In Aspergillus nidulans the Suppressors suaA and suaC Code for Release Factors eRF1 and eRF3 and suaD Codes for a Glutamine tRNA

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ABSTRACT In Aspergillus nidulans, after extensive mutagenesis, a collection of mutants was obtained and four suppressor loci were identified genetically that could suppress mutations in putative chain termination mutations in different genes. Suppressor mutations in suaB and suaD have a similar restricted spectrum of suppression and suaB111 was previously shown to be an alteration in the anticodon of a gln tRNA. We have shown that like suaB, a suaD suppressor has a mutation in the anticodon of another gln tRNA allowing suppression of UAG mutations. Mutations in suaA and suaC had a broad spectrum of suppression. Four suaA mutations result in alterations in the coding region of the eukaryotic release factor, eRF1, and another suaA mutation has a mutation in the upstream region of eRF1 that prevents splicing of the first intron within the 5’UTR. Epitope tagging of eRF1 in this mutant results in 20% of the level of eRF1 compared to the wild-type. Two mutations in suaC result in alterations in the eukaryotic release factor, eRF3. This is the first description in Aspergillus nidulans of an alteration in eRF3 leading to suppression of chain termination mutations.

Isolation of genetic suppressor mutations of chain termination mutations (and anti-suppressors) has proved to be a very powerful tool for identifying components of the translation machinery in a range of model eukaryotic organisms including Saccharomyces cerevisiae, Schizosaccharomyces pombe (Hawthorne and Leupold 1974), Podospora anserina (Coppin-Raynal and Dequard-Chablat 1988), Caenorhabditis elegans (Hodgkin 2005), and Aspergillus nidulans (Martinelli 1994). The early genetic screens made predictions about the possible function of the suppressor genes from phenotypic observations and, much later, these predictions have been confirmed by the molecular data; however, to date this has not been accomplished with *Aspergillus nidulans*. The mechanism of chain termination is still poorly understood, and it is of interest not only from a fundamental scientific standpoint as it is ubiquitous in all cells, but also from a practical point of view because therapies are being developed to allow read-through of premature chain termination mutations in disease genes (Bidou et al. 2012; Bordeira-Carrico et al. 2012), and vector selection systems are being developed that have a nonsense mutation in the recipient strain and a suppressor mutation in the vector rather than using an antibiotic resistance gene for selection (Oliveira and Mairhofer 2013). In all the organisms where chain termination suppressors have been isolated and characterized at the molecular level, the release factors eRF1 and eRF3 have been found to be essential for polypeptide chain termination. Mutations in either of these proteins can lead to read-through of chain termination mutations (Gagny and Silar 1998; Inge-Vechtomov et al. 2003), but the release factors have other roles in cell organization and cell cycle progression, and characterization of the suppressors from a range of organisms may give important insights into these additional functions (Inge-Vechtomov et al. 2003).

In *Aspergillus nidulans*, four suppressor loci, suaA, suaB, suaC, and suaD (suaD = suppressor in *Aspergillus*) were isolated by co-reversion of mutations in unrelated genes (Roberts et al. 1979). These suppressors were allele-specific and gene-unspecific and were thought to act on...
nonsense mutations because of the lack of any residual function in
the gene products of the suppressible mutant strains. Subsequent work
extended the number of co-suppressible alleles, but the number of
new suppressor loci was limited and these have not been characterized
further (Al Taho et al. 1984; Martinielli et al. 1984; Sealy-Lewis 1987).
The suppressors in the suaB and suaD loci had a restricted pattern of
suppression and were semi-dominant to the wild-type suppressor allele,
whereas mutations in suaC and suaD had a wider spectrum of suppres-
sion and were recessive or semi-dominant depending on the suppressible
allele being studied. The suaA and suaC alleles also had a number of
pleiotropic alterations, like the omnipotent suppressors in Saccharomyces
cerevisiae, that were able to suppress all three classes of chain termination
mutation. These included cold sensitivity, slow growth in conditions
not requiring the action of the suppressor, poor conidial viability, low-
ered fertility, and increased sensitivity to aminoglycoside antibiotics
(Martinielli 1994). The behavior of the two classes of suppressor led to
the hypothesis that the suaB and suaD mutations were acting by alter-
ations in tRNA whereas the suaA and suaC mutations were acting by
alterations in ribosomal proteins or release factors. The properties of
the suaA and suaC suppressor strains have similarities to the SUP45 and
SUP35 omnipotent suppressor strains in Saccharomyces cerevisiae and
the sua2 and suaI suppressors in Podospora anserina that have been shown
to code for the release factors eRF1 and eRF3, respectively (Frolova et al.
1994; Stansfeld et al. 1995; Gagny and Silar 1998). The suaB111 muta-
tion was identified as a G-to-A alteration in the anticodon CUG of
a glutamine tRNA leading to recognition of UAG as a sense codon
(Espeso et al. 2005), but the mechanism of action of the other suppress-
ers has remained unidentified. In this article, we identify functions for
suaA, suaC (eRF1 and eRF3), and suaD (glutamine tRNA).

**MATERIALS AND METHODS**

_**A. nidulans** strains, media, growth conditions, and
manipulations_

Aspergillus media and growth conditions were as described by Cove
(1966). The scoring of the suppressible alleles in alX, sb and niaD, are
described by Sealy-Lewis (1987). Genetic techniques were as described
by Clutterbuck (1974). The strains used are listed in Supporting

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**Table 1 Alleles with known chain termination sequence changes**

| Mutant Allele | Nucleotide Change | Amino Acid Affected | Amino Acid Change | Reference |
|---------------|-------------------|---------------------|-------------------|-----------|
| alX4a         | CAG to TAG        | Gin 409             | Gin to TAG        | This study |
| alcR125b      | TGG to TAG        | Trp 149             | Trp to TAG        | Dr. B. Felenbok, personal communication |
| areA600       | TCG to TAG        | Ser 646             | Ser to TAG        | Kudla et al. (1990) and Langdon et al. (1995) |
| areA601       | AAA to TAA        | Lys 206             | Lys to TAA        | Prof. H. N. Arst, personal communication |
| aldA67        | UGG to UGA        | Trp 131             | Trp to TGA        | Flippini et al. (2001) |
| acuH13        | CAG to TAG        | Gin 134             | Gin to TAA        | Martinez et al. (2007) |
| acuH31        | CAG to TAG        | Gin 26              | Gin to TAA        | Martinez et al. (2007) |
| acuH20        | TGG to TAG        | Trp 254             | Trp to TAG        | Martinez et al. (2007) |
| ngA1          | TTA to TGA        | Leu 269             | Leu to TGA        | Han et al. (2005) |
| palC143       | TTA to TGA        | Leu 223             | Leu to TGA        | Tilburn et al. (2005) |
| palF15        | TTA to TGA        | Leu 189             | Leu to TGA        | Herranz et al. (2005) |
| palB7         | GGA to TGA        | Gly 791             | Gly to TGA        | Peñas et al. (2007) |
| palB513       | TTA to TGA        | Leu 552             | Leu to TGA        | Peñas et al. (2007) |
| brlA23        | GAG to TAG        | Gin 317             | Gin to TAA        | Griffith et al. (1999) |
| brlA17        | GAA to TAA        | Glu 118             | Glu to TAA        | Griffith et al. (1999) |
| brlA19        | TAC to TAA        | Tyr 395             | Tyr to TAA        | Griffith et al. (1999) |
| brlA4         | CAA to TAA        | Gin 334             | Gin to TAA        | Griffith et al. (1999) |

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A

**Table S1. Gene symbols are as previously described (Clutterbuck 1993, 1997) and the characterized sequence changes of
suppressible alleles are listed in Table 1.**

**Molecular techniques**

Standard molecular techniques are described by Sambrook et al.
(1989). Aspergillus genomic DNA was prepared as described by Jones
and Sealy-Lewis (1989). DNA was extracted from the strains [H3,
H103, H44, H44(27), H44(23), H44(32), H7, H7 rev16] and PCR
primers (Table S2) were designed to amplify overlapping sections of
the entire coding region of the genes. The same primers were used for
sequencing the PCR products. The coding region of both genes was
sequenced on both strands. Where a change was identified compared
with the sequence in the database, the wild-type was sequenced in that
region to confirm the change. RT-PCR was performed using the GE
Healthcare Illustra Ready-to-Go RT-PCR beads. The PCR products
to eRF1 and the tubulin controls were standardly run on 1.3% TAE
tagrose gels or 5% TBE polyacrylamide gels. Crystal structure
prediction of eRF1 and eRF3 was achieved through EsyPred3D
Web server (http://www.fundp.ac.be/sciences/biologie/urbm/bioinfo/
esypred/) and Swiss-model (http://swissmodel.expasy.org/).

**Transformation experiments, protein analyses, and cell imaging**

Transformation of Aspergillus was performed as described by Tilburn
et al. (2005). Strains expressing GFP C-terminally tagged fusions of
wild-type or mutant SuaA protein were generated as described by Yang
et al. (2004). Transformation DNA cassettes were obtained by fusion
PCR procedures of three fragments comprising, in the following order,
the 5'-UTR/suaACDS, gfp/riboBAf and 3'-UTR region of suaA, as
described in Figure 1. Strains MAD1427 and H1885 were used to
generate strains expressing SuaA-GFP, and H1888 and H1884 were used to
express SuaA105-GFP and SuaA23-GFP proteins, respectively.

The duaA23 mutation was re-introduced into the suppressible
strain H44 by transformation. The fragment containing the duaA23
allele was PCR-amplified using gDNA from H44(23) as the template
and primer pairs duaA1 and duaA2. Transformants were positively
Protein extraction followed the alkaline lysis extraction procedure (Hervás-Aguilar and Peñalva 2010). For Western blotting, usually 10 μl of total protein extracts were electrophoresed in 10% SDS-polyacrylamide gels and subsequently transferred to nitrocellulose filters using TransBlot TurboTransfer System (Bio-Rad). GFP tagged proteins were detected using mouse anti-GFP (1/5000; Roche). Actin, detected with mouse anti-γ actin antibody (1/50,000; ICN Biomedicals), was used as loading control. Peroxidase-conjugated goat anti-mouse IgG immunoglobulin (Jackson Immunoresearch Laboratories) at 1/4000 was used as a secondary antibody. Peroxidase activity was detected with the Amersham Biosciences ECL kit.

For all microscopy experiments, supplemented watch minimal medium was inoculated with conidia, and cells (mostly germings) were observed after incubation at 25°C for 16 hr (Peñalva 2005) using uncoated glass-bottom dishes (MatTek Corporation, Ashland, MA). Fluorescence images were acquired with an upright Eclipse 80i microscope (Nikon, Melville, NY) equipped with Brightline GFP-3035B (Semrock, Rochester, NY), a 100-W mercury lamp epifluorescence module, Uniblitz (Rochester, NY) external shutter, a 60x 1.40-numerical aperture (NA) plan apochromat objective, and an ORCA ERG camera (Hamamatsu, Bridgewater, NJ).

**RESULTS AND DISCUSSION**

### suAD codes for a glucose tRNA

The suAD gene maps on linkage group VII and is linked to alcA/R. The suppressors, suAD103 and suAD108, have identical specificity to the suaB111 suppressor, and this was found to be an alteration in a gln tRNA (Sealy-Lewis 1987; Espeso et al. 2005). Analysis of the annotated database revealed a single tRNA on the left arm of chromosome VII distal to alcA/R on contig 1.168, which was a gln tRNA. Sequencing of the gln tRNA for suAD103 and suAD108 revealed the same nucleotide change in both mutants, leading to an anticodon change so that the UAG codon could be recognized as a sense codon. Thus, both the suaB and suAD suppressor mutations are in gln tRNAs (Table 2). Consistent with this, suaB111 and suAD103/108 suppress alX4, which contains a premature UAG stop codon, although the alcR125 and areA600 mutations also contain UAG stop codons but are not suppressed (Sealy-Lewis 1987). The insertion of glutamine would

### Table 2 Sequence changes in suppressors

| Gene   | Gene Product | Accession Number | Nucleotide Change | Gene Product Alteration |
|--------|--------------|------------------|-------------------|------------------------|
| suaB111<sup>a</sup> | gln tRNA AN9659 | contig 161 (CTG to CTA) | Anticodon change recognizes UAG |
| suaD103 | gln tRNA AN9669 | contig 168 (CTG to CTA) | Anticodon change |
| suaD108 | gln tRNA AN9669 | contig 168 (CTG to CTA) | Anticodon change recognizes UAG |
| suaA105 | eRF1 AN8853 | −209 G to A | Alteration in upstream region |
| suaA101 | eRF1 AN8853 | (TCC to TAC) C104A | S3SY, N113K |
| suaA23  | eRF1 AN8853 | (AAC to AAG) C452G | |
| suaA32  | eRF1 AN8853 | (AAA to AAC) A656C | K181N |
| suaA27  | eRF1 AN8853 | (ATT to TCT) A978T, T979C | I289S |
| suaC109 | eRF3 AN2080 | (TAT to AAT) T1186A | Y396N |
| suaC107 | eRF3 AN2080 | (TAT to AAT) T1186A | Y396N, N415H |
| suaC500 | eRF3 AN8853 | (AAC to AAG) A1243C | E117K |
| snpA6<sup>b</sup> | eRF1 ANIA_08853 | (GGT TOAGT) G906A | G265S |

<sup>a</sup>Espeso et al. (2005).
<sup>b</sup>Han et al. (2005).
<sup>c</sup>Martinez et al. (2007).
<sup>d</sup>ANIA_O8853 is the prefix given to the suaE7 mutation (aspergillusgenome.org).

**Figure 1** Generation of recombinant strains expressing GFP-tagged versions of SuaA. The cartoon (A) denotes the eRF1 locus (AN8853 on chromosome 1) indicating the position of the introns. The arrows show the positions of the single base changes for suaA105 (in the splice site for the upstream ORF) and suaA23 (in the coding region of the third exon). The cartoon below shows the DNA cassettes generated by fusion PCR used in transformation (constructs were either wild-type or contained the suaA23 or suaA105 mutation) together with the primer pairs that were used (green arrows). The cartoons in (B) show the strains transformed and the resultant status of the gene after recombination into the homologous eRF1 locus; on the top line the strains transformed with the wild-type suaA<sup>A</sup> construct, MAD1427 (pyrG89, pabaA1; argB2; ΔnkuA: argB; riboB2) or H1885(yA2 pantoB100; sb43; alaD67 riboB2); on the second line: the suaA23 construct transformed into H1888 (pabaA1; yA2 pantoB100; alX4; riboB2; suaA23); on the third line: the suaA105 construct transformed into H1884 (alX4 suaA105; riboB2).

selected on glucose minimal medium containing allantoin 10 mg/100 ml (0.6 mM) as a nitrogen source and further purified to homokaryosis, and the presence of the suaA23 mutation in several transformants was verified by sequencing.
restore the wild-type amino acid to allantoinase (Alx), but for the other two mutated proteins there would be a substitution of glutamine for tryptophan in AlcR and glutamine for serine in AreA600, which are presumably non-functional. There are 174 tRNAs annotated (Iriarte et al., 2012), and of these seven are glutamine tRNAs. There are two glutamine tRNAs that recognize the codon CAA and five that recognize the preferred codon CAG (Lloyd and Sharp 1991; Iriarte et al. 2012). The suppressor tRNAs that have been selected independently (suaB111, suaD103, and suaD108) are all in tRNAs that recognize CAG codons.

**suaA codes for the release factor eRF1**

The suaA mutations were closely linked to each other and were shown not to recombine in large numbers of progeny, making it likely that they were all mutations within the same gene (Sealy-Lewis 1987). suaA mapped on chromosome III and was closely linked to phenA in the region of eRF1. suaA101, suaA27, suaA32, and suaA23 all showed nucleotide changes in the coding region of eRF1 (ANIA 8853 version 5) (Table 2, Figure 1) compared to the wild-type sequence and the likelihood of there being a mutation in suaA in independent isolates if this is not the gene responsible for the phenotype must be very small. To confirm that a suaA mutation is responsible for the suppression, strain H44 (pabaA1; alX4; alcR125; niaD500;-fwA1) was transformed with a fragment containing suaA23 and allantoin-utilizing transformants were selected. The transformants were also suppressed for the alcR125 and niaD500 mutations showing suppressed growth on ethanol medium and nitrate medium, respectively (Figure 2).

Viable gene replacements of wild-type suaA with suaA+ tagged with GFP (in strain H1888: yA2 pantoB100; riboB2; alX4; sb43; aldA67) were obtained that expressed the SuaA-GFP fusion showing that the GFP tag does not interfere with the function of eRF1 in chain termination. SuaA-GFP showed a preferential cytoplasmic localization where its activity is expected for translation termination (Figure 3A). Interestingly, nuclei were not fluorescent, suggesting the presence of a nuclear-export system acting on SuaA to ensure exclusion of the translational machinery from the nucleoplasm in this fungus.

To investigate further the suaA23 suppressor function, a construct containing the 5'UTR and suaA23 coding region fused to the gfp/riboB4 cassette was used to transform strain H1888: yA2 pantoB100

![Figure 2](image)

**Figure 2** Growth testing of strains on 1% glucose minimal medium with 5 mM ammonium tartrate (left) or 0.6 mM allantoin as nitrogen source (right). The strains are control strains (only relevant genotype shown). 1. Wild-type; alX4 suaA+. 2. alX4 suaA105. 3. alX4 suaA32. 4. alX4 suaA27. 5. alX4 suaA23. Individual transformants: 4, 5, 6, 7 of H44 (alX4 containing strain transformed with a fragment containing the suaA23 mutation; see Materials and Methods).

![Figure 3](image)

**Figure 3** Localization and expression levels of SuaA/eRF1-GFP fusion. (A) Fluorescence images of cells of a transformant expressing SuaA-GFP fusion (H1888 transformed with the suaA::gfp construct; see Figure 1, MAD4903). (B) Western blot showing levels of SuaA-GFP fusion in total protein extracts of two transformants of suaA105::gfp (transformant 1 is MAD4904) compared with a suaA+::gfp transformant (MAD4903). Graph shows relative intensity of SuaA-GFP detection bands compared to actin levels.
Adenosines in the third and fourth positions of the tetrapeptide (Fan-Minogue et al. 2008; Bulygin et al. 2011). Analogous studies using similar derivatives of guanine have shown that in addition to association with the YxGxxxF motif, the Thr-32 in the conserved 31-GTx-33 motif is the major target for cross-linking (Bulygin et al. 2010).

Molecular modeling suggests that recognition of the UAG/UGA and UGA in stop codons is associated with a different conformation of eRF1 (Fan-Minogue et al. 2008). suaA23 has a change within the conserved YxGxxxF region and can suppress the UAG mutations within alx4 and areA600 but is still able to terminate at the UAA mutation. An interpretation of these results is that the change in the YxGxxxF motif is able to discriminate between the UAG and UAA codon. In the case of sua101, where there are two changes in the protein, it is not possible to deduce whether both of the changes are necessary for the phenotype, but the change S35Y produces a change in the conserved residue that was implicated as being important in human eRF1 for binding guanines (31-GTx-33 human numbering) and 33-GTS-35 in A. nidulans (Kryuchkova et al. 2013). The S35 residue has been changed and the sua101 strain is unable to terminate at the UAG codons within alx4 and areA600, but it is able to terminate at the UAA codon. The alterations in eRF1 that lead to suppression reduce the efficiency of termination, but the protein must retain some function because there is only one copy of the termination factors eRF1 and eRF3 in A. nidulans, and termination must still be able to occur at normal stop codons. The context of the stop codon is very important. Where stop codons occur through mutation within a coding sequence that are not in a preferred context, these mutations will be more subject to read-through by the altered release factors. Suppression at these codons is dependent on the natural suppressor activity of normal tRNAs; which tRNA will be inserted is presumably a competition between these naturally occurring tRNAs and the release factors (Beier and Grimm 2001).

suA23 has a change in domain 2 (K181N) in the region of the GGQ domain that plays a role in the interaction of eRF1 with the peptidyl transferase center (Frolova et al. 2000) and is also close to the region implicated in the stimulation of the GTPase activity of eRF3 (Cheng et al. 2009). sua27 has a change in domain 3 in the β-sheet (I289S) (Figure 5 and Figure 6). In contrast to sua101-containing and sua23-containing strains, sua23 and sua27 suppress alx4, areA600, and areA601 mutations, thus having specificity for both UAG and UAA mutations. The sua23 and sua27 mutations are less likely to affect the discrimination between the three stop codons but would be more likely to generally reduce the efficiency of chain termination, and so they are able to suppress both the UAG and UAA mutations. The mutation in sua27 is in a conserved amino acid very close to the start of domain 3 that interacts with eRF3. The hydrophobic amino acids in Schizosaccharomyces pombe eRF1: Phe288, Ile291, Tyr298, Phe300, and Phe405 have been implicated in eRF3 interaction (amino acids within A. nidulans -5). From work in S. cerevisiae, it has been found that the structural changes within eRF1 can result in alterations in the level of eRF1, but the structural change itself can alter the read-through observed (Hatin et al. 2009; Merritt et al. 2010). We cannot distinguish between these alternatives in this study. It is evident, however, that mutations within eRF1 can lead to suppression of both UAG and UAA mutations. suaA23, suaA105, suaA101, and suaA32 suppressor strains were crossed to the UGA containing aldA67 mutant strain (Table 1) and there was no suppression of the aldA mutant phenotype at 37°C, but at 25°C some suppression was observed in the suaA23 aldA67 (but not suaA101 aldA67), suaA32 aldA67, and suaA105 aldA67 double mutant, showing that eRF1 acts on UGA as well as UAG and UAA mutations.

Figure 4 Growth tests of wild-type (suA105 or suA23 alx4) in comparison with transformants of an alx4 containing strain with suA23 or suA105:gfpl constructs (see Materials and Methods). (Left) 1% glucose medium and 5 mM ammonium tartrate. (Right) 1% glucose medium with 0.6 mM allantoin as nitrogen source.
The three-dimensional structure of SuaA from *A. nidulans* has been modeled using the crystal structure of eRF1 from *H. sapiens* (PDB ID: 3CT9) as a template through SWISS-MODEL. There is 77% identity between the amino acid sequences of eRF1 from *A. nidulans* and *H. sapiens*. The coordinates for the changes in *snpA1* and *supE6* were taken from Han et al. (2005) and Martinez et al. (2007). The amino acid changes have been highlighted in the following colors: green, *suaA23* (N131I); red, *suaA101* (S35Y, N113K); purple, *snpA6* (E117K); yellow, *supE6* (G265S); blue, *suaA32* (G265S); and white, *suaA27* (I289S). (Left) All the *suaA* mutations on eRF1. (Right) Magnified images of all or parts of domains 1, 2, and 3 to show the positions of the amino acid changes more clearly.

There were two previous publications in which suppressor mutations in eRF1 were described in *A. nidulans*. In the first article, Han et al. (2005) described a mutation in the pantothenyl transferase, *ngA1*, that resulted in a lack of pigmentation in the conidia and the hyphae that could be suppressed by a conditional mutation in a second gene, *snpA6*, at 37° and 42°, but not at 25°. The *snpA6* suppressor was shown to involve an E117K mutation in domain 1 eRF1 (Figure 5) of a UGA stop codon in *ngA1*. Martinez et al. (2007) described a suppressor, *supE7*, of two UAG mutations in the acetyl carnitine transferase carrier protein (*acaH13* and *acaH31* but not *acaH20*) that involved a change in domain 2 of eRF1 (G265S). It was proposed that this amino acid change might affect the conformational properties of the hinge region between domains 2 and 3, interfering with ribosome binding or peptidyl transferase activity (Figure 5). *supE7* also suppressed UAA and UGA mutations in *pol*+ strains (Martinez et al. 2007). The *supE7* suppressor mapped on chromosome III and had very similar properties to the *suaA* and *suaC* suppressors, but it was found by a cross with a *suaA101* to be unlinked to *suaA101*. Because the four *suaA* mutations have sequence changes in eRF1, all these mutations are in eRF1 like *supE7* (it seems likely that the cross data were incorrect but it has not been possible to repeat this cross because the *supE7* strain is no longer viable) and the *suaA* mutations can suppress all three classes of chain termination codons. We propose that *suaE7* and *snpA6* are reclassified as *suaA* mutations, because the *suaA* mutations were the first to be described and *suaA* is located on the genetic map. The only alteration that was found in the *suaA105* strain was in the region upstream of the coding region of the genes at −209 with respect to the start codon. This coincides with an alteration in the 5' slice site, GT to AT, in the upstream ORF. We predicted that this would lead to a failure of splicing of the intron in the mutant that could affect the initiation of translation leading to lower levels of expression of eRF1. In RT-PCR of the wild-type and mutant RNA with a forward primer that split the first intron and a reverse primer that was after the second intron, only a PCR product was seen for the wild-type amplification, and sequencing of this product confirmed the position of the three introns as annotated. A PCR amplification where the forward primer was within the first intron and the reverse primer split the second intron in the coding sequence produced a product for the *suaA105* strain, but not for the wild-type strain, and sequencing of the PCR product confirmed the presence of an unspliced 5' intron but correct splicing of the first two introns in the coding sequence (data not shown). Epitope (-GFP) tagging of the wild-type and mutant proteins resulted in reduced expression in the *suaA105* strain (Figure 3B). As described for other constructs, a genomic fragment containing *suaA105* mutation was fused to the *gfp/riboB* cassette and was used to transform strain H1884 (Table S1) to prototrophy on riboflavin medium. A feasible explanation for the effect of altered mRNA processing due to *suaA105* mutation is at the protein levels, because a 20% reduction is observed in the mutant background compared to wild-type. Lower levels of expression of eRF1 could lead to suppression by the natural tRNAs that are able to translate chain termination codons as sense, because they would be able to compete more efficiently for translation of the termination codons. However, the *suaA105::gfp* transformants did not show suppression of *alcR125* (Figure 4), suggesting an interference of GFP in the functionality of this low-expressed but wild-type SuaA tagged protein.

**suaC codes for eRF3**

The *suaC109* suppressor–containing strain is cold-sensitive (fails to grow at 25°). *suaC* maps on linkage group VII, shows linkage to *choA* (data not shown), and has a similar broad spectrum of suppression to the *suaA* suppressor strains. *suaC109* suppresses the UAG mutations in *alX4*, *alxB125*, and *arcA600*, as well as the UAA mutation in *arcA601* (Sealy-Lewis 1987) and *brlA17*, but not the UAA mutations...
in brlA19 or brlA4 or the UAG mutation in brlA24 (Griffith et al. 1999). suA109 strains were crossed with UGA containing pal2 strains palC143, palF15, palB7, and palB513 (mutations that affect pH regulation) in an attempt to establish whether suaC109 could suppress UGA mutations, but the crosses were infertile. The location of suaC on the genetic map was in the region of eRF3, and so DNA extracted from the suaC109 strain was sequenced and a single change T1186A (Y396N) was found in the sequence predicted to encode eRF3, AN2080. The sequence as annotated has one intron and encodes a protein of 708 amino acids. The eRF3 comprises two domains: the N and M regions (amino acids 1–253 in S. cerevisiae) and the C region (amino acids 254–685) that contain a GTPase fold (amino acids 254–479) and also interact with eRF1 (Kong et al. 2004). Crystallography studies between the eRF1 from H. sapiens and S. pombe and the C-terminus of the eRF3 from S. pombe, which lacks the GTPase domain, has shown that there is an interaction between the C-terminus of eRF3 and the C-terminus of eRF3, but there is additional evidence from small-angle X-ray scattering analysis that an interaction between domain 2 (R192 H. sapiens) of eRF1 is required for stimulation of the GTP-binding and hydrolysis activities of eRF3 (Cheng et al. 2009).

There is considerable variability between eRF3s in the N and M domains in different eukaryotes, but the C-terminal domain is highly conserved. The A. nidulans eRF3 shows no similarity with the ERF3 from H. sapiens in the first 269 amino acids, but thereafter it has 53% identity. S. cerevisiae has no identity over the first 53 amino acids but has 48% identity thereafter, and S. pombe shows similarity over 99% of the protein with 49% identity. In S. cerevisiae, the N-terminal portion of the protein has been shown to be important for the cytoplasmically inherited prion determinant (PSI+), which when aggregated impairs termination and acts as a suppressor of nonsense mutations (Ter-Avanesyan et al. 1994). The N-terminal region contains four tandem repeats of the sequence, PQQGRQQYN, similar to mammalian prion repeats (Stansfield and

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**Figure 6** Multiple alignment of eRF1 sequences. ClustalW alignment of eRF1 from H. sapiens NP_004721, S. cerevisiae NP_009701.3, S. pombe NP_594680.1, and A. nidulans CBF7774 [AN8853 version 5 (aspergillusgenome.org)]. This version of the protein differs from that used by Han et al. (2005) because the most recent annotation includes further modifications of the transcript, which includes an intron in both the 3’ and 5’ non-coding regions and an additional intron at the 3’ end of the coding region, resulting in a protein that is 10 amino acids shorter at the C-terminus. *The positions where there is a fully conserved residue; () indicates that one of the following “strong” amino acids is conserved: STA NEQK, NHQK, NDEQ, MIL, MILF, HY, or FWY; and () denotes that one of the following “weaker” amino acids is conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NEQHRK, FVLM, or HFY. Domain 1 of the protein using the H. sapiens numbering of the sequence is from amino acids 4 to 140, domain 2 is from amino acids 144 to 276, and domain 3 is from 279 to 417. Conserved functional motifs have been boxed and the mutational positions of the suppressors in suaA have been highlighted in the A. nidulans sequence, with the mutation indicated above the alignment.
Tuite 1994; Lindquist 1997). This sequence is missing from the sequence in *S. pombe* and *A. nidulans*, but *S. pombe* contains a region that is rich in repeats of APST (Ito et al. 1998) that, again, is not a feature of the *A. nidulans* sequence. Examination of the *A. nidulans* eRF3 for repeated elements using REPRO (http://www.ibi.vu.nl/programs/reprowww/) did not reveal any obvious repeats, but there was a preponderance of Glu, Ala, and Tyr amino acids between amino acids 53 to 156 in the N-terminus that would produce a very

Figure 7 Multiple alignment of ERF3. Alignments using ClustalW are shown for *A. nidulans* XP_659684.1 (locus tag AN2080), *S. cerevisiae* AFD29160.1, *S. pombe* NP_588225, and *H. sapiens* ERF3A NP-002085.2. There are two genes in mammals that code for eRF3, eRF3A, and eRF3B, and they differ in their N-termini. We have used eRF3A for the alignment because the expression levels of this gene have been shown to control the formation of the termination complex (Chauvin et al. 2005). The explanation of conservations underneath the alignment is explained in Figure 6. Amino acid changes in the suppressor strains are indicated above the alignment with the amino acid that has change shaded. There are repeated sequences reported for both *S. cerevisiae* and *S. pombe*, and these are underlined in the respective sequences (see text for references).
hydrophilic flexible region. In the meiotic analysis of many nonsense suppressor mutations in \textit{A. nidulans}, there has been never been any evidence of cytoplasmic inheritance as exhibited by the PSI* strain in \textit{S. cerevisiae} and, therefore, no evidence of prion formation (H. M. Sealy-Lewis, unpublished data).

The alteration in eRF3 in \textit{suaC109} was in the C-terminus and has been located using the crystal structure of \textit{S. pombe} for modeling (Table 2, Figure 7 and Figure 8). We also sequenced a mutant strain that was selected as a revertant of the cold-sensitive phenotype of \textit{suaC109}. The \textit{alx4} and \textit{sba4} mutations were still suppressed to some extent in the revertant strain at both 37°C and 25°C. In an outcross between the \textit{suaC109} revertant strain and the wild-type, there was no segregation of the cold-sensitive phenotype and we concluded that the mutations were allelic. Sequencing of the \textit{suaC109} revertant strain revealed the change observed in \textit{suaC109} together with a second change (N415H). The revertant of the \textit{suaC109} phenotype has been renamed \textit{suaC500}. Both changes are thus in the C-terminus (Table 2, Figure 7 and Figure 8). The revertant still had suppressor activity but was cold-insensitive. The cold sensitivity was thus a property of the altered eRF3 and not a consequence of a read-through product in an unrelated gene. Both \textit{suaA} and \textit{suaC} mutations result in a number of pleiotropic changes, which could result from read-through of proteins; in addition, it has been suggested for both eRF1 and eRF3 that they might have a translation-independent role, because eRF1 and the myosin-light chain have been shown to interact in \textit{S. cerevisiae} and mutations in eRF1 can suppress defects in cytokinesis (Valouev et al. 2004). The N-terminal region of eRF3 in \textit{S. cerevisiae} has also been shown to interact with other proteins such as Ltt1p (a protein of unknown function) and Slap1, which is involved in cytoskeletal assembly; this suggests a translation-independent role for eRF3 (Bailleul et al. 1999; Urakov et al. 2001).

The reason for the cold sensitivity in the \textit{suaC109} strain is unknown, but it could involve a failure to assemble with eRF1, GTP, or the ribosome at lower temperatures, as has been described for ribosome assembly in \textit{E. coli} (Guthrie et al. 1969). This could be reversed by further changes within the eRF3 molecule. Also, in \textit{S. cerevisiae} it has been shown that the GTPase domain of eRF3 interacts with Upf1p, which is involved in nonsense-mediated decay, and any changes that interfere with their interaction could also lead to nonsense suppression (Amrani et al. 2006; Ivanov et al. 2008). Genome-wide interaction studies have identified a large number of interactions between eRF3 and eRF1 and other proteins in \textit{S. cerevisiae} (von der Haar and Tuite 2007), and there may be other proteins that interact with the GTPase domain of eRF3.

CONCLUSION

The suppressor mutations in eRF1 are found in all three domains of the protein but in eRF3 they are confined to the conserved C-terminal domain, which is similar to the findings in \textit{S. cerevisiae} (Merritt et al. 2010). The \textit{suaA} and \textit{suaC} suppressor strains were characterized by the fact that each mutant has a distinct but unique phenotype, and it is clear that the diverse interactions of eRF1 and eRF3 can lead to these properties. With regard to the \textit{suaA} suppressors, they can result in temperature-sensitive phenotypes for some suppressed proteins but not others [e.g., \textit{suaA27} and \textit{araC600} or \textit{suaA101} and \textit{amdS1005} result in a phenotype where growth is stronger at 25°C than 37°C (Sealy-Lewis 1987)]. This suggests that the same amino acid is not inserted at the stop codon for the different suppressor alleles and implies that the different eRF3 mutants compete differently with the natural suppressor tRNAs in the cell. The molecular details of how the release factors interact both with each other and with other factors on the ribosome should yield further insights into the process.

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