# Mitochondrial cysteinyl-tRNA synthetase is expressed via alternative transcriptional initiation regulated by energy metabolism in yeast cells

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Eukaryotes typically utilize two distinct aminoacyl-tRNA synthetase isoforms, one for cytosolic and one for mitochondrial protein synthesis. However, the genome of budding yeast (*Saccharomyces cerevisiae*) contains only one cysteinyl-tRNA synthetase gene (*YNL247W*, also known as *CRS1*). In this study, we report that *CRS1* encodes both cytosolic and mitochondrial isoforms. The 5′ complementary DNA end method and GFP reporter gene analyses indicated that yeast *CRS1* expression yields two classes of mRNAs through alternative transcription starts: a long mRNA containing a mitochondrial targeting sequence and a short mRNA lacking this targeting sequence. We found that the mitochondrial Crs1 is the product of translation from the first initiation AUG codon on the long mRNA, whereas the cytosolic Crs1 is produced from the second in-frame AUG codon on the short mRNA. Genetic analysis and a ChIP assay revealed that the transcription factor heme activator protein (Hap) complex, which is involved in mitochondrial biogenesis, determines the transcription start sites of the *CRS1* gene. We also noted that Hap complex–dependent initiation is regulated according to the needs of mitochondrial energy production. The results of our study indicate energy-dependent initiation of alternative transcription of *CRS1* that results in production of two Crs1 isoforms, a finding that suggests Crs1's potential involvement in mitochondrial energy metabolism in yeast.

Aminoacyl-tRNA synthetase (ARS)[2] is an ancestral housekeeping enzyme found in all organisms. ARSs are essential for precise translation of the genetic code and required for correct ligation of an amino acid to its cognate tRNA, thereby forming aminoacyl-tRNA (1). These aminoacyl-tRNAs are used as substrates for codon recognition and polymerization of amino acids in ribosomes. Prokaryotes typically have 20 ARSs that are responsible for amino acid aminoacylation and production of aminoacyl-tRNAs. In eukaryotes, the translation events occur in both the cytosol and mitochondria (2, 3). Hence, eukaryotes commonly use two distinct sets of ARSs: one functioning in the cytosol and the other operating in mitochondria. Mitochondrial ARSs are encoded by nuclear DNA, are translated in the cytosol, and are transported to organelles (mitochondria), unlike cytosolic ARSs. Mitochondrial ARSs are generally encoded by distinct genes different from those encoding cytosolic ARSs (4, 5). Some ARSs are encoded by single genes. All ARS genes can typically be distinguished on the basis of phylogenetic analyses, which shows that most mitochondrial ARSs derive from proteobacterial ARSs, but, in some cases, the proteobacterial endosymbiotic origins of the mitochondrial ARSs are unknown, possibly because of gene replacements, intranuclear gene duplications, and horizontal gene transfers (6). The exact alternative mechanisms of substitution for such missing ARS genes have remained unclear.

Some ARSs reportedly function in physiological processes other than translation, called moonlighting functions (7, 8). For example, although Cys-tRNA synthetase (CysRS, also called CARS) mediates the translation process by catalyzing Cys-tRNA synthesis (*i.e.* a canonical function), our earlier study showed that CysRS has another important function: to catalyze the biosynthesis of cysteine persulfides; *i.e.* it acts as a cysteine persulfide synthase (CPERS) (9). Therefore, the CPERS activity of CysRS is a new function of ARSs beyond translation, which is, in fact, the major pathway for production of endogenous reactive persulfides that is observed in many organisms. In the context of this unique persulfide function, we recently found that cysteine persulfides produced by CysRS in mitochondria can

This article contains Figs. S1–S4, Tables S1 and S2, and experimental procedures.
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[2] The abbreviations used are: ARS, aminoacyl-tRNA synthetase; CysRS, cysteinyl-tRNA synthetase; CPERS, cysteine persulfide synthase; MTS, mitochon-drial targeting signal; cDNA, complementary DNA; 5′-RACE, rapid amplification of 5′ cDNA ends; qPCR, real-time quantitative PCR; RSC, chromatin structure–remodeling complex; ANOVA, analysis of variance; L, log phase; ES, early stationary phase; LS, late stationary phase; Hap, heme activator protein; Ct, threshold cycle number.

support and up-regulate mitochondrial biogenesis and bioenergetics in eukaryotes (9, 10). Therefore, mitochondrial transport and localization of CysRS are presumably crucial steps for energy metabolism in addition to translation in mitochondria, although this view is still hypothetical and needs to be verified.

Nuclearly encoded mitochondrial proteins commonly contain targeting signals at their N termini, called mitochondrial targeting signals (MTSs), which localize them in mitochondria; these MTSs constitute proteolytically cleavable sequences or N termini of mature proteins that remain after being transported and located in mitochondria (11, 12). MTSs manifest great variety in their lengths and sequences (13). In fact, no sequence conservation or structural motifs of MTSs have been identified for mitochondrial proteins. The only common feature of MTSs is that they contain basic, hydroxylated, and hydrophobic residues that confer a tendency to form an $\alpha$-helices (13). This amphipathic nature is thought to play an important role in the recognition of mitochondrial receptors for transportation.

The genome of *Saccharomyces cerevisiae* lacks specific mitochondrial ARSs catalyzing aminoacylation of Ala, Gln, His, Val, and Cys for tRNA. In these cases, both cytosolic and mitochondrial isoforms are encoded and translated by a single gene via different mechanisms: alternative transcription initiation, as shown for the His-tRNA synthetase (14, 16) and Val-tRNA synthetase (15), and alternative translation started at a noncanonical initiation codon, as documented for the Ala-tRNA synthetase (17). These alternative pathways for aminoacyl-tRNA synthesis allow expression of MTS-containing ARSs (mitochondrial types) and ARSs without MTS (cytosolic types) from single genes of individual ARSs. However, the regulatory mechanism for the alternative yeast CysRS protein biosynthesis remains unclear.

In this study, we investigated the mitochondrial localization mechanism of CysRS (gene *YNL247W*, called *CRS1* here). Our study indicates that, although *CRS1* encodes both cytosolic and mitochondrial isoforms, transcription and translation of the mitochondrial CysRS are activated by an alternative transcription initiation of the single *CRS1* gene. This translational activation of *CRS1* is governed by a key transcription factor, *i.e.* a heme activator protein (Hap) complex that critically regulates the expression of many genes encoding proteins involved in energy metabolism and biogenesis in yeast mitochondria. Our data therefore demonstrate mitochondrial expression of Crs1 in this yeast that is specifically controlled by the Hap complex, a master regulator of mitochondrial bioenergetics and biogenesis in this eukaryotic organism *in vivo*.

## Results

The genome of the yeast has only one CysRS gene (*CRS1*), which suggests that *CRS1* may encode both cytosolic and mitochondrial isoforms. To investigate the subcellular localization of CysRS, we first prepared a mutant yeast strain by inserting a GFP cDNA into the 3′ site of *CRS1* in the genome of the yeast (hereafter called WT-GFP), which resulted in production of the fusion protein Crs1-GFP. This mutant yeast was used for live-cell imaging, which revealed a clear GFP signal for Crs1-GFP in both the cytosol and mitochondria (Fig. 1*A*). Western blot anal-

ysis confirmed expression of Crs1-GFP in both cytosolic and mitochondrial fractions (Fig. 1*B*). Earlier studies showed that a single gene encoded both cytosolic and mitochondrial isoforms, which are expressed by one of two different mechanisms: alternative transcription or translation starting at the noncanonical start codon (ACG) (14–17). However, the *CRS1* sequence in this yeast contained no in-frame noncanonical start codon. Hence, we sought to determine the possible involvement of an alternative transcription for the *CRS1* gene in mitochondrial Crs1 expression. To determine the transcription start sites of *CRS1*, we used the rapid amplification of 5′ cDNA ends method (5′-RACE) with mRNA extracted from WT cells. 5′-RACE indicated that *CRS1* mRNAs were largely divided into two types, long and short mRNAs, in terms of the translation initiation codon (AUG) (Fig. 1*C*). The long mRNA contained an additional 39 bp (ATGAATATCTTCATAAAAGCCCTGAGAAGATATACTATA) at the 5′ position compared with the short mRNA. The N-terminal peptide (MNIFIKALRRYTI) translated from this additional sequence had basic and hydrophobic residues. A protein secondary structure prediction software (JPred 4 http://www.compbio.dundee.ac.uk/jpred4)[3] (18) indicated that the peptide formed amphipathic $\alpha$-helices, which are common features of MTSs; that is, the long Crs1 isoform thereby translated was probably transported into mitochondria, whereas the short Crs1 isoform may be located in the cytosol. For analysis of the functions of the additional N-terminal peptide, a plasmid containing the long *CRS1* and one containing the short *CRS1*, each with the alcohol dehydrogenase 1 (ADH1) promoter, were constructed and expressed in WT cells (Fig. 1*D*). Laser confocal microscopy analysis showed that Crs1 (Crs1full) that was translated from the long *CRS1* was located only in mitochondria. The N-terminal truncated Crs1 (Crs1ΔN13) that was translated from the short *CRS1*, however, remained only in the cytosol. Western blot analysis also indicated that Crs1full was located only in mitochondria, but Crs1ΔN13 remained only in the cytosol, confirming the results of the fluorescence microscopy analysis (Fig. 1*E*).

To perform additional analyses of the N-terminal sequence, we produced a Crs1 N-terminal truncated strain (ΔN13-GFP) from WT-GFP by using the CRISPR-Cas9 system (Fig. S1*A*). Confocal microscopy analysis showed that Crs1 in WT-GFP was found in both the cytosol and mitochondria but that the N-terminal truncated Crs1 signal in ΔN13-GFP occurred only in the cytosol (Fig. 2*A*). Western blot analysis confirmed that the N-terminal truncated Crs1 in ΔN13-GFP was located only in the cytosol (Fig. 2*B*). Next we investigated the translation activity in mitochondria of ΔN13-GFP by analyzing the expression of mitochondrial genome-encoded genes. Western blotting for ΔN13-GFP indicated that the expression of mitochondrial porin (Por1), a nuclearly encoded gene, was intact, whereas the expression of subunit II of cytochrome *c* oxidase (Cox2), a mitochondrially encoded gene, was completely nullified (Fig. 2*C*). Moreover, we tested the growth of ΔN13-GFP in glucose or glycerol as a carbon source. When yeast is cultured in fermentable carbon sources (glucose), energy production
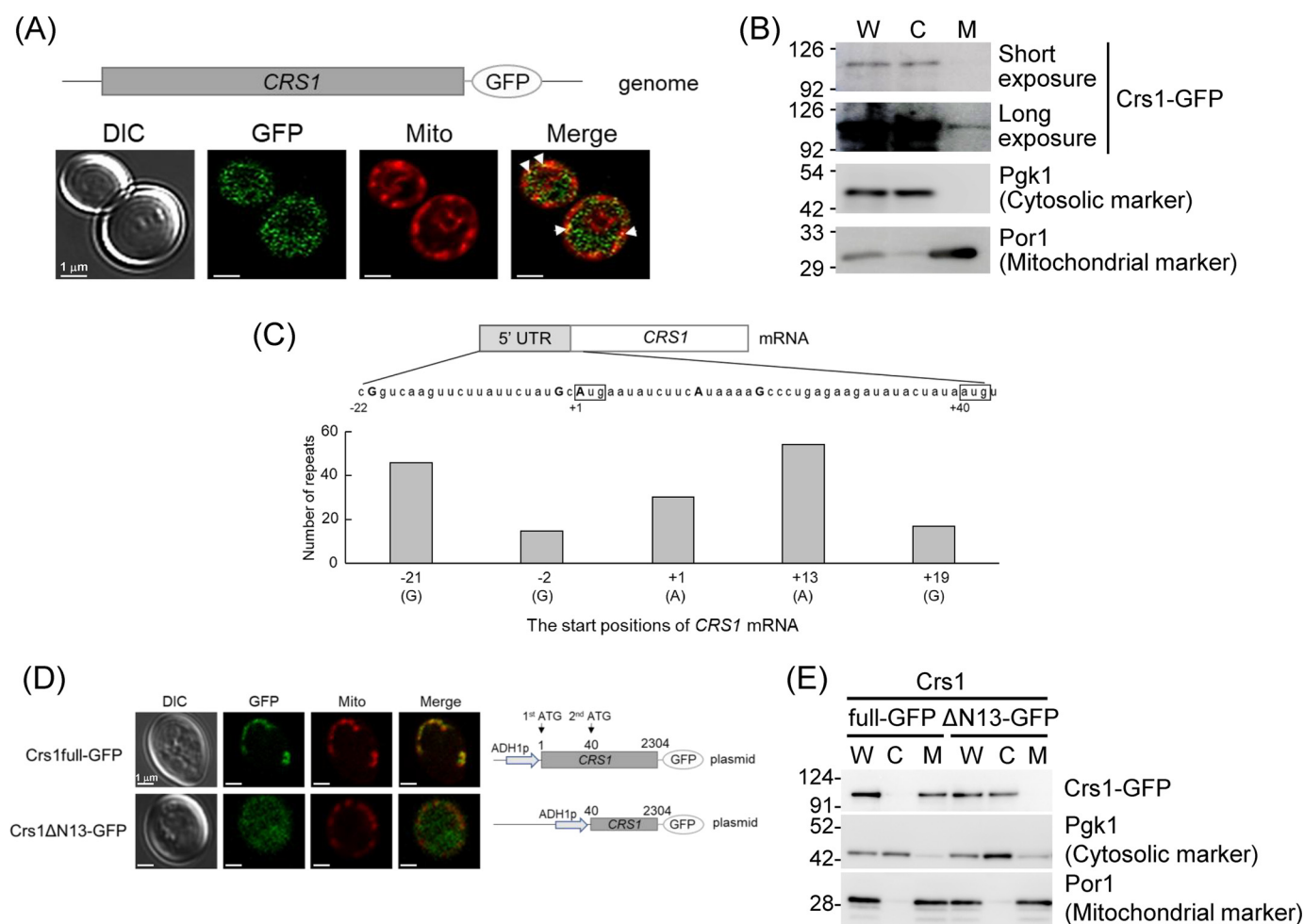
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**Figure 1. Dual localization of Crs1.** *A*, fluorescence microscopy images of WT-GFP. The *top schematic* represents the genome structure of the WT-GFP strain used in this study. MitoTracker Red was used to stain the mitochondria. The *arrowheads* point to colocalization of the Crs1-GFP and mitochondrial signals. *DIC*, differential interference contrast; *Mito*, mitochondria. *B*, Western blot analysis of the whole (*W*), cytosolic (*C*), and mitochondrial (*M*) fractions. The cells were grown in YPGly to log phase, and then the purified mitochondrial fractions were isolated. Protein samples (5 μg) were prepared for detection of Crs1-GFP, Pgk1, and Por1. *C*, transcription start site analysis of *CRS1*. Multiple transcription start sites were determined via 5′-RACE analysis (*n* = 150). The *x* axis indicates the location of each mRNA start site and the *y* axis the number of times the sequence was found. The schematic at the *top* represents part of the *CRS1* mRNA. The *uppercase letters* indicate the nucleotides of the transcription start sites, and the *boxes* indicate the nucleotides comprising the start codons. The translation start site in the database (RefSeq ID NM_001183085.1) is indicated by +1. *D*, localization analysis of Crs1 expressed with a nonoriginal promoter. WT cells harboring the pCRS1full-GFP or pCRS1ΔN13-GFP were found using fluorescence microscopy. pCRS1full-GFP is a plasmid that expresses Crs1 tagged with GFP at the C terminus plus the ADH1 promoter. pCRS1ΔN13-GFP is a plasmid that expresses the N-terminal truncated Crs1 tagged with GFP plus the ADH1 promoter. The schematic on the *right* represents the plasmid structures of pCRS1full-GFP and pCRS1ΔN13-GFP used here. MitoTracker Red was used to stain mitochondria. *E*, Western blot analysis of Crs1 expressed with a nonoriginal promoter. WT cells harboring pCRS1full-GFP or pCRS1ΔN13-GFP were grown in SG without uracil to log phase, and the whole, cytosolic, and mitochondrial fractions were isolated. Protein samples (10 μg) were prepared for detection of Crs1-GFP, Pgk1, and Por1.

occurs mainly via glycolysis, independent of mitochondrial respiration (19). In contrast, energy production for growth in nonfermentable carbon sources (glycerol) is well-known to depend completely on mitochondrial oxidative phosphorylation. ΔN13-GFP cells grew normally in glucose medium but showed defective growth in glycerol medium, which indicated that mitochondrial oxidative phosphorylation was impaired and insufficient to maintain cellular bioenergetics because of the loss of translation activity in mitochondria (Fig. 2D). These results suggest that *CRS1* encodes both cytosolic and mitochondrial isoforms via alternative transcription from a single gene and that the N-terminal peptide in the protein translated from the long mRNA probably functions as an MTS.

When yeast cells are grown in medium containing glucose, they metabolize glucose predominantly by glycolysis, which releases ethanol into the medium. When the glucose supply becomes limited, cells undergo a diauxic shift characterized by a reduced growth rate and by switching metabolism from glycolysis to aerobic utilization of ethanol via the tricarboxylic acid cycle and oxidative phosphorylation in the mitochondria (20–22). Basically, although yeast growth is independent of mitochondria before the diauxic shift, it depends fully on mitochondria after the diauxic shift (Fig. S2). We used real-time quantitative PCR (qPCR) to develop a system to detect the long mRNA (mitochondrial mRNA) of *CRS1* (Fig. S3). This qPCR analysis showed that the mitochondrial mRNA level increased as yeast cells grew on glucose medium (Fig. 3A). In fact, the mitochondrial *CRS1* mRNA level increased rapidly from early stationary phase after the diauxic shift was initiated. We also found that mitochondrial mRNA expression increased even
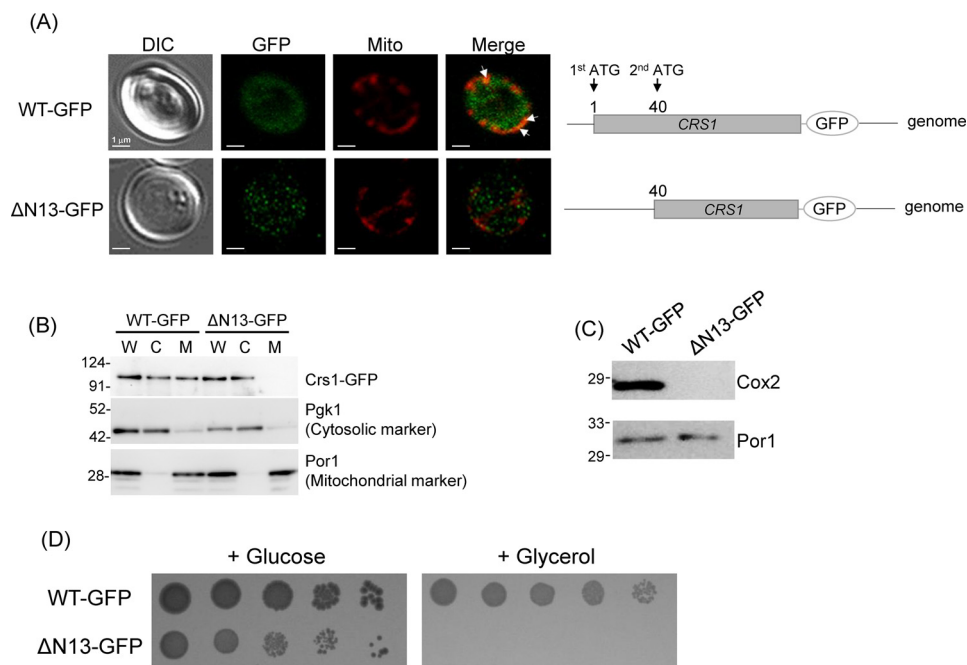
**Figure 2. Analysis of an N-terminal truncated mutant of Crs1.** *A,* fluorescence microscopy images of WT-GFP and ΔN13-GFP. Mito-ID Red was used for mitochondrial staining because MitoTracker Red cannot be applied to respiration-deficient mutants. The *arrowheads* indicate colocalization of the Crs1-GFP signal with the mitochondrial signal. The schematic on the *right* shows the genome structures of WT-GFP and the N-terminal truncated mutant (Δ*N13-GFP*). *DIC,* differential interference contrast; *Mito,* mitochondria. *B,* Western blot analysis of Crs1 localization in WT-GFP and ΔN13-GFP. WT-GFP and ΔN13-GFP were grown in SG to log phase, and the whole (*W*), cytosolic (*C*), and mitochondrial (*M*) fractions were isolated. Protein samples (10 μg) were prepared for detection of Crs1-GFP, Pgk1, and Por1. *C,* Western blot analysis of the cell extracts from WT-GFP and ΔN13-GFP. Protein samples (10 μg) were prepared for detection of Cox2, subunit II of cytochrome *c* oxidase (a mitochondrial genome-encoded gene), and Por1 (a nuclearly encoded gene). *D,* growth of WT-GFP and ΔN13-GFP in glucose or glycerol medium. Cells were grown in glucose medium and then spotted on glucose or glycerol medium.

during the log phase of growth in culture medium containing glycerol as a nonfermentable carbon source (Fig. 3*A*). These results thus indicated that regulation of mitochondrial mRNA transcription depended on the energy metabolism demand of the cells. Several reports proposed that mitochondrial bioenergetics and biogenesis were regulated by some transcription factors, especially Hap2, Rtg1, and Mig2 (23, 24). We thus investigated whether these factors were involved in the transcription of mitochondrial *CRS1*. As Fig. 3*B* shows, deletion of *HAP2* caused loss of the increased mitochondrial *CRS1* mRNA depending on the growth phase, whereas Δ*rtg1* and Δ*mig2* showed results similar to that of the WT. In addition, the absence of any subunits of the Hap complex led to a defect in the increase of mitochondrial *CRS1* mRNA expression (Fig. 3*C*). We searched the Hap complex–binding site (CCAAT) upstream of the *CRS1* gene (chromosome XIV 182074_182078) via sequence analysis (25) and constructed a strain (Δ*hap-BS*) that is deficient in the Hap complex–binding site in the genome by using the CRISPR-Cas9 system (Fig. S1*B*). Δ*hap-BS* thus obtained was defective in the increase in mitochondrial *CRS1* mRNA, as were the disrupted mutants of the *HAP* gene (Fig. 3*C*). Next we observed the effect of overexpression of *HAP4*, the gene for the catalytic subunit of the Hap complex, on the transcription of mitochondrial *CRS1* mRNA. Fig. 3*D* shows that mitochondrial *CRS1* mRNA in *HAP4*-overexpressing cells was transcribed even during the log phase of growth in culture medium containing glucose. We then used a ChIP assay with Hap2 for Hap complex–binding sites upstream of the *CRS1* gene. Fig. 3*E* shows that Hap2 bound to the Hap complex–binding site only during late stationary phase in glucose

medium. In contrast, during growth in glycerol medium, Hap2 bound to the Hap complex–binding site during any phase, even log phase. These results therefore indicate that the Hap complex may regulate the transcription start site of *CRS1* mRNA by directly binding to the promoter of the *CRS1* gene.

A transcription initiation site is determined via different steps, including chromatin remodeling (26). Imamura *et al.* (27) showed that the chromatin structure–remodeling complex (RSC) subunit Rsc2, a component of the ATP-dependent RSC, physically interacts with the Hap complex and regulates mitochondrial functions. As Fig. 3*F* indicates, the Δ*rsc2* strain showed a loss in the increase in mitochondrial *CRS1* mRNA, as did the disrupted mutants of the *HAP* gene. We then tested the growth in medium containing glucose or glycerol (Fig. 3*G*). All strains showed almost normal growth in glucose medium. The ΔN13-GFP and Δ*hap2* strains, however, exhibited a complete loss of growth in glycerol medium. Also, growth of the Δ*hap-BS* and Δ*rsc2* strains was much slower than that of the WT-GFP strain. These results suggest that Hap and RSC cooperate to determine the transcription start site of *CRS1*, which is essential for mitochondrial energy metabolism and biogenesis.

## Discussion

Yeast has a predominantly fermentative metabolism. When grown in medium containing glucose as the carbon source, yeast cells repress their respiratory metabolism to a point where all glucose has been consumed, which leaves only ethanol as a carbon source. To utilize ethanol, yeast cells reprogram their metabolism, a phase known as the diauxic shift (20–22). During the diauxic shift, the cells regulate mitochondrial bioenergetics
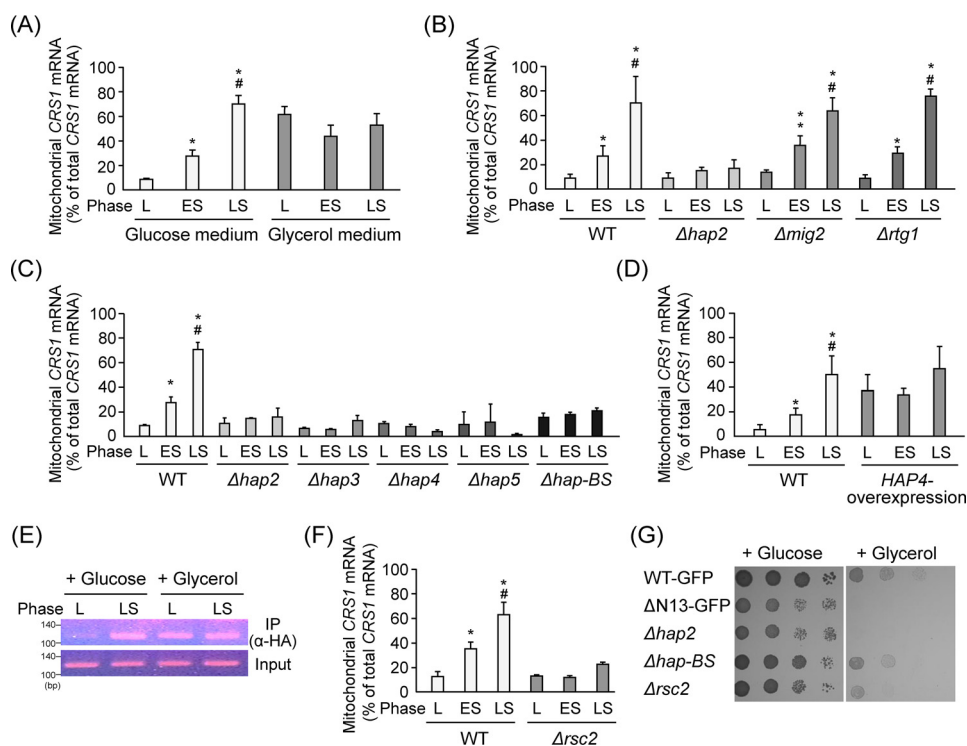
**Figure 3. Hap complex–dependent regulation of transcription start sites.** *A,* percentage of mitochondrial *CRS1* mRNA for growth in glucose or glycerol medium. Cells were grown in glucose (YPD) or glycerol medium (YPGly) and harvested at log phase (L), early stationary phase (ES), and late stationary phase (LS) (Fig. S2). The percentage of mitochondrial *CRS1* mRNA was determined using qPCR with total RNA. Results are presented as means ± S.D. (*n* = 5). Statistical significance was determined by one-way ANOVA with Tukey's test. *, *p* < 0.05 (*versus* the L result); #, *p* < 0.05 (*versus* the ES result). *B,* percentage of mitochondrial *CRS1* mRNA in mitochondrial biogenesis–related gene mutants. WT, Δ*hap2*, Δ*mig2*, and Δ*rtg1* cells were grown in glucose medium (YPD) and harvested at L, ES, and LS. The percentage of mitochondrial *CRS1* mRNA was determined using qPCR with total RNA. Results are presented as means ± S.D. (*n* = 3). Statistical significance was determined by one-way ANOVA with Tukey's test. *, *p* < 0.05 (*versus* the L result); #, *p* < 0.05 (*versus* the ES result). *C,* percentage of mitochondrial *CRS1* mRNA in Hap complex–related gene mutants. WT, Δ*hap2*, Δ*hap3*, Δ*hap4*, Δ*hap5*, and Δ*hap-BS* cells were grown in glucose medium and harvested at L, ES, and LS. The percentage of mitochondrial *CRS1* mRNA was determined using qPCR with total RNA. Results are presented as means ± S.D. (*n* = 5). Statistical significance was determined by one-way ANOVA with Tukey's test. *, *p* < 0.05 (*versus* the L result); #, *p* < 0.05 (*versus* the ES result). *D,* percentage of mitochondrial *CRS1* mRNA in *HAP4*-overexpressing cells. WT harboring the pAG426GPD-ccdB or pAG426GPD-HAP4 were grown in glucose medium (SC medium without uracil) and harvested at L, ES, and LS. The percentage of mitochondrial *CRS1* mRNA was determined using qPCR with total RNA. Results are presented as means ± S.D. (*n* = 3). Statistical significance was determined by one-way ANOVA with Tukey's test. *, *p* < 0.05 (*versus* the L result); #, *p* < 0.05 (*versus* the ES result). *E,* ChIP with the *CRS1* promoter. Cells with the HA tag inserted into the 3′ site of *HAP2* in the genome were grown in glucose (YPD) or glycerol (YPGly) medium and harvested at L and LS. Binding of Hap2 to the promoter of *CRS1* was determined by means of a ChIP assay with HA antibody. *Input* represents PCR results before immunoprecipitation (*IP*). *F,* percentage of mitochondrial *CRS1* mRNA in *RSC2* mutants. WT and Δ*rsc2* cells were grown in glucose medium (YPD) and harvested at L, ES, and LS. The percentage of mitochondrial *CRS1* mRNA was determined using qPCR with total RNA. Results are presented as means ± S.D. (*n* = 5). Statistical significance was determined by one-way ANOVA with Tukey's test. *, *p* < 0.05 (*versus* the L result); #, *p* < 0.05 (*versus* the ES result). *G,* growth of WT-GFP, ΔN13-GFP, Δ*hap2*, Δ*hap-BS*, and Δ*rsc2* in glucose (YPD) or glycerol (YPGly) medium. Cells were grown in glucose medium and spotted on glucose or glycerol medium.

and biogenesis-associated genes and catabolize ethanol via the tricarboxylic acid cycle and oxidative phosphorylation in mitochondria. Some of the genes regulated during the diauxic shift contain mitochondrial ARSs because all mitochondrial genome-encoded proteins are involved in oxidative phosphorylation. Several researchers have proposed that the Hap complex is a key transcription factor involved in the diauxic shift and that it regulates the expression of many genes encoding proteins involved in energy production in mitochondria (28–30). The Hap complex, known as the CCAAT-binding factor, consists of four components: Hap2, Hap3, Hap4, and Hap5. Formation of the Hap3 and Hap5 heterodimer creates a scaffold for Hap2 association that results in formation of the Hap2/Hap3/Hap5 heterotrimer, which can bind to CCAAT sequences on promoters (31). Hap4 is the activator of the complex. The absence of any subunits of the Hap complex leads to defects in the diauxic shift and growth in nonfermentable medium (*i.e.* ethanol or glycerol) (31, 32). Therefore, the Hap complex is the master regulator of mitochondrial biogenesis.

In this study, we investigated a mechanism for the generation of a mitochondrial isoform of yeast CysRS (Crs1). This study indicates that the single gene *CRS1* expresses two groups of mRNAs through alternative transcription starts. These mRNAs encode two alternative in-frame initiation codons. The mitochondrial Crs1 is translated from the first initiation AUG codon on the long mRNA (mitochondrial mRNA) of *CRS1*, whereas the cytosolic form is translated from the second in-frame AUG codon on the short mRNA (Fig. 4). The mitochondrial Crs1 therefore has the same polypeptide sequences as the cytosolic Crs1, except for a 13-amino-acid sequence that functions as the MTS at the N terminus. We also found that Hap and RSC likely interact with each other and regulate the transcription start sites of *CRS1*, which depends on the energy metabolic demand in mitochondria.

Fig. 1*D* shows that the *CRS1* expressed from a plasmid with a defined promoter (the ADH1 promoter) starting upstream of the first ATG produces only the mitochondrial isoform, not the cytosol isoform; that is, the ADH1 promoter cannot transcribe
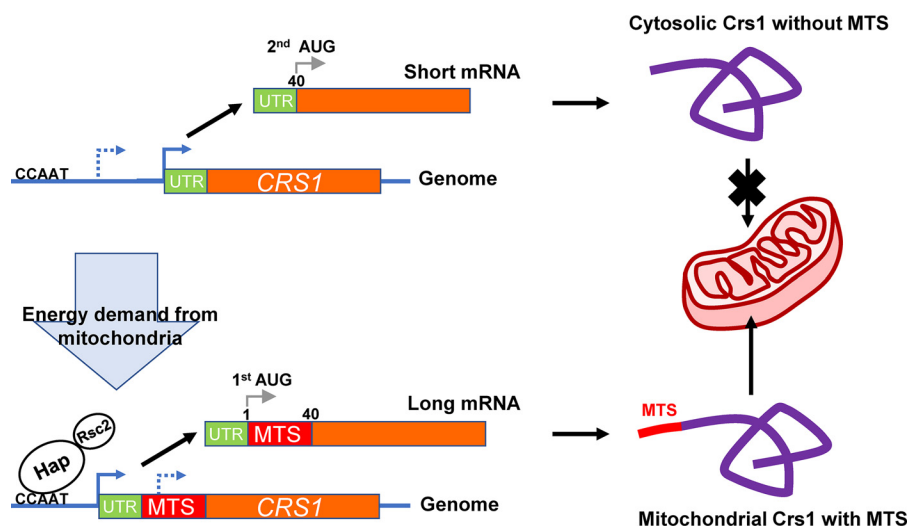
**Figure 4. Dual targeting mechanism of Crs1.** *CRS1* is transcribed from a single gene with different transcription start sites, which leads to the creation of mRNAs of different lengths: long and short. The long mRNA harboring the first upstream AUG is translated to the Crs1 protein with the MTS, which transports Crs1 to mitochondria. The short mRNA lacking the first AUG is translated from the second AUG to the Crs1 protein without the MTS. This type of Crs1 remains in the cytosol. The need for a specific mitochondrial function governs whether the Hap complex binds to the CCAAT sequence on the *CRS1* promoter, which regulates the expression of the long (mitochondrial) mRNA.

the short mRNA of *CRS1*. The *CRS1* promoter may thus have a particular function that regulates the expression of dual isoforms. In fact, our research showed that the Hap complex is important for the expression of mitochondrial mRNA of *CRS1*. The Hap complex is well-known to be a key transcription factor involved in the diauxic shift and thereby controls the expression of many genes, which, in turn, may regulate the proteins that mediate energy production in mitochondria (20–22). Apart from the fact that Crs1 is essential for the translation system in yeast mitochondria, its unique moonlighting function as a CPERS is understood better in view of the energy metabolism as described in this study, which revealed that the Hap complex and Rsc2 are important for expression of the mitochondrial mRNA of *CRS1*. The RSC functions to rearrange chromatin from a condensed state to a transcriptionally accessible state, which allows gene expression (33). We therefore propose, for the first time, a regulation mechanism operated by the Hap complex and Rsc2 that involves chromatin remodeling, which may depend on the demands of mitochondrial energy metabolism. For example, the Hap complex has been suggested to be activated by binding directly to Rsc2 (27), which may induce up-regulation of the promoter activity for mitochondrial Crs1 expression. This activation process may be mediated by the chromatin remodeling activity of the RSC complex containing Rsc2, which may cause a structural alteration of chromatin from heterochromatin to euchromatin and result in the induction of mitochondrial *CRS1* mRNA.

We found that *CRS1* encodes both cytoplasmic and the mitochondrial enzymes via alternative use of two in-frame translation initiators. The same transcriptional strategy is also used by yeasts for other single genes that encode both cytoplasmic and mitochondrial proteins. Examples include at least three yeast tRNA-processing enzymes encoded by *TRM1*, *MOD5*, and *CCA1* and two aminoacyl tRNA-synthetases encoded by *HIS1* and *VAS1* (14–16, 34). Each of these genes contains more than one in-frame ATG codon and encodes two or more protein products localized in distinct compartments (*i.e.* the cytosol

and mitochondria). Because all of these genes are somehow involved in the translation system, they are likely to be regulated by the Hap complex and Rsc2. In fact, the sequence upstream of *TRM1* and *VAS1* contains the Hap complex–binding site, as found by searching the YEASTRACT database (http://www.yeastract.com)[3] (25). This study may therefore warrant additional investigation to obtain a better understanding of alternative gene expression with dual localization of ARSs and mitochondrial proteins in general for other organisms, such as plants.

## Experimental procedures

### Yeast strains, medium, and plasmids

Table S1 summarizes the yeast strains (*S. cerevisiae* BY4742), and Table S2 lists the primers used in this study. The growth media used were yeast extract-peptone-dextrose (YPD), yeast extract-peptone-glycerol (YPGly), synthetic complete (SC), and synthetic complete with galactose (SG) (see supplemental experimental procedures). To construct a chromosomally GFP-tagged *CRS1* (*YNL247W*) strain (WT-GFP), an integration cassette containing a C-terminally positioned GFP tag was amplified by PCR (35). To construct a mutant (ΔN13-GFP) that chromosomally expresses N-terminal 13-residue (39 bp) truncated *CRS1* and a strain (Δ*hap2-BD*) lacking the Hap2-binding site (CCAAT, chromosome XIV 182074_182078) on the *CRS1* promoter, the CRISPR-Cas9 system was used (36). For the construction of Δ*hap2-*, Δ*mig2-*, Δ*rtg1-*, Δ*hap3-*, Δ*hap4-*, and Δ*hap5*-disrupted strains, gene-specific deletion cassettes were prepared from the BY4741-derived deletion library (EUROSCARF) by using PCR. To perform the ChIP assay for Hap2, an HA tag–inserted strain (Y800 HAP2-HA) was purchased from Horizon Discovery. All plasmids used in this study were constructed using Gateway technology (Thermo Fisher Scientific, Rockland, IL). The codon-modified *CRS1* (Fig. S4) was designed using GeneOptimizer expert software (37) to keep the plasmid containing the *CRS1* sequence in *Escherichia*

*coli* stable. The detailed procedures are described in the supplemental experimental procedures.

## Fluorescence microscopy images

WT-GFP and ΔN13-GFP cells were grown to early stationary phase in filter-sterilized YPD medium at 30 °C. WT cells harboring pCRS1full-GFP or pCRS1ΔN13-GFP were grown to early stationary phase in filter-sterilized SC medium without uracil at 30 °C. After mitochondrial staining (described below), cells were harvested, washed with PBS, and viewed with a Nikon EZ-C1 confocal laser microscope (Tokyo, Japan).

## Mitochondrial staining

Cultured cells were subjected to 40 nM MitoTracker Red CM-H$_2$Xros (Thermo Fisher Scientific) in medium for 20 min. For the respiration-deficient strain (ΔN13-GFP), cells were subjected to Mito-ID Red detection reagent (Enzo Life Sciences, Farmingdale, NY), diluted 1:2500, for 30 min (38).

## Mitochondrial isolation

Cells were grown in YPGly or SG with or without uracil to log phase, and crude mitochondrial fractions were isolated as described previously (39). The soluble fractions obtained by this process were filtered through a 0.22-mm filter and used as cytosolic fractions for Western blot assays. For isolation of purified mitochondrial fractions, the crude fractions were layered onto an OptiPrep (Axis-Shield, Oslo, Norway) gradient (a discontinuous gradient of equal volumes of 2%, 8%, 14%, 20%, and 25% iodixanol) in an isotonic buffer (0.6 M sorbitol and 20 mM HEPES-KOH (pH 7.4)). The OptiPrep gradient was centrifuged at 12,000 × $g$ for 2 h, and then the purified mitochondrial fractions were fractionated (40).

## Western blot analysis

Western blot analysis was performed with anti-GFP (Roche, a mixture of clones 7.1 and 13.1), anti-Pgk1 (Pgk1, 3-phosphoglycerate kinase; Abcam, Cambridge, UK; clone 22C5D8), anti-Por1 (Por1, mitochondrial porin; Thermo Fisher Scientific; clone 16G9E6BC4), and anti-Cox2 (Abcam, clone 4B12A5) as described previously (41).

## Determination of transcription start sites

Transcription start sites of *CRS1* were determined via 5′-RACE. WT cells were grown to early stationary phase in YPD medium at 30 °C. Total RNA was extracted by using TRI reagent with Direct-zol RNA MicroPrep (Zymo Research, Irvine, CA), and poly(A)$^+$ RNA was isolated from the total RNA using the Oligotex-dT30 Super mRNA Purification Kit (Takara Bio) according to the manufacturer's instructions. To determine the transcription start sites, circular first-strand cDNA–mediated 5′-RACE was employed with poly(A)$^+$ RNA using the 5′-full RACE core set (Takara Bio) and the following primers: YNL247W RT primer with 5′-phosphate for gene-specific reverse transcription, YNL247W S1 and YNL247W A2 for the first round of PCR, and YNL247W A1 and YNL247W S2 for the second round of PCR. The second PCR products were cloned into the pTA2 vector (TOYOBO), and 150 sequences were determined.

## Growth test

Cells were grown for 2 days in YPD medium and diluted to an optical density of 1.0 with water. Aliquots of 2 $\mu$l of 10-fold serial dilutions were spotted on YPD or YPGly plates and incubated for 3–4 days at 30 °C.

## Detection of mitochondrial CRS1 mRNA

Cultured cells were disrupted using the Multi-Beads Shocker (YASUI Kikai, Osaka, Japan) with 0.5-mm glass beads, and total RNA was extracted with the NucleoSpin RNA Plus Kit (Takara Bio) according to the manufacturer's instructions. cDNA was synthesized from total RNA with the PrimeScript RT Reagent Kit (Takara Bio). The relative abundance of mitochondrial *CRS1* mRNAs in total *CRS1* mRNA was quantified by means of qPCR with the CFX Connect Real-Time System (Bio-Rad) and SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The following primer sets were used in this analysis (Fig. S3): YNL247W qPCR long form forward and YNL247W qPCR long/short reverse for detection of mitochondrial *CRS1* mRNAs (PCR efficiency 98.6%) and YNL247W qPCR short form forward and YNL247W qPCR long/short reverse for detection of total *CRS1* mRNAs (PCR efficiency 97.5%). The following PCR protocol was used: 95 °C for 4 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 30 s. The values of the threshold cycle number (Ct) of the total *CRS1* mRNAs and the mitochondrial *CRS1* mRNAs were determined for each separate qPCR run. The ΔCt (Ct (total *CRS1* mRNAs) − Ct (mitochondrial *CRS1* mRNAs)) represents the relative abundance. The quantitative results were expressed as the percentage of mitochondrial *CRS1* mRNAs by $2^{\Delta Ct}$.

## ChIP assay

The Y800 HAP2-HA strain was cultured in YPD or YPGly medium, and the ChIP assay was performed with anti-HA magnetic beads (Thermo Fisher Scientific) as described by Dedon *et al.* (42).

## Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's test was used for multiple comparisons with GraphPad Prism 7 (GraphPad Software). $p < 0.05$ was accepted as the level of statistical significance.

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