Asc1, homolog of human RACK1, prevents frameshifting in yeast by ribosomes stalled at CGA codon repeats

ANDREW S. WOLF[1] **and ELIZABETH J. GRAYHACK**[1,2]

[1]Department of Biochemistry and Biophysics, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642, USA
[2]Center for RNA Biology, University of Rochester, Rochester, New York 14642, USA

ABSTRACT

Quality control systems monitor and stop translation at some ribosomal stalls, but it is unknown if halting translation at such stalls actually prevents synthesis of abnormal polypeptides. In yeast, ribosome stalling occurs at Arg CGA codon repeats, with even two consecutive CGA codons able to reduce translation by up to 50%. The conserved eukaryotic Asc1 protein limits translation through internal Arg CGA codon repeats. We show that, in the absence of Asc1 protein, ribosomes continue translating at CGA codons, but undergo substantial frameshifting with dramatically higher levels of frameshifting occurring with additional repeats of CGA codons. Frameshifting depends upon the slow or inefficient decoding of these codons, since frameshifting is suppressed by increased expression of the native tRNA$^{Arg(ICG)}$ that decodes CGA codons by wobble decoding. Moreover, the extent of frameshifting is modulated by the position of the CGA codon repeat relative to the translation start site. Thus, translation fidelity depends upon Asc1-mediated quality control.

Keywords: translation stalls; reading frame; ribosome; frameshift; Asc1; Rack1

INTRODUCTION

Ribosomes stall due to a variety of translational impediments, including structures in the mRNA (Doma and Parker 2006), the absence of stop codons (Frischmeyer et al. 2002; van Hoof et al. 2002), the presence of inhibitory amino acid combinations (Gutierrez et al. 2013; Woolstenhulme et al. 2013), or the presence of inhibitory codon combinations (Letzring et al. 2010; Seidman et al. 2011). Such sequences may substantially impact downstream translation. For instance, in the yeast *Saccharomyces cerevisiae*, a single CGA–CGA codon pair is translated inefficiently, which depending on its location can cause a 30%–50% reduction in expression of downstream sequences (Letzring et al. 2010).

In many cases, cells follow a kingdom-dependent program to escape the bottleneck caused by the stalled ribosome. In bacteria, such as *Escherichia coli*, stalled ribosomes are sometimes rescued by the *trans*-acting tmRNA·SmpB system (Moore and Sauer 2007), but may also undergo translational recoding, in particular +1 frameshifting (Seidman et al. 2011). Errors in which ribosomes switch reading frames generally occur at extremely low rates, 3×10^{-5} (Kurland 1992), and are almost certain to result in a nonfunctional polypeptide. However, particular codon combinations that create a slippery sequence are one of the key elements in programmed frameshift sites, at which ribosomes transfer reading frames at much higher frequencies (Dinman 2012).

In eukaryotes, quality control systems stop translation at the stall site, recycle the ribosomes and often cause degradation of the nascent peptide and mRNA (Brandman et al. 2012; Shoemaker and Green 2012). In *S. cerevisiae*, the Asc1 protein, which binds to the small ribosomal subunit (Gerbasi et al. 2004; Sengupta et al. 2004; Coyle et al. 2009; Rabl et al. 2011), affects the response to some stalled ribosomes, since deletion of *ASC1* allows read-through of particular inhibitory sequences (Kuroha et al. 2010; Brandman et al. 2012) including internal CGA codon repeats (Letzring et al. 2013). Asc1, the highly conserved yeast ortholog of human Receptor of Activated Kinase 1 (RACK1), binds tightly to the eukaryotic 40S ribosome (Gerbasi et al. 2004) near the mRNA exit site (Sengupta et al. 2004; Rabl et al. 2011) contacting ribosomal proteins, rpS16e, rpS17e, and rpS3e (Rabl et al. 2011). Human RACK1, a member of the Trp-Asp (WD) repeat family, integrates numerous signaling pathways with translation regulation (Adams et al. 2011), for instance, by mediating phosphorylation of eIF6 by protein kinase C to promote joining of the ribosomal subunits

(Ceci et al. 2003). Yeast Asc1 likewise has pleiotropic effects on the cell (Rachfall et al. 2013), at least some of which are caused by its interaction with the ribosome (Coyle et al. 2009); $asc1$-Δ mutants de-repress the general amino acid control genes in rich media (Hoffmann et al. 1999), alter expression of several signaling pathways (Rachfall et al. 2013), and fail to recruit the mRNA binding protein Scp160 to the ribosome (Baum et al. 2004). Moreover, Asc1 protein levels are modulated, decreasing substantially during hypoxic stress (Bruckmann et al. 2009), perhaps indicating that the response to ribosome stalls is regulated. Despite this body of knowledge, it is unknown what happens to translation elongation if Asc1 fails to stop the stalled ribosome.

There is substantial evidence that quality control and its components are important for the health of the cell. In yeast, lack of Dom34, which cooperates with Hbs1 and Rli1 to rescue arrested ribosomes (Shoemaker and Green 2011; Tsuboi et al. 2012), results in an accumulation of nontranslating ribosomes in the 3′ UTRs of mRNAs (Guydosh and Green 2014) and in synthetic genetic interactions indicative of a limiting ribosome supply (Bhattacharya et al. 2010; Balagopal and Parker 2011). In mice, mutations in Ltn1, an E3 ubiquitin ligase (Wilson et al. 2007; Bengtson and Joazeiro 2010) and a member of a ribosome quality control complex (RQC) (Brandman et al. 2012), result in neurodegeneration (Chu et al. 2009). However, we do not know if the activity of the quality control systems to end translation is important to prevent aberrant translation by the ribosome.

Since stalled ribosomes in *Escherichia coli* sometimes frameshift (Seidman et al. 2011), we considered that Asc1 might not only restrict continued translation by ribosomes paused at internal CGA codon repeats (Letzring et al. 2013), but might also prevent recoding events. We provide evidence here that, in the absence of Asc1 protein, ribosomes stalled at an internal CGA codon repeat undergo substantial frameshifting. Nearly 40% frameshifting occurs in an $asc1$-Δ strain during translation of an artificial construct with six adjacent CGA codons, a level observed only in a fraction of programmed frameshift events (Dinman 2012). Furthermore, the length of the nascent polypeptide modulates the extent of frameshifting, as well as CGA-mediated translation inhibition. We conclude that the Asc1 protein (or proteins recruited by Asc1) constrains read-through by ribosomes stalled at internal CGA repeats in order to inhibit promiscuous changes in reading frame at this stall. Thus, translation fidelity is dependent upon Asc1-mediated quality control.

RESULTS

Ribosomes lacking Asc1 frameshift at CGA codon repeats

We assessed frameshifting at a $(CGA)_4$ codon repeat in yeast, using *Renilla* luciferase-codon insert-GFP fusion reporters (Fig. 1A), in which both the codon insert and the reading frame of GFP were varied. We refer to these reporters throughout the manuscript by the codon repeat and reading frame insert. For example, a *Renilla* luciferase-$(CGA)_4$+1-GFP reporter containing four CGA codons and an extra nucleotide to place the downstream GFP out of frame relative to the *Renilla* luciferase is called a $(CGA)_4$+1 reporter. In wild-type yeast, ribosomes paused at internal CGA repeats (Letzring et al. 2013) do not frameshift at an appreciable level, based on measurements in this construct (Fig. 1B). Thus, we asked if reading frame maintenance might be controlled by quality control components, such as Asc1.

We find that, in the absence of Asc1, ribosomes translating a $(CGA)_4$+1 reporter undergo ~11% frameshifting at CGA codon repeats (Fig. 1B), which is estimated as the percentage of GFP/RFP from the $(CGA)_4$+1 construct (4.6 ± 0.04) minus GFP/RFP background (0.4) relative to the GFP/RFP from the in-frame $(AGA)_4$ construct (38.6 ± 0.4) minus GFP/RFP background (0.4). This 11% estimate of frameshifting is a minimal estimate of the percentage of ribosomes that frameshift, since it is relative to the GFP/RFP from the in-frame $(AGA)_4$ construct, which represents the maximum translation efficiency for this polypeptide. Frameshifting is not observed from either the $(AGA)_4$+1 construct, or from any construct in the −1 frame (Fig. 1B), since GFP/RFP from all of these constructs is at or near background levels of 0.4. Similarly, we observe ~18% frameshifting in an $asc1$-Δ mutant with a $(CGA)_4$+1 insertion in a *Renilla* luciferase-firefly luciferase reporter (Fig. 1C); with this reporter, no expression is detected with an $(AGA)_4$+1 insert in either the wild-type or the $asc1$-Δ strain, and little expression is detected with the $(CGA)_4$+1 insert in the wild-type strain. Furthermore, frameshifting in the $(CGA)_4$+1 construct is due to lack of Asc1, since it is completely suppressed by a plasmid-borne copy of the *ASC1* gene, even one without the *ASC1* intron, which encodes *snR24* (Fig. 1D; Kiss-László et al. 1996).

Improved CGA decoding inhibits frameshifting

We find that frameshifting depends upon inefficient decoding of the CGA codons since tRNAs that suppress the expression defect of CGA codon repeats (Letzring et al. 2010) also suppress frameshifting. CGA-mediated frameshifting is eliminated by expressing the anticodon mutated tRNA[Arg(UCG)] (which forms W:C base pairs with CGA) (Fig. 2A), and is markedly reduced by overproducing the native tRNA[Arg(ICG)] on a multicopy plasmid (Fig. 2B), since GFP/RFP from the $(CGA)_4$+1 construct is reduced from 5.1 without tRNA to 0.9 with additional copies of tRNA[Arg(ICG)]. Increasing the quantity of the native tRNA does not alter its function in the ribosome (either the codon–anticodon interactions or translocation efficiency of the tRNA), but is likely to increase the rate of translation elongation by increasing the rate of A site acceptance (due to tRNA availability). Thus, we infer that frameshifting in the $asc1$-Δ mutant depends

critically on a slow rate of translation through the CGA codon repeat.

To determine if GFP measured from frameshifted *Renilla* luciferase-GFP constructs is due to translation of a *Renilla* luciferase-codons-HA epitope-GFP fusion protein or to internal initiation at the HA epitope-GFP, we examined the size of the frameshifted polypeptide by SDS-PAGE and antibody detection. A full-length fusion protein is the primary polypeptide detected from all constructs, including the (CGA)$_4$+1 construct in the *asc1-Δ* strain (Fig. 2C). Furthermore, the intensity of the Western signal from the frameshifted polypeptide in the *asc1-Δ* strain (Fig. 2C, lane h) is similar to that in lanes with comparable GFP/RFP activ-

ity units (Fig. 2C, lanes e and j). Thus, we conclude that the Asc1 protein directly or indirectly prevents a stalled ribosome from moving to another reading frame. We also observe trace amounts of a smaller polypeptide that migrates similarly to the 3C-HA-His6-superfolder GFP protein (lacking *Renilla* luciferase) in extracts from the *asc1-Δ* strain expressing the (CGA)$_4$+1 construct (Fig. 2C, lane h); this is most likely due to internal initiation at an AUG in the +1 frame located 10 codons upstream of the start of the CGA repeat.

We considered that the frameshifting observed in the *asc1-Δ* mutant might actually be due to a failure of other known quality control systems, caused by the absence of *ASC1*. For instance, Asc1 is implicated in recruitment of the ribosome quality control (RQC) complex, in which the E3 ubiquitin ligase Ltn1 is not only a member but is required for assembly of the complex (Bengtson and Joazeiro 2010; Brandman et al. 2012). If recruitment of the RQC complex actually stops translation and prevents frameshifting, we would expect to observe frameshifting in an *ltn1-Δ* mutant; however, we do not observe any detectable frameshifting in an otherwise wild-type strain bearing a deletion in *LTN1* (Fig. 3A). No direct evidence exists that Asc1 recruits other quality control proteins, such as Dom34 and Hbs1, which are involved in recycling stalled ribosomes (Doma and Parker 2006; Shoemaker et al. 2010), or Nam7 and Nmd2, which are involved in nonsense-mediated decay (Isken and Maquat 2007), but we also tested these mutants and did not find evidence of significant frameshifting in any of these single mutants (Tables 1, 2). We noted that GFP/RFP from the in-frame *Renilla* luciferase-(AGA)$_4$-GFP was increased in the *nam7-Δ* and *nmd2-Δ* strains, suggesting that our *Renilla* luciferase-GFP construct might be a natural target of the NMD pathway (Table 2). To determine if mRNA
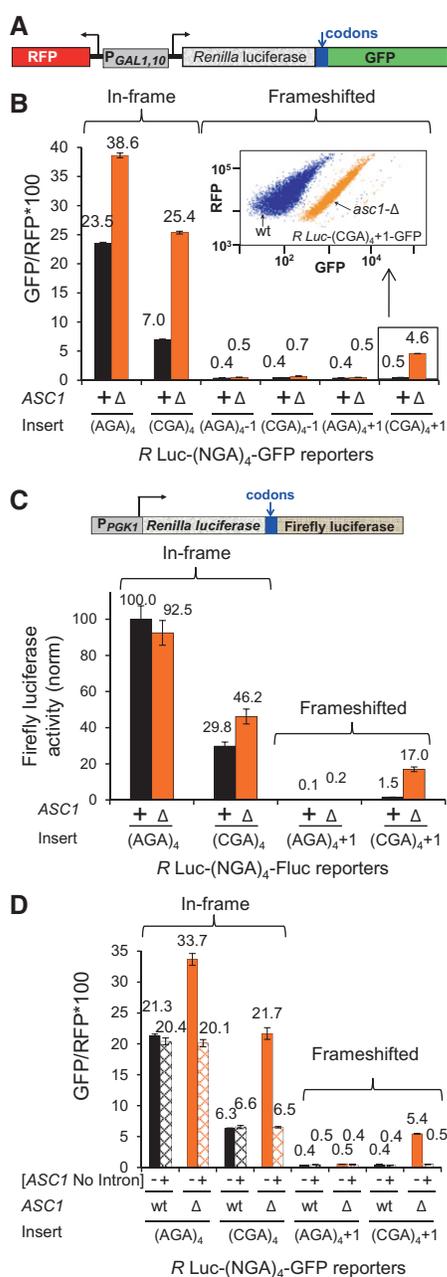
FIGURE 1. Ribosomes lacking Asc1 frameshift at CGA codon repeats. (*A*) Schematic of GFP Frameshifting Reporter. In this variant of the RNA-ID reporter (Dean and Grayhack 2012; Letzring et al. 2013), the reading frame of GFP is varied by insertion of 1 or 2 nt immediately downstream from the codon insert. Noise is minimized by independent measurement of RFP to obtain GFP/RFP and by integrating the reporter into the chromosome. (*B*) Expression of GFP in the +1 frame is observed in the *asc1-Δ* strain with a (CGA)$_4$+1 codon insert but not with an (AGA)$_4$+1 insert. Quantification of median GFP/RFP from in-frame and frameshifted reporters with (CGA)$_4$ and (AGA)$_4$ inserts in wild-type and *asc1-Δ* strains. A scatter plot of flow cytometry of GFP versus RFP for (CGA)$_4$+1 reporters in wild-type (blue) and *asc1-Δ* (orange) strains is shown. (NGA)$_4$ indicates either four Arg AGA or four Arg CGA codons inserted in-frame with *Renilla* luciferase. Values are derived from flow cytometry of four individual strains, and standard deviations are indicated. (*C*) Expression of firefly luciferase in the +1 frame is observed from a *Renilla* luciferase-firefly luciferase fusion protein with a (CGA)$_4$+1 insert in an *asc1-Δ* strain. A diagram of the *Renilla* luciferase-firefly luciferase fusion reporter is shown. Firefly luciferase activity is normalized to firefly luciferase activity of the *R* Luc-(AGA)$_4$-F Luc reporter in the wild-type. (*D*) A plasmid-borne copy of the *ASC1* gene, lacking the intron encoding *snR24*, complements the effects of the *ASC1* deletion on translational frameshifting and on suppression of CGA inhibition.

A

B

C
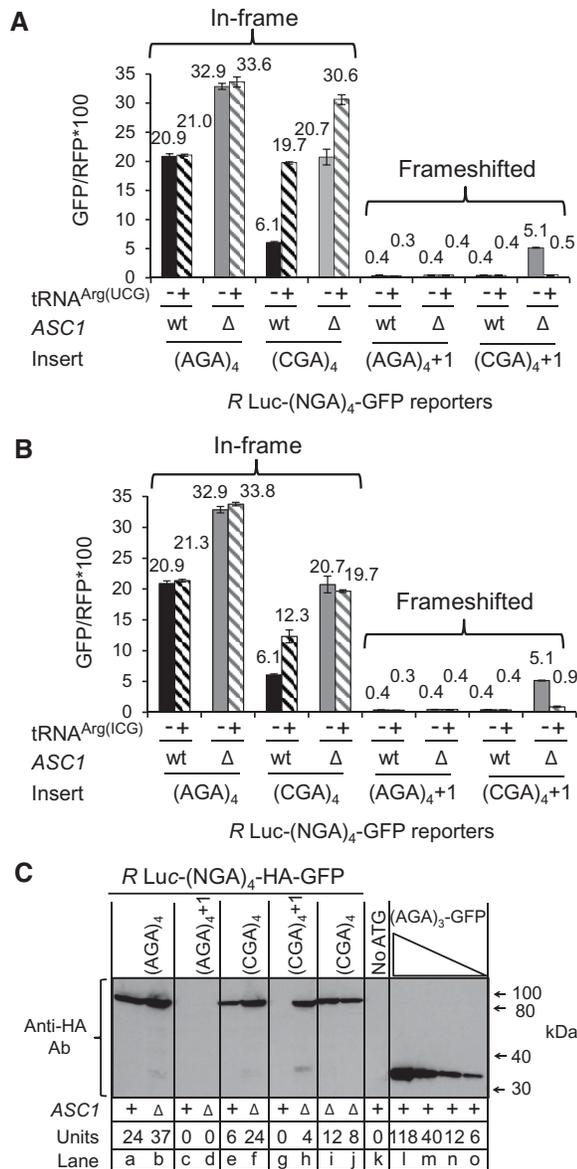
FIGURE 2. Frameshifting depends upon inefficient decoding of CGA codons and yields a full-length polypeptide. Expression of the nonnative anticodon mutated tRNA$^{Arg(UCG)}$ (A) or the native tRNA$^{Arg(ICG)}$ (B) on a 2μ plasmid suppresses frameshifting in the (CGA)$_4$+1 construct in an asc1-Δ mutant. Median GFP/RFP expression of the indicated R Luc-(NGA)$_4$-GFP constructs in the indicated wild-type and asc1-Δ strains. (C) CGA-mediated frameshifting yields a full-length fusion protein. Yeast extracts containing indicated units of GFP/RFP were resolved by SDS-PAGE and probed with an anti-HA antibody to detect GFP; the HA epitope is encoded just downstream from the frameshift site in-frame with GFP. The sample in lane k is derived from a GFP construct lacking an initiating methionine, while samples in lanes l–o are derived from yeast extracts expressing a 3C-HA-His6-superfolder GFP protein (lacking Renilla luciferase), derived from pEKD1024 (Dean and Grayhack 2012). Relative amounts of input protein were varied in lanes l–o and lanes f, i, and j, as indicated.

stabilization played a role in (CGA)$_4$+1 frameshifting in the asc1-Δ mutant, we examined GFP/RFP expression and GFP/actin mRNA levels (by qPCR) from (AGA)$_4$ and

(CGA)$_4$+1 constructs in nmd2-Δ single mutant and in the nmd2-Δ asc1-Δ double mutant. The differences in GFP mRNA levels between the (CGA)$_4$+1 constructs in the nmd2-Δ mutant and the nmd2-Δ asc1-Δ mutant strains are relatively small (approximately twofold) and cannot account for the differences in frameshifted GFP expression (GFP/RFP

A

B

C
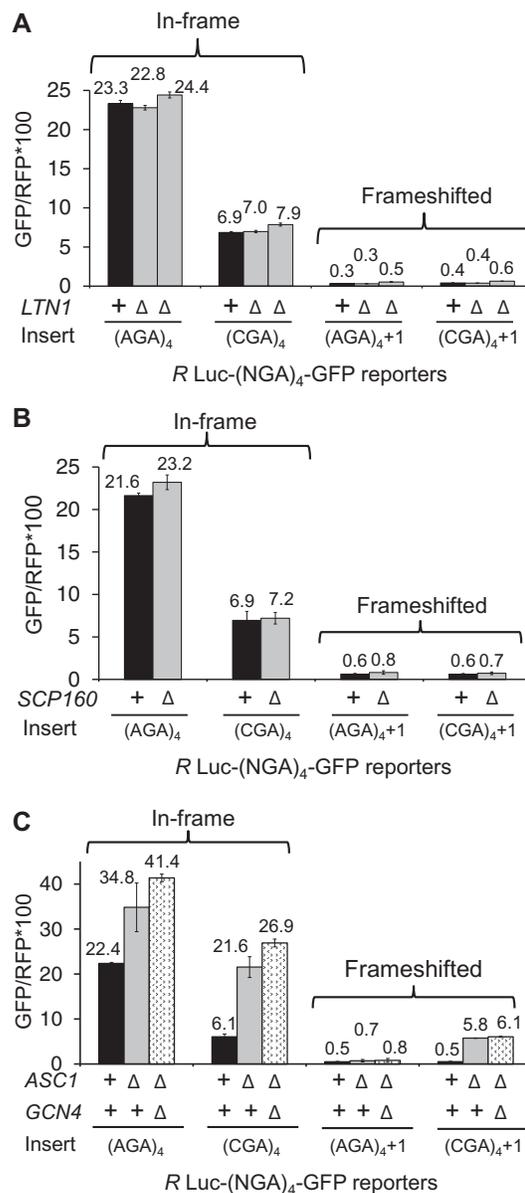
FIGURE 3. Frameshifting is not observed in ltn1-Δ or scp160-Δ mutants and is not impaired in an asc1-Δ gcn4-Δ mutant. (A) Deletion of LTN1 does not stimulate frameshifting. GFP/RFP expression from in-frame and frameshifted reporters with (CGA)$_4$ and (AGA)$_4$ inserts in wild-type, and two independently constructed ltn1-Δ strains. (B) Deletion of SCP160 does not stimulate frameshifting. GFP/RFP expression from in-frame and frameshifted reporters with (CGA)$_4$ and (AGA)$_4$ inserts in wild-type, and scp160-Δ strains. (C) Frameshifting is not dependent upon activation of the GCN4 pathway. GFP/RFP expression from in-frame and frameshifted reporters with (CGA)$_4$ and (AGA)$_4$ inserts in wild-type, asc1-Δ and double mutant asc1-Δ gcn4-Δ strains.

TABLE 1. Deletion of *DOM34* or *HBS1* does not stimulate frameshifting

	GFP/RFP × 100			
Strain	$(AGA)_4$	$(CGA)_4$	$(AGA)_4+1$	$(CGA)_4+1$
wt	22.5 ± 1.1	7.0 ± 0.6	0.48 ± 0.03	0.61 ± 0.03
dom34-Δ	23.0 ± 0.5	8.7 ± 0.3	0.57 ± 0.07	0.64 ± 0.06
hbs1-Δ	22.8 ± 1.0	7.9 ± 0.3	0.48 ± 0.04	0.51 ± 0.03

of 0.6 in the *nmd2-Δ* mutant versus 8.1 in the *nmd2-Δ asc1-Δ* mutant [Table 3]). Furthermore, no frameshifting is observed in strains with deletions in either of two other genes implicated in CGA codon read-through: *HEL2*, a putative E3 ubiquitin ligase (Brandman et al. 2012; Letzring et al. 2013), or *RPL1B*, encoding one of two copies of ribosomal L1 protein (Table 4; Letzring et al. 2013).

We considered that frameshifting might be dependent upon other known interactions of the Asc1 protein or known effects caused by mutating *ASC1*. However, deletion of *SCP160*, an mRNA binding protein recruited to the ribosome by Asc1 (Baum et al. 2004), is not sufficient to cause frameshifting (Fig. 3B). Furthermore, frameshifting does not require the function of the Gcn4 pathway, which is induced in rich media in the *asc1-Δ* mutant, since frameshifting is not reduced in the double *asc1-Δ gcn4-Δ* mutant (Fig. 3C; Hoffmann et al. 1999). Thus, frameshifting in the *asc1-Δ* strain is not simply explained by known quality control systems, or altered CGA decoding, or known Asc1 interactions.

Frameshifting depends upon CGA codon dosage

To determine if frameshifting is primarily dependent upon the number of CGA codons, or if there are additional constraints upon frameshifting, we examined the effects of increasing numbers of adjacent CGA codons on both in-frame and frameshifted expression of GFP/RFP in the wild-type and *asc1-Δ* strains (Fig. 4). We have previously noted that adjacent CGA–CGA codons (CGA codon pairs) are far more efficient inhibitors of expression than individual CGA codons. Thus, we increased CGA codon pairs by adding adjacent CGA codons (Fig. 4A). (Note each additional CGA codon [after two CGA codons] adds another CGA codon pair: a CGA_1–CGA_2–CGA_3 sequence includes two CGA codon pairs since the ribosome first translates CGA_1–CGA_2 and then CGA_2–CGA_3.)

In-frame expression of GFP/RFP decreases progressively with increasing numbers of CGA codon pairs in both strains (Fig. 4B) and fits a single exponential with exponential coefficients of inhibition (k_{TI}) (Letzring et al. 2010) that are consistent with differences in the effectiveness of the CGA inhibition in the wild-type ($k_{TI} = -0.44$) and *asc1-Δ* ($k_{TI} = -0.12$) strains. We note that the best fit occurs if the exponential is fit to CGA codon pairs. Frameshifted GFP/RFP increases progressively with increasing numbers of CGA codon

pairs in the *asc1-Δ* strain (and also fits a single exponential), although there is little or no indication of frameshifting with this construct in the wild-type strain (Fig. 4C). Frameshifted GFP/RFP reaches a maximum of 14.9 with six adjacent CGA codons, equivalent to ∼38% frameshifted product (relative to the in-frame AGA control). Thus, CGA-mediated frameshifting is limited primarily by the number of adjacent CGA codons.

We noted that frameshifting with two adjacent CGA codons, a single CGA–CGA pair, is relatively low (GFP/RFP is 0.8 ± 0.05), but is greater than GFP/RFP observed from either the $(AGA)_6+1$ construct or the identical $(CGA)_2(AGA)_4+1$ construct in the wild-type strain (GFP/RFP is 0.6 ± 0.04) (Fig. 4C). We also noted that GFP/RFP was higher from a construct with two CGA–CGA pairs that are separated from each other by two AGA codons (GFP/RFP is 1.2) (Fig. 4D), although even more frameshifting is seen with three adjacent CGA codons (GFP/RFP is 4.0). To establish whether or not frameshifting occurs at a single CGA–CGA codon pair, we compared GFP/RFP expression in a set of Arg3 constructs encoded with $(AGA)_3+1$, $[(CGA)_2(AGA)+1]$ and $(CGA)_3+1$ in both an *asc1-Δ* mutant and in an *nmd2-Δ asc1-Δ* mutant, subjecting the resulting values to Welch's 1-tailed *t*-test. In both strains, frameshifted GFP/RFP from the $[(CGA)_2(AGA)+1]$ construct was significantly greater than the corresponding frameshifted GFP/RFP from the $(AGA)_3+1$ construct (Table 5) with *P* values $<1 \times 10^{-5}$. Based on these results, we conclude that ribosomes lacking Asc1 do frameshift at a CGA–CGA pair, albeit inefficiently, and thus that frameshifting could occur in the 26 yeast genes with CGA–CGA codon pairs (Tumu et al. 2012).

The nascent polypeptide length modulates frameshifting, as well as CGA-mediated inhibition

We considered that the effects of Asc1 on frameshifting, as well as on suppression of CGA-mediated inhibition, might depend upon the distance between the CGA codon repeat and the translation start site, since we had previously observed that deletion of *ASC1* improved read-through of CGA codon repeats at amino acid 318, but not at amino acid 4 (Letzring et al. 2013). To investigate this, we altered the location of the codon insert by encoding distinct segments of the amino-terminal domain (NTD) of the *GLN4*

TABLE 2. Inactivation of nonsense-mediated decay does not stimulate frameshifting

	GFP/RFP × 100			
Strain	$(AGA)_4$	$(CGA)_4$	$(AGA)_4+1$	$(CGA)_4+1$
wt	28.9 ± 0.5	9.4 ± 0.5	0.61 ± 0.04	0.60 ± 0.02
nam7-Δ	54.6 ± 0.5	14.2 ± 0.4	0.77 ± 0.03	0.67 ± 0.18
nmd2-Δ	55.2 ± 0.6	15.0 ± 0.5	0.67 ± 0.11	0.80 ± 0.06

TABLE 3. GFP/RFP and GFP/actin mRNA in *nmd2-Δ* and *nmd2-Δ asc1-Δ* strains

	GFP/RFP × 100		GFP/actin mRNA	
Strain	$(AGA)_4$	$(CGA)_4+1$	$(AGA)_4$	$(CGA)_4+1$
nmd2-Δ	48.2	0.6	0.73 ± 0.05	0.61 ± 0.09
nmd2-Δ asc1-Δ	49.1	8.1	0.72 ± 0.04	1.25 ± 0.04

gene upstream of these codons. The *GLN4* NTD is highly expressed in yeast and forms a well-structured domain with several distinct α helices (Grant et al. 2011), allowing us to position the CGA codons between α helices (Fig. 5A). Control experiments show similar levels of expression of each of the *GLN4*-$(AGA)_4$-GFP fusion proteins (Table 6).

We find that the distance between the initiating methionine and the CGA codons influences three aspects of CGA-mediated effects on translation. First, although four adjacent CGA codons are inhibitory at every position in a wild-type strain, in-frame translation of GFP downstream from four CGA codons (relative to the identical construct with four AGA codons) increases approximately linearly as the insertion position is increased, up to *GLN4* amino acid 44, in both the wild-type and *asc1-Δ* strains. Each construct is designated by the amino acid position in *GLN4* after which the CGA or AGA codons are inserted. Expression of the $(CGA)_4$ construct with insertion beginning after amino acid 5 results in 2.9 units GFP/RFP (3.2 units in the *asc1-Δ* strain) (after subtraction of 0.58 units background) and is 3.7% (5.9% in the *asc1-Δ*) that of the $(AGA)_4$ construct (Fig. 5B). Expression of the $(CGA)_4$ construct in which the CGA codons are inserted after *GLN4* amino acid 44 increases to GFP/RFP of 27.8 (26.9), which is 38.4% (40.3%) that of the corresponding $(AGA)_4$ construct (Fig. 5B). Second, suppression of CGA-mediated inhibitory effects by the *asc1-Δ* mutation is not apparent until CGA codons are positioned after amino acid 62 (31.8 GFP/RFP [58.3%] in *asc1-Δ* versus 12.3 [19.0%] in the wild-type strain) (Fig. 5B). The observation that suppression by *asc1-Δ* is not observed until amino acid 62 is consistent with the idea that CGA codons near the initiating methionine inhibit expression in a manner that is independent of Asc1-mediated effects. Thus, there are likely two modes of codon-mediated inhibition.

Third, frameshifting in the *asc1-Δ* strain is detectable (albeit minimally) with $(CGA)_4+1$ inserted after amino acid 15, and increases steadily with $(CGA)_4+1$ inserts after amino acid 25, 44, 62, and 99 (Fig. 5C). GFP/RFP levels rise from 0.16 GFP/RFP (0.3%) at amino acid 5 to 10.7 GFP/RFP (16.2%) (Table 6). Controls reveal very little, if any, (<0.26 GFP/RFP) frameshifting with any construct in the wild-type strain or with the $(AGA)_4$ codon insert in either strain (Table 6). Moreover, if we consider the fraction of GFP/RFP that derives from frameshifting at CGA codons relative to the total GFP/RFP from both in-frame and frameshifted

$(CGA)_4$ constructs, we find an increase in the percentage of frameshifted GFP/RFP with distance from the initiating methionine (Fig. 5D), at least over this range of amino acids. We presume that frameshifting has reached a maximum by amino acid 99 since frameshifting is similar at amino acid 318 in the *Renilla* luciferase-GFP constructs. Thus, among ribosomes that continue translation past CGA codons, frameshifting appears to be the more favored outcome as the distance from the initiating methionine increases.

DISCUSSION

We have provided evidence that the Asc1/RACK1 protein plays a role in reading frame maintenance, a critical feature of translation, and infer that Asc1-mediated cessation of translation is important to avoid synthesis of aberrant proteins through frameshifting, although the precise nature of the frameshifting event (+1 or −2) (Fang et al. 2012) that occurs in the *asc1-Δ* mutant is unknown. Moreover, the effects of the *asc1-Δ* mutant may be physiologically relevant, since Asc1 protein levels are decreased substantially during hypoxic stress (Bruckmann et al. 2009), and since frameshifting is detectable (albeit minimally) at CGA–CGA codon pairs, which are found in 26 yeast genes, with two occurrences of CGA–CGA pairs in one gene (*BUD8*). In most cases, the CGA codons are located well into the coding sequence, median position beginning at codon 209 and in only five genes are the CGA–CGA codon pairs found in the first 50 codons. Furthermore, the finding that frameshifting occurs at CGA–CGA codon pairs leads to speculation that such frameshifting occurs at some other inhibitory codon combinations.

Decoding at programmed frameshifts and at CGA codon repeats share two features that may be key to ribosomal frameshifting at CGA repeats. First, both types of frameshifting are associated with ribosome stalls. Thus, −1 and +1 programmed frameshifting events depend upon a ribosome stall, mediated either by codons at the frameshift site, by structures downstream from the frameshift site or by sequences upstream of the frameshift site (Dinman 2012), although stalling is not sufficient to cause frameshifting (Tu et al. 1992; Somogyi et al. 1993; Dinman 2012). Similarly, ribosomes

TABLE 4. Deletion of *HEL2* or *RPL1B* does not stimulate frameshifting

	GFP/RFP × 100			
Strain	$(AGA)_4$	$(CGA)_4$	$(AGA)_4+1$	$(CGA)_4+1$
wt (1)[a]	22.5 ± 1.1	7.0 ± 0.6	0.48 ± 0.03	0.61 ± 0.03
hel2-Δ	24.1 ± 0.2	14.9 ± 0.3	0.47 ± 0.04	0.72 ± 0.08
wt (2)[a]	24.5 ± 0.5	7.1 ± 0.2	0.40 ± 0.04	0.49 ± 0.02
rpl1b-Δ	32.7 ± 0.2	14.9 ± 0.2	0.39 ± 0.05	0.47 ± 0.01

[a]GFP/RFP values were independently measured in wt strains in the same experiment with *hel2-Δ* mutant (1) and with the *rpl1b-Δ* mutant (2).
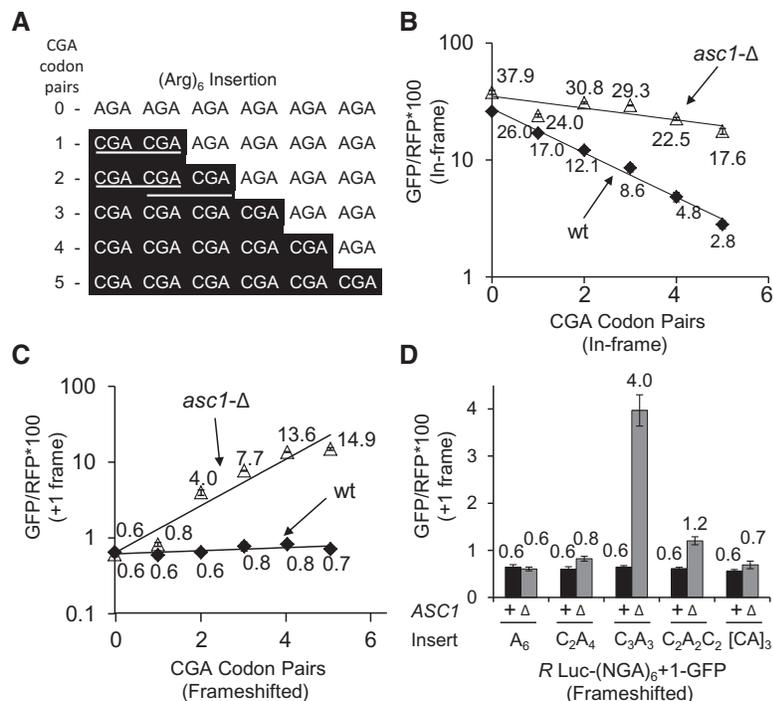
FIGURE 4. Frameshifting efficiency rises with increased CGA–CGA codon pair dosage. (*A*) Schematic of constructs made to measure the dose dependence of CGA codon pairs on expression and frameshifting. CGA codon pairs are denoted with the white lines for the $(CGA)_2(AGA)_4$ and $(CGA)_3(AGA)_3$ constructs, illustrating that addition of a third CGA codon results in two CGA–CGA codon pairs. (*B*) Exponential decrease in GFP/RFP as function of CGA–CGA codon pair dosage for the in-frame *Renilla* Luciferase-GFP reporter in wild-type and *asc1-Δ* strains. (*C*) Exponential increase in GFP/RFP from the +1 frameshifted *Renilla* Luciferase-GFP reporter as function of CGA–CGA codon pair dosage in the *asc1-Δ* strain. Frameshifting is not observed in the wild-type strain. (*D*) Comparison of frameshifting with a single $(CGA)_3$ triple, with one or two separated $(CGA)_2$ pairs. AGA codons are designated as A and CGA codons are designated as C.

also stall at CGA codon repeats as evidenced by mRNA cleavage at these sites (Chen et al. 2010; Letzring et al. 2010), by detection of the nascent polypeptide arrested at the CGA repeat and by destruction of the nascent polypeptide by Ltn1 (Letzring et al. 2013), a member of the RQC complex (Kuroha et al. 2010; Brandman et al. 2012). This ribosome stalling may mechanistically aid frameshifting. Ribosomes stalled at the −1 programmed frameshifting site in the *E. coli* dnaX gene undergo fluctuations between hybrid and classical states due to an energy barrier to translocation (Kim et al. 2014). Since it is known that yeast ribosomes can be found in two distinct conformations at different codons (Lareau et al. 2014), it is reasonable to speculate that ribosomes at CGA codon repeats might also undergo fluctuations between states.

Second, both classes of frameshifting may require a poorly decoded P site codon. In several examples, +1 programmed frameshifting depends critically on a poorly decoded P site codon, such as CUU or GCG, each decoded by wob-

ble (Belcourt and Farabaugh 1990; Baranov et al. 2004; Dinman 2012). CGA codons, like CUU and GCG, are inefficiently decoded, and require an I·A wobble interaction, which results in distortion of the tRNA anticodon in *Thermus thermophilus* 30S ribosomal subunits (Murphy and Ramakrishnan 2004). Moreover, we know that I·A wobble decoding of CGA codons results in poor decoding in both the A and the P sites of the ribosome, since CGA–CGA codon pairs are synergistically inhibitory (Letzring et al. 2010), and since frameshifting requires a CGA–CGA pair. Thus poor decoding of CGA in the P site is implicated in frameshifting at CGA codon repeats. However, CGA repeats lack some sequences that facilitate frameshifting, such as the Ty1 +1 frameshift site, which has a CUU A sequence on which tRNA$^{Leu(UAG)}$ can slip from CUU to UUA (Belcourt and Farabaugh 1990). On the other hand, there are +1 programmed frameshifting sites, such as Ty3, that lack these slippery sequences (Guarraia et al. 2007).

The specific manner in which Asc1 blocks read-through of CGA codons and prevents frameshifting is unknown. Although it is unproven that the loss of reading frame maintenance and suppression of CGA inhibitory effects are a direct consequence of translation by ribosomes lacking Asc1 protein, as compared with altered expression of another gene in the *asc1-Δ* mutant, this is the most parsimonious explanation of these events. Furthermore, the known interactions of Asc1 with ribosomal protein rpS3e could provide a clue to the manner in which the stall state may be transmitted to Asc1. In bacteria, rpS3p is found in the mRNA channel near a conserved base in the 16S rRNA that interacts with mRNA and undergoes conformational rearrangements during the elongation cycle; this base in 16S rRNA has been proposed to

TABLE 5. Comparison of expression and frameshifting with Arg3 constructs

Construct	GFP/RFP × 100			
	wt	*asc1-Δ*	*nmd2-Δ*	*nmd2-Δ asc1-Δ*
$(AGA)_3$	25.87 ± 0.85	38.59 ± 0.83	44.71 ± 0.60	43.42 ± 0.90
$(CGA)_2(AGA)$	15.81 ± 0.24	23.17 ± 0.98	21.83 ± 0.11	23.95 ± 0.54
$(AGA)_3$+1	0.41 ± 0.03	0.50 ± 0.05	0.57 ± 0.03	0.62 ± 0.07
$(CGA)_2(AGA)$+1	0.46 ± 0.04	0.65 ± 0.04	0.62 ± 0.04	0.98 ± 0.09
$(CGA)_3$+1	0.51 ± 0.04	2.91 ± 0.18	0.65 ± 0.04	4.77 ± 0.27
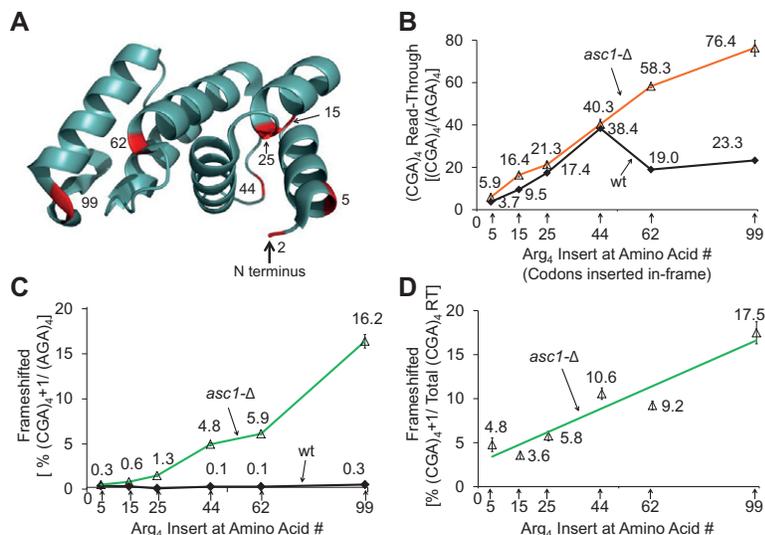
FIGURE 5. The length of coding sequence upstream of the CGA codon insert modulates CGA-mediated frameshifting and inhibition. (*A*) Structure of the Gln4 NTD (amino acids 1–187) based on the crystal structure (PDB ID: 3TL4) (Grant et al. 2011), indicating the location of the C termini used in constructs in *B–D*. Effects of the length of the nascent polypeptide preceding the CGA codons on CGA-mediated inhibition (*B*) and frameshifting (*C,D*) in wild-type and *asc1-*Δ yeast strains. (CGA)$_4$-GFP and (AGA)$_4$-GFP were inserted downstream from the indicated number of amino acids derived from the *GLN4* NTD, starting at the native *GLN4* AUG. In *B*, read-through for each in-frame (CGA)$_4$ construct is reported as the percentage of the expression of the in-frame (AGA)$_4$ construct with the identical leader ([GFP/RFP from (CGA)$_4$ construct/ GFP/RFP from (AGA)$_4$ construct] × 100). In *C*, expression of the frameshifted (CGA)$_4$+1 constructs are reported as the percentage of the in-frame (AGA)$_4$ construct with the identical leader. In *D*, expression of the frameshifted (CGA)$_4$+1 constructs in the *asc1-*Δ strain are reported as the percentage of total GFP/RFP from read-through (RT) at CGA codons, that is GFP/RFP from the in-frame (CGA)$_4$ construct plus GFP/RFP from the frameshifted (CGA)$_4$+1. GFP/RFP expression of each construct is reported in Table 6.

act as a pawl to hold the mRNA in place (Zhou et al. 2013). In eukaryotes, rpS3e contains additional sequences, so that rpS3e traverses the small subunit from the mRNA channel to RACK1 (near the 40S beak) (Rabl et al. 2011). Thus, changes in rpS3e might occur as the ribosome shifts between states, and such changes could affect the interaction between rpS3e and Asc1, allowing Asc1 to stop translation.

We also found that three phenomena, CGA-mediated inhibition in wild-type yeast, CGA-mediated frameshifting and suppression of CGA-mediated inhibition in *asc1-*Δ mutants, all depend upon distance from the initiating methionine. Since CGA codons near the initiating methionine inhibit translation in a manner independent of Asc1 protein, while much of the inhibition by CGA codon repeats downstream from amino acid 62 depends upon Asc1 protein, there are likely to be at least two mechanisms by which CGA codon repeats inhibit translation. In contrast to CGA-mediated inhibition of translation, frameshifting at the CGA codons always requires the absence of Asc1 protein.

There are at least two models that would explain the Asc1-independent inhibition by CGA codons in the first 40–60 amino acids, each of which might also explain how inhibitory effects wane with increasing distance from the initiating methionine. The first model is based on results from Chu et al.

(2014) who suggest that slowly elongating ribosomes may restrict initiation by an incoming ribosome; that is, that codon-mediated effects on the translation elongation rate could result in reduced rates of translation initiation. In this model, slow translation of CGA codons closer to the initiation site (in which stalled ribosomes would directly affect the next initiating ribosome) would be more inhibitory than slow translation further from the initiation site; a feedback mechanism of this type would be independent of Asc1 protein and might only operate near the initiation site. The second model is based on the idea that the efficiency of the peptidyl transferase reaction is modulated by the nascent polypeptide within the exit tunnel, an idea for which there is precedent from nascent peptide sequences that stall the ribosome (Ramu et al. 2011; Martinez et al. 2014), and evidence for contact between the nascent polypeptide and 28S rRNA at the peptidyl transferase center (Bhushan et al. 2010). In this model, which is similar to the promoter clearance step in transcription, the ribosome becomes committed to continued elongation as the exit tunnel fills, and thus CGA codons become less inhibitory as the exit tunnel fills. The exit tunnel accommodates 30–40 amino acids in unfolded form and up to 72 amino acids in an α-helical fold (Malkin and Rich 1967; Blobel and Sabatini 1970; Kramer et al. 2001); the large effects on modulating CGA codon inhibition occur in this same range. It is intriguing that suppression caused by loss of Asc1 occurs at a

TABLE 6. Expression of the *GLN4*-(NGA)$_4$-GFP fusion proteins

Strain	Amino acid	GFP/RFP × 100			
		(AGA)$_4$	(CGA)$_4$	(AGA)$_4$+1	(CGA)$_4$+1
wt	5	78.8 ± 3.3	3.5 ± 0.3	0.69 ± 0.08	0.71 ± 0.12
*asc1-*Δ	5	54.4 ± 5.8	3.8 ± 0.4	0.64 ± 0.03	0.74 ± 0.04
wt	15	76.0 ± 5.4	7.8 ± 1.0	0.60 ± 0.02	0.67 ± 0.09
*asc1-*Δ	15	48.5 ± 2.3	8.4 ± 0.7	0.60 ± 0.04	0.87 ± 0.02
wt	25	60.2 ± 2.8	11.0 ± 0.1	0.43 ± 0.04	0.51 ± 0.09
*asc1-*Δ	25	49.5 ± 2.9	11.0 ± 0.4	0.64 ± 0.02	1.22 ± 0.06
wt	44	72.8 ± 1.9	28.3 ± 0.6	0.56 ± 0.19	0.63 ± 0.11
*asc1-*Δ	44	67.4 ± 2.1	27.5 ± 4.4	0.64 ± 0.08	3.77 ± 0.13
wt	62	65.3 ± 2.6	12.9 ± 1.5	0.46 ± 0.05	0.63 ± 0.10
*asc1-*Δ	62	55.2 ± 0.6	32.4 ± 0.6	0.64 ± 0.14	3.82 ± 0.13
wt	99	86.7 ± 3.4	20.6 ± 1.6	0.82 ± 0.02	0.84 ± 0.10
*asc1-*Δ	99	66.7 ± 3.0	51.1 ± 3.9	0.73 ± 0.09	11.28 ± 0.35

point where most of the exit tunnel would be filled. Subsequent experiments are needed to shed light on the mechanism by which Asc1 mediates CGA inhibition and prevents loss of reading frame.

MATERIALS AND METHODS

Strains, plasmids, and oligonucleotides

Strains, plasmids, and oligonucleotides used in these studies are listed in Supplemental Tables S1–S3, respectively. The yeast strain BY4741 (*MAT*a *his3*Δ1, *leu2*-Δ0, *met15*-Δ0, *ura3*-Δ0) (Open Biosystems) was the parent strain for all yeast manipulations. Yeast strains bearing deletions of *NAM7*, *NMD2*, and *SCP160* were constructed by PCR amplification of the *kan*[R] cassette from the systematic deletion collection (Open Biosystems). Deletions of *DOM34* and *HBS1* were constructed by amplification of the bleo[R] cassette from pUG66 (Gueldener et al. 2002) with oligonucleotides homologous to the 5′ and 3′ ends of the genes. Yeast strains with deletions in *RPL1B*, *HEL2*, *LTN1*, and *GCN4* have been described (Letzring et al. 2013).

The yeast strain AW768 bearing the *asc1*-Δ::sp*HIS5*, and the plasmid p*URA3*, *ASC1* was made by first creating the *asc1*-Δ::sp*HIS5* cassette using pUG27 (Gueldener et al. 2002) and primers homologous to the 5′ and 3′ ends of *ASC1* (OW125, OW126), and then integrating this cassette into an *asc1*-Δ::kanMX strain (AW714) derived from the corresponding knockout strain in the systematic deletion collection (Open Biosystems). Then, the *asc1*-Δ::sp*HIS5* cassette with flanking regions was obtained by genomic PCR of a His[+] isolate, and integrated into a strain AW765 (which is derived from BY4741 and carries the plasmid *URA3* 2µ *ASC1* plasmid, pEAW076). Transformants were screened for colonies that retained their His[+] phenotype after removal of the [*ASC1*] covering plasmid by FOA treatment. The resulting strain AW768 (*MAT*a *his3*-Δ1, *leu2*-Δ0, *met15*-Δ0, *ura3*-Δ0, *asc1*-Δ::sp*HIS5*, p*URA3*, *ASC1*) was verified by confirmation PCR, and a test for sensitivity to cycloheximide.

The plasmid vector pEAW012, in which *Renilla* luciferase is fused to GFP under P$_{GAL1}$ control, was obtained by PCR amplification of the *Renilla* luciferase gene from pDL202 (Letzring et al. 2010) using oligonucleotides OW090 and OW091, which was inserted into the Pac1 site of pEKD1024 (Dean and Grayhack 2012) using LIC cloning; this regenerated a Pac1 site as well as a LIC site for use in cloning codon inserts as *Renilla* luciferase-GFP fusion proteins. Oligonucleotides carrying codon sequences were annealed and inserted in-frame or out of frame with GFP by insertion into the PacI and BbrPI sites of pEAW012 using LIC.

Plasmids containing truncated *GLN4* (Grant et al. 2011) fused to GFP were obtained by cloning the *GLN4*-codon inserts (with or without a frameshifting nucleotide) into the PacI and BbrPI sites of pEKD1024 using LIC cloning (Dean and Grayhack 2012). The *GLN4* sequences corresponding to amino acids 1–5 and 1–15 were obtained by annealing oligonucleotides with the sequences to be inserted. The *GLN4* sequences encoding amino acids 1–25, 1–44, 1–62, 1–99 were obtained by PCR amplification from plasmid pJE1012a using the forward primer OJW011 and the reverse oligonucleotide corresponding to the 3′ end of *GLN4* (OJY12-OJY19 and OW212-OW219).

The *ASC1* 2µ *URA3* plasmid (pEAW076) was constructed by cloning the *ASC1* gene with flanking sequences (−326 to +434)

into pECB194 (derived from AVA577) (Alexandrov et al. 2004). The *ASC1*-Δ*intron URA3* Cen plasmid pEAW141 was constructed from two fragments joined by LIC methods, followed by ligation and PCR amplification of the joined fragments. Sequence from −496 to +537, directly upstream of the intron, were amplified with oligonucleotides OW119 and OW121, while sequences from 811, immediately downstream from the intron, through the coding region to +537 downstream from the TAA stop codon were amplified with oligonucleotides OW122 and OW123. The oligonucleotide OW122 had an additional 12 bases on the 5′ end, to generate homologous ends with the upstream PCR product. Twelve base single stranded homologous ends on each PCR product were generated by treatment with T4 DNA Polymerase, subsequently annealed and ligated, which enabled PCR amplification of the entire *ASC1* coding sequence with flanking regions (−482 to +445) using primers OW120 and OW124. The PCR product was cloned into pAVA577 (Alexandrov et al. 2004) using LIC cloning to make pEAW141.

Flow cytometry

Yeast strains bearing the *R* Luc-(NGA)$_x$-GFP, GFP or *GLN4*-(NGA)$_4$-GFP reporters were constructed by integrating each Stu1-digested reporter into the *ADE2* locus (Dean and Grayhack 2012) and selecting for growth on SD-Met-Ura to retain the *ASC1* plasmid. Transformants were streaked onto SD-Met-Ura, grown overnight in YPD or SD-Met media, and streaked for single colonies onto SD-Met + FOA for 2–3 d. FOA treated single colonies were grown in YP + Raff + Gal + Ade at 30°C to an OD$_{600}$ of 0.6–1.5 and analyzed using flow cytometry. Strains bearing *LEU2* plasmids followed a similar treatment except that they were streaked onto SD-Leu + FOA plates, and grown in S-Leu + Raff + Gal + Ade at 30°C for flow cytometry. Analytical flow cytometry and subsequent data analysis were performed as previously described (Dean and Grayhack 2012). Mean GFP/RFP values and their standard deviations reported in the bar graphs are obtained from the median GFP/RFP from measurements of three (and usually four) independent yeast transformants.

Protein expression analysis

Western analysis of the *R* Luc-(NGA)$_4$-GFP and (AGA)$_3$-GFP with anti-HA antibody was performed as described previously (Gelperin et al. 2005).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

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