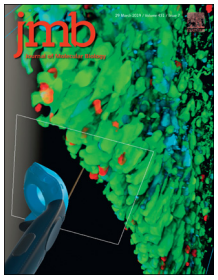

Alternative Translation Initiation at a UUG Codon Gives Rise to Two Functional Variants of the Mitochondrial Protein Kgd4

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Abstract

Kgd4 is a novel subunit of the mitochondrial α-ketoglutarate dehydrogenase complex (KGDH). In yeast, the protein is present in two forms of unknown origin, as there is only one open reading frame and no alternative splicing. Here, we show that the two forms of Kgd4 derive from one mRNA that is translated by employing two alternative start sites. The standard, annotated AUG codon gives rise to the short form of the protein, while an upstream UUG codon is utilized to generate the larger form. However, both forms can be efficiently imported into mitochondria and stably incorporate into KGDH to support its activity. Translation of the long variant depends on sequences directly upstream of the alternative initiation site, demonstrating that translation initiation and its efficiency are dictated by the sequence context surrounding a specific codon. In summary, the two forms of Kgd4 follow a very unusual biogenesis pathway, supporting the notion that translation initiation in yeast is more flexible than it is widely recognized.

Introduction

We recently identified the yeast mitochondrial protein Kgd4 as a novel subunit of the citric acid cycle enzyme complex α-ketoglutarate dehydrogenase (KGDH) [1]. The conserved role of Kgd4 and its mammalian homologs is to recruit the E3 subunit to the core of KGDH consisting of the E1 and the E2 subunits, acting as a stability factor for the fully assembled complex. In yeast mitochondria, Kgd4 is present in two forms and it remained unclear how both variants are produced. Because *KGD4* does not contain any introns, alternative splicing cannot be responsible for the two variants of its gene product. Here, we set out to identify the origin of the two Kgd4 isoforms and found that biogenesis of the large form employs an UUG codon that is present 90 nucleotides upstream and in frame of the annotated start-codon. Recognition of this alternative start codon critically depends on the sequence context preceding the UUG codon, thereby demonstrating that the sequence context can be more relevant for the initiation efficiency than the identity of the start codon.

Results

Two Kgd4 forms that differ in their N-termini

Kgd4, the product of open reading frame *YFR049W*, is present as two isoforms in yeast mitochondria with one long (Kgd4L) and one short variant (Kgd4S) that have an apparent molecular weight of 20 or 15 kDa, respectively (Fig. 1). To determine whether both forms differ in their N- or C-termini, we generated yeast strains carrying either a His7-tag before the stop codon of *KGD4* at its authentic chromosomal location. Consequently, the tagged forms of Kgd4 are expressed under the control of the native *KGD4* promotor. Western blot analyses revealed that also the tagged variants of Kgd4 are expressed as a long and a short form (Fig. 1A). Both isoforms of Kgd4-His7

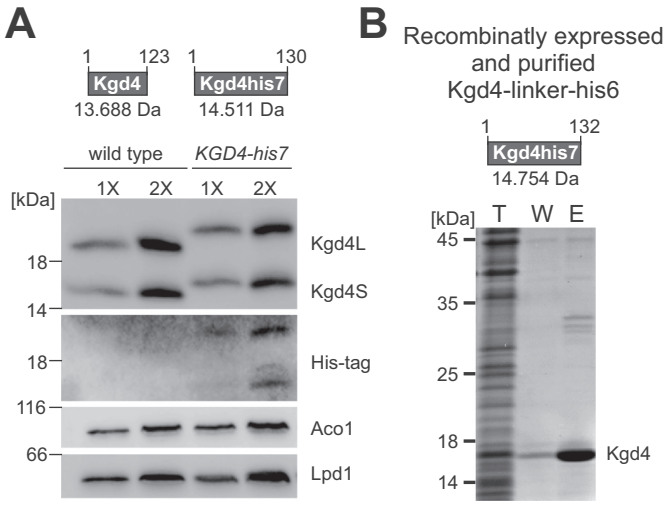

Fig. 1. Kgd4 is present in two isoforms that differ in their N-termini. (A) Yeast strains were generated that expressed a C-terminally His tagged form of Kgd4 from the authentic chromosomal location. The mitochondria containing the indicated variants of Kgd4 were lysed, separated on SDS-PAGE, and analyzed by Western blotting with the indicated antibodies. Aco1 and Lpd1 serve as controls. (B) The annotated open reading frame of Kgd4 was cloned into pET28, recombinantly expressed, and purified. T, total; W, wash fraction; E, eluate.

are present in roughly similar quantities, revealing efficient production of both forms. Because the C-termini are identical, we concluded that the two variants of Kgd4 differ in their N-termini. Finally, we recombinantly expressed and purified the annotated open reading frame of Kgd4 with a short linker followed by a His6 tag (Fig. 1B). This protein had an apparent molecular weight of 16.5 kDa, which is close to that of the short form. Because mitochondrial targeting sequences are typically removed upon import by the mitochondrial processing peptidase, the similar sizes of the recombinantly expressed proteins and Kgd4S suggest that Kgd4S in mitochondria does not contain a cleavable mitochondrial import signal.

An alternative start-codon accounts for the production of Kgd4L

The annotated size of Kgd4 predicts a protein of 123 amino acids (13.7 kDa), which is substantially smaller than the apparent molecular weight of Kgd4L of around 20 kDa (Fig. 1). Sequencing of the 5′-untranslated regions of the yeast transcriptome [2,3] only revealed evidence for a single mRNA species encoding Kgd4, which contains 160 nucleotides before the start codon. Interestingly, additional coding sequence that is not interrupted by a stop codon and that is in frame with the reading frame of *KGD4* is present upstream of the annotated start codon. It has been shown that translation in yeast can also occur at non-methionine codons, but with lower efficiency [4]. In fact, all codons that differ from AUG by one nucleotide (with the exception of AAG and AGG) can serve as alternative translation initiators. Screening of the upstream coding sequence of Kgd4 revealed several candidate codons for non-AUG translation initiation [4]. Among them, UUG, allows for a 50% initiation efficiency as compared to AUG [4]. The UUG at −90 could be one of the most distal alternative start codon (Fig. 2A). The encoded protein would be 30 amino acids longer than the annotated sequence and the additional stretch has a high probability for mitochondrial protein import; hence, the protein produced from this UUG codon could therefore well be the source of the Kgd4L.

Plasmid-expressed forms of Kgd4l and Kgd4s correspond to the wild-type isoforms

In order to test whether Kgd4L indeed arises from translation initiation at UUG from position −90, we employed two different approaches. First, we cloned

Fig. 2. An alternative start-codon could account for the long form of Kgd4. (A) The RNA sequence surrounding the annotated AUG codon of yeast *KGD4* is depicted. The UUG codon at −90 could serve as alternative initiation site. The resulting translation product has a predicted import probability of 0.8497, while that of the protein starting from the annotated start codon is 0.7442 [5]. (B) Two plasmids were constructed allowing to synthesize *in vitro* Kgd4S or Kgd4L in the presence of [^{35}S]-methionine. Both forms were imported into a protease-resistant location in isolated, energized mitochondria. PK, proteinase K; Δψ, membrane potential; prec, precursor protein. (C) Individual expression of Kgd4L or Kgd4S in *kgd4Δ* cells reveals that both isoforms indeed stem from alternative usage of start codons. Both *KGD4* forms were cloned into a yeast expression vector and used to transform a *kgd4Δ* strain. Proteins of these strains were extracted and analyzed by Western blotting. Aco1 serves as loading control. (D) Schematic depiction of the fusion between *KGD4* flanking regions and GFP coding sequences. The mRNAs produced from the different variants are depicted. (E) Western blot analysis of cells expressing GFP under the control of *KGD4* flanking regions. (F) Fluorescence microscopy of cells expressing the different GFP forms. Cells were counterstained with mitotracker to indicate the distribution of mitochondria. Scale bar is 5 μm.

both forms into a plasmid enabling *in vitro* translation in the presence of [^{35}S]-methionine. The construct producing Kgd4L contained the regular Kgd4 sequence but lacked the annotated start codon that was changed to an alanine codon. In addition, it harbored the upstream coding sequence with the hypothetical

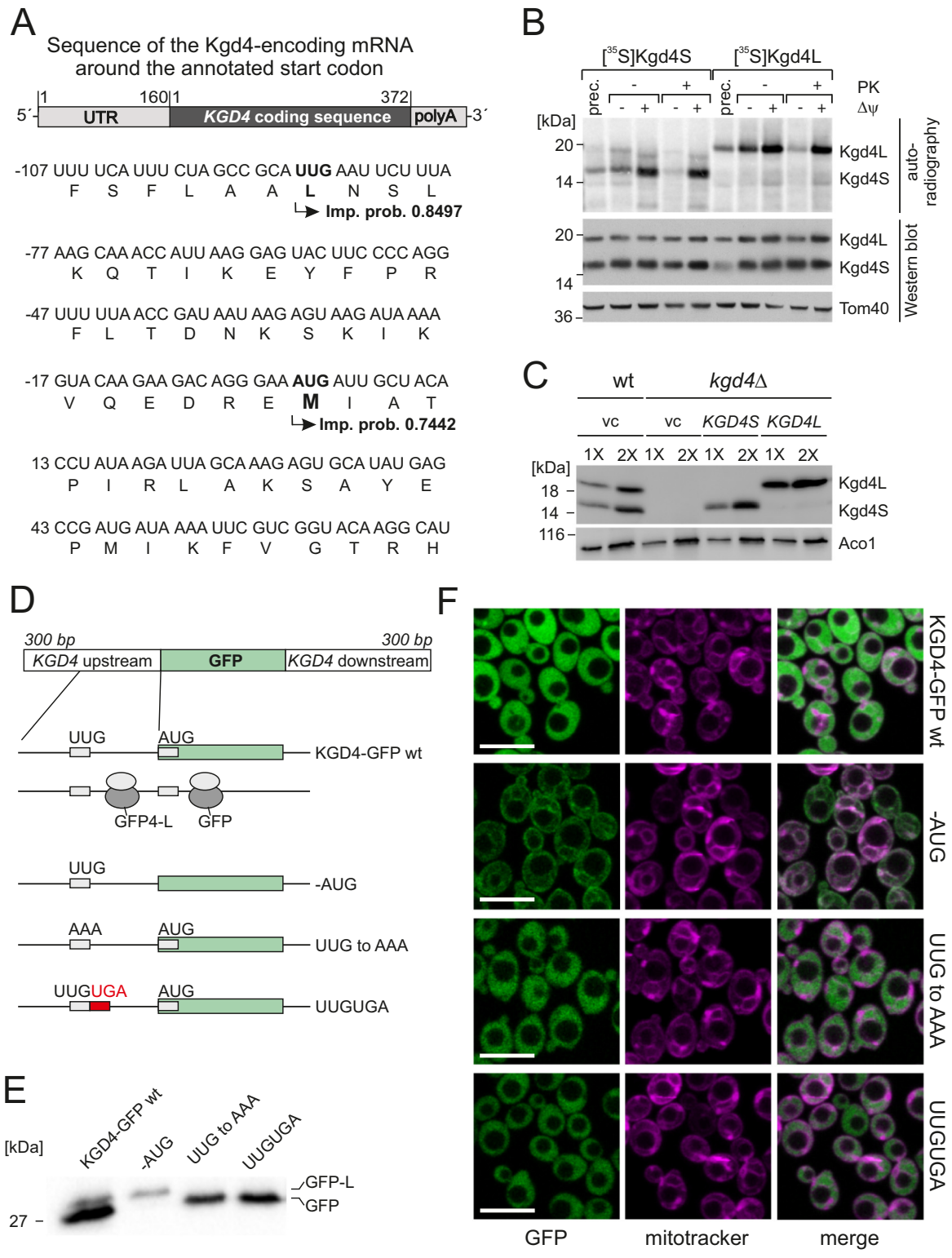

Fig. 2 (*legend on previous page*)

alternative start UUG changed to AUG. The construct designed to express Kgd4S contained the unaltered annotated Kgd4 coding sequence. The *in vitro* translations resulted in two radioactively labeled proteins that had similar sizes as the authentic Kgd4 forms (Fig. 2B). When exposed to energized isolated mitochondria, both proteins were imported into the mitochondrial matrix in a membrane potential dependent manner (Fig. 2B), in line with the predicted probability of both forms for mitochondrial import (Fig. 2A). Importantly, none of these proteins were proteolytically cleaved upon import, indicating the absence of a cleavable mitochondrial targeting signal in both proteins. Next, we generated two different plasmids for expression of the two variants in a yeast strain lacking *KGD4*. As expected, the coding sequence containing the annotated start AUG gave rise to the Kgd4S (Fig. 2C). When we changed the UUG at −90 to AUG and expressed this construct, exclusively Kgd4L was detected (Fig. 2C).

To further test and characterize the cryptic start site present in the 5′-UTR of the KGD4 mRNA, we constructed a gene expressing a chimeric construct consisting of 300 bp upstream of the Kgd4-encoding open reading frame followed by sequences encoding GFP. In this construct, designated KGD4-GFP wt, two different variants of GFP can be produced, namely, normal GFP and a form that contains the additional N-terminal segment deriving from the upstream initiation event (Fig. 2D and E). As expected, GFP was evenly distributed in the cytoplasm of yeast cells, as revealed by confocal microscopy (Fig. 2F). When the start codon of GFP was removed, only GFP-L was produced (Fig. 2E) that was at least partially localized to mitochondria (Fig. 2F). We next mapped more directly the alternative start site by two overlapping approaches. First, we replaced the near-cognate UUG codon by AAA, and second, we inserted directly after the UUG codon the stop codon UGA (Fig. 2D). As predicted, these mutations abrogated the production of GFP-L and resulted in a robust cytosolic GFP signal in the yeast cells (Fig. 2F). Taken together, these data confirm that Kgd4L is the product of alternative initiation at an UUG codon in the 5′-UTR of the *KGD4* mRNA.

Kgd4S and Kgd4L both contribute to KGDH activity and stability

We next asked if both Kgd4 forms are equally efficient in supporting the function of KGDH. The main molecular function of Kgd4 is to recruit the E3 subunit Lpd1 into the complex [1]. To this end, it has a C-terminal domain that interacts with the E1 subunit

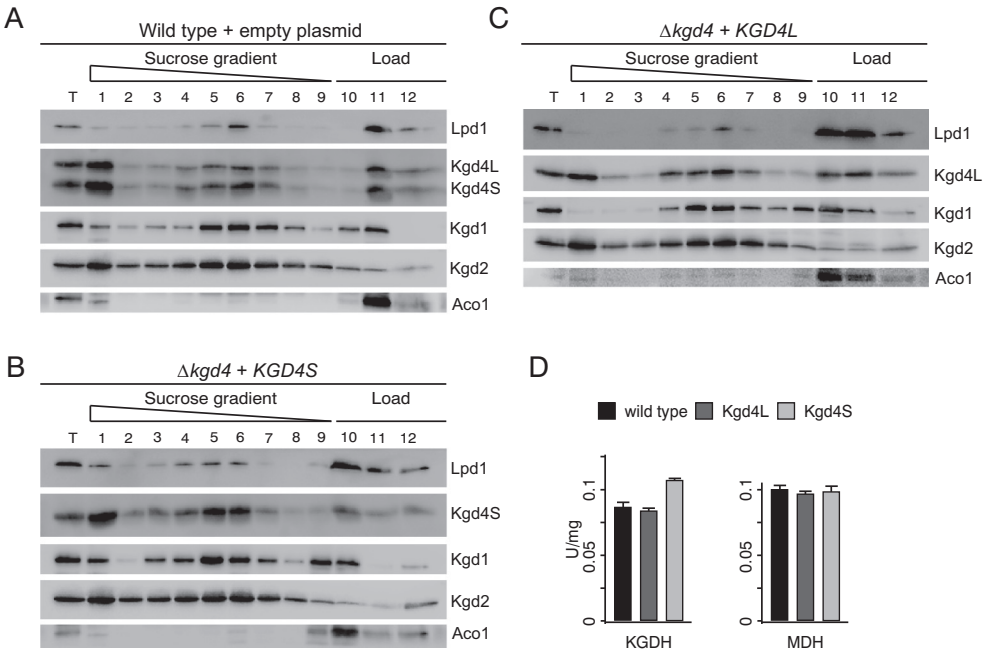

Fig. 3. Kgd4S and Kgd4L both contribute to KGDH activity and stability. (A) Kgd4L and Kgd4S co-migrate with subunits of KGDH. Mitochondria from the wild type transformed with an empty plasmid were lysed, separated on a linear sucrose gradient and analyzed by Western blotting. (B) Kgd4S co-migrates with KGDH on sucrose gradients. Isolated mitochondria from a *kgd4Δ* strain expressing Kgd4S were processed as in panel A. (C) Kgd4L co-migrates with KGDH on sucrose gradients. Isolated mitochondria from a *kgd4Δ* strain expressing Kgd4L were processed as in panel A. (D) KGDH or MDH activity of cells containing either the short or the long Kgd4 form. The specific activity of either KGDH or MDH of three independent experiments is depicted.

Kgd1 and an N-terminal domain that interacts with Lpd1 [1], and both domains are present in Kgd4S and Kgd4L. We first set out to analyze how both forms interact with KGDH and prepared mitochondria from wild type or the *kgd4Δ* strain expressing either Kgd4S or Kgd4L. When lysates of these mitochondria were separated by centrifugation on linear sucrose gradients, both forms co-migrated with KGDH (Fig. 3A–C). Importantly, both Kgd4S and Kgd4L were able to recruit the E3 subunit Lpd1 into the complex (Fig. 3A–C) demonstrating that the main function of Kgd4 is preserved in both isoforms. Consistently, mitochondria from strains expressing Kgd4L had KGDH activity that was similar to the wild type, while Kgd4S results in slightly increased specific KGDH activities (Fig. 3D). The activity of malate dehydrogenase (MDH) that served as a control was not changed (Fig. 3D).

Sequences directly preceding the alternative start site determine efficiency of initiation

Next we asked which role the sequence context around the alternative start site plays in determining translation efficiency from this second initiation site. Generally, the translation start site is identified in eukaryotes through a mechanism by which the 48S pre-initiation complex scans the mRNA from the CAP in 3′ direction to find a start codon. Once the complex encounters a codon with sufficient base-pairing to the initiator methionyl-tRNA, the ribosome assembles and translation starts. The sequence context directly upstream of the standard AUG codon determines translation efficiencies [6,7], possibly leading to a pause of the 48S complex on this stretch to facilitate codon–anticodon interaction. We therefore hypothesized that the sequence context surrounding the UUG codon is important for the production of Kgd4L. However, the sequence upstream of the UUG codon responsible for production of Kgd4L (Fig. 4A) is far from the optimal, A-rich sequences found in front of highly translated mRNAs [6,7]. This suggests that sequence context in broader terms determines usage of the UUG codon. To test the significance of the sequence context for initiation efficiency, we exchanged the 10 nucleotides upstream of the UUG codon with sequences shown to lead to either low or high translation levels [7]. These variants and the wild type sequence were expressed from an integrative plasmid containing the authentic promotor. As predicted, changing the sequence context to a variant with low translation efficiency abrogated production of Kgd4L and increased production of Kgd4S, while one with high translation efficiency lead to the production of Kgd4L to similar quantities as from the wild type mRNA (Fig. 4B). We therefore concluded that the nucleotide sequence directly preceding the UUG codon determines the levels by which the UUG codon is used for initiation and that in this case initiation at the annotated AUG is abrogated by the efficient translation from the UUG codon.

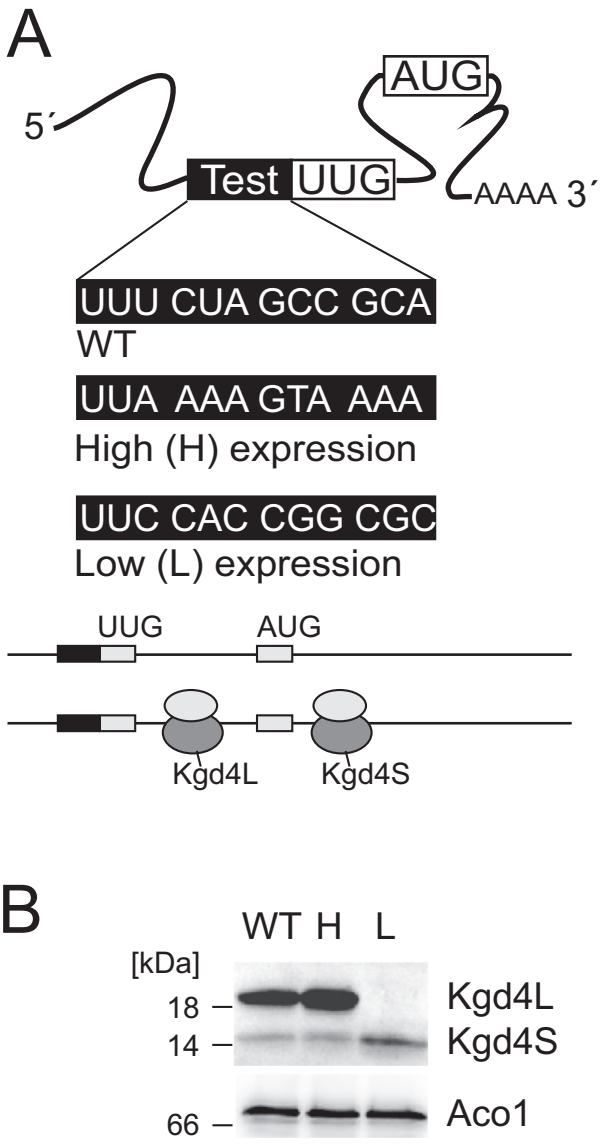

Fig. 4. The sequence context upstream of the UUG codon determines production of Kgd4L. (A) The sequence directly preceding the UUG codon was replaced by a 10 nucleotide sequence shown to be in front of ORFs with either low (L) or high expression levels [7] to influence the relative abundance of Kgd4S or Kgd4L synthesis. (B) The constructs presented in panel A were cloned into an integrative plasmid that was used to transform a *kgd4Δ* strain. Proteins were extracted, separated on SDS-PAGE, and analyzed by Western blotting.

Discussion

In the present study, we describe a novel example of non-AUG translation initiation in *Saccharomyces*

cerevisiae. The *KGD4* mRNA gives rise to two proteins, which differ by 30 amino acids at their N-terminus. The short version originates from the annotated start-AUG. Translation of the long version starts from an UUG at position −90. Interestingly, both versions of Kgd4 are imported into mitochondria but are not cleaved by mitochondrial processing peptidase. The fact that they exhibit substantial steady-state protein levels points to a robust translation initiation not only at the annotated AUG but also at UUG −90.

Translation initiation at codons that differ from AUG in one nucleotide is a frequently occurring phenomenon in higher eukaryotes [8,9] that contributes to protein diversification similarly as alternative mRNA splicing [10–12]. Specifically, translation initiation at alternative start-codons can modulate the relative levels of distinct protein isoforms [13]. In contrast, there are only few documented examples of translation initiation at alternative start codons in yeast. So far, this has been described for *GRS1* [14], *ALA1* [15] and *HFA1* [16] in *S. cerevisiae* and for *CARP2A* in *Candida albicans* [17]. Grs1 is a glycyl-tRNA synthetase, which has a cytoplasmatic and a mitochondrial isoform. The cytoplasmatic protein is translated from a traditional AUG codon, while translation of the mitochondrial isoform starts at an UUG codon which is located upstream and in-frame on the same open reading frame. The 23-amino-acid extension of the mitochondrial isoform can also independently act as a mitochondrial targeting sequence, underlining the distinct functions of the two transcripts [15]. Also in the case of *ALA1*, which encodes the alanyl-tRNA synthetase, the shorter translation product is initiated at an AUG codon and represents the cytosolic form of the protein, while the longer translation product, which is initiated at two redundant upstream in-frame ACG codons, is imported into mitochondria [14]. Similarly, an AUU at position −372 relative to the annotated AUG in *HFA1* is used to express a mitochondrially targeted variant of the acetyl-coenzyme A carboxylase [16]. Hence, these cases of translation initiation at non-AUG codons have significance for the cellular localization of the respective proteins. This is not the case for Kgd4. Here, both isoforms are imported into mitochondria where they interact with the same enzyme-complex and support KGDH activity *in vitro* similarly, while Kgd4 homologs in other species like humans occur in only one isoform [1].

The efficiency of translation at non-AUG codons strongly depends on the sequence context and, more specifically, on three upstream-neighboring nucleotides [13]. The nucleotide at −93 in the Kgd4-encoding mRNA is a G. Consistently, a purine at position −3 relative to the non-AUG start-codon has been shown to compensate for the weaker base-pairing between the initiator t-RNA and the non-AUG codon [8,18]. We have here exchanged the entire 10 nucleotides prior to the UUG codon and found that this stretch determines the efficiency by which UUG is used for initiation. During initiation, the 48S pre-initiation complex scans the mRNA for a codon that can efficiently base-pair with the anticodon of the initiator tRNA. It is likely that the geometry by which mRNA and the small ribosomal subunit interact influences the efficiency by which base-pairing can occur. Such a notion is supported by the recent finding that the protein Rps26A from the yeast small ribosomal subunit is involved in the specific interaction between the sequence preceding the start codon and the ribosome to modulate the affinity of ribosomes to mRNAs [19]. Our findings that the sequence context surrounding a near cognate codon can be more important than the identity of the codon itself, likely explains translational plasticity, as revealed, for example, by ribosome profiling [20,21]. This can generate variability in the proteome, allowing sequences that turn out to be beneficial to be fixed through selection. Hence, the observation that Kgd4 is translated from two different start-codons demonstrates that translation–initiation in yeast is more flexible than generally anticipated, creating proteins that are difficult to predict by bioinformatic tools.

Materials and Methods

Yeast strains and growth media

All strains used in this study were isogenic to the wild-type strain YPH499. Yeast cultures were grown at 30 °C in YP (1% yeast extract, 2% peptone) medium supplemented with 2% dextrose (D), 2% galactose (Gal), or 2% glycerol (G). His7- or Protein A-tagged variants of Kgd4 were generated by directed homologous recombination as described [22]. *KGD4* was disrupted using a kanamycine resistance cassette (*KanMX*). Kgd4L and Kgd4S were generated by site-directed mutagenesis and cloned into pYX142 plasmids or pGEM4. Likewise, the variants expressing Kgd4 or GFP from mRNAs with changed composition in the 5′-UTR were cloned into the integrative plasmid pRS305, including the full promotor and terminator region of the wild-type *KGD4* gene.

Purification of Kgd4-His6

E. coli BL21 (DE3) cells containing plasmid pET21b-Kgd4 were grown in LB_{AMP} at 37 °C to an OD_{600} of 0.6. Cells were induced with 1 mM IPTG for 4 h at 30 °C after which they were washed with water and lysed by sonication in buffer A [1% Triton X-100, 150 mM NaCl, 40 mM imidazole (pH 7.4), 2 mM PMSF, 1 × complete protease inhibitors, 20 mM Tris (pH 8.0)]. After a clarifying spin for 10 min at 25,000*g*, 4 °C, tagged proteins were purified at 4 °C

on NiNTA beads (Quiagen, Germany). The beads were washed three times with buffer B [0.1% Triton X-100, 150 mM NaCl, 65 mM imidazole, 20 mM Tris (pH 8.0)] and eluted with 500 mM imidazole pH 7.4.

KGDH activity assay

Isolated mitochondria were lysed in buffer containing 1% Triton X-100 and 50 mM KPi (pH 7.4) for 10 min at 4 °C. After a clarifying spin for 10 min at 25,000*g* and 4 °C, the lysate was mixed with 400 µl assay buffer [2.5 mM NAD, 0.2 mM thiamine pyrophosphate, 0.1 mM coenzyme A, 0.3 mM dithiothreitol, 1 mM $MgCl_2$, and 50 mM KPi (pH 7.4)]. The reaction was started by the addition of 5 mM α-ketoglutarate. KGDH activity was followed by measuring the NADH production at 340 nm.

MDH activity assay

Isolated mitochondria were adjusted to a concentration of 1 mg/ml in isotonic buffer [0.6 M sorbitol, 20 mM Hepes (pH 7.4)]. To start the reaction, 200 µl of this solution was added to 1 ml assay buffer [2.5 mM NAD, 37 mM DL-malate, 50 mM glycine (pH 10.0)]. MDH activity was followed by measuring the NADH production at 340 nm.

Fractionation of mitochondrial protein complexes on linear sucrose gradients

Isolated mitochondria were lysed for 15 min at 4 °C in lysis buffer [1% Triton X-100, 150 mM KCl, 20 mM Hepes (pH 7.4), 1 mM PMSF and 1× complete Protease Inhibitor Mix (Roche)]. After a clarifying spin for 10 min at 25,000*g*, 4 °C, the extract was layered on top of a continuous 4 ml sucrose gradient [0.3–1 M sucrose, 0.1% Triton X-100, 25 mM KCl, 20 mM Hepes (pH 7.4), 1 mM PMSF] and centrifuged for 1 h and 15 min at 484,183*g* and 4 °C in an SW60 Ti rotor (Beckman Coulter, Fullerton, CA). Fractions were collected and proteins were precipitated with 12% trichloroacetic acid.

In vitro import of preproteins into mitochondria

For generation of [^{35}S]-methionine-radiolabeled preproteins, the TNT coupled kit (Promega) was used. Precursor proteins were precipitated with saturated ammonium sulfate solution and denatured in urea buffer [8 M urea, 30 Mops–KOH (pH 7.2), 20 mM DTT]. Isolated mitochondria and precursor proteins were incubated in import buffer [10 mM Mops–KOH (pH 7.2), 3% (w/v) bovine serum albumin, 250 mM sucrose, 5 mM $MgCl_2$, 80 mM KCl, 5 mM KP_i] supplied with 2 mM ATP and 2 mM NADH at 30 °C for 45 min. For dissipation of the membrane potential 1 µM valinomycin, 20 µM oligomycin, and 8 µM antimycin A was added to the reactions. Samples were treated with 100 µg/ml proteinase K for 10 min on ice followed by 10-min incubation with 2 mM PMSF. Mitochondria were reisolated and washed with SEM buffer [250 mM sucrose, 1 mM EDTA, 10 mM Mops–KOH (pH 7.2)]. Samples were analyzed by SDS-PAGE and digital autoradiography (PhosphorImager; Molecular Dynamics).

Fluorescence microscopy

To study the intracellular localization of the GFP signal, 2×10^7 cells were harvested after 8 h growing at 28 °C in YPD, washed once in PBS, and immediately visualized using a Zeiss LSM800 laser scanning confocal microscope equipped with a Plan-Apo 63x/1.40 oil immersion objective. Mitochondrial morphology was observed using the fluorescent dye Mitotracker Red CMXRos (Invitrogen). For this purpose, 2×10^7 cells were harvested and resuspended in 500 µl PBS containing 200 nM Mitotracker Red CMXRos. After incubation for 10 min at room temperature in the dark, cells were washed in PBS and analyzed as described above. All microscopic pictures were analyzed and processed with the open-source software Fiji [23].

Miscellaneous methods

Mitochondria were isolated as previously described [22].

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†M.H.and M.N. contributed equally to this study.

Abbreviations used:
KGDH, α-ketoglutarate dehydrogenase; MDH, malate dehydrogenase.

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