenesis must be dynamic and rapid to adapt to a cell's metabolic state. Throughout ribosome biogenesis, proteins involved in each maturation step are released after they act. A small fraction of known ribosome biogenesis factors accompanies the pre-60S and pre-40S subunits to the cytoplasm, where the factors are released and then recycled back to the nucleus; for simplicity, Figure 1 illustrates only the export component of the process.

An important export protein is Crm1, which requires an export adapter to move the pre-60S ribosomal subunit out of the nucleus. The role of an export adapter is to connect the complex being transported with the transport protein and to provide the nuclear export sequence (NES) required for interaction with the transport protein. Export also requires a G-protein coupled with GTP called Ran-GTP that binds to the Crm1 export complex; after exit from the nucleus, Ran-GTP is hydrolyzed to Ran-GDP, which allows release of the pre-60S complex or other cargo (Hedges *et al.* 2005) (Figure 2). A different export complex, Mex67/Mtr2, is required for mRNA export as well as for pre-60S (Yao *et al.* 2007) and pre-40S export (Faza *et al.* 2012). While multiple pre-60S-specific export adapters for Crm1-dependent export have been identified, a pre-40S-specific export adapter has not. A search for mutants that are temperature sensitive for pre-40S export yielded only 60S export factors (Moy and Silver 2002). A possible Crm1 adapter for export of pre-40S ribosomal subunits is Ltv1, the protein of interest in this study.

Previous findings from the Lycan Laboratory

The Lycan Laboratory initially characterized Ltv1 function through its role in ribosome biogenesis in response to environmental stress (Loar *et al.* 2004). In yeast, Ltv1 interacts with RpS3, a ribosomal protein that is part of the 40S subunit (Ito *et al.* 2001; Merwin *et al.* 2014), and with Yar1, a cytoplasmic chaperone for RpS3 (Loar *et al.* 2004; Koch *et al.* 2012). To examine the role of Ltv1 in ribosome

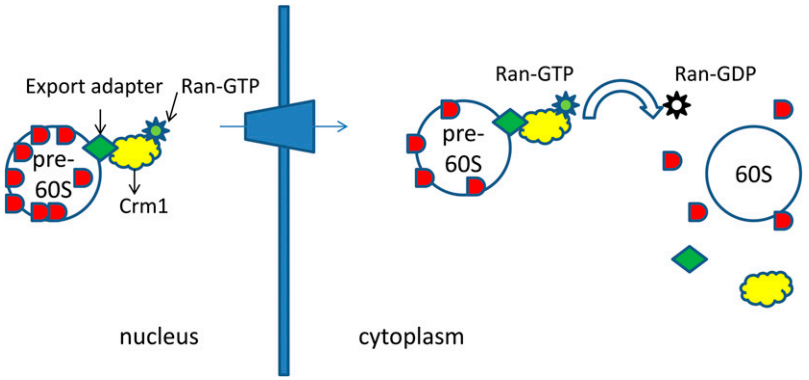

Figure 2 Export of the pre-60S ribosomal subunit requires an export adapter, Crm1, and Ran-GTP. Many protein factors ( ) are associated with the pre-60S ribosomal subunit in the nucleus. The export adapter ( ) provides a link between the pre-60S subunit and the Crm1 export complex ( ). Ran-GTP ( ) is bound to the Crm1 export complex and, after export from the nucleus, is hydrolyzed to Ran-GDP, allowing release of the 60S ribosomal complex.

biogenesis, the authors looked at the association of Ltv1 with ribosomal subunits in cells containing a tagged version of Ltv1 using polysome profiling, which separates ribosomal subunits from each other and from actively translating ribosomes, called polysomes, by sucrose density gradient centrifugation (see *Ribosome/polysome analysis by sucrose gradient centrifugation* for a description). Results showed that Ltv1 co-sediments with 40S particles but not with polysomes (Loar *et al.* 2004); it is also part of a cytoplasmic 40S complex (Schäfer *et al.* 2003). Recent work suggests that Ltv1 dissociates from the 40S complex shortly after entering the cytoplasm (Strunk *et al.* 2011), supporting a possible role for Ltv1 in pre-40S transport. The authors further analyzed the function of Ltv1 by testing the effects of deleting the *Ltv1* gene on ribosome biogenesis. The polysome profiles of cells with a deletion of Ltv1 show fewer 40S subunits and more free 60S subunits compared with wild-type cells, indicative of a 40S biogenesis defect (Loar *et al.* 2004). In addition, cells with deletions in the *Ltv1* gene show a delay in pre-40S subunit export (Seiser *et al.* 2006).

Ltv1 shuttles between the nucleus and cytoplasm and accumulates in the nucleus when Crm1 is inhibited (Seiser *et al.* 2006). In a previous study, researchers identified a sequence in Ltv1 that resembled a Crm1-dependent nuclear export signal (Fassio *et al.* 2010). Deletion of the sequence was deleterious to cells; however, although the mutants showed a pre-40S subunit maturation defect, they did not show accumulation of the mutant Ltv1 in the nucleus, which would be expected for a transport mutant (Fassio *et al.* 2010). Thus, there was conflicting evidence about whether Ltv1 is an export adaptor for pre-40S subunits.

Merwin *et al.* (2014) identified a leucine-rich nuclear export signal in Ltv1 that is necessary for both Ltv1 export and interaction with Crm1 and is different from the sequence they described previously (Fassio *et al.* 2010). Overexpression of Ltv1 lacking this signal in an otherwise wild-type strain is dominant negative, meaning that an excess of the mutant protein is deleterious to cells, despite the presence of the wild-type Ltv1 protein. The reason why it is deleterious appears to be that the mutant protein causes the nuclear accumulation of an essential 40S ribosomal component. Overall, pre-40S export, however, is not affected. In addition, if Ltv1 lacking the nuclear export signal is expressed at endogenous levels in a strain deleted for the *Ltv1* gene, it can complement the 40S biogenesis defect, indicating that it is functional despite not having the nuclear export signal. Therefore, Ltv1 function must be fully redundant with other export factors.

Yeast as a model system

The yeast *Saccharomyces cerevisiae*, baker's yeast, has been very useful for studying ribosome biogenesis as well as other fundamental cellular pathways because the genes and proteins involved are highly conserved with those in more complex organisms (reviewed in Botstein and Fink 2011 and Duina *et al.* 2014). Yeast are amenable to a variety of experimental approaches, including genetic, cell biological, and biochemical, all of which the Lycans Laboratory has employed in its investigations. The protein of interest in this paper, Ltv1, has a human homolog, hLtv1, that is a component of late pre-40S particles in human cells (Zemp *et al.* 2009), supporting the utility of yeast as a eukaryotic model for understanding ribosome biogenesis in humans and how defects in the process can lead to various human diseases.

Unpacking the Work

The big question and experimental strategy

The authors were interested in how the pre-40S ribosomal subunit exits the nucleus. They proposed that (1) pre-40S export relies on distributed or partially redundant pathways or (2) a necessary adapter plays an essential role in some other aspect of ribosome biogenesis that masks its role in export. The authors' previous work suggested that Ltv1 plays a role in pre-40S export from the nucleus. They probe this possibility further in this study.

Specific research questions

Merwin *et al.* (2014) asked if the main role of Ltv1 is in export of pre-40S subunits from the nucleus because earlier research indicated that it associates with pre-40S subunits in the nucleus and is released shortly after export to the cytoplasm. The authors hypothesized that if Ltv1 acts as an export adapter, could they identify a Crm1-dependent NES in Ltv1? They found such a sequence at the C-terminus of Ltv1, which they verify by inserting it in place of the known NES

in the Crm1 adapter protein Nmd3 (figures 1 and 2 of Merwin *et al.* 2014). If this sequence is important for export of pre-40S subunits from the nucleus, does deletion of the NES in Ltv1 prevent such transport? How did the authors determine that the sequence functions not only in export but also specifically in Crm1-dependent export? They tested for loss of export by fluorescence microscopy of GFP-tagged versions of Ltv1 and for loss of Crm1 interaction through yeast two-hybrid assays (figure 3 of Merwin *et al.* 2014; see Figure 4 and its explanation). The authors also expressed either wild-type Ltv1 or Ltv1 lacking the NES (Ltv1ΔNES) at high levels through the use of a galactose-inducible promoter and found that Ltv1ΔNES has a dominant negative phenotype, indicating that the overexpressed Ltv1 lacking the NES interferes with the function of the wild-type Ltv1 protein. What is the underlying cause of the dominant negative phenotype? The authors address this question in the remainder of the article, specifically whether Ltv1ΔNES affects pre-40S export or localization of proteins with which Ltv1 interacts. Their results help them to define a function for Ltv1 in small-subunit biogenesis.

Experimental tools

Ribosome/polysome analysis by sucrose gradient centrifugation: One of the key questions the authors asked was whether the dominant negative phenotype of the overexpressed Ltv1 with the deleted nuclear export signal (Ltv1ΔNES) could be explained by a defect in ribosome biogenesis, specifically of the 40S subunit. The experimental method to examine ribosomal subunits and actively translating ribosomes (polysomes) is sucrose density gradient centrifugation. Specifically, the authors employ rate zonal centrifugation, in which particles sediment in the gradient as a band, and the location depends on particle size, shape, and density. The gradient is discontinuous, with the sucrose concentration increasing from top, 10% sucrose, to bottom, 50% sucrose (Marks 2001) (Figure 3) [see Esposito *et al.* (2010) for a demonstration of the method].

Cycloheximide is an inhibitor of protein synthesis in eukaryotes, blocking translation elongation, and it is added to stop translation, providing a snapshot of ribosomes at a given point in the growth of the cells. The rRNA in the ribosomes absorbs at 254 nm, providing a way to assay the location of ribosomes in the gradient. It is also possible to precipitate the proteins in each fraction and then analyze them by Western blot analysis [see figure 6C of Merwin *et al.* (2014) for an example]. Subunit terminology is based on the protein sedimentation rate in a density gradient, with higher numbers indicating faster sedimentation and larger molecular weights; the unit for sedimentation coefficient is the Svedberg (S), with one Svedberg equivalent to 10^{-13} sec.

Yeast two-hybrid assay: Merwin *et al.* (2014) use the yeast two-hybrid assay to test whether or not the consensus NES they identified in Ltv1 is required for interaction with the nuclear export receptor Crm1 (figure 3 of Merwin *et al.* 2014). The assay relies on the modularity of the Gal4 transcription factor, with one domain responsible for DNA binding (GBD) and the other domain required for transcriptional activation through interaction with transcriptional machinery (GAD); both domains are required to get transcriptional activation (Figure 4). To test whether Ltv1interacts with Crm1, the authors fused different forms of Ltv1 (full length or truncated) to the GAD sequence in a vector harboring a gene required for growth on plates lacking leucine and fused the Crm1 sequence to the GBD sequence in a vector expressing a gene required for growth on media lacking tryptophan. They transformed both plasmids into a yeast strain auxotrophic for both leucine and tryptophan, and cells containing the plasmids can grow on plates lacking both amino acids (-leu/-trp). The strain also has reporter genes that measure whether or not the proteins of interest interact: *HIS3*, *ADE2*, and *lacZ*, each of which is under the control of a Gal4 upstream activation sequence (UAS) and therefore requires both domains of the Gal4 transcription factor to activate transcription (Figure 4). If Ltv1 and Crm1 interact, they bring the two domains of the Gal4 transcription factor, GBD and GAD, together, which activate *HIS3*, *ADE2*, and *lacZ* expression, permitting growth on plates lacking leucine, tryptophan, histidine, and adenine. The authors assayed *ADE2* activation in one experiment (figure 3 of Merwin *et al.* 2014; see hypothetical scenario in Figure 4) and *HIS3* in another (figure 9 of Merwin *et al.* 2014). Negative control vectors contain the GBD and GAD sequences but neither Ltv1 nor Crm1 to confirm that the two domains of the Gal4 transcription factor will not be able to activate

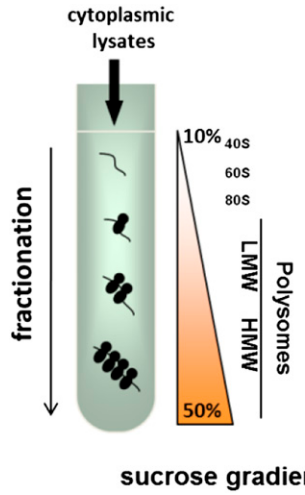

Figure 3 Sucrose density gradient centrifugation. The gradient is from 10% sucrose at the top to 50% at the bottom. LMW, low molecular weight; HMW, high molecular weight. Whole-cell lysates are prepared and layered carefully on top of the gradient. As indicated, individual ribosomal subunits or the complete ribosome is less dense than polysomes and therefore ends up near the top of the gradient after centrifugation (from Abdelmohsen 2012).

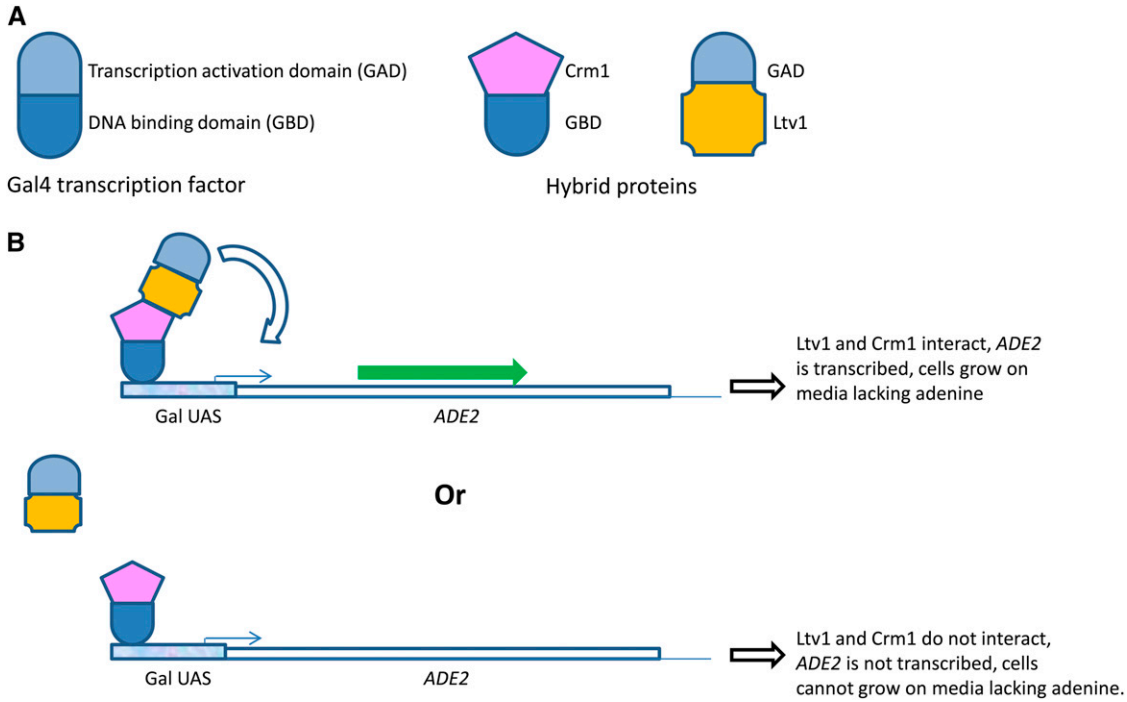

Figure 4 The yeast two-hybrid system. A. (Left) Representation of the yeast Gal4 transcription activator with the DNA-binding (GBD) and transcription-activation (GAD) domains colored in different shades of blue, as indicated. (Right) Representations of two hybrid proteins with Ltv1 fused to the Gal4 activation domain (GAD) and Crm1 fused to the Gal4 DNA-binding domain (GBD). B. (Top) Hypothetical scenario in which Ltv1 and Crm1 interact, leading to expression of *ADE2* and (bottom) hypothetical scenario in which Ltv1 and Crm1 do not interact (adapted from Duina *et al.* 2014 and James *et al.* 1996).

ADE2 or *HIS3* transcription in the absence of the two proteins of interest interacting.

Connections to Genetics Concepts

The article highlights a number of important genetics concepts as applied to studying ribosome biogenesis. Figure 1 of Merwin *et al.* (2014) illustrates complementation of the *Δltv1* strain by a tagged Ltv1, and this experiment could lead to a discussion of what complementation of a mutant phenotype means. A concept that students may find challenging is a dominant negative phenotype and how to interpret it. Figure 4A of Merwin *et al.* (2014) shows that Ltv1 lacking the NES is dominant negative when overexpressed, and then in following experiments the authors investigated cellular mechanisms underlying the growth phenotype. The experiment can lead to a discussion of inducible expression. Later experiments showed rescue of the mutant phenotype, and students can see why the results shed light on the mechanism. The authors employed the yeast two-hybrid assay, which is a useful approach to investigating protein-protein interactions and allows for discussion of auxotrophic markers.

The details of ribosome biogenesis are not normally covered in depth in a genetics course, but students are familiar with the concepts of transport and translation, and the article can be used to illustrate the connections between topics normally separated in the curriculum, with translation discussed in molecular genetics and transport associated with cell biology.

Suggestions for Classroom Use

Most of the experiments in this article were done by undergraduate students, making this article of special interest to undergraduates and highlighting their ability to contribute to the scientific research enterprise. Instructors may assign this Primer up to the section on experimental methods and then have students turn to the article by Merwin *et al.* (2014), working through the article outside class. Depending on the size of the class or discussion section, pairs or teams of students may be responsible for presenting one of the figures or part of a figure in depth to the class, putting the experiment into the context of the article and the figure preceding or following it. Each student reads the entire article, using the detailed discussion questions to help determine how to present the assigned figure in class.

Detailed questions

1. Why did the authors test two different GFP-tagged vectors in figure 1B of Merwin *et al.* (2014)?
2. Why is it important to show the information in figure 1B of Merwin *et al.* (2014) before interpreting the information in figure 1C of Merwin *et al.* (2014)?

3. What is the purpose of the experiment in figure 2 of Merwin *et al.* (2014)? How does it support the results shown in figure 1C of Merwin *et al.* (2014)? Can a region of a protein be necessary but not sufficient for a process?
4. Figure 3A of Merwin *et al.* (2014) shows a schematic of the Ltv1 protein and the putative NES sequence at the C-terminus. What mutations do the authors make to test their predictions about the NES? In figure 3B of Merwin *et al.* (2014), why did the authors tag the Ltv1 proteins with GFP? What do the results in figure 3B of Merwin *et al.* (2014) indicate about the role of the C-terminal sequence in Ltv1 export? What additional information do the data in figure 3C of Merwin *et al.* (2014) provide regarding the role of the putative NES in Ltv1?
5. The title of figure 4 of Merwin *et al.* (2014) is "Ltv1-ΔNES overexpression is dominant negative." What data in figure 4 of Merwin *et al.* (2014) specifically support this title? Why do the authors say that it is dominant?
6. In figure 4B of Merwin *et al.* (2014), how do the authors detect RpS3? How do the left and right panels differ? Are these cells grown in glucose or in galactose? What do these data allow the authors to conclude? Suggest an additional control for the experiment.
7. Compare the polysome profiles in figure 4C of Merwin *et al.* (2014). How do the Ltv1 C-terminal mutants compare with wild-type Ltv1? Is this result what you expected, given the results in figure 4B of Merwin *et al.* (2014)?
8. In figure 5A of Merwin *et al.* (2014), why did the authors assay the proteins indicated, and how do their results support the figure title? What is ITS1, shown in figure 5B of Merwin *et al.* (2014), and why did the authors assay it?
9. What was the purpose of changing the promoter/enhancer driving GFP-Ltv1 expression from GAL1 to MET25 to LTV1 (figure 6 of Merwin *et al.* 2014)? How did the results in figure 6 of Merwin *et al.* (2014) modify the authors' hypothesis regarding Ltv1 function?
10. Figure 7 of Merwin *et al.* (2014) presents two approaches to investigating protein-protein interactions. What are the benefits and limitations to each approach, and why do the authors use both?
11. Why did the authors test whether RpS3/Yar1 overexpression could rescue the Ltv1ΔNES phenotype? What do the results in figure 8 of Merwin *et al.* (2014) show?
12. How do the results in figure 9 of Merwin *et al.* (2014) add to the conclusions of figure 8 of Merwin *et al.* (2014) regarding understanding the Ltv1ΔNES overexpression phenotype?

Big picture topics and questions

1. While multiple pre-60S-specific export adapters for Crm1-dependent export have been identified, a pre-40S-specific export adapter has not. Why would it be more straightforward to identify pre-60S export factors compared with pre-40S export factors?
2. The authors suggest that the export function of Ltv1 may be functionally redundant with other pre-40S export factors. Identify other examples of functional redundancy in cells and why such redundancies are retained in evolution.
3. Illustrate polysome profile results that would have supported the hypothesis that Ltv1 is an essential factor for pre-40S export (*i.e.*, how would the results differ from those in figure 4 of Merwin *et al.* 2014). What other questions can polysome profiling address?
4. Compare information gained from mutations that lead to the following phenotypes: temperature sensitive, dominant negative, complementation. What information does each type of mutant provide?

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