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Identifikace kritických faktorů určujících další osud ribosomu po ukončení translace.

Identification of critical factors determining post-termination decision programming.

Disertační práce

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Molecular and Cell Biology, Genetics and Virology

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Doctoral thesis

Supervisor: Res. prof. Leoš S. Valášek, Ph.D.

Prague, 2025

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Differential effects of 40S ribosome recycling factors on reinitiation at regulatory uORFs in GCN4 mRNA are not dictated by their roles in bulk 40S recycling

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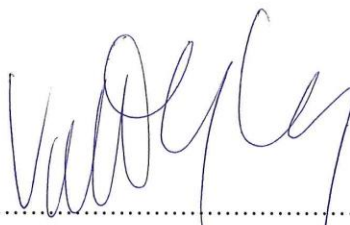
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LIST OF ABBREVIATIONS

4EBP1	eukaryotic translation initiation factor 4E-binding protein 1
ABCE1	ATP-binding cassette sub-family E member 1
ADP	adenosine diphosphate
ATF4	activating transcription factor 4
ATF5	activating transcription factor 5
ATP	adenosine triphosphate
ASNS	asparagine synthetase
AZIN1	antizyme inhibitor 1
Bd	body
CDKN1A	cyclin dependent kinase inhibitor 1A
CDS	coding sequence
circRNA	circular RNA
CFTR	CF transmembrane conductance regulator
CHOP	C/EBP homologous protein
CK2	casein kinase 2
CP	central protuberance
CReP	constitutive revertor of eIF2 α phosphorylation
CSFV	classical swine fever virus
CTD	C-terminal domain
DBP5	DEAD-box protein 5
DC	decoding center
DENR	density-regulated protein
DOM34	duplication of multilocus region
eEF	eukaryotic elongation factor
eIF	eukaryotic initiation factor
EPRS	glutamyl-prolyl-tRNA synthetase
eRF	eukaryotic release factor
EV	empty vector
FIV	feline immunodeficiency virus
GADD34	growth arrest and DNA damage-inducible protein 34
GCN2/4	general control nonderepressible 2/4
GDP	guanosine diphosphate
GST	glutathione S-transferase
GTP	guanosine triphosphate
h.c.	high copy
<i>H. sapiens</i>	<i>Homo sapiens</i>
HBS1	Hsp70 subfamily B suppressor
HBS1L	HBS1 like translational GTPase
HCR1	high-copy suppressor of Rpg1
HCV	hepatitis C virus
HEK293T	human embryonic kidney cells

HIV	human immunodeficiency virus
HLH	helix loop helix
HRI	heme-regulated inhibitor
IBTK α	inhibitor of Bruton tyrosine kinase
IRES	internal ribosomal entry site
ISR	integrated stress response
LSU	large subunit
m ⁶ A	6-methyladenosine
m ⁷ G	7-methylguanosine
MCTS1	malignant T-cell amplified sequence 1
METTL3	methyltransferase 3
Met-tRNA _i ^{Met}	methionyl initiator tRNA
MFC	multi-initiation factor complex
mORF	main open reading frame
MPN	<i>Mpr1-Pad1-N-terminal</i>
mRNA	messenger RNA
MS	mass spectrometry
NBD	nucleotide binding domain
NIP1	nuclear import 1
NMD	nonsense-mediated decay
NTD	N-terminal domain
NTT	N-terminal tail
OD	optical density
ORF	open reading frame
PABP	poly-A binding protein
PCI	proteasome COP9/signalosome eIF3
PERK	(PKR)-like endoplasmic reticulum kinase
PIC	preinitiation complex
PKR	protein kinase R
post-TC	post-termination complex
PRT1	protein synthesis 1
Pt	platform
PTC (1)	peptidyl transferase center
PTC (2)	premature termination codon
PTC (3)	post-termination complex
RAC	ribosome-associated complex
RAI1	RAT1-interacting protein
RAT1	ribonucleic acid trafficking
REI	reinitiation
RLI1	RNase L Inhibitor
RPE	reinitiation promoting element
RPL	ribosomal protein large subunit
RPP	ribosomal protein P
RRM	RNA recognition motif

RPS	ribosomal protein small subunit
rRNA	ribosomal RNA
<i>S.c.</i>	<i>Saccharomyces cerevisiae</i>
s.c.	single copy
SC-RT	stop codon readthrough
SL	stem loop
SSU	small subunit
SUI1	suppressor of initiator codon
SQSTM1	sequestosome-1
TC	ternary complex
TCP-seq	translational complex profiling-sequencing
TMA20/22/64	translation machinery associated 20/22/64
TRIB3	tribbles homologue 3
tRNA	transfer RNA
uORF	upstream open reading frame
UPR	unfolded protein response
UTR	untranslated region
WRS	tryptophanyl tRNA synthetase
YLC	yeast-like core
ZUO1	zuotin

ABSTRACT

Translation in eukaryotic organisms is carried out in the translational cycle through four distinct steps – initiation, elongation, termination and ribosome recycling. Each of these steps is tightly regulated through both *cis*- and *trans*-acting factors, many of which are yet unknown. Translation reinitiation (REI) is a detour from this cycle – during some translational events, the recycling step after termination is incomplete, and the ribosome continues downstream on the same mRNA to initiate a new event. It was the general aim of thesis to identify new *cis*- and *trans*-acting elements influencing this post-termination decision programming (i.e. whether to recycle or reinitiate), and the mechanisms behind their function.

Recycling of the 40S ribosomal subunit in yeast involves the Tma20/Tma22 and Tma64 proteins, which are homologues of mammalian MCTS1/DENR and eIF2D. MCTS1/DENR have been shown to promote REI after short upstream open reading frames (uORFs) through 40S recycling, especially at short uORFs with penultimate codons dependent on these factors for bulk recycling. We investigated the function of the Tma20/22/64 proteins on regulatory short uORFs in yeast, using a *GCN4*-based reporter system. These reporters contained either uORFs that promote REI (uORF1) or inhibit REI (promote recycling; uORF4). We found that the Tma20/22/64 proteins generally inhibit REI at uORF4 with various penultimate codons, regardless of their Tma-dependence for bulk recycling. The Tma factors also had no effect on REI after uORF1, regardless of the penultimate codon of the uORF. Converting these uORFs to AUG-stop elements (which are DENR-dependent) also conferred no Tma-dependence. Therefore, effects of the Tma proteins are not dictated by the Tma-dependence of the codon in bulk recycling but vary depending on the REI potential of the uORF and its penultimate codon.

The transcription factor ATF4 is the master regulator of the mammalian integrated stress response. Previously, it was thought that its translational regulation fits the delayed REI model, where two conserved uORFs in *ATF4*'s 5' UTR play a key role. However, evidence has been accumulating that many other elements on the *ATF4* mRNA contribute to the mechanism behind its translation, which prompted us to investigate several of them. We found a conserved stem loop in the uORF2/CDS overlap with an alternative near-cognate CUG in the near vicinity, which causes ribosome queuing upstream and influences rate of translation of uORF2. These elements would explain the contradictory results that the inhibitory uORF2 can also be translated under stress. We also identified two conserved potential modification sites with antagonistic roles and proved that the canonical ATF4 start site is substantially leaky scanned. Thus, we demonstrated that the *ATF4* mRNA has deeper layers of regulation than previously thought, which might shed more light on its biological function in the future.

Finally, I performed a screening of potential REI/recycling factors in yeast, using a *GCN4*-based reporter system and a library of yeast deletion strains. For further analysis, I selected three candidates – Rai1 (a decapping/decay factor, candidate REI factor) and Ssz1/Zuo1 (two ribosome-associated factors that form a heterodimer, candidate recycling factors). In my phenotypical and biochemical analysis of these factors, I found a potential functional and physical interaction between Ssz1 and Rai1. I have also demonstrated that Rai1 is associated with ribosomes. Additionally, our pulldown-Mass Spectrometry experiments have revealed

several interaction partners of interest that could shed more light on the mechanism of function of these factors. This will be the subject of interest in further work on the project.

My ongoing work is now dedicated to full characterization of these three factors and their role in translation regulation.

ABSTRAKT

Translace v eukaryotických organismech probíhá v rámci translačního cyklu ve čtyřech různých krocích – iniciace, elongace, terminace a recyklace ribozomů. Každý z těchto kroků je přesně regulován prostřednictvím *cis*- i *trans*-působících faktorů, z nichž mnohé jsou dosud neznámé. Reiniciace translace (REI) je odbočkou z tohoto cyklu – během některých translačních událostí je recyklační krok po terminaci neúplný a ribozom pokračuje 3' směrem na stejnou mRNA, aby zahájil nový cyklus translace. Obecným cílem dizertační práce bylo identifikovat nové *cis*- a *trans*-působící faktory určující toto rozhodování ribozomu po ukončení translace (t.j. zda recyklovat, nebo reinicizovat) a mechanismy stojící za jejich funkcí.

Recyklace ribozomální podjednotky 40S u kvasinek zahrnuje proteiny Tma20/Tma22 a Tma64, které jsou homology savčích MCTS1/DENR a eIF2D. Bylo prokázáno, že MCTS1/DENR podporují REI po krátkých otevřených čtecích rámcích před start kodonem (uORF) prostřednictvím recyklace 40S, zejména u krátkých uORF s předposledními kodony závislými na těchto faktorech pro hromadnou recyklaci 40S. Zkoumali jsme funkci proteinů Tma20/22/64 na regulačních krátkých uORF u kvasinek pomocí reportérového systému založeného na *GCN4*. Tyto reportéry obsahovaly buď uORF, které podporuje REI (uORF1), nebo inhibuje REI (podporují recyklaci; uORF4). Zjistili jsme, že proteiny Tma20/22/64 obecně inhibují REI u uORF4 s různými předposledními kodony bez ohledu na jejich závislost na Tma pro hromadnou recyklaci. Faktory Tma také neměly žádný vliv na REI po uORF1 bez ohledu na předposlední kodon uORF. Převedení těchto uORF na AUG-stop elementy (které jsou závislé na DENR) rovněž nepřinášelo žádnou závislost na Tma. Účinky proteinů Tma tedy nejsou dány závislostí kodonu na Tma při hromadné recyklaci, ale liší se v závislosti na reiniciačním potenciálu uORF a jeho předposledního kodonu.

Transkripční faktor ATF4 je hlavním regulátorem integrované stresové odpovědi savců. Dříve se předpokládalo, že jeho translační regulace odpovídá modelu zpožděné REI, kde klíčovou roli hrají dva konzervované uORF v 5' UTR *ATF4*. Hromadí se však důkazy, že k mechanismu translace *ATF4* přispívá mnoho dalších prvků na mRNA, což nás přimělo prozkoumat několik z nich. Nalezli jsme konzervovanou vlásenku se smyčkou v překryvu uORF2/CDS s alternativním blízce kognátní CUG v blízkém okolí, který způsobuje řazení ribozomů do fronty před vlásenkou a ovlivňuje translaci uORF2. Tyto prvky by vysvětlovaly rozporuplné výsledky, že inhibiční uORF2 může být překládán i při stresu. Identifikovali jsme také dvě konzervovaná potenciální modifikační místa s antagonistickou rolí a prokázali jsme, že kanonické startovací místo ATF4 podléhá “leaky scanningu” – jeho start kodon je často pročitán a translace není zahájena. Prokázali jsme tedy, že mRNA *ATF4* má hlubší vrstvy regulace, než se dosud předpokládalo, což by mohlo v budoucnu pomoci odhalit více o biologické funkci tohoto faktoru.

Nakonec jsem provedla screening potenciálních REI/recyklačních faktorů u kvasinek pomocí reportérového systému založeného na *GCN4* a knihovny delečních kmenů kvasinek. Pro další analýzu jsem vybral tři kandidáty - Rai1 (faktor odstraňující čepičku mRNA a účastník se degradace RNA, kandidát na faktor ovlivňující REI) a Ssz1/Zuo1 (dva faktory asociované s ribozomem, které tvoří heterodimer; kandidáti na recyklační faktory). Při fenotypové a

biochemické analýze těchto faktorů jsem zjistila potenciální funkční a fyzikální interakci mezi Ssz1 a Rai1. Prokázala jsem také, že Rai1 je spojen s ribozomy. Kromě toho naše experimenty skrze pull-down/hmotnostní spektrometrii odhalily několik zajímavých interakčních partnerů, kteří by mohli odhalit víc o mechanismu funkce těchto faktorů. To bude předmětem zájmu další práce na projektu.

Moje současná práce se nyní věnuje úplné charakterizaci těchto tří faktorů a jejich role v regulaci translace.

INTRODUCTION

Translation

Translation is protein synthesis from an mRNA template and has a fundamental place in the process of gene expression. Moreover, its regulatory mechanisms are uniquely important in the realm of post-transcriptional gene regulation, which has steadily been gaining more and more prominence around the scientific field of gene regulation. It is carried out by small molecular machines dubbed ‘ribosomes’, which use ribosomal proteins and ribosomal RNA (rRNA) as their main building blocks and catalyze the joining of individual amino acids into peptides.

Mirroring the similarly key process of transcription, the translational cycle is comprised of three distinct phases: initiation, elongation and termination, which are followed by ribosome recycling. This introduction will briefly describe the ribosome and its function, and the key processes in translation, then followed by descriptions of ribosome-associated factors that guide and regulate these events.

Ribosome structure and function

In eukaryotes, ribosomes are comprised of a 40S small subunit (SSU) and a large 60S subunit (LSU), that join to form the 80S ribosome. The 18S rRNA along with 33 ribosomal proteins form the 40S subunit, whereas 60S subunit is formed by three rRNAs (25S/28S, 5.8S and 5S) in addition to 46 (yeast) or 47 (human) ribosomal proteins. During protein synthesis, mRNA is bound by the 40S subunit, which contains the decoding center (DC) which pairs mRNA codons with their corresponding cognate tRNAs. In contrast, the 60S subunit contains the peptidyl transferase center (PTC), which catalyzes peptide bond formation in the emerging polypeptide chain. This polypeptide leaves the ribosome through the peptide exit tunnel of the 60S subunit. The PTC is formed by rRNA, which classifies ribosomes as ribozymes (Dorner et al. 2023; Nissen et al. 2000).

First atomic resolution structure of the *S. cerevisiae* yeast eukaryotic ribosome was solved in 2011 by the Yusupov group; this model contains 90.5% of all ~13,000 ribosomal protein residues and 95.5% of ~5500 rRNA residues, along with ~2000 metal ions (Ben-Shem et al. 2011) (Figure 1). Eukaryotic-specific parts (rRNA expansion segments, proteins absent in bacteria and eukaryotic-specific domains within conserved proteins) are located on the ribosomal surface, enveloping an evolutionarily conserved core.

Translation initiation

Translation initiation stands at the beginning of the whole translational cycle and as such, it is tightly regulated. It is the process of recognizing the AUG start codon in mRNA by methionyl initiator tRNA (Met-tRNA_i^{Met}). Translation of most eukaryotic mRNAs begins with a scanning mechanism, where the small subunit is first loaded with initiator tRNA in a ternary complex (TC) with GTP-bound eukaryotic Initiation Factor (eIF) 2 in a reaction promoted by eIF1, eIF1A, eIF5 and the multisubunit complex eIF3. This forms a 43S preinitiation complex (PIC), which in turn is recruited to the 5' end of the mRNA by the eIF4F complex, forming the 48S PIC. The 5' UTR of the mRNA is then scanned base-by-base for complementarity to the anticodon of Met-tRNA_i^{Met} as the codons of the mRNA enter the decoding site of the small subunit. AUG recognition triggers hydrolysis of GTP and release of eIF2-GDP from Met-tRNA_i^{Met} which is followed by joining of the 60S large subunit, prompting formation of an 80S initiation complex (reviewed in Hinnebusch 2017, Brito Querido et al. 2023).

While most ribosomes scanning the 5' UTR of mRNA initiate translation at the first AUG codon, a fraction of them may continue scanning and initiate at a start codon downstream, in a process called “leaky scanning”. This is more prominent when the first start codon is in a poor Kozak context or under conditions of heightened global starting codon stringency (caused by elevated levels of eIF1 or reduced levels of eIF5) (Dever et al. 2023). Near-cognate start codons that differ from AUG by one nucleotide can also serve as lower-efficiency start codons, with different preferences in different species (Kozak 1989, Koltz et al. 2009, Wei et al. 2013, Dever et al. 2023). Additionally, start codon selection can also be influenced by mRNA secondary structures, e.g. a stem-loop or hairpin structure downstream of start codons with poor context can enhance translation initiation (Kozak 1990, Wang et al. 2022).

Translation reinitiation

After start codon recognition, the 80S initiation complex proceeds to carry on into elongation, followed by termination and complete recycling of the ribosome to be used in another round of translation. However, in specific cases termination is followed by incomplete recycling (or blockage of recycling) and another initiation event occurs downstream of the stop codon on the same mRNA molecule. This process is called translation reinitiation – REI (reviewed in Gunisova et al. 2017).

Translation REI serves multiple purposes in translational regulation. One of them is an increase of coding capacity of genomes through production of multiple proteins from a single mRNA (this occurs widely in prokaryotes and viruses, organisms with tightly packed genomes). Another is merely regulation of translation of downstream main ORF through translation of uORFs. These ORFs are located upstream of the main CDS, in the 5' UTR of the corresponding transcripts. Their peptide product is often not physiologically important and these uORFs serve mainly as means of control over the translation rate of downstream main ORF (mORF), usually in an inhibitory manner (Gunisova et al. 2017).

Mechanistically, REI can be achieved either by (i) incomplete post-termination complex (post-TC) recycling, allowing only dissociation of 60S subunit by ABCE1/Rli1, followed by release

of deacylated tRNA from the P-site of the post-termination 40S to allow further downstream scanning and canonical initiation, or (ii) blocking the whole recycling process, in which case REI occurs with the post-TC in a manner that may or may not require release of the deacylated tRNA (Gunisova et al. 2017).

The most widely recognized type is REI after short uORFs, whose occurrence is relatively widespread throughout eukaryotic genomes, being found in ~13 % of yeast transcripts and ~49% of human transcripts, respectively (Calvo et al. 2009, Lawless et al. 2009). However, one needs to distinguish between inhibitory uORFs that simply impair ribosome access to the main ORF and uORFs that enable translational regulation in response to change in cellular conditions – please keep in mind that not all uORFs are regulatory (Dever et al. 2023).

The delayed REI model of translational control – *GCN4*

Arguably, the most prominent example of translational control through regulatory uORFs is translation of the Gcn4 protein in *S. cerevisiae*, which is a transcription factor that regulates the yeast's response to stress conditions. Under stress conditions, eIF2 α is phosphorylated and eIF2-P is unable to form ternary complexes (reviewed in Hinnebusch 2005).

The *GCN4* 5' UTR contains four short uORFs and this mRNA is very sensitive to TC levels that are changing in yeast cells due to different nutrient conditions. The first uORF (uORF1) is efficiently translated under both nutrient rich and starvation conditions, and generally permissive for REI - the 40S post-termination complex after terminating at uORF1 remains bound to the mRNA and scans downstream for the next AUG. In non-stressed cells (high TC levels), most ribosomes rebind the TC before reaching one of the last two uORFs (uORF3 and uORF4). These uORFs however, do not support efficient REI, therefore ribosomes terminating at these two uORFs undergo full recycling of both ribosomal subunits and do not reach the main *GCN4* coding sequence - Gcn4p expression remains stably low. In contrast, under starvation stress conditions, the Gcn2 kinase senses low amino acid levels and phosphorylates the alpha subunit of eIF2, which suspends formations of new TCs in the cytoplasm and downregulates global translation rates. Thus, scanning ribosomes are unable to acquire new TCs before reaching uORFs 3 and 4, effectively bypassing them. A large portion of the scanning ribosomes is then able to reacquire the TC downstream of uORF4 and upstream of the *GCN4* start codon, triggering expression of the Gcn4 protein (Mueller and Hinnebusch 1986, Dever et al. 1992, Gunisova et al. 2016). While uORF1 is the main REI-promoting driver of the *GCN4* 5' leader, uORF2 serves as a backup (or fail-safe) to capture ribosomes that leaky scan past uORF1 and stands at ~80-90% of uORF1 REI activity (Gunisova and Valasek 2014, Gunisova et al. 2016).

Mechanistically, these regulatory uORFs owe their functions to several *cis*- and *trans*-acting elements. It has been determined that uORF1 and uORF2 owe much of their exceptional REI activity to 5' sequences, which are required for REI past them (Grant et al. 1995). These sequences contain specific REI-promoting elements (RPEs), which form the 5' enhancer with a specific secondary structure arrangement (Munzarova et al. 2011, Gunisova and Valasek 2014). Furthermore, the eIF3a/TIF32 subunit of eIF3 also stimulates REI past these uORFs through a functional interaction with its RPEs (Szamecz et al. 2008, Munzarova et al. 2011). REI also seems to be potentiated by sequences immediately following uORF1 and by

penultimate (second to last) codon identity of uORFs 1 and 2 (Grant and Hinnebusch 1994, Gunisova et al. 2016). In contrast, the REI-inhibitory activity of uORF3 and uORF4 is influenced by both their codon identity and their 3' nucleotide context. While uORF3 exerts REI activity at around 13-18% of uORF1, uORF4 REI activity drops down to 3-5% of uORF1 (Munzarova et al. 2011, Gunisova and Valasek 2014). This difference arises partially due to a more efficient termination context at uORF3 (due to an A base following stop codon), allowing for more REI at *GCN4* mORF, whereas uORF4's stop codon is followed by a C, which promotes stop codon readthrough. This results in smaller rate of REI at *GCN4* mORF (Gunisova et al. 2016). An overview of these determinants can be found in Figure 2.

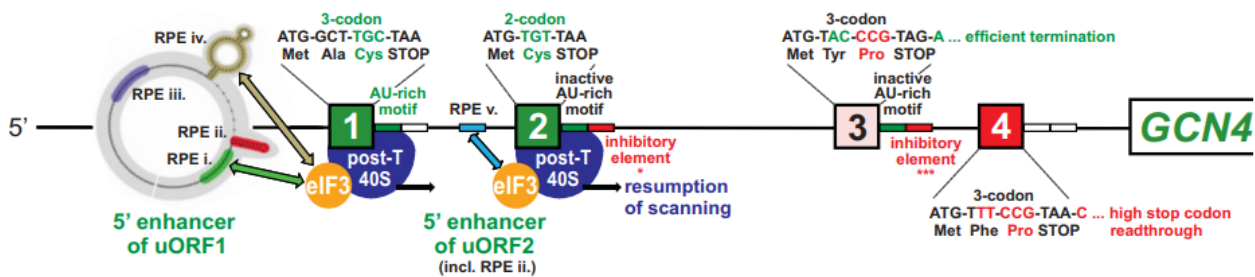


Figure 2: Summary of all *cis*-determinants that either promote or inhibit reinitiation on *GCN4* after translation of its four short uORFs. Green color-coding generally indicates stimulatory effects of the corresponding *cis*-factors on efficiency of REI, whereas red color-coding indicates inhibitory effects (with the exception of RPE ii. of uORF1, which is also stimulatory); the number of asterisks below the inhibitory elements of the uORF2 and uORF3 3' sequences illustrates the degree of their inhibition as determined experimentally. Reprinted with permission from Gunisova et al. 2016.

The delayed REI model of translational control – *ATF4*

Another example of delayed REI is mRNA of the mammalian transcription factor *ATF4* – a major player in integrated stress response (ISR) (reviewed in Pakos-Zebrucka et al. 2016). Similarly to yeast *Gcn4*, the mammalian transcription factor *ATF4* is an effector of this pathway, activating transcription of stress-response genes. Additionally, it similarly relies (at least partially) on the delayed REI mechanism.

Phosphorylation of eIF2 in mammals is at the heart of the ISR. There are four kinases that phosphorylate eIF2 α , leading to formation of fewer ternary complexes – PERK (activated by ER stress – unfolded protein response), PKR (activated by viral infection or oxidative/ER stress), GCN2 (activated by amino acid starvation, as in yeast), HRI (activated during hematopoiesis by low iron levels, or other stresses such as proteasome inhibitor or arsenite exposure) (Donnelly et al. 2013). This causes induction of *ATF4* expression through a mechanism described below, which further leads to activation of downstream effectors that affect transcription regulation (CHOP, ATF5), amino acid synthesis and transport (ASNS), protein synthesis (EPRS, WRS, 4EBP1), turnover (SQSTM1/p62, IBTK α), cell cycle (CDKN1A/p21) and even feedback control of the ISR, which leads to dephosphorylation of eIF2 α , so the stress response does not reach unacceptable levels (GADD34, TRIB3) (Wek et

al. 2023). These (and more) effectors help balance the delicate decision making between proteostasis collapse/cell death and adaptive proteostasis that leads to cell survival (Wek et al. 2023). Moreover, ATF4 is not the only gene whose expression is affected by the eIF2 α phosphorylation – other examples include the ISR effectors GADD34, CHOP and CReP (Wek et al. 2023). This robust interconnectedness is one of the intricacies of this tightly regulated process. And for a good reason – dysregulation of the ISR contributes to several pathological states. Mutations leading to hyperinduction of ISR are a contributing factor to pathogenesis of diabetes, vanishing white matter disease, Charcot-Marie-Tooth disease or epilepsy and autism. In contrast, mutations that lead to hypoinduction contribute to Wolcott-Rallison syndrome or Pulmonary venoocclusive disease (Wek et al. 2023). Of course, dysregulation of ISR also influences the pathogenesis of cancer or several metabolic, immune and cognitive diseases (Lines et al. 2023).

The *ATF4* 5' UTR contains two uORFs – a short uORF1, which is permissive for REI, and a longer uORF2, which partially overlaps the main ATF4 coding sequence (Vattem and Wek 2004, Lu et al. 2004). The original model of ATF4 regulation posits that in physiological conditions (normal TC levels), ribosomes translate uORF1 (which is REI-permissive) and after termination, the 40S post-termination complexes continue scanning further downstream, reacquire TC and proceed to initiate at uORF2, which overlaps with the main CDS and is in -1 frame. After termination at uORF2, ribosomes simply dissociate from the mRNA and levels of ATF4 expression stay low. However, in the event of cellular stress and subsequent ISR activation, global TC levels are reduced and after translation of uORF1, scanning ribosomes are unable to reacquire TC early enough to initiate at uORF2, effectively bypassing it. A portion of ribosomes then manages to reacquire TC between uORF2 and mORF start codons, initiating at the mORF and inducing translation of ATF4 protein (Vattem and Wek 2004, Lu et al. 2004). This model bears stark similarities to the *GCN4* model of translational regulation. In fact, the resemblances do not end here – similarly to *GCN4*'s uORFs 1 and 2, flanking sequences of *ATF4*'s uORF1 work to boost REI-permissivity and contain structural features that resemble *GCN4*'s 5' enhancer. Moreover, eIF3 also promotes REI here in a manner similar to its function in *GCN4*'s delayed REI (Hronova et al. 2017).

However, in the case of ATF4, there is growing body of evidence beyond this delayed REI mechanism, including a potential methylation site in the uORF2 region and alternative start site of ATF4 translation (Zhou et al. 2018). Substantial translation of uORF2 under stress conditions has also been reported (Andreev et al. 2015, Starck et al. 2016). Furthermore, a 'start-stop' element (comprised of only a start codon and stop codon) preceding uORF1, which will be referred to as uORF0, may represent an additional inhibitory element to *ATF4* expression (Wagner et al. 2020). One of the aims of this thesis will be investigation of potential *cis*-acting elements influencing ATF4 translational control.

Translation elongation

Translation elongation – the process of polypeptide synthesis by the ribosome – is conserved across all kingdoms of life and shares its key steps between eukaryotes and bacteria. During translation initiation, the anticodon of Met-tRNA_i^{Met} is base paired with the start codon of the mRNA in the P site. Elongation commences with delivery of the cognate elongating aminoacyl-

tRNA to the A site of the ribosome (reviewed in Dever et al. 2018). The eukaryotic Elongation Factor (eEF) 1A is activated upon GTP binding and forms a ternary complex with an aminoacyl-tRNA. Then, this ternary complex binds in the ribosomal A site, and the codon-anticodon base-pairing interactions trigger GTP hydrolysis by eEF1A. Then, this complex is released and the aminoacyl-tRNA is accommodated into the A site (Dever et al. 2018) in a process called decoding and proof-reading. A site accommodation is accompanied by a structural rearrangement of the small subunit dubbed ‘subunit rolling’ (Budkevich et al. 2014), which also reduces the rate at which decoding occurs, enhancing quality control of decoding by providing more time for near- and non-cognate tRNAs to dissociate (Holm et al. 2023).

Following this, peptide bond formation with the peptidyl-tRNA in the P site occurs rapidly in the PTC, which is composed of 23/28S rRNA (in prokaryotes and eukaryotes) elements and well conserved between bacteria and eukaryotes. The PTC functions by positioning the peptidyl- and aminoacyl-tRNAs for efficient catalysis (Dever et al. 2018, Ben-Shem et al. 2010), aided by eIF5A, which is thought to further induce a favorable positioning of the substrates (Dever et al. 2018, Schmidt et al. 2016).

The nascent peptide is transferred from the peptidyl-tRNA in the P site to the amino group of the A site aminoacyl-tRNA to form a new extended peptidyl-tRNA. This is accompanied by repositioning the tRNAs and subunit rotation (Moazed and Noller 1989, Budkevich et al. 2012). Translocation of the tRNAs to E and P sites is promoted by the eEF2, which binds in the A site, where it is thought to unlock the decoding “interaction” of the ribosomal decoding center with the codon-anticodon duplex in the A site (Dever et al. 2018, Taylor et al. 2007). In the same fashion as has been described in bacterial studies (Ratje et al. 2012, Ramrath et al. 2013), the movement of the tRNAs into P and E sites is complemented by swiveling of the small subunit head and anticlockwise rotation of the small subunit relative to the large subunit (Djumagulov et al. 2021, Ranjan et al. 2021). Deacylated tRNA is then slowly released from E site of ribosomes. In yeast, this release is catalyzed by eEF3, which is – however – absent from higher eukaryotes (Ranjan et al. 2021).

An overview of the elongation pathway can be found in Figure 3.

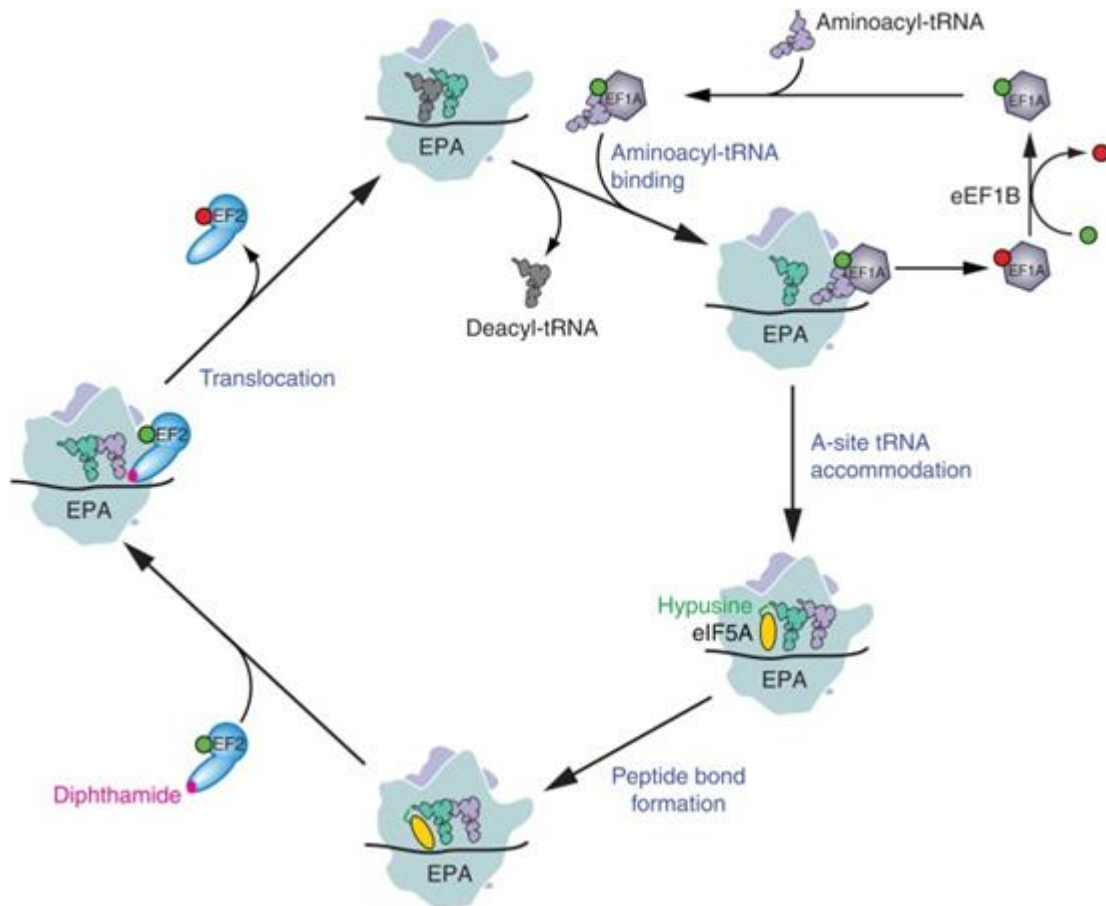


Figure 3: Model of the eukaryotic translation elongation pathway. At the top, an eEF1A•GTP•aminoacyl-tRNA ternary complex binds to the A site of an 80S ribosome with the anticodon loop of the tRNA in contact with the mRNA. Following GTP hydrolysis and release of an eEF1A•GDP binary complex, the aminoacyl-tRNA is accommodated into the A site, and the eEF1A•GDP is recycled to eEF1A•GTP by the exchange factor eEF1B. During catalysis of peptide bond formation, the A- and P (peptidyl)-site tRNAs shift into hybrid states with the acceptor ends of the tRNAs moving to the P and E sites, respectively. Substrate positioning for peptide bond formation is aided by binding of the factor eIF5A and its hypusine modification (green) in the E site. Following peptide bond formation, the factor eEF2•GTP with its diphthamide modification (magenta) binds in the A site and promotes translocation of the tRNAs into the canonical P and E sites. Following release of the deacylated tRNA from the E site, the next cycle of elongation commences with binding of the appropriate eEF1A•GTP•aminoacyl-tRNA to the A site. Throughout, GTP is depicted as a green ball and GDP as a red ball; also, the large ribosomal subunit (light blue) is displayed transparently to enable visualization of the tRNAs, factors, and mRNA bound to the decoding center at the interface between the large and small subunits and of tRNAs, interacting with the peptidyl transferase center in the large subunit. Note, however, that the positions of the mRNA, tRNAs, and factors are drawn for clarity and are not meant to specify their exact places on the ribosome. Reprinted with permission and edited from Dever et al. 2018.

Translation termination

Translation termination commences upon recognition of the stop codon in the A site by eukaryotic Release Factor (eRF) 1. This factor also contains a universally conserved GGG motif that induces release of the nascent polypeptide from peptidyl-tRNA in the P site (reviewed in Hellen 2018).

Stop codon recognition is influenced by multiple interactions between stop codon along with its surrounding nucleotides, and both 18S rRNA and eRF1 to certain extent resemble the codon-anticodon minihelix. Recognition of stop codons likely occurs via multiple steps involving RNA compaction in the A site, conformational changes in key 18S rRNA decoding bases, and exposure of the residues of the TASNKS and GTS motifs in eRF1 and other localized eRF3-induced changes (Hellen 2018, Fan-Minogue et al. 2008; Wong et al. 2012; Kryuchkova et al. 2013). The GTPase eRF3 helps accommodate eRF1 in the PTC after GTP hydrolysis (which is aided by eRF1 itself) (Cheng et al. 2009, Bulygin et al. 2016), while eRF3 also stimulates eRF1's peptide release activity, thus coupling stop codon recognition and peptidyl-tRNA hydrolysis by eRF1 (Alkalaeva et al. 2006, Salas-Marco & Bedwell 2004, Hellen 2018). Apart from enhancing the rate of termination, eRF3 could potentially also increase its fidelity by introducing “kinetic proofreading” through irreversible GTP hydrolysis step between stop codon recognition and peptidyl-tRNA hydrolysis (Hellen 2018).

The mechanism of peptide release is mediated by the GGG motif of eRF1. Conformationally, after eRF3-bound GTP hydrolysis and eRF3 departure from the ribosome, the accommodated eRF1 positions GGG motif in the PTC next to the CCA end the P-site peptidyl tRNA, adjacent to the bond between tRNA and nascent polypeptide (Preis et al. 2014). We can infer the mechanism from bacteria, where Release Factors (that are otherwise unrelated to eRF's) also contain a conserved GGG motif. Bacterial GGG motif in the PTC induces conformational changes that expose the peptidyl-tRNA ester bond to nucleophilic attack by water, promoting cleavage and release of nascent polypeptide (Hellen 2018, Jin et al. 2010, Kazemi et al. 2016).

Overview of translation termination can be found in Figure 4.

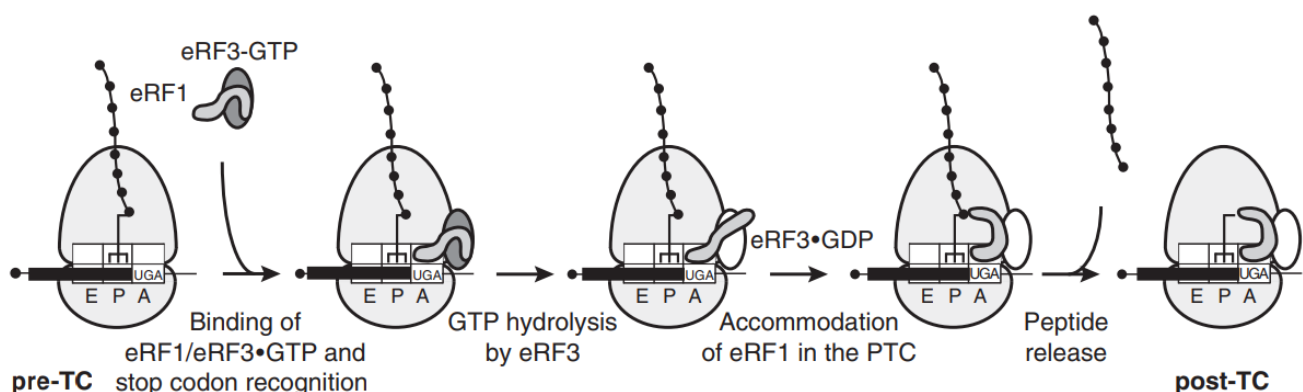


Figure 4: Outline of the termination process. The pretermination complex (pre-TC) contains peptidyl-transfer RNA (tRNA) in the P site. The eRF1/eRF3–guanosine triphosphate (GTP) complex binds to the A site of the pre-TC, and eRF1 recognizes the stop codon, which, with

the +4 nucleotide, binds in a pocket formed by eRF1 and the 40S subunit. eRF1's M domain dissociates from eRF3's Switch I/Switch II elements, and after GTP hydrolysis by eRF3, accommodates in the peptidyl-transferase center (PTC), inducing peptide release. eRF1 and possibly eRF3-guanosine diphosphate (GDP) remain associated with post-termination complexes (post-TCs). Reprinted with permission from Hellen 2018.

Stop codon readthrough

The process of termination is, however, not 100% efficient. One of the most investigated mechanisms of termination suppression is stop codon readthrough (SC-RT). In this process, a near-cognate aminoacyl tRNA (can mispair with position 1 and 3 of the stop codon sequence) is accommodated in the A-site of the ribosome instead of eRF1. This leads to erroneous decoding of the stop codon as a sense codon and the polypeptide is C-terminally extended until the next stop codon. SC-RT thus relies on the competition between a near-cognate tRNA and eRF1 for stop codon recognition (Dabrowski et al. 2015).

This process can be spontaneous (or basal) or programmed in different environmental conditions. The efficiency of basal SC-RT is influenced by various elements. In particular, the stop codon itself plays a role, with UGA > UAG > UAA ordered in terms of highest to lowest readthrough efficiency. The +4 base (with first nt of the stop codon marked as +1) also has strong influence on SC-RT, e.g. with C at the +4 position exerting the highest RT potential at UGA and UAA codons. (Dabrowski et al. 2015, Bonetti et al. 1995, Beznoskova et al. 2015, McCaughan et al. 1995). In addition, several motifs exist that are known to stimulate SC-RT in both viral RNAs and the eukaryotic genome (Beier & Grimm 2001, Fearon et al. 1994, Loughran et al. 2014), sometimes even with clinical relevance in humans (Pacho et al. 2011).

Interestingly, 11% of inheritable genetic diseases are caused by nonsense mutations – single base pair substitutions, which convert a sense codon in a premature termination codon (PTC), resulting in truncated proteins and reduction of mRNA levels of these particular transcripts due to PTCs triggering the Nonsense-Mediated mRNA Decay (NMD) pathway (Lombardi et al. 2020). Thus, the mechanism of SC-RT has gained major interest as a druggable target. Apart from readthrough inducing drugs, there have also been efforts to engineer specific tRNA therapeutics with high activity and safety (Lombardi et al. 2020, Collier & Ignatova 2024).

Ribosome recycling

Recycling is the last phase of the translational cycle. In this process, the 60S subunit is split from the 80S post-TC and the remaining 40S post-TCs is further recycled to be used in the next round of translation. A failure of this process may result in increased REI, as described in the corresponding chapter.

Subunit splitting is mediated by yeast Rli1 (ABCE1 in mammals), which is an essential ATPase that interacts with eRF1 to facilitate recycling (Pisarev et al. 2010, Preis et al. 2014, Young et al. 2015). In ABC proteins (such as Rli1/ABCE1), hydrolysis of ATP and release of ADP leads to conformational changes that promote structural rearrangement of the affected molecules and their binding partners (Reese 2009). In the case of Rli1/ABCE1, ATP hydrolysis is accompanied by structural rearrangement that leads to change in Rli1/ABCE1 conformation

and shifting of eRF1 (Preis et al. 2014). These changes could destabilize the intersubunit bridges, leading to the subunit splitting step of recycling (Hellen 2018). In vitro, subunit splitting can be mediated solely by eRF1:eRF3, as well as ribosome rescue factors Dom34 (mammalian Pelota) and Hbs1 (mammalian HBS1L), which are part of the No-Go Decay pathway and are homologous to eRFs (Shoemaker et al. 2010). However, addition of Rli1 greatly stimulates this activity (Shoemaker & Green 2011). In vivo, depletion of Rli1 in yeast leads to 80S ribosome accumulation at stop codons in 3' UTRs of virtually all yeast genes. In fact, this recycling defect also leads to aberrant REI in the 3' UTR. Deletion of the aforementioned rescue factor Dom34 further increases this ribosome accumulation, suggesting Dom34 is critical for rescue of ribosomes that escaped the normal recycling process (Young et al. 2015). Moreover, the eIF3-associated factor eIF3j (yeast Hcr1) has been shown to promote subunit splitting as an accessory factor of Rli1. This factor is involved in many processes, including AUG selection (Elantak et al. 2010), recruitment of other eIF3 components to the 40S subunit during initiation (Aylett et al. 2015) and finally, translation termination and stop codon readthrough (Beznoskova et al. 2013, 2015). Its involvement in 60S subunit recycling has been shown *in vivo* in yeast, where the phenotype of *HCR1* deletion was consistent with a defect in 80S recycling that can be rescued by *RLI1* overexpression. Curiously, in the same study, *RLI1* expression was also shown to increase in the absence of Hcr1, revealing a feedback mechanism (Young & Guydosh 2019).

After subunit splitting, the 40S post-TC remains bound to the mRNA, with deacylated tRNA bound to its P-site. For the subunit to get recycled (or REI further downstream), this P-site tRNA needs to be vacated from the ribosome. To complete the process, the 40S subunit dissociates from the mRNA. This is thought to occur through various possible means. The most thoroughly investigated mechanism relies on factors Tma20 (yeast)/MCTS1 (mammals, also known as MCT-1 or MCT1), Tma22/DENR (which form the DENR:MCTS1 heterodimer) and Tma64/eIF2D (a longer protein with two halves homologous to the DENR:MCTS1 heterodimer). These factors have been shown *in vivo* to promote recycling of the 40S subunit in yeast (Young et al. 2018, 2021) and to promote REI via ribosome recycling of the 40S subunit in mammals (Bohlen et al. 2020).

The other suggested pathway of P-site tRNA recycling/40S dissociation from the mRNA involves initiation factors eIF1, eIF1A and eIF3j, but has only been proven through a reconstituted *in vitro* system. In this model, eIF1 and eIF1A promote release of deacylated tRNA, while eIF3j promotes release of 40S post-TC from the mRNA (Pisarev et al. 2007). It is interesting to note that while Rli1/ABCE1 is an essential factor indispensable for 60S recycling, Tma20/MCTS1, Tma22/DENR and Tma64/eIF2D are not – this could suggest that there is a degree of redundancy between the “eIF” and “DENR” pathways in 40S recycling (that is not present in 60S recycling), or that the spontaneous rate of dissociation may be adequate to maintain cellular function (Young & Guydosh 2022).

Overview of recycling pathways can be found in Figure 5.

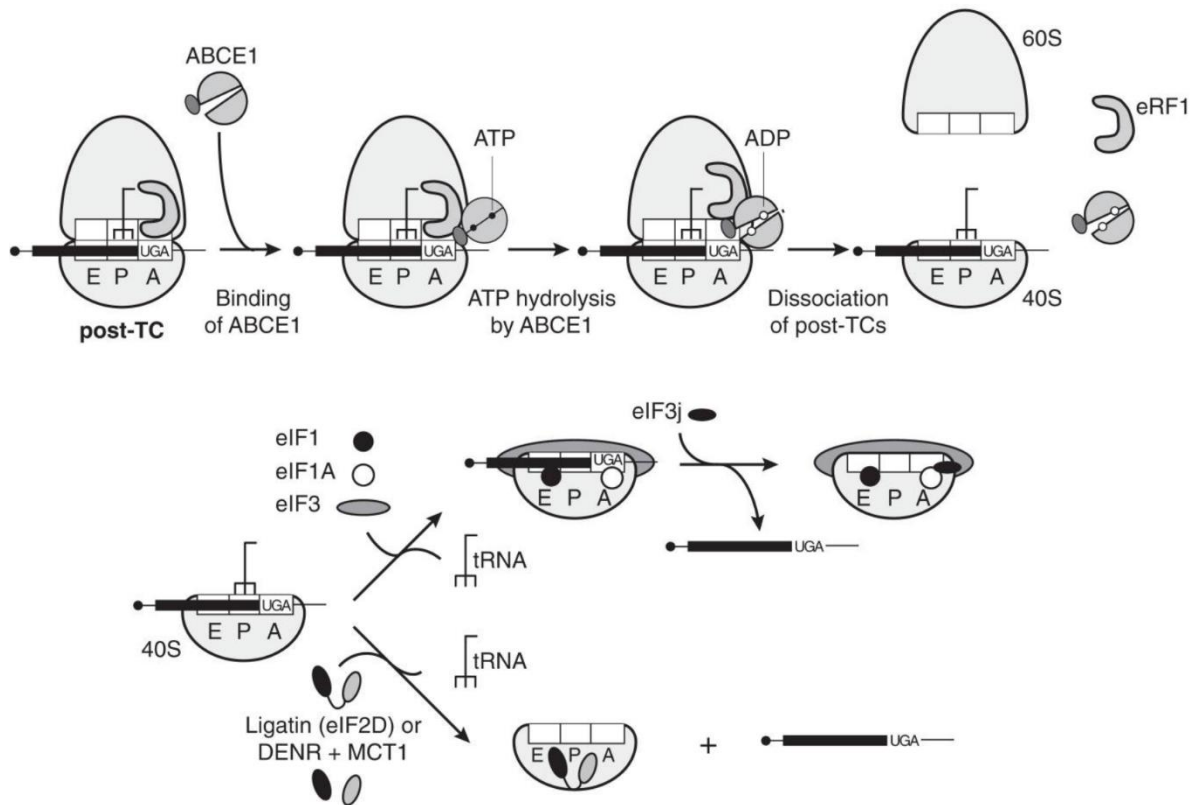


Figure 5: Upper half: Model for ribosome recycling (Pisarev et al. 2010), in which ABCE1 binds to eRF1 on the post-termination complex (post-TC), and ATP hydrolysis leads to a power stroke that splits the post-termination ribosome, yielding a 60S subunit, eRF1, ABCE1, and a 40S subunit still bound to messenger mRNA and deacylated tRNA. Lower half: Outline of the release process mediated by initiation factors 1, 1A, 3, and 3j, by eIF2D, or by MCT1 and DENR. Reprinted with permission and adapted from Hellen 2018.

Trans-acting translation factors

In this chapter, many of the factors influencing the mechanisms introduced in the last chapter will be further described. Since the experimental work in this thesis is focused on translation REI and ribosome recycling, this chapter will be limited to initiation and recycling factors, with special emphasis on 40S recycling. It includes information about both yeast and mammalian factors, including a comparison of the differences between these two systems.

(Re)initiation factors

The eIF4F complex

The heterotrimeric eIF4F complex is composed of three distinct members – eIF4A (DEAD-box RNA helicase protein), eIF4E (m⁷G cap binding protein) and eIF4G (scaffolding protein). Together, these proteins are recruited to the mRNA (Figure 6), while also aiding mRNA circularization through the interaction of eIF4G with PABP (poly-A binding protein), which also enhances eIF4F's affinity for the mRNA cap (Brito Querido et al. 2024).

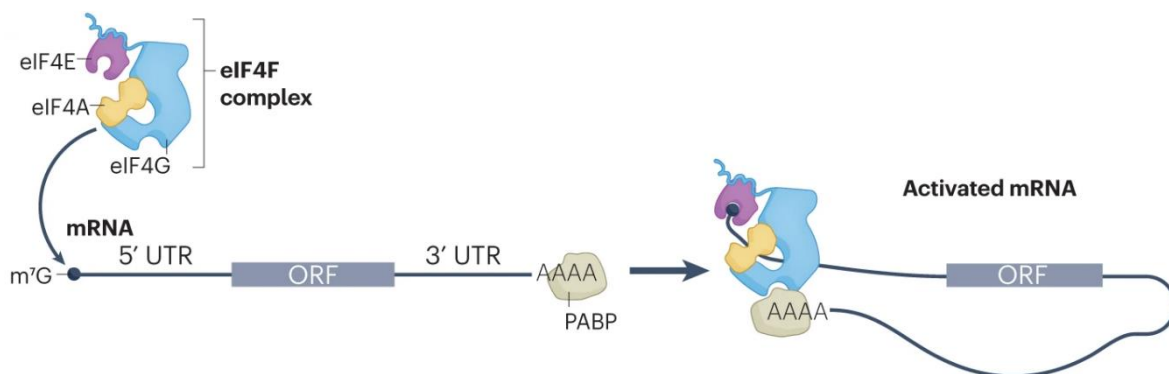


Figure 6: Recruitment of eukaryotic translation initiation factor 4F (eIF4F) to mRNA through eIF4E–7-methylguanosine (m⁷G) cap and eIF4G–polyadenylate-binding protein (PABP)–poly(A) interactions. Reprinted with permission and adapted from Brito Querido et al. 2023.

The eIF4A helicase is a bit peculiar – it is present in excess in the cell compared to other initiation factors, and most of eIF4A in the cell is not part of the eIF4F complex (Kulak et al. 2014). eIF4A may also exhibit 4F-independent activity through forming its own interaction with mRNA (Wang et al. 2022, Brito Querido et al. 2024). Interestingly, eIF4A is very non-processive on its own, but when coupled with accessory factor eIF4G and eIF4B (a factor that is not part of the eIF4F complex), it functions as an ATP-dependent processive helicase (Garcia-Garcia et al. 2016).

There are two main proposed mechanisms of eIF4F-dependent recruitment of mRNA – the slotting and threading model (Figure 7). As summarized in Fig. 7 legend, in the slotting model, the 5' UTR is laterally inserted into the mRNA channel, thus the eIF4F complex is positioned upstream of the 43S complex. However, the start codon would need to be located ~40 nt downstream from the cap. In the threading model, eIF4F binds to 43S PIC at the mRNA entry site – once 43S PIC is attached, eIF4E would have to dissociate from the cap to allow the mRNA

to thread into 40S, presumably driven by helicase activity of eIF4A. It is predicted that this way, the 43S can scan from the very first nucleotide of the 5' UTR (Brito Querido et al. 2024).

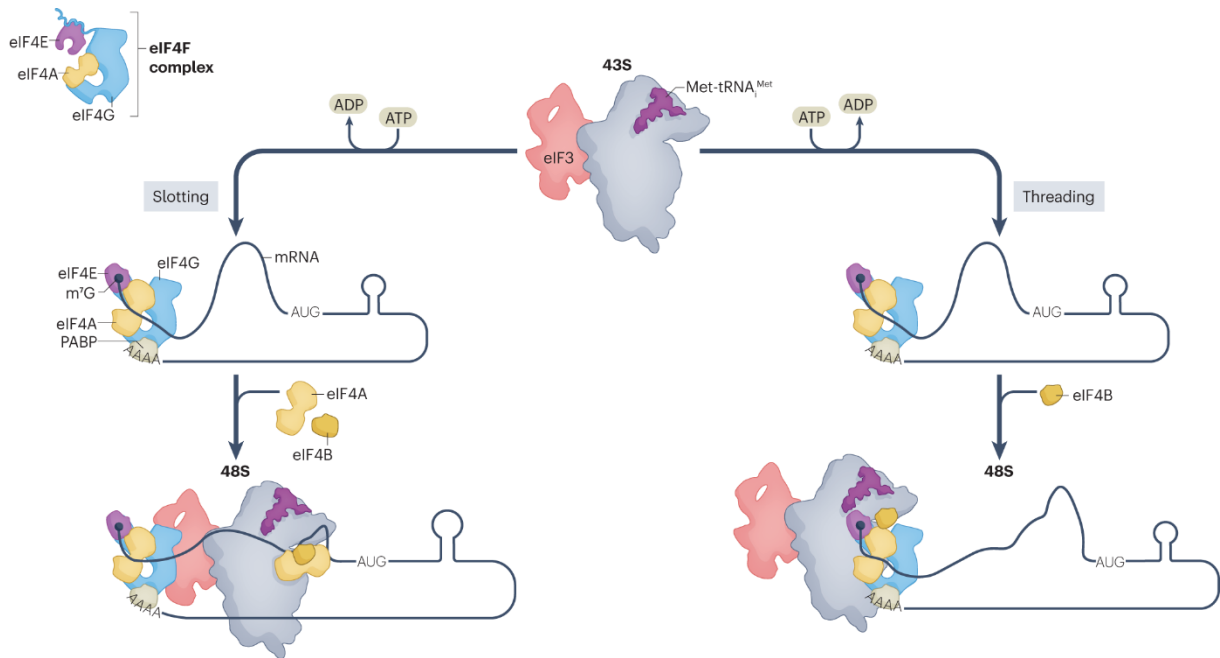


Figure 7: Two main mechanisms of eukaryotic translation initiation factor 4F (eIF4F)-dependent recruitment have been proposed: the slotting model (left) and the threading model (right). Both mechanisms would require ATP hydrolysis for recruitment. In the slotting model, eIF4F binds upstream of the ribosome, near the mRNA channel exit site in the 40S complex, and the mRNA is laterally slotted into the channel. A second molecule of eIF4A (in addition to the one in eIF4F) and its cofactor eIF4B bind on the opposite side of the 43S pre-initiation complex (43S) at the mRNA entry site to increase recruitment efficiency. Thus, eIF4A–eIF4B has an eIF4F-independent role during recruitment and scanning. The scanning model requires a minimum distance between the 5' end of mRNA and the start codon, which needs to be positioned at the P site of the ribosome, and is compatible with the length of typical 5' untranslated regions (UTRs). However, mRNAs with short 5' UTRs would have a start codon too close to the 5' end to reach the P site. Thus, these mRNAs would require backtrack scanning or an alternative mechanism for recruitment. In the threading model, eIF4F binds near the mRNA entry site, followed by the release of eIF4E from the 5' cap (not shown), thereby allowing threading of the mRNA through the mRNA channel and its scanning from the first nucleotide downstream of the cap. This model would permit translation of any mRNA regardless of the length of the 5' UTR. However, it is not compatible with recent structures of the 48S complex. Reprinted with permission from Brito Querido et al. 2024.

While the threading model is supported by biochemical data showing efficient initiation on very short 5' UTR mRNAs (Kumar et al. 2016), as well as on IRES (internal ribosomal entry site)-containing mRNAs (reviewed in Mailliot & Martin 2018) and circRNAs (Pamudurti et al. 2017, Yang et al. 2017), some structural studies do not support this model. Brito Querido et al. (2020) showed that eIF4F seems to be located on the exit side of the mRNA channel, not the entry side, supporting the slotting model. Furthermore Bohlen et al. (2020) demonstrated that eIF4E

remains associated with the ribosome after initiation with a decay half-length of 12 codons. This is in opposition with the threading model, which has the 4E dissociate, although it is unclear whether this dissociation is from the cap or from 4G. The position of eIF4F complex in the structure of human 48S supports the slotting model and suggests the start codon would have to be at least 40 nt from the 5' end of the mRNA (Brito Querido et al. 2020, Brito Querido et al. 2024).

Since the eIF4F complex seems to be positioned upstream of the 43S PIC, there is a question of how it can aid scanning. This mystery is amplified by the assertion that eIF4A may not require eIF4G or eIF4E for promoting scanning but is also active with the help of select subunits of eIF3 (Yourik et al. 2017), which are located on the opposite sites of the ribosome. Structural work has shed light on this (Brito Querido et al. 2024 (2)), revealing that there is a second eIF4A molecule located on the opposite site of 43S, at the mRNA entry site, possibly functioning separately of eIF4F, also positioned closely to eIF4B which functions as its partner in processive helicase activity (Garcia-Garcia et al. 2016). It is possible that eIF4F also serves as a Brownian ratchet (Li et al. 2012), using energy from ATP hydrolysis by 4A to move in 5'→3' direction and prevent any backward (3'→5') movement of the 43S PIC (Brito Querido et al. 2020, Abaeva et al. 2016). This way, eIF4F and the second eIF4A molecule may both work towards assuring directionality and efficiency of scanning (Brito Querido et al. 2024).

Evidence in mammals and yeast also points to a key role of eIF4F in REI, where eIF4G and 4E remain bound to 80S ribosomes as they begin translating on short uORFs (Bohlen et al. 2020, Mohammad et al. 2021).

eIF2, eIF1, eIF1A, eIF5

The factor eIF2 has a uniquely important function – it binds GTP and the initiator tRNA Met-tRNA_i^{Met}, forming a TC, which binds to the 40S subunit in order to facilitate translation initiation. It is a heterotrimer of subunits α , β and γ (Figure 8, Dever et al. 2016). The affinity of Met-tRNA_i^{Met} binding to eIF2 is controlled by guanine nucleotides, which interact with high affinity when eIF2 is bound to GTP. GTP hydrolysis and Pi release stimulated by eIF5 (Figure 8) is prompted by AUG codon recognition during the scanning process. The resulting eIF2-GDP complex has low affinity for Met-tRNA_i^{Met}, and eIF2-GDP leaves the ribosome together with eIF5 (Kapp & Lorsch 2004, Adomavicius et al. 2019, Jennings et al. 2017). The reacquisition of Met-tRNA_i^{Met} is then facilitated by another factor, eIF2B. EIF2B acts to dissociate eIF5 and then serves as a guanine nucleotide exchange factor to activate eIF2 upon AUG recognition (Adomavicius et al. 2019, Jennings et al. 2013, Jennings et al. 2017).

Binding of eIF2 TC to 40S is facilitated by factors eIF1 and eIF1A (Figure 9), which bind to the 40S subunit. eIF1 binds to the 40S near the P site and eIF1A near the A site, likely also to prevent Met-tRNA_i^{Met} binding to the A site (Hussain et al. 2014, Dever et al. 2016). The binding of these factors to the 40S subunit rotates its head, possibly providing better access for the TC to enable binding of mRNA, and thus to promote an “open” complex (P_{out}) (Figure 9). Movement of Met-tRNA_i^{Met} into the P_{in} conformation triggers the release of eIF1. Then eIF5

binds the site vacated by eIF1 and stabilizes $\text{tRNA}_i^{\text{Met}}$ interaction, with concurrent completion of GTP hydrolysis by eIF2 and Pi release (Dever et al. 2023, Llacer et al. 2018).

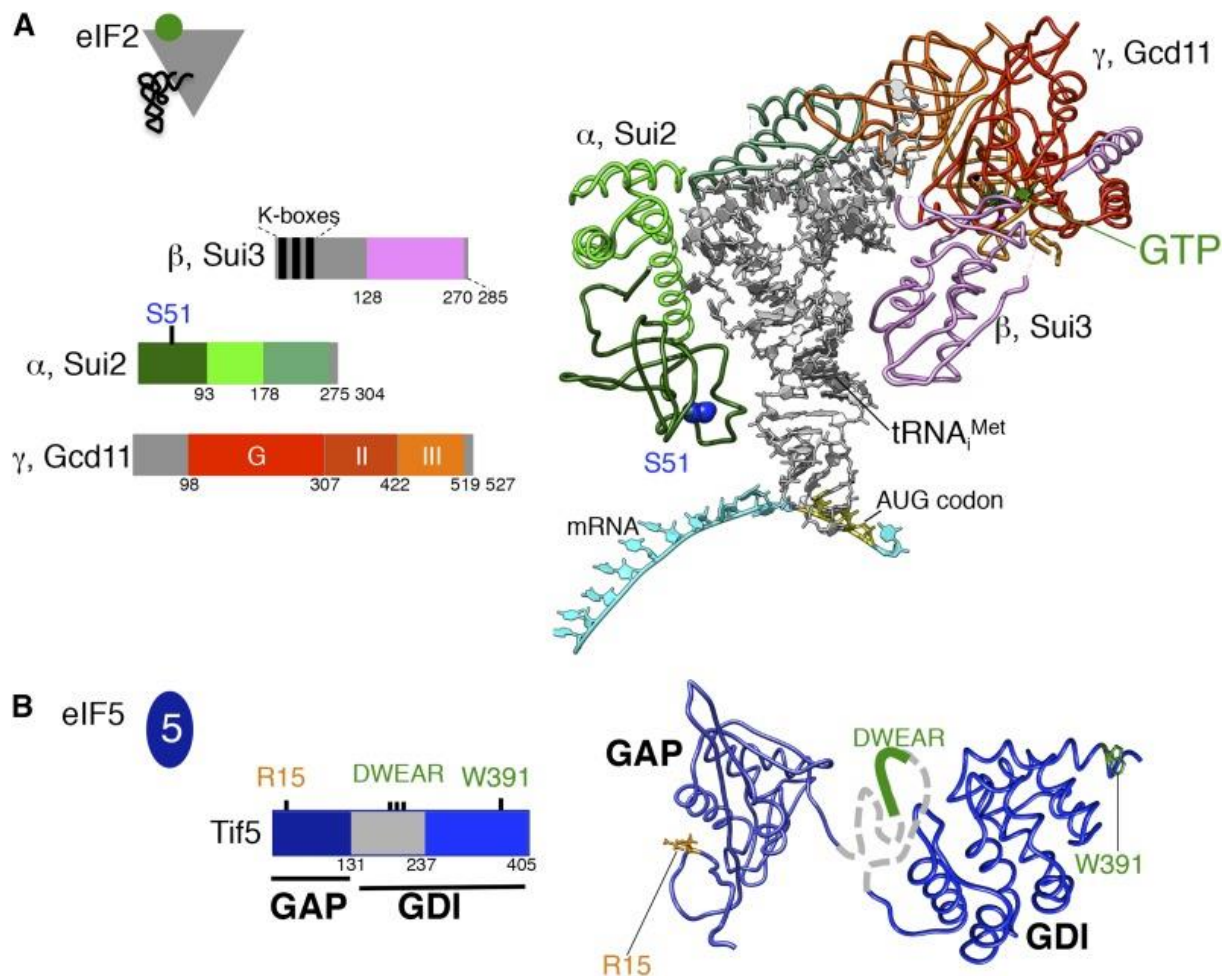


Figure 8 Schematic and structural models of eIF2 and eIF5. (A) Structural model of the eIF2–GTP–Met– $\text{tRNA}_i^{\text{Met}}$ ternary complex bound to an mRNA AUG codon (right) and cartoons depicting the eIF2 α , β , and γ subunit structural domains (left) using the same color schemes. The structural model is adapted from the structure of the yeast 48S complex (pdb 3JAP) with the 40S ribosome and other initiation factors omitted for clarity (Llacer et al. 2015). The eIF2 α residue Ser51 (blue), GTP analog (green), Met– $\text{tRNA}_i^{\text{Met}}$ (gray), and mRNA (cyan) with AUG codon (yellow) are indicated. (B) eIF5 domains and activities (left) and structural models (right) for the human GAP domain bearing R15 (pdb 2E9H) and the yeast CTD bearing W391 (pdb 2FUL) (Wei et al. 2006). Structures were drawn using Chimera software (University of California, San Francisco, UCSF). Reprinted with permission from Dever et al. 2016.

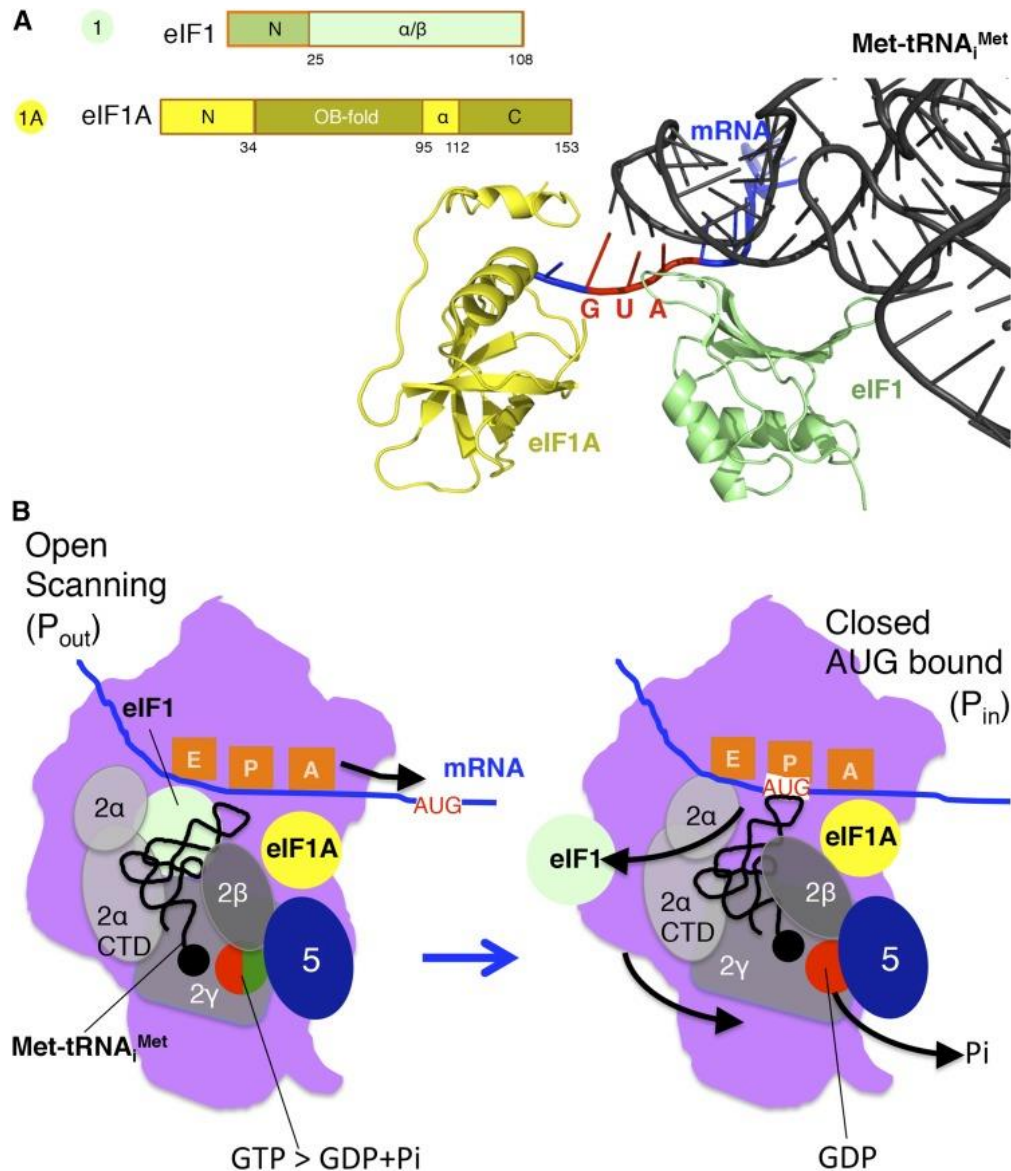


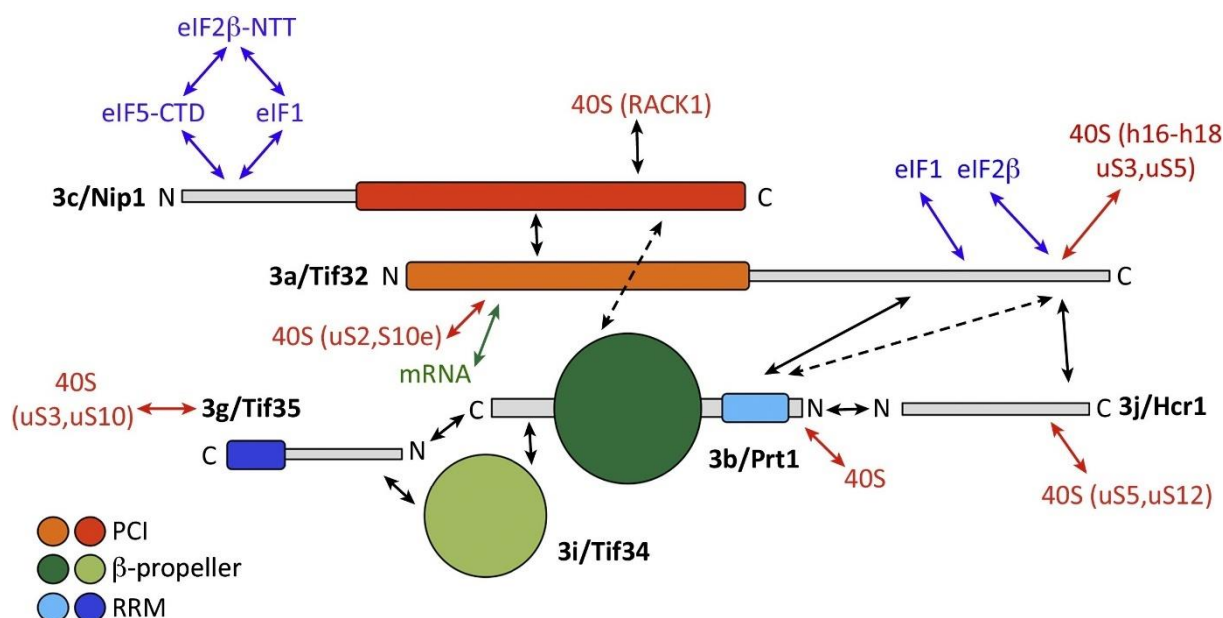
Figure 9: Schematic and structural models of eIF1, eIF1A, and AUG codon selection. (A) Structural model (right) and schematics (left) of eIF1 (green) and eIF1A (yellow) bound to the 48S PIC (pdb 3JAP) along with Met-tRNA_i^{Met} (black) and mRNA (blue, AUG codon in red), but other factors and the ribosome are removed for clarity. Structure was generated using the PyMol Molecular Graphics System (version 1.7.6.6, Schrödinger). (B) Cartoon showing approximate positions of eIFs 1 and 1A with TC and eIF5 in the open scanning conformation (left) with Met-tRNA_i^{Met} not fully engaged in the P site (P_{out}), and factor movements (black arrows) induced by AUG codon recognition (right) and the transition to the closed complex (P_{in}) signaled by movement of eIF1 that triggers Pi release prior to eIF2-GDP-eIF5 release from the PIC. Reprinted with permission from Dever et al. 2016.

However, eIF1A remains bound to the A site and helps to recruit the second GTPase eIF5B, which reorients the position of Met-tRNA_i^{Met} to enable 60S subunit joining (Lapointe et al. 2022, Dever et al. 2023). Subsequent GTP hydrolysis enables the release of these two factors and the 80S ribosome may proceed into elongation.

In contrast with yeast, in mammalian cells eIF5 is not a main part of the 43S PIC until the recognition of a start site (Brito Querido et al. 2020, Llacer et al. 2015, Hussain et al. 2014) and, according to a preprint study (Grosely et al. 2024), it binds initiation complexes only transiently – only one or two eIF5 binding events are needed for eIF2 dissociation and eIF5B association. This binding also is varied in response to the identity of the start site – binding was less frequent on initiation sites with not start site or AUC, CUG, UUG and GUG start sites, most likely due to binding kinetics of eIF5 with the anti-codon stem and loop of Met-tRNA_i^{Met}. Destabilization of eIF5 at alternative start sites might also promote eIF1 reassociation to resume scanning. This would further contribute to control of fidelity of start codon selection.

eIF3

The yeast eIF3 complex has 5 core subunits (3a/Tif32, 3b/Prt1, 3c/Nip1, 3g/Tif35, 3i/Tif34) along with the associated, non-stoichiometric factor eIF3j/Hcr1 (reviewed in Valasek et al. 2017). These subunits interact with each other along with the small ribosomal subunit and other factors. Their interaction map can be seen in Figure 10.



Trends in Biochemical Sciences

Figure 10: Interactions of Yeast eIF3 Subunits. Linear depictions of yeast eIF3 subunits with arrows showing established interactions with one another (black), other initiation factors in the MFC (blue), the 40S subunit (and specific 40S constituents in parenthesis) (red), and mRNA at the exit channel (green). The proteasome-COP9-initiation factor (PCI), β-propeller, and RRM domains, of known structure, within eIF3 subunits are indicated by filled colored rectangles or circles. Abbreviations: eIF, eukaryotic initiation factor; MFC, multi-initiation factor complex; NTT, N-terminal tail; RRM, RNA recognition motif. Reprinted with permission from Hinnebusch 2017.

As portrayed in Fig. 10, 3i and 3g form a subcomplex, and 3a and 3c form a second subcomplex. These two subcomplexes are bridged by 3b, and 3a and 3b are both able to interact with 3j. The

structure of yeast 48S PIC in closed conformation (after recognition of the start codon) with eIF3 and other initiation factors labeled, can be seen in Figure 11.

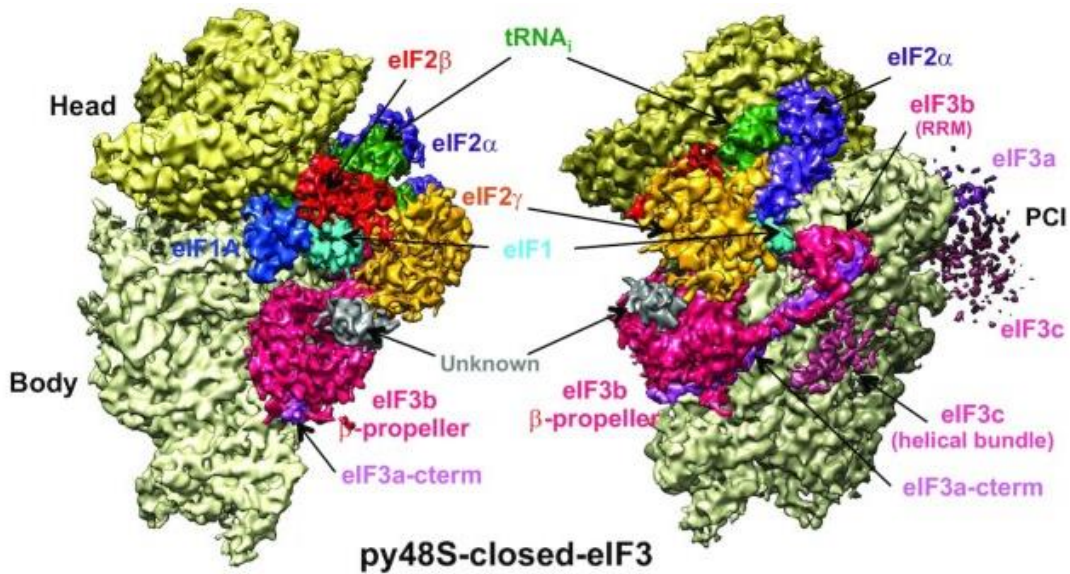


Figure 11: Cryo-EM maps (at 5.8 Å, threshold of 0.05) of the py48S-closed-eIF3 PIC shown in two orientations, with labelled select eIFs. Reprinted with permission from Llacer et al. 2021.

Structurally and functionally, eIF3 is the largest and most complex of all eIFs, and it promotes nearly every step of translation initiation (Valasek et al. 2017). It has a role in subunit separation (Kolupaeva et al. 2005), recruitment of TC to the 43S PIC, and 48S PIC assembly by mRNA recruitment (Valasek et al. 2004, 2002, Karaskova et al. 2012, Aitken et al. 2016, Mitchell et al. 2010). eIF3 also controls the processivity of scanning (Valasek et al. 2004, Karaskova et al. 2012, Aitken et al. 2016, Llacer et al. 2021). In addition, eIF3 stays bound to 80S complexes during early elongation at short uORFs, helping to promote translation REI (Mohammad et al. 2017, Munzarova et al. 2011).

The core eIF3 factors may also play a role in ribosome recycling, as outlined in the previous chapter on recycling – *in vitro*, eIF3 along with eIF1, 1A and 3j were sufficient to promote complete recycling of post-TC even in the absence of *bona fide* recycling factors (Pisarev et al. 2007). Along with eIF3j, eIF3 has been shown to control stop codon readthrough (Beznoskova et al. 2013, 2015).

Unlike its yeast counterpart, the mammalian eIF3 complex (with a molecular mass of 804 kDa) has 12 subunits (3a, 3b, 3c, 3d, 3e, 3f, 3g, 3h, 3i, 3k, 3l, 3m). These subunits assemble into distinct subcomplexes – a, c, e, f, h, k, l, m form the so-called octamer; b, g and i form the Yeast-like Core together with the C-terminal region of a, and the d subunit attaches to eIF3 via 3e (Valasek et al. 2017). Overview of this assembly of subunits along with some of its interaction partners can be found in Figure 12.

Apart from the roles in translation initiation already mentioned for yeast eIF3, mammalian eIF3 plays a significant role in noncanonical initiation events, usually in conditions where canonical initiation is inhibited (reviewed in Brito Querido et al. 2024), such as:

- IRES-dependent initiation
- m⁶A-dependent initiation
- eIF3d-dependent initiation

IRESs are *cis*-acting RNA elements with complex secondary structures that were first described in viral mRNAs (Pelletier and Sonenberg 1988). These viral IRESs are often used to bypass stress induced shutdown of cap-dependent initiation while eliminating the need for a 5' m⁷G cap or some initiation factors, and viruses are thus able to evade innate immunity response such as eIF2 phosphorylation (Mailliot and Martin 2018, Brito Querido et al. 2024). Four classes of viral IRESs have been described – type I and II require all initiation factors apart from eIF4E, type III requires only eIF1A, eIF2, eIF5 and eIF5B, and type IV requires no canonical initiation factors (Mailliot and Martin 2018, Brito Querido et al. 2024). eIF3 specifically plays a role in initiation from specific IRESs, such as HCV-like IRESs contained in Hepatitis C Virus (HCV) and Classical Swine Fever Virus (CSFV) mRNA. For example, eIF3 is required for initiation from the HCV IRES (Buratti et al. 1988). Interestingly, structural studies of the CSFV virus IRES with eIF3 have shown that the HCV-like IRES might even displace eIF3 from its position in the 43S PIC, inhibiting formation of new 43S PIC and thus making the translation apparatus favor viral mRNAs (Hashem et al. 2013). However, in the case of the intracellular X-linked Inhibitor of Apoptosis IRES, binding of eIF3 to the IRES seems to potentiate the ribosomal recruitment to this IRES, promoting the IRES-driven translation (Thakor et al. 2016). Thus, it seems like eIF3 might have both stimulatory and inhibitory effects on IRES translation (Valasek et al. 2017). Interestingly, in the case of the HIV virus, while not directly IRES related, eIF3d subunit seems to inhibit replication of the virus and is a target for the HIV protease (Jäger et al. 2011), also through modulation of T cell function (Pan et al. 2019).

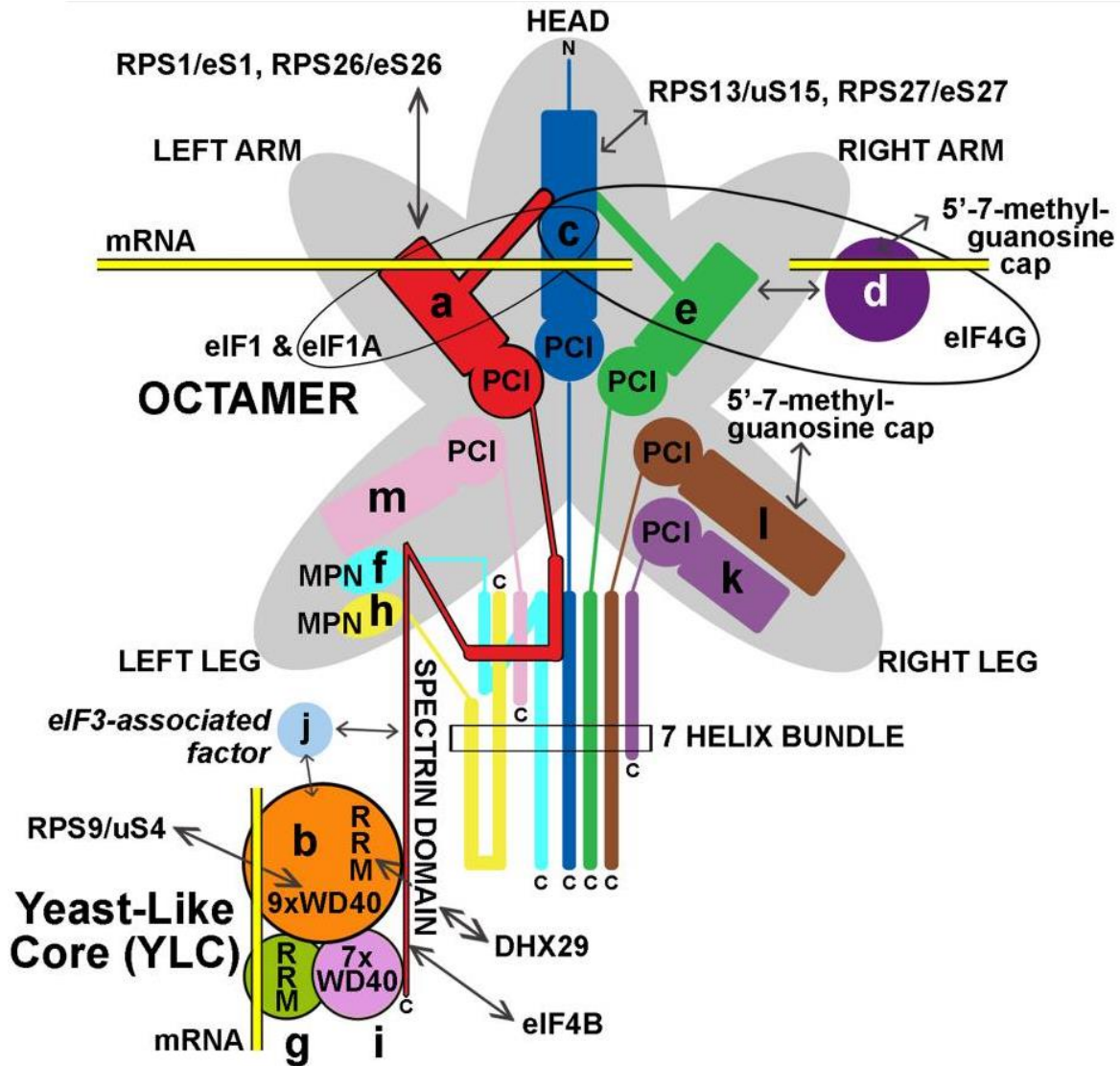


Figure 12: A schematic model of human eIF3. The eIF3 subunits forming the PCI (*proteasome, COP9/signalosome, eIF3 - domain*) / MPN (*Mpr1-Pad1-N-terminal – domain*) octamer with are indicated by the grey background. The rectangle marks the seven α -helices involved in formation of the 7-helix bundle. The Yeast-Like Core (YLC) comprising the eIF3 subunits a, b, g and is depicted and so is the eIF3-associated factor eIF3j with arrows indicating its contacts with other eIF3 subunits. The upper right-hand side arrow indicates the interaction between eIF3e and eIF3d that attaches eIF3d to the rest of eIF3. Arrows indicate all known interactions of eIF3 domains with other eIFs, ribosomal proteins and mRNA. Adapted from Valasek et al. 2017.

M^6A – the methylation of adenosine at position N6 is the most common internal mRNA modification, implicated in controlling RNA stability, and is present all throughout the mRNA – 5' UTR, CDS and 3' UTR (Brito Querido et al. 2024). It has also been proposed to affect translation initiation. M^6A both enhances cap-dependent initiation (Meyer et al. 2015) and might specifically interact with eIF3, also with involvement of auxiliary m^6A writers and readers (Choe et al. 2018, Di Timoteo et al. 2020). The writer METTL3 might interact with m^6A in the 3' UTR while also interacting with eIF3h (Choe et al. 2018).

eIF3d is an eIF3 subunit that exhibits m⁷G cap binding activity and is used in a cap-dependent pathway of initiation independent of eIF4E, such as initiation at the *c-Jun* mRNA, which contains an eIF4E-binding inhibitory element (Lee et al. 2016), or translational reprogramming under metabolic stress, which requires CK2-mediated phosphorylation near the eIF3d cap-binding pocket (Lamper et al. 2020). In fact, blocking eIF4E1 results in a subset of mRNAs that are still translated efficiently and bind to eIF3d via its cap-binding pocket, enabling cap-dependent initiation (Roiuk et al. 2024). Interestingly, eIF3l has also been shown to interact with the m⁷G cap, but with currently unknown function (Kumar et al. 2016).

These are just some examples of eIF3's role in translation, but due to the broad spectrum of eIF3's functions, we can infer that much more remains to be learned. eIF3 is even becoming a potential target of therapies against multiple diseases (Wolf et al. 2020).

Recycling factors

Rli1

As outlined in the chapter on recycling, yeast Rli1/ABCE1 is the main factor driving subunit splitting and 60S recycling after translation termination (additionally, eIF3j has been shown *in vivo* to aid Rli1 in 60S recycling (Young and Guydosh 2019)). It's an ATP-binding cassette (thus the alternative nomenclature ABCE1, which is used for its mammalian counterpart), which means it contains two nucleotide binding domains (NBD) that form sandwich-like nucleotide-binding sites for two ATP molecules. Additionally, there's a Helix-Loop-Helix (HLH) motif in the first nucleotide binding domain, and a unique FeS domain, containing two [4Fe-4S]²⁺ clusters, which is connected to the core protein with a specific β -sheet called the cantilever arm, along with a cantilever hinge flexible linker (Heuer et al. 2017, Hellen 2018).

A scheme of ABCE1 domains can be seen in Figure 13.

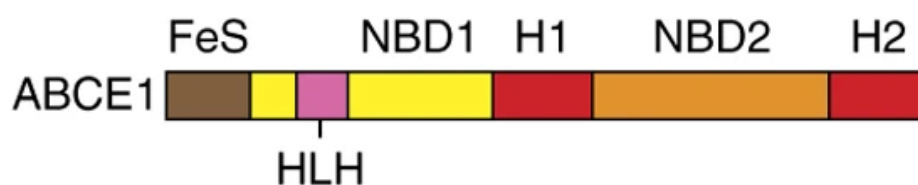


Figure 13: Domain structure of ABCE1/Rli1. Adapted and reprinted with permission from Heuer et al. 2017.

Post-peptidyl release, Rli1/ABCE1 still in complex with eRF1 to facilitate subunit splitting (Figure 14). The CTD of eRF1 contacts the FeS domain of ABCE1, and its central domain with the GGQ motif contacts the P-site tRNA (Preis et al. 2014). In fact, Rli1 contributes to peptide release more than eRF3 when working in concert with eRF1 and eRF3 (Shoemaker and Green, 2011).

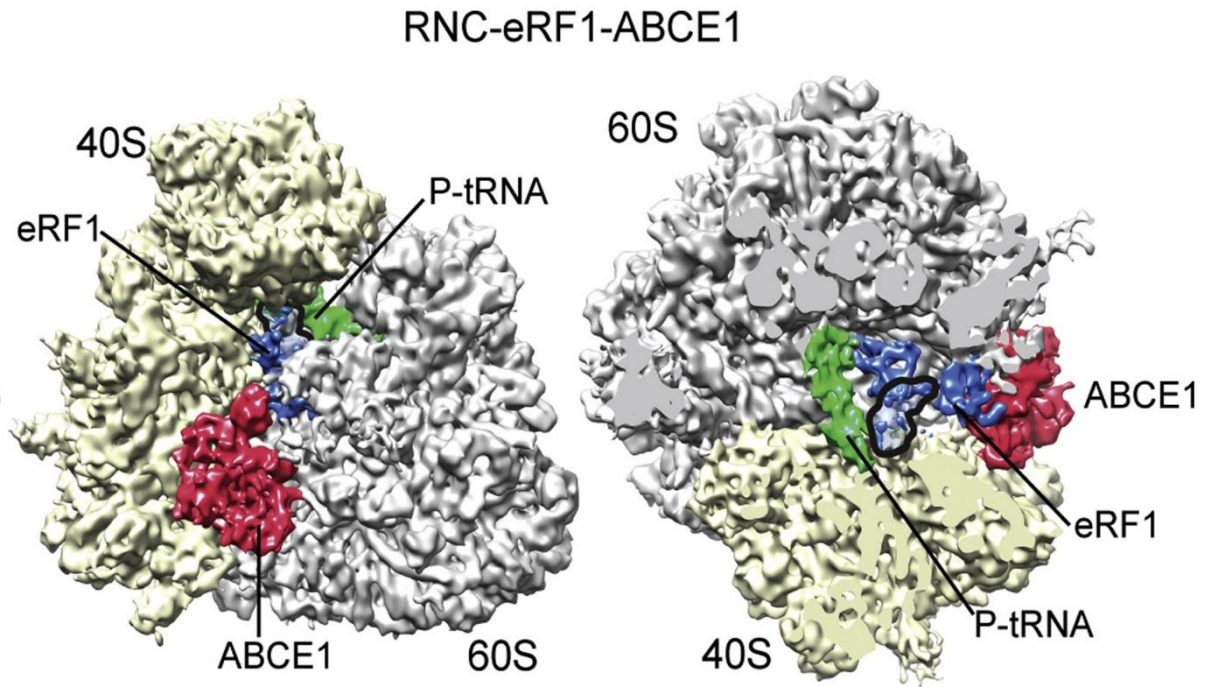


Figure 14: Side and top views of the 80S ribosome post-TC with eRF1 and Rli1 (ABCE1). Density attributed to eRF1 occupies the A site and position of the flexible NTD of eRF1 is outlined with a black line. Adapted from Preis et al. 2014.

Apart from the interaction with eRF1, Rli1/ABCE1 also contacts several ribosomal proteins and rRNA helices (Heuer et al. 2017). Based on comparisons of pre-subunit splitting and post-splitting states of Rli1, subunit splitting can be divided into two phases. In the first phase, FeS domain in contact with eRF1 CTD possibly pushes eRF1 into the intersubunit space upon closure of NBDs (in the case of rescuing stalled ribosomes, eRF1 is substituted by Pelota/yeast Dom34). In the second phase, FeS domain can complete its transition by fully rotating into its new position on the 40S subunit. Moreover, the new position of Rli1 would possibly clash with intersubunit bridges between 40S and 60S subunits, explaining its anti-association property (Heuer et al. 2017). Model of this splitting process can be seen in Figure 15.

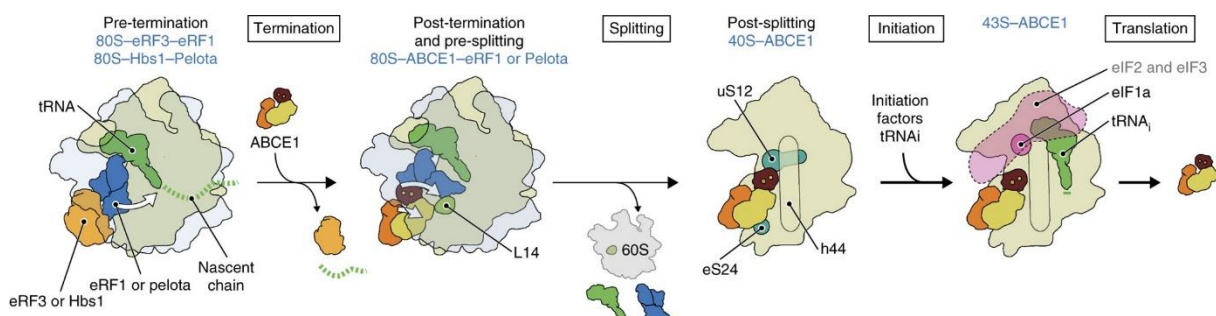


Figure 15: After recognition of a terminating or stalled ribosome by eRF1 and eRF3, or Pelota and Hbs1, and dissociation of the GTPases eRF3 or Hbs1, ABCE1 binds to these A-site factor-containing complexes in an intermediate, semi-closed conformation. Splitting may be promoted

in two steps. First, closure of the NBDs drives the FeS cluster domain toward the A-site factor. This leads to an immediate force exerted on eRF1 or Pelota, acting as a wedge to destabilize the intersubunit bridges. Second, once the 40S subunit is idle, reassociation of the 60S subunit is blocked, since the FeS cluster domain adopts a position that prevents formation of intersubunit bridge B5 between uL14 and h44 of the 18S rRNA. ABCE1 is also likely to be a transient component of the 43S pre-initiation complex (containing eIF1a, eIF2, eIF3, and initiator tRNA). However, the exact role of ABCE1 in these complexes is still elusive. Reprinted with permission from Heuer et al. 2017.

ABCE1

The process of 60S recycling seems to be well conserved between yeast and mammals, with little significant differences found between ABCE1 and Rli1, or archaeal homologues for that matter. Interestingly, mammalian ABCE1 was first identified as an inhibitor of RNase L (then called RLI; Bisbal et al. 1995). This protein takes part in an immune response against viruses through its recognition of dsRNA, leading to degradation of viral and cellular mRNAs (reviewed in Koul et al. 2024). ABCE1 also regulates RNase L-induced autophagy in viral infections – cells with depleted ABCE1 have increased RNase L activity and enhanced autophagy after activation of the antiviral Interferon response (Ramnani et al. 2021). However, in an opposing manner, ABCE1 has also been found to act as a positive regulator of exogenous RNA decay involving RNase L (Nogimori et al. 2021). ABCE1 itself also plays a role in assembly of various virions, such as HIV-1 (Robinson et al. 2014), FIV (Feline Immunodeficiency Virus) (Reed et al. 2018), and possibly also the rabies virus (Lingappa et al. 2013) and a related rhabdovirus, the Chandipura virus (Rajasekharan et al. 2015).

Unsurprisingly, ABCE1 also plays a role in the progression of many cancers – an observational study has found elevated ABCE1 expression at both mRNA and protein level in most cancers. Additionally, with some cancers, high levels of ABCE1 lead to worse patient prognosis (Yu et al. 2022, Liu et al. 2021) and it's been linked to multidrug resistance in cancer (Marques et al. 2024). The diverse molecular mechanisms behind the various functions have thus become of clinical interest in developing new targeted therapeutics (Gao et al. 2010, Lingappa et al. 2013, Pillar et al. 2019, Tong et al. 2024).

Tma20/Tma22/Tma64

These three 40S recycling factors are homologues of mammalian MCTS1, DENR and eIF2D/Ligatin, which will be discussed in the next subchapter. The next steps of recycling after subunit splitting consist of removal of the deacylated P-site tRNA and dissociation of mRNA from the 40S subunit. Either the heterodimer formed by DENR/MCTS1 or eIF2D alone were found to carry out this activity *in vitro* (Skabkin et al. 2010), prompting further investigation into their yeast counterparts. These factors share their basic domain structure (Figure 16). Both Tma64/eIF2D and Tma22/DENR contain a SUI1 (eIF1) domain fold, which binds to the same ribosomal site on the 40S subunit as eIF1 (Skabkin et al. 2010). The PUA domain of Tma20/MCTS1 and Tma64/eIF2D on the other hand binds tRNAs with different affinities (Ahmed et al. 2018). While there are no experimental data available on structure of the yeast

Tma proteins, there are studies on their function in ribosome recycling. Deletion of these three genes from the yeast genome results in accumulated unrecycled 40S subunits at stop codons on a genome-wide scale. This phenotype is consistent with defects in 40S recycling, which is furthermore supported by the fact that *RLI1* overexpression does not rescue this defective phenotype. Interestingly, another feature of this phenotype is the specific ‘Tma-dependency’ of certain penultimate codons for bulk recycling.

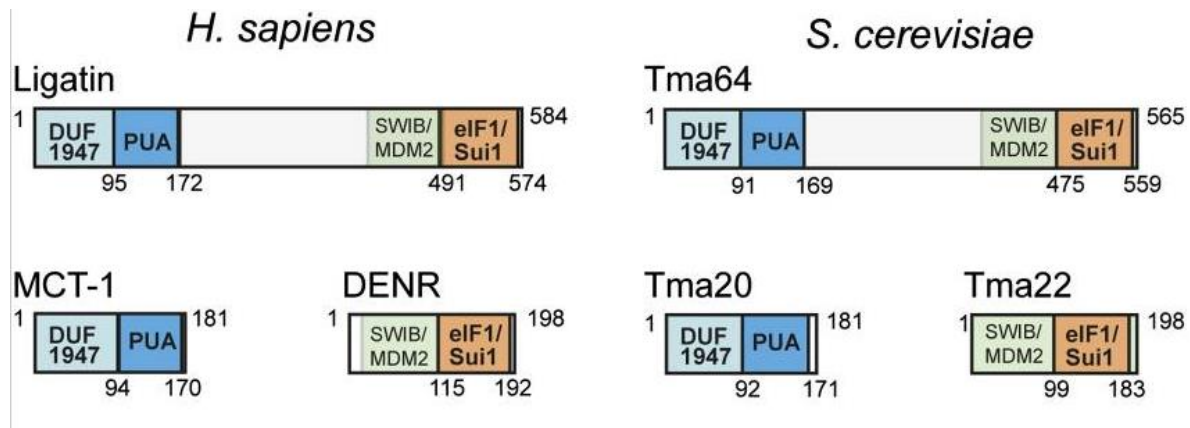


Figure 16: Domain organization of Homo sapiens Ligatin/eIF2D, MCT-1, and DENR and their S. cerevisiae orthologs Tma64, Tma20, and Tma22. Reprinted with permission from Skabkin et al. 2010.

This means that some last sense codons before the stop codon of an ORF display a higher ratio of accumulated unrecycled 40S subunits (in the deletant strains compared to the WT) than others (Figure 17). The mechanism behind this phenotype is not known, but it may be due to the interactions between the Tma factors and residual P-site tRNAs (Young et al. 2018, 2021).

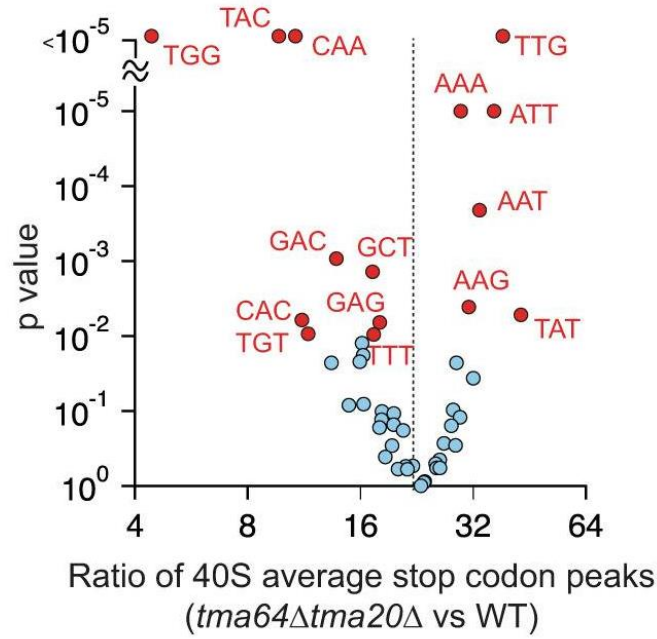


Figure 17: Average ratio of 40S stop codon peaks (*tma64Δtma20Δ* vs WT) are plotted against their significance (p value) as determined by bootstrapping for penultimate codons with at least ten occurrences. Significant codons (DNA sequences) are indicated (red) at the 99th confidence interval. Dotted line indicates average increase across the entire transcriptome of 23.4-fold. Reprinted with permission from Young et al. 2021.

Additionally, the Tma20/Tma22 heterodimer has been found to carry out the majority of 40S recycling work in a cell, with Tma64 playing a minor, perhaps only supportive role (Young et al. 2021). Interestingly, a mutation in mammalian DENR that is associated with autism in humans has been found (in a homologous position in yeast Tma22) to also impair recycling activity in yeast (Young et al. 2021).

Interestingly, there have also been observations that the *tma64Δtma20Δ* strain undergoes translation reprogramming, wherein translation efficiencies (TEs) of the most efficiently translated ‘strong’ mRNAs increase, while those of ‘weak’ mRNAs decline. It is possible that this is due to the decreased availability of free 40S subunits for 43S PIC formation, and this phenotype is similarly reproducible through 43 PIC limitation through other means (Gaikwad et al. 2021). Recently, a genetic screen has identified Tma20 and Tma22 as potential players in NMD (Pacheco et al. 2024), where perturbation in 40S recycling caused by loss of these factors correlated with a loss of NMD, similarly to mammalian perturbation in 60S recycling leading to a similar loss.

While the evidence for the Tma proteins’ role in recycling is quite clear, it is still unclear how the changes in 40S recycling influence downstream translation REI after uORFs. It will be one of the aims of this thesis to investigate the influence of *TMA20/22/64* deletions on REI after short uORFs, with respect to Tma-dependency of different penultimate codons.

DENR, MCTS1 and eIF2D

The mammalian homologues of the yeast Tma22, Tma20 and Tma64 proteins have a very similar domain structure (see Figure 16), however they have been subject to more structural studies, shedding more light on their function.

As mentioned previously, DENR contains an eIF1-like SUI domain fold, which binds to the ribosome in a similar manner to eIF1 near the ribosomal P site, and it may contact or stabilize tRNAs in the P site. On the other hand, MCTS1 binds to the 40S subunit in a position that is incompatible with the position of eIF3a and eIF3c subunits of eIF3 (Figure 18). While this remains only a possibility due to some ambiguity in the eIF3 binding positions, this suggests that REI and alternative initiation activity of the DENR/MCTS1 heterodimer might not require eIF3 at all or might even be in direct competition with it (Lomakin et al. 2017). The binding of DENR with MCTS1 might additionally also provide a platform for binding and stabilization of P site tRNAs (Lomakin et al. 2017). This is supported by biochemical data that have shown that DENR/MCTS1 can directly bind tRNAs *in vitro*, with varying affinities for different tRNAs (Ahmed et al. 2018).

Functionally, DENR and MCTS1 promote REI after short uORFs and even start-stops, including uORFs in the 5' UTR of *ATF4* (Bohlen et al. 2020, Schleich et al. 2017). However, it has also been found that knockout of DENR introduces a 40S recycling defect into cells, which leads to accumulation of unrecycled 40S subunits at stop codons. This suggests that DENR/MCTS1 in mammals promotes REI after short uORFs through 40S recycling, particularly through P site tRNA removal from the small subunit while the 40S stays bound to the mRNA. Similarly to the yeast Tma proteins, some penultimate codons display DENR-dependency in mammalian cells, i.e. these codons preferentially accumulate more unrecycled 40S subunits after DENR knockout than other codons (Bohlen et al. 2020). While the exact mechanism of this dependency is not known, DENR/MCTS1 or eIF2D have been shown to promote dissociation of deacylated tRNAs from the 40S, and dissociation of the 40S from the mRNA *in vitro* (Skabkin et al. 2010), as well as to bind tRNAs directly with various affinities (Ahmed et al. 2018). In addition to this, some tRNAs are known to dissociate from the 40S post-TC more readily than others (Skabkin et al. 2013). Thus, we could speculate that this DENR-dependence might be caused by higher affinity of these factors to some deacylated tRNAs during the recycling process, which need them for successful dissociation from 40S post-TC. Failure to do so could lead to preferential accumulation of unrecycled subunits over the stop codons with penultimate codons belonging to these tRNAs over others (Bohlen et al. 2020).

eIF2D is a close homologue of DENR/MCTS1 and loss of function of eIF2D has shown an additive effect to DENR depletion (Bohlen et al. 2020). However, this protein itself also has separate functions from 40S recycling/REI, mainly involved in alternative initiation. eIF2D is able to deliver both initiator and elongator Met-tRNA^{Met} into the P site in a GTP-independent way, and able to form a 48S complex without eIF1A, eIF3 and eIF4 *in vitro* (Dmitriev et al. 2010). It can also aid in HCV IRES translation initiation (Niepmann and Gerresheim 2020), or enteroviral translation (Kim et al. 2023). Even some human mRNAs require eIF2D for

translation initiation from internal IRESs, such as the *Hoxa3* mRNA, which contains an IRES as a negative regulatory element regulating the translation of the main ORF (Alghoul et al. 2021).

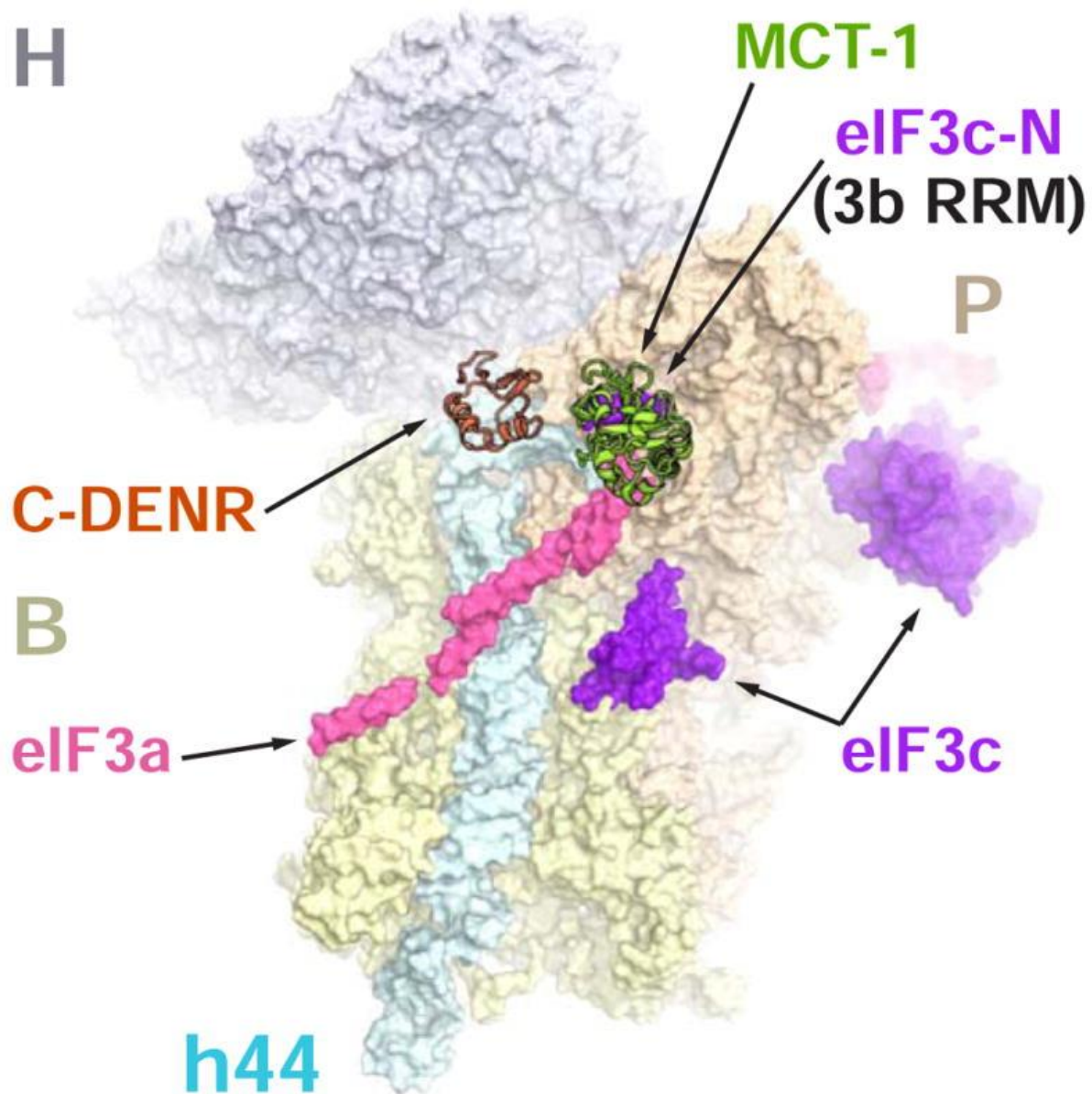


Figure 18: The crystal structure of the human 40S-DENR-MCTS1 (MCT-1) complex was superposed with the structure of the partial yeast 48S complex in closed conformation (PDB ID 3JAP). Only eIF3a (pink) and eIF3c (purple) subunits from the yeast structure are shown. Electron density corresponding to eIF3a and eIF3c-N can also be attributed to eIF3b RRM (PDB ID 5K1H). C-DENR and MCTS1 are shown in coral and green, respectively. H, head; B, body; P, platform; h44, helix 44 - domains of the 40S subunit. Reprinted from Lomakin et al. 2017.

Both DENR and eIF2D have been implicated in several physiological and pathological processes. DENR or MCTS1 have been suggested as a biomarker for Parkinson's disease (Wu et al. 2024), major depressive disorder (Christodolou et al. 2023) and various cancers (Wang et al. 2019, Zhang et al. 2022, Ma et al. 2023; also reviewed in Shyrokova et al. 2021). The heterodimer is also involved in regulation of cell cycle (von Hohenberg et al. 2022), circadian rhythm (Castelo-Szekely et al. 2019), immune response (Chen et al. 2022, Bohlen et al. 2023) or neural development (Haas et al. 2016). eIF2D has also become of similar interest, as a potential biomarker in cancers (Lin et al. 2020, Golob-Schwarzl et al. 2020, Basu et al. 2019) or schizophrenia (Gilabert-Juan et al. 2019).

Comparison of yeast and mammalian factors

Tables comparing mammalian and yeast initiation and recycling factors and their different functions can be found below.

TABLE 1: Eukaryotic initiation factors

FACTOR	Yeast gene name (<i>S.c.</i>)	Yeast main function	Mammalian gene name (<i>H.s.</i>)	Mammalian main function
eIF4A	<i>TIF1/2</i>	mRNA helicase	<i>EIF4A1/2/3</i>	mRNA helicase
eIF4E	<i>TIF45/CDC33</i>	cap-binding	<i>EIF4E</i>	cap-binding
eIF4G	<i>TIF4631/4632</i>	eIF4 scaffold	<i>EIF4G1</i>	eIF4 scaffold
eIF2	<i>SUI2/3/4 (GCD11)</i>	tRNA _i ^{Met} delivery	<i>EIF2S1/2/3</i>	tRNA _i ^{Met} delivery
eIF1	<i>SUI1</i>	scanning	<i>EIF1</i>	scanning
eIF1A	<i>TIF11</i>	scanning	<i>EIF1AX</i>	scanning
eIF5	<i>TIF5</i>	scanning; GTP exchange on eIF2	<i>EIF5</i>	scanning; GTP exchange on eIF2
eIF3a	<i>TIF32/RPG1</i>	mRNA/tRNA _i ^{Met} loading; REI	<i>EIF3A</i>	REI, initiation
eIF3b	<i>PRT1</i>	REI, initiation	<i>EIF3B</i>	initiation
eIF3c	<i>NIP1</i>	initiation	<i>EIF3C</i>	initiation
eIF3d	-	-	<i>EIF3D</i>	initiation
eIF3e	-	-	<i>EIF3E</i>	initiation
eIF3f	-	-	<i>EIF3F</i>	initiation
eIF3g	<i>TIF35</i>	REI, initiation	<i>EIF3G</i>	initiation

eIF3h	-	-	<i>EIF3H</i>	initiation
eIF3i	<i>TIF34</i>	REI, initiation	<i>EIF3I</i>	initiation
eIF3k	-	-	<i>EIF3K</i>	initiation
eIF3l	-	-	<i>EIF3L</i>	initiation
eIF3m	-	-	<i>EIF3M</i>	initiation
eIF3j	<i>HCRI</i>	60S recycling, SC-RT	<i>EIF3J</i>	initiation, recycling

Table 1: A summary table listing eukaryotic initiation factors discussed in the previous chapters, their mammalian and yeast gene names and main function. Since i.) most biochemical research on eIF3 functions was done on yeast, and ii.) each eIF3 subunit has several multifaceted roles, the function of most eIF3 subunits in mammals or yeast was listed as initiation or REI, initiation. *S.c.* = *Saccharomyces cerevisiae*, *H.s.* = *Homo sapiens*

TABLE 2: Recycling factors

Yeast name (gene name; <i>S.c.</i>)	Yeast main function	Mammalian name (gene name; <i>H.s.</i>)	Mammalian main function
Rli1 (<i>RLI1</i>)	subunit splitting, 60S recycling	ABCE1 (<i>ABCE1</i>)	subunit splitting, 60S recycling
Tma20 (<i>TMA20</i>)	40S recycling	MCTS1 (<i>MCTS1</i>)	40S recycling, REI
Tma22 (<i>TMA22</i>)	40S recycling	DENR (<i>DENR</i>)	40S recycling, REI
Tma64 (<i>TMA64</i>)	40S recycling (accessory)	eIF2D (<i>EIF2D</i>)	alternative initiation, REI

Table 2: A summary table of recycling factors described in the previous chapters in yeast and mammals, their yeast and mammalian gene names and main function. *S.c.* = *Saccharomyces cerevisiae*, *H.s.* = *Homo sapiens*

Introduction into the *GCN4* reporter system

While the delayed REI model of *GCN4* translational control was already introduced earlier, this chapter will describe the β -galactosidase reporter system derived from this gene, making use of its 5' UTR and its mechanism of translational regulation. I also explain the way I harnessed the power of this system to shed light on REI and recycling processes in the yeast cell.

To briefly recap this mechanism, the *GCN4* 5' leader contains four short uORFs – first two of these (uORF1 and uORF2) are permissive for REI after their translation, the next two (uORF3 and uORF4) are not and promote recycling after their translation. Under physiological conditions, the ribosome translates uORF1 and then reacquires the TC in time to reinitiate at uORF3 or 4. uORFs 3 and 4 are not REI-permissive, most of the ribosomes are recycled and there is very little expression of the main *GCN4* CDS. Under amino acid starvation stress conditions (where eIF2 α is phosphorylated and levels of TC are low), the ribosome usually translates uORF1 but does not manage to acquire TC in time to reinitiate at uORF3/4, and reinitiates at the main *GCN4* CDS, which leads to a robust induction of Gcn4 protein translation.

While this system was first used to investigate the mechanism of delayed REI of *GCN4* translational control (reviewed in Hinnebusch 2005, updated in Gunisova and Valasek 2014, Gunisova et al. 2016), it has also been used to determine some more general rules of REI in yeast (Gunisova et al. 2016). The *GCN4*-based constructs I used are all based on the original *GCN4-lacZ* construct p180 (Hinnebusch 1985), which contains the whole ~600 nt 5' leader of *GCN4* and 55 codons of its coding sequence fused with the *LacZ* coding sequence. This gene codes for the β -galactosidase enzyme of *E. coli* that can be used for β -galactosidase reporter assays. In contrast to the p180 construct containing the whole *GCN4* leader with all four uORFs, I used constructs with uORF1-only (p209) and uORF4-only (p226) (with the other uORF start codons mutated out with point mutations) (Valasek and Gunisova 2014; Figure 19) for the following purpose.

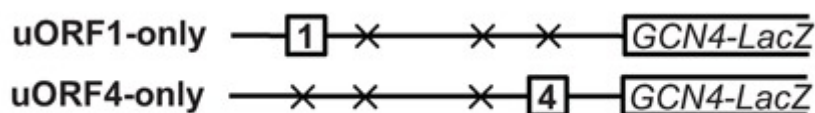


Figure 19: Scheme of uORF1-only and uORF4-only constructs I used in this work. The constructs contain only either uORF1 or uORF4, and the other uORF start codons are mutated out by point mutations (indicated by X). Reprinted with permission and edited from Gunisova and Valasek 2014.

It has been shown that impairments in REI (for example by mutations in eIF3 subunits a/tif32- Δ 8 or g/tif35-KLF), lower the reporter activity from the uORF1-only construct, reflecting the REI defect (Cuchalova et al. 2010, Gunisova and Valasek 2014). Logically, with uORF4 we could infer that defects in ribosome recycling would lead to increased REI after uORF4, increasing the reporter activity. First, I used this system to screen for new factors influencing REI and ribosome recycling using selected yeast deletion strains. To rule out any effects of these deletions on general translation initiation, I used a “uORF-less” reporter construct (with

all four uORFs mutated out) and normalized the deletion strain reporter activities to the activity of this construct (this normalization is further described in Methods and Materials).

In a similar manner, constructs based on both uORF4-only and uORF1-only constructs were used to assess the contribution of Tma20/Tma22 and Tma64 proteins to ribosome recycling and translation REI.

AIMS

Translational regulation is one of the main cornerstones of post-transcriptional gene regulation. It has the advantage of being rapid and it is also multi-layered and influenced by a myriad of *cis*- and *trans*-acting elements. This work will overall be focused on the issue of post-termination decision programming, meaning factors influencing the post-termination mechanisms of translation REI and ribosome recycling. While we have a good idea about the basic mechanisms of REI in yeast, much remains to be determined about mechanisms behind ribosome recycling, and auxiliary factors that may influence REI.

In mammalian cells, ATF4 (homologue of yeast Gcn4) is a master regulator of integrated stress response. While its basic regulation is similar to the delayed REI model of Gcn4, there are some additional elements coming into play here, influencing the resulting ATF4 expression. Another goal of this work was to determine some of the *cis*-acting elements contributing to translation regulation of the *ATF4* mRNA done in HEK293T cells using a quantitative Western Blotting system and constructs with various mutations of the *ATF4* 5' UTR, and CDS containing an ATF4-HA fusion.

The individual aims of this thesis were:

1. to screen for new translation REI or ribosome recycling factors in yeast using a reporter system and a yeast deletion library
2. to characterize the role of the identified factors using biochemical methods
3. to determine how the 40S recycling factors Tma20, Tma22 and Tma64 influence REI after short uORFs in yeast and the interplay between these factors
4. to determine how 'Tma-dependency' of penultimate codons influences REI after short uORFs in yeast strains expressing or lacking Tma20, Tma22 and Tma64
5. using a quantitative Western Blotting approach, to determine which *cis*-acting elements on the *ATF4* mRNA leader influence ATF4 expression in mammalian cells

METHODS AND MATERIALS

This chapter pertains to methods used in the Unpublished Results section. For methodological details of other results, please see the corresponding publications.

β -galactosidase reporter assays

β -galactosidase activities were assayed in whole cell extracts (WCEs) as described in Jendrichova et al. 2024. Briefly, cells were transformed with plasmids bearing *GCN4-lacZ* fusion reporters and grown in synthetic SD media to mid-log phase. Cells were centrifuged, washed with deionized water and broken using glass beads in Breaking Buffer (100 mM Tris 8.0, 4% Glycerol, 1 mM β -mercaptoethanol), then clarified by centrifugation (18,400g/30 min/4 °C). β -galactosidase activity (measured as nanomoles of o-nitrophenyl-3-Dgalactosidase cleaved per minute per milligram of protein) was normalized to protein concentration of the WCE, which was measured by Bradford assay with BSA as standard.

The normalized β -galactosidase activity for each reporter construct in transformants of deletion strains was determined by dividing the unnormalized β -galactosidase activities by a normalization factor. This factor was calculated separately for each individual experiment by assaying the activity of the “uORF-less” reporter (p227) in WT and deletion strains in four transformants under the same conditions as uORF1-only/uORF4-only reporters. Then, the mean of each deletion strain “uORF-less” activities was divided by the mean of WT “uORF-less” activities, determining the normalization factor.

Plasmids and yeast strains

All plasmids and primers used are listed in Table 3 and Table 5, respectively, at the end of the Methods and Materials section. Cloning was carried out in the DH5 α *E. coli* strain.

The plasmids were constructed as follows: pKJ64 was constructed by replacing the *PstI*-*BamHI* fragment of YEplac181 by synthesized DNA fragment 1 (all fragments are available in Supplementary Table S1). pKJ65 was constructed by replacing the *HindIII*-*EcoRI* fragment of YEplac181 by synthesized DNA fragment 2. pKJ66 was constructed by replacing the *HindIII*-*FseI* fragment of pGL4-CMV by a fragment amplified by PCR from yeast cDNA with primers KJ110 and KJ111. pKJ67 was constructed by replacing the *EcoRI*-*FseI* fragment of pGL4-CMV by a fragment amplified by PCR from yeast cDNA with primers KJ112 and KJ113. pKJ68 was constructed by replacing the *BamHI*-*Sall* fragment of pGEX-5-X-3 with a fragment amplified by PCR from yeast cDNA using primers KJ108 and KJ109. pKJ72 and pKJ73 were generated by subcloning the synthesized fragments from pKJ64 and pKJ65 into the YCplac111 backbone, as the multicloning site in YEplac181 and YCplac111 is identical.

Yeast strain creation

All yeast strains (incl. their genotypes) and primers used are listed in Table 4 and Table 5, respectively, at the end of the Methods and Materials section.

Yeast deletion strains lacking candidate translation factor genes were sourced from the Euroscarf yeast deletion collection. Yeast double deletion strains were constructed using deletion cassettes carrying the *hphNT1* and *natMX6* genes, carried by plasmids pZC3 and pZC4. The deletion cassettes for *SSZ1* deletion were amplified using PCR from their corresponding plasmids, using primers KJ114 and KJ115. To create YKJ12 (*rai1Δ ssz1Δ*) and YKJ9 (*ssz1Δ zuo1Δ*), the parental yeast strains EUR3 (*rai1Δ*) and EUR78 (*zuo1Δ*) were then transformed with the deletion cassettes targeting *SSZ1* to confer antibiotic resistance and selected using Nourseothricin (Jena Bioscience), or Hygromycin B (Carl Roth GmbH), respectively, on YPD agar medium. Incorporation of the cassettes was verified by colony PCR (Looke et al. 2011) using confirmation primers KJ116 and PB238.

Yeast spot assay

Yeast strains were spotted onto minimal synthetic defined (SD) medium plates in five serial 10-fold dilutions (starting with OD₆₀₀ 0.5) and grown for 48 h at 30 °C. For Rli1 knockdown, YPD plates were used and doxycycline (Sigma Aldrich; 0.5 µg/ml) was added to the medium.

Polysome profiling and fraction collection

For polysome profiling, yeast cultures were grown to OD₆₀₀ ~ 0.8 in YPD media at 30 °C 230rpm. Then, they were treated with cycloheximide (Sigma Aldrich; 50 µg/1 ml of culture) for 10 minutes and immediately put on ice for 5 minutes. The pellets were washed with dH₂O and then resuspended in 1 ml of GA⁺ buffer (20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 5 mM NaF, 50 µg/ml cycloheximide). After centrifugation, the pellet was again resuspended in 300 µl of GF buffer (20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, 5 mM NaF, 50 µg/ml cycloheximide, C0mplete EDTA-free Protease Inhibitor mix (Roche)) and cells were broken using glass beads. Afterwards, the lysate was clarified at 13,500g/10 minutes/4 °C. 8 AU OD₂₆₀ were loaded on a 5-50% sucrose gradient and separated by ultracentrifugation at 39,000rpm for 2.5 hours using the SW41Ti rotor. The gradients were scanned at A254 nm using the Biocomp Gradient Station™ (Biocomp Instruments).

For fraction collection, yeast strains transformed with FLAG-Ssz1 and FLAG-Rai1 plasmids were grown in SD media to OD₆₀₀ ~ 0.9 at 30 °C 230rpm. Then, cells were put on ice and 1% formaldehyde (Sigma Aldrich) was added as a crosslinking agent and incubated for 60 minutes on ice. Afterwards, 10 ml of 2.5M Glycine/100 ml of culture was added. The pellets were then resuspended in GF buffer, centrifuged, again resuspended in 600µl of GF buffer and broken using glass beads. 20 AU OD₂₆₀ were loaded on a 5-50% sucrose gradient and separated by ultracentrifugation at 39,000rpm for 2.5 hours using the SW41Ti rotor. Fractions were collected using the Biocomp Gradient Station™ (Biocomp Instruments). Each fraction was precipitated with 96% Ethanol (2x volume of the fraction) at -20 °C overnight, then centrifuged at 21,000g/1 hour/4 °C. The resulting pellets were then resuspended in 1x Laemmli Sample Buffer (BioRad) and used for Western Blot analysis.

Western Blot

Samples were resolved through SDS-PAGE followed by Western Blotting. The primary antibodies used were anti-FLAG M2 (Sigma Aldrich, cat# F1804, lot# 080M6035; dilution 1:1000), anti-Rps26 (sourced from Vikram Panse, University of Zurich, Switzerland; dilution 1:1000) and anti-TIF32 (sourced from Lon Phan, NCBI, USA; dilution 1:1000). The secondary antibodies used were Amersham ECL™ Anti-Mouse IgG HRP-linked (Cytiva, cat# NA931V, lot# 17853437, dilution specified in corresponding figure legends) and Amersham ECL™ Anti-Rabbit IgG HRP-linked (Cytiva, cat# NA934V, lot# 18227506, dilution specified in corresponding figure legends). The signal was developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged using G-Box (Syngene).

Protein expression and purification

The *E.coli* strain BL21 Star (DE3) (Invitrogen) was used for expression and purification of GST-tagged Ssz1 protein. Expression of GST-Ssz1 was induced by 10 mM IPTG (Serva) overnight at 16 °C 230rpm in LB media. Pellet was washed in cold dH₂O and resuspended in 5 ml of cold PBS (pH 7.5, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄). Cells were broken using glass beads and 1.5 % Triton X-100 (Sigma Aldrich) was added. The mixture was incubated on a rotator for 30 minutes at 4 °C and then centrifuged at 21,000g/10 minutes/4 °C. Gluthathione Sepharose beads (50% slurry; GE Healthcare) were washed 3x with PBS and 400µl of the slurry was added into the clarified cell lysate. The lysate and beads were then incubated for 30 minutes at RT. Afterwards, the beads were washed 3x with cold PBS, aliquoted and frozen at -80 °C for use in the GST pulldown.

GST pulldown

³⁵S-labelled proteins were produced using the TnT® Quick Coupled Transcription/Translation System (Promega) according to vendor instructions. GST-Ssz1 on Sepharose beads was incubated with ³⁵S-Rai1 and ³⁵S-Zuo1 for 2 hours at 4 °C in 300µl of Buffer B (20 mM HEPES (pH 7.5), 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 0.05% Igepal CA-630, 1mM DTT, 1% fat-free milk) and then washed 3x with PBS. The proteins were separated by SDS-PAGE, gels were first stained with Simply Blue™ SafeStain (Invitrogen) and then subjected to autoradiography.

FLAG pulldown and Mass Spectrometry analysis

Yeast strains transformed with FLAG-Ssz1 or FLAG-Rai1 plasmids (or with no plasmid as negative control) were grown in SD media to OD₆₀₀ ~ 0.8 at 30 °C 230rpm. The pellets were washed with 25 ml of cold FLAG buffer (20 mM TRIS (pH 7.5), 200 mM KCl, 5 mM MgAc), then weighed and resuspended in cold FLAG buffer with protease inhibitors (Aprotinin 1µg/ml, Leupeptin 1µg/ml, Pepstatin 1µg/ml, 1 mM PMSF, C0mplete EDTA-free Protease Inhibitor mix (Roche), 1 mM DTT). The amount of buffer used was 1 ml/g of pellet weight. The cells were broken using glass beads and the lysate was subsequently cleared 2x at 18,400g/10 min/4 °C. For the pulldown, ANTI-FLAG® M2 Affinity Gel (Milipore) beads slurry was washed 3x

with cold FLAG buffer (with protease inhibitors). Afterwards, whole cell lysate was added (beads slurry amount and total protein amount for each reaction will be specified later) to the beads and incubated for 2 hours at 4 °C on a rotator. Then, the beads were washed 3x with 1 ml of cold FLAG buffer with 0.2% Triton X-100 (Sigma Aldrich), 1x with cold FLAG buffer without Triton X-100 and 2x with cold 50 mM TRIS (pH 8.0) and finally resuspended in 50µl of 50 mM TRIS (pH 8.0) to be sent for Mass Spectrometry (MS) analysis. For FLAG-Rai1 and its corresponding negative control, 2.5µl of beads slurry and 0.2mg of total protein was used. For FLAG-Ssz1 and its corresponding negative control, 10µl of beads slurry and 0.2mg of total protein was used. Total protein concentrations were determined using Bradford assay.

MS was carried out using LC/MS by OMICS Mass Spectrometry Core Facility in BIOCEV, Vestec, Czech Republic.

Statistical analysis

Statistical significance was determined as in Jendruchova et al. 2024. For all reporter assays, all data were tested for normality by the Shapiro–Wilk test. Based on the normality test, data were compared either by the parametric, unpaired, two-tailed Welch’s *t* test, or by the non-parametric Mann–Whitney test; **** indicates $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns non-significant. All individual data points are shown as part of bar plots. The SD was calculated from all biological replicates for each construct in each strain. Statistical analysis and visualization were performed in GraphPad Prism, version 9.4.1 (GraphPad Software). All replicates shown in the reporter assays graphs were independent transformants—biological replicates.

For Mass Spectrometry volcano plot creation, the VolcanoR tool was used (Goedhart and Luijsterburg 2020).

TABLE 3: Plasmids used.

Plasmid:	Description:	Source:
p209	<i>GCN4</i> 5' UTR with only ORF1 (containing AUG mutations in uORFs2-4) and <i>GCN4</i> CDS fused with <i>LacZ</i> ("uORF1-only")	Grant et al. 1994
p226	<i>GCN4</i> 5' UTR with only ORF4 (containing AUG mutations in uORFs1-3) and <i>GCN4</i> CDS fused with <i>LacZ</i> ("uORF4-only")	Mueller and Hinnebusch 1986
p227	<i>GCN4</i> 5' UTR containing AUG mutations in uORFs1-4 of and <i>GCN4</i> CDS fused with <i>LacZ</i> ("uORF-less")	Miller and Hinnebusch 1989
pKJ64	YEplac181 backbone (high copy; Gietz and Sugino 1988) with SSZ1-6x Gly-3x FLAG fusion and 500 bp 5' and 3' up/downstream from CDS	This thesis
pKJ65	YEplac181 backbone (high copy; Gietz and Sugino 1988) with RAI1-6x Gly-3x FLAG fusion and 500 bp 5' and 3' up/downstream from CDS	This thesis
pKJ66	RAI1 CDS in pGL4-CMV backbone (Wagner et al. 2014) for in vitro transcription/translation	This thesis
pKJ67	ZUO1 CDS in pGL4-CMV backbone (Wagner et al. 2014) plasmid for in vitro transcription/translation	This thesis
pKJ68	GST-SSZ1 N-terminal fusion in pGEX-5X-3 backbone (GE Healthcare)	This thesis
pKJ72	YCplac111 backbone (low copy; Gietz and Sugino 1988) with RAI1-6x Gly-3x FLAG fusion and 500 bp 5' and 3' up/downstream from CDS	This thesis
pKJ73	YCplac111 backbone (low copy; Gietz and Sugino 1988) with SSZ1-6x Gly-3x FLAG fusion and 500 bp 5' and 3' up/downstream from CDS	This thesis
pZC3	pFA6 backbone containing <i>hphNT1</i> resistance marker-based deletion cassette	Carter and Delneri 2010
pZC4	pFA6 backbone containing <i>natNT2</i> resistance marker-based deletion cassette	Carter and Delneri 2010

YEplac181	Yeast episomal plasmid with a LEU2 marker	Gietz and Sugino 1988
YCplac111	Yeast centromeric plasmid with a LEU2 marker	Gietz and Sugino 1988
pGL4-CMV	CMV promoter inserted into pGL4.10 backbone (Promega)	Wagner et al. 2014
pGEX-5X-3	Bacterial vector for expressing GST fusion proteins with a Factor Xa site.	GE Healthcare

Table 3: A table of plasmids used along with their Description and Source of each plasmid.

TABLE 4: Yeast strains used.

Name:	Genotype	Parental strain:	Source:
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	-	Euroscarf
EUR3 (<i>rai1Δ</i>)	<i>MATa, his3Δ1; leu2Δ0; met 15Δ0; ura3Δ0; YGL246c::kanMX4</i>	BY4741	Euroscarf
EUR43 (<i>ssz1Δ</i>)	<i>MATa, his3Δ1; leu2Δ0; met 15Δ0; ura3Δ0; YHR064c::kanMX4</i>	BY4741	Euroscarf
EUR78 (<i>zuol1Δ</i>)	<i>MATa, his3Δ1; leu2Δ0; met 15Δ0; ura3Δ0; YGR285cc::kanMX4</i>	BY4741	Euroscarf
YKJ9 (<i>ssz1Δ zuol1Δ</i>)	<i>MATa, his3Δ1; leu2Δ0; met 15Δ0; ura3Δ0; YGR285cc::kanMX4; YHR064c::natMX6</i>	EUR78	This thesis
YKJ12 (<i>rai1Δ ssz1Δ</i>)	<i>MATa, his3Δ1; leu2Δ0; met 15Δ0; ura3Δ0; YGL246c::kanMX4; YHR064c::hphNT1</i>	EUR3	This thesis
YKJ3 (<i>tma20Δ tma22Δ tma64Δ</i>)	<i>MATa; his3D1; leu2D0; met15D0; ura3D0; YER007C-A::kanMX4, YDR117C::natMX6, YJR014W::hphNT1</i>	BY4741	Jendrichova et al. 2024
H509 (Tet-Rli1)	<i>mat@; ade2; can1-100; his3-11; his3-15; leu2-3; leu2-112; trp1-1; ura3-1; KAN^R tetO7Δ::YDR091C</i>	W303	Kispal et al. 2005

H86 (W303)	<i>mat@; ade2; can1-100; his3-11; his3-15; leu2-3; leu2-112; trp1-1; ura3-1</i>	-	gift from Ted Young
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Table 4: A table of yeast strains used along with their Genotype, Parental strain and Source of each strain.

TABLE 5: Primers used.

Primer:	Sequence:
KJ108	TCG TGG GAT CCA GAT GTC CTC TCC AGT GA
KJ109	CTC GAG TCG ACC TAT AAT TCA CCC TTT AC
KJ110	GTA CCA AGC TTA CCA TGG GTG TTA GTG CAA AT
KJ111	GAA GCG GCC GGC CTT ATT TCA AAG ATT TTC TC
KJ112	CTG CAG AAT TCA CCA TGT TTT CTT TAC CTA CC
KJ113	GAA GCG GCC GGC CTC ACA CGA AGT AGG ACA AC
KJ114	ATT GAG ATC TCT ACT ACC ACA ATC ACA GTT AAA ATT ACA AAT AA G CTA TAG AAC GCG GCC GCC AG
KJ115	TAT GTA TTT TTC TAT ATA CGT ATA CAT ACC GTT TTT CTT AGA GCG CTC ACT ATA GGG AGA CCG GCA G
KJ116	AGCCATTCATAACTCATTCC
PB238	GCA GCG AGG AGC CGT AAT

Table 5: A table of primers used.

RESULTS

Publication summaries

Differential effects of 40S ribosome recycling factors on reinitiation at regulatory uORFs in GCN4 mRNA are not dictated by their roles in bulk 40S recycling

Jendruchova K*, Gaikwad S*, Poncova K, Gunisova S, Valasek LS, Hinnebusch AG
* these authors contributed equally

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IF (2023): 5.2

This study is focused on the yeast 40S recycling factors Tma20/MCTS1, Tma22/DENR and Tma64/eIF2D, which have been shown to recycle 40S subunits *in vivo* (Young et al. 2021, 2018). Upon Tma-deletion, unrecycled 40S subunits tend to accumulate at stop codons, at some codons preferentially over others ('Tma-dependency'). Using the *GCN4*-based β -galactosidase reporter system as a tool, we investigated both the interplay between these three Tma factors, and the influence of these factors on REI after short uORFs (since it is known their mammalian counterparts promote REI after short uORFs (Schleich et al. 2017, Bohlen et al. 2020)) with different penultimate codons.

GCN4's uORF4 allows us to examine the effect of Tma deletion on recycling in the cell, as outlined in the Introduction. First, based on the Young et al. 2021 study, we chose a Tma-hyperdependent (TTG) and hypodependent (TGG) codon and mutated the penultimate codon of uORF4 in a uORF4-only construct to either of these codons. We found that single deletion of *TMA20* and *TMA22*, but not *TMA64*, lead to sizable increase of REI after uORF4 versus WT at the Tma-hyperdependent codon, but not so much at the hypodependent codon. We consider this increase in reporter activity a manifestation of the 40S recycling defect. Additionally, we found that double deletion of *TMA20/TMA22* results in a lower increase of reporter activity than *TMA20/TMA64* or triple deletion of all of these factors. This suggests that Tma64 plays a supportive role, while Tma20 or Tma22 bear the brunt of the recycling activity. Growth assay has shown that deletion of either *tma20Δ tma64Δ* or *tma20Δ tma22Δ tma64Δ* do not result in a significant growth defect.

We then created a set of constructs with uORF4 penultimate codons exchanged for either Tma-hypodependent, Tma-average (not significantly hyper- or hypodependent) or Tma-hyperdependent codons, and performed reporter assays in WT, *tma20Δ tma22Δ* and *tma20Δ tma22Δ tma64Δ* strains. Curiously, we found that most penultimate codons conferred increased REI and there was no significant difference in average reporter expression between Tma-average and Tma-hyperdependent or Tma-hypodependent codons groups. Some hypodependent or average codons can even reach reporter expression levels on par with hyperdependent codons, or vice versa. Moreover, certain codons showed higher reporter expression levels in *tma20Δ tma22Δ* deletant than in the triple deletant.

Next, we created a set of uORF1-only constructs with the uORF1 penultimate codon exchanged for Tma-average, hyper- or hypodependent codons. Since uORF1 is primed for REI instead of recycling, this setup is more similar to mammalian uORFs, where DENR promotes REI and its knockout results in reduction of REI (Bohlen et al. 2020). We found that in the *tma20Δ tma64Δ* strain, only three out of twenty-nine penultimate codons have shown a significant reduction in reporter expression versus WT, two of them Tma-average and one Tma-hyperdependent. We also observed a small significant difference between reporter expression of hypodependent and average codons, but not hyperdependent and hypodependent or hyperdependent and average.

Next, we explored whether start-stop (uORFs comprised only of a start and stop codon) elements in yeast are dependent on the Tmas for REI, as they are in mammalian cells (Schleich et al. 2017, Bohlen et al. 2020). We converted uORF1/3/4 in uORF1/3/4-only constructs into start-stops and measured their reporter activity. We found that all three start-stops led to a significant reduction in REI past them (resulting in lower reporter activity) compared to native uORFs, but there was little difference between WT and *tma20Δ tma64Δ* strains, suggesting that yeast start-stop elements are not Tma-dependent.

In summary, we found that most replacements of the uORF4 penultimate codon confer increased REI in mutants lacking both Tma64 and the Tma20/Tma22 heterodimer compared to the native uORF4. This suggests a codon-dependent role for the Tma factors in dissociating 40S post-termination complexes at this uORF, even for penultimate codons that were shown to be Tma-hypodependent for recycling. This implies that the deacylated tRNA can dissociate at both Tma-hyperdependent and Tma-hypodependent penultimate codons even in the absence of Tmas, and the resulting loss of Tma-catalyzed recycling of the empty 40S subunit allows for elevated REI at uORF4. In contrast to findings on short REI-permissive uORFs in mammalian cells (and *ATF4* uORF1 in particular) (Bohlen et al. 2020), we found that only one of six Tma-hyperdependent penultimate codons introduced at *GCN4* uORF1 showed reduced REI in *tma20Δ tma64Δ* cells. This indicates that dissociation of the deacylated tRNA and subsequent REI can generally proceed without Tma factors at uORF1, even at penultimate codons where these proteins enhance bulk 40S recycling. We, again, attribute this to Tma-independent tRNA release. Also at odds with findings on mammalian MCTS1/DENR, converting uORF1 to a start-stop element did not confer Tma-dependent REI, and converting uORF4 to a start-stop element decreased REI and diminished the contribution of Tma factors in suppressing REI. Overall, the influence of Tma factors on REI at short regulatory uORFs in yeast cells differs considerably from that observed for mammalian MCTS1/DENR.

Author contribution: 70% experimental, 30% writing

I am the first co-first author of this paper. I cloned of all uORF4 plasmids (Fig. 3 and Fig. 4) and measured corresponding reporter assays, start-stop reporter assays (Fig. 7) and completed statistical analysis of the reporter data. I created *tma20Δ tma22Δ* and *tma20Δ tma22Δ tma64Δ* yeast strains and performed growth assays. I also created and assembled the figures (except Fig. 5 and 8), drafted the first draft of this manuscript and contributed to its editing.

Stem-loop-induced ribosome queuing in the uORF2/ATF4 overlap fine-tunes stress-induced human ATF4 translational control

Smirnova AM, Hronová V, Mohammad MP, Herrmannová A, Gunišová S, Petráčková D, Halada P, Coufal Š, Świrski M, Rendleman J, Jendruchová K, Hatzoglou M, Beznosková P, Vogel C, Valášek LS.

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IF (2023): 7.5

While ATF4 translational control is one of the examples of a delayed REI model, a growing body of evidence suggests alternative mechanisms of its regulation, as outlined in the introduction. Thus, we conducted a complex bioinformatics and experimental mutational analysis of the human *ATF4* 5' UTR to uncover additional *cis*-acting features using a newly developed reporter assay based on a quantitative Western Blot method.

Using reporter constructs with solitary elements of the 5' UTR (start-stop/uORF0, uORF1, uORF2) in HEK293T cells with no stress treatment or Thapsigargin (Tg)-induced stress treatment, we confirmed that the start-stop element (which was not included in the previous models of delayed REI) at the beginning of *ATF4* 5' UTR modestly inhibits expression of ATF4. However, we also saw, contrary to previous models, that a solitary uORF2 (which should inhibit inducibility of ATF4) had a stress induction capability.

A bioinformatics analysis uncovered several interesting potential *cis*-acting features. The most notable was a conserved stem loop (SL3) roughly in the middle of the uORF2/ATF4 CDS overlap, which has a hairpin structure and could be inhibitory. This analysis also predicted four potential sites of m⁶A modification within corresponding motifs. Interestingly, the *ATF4* CDS begins with two AUGs in close proximity, and a third one 16 codons downstream of the first AUG. AUG1 overlaps with one of the methylation sites and AUG3 is in weak context and located in the SL3 region. Other predicted methylation sites are located within uORF2 (two sites) and near the SL3. We created an array of mutant constructs and evaluated physiological importance of these elements in a large-scale screen.

SL3 was one of the elements whose mutation had an observable effect on ATF4 expression, leading to an increase in expression, whether under no stress or in Tg-stressed cells. Thus, SL3 seems to be an inhibitory element that might delay scanning/translating ribosomes and prompt them to form a queue. Moreover, eliminating SL3 along with uORF2 resulted in even more robust increase in ATF4 expression, suggesting these elements act in concert to further inhibit ATF4 translation. Interestingly, there is a CUG near-cognate codon (in frame with ATF4 CDS) shortly upstream of SL3, which could serve as an initiation site for ribosomes slowed by SL3. Replacing this CUG by an CUA codon significantly reduced ATF4 expression (by 20%) in both stress and non-stress conditions but combining the SL3 mutation with the CUG mutation nullified this effect, suggesting a functional interplay between these elements. To test our queuing hypothesis, we conducted a ribosome protection assay and a bioinformatic analysis of ribo-seq data from ribosome profiling experiments), which both supported our assertion that

SL3 pauses ribosomes and prompts their queueing under both stress and non-stress conditions. This might lead to higher uORF2 expression (since these elements are located within the uORF2 region) even in non-stress conditions. Our experiments also showed substantial leaky scanning past ATF4's AUG1, resulting in formation of shorter ATF4 variants.

Other elements we investigated were the potential methylation sites in the *ATF4* mRNA – A235 and A326. Our T3 ligation assay (which investigates modification status of nucleotides) uncovered a potential mRNA modification at site A235 in native *ATF4* transcripts. Mutating A235 in our reporter set up had a significant stimulatory effect on *ATF4* expression under non-stress conditions. While A326 was not confirmed to be modified, reporter assays have shown that it stimulates ATF4 expression, and it is located within SL3. Interestingly, the double mutation of A326 and SL3 abolished the stimulatory effect of SL3 mutation, but only under non-stress condition, indicating that A326 has a stimulatory role under non-stress conditions and acts in concert with SL3.

Overall, our work has presented a new extended model of ATF4 translational control and introduced new evidence to challenge some of its old paradigms. We uncovered a layer of control imposed by a stem loop SL3, which cooperates with a nearby near-cognate codon, possibly inducing ribosome queueing leading to higher uORF2 translation even under stress. We also confirmed the presence of a modification at the A235 site (where an m⁶A modification was observed in mice) that also contributes to translational control of ATF4, and discovered a new potential modification site A326, which cooperates with SL3. Based on mounting evidence from the literature, it is now established that the precise mode of ISR induction through many pathways likely has distinct downstream effects that manifest in highly nuanced ATF4 translational control followed by unique modes of ATF4-mediated transcriptional responses. It seems plausible that a complex network of translation regulatory modes, as described here, is required to adjust ATF4 expression to a variety of conditions and tissues. Given the involvement of deregulated ATF4 expression in various pathologies and cancers, the complexity of its translational control should be kept in mind when considering targeted therapies.

Author contribution: 5%

I am one of the co-authors of this study. I cloned the constructs and conducted reporter analysis using the Jess™ Simple Western system for Figure 7B to help determine the influence of potential modification sites on ATF4 translational control. I also contributed to editing of the manuscript.

Unpublished results

Screening for new factors influencing REI and recycling

One of the aims of this work was to search for new factors influencing translation REI and/or ribosome recycling. For this purpose, I used a library of yeast strains deleted for selected factors and utilized the *GCN4*-based reporter system to assess defects in either REI or recycling.

The selection of the candidate factors was based on several criteria. The first was an experimental screening (Gunisova, unpublished data), where the RPEs of the *GCN4* 5' UTR were *in vitro* transcribed and labeled with biotin. These biotinylated RPEs were then captured with streptavidin beads and incubated with yeast lysates. After several washes and subsequent elution, proteins bound to RPEs underwent Mass Spectrometry (MS) analysis. Another criterium was a literature search. The final short-listed candidates I tested are shown below:

TABLE 6: Tested candidates.

SYSTEMATIC GENE NAME	STANDARD GENE NAME ^a
YCR051w	YCR051W
YDR388w	RVS167
YGL246c	RAI1
YGL049c	TIF4632
YLR134w	PDC5
YGL213c	SKI8
YDR385w	EFT2
YDL224c	WHI4
YGL195w	GCN1
YGL078c	DBP3
YHR121w	LSM12
YJL080c	SCP160
YKR048c	NAP1
YJL138c	TIF2
YOR323c	PRO TWO
YOR091w	TMA46

YJL124c	LSM1
YNL208w	YNL208w
YHR086w	NAM8
YGR086c	PIL1
YGR063c	SPT4
YNL197c	WHI3
YPL119c	DBP1
YIL137c	TMA108
YOR133w	EFT1
YPL184c	MRN1
YOR198c	RUP1
YOR252w	TMA16
YGR173w	RBG2
YKL001c	MET14
YKL149c	DBR1
YDL110c	TMA17
YGR210c	YGR210C
YHR064c	SSZ1
YDR293c	SSD1
YDL051w	LHP1
YNL147w	LSM7
YOR276w	CAF20
YMR074c	SDD2
YGR252w	GCN5
YPL004c	LSP1
YGR250c	RIE1

YGR093w	DRN1
YBR212w	NGR1
YLR327c	TMA10
YLR262c-a	TMA7
YGR285c	ZUO1

Table 6: The list of short-listed tested candidates for novel REI or recycling factors.
^a in case of no standard name available, systematic name is listed instead.
Both systematic and standard names are sourced from the *Saccharomyces* Genome Database (Wong et al. 2023).

All deletion strains originated from the Yeast Deletion Collection (Giaever and Nislow 2014) available at Euroscarf. I used plasmids p209 (“uORF1-only”, Grant et al. 1994) and p226 (“uORF4-only”, Mueller and Hinnebusch 1986) for the screening purposes, and their reporter activity was normalized to activity of p227 (“uORFless”, Miller and Hinnebusch 1989). I transformed the WT BY4741 strain and the deletion strains with each of the plasmids and performed β -galactosidase reporter assays. Based on the preliminary screening results (preliminary data not shown) and further literature search, we picked the following candidates for further analysis:

- a) Ssz1 and Zuo1
- b) Rai1

The yeast proteins Ssz1 and Zuo1 form a heterodimer which is also known as the RAC (Ribosome Associated Complex). It was first discovered and found to bind to the ribosomes by Gautschi et al. in 2001. This heterodimer interacts with the nearly identical Ssb1/2 chaperones, and this triad acts in concert to perform co-translational folding of the nascent peptide chains (Gautschi et al. 2002, Huang et al. 2005, Zhang et al. 2020, Kišonaitė et al. 2023). This heterodimer, however, also appears to regulate an array of other functions – it has a role in -1 programmed frameshifting (Muldoon-Jacobs and Dinman 2006), stop codon readthrough (Rakwalska and Rospert 2004), rRNA processing (Albanèse et al. 2010), pleiotropic drug resistance (Eisenman and Craig 2004, Prunuske et al. 2012), yeast prion formation (Shorter and Lindquist 2008, Kiktev et al. 2015, Son and Wickner 2020, Kelly et al. 2023) and quorum sensing, moonlighting as a transcriptional activator (Prunuske et al. 2012).

Structurally, Zuo1 binds to ribosomal proteins (Rpl31, Rpl22) and helix 24 of rRNA near the peptide exit tunnel (Peisker et al. 2008, Lee et al. 2016) and was also found to bind to the helix 44 of rRNA in the decoding center of the 40S subunit (Lee et al. 2016, Chen et al. 2022). Ssz1 contacts Zuo1 (first helix of its NTD), the ribosomal protein Rpl26/uL24 and rRNA helix 7 in certain conformations (Chen et al. 2022).

Considering these two proteins are associated with the ribosome and are known to perform several very well described roles in translation regulation, I investigated their potential roles in

ribosome recycling or REI, and I found significant >1.5-fold increase in uORF4 reporter activity (Fig. 20B+C).

Another factor of interest is Rai1. This factor's functions are often performed in complex with its main binding partner Rat1 (an exonuclease that localizes exclusively to the nucleus) – Rai1 acts as a cofactor to Rat1. This heterodimer or Rai1 alone regulates mRNA degradation (Das et al. 2003), transcription termination (Kim et al. 2004, El Hage et al. 2008, Park et al. 2015), surveillance of aberrantly capped mRNAs and decapping (Xiang et al. 2009, Jiao et al. 2010, Ya-Fan Wang et al. 2015), including non-canonical NAD caps (Zhang et al. 2020) and possibly FAD caps (Doamekpor et al. 2020), and also rRNA processing (Fang et al. 2005, Sydorsky et al. 2003).

Rai1 is localized both to the cytoplasm and nucleus, while Rat1 is localized strictly to the nucleus. It has been shown that in a mutant strain with non-functional Rat1, Rai1 re-localizes predominantly into the cytoplasm (Sydorsky et al. 2003).

While Rai1 has known implication role in translation so far, our results indicate it could be a potential REI factor (Fig. 20D+E). I found significant 50% reduction in REI after uORF1 (Fig. 20D). Considering the existing links between RNA degradation pathways and translation regulation, I examined this factor further, too.

Analysis of potential novel factors

Genetic and functional interactions

I first characterized the phenotype and genetic interactions of all selected factors. Since Zuo1 and Ssz1 form a heterodimer, I created a double deletion strain for both genes for further work. Based on reports of a physical interaction between Rai1 and Ssz1 found in a yeast proteome survey (Gavin et al. 2006), I also investigated any genetic or functional interaction between these two factors by creating a double deletion strain of *RAI1* and *SSZ1*. First, I analyzed the growth phenotype of the WT strain and strains deleted for *RAI1*, *SSZ1*, *ZUO1* and both *SSZ1* with *ZUO1/RAI1* using the yeast spot assay (Figure 20A).

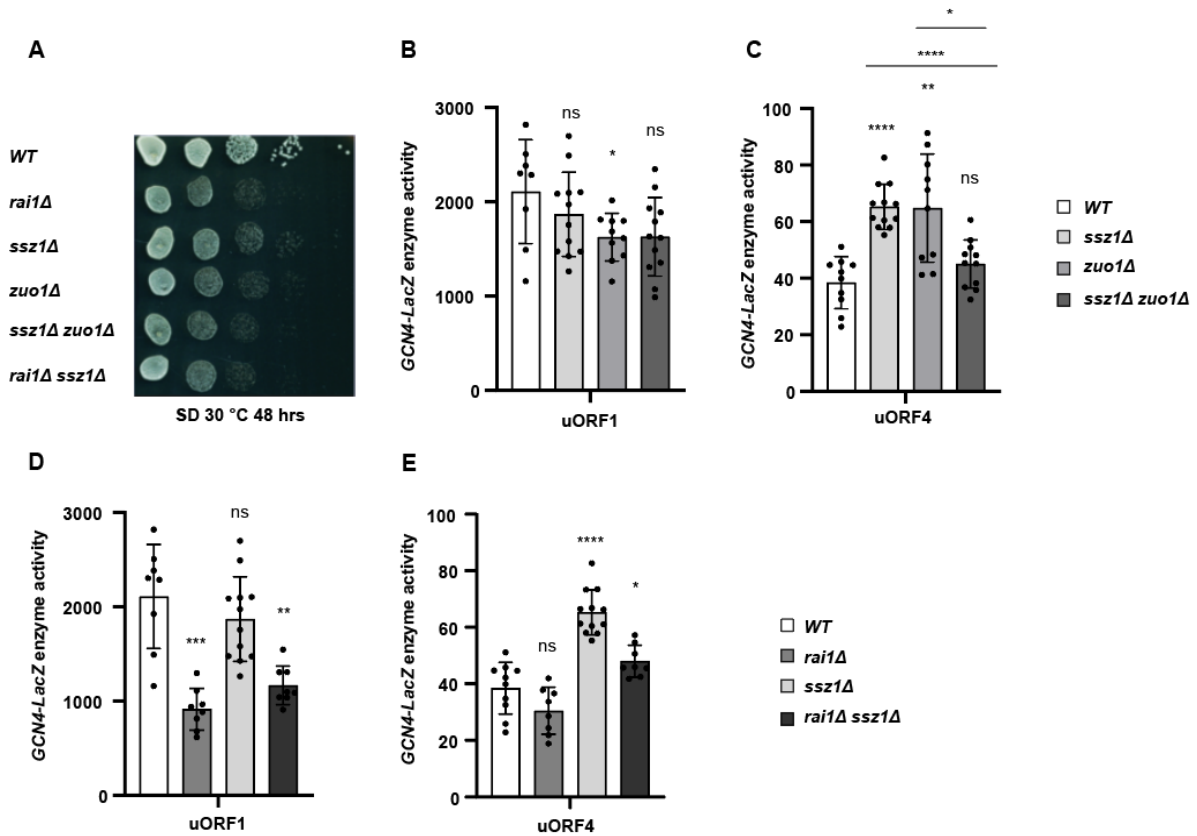


Figure 20: Impact of *rai1*, *ssz1*, *zuo1* single and double deletion effects on growth phenotype and reporter expression. **A.** The cultures were first diluted to $OD_{600} = 0.5$, then serially diluted (10x) and spotted onto SD plates. The plates were grown at 30 °C for 48 hours. **B.** WT, *ssz1Δ*, *zuo1Δ* and *ssz1Δ zuo1Δ* yeast strains were transformed with plasmid containing uORF1 and *GCN4-LacZ* fusion. Reporter activity was assayed in at least 5 independent transformants and normalized as described in Methods and Materials. Data are represented as normalized mean values $\pm$ SD with points denoting each individual reporter value. Statistical analysis was performed using two-tailed unpaired Welch's t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns not significant. **C.** Same analysis as in (B), but the strains were transformed with the uORF4-only plasmid. **D.** WT, *rai1Δ*, *ssz1Δ* and *rai1Δ ssz1Δ* yeast strains were transformed with plasmid containing only uORF1 and a *GCN4-LacZ* fusion. Reporter activity was assayed in at least 5 independent transformants and normalized as described in Methods and Materials. Data are represented as normalized mean values $\pm$ SD with points denoting each individual reporter value. Statistical analysis was performed using two-tailed unpaired Welch's t test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns not significant. **E.** Same analysis as (D), but the strains were transformed with uORF4-only plasmid.

All deletion strains exhibited a slow growth phenotype at 30 °C on SD media, underscoring the importance of these factors for cell growth and fitness. The phenotype was more severe in case of the *rai1Δ*, *zuo1Δ* and double deletion strains than in the case of *ssz1Δ* (Fig. 20A).

There was no significant difference in *GCN4-LacZ* expression after uORF1 between double deletion and WT strains (Fig. 20B). In strains deleted for either *SSZ1* or *ZUO1*, *GCN4-LacZ* expression after uORF4 is significantly increased (>1.5-fold), whereas the *ssz1Δ zuo1Δ* double deletion strain a much smaller non-significant increase can be seen (Fig. 20C). Moreover, there was also a significant difference in activity after uORF4 between both single *ssz1Δ/zuo1Δ* strains versus the *ssz1Δ zuo1Δ* strain (Fig. 20C). The *rai1Δ ssz1Δ* strain seems to exhibit a significant, almost 2-fold decrease in reporter expression after uORF1 compared to the WT, only a slightly smaller decrease than the single deletion *rai1Δ* strain (Fig. 20D). In contrast, the *rai1Δ ssz1Δ* strain exhibits only a modest, but still significant increase in reporter expression after uORF4 (Fig. 20E). This is also in contrast with the *rai1Δ* strain, where there was no significant difference found in reporter expression after uORF4 versus the WT (Fig. 20E). This phenotype would suggest a.) a complete dominance of the *RAI1* deletion over REI and b.) deletion of *RAI1* somehow partially rescues a potential recycling defect visible after *SSZ1* deletion. This may point to a potential functional interaction.

Next, I created plasmids carrying the *RAI1* and *SSZ1* genes fused C-terminally with a 6x Gly linker and 3x FLAG tag. These fusions were flanked by 500 nt of the natural 5' UTR of the corresponding gene upstream from the start codon and 500 nt of the 3'UTR downstream from the stop codon. YEplac181 (high copy; Gietz and Sugino 1988) and YCplac111 (single copy; Gietz and Sugino 1988) were used as shuttle vectors. First, I performed spot assays to test growth complementation by the tagged proteins (Fig. 21).

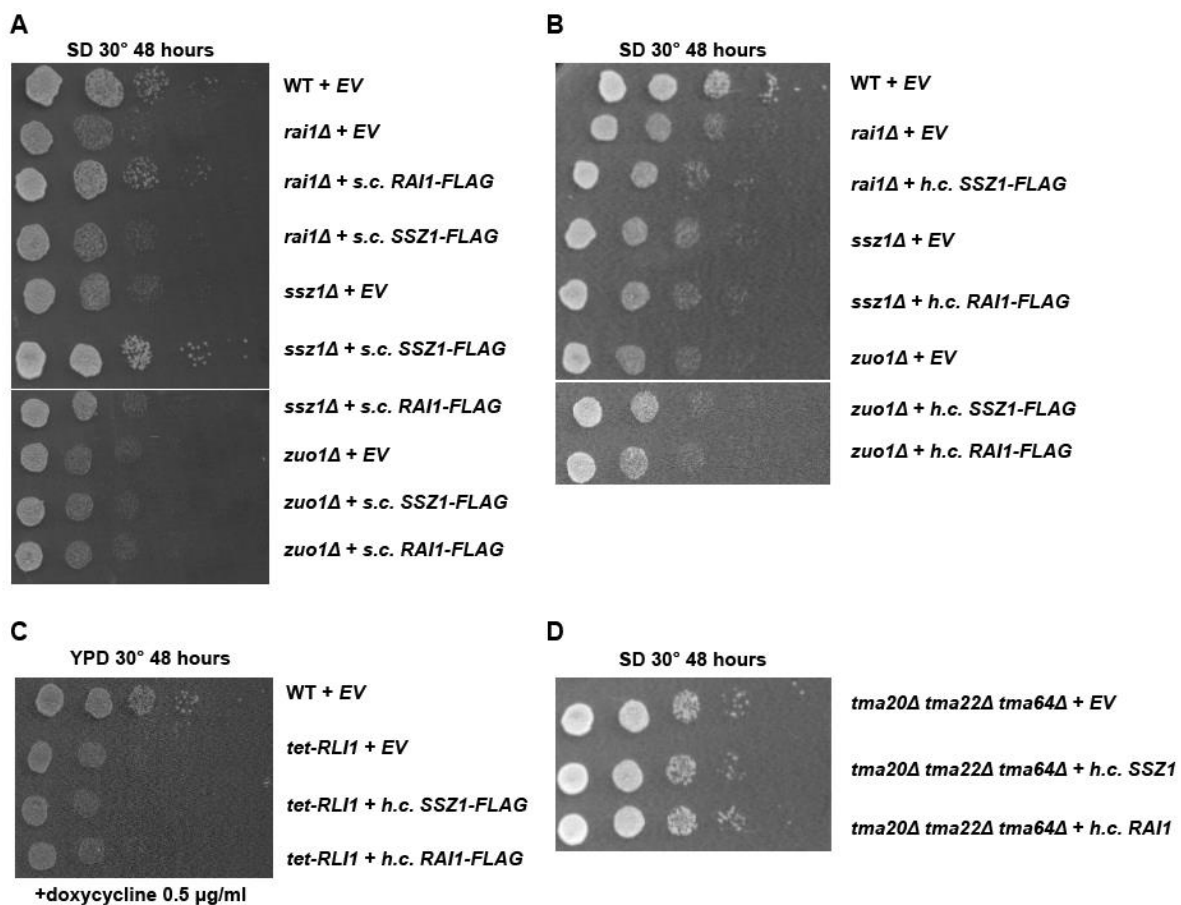


Figure 21: Growth complementation spotting assays. **A.** WT, *rai1Δ*, *ssz1Δ* and *zuo1Δ* strains were transformed with either the empty vector (EV) YCplac111 or the single copy (s.c.) plasmids carrying either *SSZ1-FLAG* or *RAI1-FLAG* fusions. The cultures were first diluted to OD₆₀₀ = 0.5, then serially diluted (10x) and spotted onto SD plates. The plates were grown at 30 °C for 48 hours. **B.** WT, *rai1Δ*, *ssz1Δ* and *zuo1Δ* strains were transformed with either the EV YEplac181 or the high copy (h.c.) plasmids carrying either *SSZ1-FLAG* or *RAI1-FLAG* fusions. The cultures were first diluted to OD₆₀₀ = 0.5, then serially diluted (10x) and spotted onto SD plates. The plates were grown at 30 °C for 48 hours. **C.** WT and *tet-RLII* strains were transformed with either the EV YEplac181 or the high copy (h.c.) plasmids carrying either *SSZ1-FLAG* or *RAI1-FLAG* fusions. The cultures were first diluted to OD₆₀₀ = 0.5, then serially diluted (10x) and spotted onto a YPD plate with doxycycline (0.5 µg/ml). The plate was grown at 30 °C for 48 hours. **D.** Deletion strain *tma20Δ tma22Δ tma64Δ* was transformed with either the EV YEplac181 or the high copy (h.c.) plasmids carrying either *SSZ1-FLAG* or *RAI1-FLAG* fusions. The cultures were first diluted to OD₆₀₀ = 0.5, then serially diluted (10x) and spotted onto an SD plate. The plate was grown at 30 °C for 48 hours. This assay was performed along with the assay from (B) and the WT in (B) is also the corresponding WT for this assay.

The FLAG-tagged fusion proteins complemented growth defects in the corresponding deletion strains. However, we saw no growth complementation for either *rai1Δ* with *SSZ1-FLAG*, or for *ssz1Δ* with *RAI1-FLAG*. Moreover, growth defect in the *zuo1Δ* strain was not complemented by overexpression of either protein (Fig. 21A). Overexpression from the high copy plasmids had no effect either (Fig. 21B).

Next, I performed a growth complementation assay with an inducible knockdown strain for the essential 60S recycling factor Rli1. I used an inducible depletion strain *Tet-RLII* (Kispal et al. 2005) with *RLII* under an inducible-repressible tetracycline promoter (Fig. 21C). For another candidate interactor, I used a triple deletion strain of major 40S recycling factors Tma20/Tma22/Tma64 (Jendrichova et al. 2024) (Fig. 21D). However, neither the overexpression of *SSZ1-FLAG*, nor *RAI1-FLAG* rescued the growth defect originating from the knockdown of *RLII* (induced by doxycycline) (Fig. 21C). The *tma20Δ tma22Δ tma64Δ* strain does not exhibit a slow growth phenotype, and overexpression had no effect on its growth whatsoever (Fig. 21D).

Translation phenotype

To assess the general translation phenotype induced by single or double deletion of *RAI1*, *SSZ1* and *ZUO1*, I performed polysome profiling using cycloheximide as an elongation freezing compound (Figure 22).

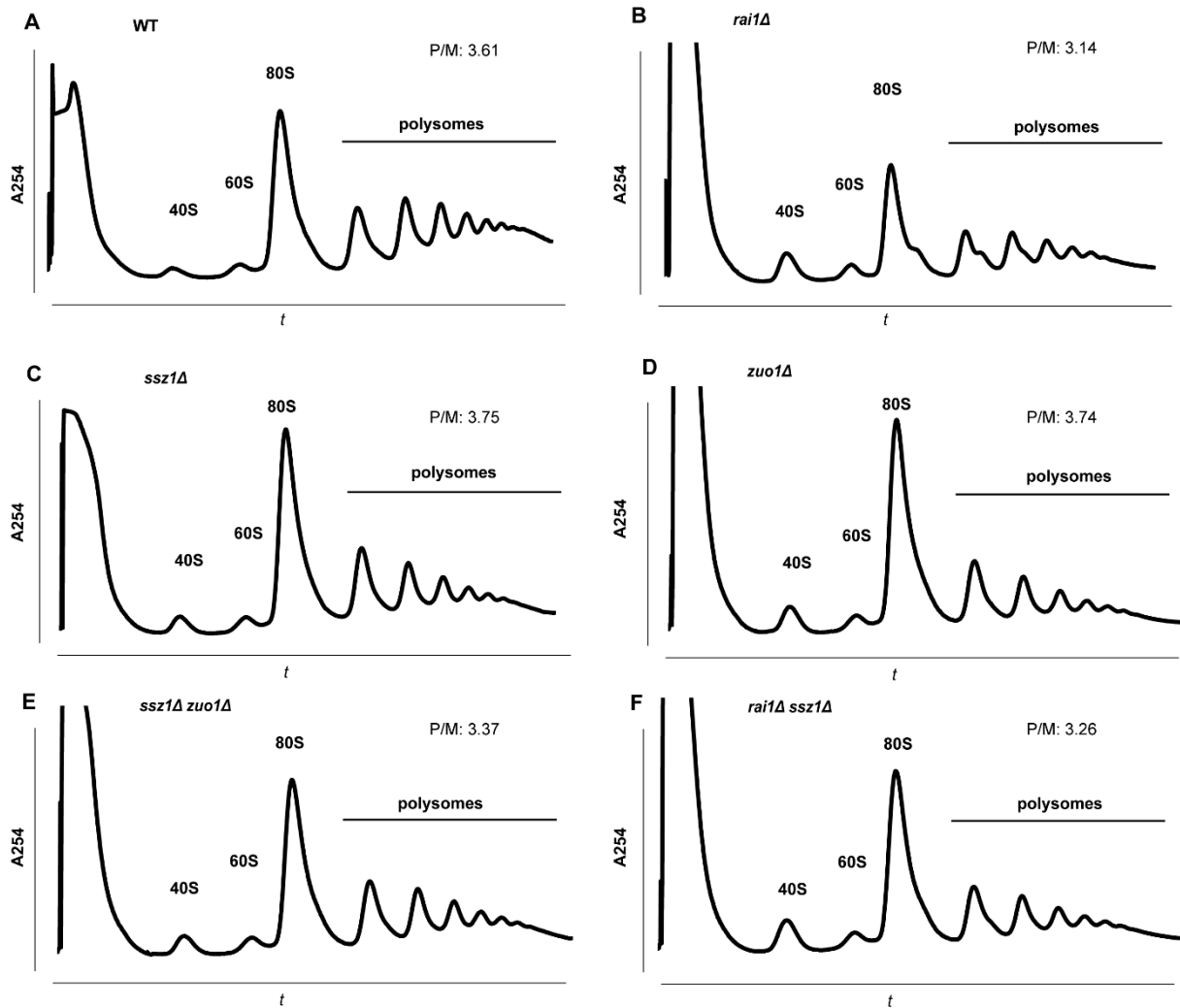


Figure 22: Polysome profiles of wild type and deletion strains. Yeast strains were grown to $OD_{600} \sim 0.8$ in SD media, treated with cycloheximide and WCEs were prepared. 8U of RNA were layered on 5-50% sucrose gradients and separated by ultracentrifugation at 39,000rpm for 2.5 h (using an SW41 Ti rotor). Gradients were scanned at 254 nm to visualize ribosomal species. Positions of 40S, 60S, 80S and polysomal species are indicated and P/M ratios are displayed in the upper right corner. Strains used: **A.** WT BY4741 **B.** *rai1Δ* **C.** *ssz1Δ* **D.** *zuo1Δ* **E.** *ssz1Δ zuo1Δ* **F.** *rai1Δ ssz1Δ*.

Briefly, polysome profiling is a well-established method that is routinely used to monitor translational fitness of cells - it separates free 40S and 60S subunits from monosomes and polysomes through separation on a sucrose gradient and visualizes their amounts as ‘peaks’ with different heights. To prevent any ‘run off’ of polysomes during sample preparation, a stabilizing agent is used, such as cycloheximide or formaldehyde (Pospisek and Valasek 2013). As can be seen in Fig. 22B, the *rai1Δ* strain exhibited so-called ‘halfmers’ that can be seen as trailing shoulders of 80S monosomes and polysomes. Single deletion *rai1Δ* and the double deletions of *ssz1Δ zuo1Δ* and *rai1Δ ssz1Δ* exhibited a mild polysome runoff (indicated by a lower P/M ratio), indicating a modest reduction of initiation rates (Fig. 22B/E/F). Single deletion *rai1Δ* and *zuo1Δ* and double deletion *rai1Δ ssz1Δ* strains seem to have a reduced

number of 60S subunits as judged from the higher 40S peak, which could indicate a defect in 60S biogenesis (Fig. 22B/D/F).

The Ssz1:Zuo1 heterodimer has been already found to be associated with ribosomes (Lee et al. 2016, Chen et al. 2022). While Rai1 seems to influence translation, its direct association with ribosomes has not yet been determined. To analyze this and confirm Ssz1 association with ribosomes in our setup, I transformed the *railΔ* and *ssz1Δ* strains with their corresponding *RAI1-FLAG/SSZ1-FLAG* s.c. plasmid, collected fractions from sucrose gradients and performed Western blotting to determine their presence in each fraction (Figure 23). The polysome profiles look different compared to those in Figure 22 due to the use of 1% formaldehyde as a crosslinking agent. Rai1-FLAG was detected mainly in the fractions containing free 40S and 60S subunits, weakly in the 80S fractions, but did not associate with translating polysomes (Fig. 23A). Ssz1-FLAG was detected mainly in fractions containing free 40S subunits, but also associated weakly with free 60S subunits, 80S monosomes and translating polysomes (Fig. 23B).

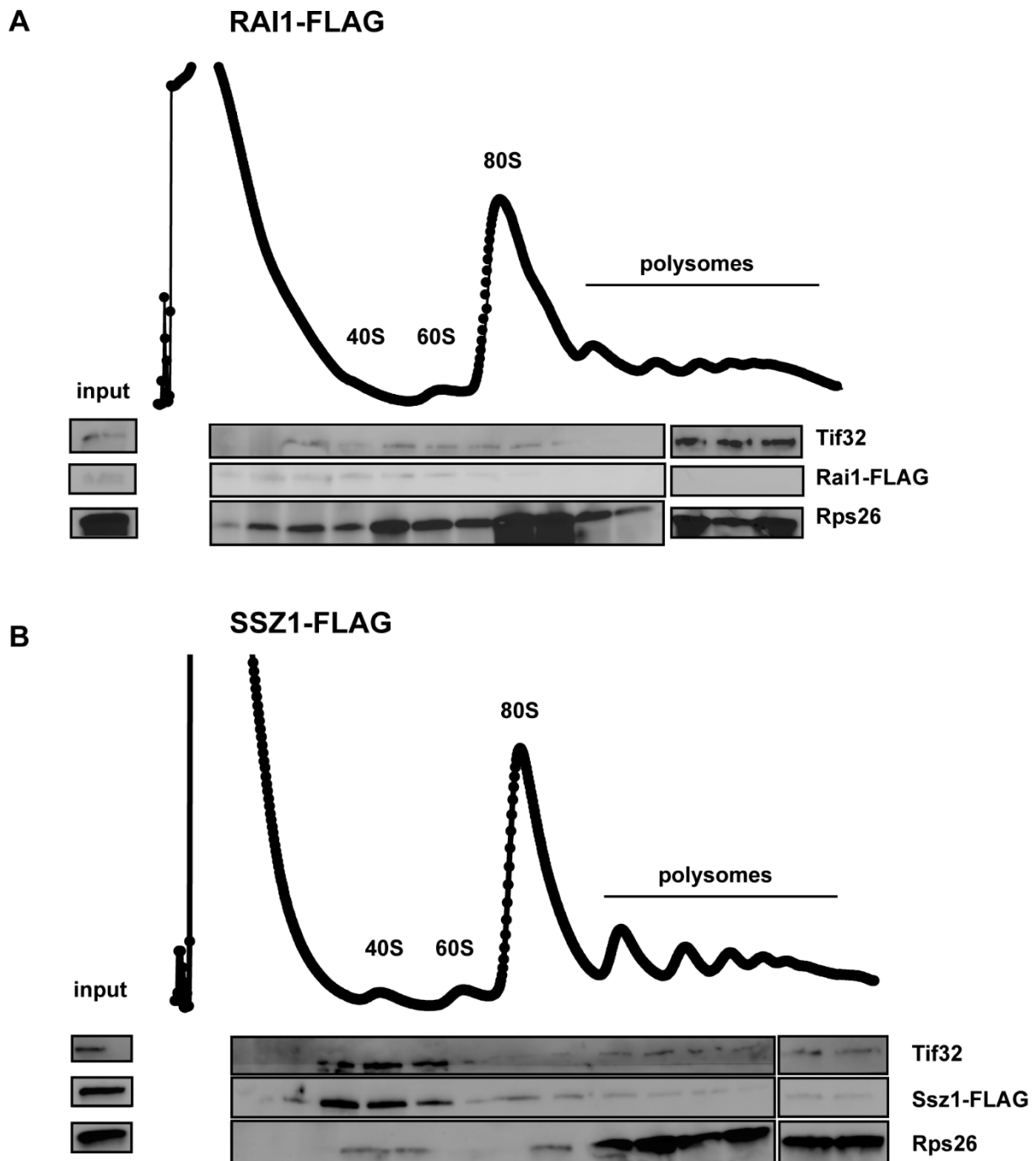


Figure 23: Association of Rai1 and Ssz1 with ribosomes. Yeast cultures transformed with appropriate plasmids were grown in SD media to $OD_{600} \sim 1.0$, crosslinked with 1% formaldehyde and WCEs were prepared. 20 U of RNA were layered on 5-50% sucrose gradients and separated by ultracentrifugation at 39,000rpm for 2.5 h (using an SW41 Ti rotor). Gradients were scanned at 254 nm to visualize ribosomal species, fractions were collected, precipitated with 96% EtOH and subjected to Western Blot analysis. Positions of 40S, 60S, 80S and polysomal species are indicated. **A.** *rai1Δ* strain was transformed with a single copy plasmid carrying a *RAI1-FLAG* fusion. Rai1-FLAG was detected using α -FLAG antibody (1:1000 primary Ab), as controls we used eIF3a/Tif32 (1:1000 primary Ab) and Rps26 (1:1000 primary Ab). Secondary antibodies were diluted 1:5000. **B.** *ssz1Δ* strain was transformed with

a single copy plasmid carrying a *SSZ1-FLAG* fusion. Ssz1-FLAG was detected using α -FLAG antibody (1:1000 primary Ab, 1:2500 secondary Ab), as controls we used eIF3a/Tif32 (1:1000 primary Ab, 1:2500 secondary Ab) and Rps26 (1:1000 primary Ab, 1:7500 secondary Ab).

Physical interactions

To get more insight into the potential function of these factors, I investigated the physical interactions of these factors with potential binding partners. First, since a physical interaction was found between Ssz1 and Rai1 in a yeast proteome survey (Gavin et al. 2006), I verified this interaction in an *in vitro* GST-pulldown setup. Using pGEX-5X3 (GE Healthcare) as a backbone, I created a *GST-SSZ1* fusion, induced its expression in *E.coli* and purified the GST-Ssz1 protein from bacterial cultures on Gluthathione sepharose beads. I also cloned the *RAI1* CDS and *ZUO1* CDS into a pGL4-CMV backbone (Wagner et al. 2014) and used an *in vitro* transcription/translation system incorporating ^{35}S -labelled methionine into Rai1 and Zuo1 proteins. Then, I performed a pull-down assay followed by autoradiography (using Zuo1 as a positive control) (Figure 25).

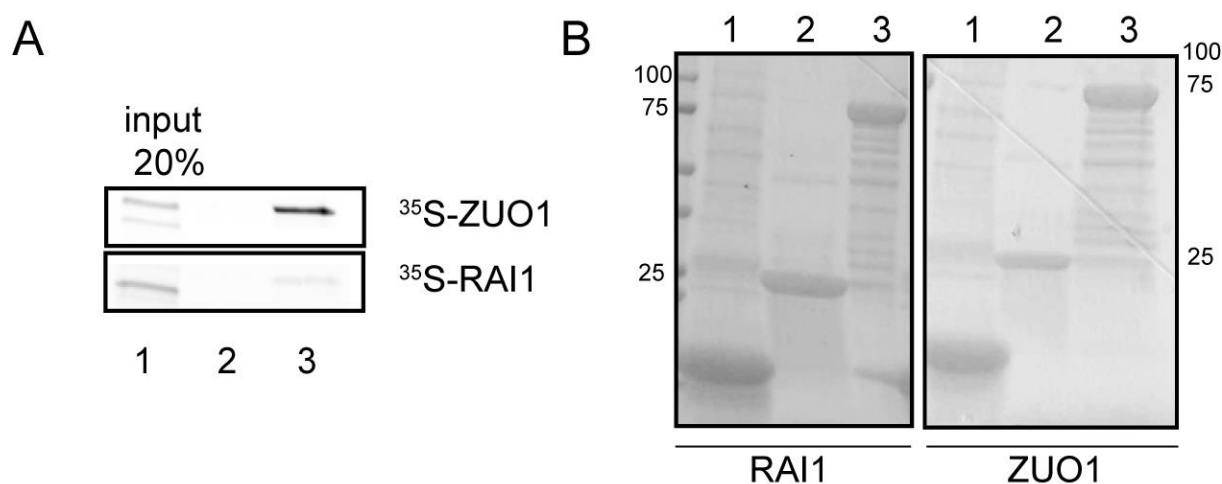


Figure 24: GST-pulldown of GST-SSZ1 and ZUO1/RAI1. **A.** Pulldown reaction of GST-SSZ1 with either radioactively labelled Zuo1 (line 1) or Rai1 (line 2) as prey, using GST as a negative control, imaged through autoradiography. Column 1 represents 20% of input, column 2 represents pulldown with GST alone as bait and column 3 represents pulldown with GST-SSZ1 as bait. **B.** Stained SDS-page gel loaded with reactions from columns 1, 2 and 3 for each set of reactions (with RAI1 or ZUO1). The radioactively labelled proteins are not visible as distinct bands on these gels, not even as input in column 1, simply due to the fact that the *in vitro* transcription/translation reaction does not produce enough material for the sensitivity of this staining. The biggest band in column 1 are most likely small ribosomal proteins from rabbit reticulocytes. The biggest bands in columns 2 and 3 are GST or GST SSZ1, respectively.

Fig. 24A shows pulldown reactions using control GST/GST-SSZ1 as bait and radioactively labelled ^{35}S -ZUO1 (line 1) or ^{35}S -RAI1 (line 2) as prey. Column 1 represents 20% of input reaction, column 2 represents pulldown with GST alone (as a negative control) and column 3 represents pulldown with GST-SSZ1. We detected a strong interaction between Ssz1 and Zuo1, its canonical binding partner in the RAC. A weak interaction between Ssz1 and Rai1 was also

observed, confirming the results from Gavin et al. 2006. Stained gels loaded with the reactions from columns 1-3 are shown in Fig. 24B.

Next, I explored other physical interactors of these factors on a large scale using an MS-based approach. I transformed *rai1Δ/ssz1Δ* strains with high copy plasmids carrying *RAI1-FLAG/SSZ1-FLAG* and grew the yeast cells to OD₆₀₀ ~ 0.8 in SD media. Afterwards, I performed pulldown using FLAG beads and sent the purified complexes for MS analysis. Pulldowns from *rai1Δ/ssz1Δ* strains not carrying FLAG plasmids were used as a negative control. The results of Pulldown-MS analysis are shown in volcano plots in Fig. 25A (Rai1) and Fig. 25B (Ssz1). The range of the X axis (fold-change) of the volcano plot was adjusted for better clarity of the plot, omitting some hits with very high fold-change that would obfuscate the visibility of less abundant proteins on the plot. Examples of these highly abundant hits are Rai1/Ssz1 themselves and their canonical binding partners Rat1/Zuo1. However, the non-adjusted volcano plots are shown in Supplementary Figure S1.

It is also important to note that the Rai1 pulldown (Fig. 25A) was possibly influenced by the abundance of Leu2 pulled down (see Sup. Fig. S1A), which is the auxotrophic marker on the high copy *RAI1-FLAG* plasmid. Thus, we can see several proteins from aminoacid biosynthetic pathways as top hits in the pulldown. This was not observed in the Ssz1 pulldown (Fig. 25B, Sup. Fig. S1B), but the pulldowns for both proteins were performed in slightly different conditions (see Methods and Materials for details). Perhaps this is not unspecific binding between them due to *LEU2* overexpression, but Rai1 interacts with the Leu2 enzyme specifically. Among the most intriguing specific hits was Dbp5, which is a DEAD-box helicase that has roles in RNA decay, translation termination, RNA quality control and several other processes related to gene expression (reviewed in Querl and Krebber 2023). We also found that Rai1 pulled down several ribosomal proteins of the large subunit, most interestingly the RPP2B protein. It is a component of the ribosomal stalk (part of the 60S subunit) which is only joined to the ribosome upon subunit joining, but not at free 60S subunits (Bautista-Santos and Zinker 2014). With Ssz1 (Fig. 25B), we saw it pulled down Asc1 (orthologue of the mammalian RACK1), which was shown to be a ribosome quality control factor (Boopathy et al. 2023) and a small ribosomal protein of the small subunit implicated in multiple cellular processes (Singh et al. 2019, Schmitt et al. 2021, Wang et al. 2018). Ssz1 also interacts with several ribosomal proteins of both the large and small subunit (not all of them are labelled in the volcano plot, mostly due to the sheer abundance of hits). The most interesting hit among them was RPP0, a protein which is a permanent component of the ribosomal stalk, and it forms the stalk with the RPP2B protein (Grela et al. 2012), which I found to interact with Rai1.

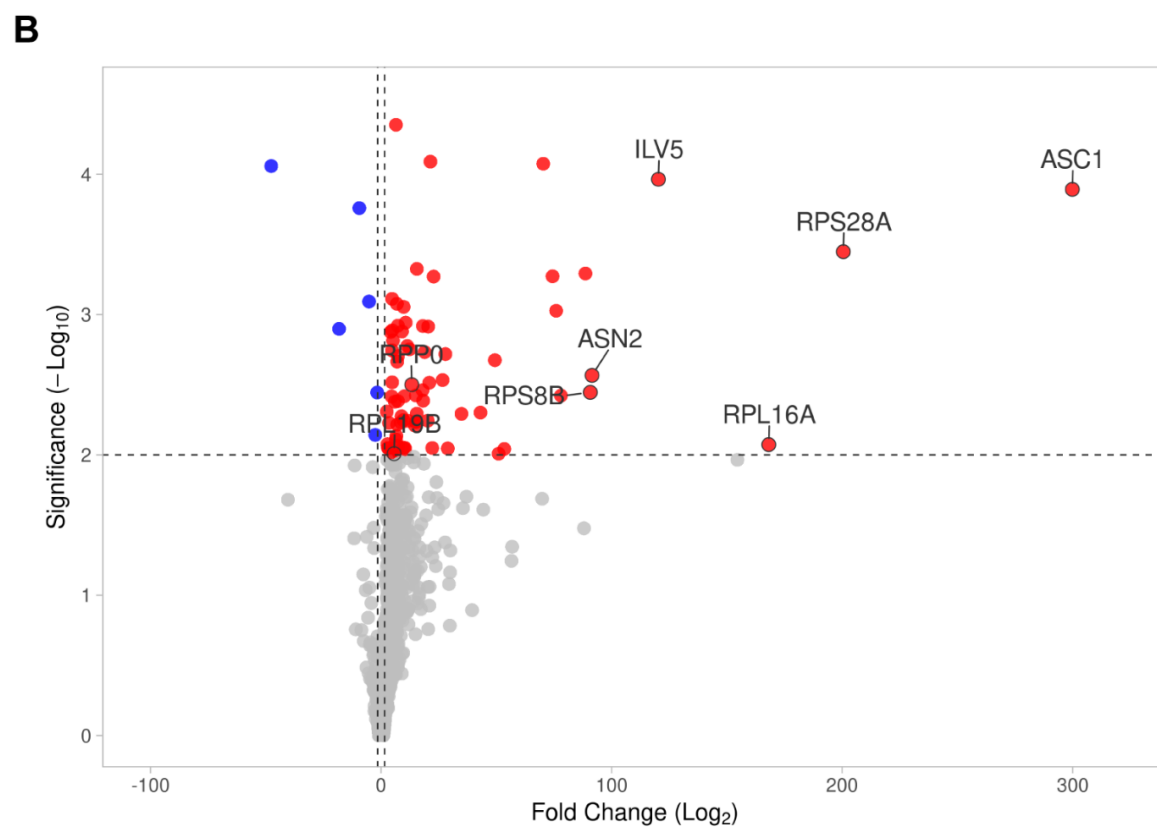
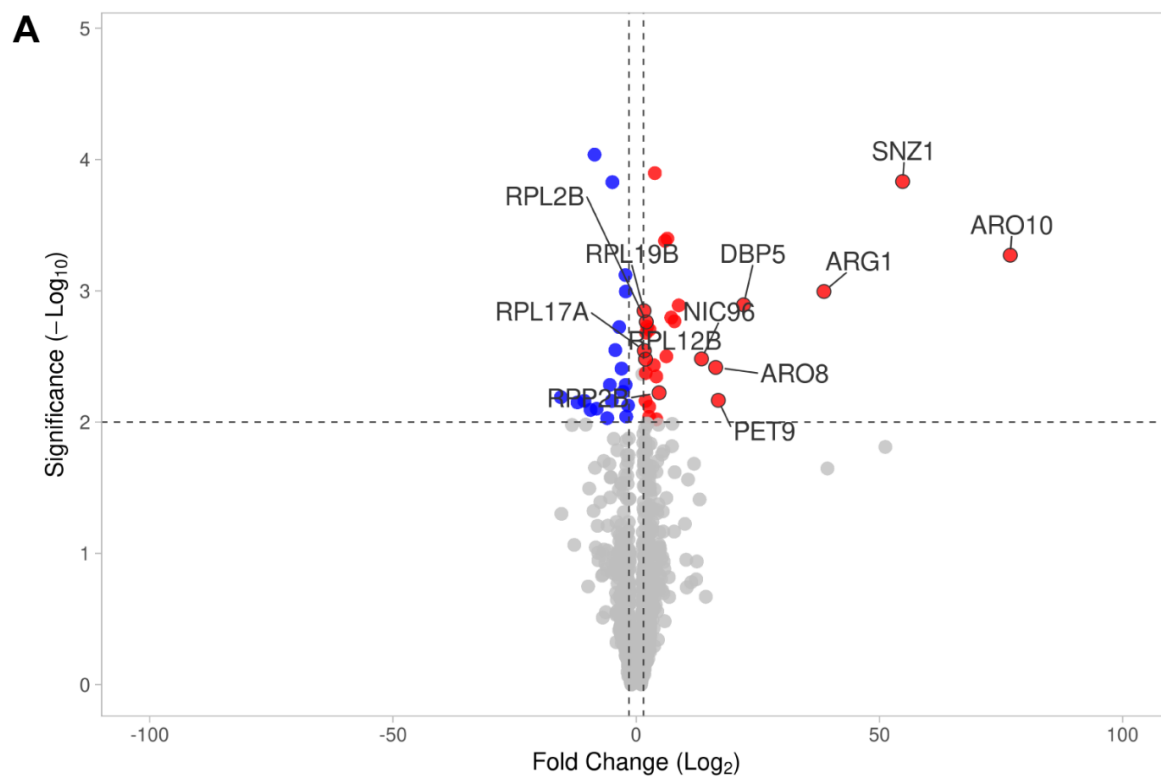


Figure 25: Volcano plot of MS analysis of pulled down complexes. **A.** Rai1-FLAG complexes were pulled down and sent for MS analysis. **B.** Ssz1-FLAG complexes were pulled down and sent for MS analysis.

DISCUSSION

Differential effects of 40S ribosome recycling factors on reinitiation at regulatory uORFs in *GCN4* mRNA are not dictated by their roles in bulk 40S recycling.

In Jendruchova et al. 2024, we used an established reporter system based on *GCN4* translational control to examine the effects of ribosome recycling factors Tma20, Tma22 and Tma64 on downstream REI after short uORFs of two classes: i) “yeast-like” uORFs, which are mostly primed for recycling (represented by *GCN4* uORF4) ii) “mammalian-like” uORFs which are mostly primed for REI (represented by *GCN4* uORF1). We examined them with consideration to different penultimate codons and their effect on downstream REI. Using genetic tools, we also dissected the functional interplay between these factors, as well as their influence on REI after start-stops.

The penultimate codon TTG^{Leu} is one of the most Tma-dependent (dubbed ‘Tma-hyperdependent’ by us), while TGG^{Trp} one of the least Tma-dependent (or ‘Tma-hypodependent’) for bulk 40S recycling according to Young et al. 2021. We picked these two codons to dissect the interplay between the Tma factors. We mutated the uORF4 penultimate codon to these two, creating two new uORF4-only constructs, and performed reporter assays in WT and deletion strains of *TMA20/22/64*. We found that single deletion of *TMA20* and *TMA22* increases REI after TTG^{Leu}, but very little after TGG^{Trp}, following the trend of this ‘Tma-dependence’. Deletion of *TMA64* impaired very little increase in REI by itself. However, deletion of *TMA64* alongside either *TMA20* or both *TMA20+TMA22* (creating the triple deletion *tmaΔΔΔ* strain) enhances the increase in REI caused by the defect induced by this deletion. This agrees well with Young et al. 2021, which found an abundance of unrecycled 40S subunits at stop codons after single deletion of either *TMA20* or *TMA22*, but not *TMA64*. The possible reason for this might be that Tma64 plays only a supportive role to Tma20/22, which bear the brunt of 40S recycling activity. The defect in recycling after their deletion subsequently manifests as increased REI in deletion strains. Looking at it from another perspective, we could also say the Tma proteins work to *suppress REI* by *promoting 40S recycling* after uORFs.

In a similar vein, we picked 12 penultimate codons which were either Tma-hypodependent, Tma-average or Tma-hyperdependent, and created 12 new uORF4 variants in uORF4-only constructs. Our reporter assays (conducted in the *tma20Δ tma22Δ* and *tmaΔΔΔ* strains) revealed that deletion of all three factors increased REI (and thus reporter activity) in all tested codons, the smallest increase being 1.23x (TGG^{Trp}) and the largest being 4.56x (AAT^{Asn}), whereas the double deletion (*tma20Δ tma22Δ*) saw a marginally smaller increase between 1.30x and 4.13x. Interestingly, these increases do not completely follow rules postulated by the Tma-dependency as the dependency for bulk recycling (Young et al. 2021); we did not find significant differences between the Hypodependent and Average groups, or between the Hyperdependent and Average groups (Fig. 26A).

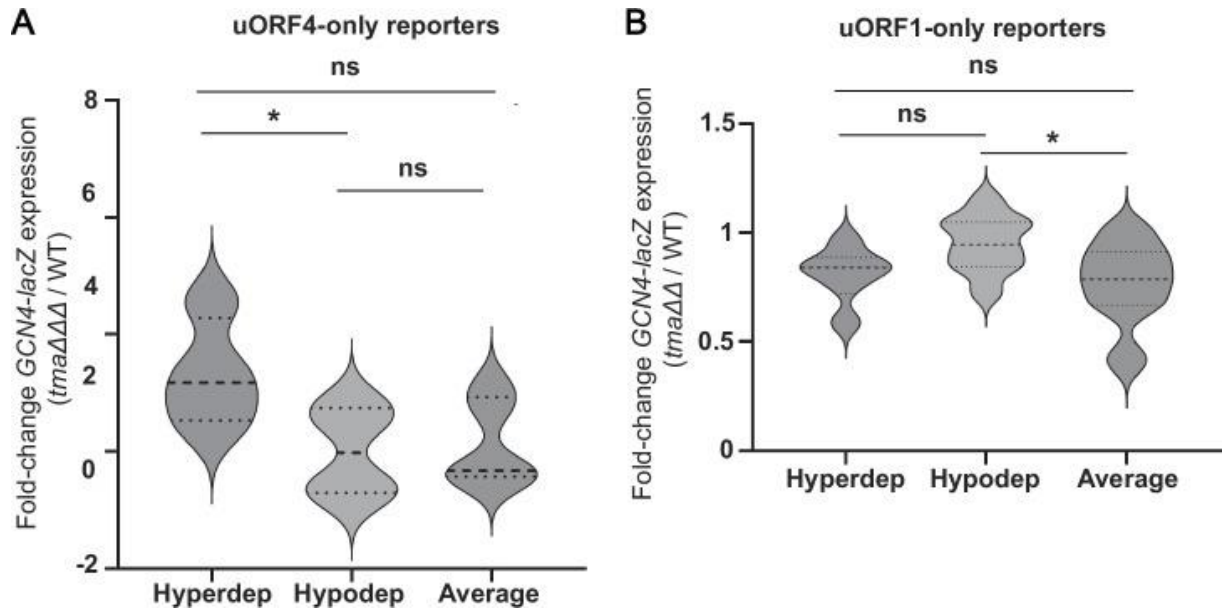


Figure 26: **A** Violin plot of the average fold-changes in expression of uORF4-only *GCN4-lacZ* reporters harboring the four Tma-hyperdependent (Hyperdep), four Tma-hypodependent (Hypodep) or five Tma-average (Average) penultimate codons in the triple deletant yeast strain *tma20Δtma22Δtma64Δ* vs. isogenic WT strain. The average fold-change of expression determined from biological replicates only was calculated for each construct from results of reporter assays. Statistical analysis was performed using two-tailed unpaired *t*-test $*p < 0.05$. **B** Violin plot of fold changes in expression of uORF1-only *GCN4-lacZ* reporters harboring the six Tma-hyperdependent, nine Tma-hypodependent and 14 Tma-average penultimate codons in *tma20Δtma64Δ* strain vs. WT strain. The average fold-change of expression determined from biological replicates was calculated for each construct from the results of reporter assays. Statistical analysis was performed using the two-tailed unpaired *t*-test. $*p < 0.05$. Reprinted with permission from Jendrichova et al. 2024.

Contrary to our expectations, some codons from the Hypodependent group could reach an increase in reporter activity on par with those in the Hyperdependent group, and certain codons from the Average group reached increases in reporter activity on par with those in either the Hypodependent, or the Hyperdependent group. We thus conclude that while the TTG^{Leu} and TGG^{Trp} codons that we used to investigate the interplay between these factors are representative of expectancies for their respective codon groups, in general, codons do not explicitly follow the Tma-dependency rules set by bulk recycling. Instead, this dependency for bulk recycling might only partially contribute to the whole picture. It is also important to note that even the least Tma-hypodependent codons according to Young et al. 2021 saw at least 4x increase in the amount of unrecycled 40S subunits at stop codons in the *tma20Δ tma64Δ* deletion strain compared to the WT, with a maximum of >32x increase in the case of the most Tma-hyperdependent codons. This does not translate directly to the increase in REI after uORF4 in our deletion strains, which saw a minimal 1.3x increase and maximum of ~4.5x increase in REI after uORF4. Interestingly, three codons even showed a higher increase in REI in the *tma20Δ tma22Δ* strain than the *tmaΔΔΔ* strain, showing there is possibly even a more complicated

mechanism at play regarding the role of Tma64, which is generally supportive, but seemingly not always.

We also examined whether the Tma factors function similarly to DENR/MCTS1 and eIF2D in mammalian cells. There, these factors promote REI after short uORFs. This is mainly due to mammalian uORFs being generally more permissive for REI due to higher retention of 40S post-TC on the mRNA, where DENR/MCTS1 could catalyze release of deacylated tRNA and promote resumption of scanning by 40S subunits. However, there is still preferential DENR-dependence of certain codons compared to others, with the assumption that DENR/MCTS1 catalyze preferentially dissociation of some tRNAs over others (Bohlen et al. 2020). To test this, we created a barrage of uORF1-only mutant constructs with exchanged penultimate codons and compared their reporter activities in the *tma20Δ tma64Δ* strain versus the WT strain. *GCN4*'s uORF1 is surrounded by RPEs which promote retention of 40S post-TC with bound eIF3 and eIF4F to impair higher REI after uORF1 (Munzarova et al. 2011, Mohammad et al. 2017, 2021). This mimics the higher rate of post-TC retention that can be found in mammalian uORFs. The expected result for Tma-dependent codons would be that upon deletion of *TMA* genes, REI would be reduced (in a similar manner to DENR-dependent codons in mammals). In our reporter setup, surprisingly, only three codons out of 29 that were examined showed significant reduction in REI after uORF1. Only one of them (TTG^{Leu}) was Tma-hyperdependent for bulk recycling (Young et al. 2021) and one of them (ATG^{Met}) was DENR-dependent in mammalian cells (Bohlen et al. 2020). Interestingly, we also found a slight increase in REI of the Hypodependent group in the *tmaΔΔ* strain compared to the WT, resulting in a significant difference in REI between the Hypodependent and Average groups, but remarkably not the Hyperdependent group (Figure 26B). This shows that even in the case of uORF1, Tma-dependency for bulk recycling does not translate into dependency for REI and the rules seem to be more complex.

An important thing to note is also another possibility – that the interaction of uORF1 RPEs with eIF3 overrides any dependency for the Tma factors for REI. This interaction that prevents 40S dissociation might provide ample time for deacylated tRNAs to dissociate from the post-TCs on their own, or by an independent pathway. Furthermore, structural analysis of DENR/MCTS1 suggests that MCTS1 might occupy the same binding site on the ribosome as eIF3a, b and c (Lomakin et al. 2017). Since the binding interface seems to be well conserved between yeast Tma20 and mammalian MCTS1 (Figure 27A), this might indicate potential competitive binding between eIF3 and the Tma20/Tma22 heterodimer, wherein eIF3 interacting with the RPEs would potentially prevent the Tma factors from influencing REI at uORF1. The comparison of the structures of these regions can be seen in Fig. 27B. Therefore, the interaction between the deacylated leucyl tRNA with the TTG^{Leu} codon might be too stable to overcome the requirement for Tma-mediated aid in its dissociation. and this could also apply to the two other codons that reduced REI after uORF1.

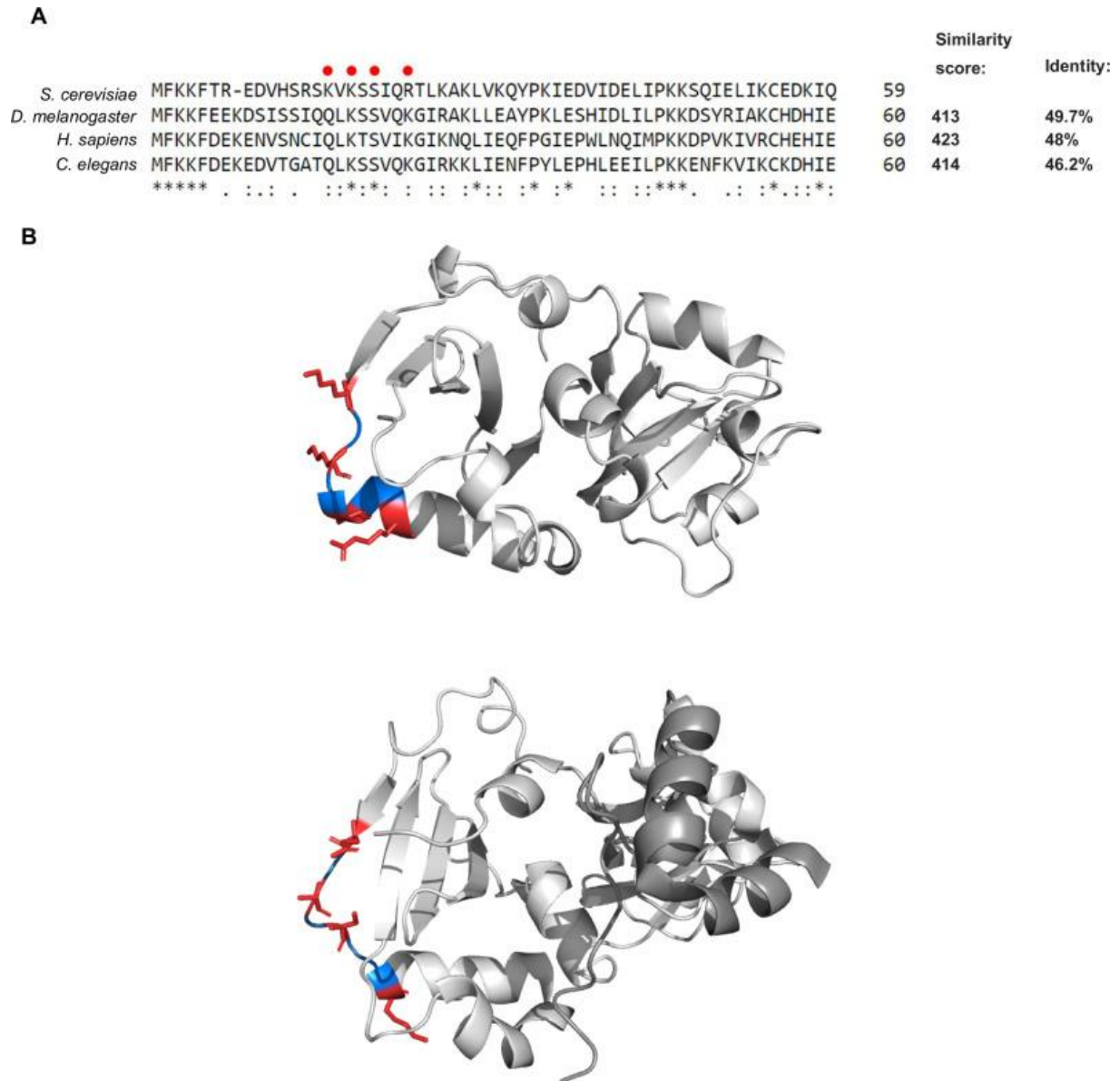


Figure 27: **A** Multiple sequence alignment was performed using Clustal Omega. This alignment illustrates the conservation of amino acid residues of the ribosome binding interface described previously (Lomakin et al. 2017) and marked here by red dots. Similarity score and identity percentage were determined using UniProt BLAST tool. **B** Comparison of predicted Tma20 structure (upper panel) and structure of MCTS1 in complex with DENR (bottom panel; MCTS1 colored in light gray, DENR colored in dark grey). Conserved residues are depicted in red and regions separating them are colored in blue. Tma20 structure was predicted using the AlphaFold3 algorithm (Abramson et al. 2024). MCTS1/DENR complex structure was published previously (Lomakin et al. 2017) (PDB accession 6MS4). PyMOL 3.0 was used for data visualization. Reprinted with permission from Jendruchova et al. 2024.

Apart from supporting REI after short uORFs, DENR/MCTS1 have been found to promote REI after start-stop elements (Bohlen et al. 2020, Schleich et al. 2017). However, we found no evidence of Tma-dependent REI or recycling at start-stops in yeast. For this, we used reporter constructs with uORF1, 3 and 4 converted into start-stop elements. While we found that these

start-stops act as “translational brakes” (universally reducing REI even in the case of the already non-permissive uORF4), there was little difference in expression between the WT and *tma20Δ tma64Δ* strains. Interestingly, we observed a massive (~50-fold) reduction in REI after start-stop1 (converted from uORF1) compared to the native uORF1, indicating that the start-stop somehow overrides the RPEs that are required for its high rate of REI. Converting uORF3 and 4 to start-stops seems to have diminished the increase in REI conferred by deletion of the *TMA* genes. Start-stops also do not display decreased REI in the *tmaΔΔ* strain like we observed with uORF1 with the ATG^{Met} penultimate codon – unsurprisingly, since the start-stops seemingly lost the capacity for 40S post-TC retention on the mRNA through RPEs. Additionally, we do not know the manner in which the Tma factors may (or may not) interact with initiator Met-tRNA_i^{Met} (as the penultimate codon in start-stops is a start codon) as opposed to the elongator methionyl tRNA that we tested with uORF1.

To summarize, we found that rules for Tma-dependent bulk recycling serve as a poor predictor for how the Tma factors influence REI after uORFs. At the REI non-permissive uORF4, all penultimate codons conferred a certain degree of increased REI in deletion strains for the Tma factors (as we would expect for a defect in recycling). This was found even in case of penultimate codons classified as Tma-hypodependent for bulk recycling. This implies that the deacylated tRNA can dissociate in the absence of the Tma factors at both Tma-hyperdependent or -hypodependent codons, but the loss of Tma-catalyzed 40S recycling of the empty 40S subunit permits increased REI at uORF4. In the case of the REI permissive uORF1, we found that only one of the tested Tma-hyperdependent codons for bulk recycling conferred significantly reduced REI in the *tmaΔΔ* strain (as we would expect for Tma-dependent REI). Two other codons that also conferred reduced REI are Tma-average for bulk recycling. This indicates that in general, REI can proceed on uORF1 in a Tma-independent way (possibly thanks to its RPEs, which might cause the Tma factors compete with eIF3 for ribosome binding). Converting the uORFs into start-stop elements showed no evidence of Tma-dependency, unlike their mammalian counterparts. Overall, we demonstrated that i.) Tma20, Tma22 and Tma64 have roles in suppression of REI which are not strictly correlated with Tma-dependency for bulk recycling, and ii.) their influence on REI after short uORFs differs from the function of their mammalian counterparts.

Stem-loop-induced ribosome queuing in the uORF2/*ATF4* overlap fine-tunes stress-induced human ATF4 translational control

While translational control of *ATF4* is heralded as one of the two main examples of the delayed REI mechanism of translational control, evidence suggests that there are more layers to it. Due to the limitations of previous models used for existing *ATF4* studies (namely focus on murine *ATF4* and the use of luciferase reporter assays), in Smirnova et al. 2024 we designed a new reporter system which is based on human *ATF4* sequence and utilizes the JessTM Simple Western quantitative Western Blot system to quantify ATF4 protein levels. This system also avoids normalization through mRNA levels, which are known to change in stress (Dey et al. 2010), or *Renilla* activity, which may change upon translational shutdown (due to general shutdown of protein production). This allowed us to investigate the *cis*-acting elements influencing ATF4 translation in detail.

Our results testing each uORF (start-stop/uORF0, uORF1, uORF2) confirmed the aspects of the original delayed REI model and showed that the start-stop acts as a repressive element. Interestingly, we also found that a uORF2-only construct is Tg stress-inducible – something that does not agree with the delayed REI model, which posits that uORF2 (which overlaps with the *ATF4* CDS) is one of the main roadblocks to ATF4 expression. Thus, initiation at uORF2, especially in conditions with low levels of TC, should ensure there is little to no translation of the main *ATF4* CDS. Starck et al. (2016) reported unexpectedly high uORF2 translation also under stress conditions, which was corroborated by ribo-seq studies (Andreev et al. 2015, Sidrauski et al. 2015). The posited explanation for the original model discrepancies was that ATF4 expression is a combination of REI and leaky scanning past these uORFs. Our reporter system confirmed these high levels of uORF2 expression.

This led us to a detailed bioinformatics examination of potential elements in the *ATF4* 5' UTR and the uORF2/*ATF4* CDS overlap for sequences and structures that would add additional layers to known regulatory mechanisms. We predicted a stem loop SL3 in the uORF2/*ATF4* overlap, four potential methylation sites with known methylation motifs, and potential alternative AUGs near the beginning of *ATF4* CDS which could serve as alternative initiation sites. After a screening of elements that had an observable effect, we picked a few for further analysis. Loosening of the SL3 stem loop resulted in both increased ATF4 expression and inducibility – suggesting it might act as a physical barrier for scanning or translating ribosomes. Loosening SL3 along with mutation of uORF2 start results in a robust increase in Tg-inducibility, suggesting these two elements act in concert to inhibit ATF4 translation. We also discovered a potential near-cognate CUG start codon 20 nt upstream from SL3, in frame with the main CDS. This position would be ideal for initiating ribosomes slowed by the SL3, since initiating 48S PICs cover ~12 nt from the mRNA entry site to the P site and ~15-25 nt downstream of the P site to the mRNA exit site (Wagner et al. 2020, 2022). Mutating this CUG to CUA reduced ATF4 expression by ~20%, offering evidence to the possibility of this CUG being an alternative initiation site for ribosomes slowed by SL3. This was further corroborated by evidence that CUG mutation and SL3 mutation at once nullify each other's effects (since CUG with its weak Kozak context can hardly serve as an initiation site without SL3 as an inhibitory element). These results bring us evidence of intriguing new elements proving significant in ATF4 regulation. In fact, this SL3-CUG interplay could also trigger i.) ribosome queuing, ii.) uORF2-to-ATF4 frameshifting. Inhibition provided by SL3 could help form a queue of 80S ribosomes translating uORF2. This queue would reduce translation rate of uORF2 and help eliminate uORF2 leaky scanning, leading to tighter repression of ATF translation under non-stress condition. Consequently, the 48S PIC which would be at the most 5' end of this queue could potentially initiate at uORF2, explaining its high translation under non-stress conditions.

A system like this is not unknown in the world of translational control – one such system is the AZIN1 system (Ivanov et al. 2018). AZIN1 is an antizyme inhibitor 1 – regulator of cellular polyamine synthesis. The 5' UTR of *AZIN1* mRNA contains a long inhibitory upstream conserved coding region with a Pro-Pro-Trp (PPW) motif which pauses ribosomes and is polyamine dependent. At low polyamine levels, the scanning ribosomes mostly skip this coding

region and translate AZIN1 instead. High polyamine levels interfere with eIF5A function and cause stalling at PPW in the region. Scanning ribosomes subsequently form a queue upstream of the PPW motif, and stimulate initiation at the coding region, further reinforcing the stall to suppress AZIN1 synthesis. This mechanism thus works to regulate polyamine metabolism based on polyamine levels in the cell. Another stalling-based regulatory mechanism can be found on the human cytomegaloviral *UL4* mRNA uORF2, where the uORF contains a terminal diproline motif which causes ribosome stalling. This seemed to result in subsequent dissociation of scanning 40S when encountering the stalled queue (Bottorff et al. 2022). Additionally, several human mRNAs seem to contain elements that cause stalling which enables the uORFs to ‘buffer’ the main CDS translation, such as *GADD34* (Young et al. 2015) and *CHOP* (Young et al. 2016). Combined with the fact that stem loops are known to reduce the rate of translation (Tuck et al. 2020), this kind of mechanism is not out of the realm of possibility for *ATF4*.

To prove this, we extended ATF4 CDS by 6x Myc tag after AUG1 (before SL3). Extended spacing between AUG and SL3 should thus mitigate the queueing effects of SL3 in an otherwise WT construct, and have no effect in case of SL3 mutation, which is what we saw. Additionally, during these experiments we also saw a shorter ATF4 variant which we contribute to leaky scanning past AUG1 of ATF4. To obtain additional evidence of ribosome queueing, we performed a cross-linking assay. We crosslinked ribosomes with mRNA, applied RNase I treatment and performed qRT-PCR on isolated RNA fragments. Here, we found an enrichment of the hypothesized queueing region over control regions. Elimination of SL3 reduced this enrichment. We combined this with analysis of published disome-seq data and ribo-seq libraries, which were consistent with queues of ribosomal species accumulated between uORF2 AUG and SL3.

Furthermore, we confirmed that one potential mRNA modification site (located within uORF2) is modified, and its mutation increases ATF4 production. Another potential modification site was not found to be modified, but its mutation to another nucleotide could further stabilize SL3, since this mutation even further reduced basal expression of ATF4. This queueing system seems to be fine-tuned for complex function, since our data show it is sensitive on spacing between SL3 and upstream start codons. An increase of this spacing relaxes ATF4 control. Additionally, a study came out after the publication of our paper that the *ATF4* mRNA is a target for the METTL16 methylase, although it is unknown which residues are modified. METTL16 knockdown reduced ATF4 expression (Zhao et al. 2025). However, this knockdown would possibly affect several target modification sites, which makes it impossible to determine what the contribution of each site would be.

ATF4 is a transcription factor that functions as a master regulator of stress response, and its deregulation has the potential to wreak havoc on the cell. ATF4 deregulation may result in deregulation of many cellular processes, including immune response (reviewed in Chen et al. 2021), aging (reviewed in Wang and Zhang 2025), neurodegeneration (reviewed in Zhang et al. 2024) or cancer (reviewed in Wu and Liang 2024). The high degree of involvement of ATF4 in these many processes might indicate a need for tight, complex and responsive translational control, especially since transcription factor function may be highly dosage sensitive (Naqvi et

al. 2023). Our discovery of new layers to this regulation may prove important in future clinical uses, including development of new therapeutic approaches to disease states.

Screening for and examination of new factors influencing REI and recycling

Another aim of this work was to search for new REI and ribosome recycling factors. Based on the preliminary pre-screening analyses described in results, I picked three candidate proteins for detailed analysis – Rai1, Ssz1 and Zuo1. The Ssz1:Zuo1 heterodimer forming the RAC complex already has an established role in translational control – it regulates processes such as co-translational folding (Gautschi et al. 2002, Huang et al. 2005, Zhang et al. 2020, Kišonaite et al. 2023), -1 frameshifting (Muldoon-Jacobs and Dinman 2006) and SC-RT (Rakwalska and Rospert 2004). The mammalian RAC complex (Hsp70L1 is a homologue of Ssz1 and MPP11 of Zuo1) seems to have the same effect on SC-RT and, importantly, mRAC complements the yeast RAC deletion (*ssz1Δ zuo1Δ*) (Jaiswal et al. 2011).

The higher uORF4-only reporter activity that I observed with the *ssz1Δ* and *zuo1Δ* single deletions strains (Fig. 20C) versus the WT could suggest a recycling defect (as we showed in Jendruchova et al. 2024). Alternatively, these two factors could be somehow preventing REI, however, I consider this to be unlikely, since the uORF1 activity in the corresponding deletion strains was not significantly increased. Nonetheless, this possibility cannot be ruled out completely due to the following reason: uORF1 is surrounded by RPEs that promote effective REI due to their interaction with eIF3 (Munzarova et al. 2011). Therefore, the RPEs could override any effect on REI after uORF1 translation that Ssz1 and Zuo1 may have, similar to what we proposed with the Tma factors (Jendruchova et al. 2024). The effect of -1 or +1 frameshifting also cannot explain my data with the uORF4-only reporter, because the main *GCN4* CDS is in +2 frame compared to uORF4. Finally, while the deletion of *SSZ1* and *ZUO1* increases SC-RT (Rakwalska and Rospert 2004), this is also unlikely to be the reason for the increased activity after uORF4 translation, since heightened SC-RT decreases the efficiency of REI past uORF4 (Gunisova et al. 2016). Therefore, I favor the recycling defect as my working hypothesis at the current stage of our knowledge.

Rai1 has been implicated in mRNA degradation (Das et al. 2003), transcription termination (Kim et al. 2004, El Hage et al. 2008, Park et al. 2015), mRNA surveillance and decapping (Ya-Fan Wang et al. 2015, Zhang et al. 2020) and rRNA processing (Fang et al. 2005, Sydorsky et al. 2003). However, these functions are largely located to the nucleus (often in cooperation with its nuclear binding partner Rat1) or the localization is currently not known. Note, that upon *rat1* loss-of-function mutation, Rai1 predominantly re-localizes to the cytoplasm (Sydorsky et al. 2003), where its roles have not been explored so far. It is only known that, unlike other decapping and decay proteins (Sheth and Parker 2007), Rai1 does not localize to yeast P bodies (Xing et al. 2020). Deletion of *RAI1* manifested ~50% decrease in the uORF1-only reporter activity when compared to the WT (Fig. 20D), suggesting that one of its cytoplasmic roles could be in promoting REI. Interestingly, the extent of this effect is similar to the effect displayed by mutations of some subunits of the eIF3 complex. The *g/tif35-KLF* mutation of the 3g subunit reduced the uORF1-only reporter activity to 52% (Cuchalova et al. 2010), and the 3a subunit mutations *a/tif32-Box6*, *-Box8*, *-Box17*, *-Box6+17*, *-Box8+17* and *-Δ8* reduced it to 36%, 53%, 47%, 34%, 30% and 29%, respectively (Munzarova et al. 2011). It is important to emphasize

that while it is likely that the *rai1Δ* deletion affects RNA decay or decapping (Doamekpor et al. 2022), which could influence the reporter mRNA levels and in turn its activity, this effect, if present, should be completely nullified due to the normalization step to the uORFless construct (see Methods and Materials) that I routinely performed.

Considering the functional interplay between these factors, no apparent difference in growth rate was observed between the single and double deletions of *SSZ1* and *ZUO1* genes. Similarly, the *rai1Δ ssz1Δ* double deletion displayed a growth defect comparable to *rai1Δ* single deletion (Fig. 20A). This is in line with *rai1Δ* deletion “dominating” over *ssz1Δ* deletion in reporter assays of the double deletion strain (Fig. 20D+E). The *GCN4-LacZ*-based reporter assays then revealed an interesting phenomenon. While the single *ssz1Δ* and *zuo1Δ* deletions manifested significantly higher uORF4 reporter activity than the WT strain, their combination virtually suppressed this effect (Fig. 20C). This could be explained by differences in the mode of action between these two factors; they do not seem to directly co-operate with each other during recycling, even though they were found to exist in a heterodimer. The fact that there is no obvious redundancy - if there were, it would not lead to a change in the phenotype between single and double deletions - opens a possibility that in the case of recycling, they may actually act outside of the heterodimer. The easiest explanation is that both proteins carry out a different, relatively independent steps of the recycling job. These steps do not necessarily depend on these factors, their action most probably only moderately increases the efficiency of these steps. Importantly, these steps do not seem to be in any sequential relationship, i.e. they can be carried out independently of each other. However, for each of them to proceed further, the clearance of the rate-increasing executor of the other steps is required. For example, their binding to the post-termination ribosome can be mutually exclusive and, therefore, once the Ssz1 job gets done, Zuo1 binds, kicks out Ssz1 (the processivity task) and takes care of its own step (the recycling task), and *vice versa*. Deleting both factors may modestly reduce the overall recycling rate, however, at the same time it removes the processivity block. Only future experiments will show what the nature of their indirect co-operation during ribosomal recycling.

In case of the *rai1Δ ssz1Δ* double deletion strain that significantly reduced the uORF1 reporter activity, akin to the single *rai1Δ* deletion (contrary to the *ssz1Δ* uORF4-specific phenotype), and increased uORF4 activity only slightly and with much lower significance compared to single *ssz1Δ* deletion (Fig. 20D+E). It seems obvious that *rai1Δ* phenotype prevails over *ssz1Δ* phenotype, and “dampens” the *ssz1Δ* effect. The reasons for this interplay are unknown at present.

Polysome profiles confirmed earlier results (Xue et al. 2000) that the deletion of *RAI1* generates ‘halfmers’ (Fig. 22B), which the authors attributed to defective 60S biogenesis due to an rRNA processing defect. A single deletion of *SSZ1* and *ZUO1* modestly reduced the amount of polysomes and a slightly increased 40S peak was observed in both deletion strains (Fig. 22C and D). In analogy to the phenotype seen in *rai1Δ* deletion strain, it might result from the 60S biogenesis defect, however, of a different kind because no halfmers were observed. Nonetheless, Zuo1 (and also Ssz1 to some extent) was shown to promote 60S biogenesis (Albanese et al. 2010). Notably, the *rai1Δ ssz1Δ* strain does not exhibit halfmers, compared to

railΔ. This somewhat contrasts with the earlier observation that Rai1 and Ssz1 promote 25S rRNA processing (Albanese et al. 2010, Weidong et al. 2024).

Using FLAG-tagged proteins of my interest, I found that Rai1-FLAG mostly associated with free 40S and 60S subunits, weakly 80S ribosomes, but not translating polysomes. This is the first time when a direct Rai1 binding to ribosomes has been demonstrated (Fig. 23A). This pattern of Rai1 association would be consistent with a factor binding to free or scanning 40S subunits, which may stay bound even to early elongating ribosomes. This mechanism would be reminiscent of yeast eIF3, which stays bound to translating ribosomes in early elongation and mutations in 3a/TIF32 and 3g/TIF35 sharply reduce the rate of REI at uORF1 (Mohammad et al. 2017, Munzarova et al. 2011). Ssz1-FLAG associated mostly with free 40S and 60S subunits, but also with 80S monosomes and translating polysomes (Fig. 23B). Interestingly, this is in accordance with published results examining Ssz1 and Zuo1 association with ribosomes by Eisenman and Craig, 2004. While Ssz1 associates with both free and scanning subunits, monosomes and translating polysomes, Eisenman and Craig (2004) found that Zuo1 associates mostly with translating polysomes and not free 40S subunits. It is pertinent to note that they used an *SSZ1* overexpression strain, whereas I used a single copy expression plasmid. The fraction-association of Zuo1 has not been tested so far. If this difference in association would be confirmed in our experimental setup, it might be another indication that Ssz1 and Zuo1 might act outside the heterodimer in the recycling process.

Using GST pull down experiments, I confirmed a direct physical interaction between Rai1 and Ssz1 (Fig. 24) (first found by Gavin et al. 2006). Subsequent large-scale pull-down using FLAG-tagged Rai1 and Ssz1 as baits followed MS analysis (Fig. 25) identified Dbp5 as a major Rai1 hit. Dbp5 has a role in many facets of RNA metabolism, translation termination and stop-codon readthrough (reviewed by Rajan and Montpetit 2020, Querl and Krebber 2023). Ssz1 was found to interact with Asc1 (yeast orthologue of mammalian RACK1) and many ribosomal proteins, of the large and small subunit, the most intriguing of which was RPP0. It is a component of the P stalk, which binds to RPP2 (Grela et al. 2012). The stalk is part of the GTPase-associated center, which is composed of conserved rRNA sarcin-ricin loop, and the stalk itself. It is a conserved ribosomal element, where stimulation of GTP hydrolysis on GTPases like elongation factors eEF1 and eEF2 takes place (Liljas and Sanyal 2018, Wawiora et al. 2017, Maracci and Rodnina 2016). Interestingly, Rai1 was found to interact with RPP2B, a factor that is also a constituent of the ribosomal P stalk. Collectively, these findings consistently revolving around the P stalk might indicate that there might be some functional connection worth of future investigation. On the other hand, we found several adjacent ribosomal proteins in our MS analysis and based on available structural data (Leiding et al. 2012, Chen et al. 2022, Ben-Shem et al. 2011), it is possible that the stalk proteins were pulled down in the FLAG-Ssz1 pulldown by their indirect association via nearby LSU proteins representing the binding sites of the RAC complex.

Future plans

- detailed analysis of the Zuo1 protein effects to complete this part of our study for all three proteins of interest

- to examine whether a triple deletion strain *ssz1Δ zuo1Δ tma20Δ* shows a more pronounced recycling defect than the *ssz1Δ zuo1Δ* strain; the question is whether the Tma proteins compensate for loss of the Ssz1/Zuo1 heterodimer
- to test the impact of our deletions on the overall translational control of *GCN4*
- to follow up on our MS analysis; binding of Rai1 to Dbp5 and the ribosomal stalk proteins
- to carry out Translational Complex Profiling (TCP-seq; Archer et al. 2016, Shirokikh et al. 2017). This method monitors positions of any type of ribosome-RNA complex transcriptome-wide. Fast, covalent fixation of translation complexes is followed by RNase footprinting of translation intermediates and their separation into complexes involving the full 80S ribosome or only the 40S subunit. The footprints are then deep sequenced separately, generating distribution profiles during elongation, as well as initiation and termination (Shirokikh et al. 2017). This helps us investigate the dynamics of the initiation and recycling phases. Using this method, we could determine the importance of our factors of interest for REI/ribosome recycling on a transcriptome-wide scale level (for example, this method was used to study DENR/MCTS1 and Tma20/Tma22 recycling factors (Bohlen et al. 2020, Young et al. 2021)).

CONCLUSION

This thesis provides new insights into mechanisms involved in the post-termination decision making process in both yeast and mammalian cells.

We discovered that the yeast 40S recycling factors Tma20/Tma22 and Tma64 (homologues of human MCTS1/DENR and eIF2D) suppress REI after short uORFs optimized for inhibiting REI with various penultimate codons, but this was not directly correlated with Tma-dependence of these penultimate codons for bulk 40S recycling. Moreover, the Tma factors have no effects on REI at short uORFs optimized for promoting REI, even after exchanging the penultimate codon for Tma-hyperdependent codons for bulk recycling. Additionally, we found that start-stop elements (uORFs comprised only of two codons – AUG-stop) are also not Tma-dependent for REI, unlike in mammalian cells, where they are dependent on DENR/MCTS1. This gave us new insights into mechanisms governing REI and recycling after short uORFs in yeast and demonstrated that effects of the Tma proteins vary depending on the uORF and penultimate codon but are not chiefly dictated by Tma-dependence of the codon in bulk recycling, unlike in mammalian cells. The found differences between yeast and human 40S recycling factors helped us get a clearer understanding of the differences in mechanisms of REI and 40S recycling between these two models.

We found new *cis*-acting elements of the *ATF4* mRNA, which further contribute to the intricacies of its translational control. ATF4 stress induction was first thought to be due to delayed REI, however evidence has been mounting that other regulatory routes influence the mechanism. We identified a conserved stem-loop SL3 in the overlapping uORF2/ATF4 region, preceded by a near-cognate CUG, which may induce ribosome queuing in the area – this would explain high uORF2 translation also under stress, which was previously thought incompatible with the delayed REI model. We also found two conserved potential RNA modification sites, one located in the SL3 area, that perform antagonistic roles. One of these sites has been shown to be modified experimentally by us. We also demonstrated substantial leaky scanning of the ATF4 translation start site. All of these newfound elements contribute to the understanding of ATF4 translational control and show it is more complex than originally described.

Finally, I conducted a screening of potential factors influencing REI or recycling, followed by analysis of the selected factors – Ssz1, Zuo1 and Rai1. I have examined genetic and physical interactions between these factors and translational fitness of cells lacking these factors. I have also demonstrated – for the first time – that Rai1 (which has been so far regarded only as an RNA degradation/quality control factor and ribosome biogenesis factor) associates with ribosomes. My pull down-MS experiments identified new interaction partners of these factors, which are the subject of my current work.

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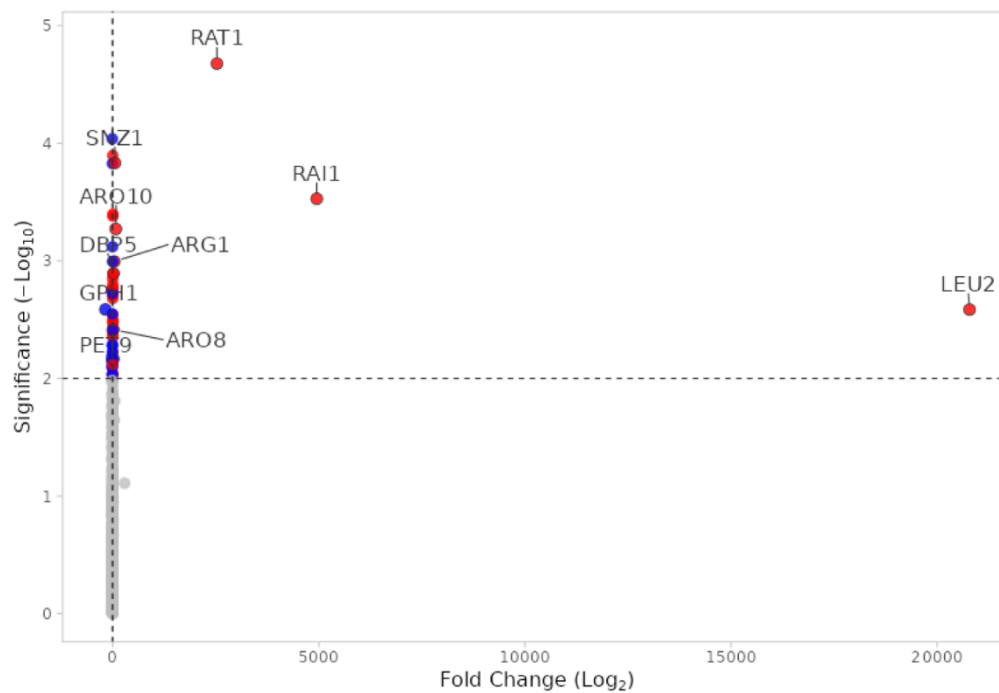
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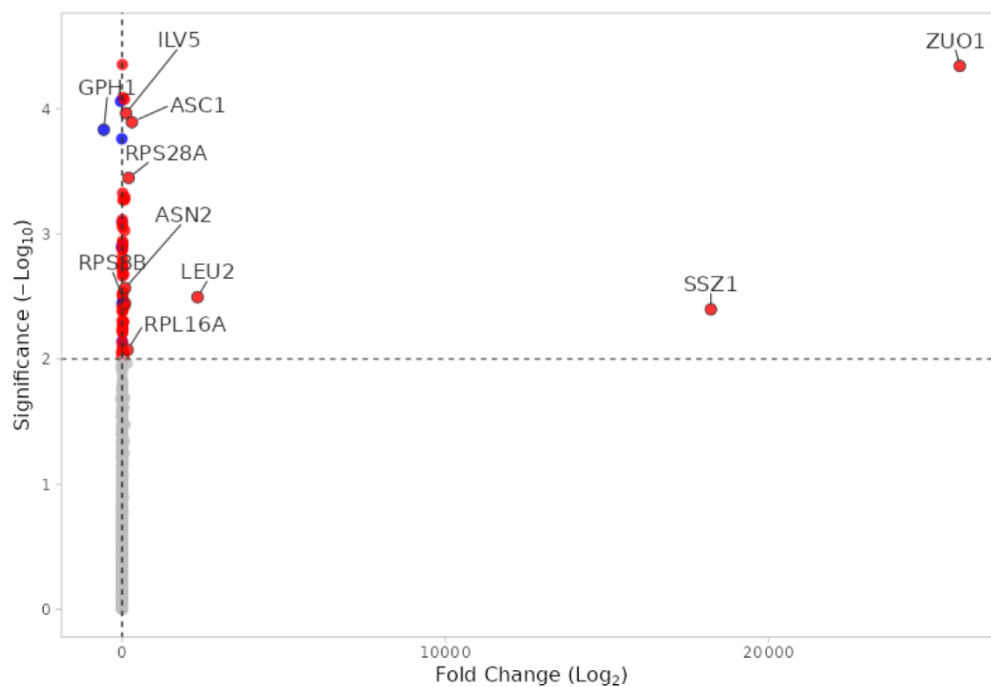
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SUPPLEMENTARY FIGURES

A



B



Supplementary Figure S1: Unadjusted volcano plot of MS analysis of pulled down complexes. **A.** RAI1-FLAG complexes were pulled down and sent for MS analysis. **B.** SSZ1-FLAG complexes were pulled down and sent for MS analysis.

SUPPLEMENTARY TABLES

SUPPLEMENTARY TABLE S1. Synthesized DNA fragments for cloning

Fragment:	Sequence:
1 (pKJ64)	GCTGCTCGTCGCTCGTCAAACTGCAGGGGAAGTTGTAATGGAAT GAACAGAAGAAACAAGGAGAAGATTGCCAGAGGTAAGGGAAGAA GAGGAAGAATGATGACAAGAGAGAATATGGACATGGGAGAAAGG TAGGCTTTGTAAGCATGGTTTACCTTTTAAACGCCCAAAGTTCAATT TAACTTAGAAATATTTATCTATATGTTTCATATATACATCTATCAATA TCACATAAAATTGCCTATCATTCTTATTTCGTTTCCTATTTGTTTCTC GAGCCATTCATAACTCATTCCGCGTAATGGGAGCGGTAAAAGATGT GCAAAATAAGTAACTATTTACGAAGTATTCATATTTTAGCTCTTTTT CTTCGTCGAATGTGATGGTGAAATTTTTTCCAAGAGATGATGAGGT CGGATAATATATGAAGTACATGGTAGTCAGCTCGCCTATACAAGAG GTTTTAAGATTACATAAATATATTGAGATCTCTACTACCACAATCA CAGTTAAAATTACAAATAAGATGTCCTCTCCAGTGATTGGTATCAC CTTTGGTAACACCTCTTCTTCTATTGCCTACATCAACCCAAAGAACG ATGTTGATGTCATTGCCAACCCAGATGGTGAGCGTGCCATTCCATC CGCTTTATCCTATGTCGGTGAAGATGAATACCACGGTGGTCAAGCT TTGCAACAATTAATCAGAAATCCTAAGAATACTATCATTAAGTTCC GTGACTTCATTGGTTTGCCATTTGACAAGTGTGATGTCAGCAAGTG CGCTAACGGTGCCCCAGCTGTCGAAGTTGATGGCAAAGTTGGATTT GTTATTTCAAGAGGCGAAGGTAAGGAAGAAAAAACTTACTGTAGAT GAAGTGGTCTCCAGACATTTAAACAGATTAAAGTTAGCCGCGGAA GATTACATCGGTTCTGCCGTAAAGGAAGCTGTATTGACAGTTCCAA CAAAGTTTCAAGTGAAGAACAAGAGACTGCACTAAAGGCTTCTGCCG CCAAAATTGGTCTGCAAATTGTTCAATTCATCAATGAACCTTCTGCT GCTTTATTAGCCACGCTGAACAATTCCCATTTGAAAAAGATGTTA ACGTTGTTGTTGCTGACTTCGGTGGTATTAGATCTGACGCTGCTGTC ATTGCCGTTTCGTAACGGTATTTTCACTATTTTGGCCACTGCTCATGA CCTCAGCTTAGGTGGTGACAATTTGGATACTGAATTAGTCGAATAT TTTGCTAGTGAGTTCCAAAAGAAGTATCAAGCCAATCCAAGAAAG AACGCTAGATCCTTGGCCAAGTTAAAGGCTAACTCTTCAATTACCA AGAAGACTTTGTCCAACGCAACTTCTGCCACTATTTCCATCGATTCC TTAGCTGATGGTTTCGACTATCACGCTTCTATCAACAGAATGAGGT ACGAATTGGTAGCTAACAAGGTCTTCGCCCAATTTTCCTCTTTCGTT GATTCTGTCATTGCCAAGGCTGAATTAGACCCATTGGACATCGATG CTGTTCTTTTACTGGTGGTGTATCATTTACTCCAAAATTAACCACT AACTTGGAATACACTTTACCAGAATCAGTCGAAATTCTTGGTCCAC AGAACAAGAACGCTTCTAACAATCCAAACGAATTAGCTGCATCCG GTGCCGCATTACAAGCAAGATTGATTAGCGATTACGATGCTGACGA ATTGGCTGAAGCTTTACAACCAGTTATCGTCAATACTCCACATTTA AAGAAGCCTATTGGTTTGATTGGTGCTAAGGGCGAATTCCACCCAG

	TATTGTTGGCTGAAACTTCGTTCCCTGTACAAAAGAAATTGACTTT GAAACAAGCCAAGGGTGATTTCTTGATTGGTGTTTACGAAGGTGAC CATCACATCGAGGAAAAGACTTTGGAGCCAATTCCAAAAGAAGAA AATGCTGAAGAGGACGATGAAAGTGAATGGTCCGACGATGAACCT GAAGTCGTCAGAGAAAACTATACACTTTGGGTACCAAGTTGATG GAATTGGGAATTAAAAACGCTAACGGTGTTGAAATTATCTTTAACA TTAACAAAGACGGTGCTTTAAGAGTCACCGCTAGAGATTTGAAAAC TGGTAATGCTGTAAAGGGTGAATTAGGTGGTGGTGGTGGTGGTGAC TACAAAGACCATGACGGTGATTATAAAGATCATGACATCGACTAC AAGGATGACGATGACAAGTAGAGCGCTCTAAGAAAAACGGTATGT ATACGTATATAGAAAAATACATACATGATGGAACCTATACAGCCCTT GAAAAAAGAACATATAAAATTAAGTCAAAAAATGCTATAGCAAGG TTATTATGGTTAGGTTTGTGACTTTCTGTTGAGTCACGTTCTATTCA AATTCATATTTTCATGTTTCCGATTTCTTTTTTTTATAGATTGGCGGCC GCTAACGGTTTCTCGAGAAAGGCTTATCGTGCCATTCAGCTTAATA CAATATATTAAAAAACAAGATACTTTATACTCACCTTTGTATGT TACGATCGATAGGAGAAATCGATAATATACGTATACGATAAAGTCT GTACAATATGACTGCACCACACAGAAGTACTATTCATATTCTTGGG TTGGGTGCCATGGGTACCGTTTGGCAGTTGACCTTTTAAGATTAC CAATGCCCTAGTTGTGCCCTTATTTAGATCACAAGAAAGACTTGCA CAATTTTCAGAAGAGGATCCACTGACTGCTAGCTG
2 (pKJ65)	ACTGACTGACTGACTGACTGAAGCTTTAATTCCTTTTGGGCCAGAT TAACCCATTTAGCAACTTGCTCTTTATCACCATTATCGAAGATGGC GGCGAATTTGCTAGTTATCTTGTTGAAGACATCTTCAGTAGCATCA TCGAGCTTGGCAGCAGGTGCTTTCTTATCGTCCACCAACTTAATAG CTATGGAGTTAGGAGCTAACGCATTCACAATACGAGCAGCAATTA GTTTCAGCATAAGCCACAATTGGGGCTTTTCCATTAATAGTCAAGGT AGATGGCATCTTGATCCCTTCTATGCTTTCCTTAACCTTTGAAAATA GTTTCGTCATTCTTATGGAAGTTATCTTCCATTTTTTTTCACTTTATCT TGCTTTTAAACGAAGAAGAAAAAAGAGTAAGCATTCGGGTAAATT GAAAAATTTACTTCGAGAATATTAGGAACAGATGAGGTCTGGTAAT GTTACTAATACTTCTGTAATATGGTGAAAGAATAGCGAAATATTAG ACCAACATAGTGTATCCCAATGGGTGTTAGTGCAAATTTGTTTCGTA AAGCAAAGAGGGTCGACAACAGCTCTGAAGCAGCCAAAGGAAATA GGATTCTATTCAAGAACTAAGGATGAAGAATATTTAATTAGCGATG ATACGAATTTGAATTACTACTATCTTCCAGACGCTGAATTGGACAG GAAGTTGGATCTTTCATCAGGATTTTCAGAAATTTAAGGATTATTAC AAAGATTTTGAAGACAGGTGTTCCCTGCGTGGGCTATTAGAAACCA TAGAAAGCTCTGAAAGACATAAAGGAAAAAAAATTAATGCTGATA TAATTACTTTTAGAGGAATAGCACGGAAATTAATTTTCATGTGCATT TGACTCTCCATCTTTTAATACAGTTGATCTTCGTATCGTTTCATTCA ACGGCCAACTTTTTATCAAGGAAGTGCCTGAAGCTGTCAATGCAGC TAAGGCTTCCTCTGCAACTGAAGCAGGGCGTAATATAAATCAAGAT

	CTGAATGTATTTACAGGGTACAAGTTTGAAACTTTGGCTACTCTTTC CAATCCGTTGCAGTATACACCCCGTGAAGTGATAGAAAAAGAAC TAAAAGGATTGTATCGCATGGAGATGAATACATTAGTGTTGTGAGA ACTGGTGTAGGTAAGTCAAGTTAATTCTTGGTGCTGAGGTGGATT GTATATTTGACTTTAAAGAAAACGGTAGAGACAATTTAAACATTA TGCCGAATTGAAATGTACGCAGCAAGTTGCGAACATATCGGATAC GCATAAGTTTGAGCGTAAACTGTTCCGGACCTGGCTGCAATGCTTC TTAGTTGGGATCCCAAGGATCATATATGGTTTTAAAGACGACCATT ATGTATTAAAACTGTGGAAGAGTTTTCAACGGAGGAAGTACCTGT TTTATTAAAGAACAATAATCCACAAGTTGGAAGTGCATGTTTAGAA GCCATTAAATGGTATGGTCTCCTTACTGAGTGGCTTTTGAAAATGA TACCAAGAGATGAGGATCCTCATTCTCAAATAAGAGCATTTAAGTT GGTTTTTCGAAAATAATCACCTAAGATTATCCGAAATTGAAGAAAGT GATGAGGAATATTCAGGTTTGATTGATGGAGAACATATATTATCGA ACGGGTTTAAGGAGTGGAGAAAATCTTTGAAAGGTGGTGGTGGTG GTGGTGACTACAAAGACCATGACGGTGATTATAAAGATCATGACA TCGACTACAAGGATGACGATGACAAGTAACAATATTCCTTTATGTC TTTCCTGCGCATATCCTCATCGACGTATGGATCTAAATAATATAAA TGTGCAGATTAGATTAAAGTTATATACACTATGCATGGCGAACCGAT TTTGTATCTTTATTATTGAGATAAACCCTGTATGATCCGAAACTCGT ATTGGTAACAAATATTATACCTATTATGCTAATTAAAATGAAAAGG AAAGATCGTATACTGAACGCTTCAAAAAAGCATTATTACCTCCG CCAGAGTACGAGCCCATGCTTTAGCCCATTTGGTAATCGGAGCCAGA CACTATTTCTTGTTTCATACATTTTGACCATTTCATTGCAACGTACCT CCAATGCTGGAACCCTCATATGAGGTTTCGCACTGGTTTTCAAATA CCTTGATTTGCAAGTTGCAATTTTATCTAATTCTTCCCTCACGTTAT GCTCCAGTTTTAAGTTAACATCGTTTCTTATCATCAAAATGAATTTA ATAGCAAGAGACAGTCCTAGAATTCAGTACTGACTGACTGACTG
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Supplementary Table S1: sequences of synthesized DNA fragments for cloning

PUBLICATIONS

1. Jendruchová K, Gaikwad S, Poncová K, Gunišová S, Valášek LS, Hinnebusch AG. 2024. Differential effects of 40S ribosome recycling factors on reinitiation at regulatory uORFs in GCN4 mRNA are not dictated by their roles in bulk 40S recycling. *Commun Biol* **7**: 1083.
2. Smirnova AM, Hronová V, Mohammad MP, Herrmannová A, Gunišová S, Petráčková D, Halada P, Coufal Š, Świrski M, Rendleman J, et al. 2024. Stem-loop-induced ribosome queuing in the uORF2/ATF4 overlap fine-tunes stress-induced human ATF4 translational control. *Cell reports* **43**: 113976.

<https://doi.org/10.1038/s42003-024-06761-x>

Differential effects of 40S ribosome recycling factors on reinitiation at regulatory uORFs in *GCN4* mRNA are not dictated by their roles in bulk 40S recycling



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Recycling of 40S ribosomal subunits following translation termination, entailing release of deacylated tRNA and dissociation of the empty 40S from mRNA, involves yeast Tma20/Tma22 heterodimer and Tma64, counterparts of mammalian MCTS1/DENR and eIF2D. MCTS1/DENR enhance reinitiation (REI) at short upstream open reading frames (uORFs) harboring penultimate codons that confer heightened dependence on these factors in bulk 40S recycling. Tma factors, by contrast, inhibited REI at particular uORFs in extracts; however, their roles at regulatory uORFs in vivo were unknown. We examined effects of eliminating Tma proteins on REI at regulatory uORFs mediating translational control of *GCN4* optimized for either promoting (uORF1) or preventing (uORF4) REI. We found that the Tma proteins generally impede REI at native uORF4 and its variants equipped with various penultimate codons regardless of their Tma-dependence in bulk recycling. The Tma factors have no effect on REI at native uORF1 and equipping it with Tma-hyperdependent penultimate codons generally did not confer Tma-dependent REI; nor did converting the uORFs to AUG-stop elements. Thus, effects of the Tma proteins vary depending on the REI potential of the uORF and penultimate codon, but unlike in mammals, are not principally dictated by the Tma-dependence of the codon in bulk 40S recycling.

Synthesis of proteins is carried out by ribosomes, molecular machines that assemble from the small (40S) and large (60S) ribosomal subunits. During translation initiation, the 40S subunit acquires the ternary complex (TC) composed of the methionyl initiator Met-tRNA^{Met}, GTP and the eukaryotic Initiation Factor (eIF) 2, forming the 43S pre-initiation complex (PIC). Upon mRNA recruitment, the 48S PIC thus formed scans the mRNA 5' leader region to locate the authentic initiation codon, generally an AUG triplet in a favorable “Kozak” sequence context. Subsequently, subunit joining occurs to produce the 80S initiation complex and elongation commences. Upon stop codon recognition, translation terminates, and the nascent peptide is released. This is followed by ribosomal recycling, i.e.,

splitting of both ribosomal subunits and dissociation of the 40S and the deacylated tRNA that decoded the last sense codon from mRNA, to start the new translational cycle (reviewed in refs. 1–4). In some cases, the recycling step is incomplete, leaving the post-termination 40S mRNA-bound, which allows it to resume traversing downstream and, upon reacquisition of the TC, reinitiate translation of a downstream ORF (reviewed in ref. 5).

Translation termination and ribosome recycling are intrinsically linked. When the stop codon enters the ribosomal A site, it is recognized by a heterodimer composed of eukaryotic Release Factors 1 and 3 (eRF1/eRF3). Stop codon recognition results in GTP hydrolysis on eRF3 followed by its release. eRF1 then catalyzes hydrolysis of the nascent polypeptide chain,

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which generates the post-termination 80S ribosome harboring deacylated tRNA base-paired with the penultimate codon in the P site⁶. The ATP-binding cassette protein Rli1/ABCE1 (yeast/mammals) facilitates the dissociation of the 60S subunit both in vitro in yeast and mammalian reconstituted systems^{7,8} and in vivo in yeast cells⁹, aided by the eIF3-associated factor eIF3^{10,11}. Studies in vitro revealed that after subunit splitting, the deacylated tRNA and mRNA are dissociated from the 40S post-termination complex by the non-canonical initiation factor eIF2D or its functionally and structurally related heterodimer MCTS1/DENR^{12,13}. DENR and MCTS1 bear sequence similarity to the N-terminal and C-terminal regions of eIF2D, respectively. The MCTS1 and the eIF2D N-terminal region harbor DUF1974 and PUA domains, whereas DENR and eIF2D's C-terminal region contain SWIB/MDM2 and SUI/eIF1 domains. eIF2D additionally contains a central winged-helix domain⁴.

Ribosome profiling studies demonstrated that the yeast orthologs of eIF2D, MCTS1 and DENR, known as Tma64, Tma20 and Tma22, respectively¹⁴, function in 40S post-termination complex recycling in vivo^{15,16}. Thus, deleting the corresponding genes led to accumulation of unrecycled 40S subunits at the majority of stop codons, with the largest defect observed in $\Delta tma20\Delta tma64$ (*tma $\Delta\Delta$*) cells. Quantifying the accumulation of unrecycled 40S subunits at all individual stop codons in *tma $\Delta\Delta$* vs. wild-type (WT) cells revealed that certain penultimate codons result in significantly higher accumulation of unrecycled 40S subunits in the mutant cells—a phenomenon dubbed Tma-dependence. Furthermore, comparison of the double deletant to single deletant strains showed that Tma64 is largely dispensable, whereas both subunits of the Tma20/Tma22 heterodimer are required for the majority of 40S recycling events in vivo. A notable consequence of defective 40S recycling in mutants lacking an intact Tma20/Tma22 heterodimer is increased REI downstream of stop codons of main ORFs within the 3' untranslated regions (3' UTRs) of mRNAs. Such non-canonical REI frequently occurs by conventional scanning of the unrecycled post-termination 40S complexes to 3' UTR-situated AUG codons, preferentially in optimum Kozak context^{15,16}. Absence of the Tma20/Tma22 heterodimer also conferred increased canonical REI downstream of the stop codons of upstream open reading frames (uORFs) in the 5' UTRs of reporter mRNAs in yeast cell-free translation extracts¹⁵.

Whereas yeast Tma20/Tma22 were shown to inhibit REI, work on their mammalian orthologs indicated that human DENR conversely promotes REI after translation of certain uORFs, including 1aa-long uORFs comprised of only a start and stop codon, referred to here as “start-stops”, with their ATG start codons in optimum Kozak context^{17,18}. In the absence of DENR/MCTS1, mRNAs containing start-stops exhibit reduced translation of the downstream main ORF. DENR-dependence for REI was also established for certain other short uORFs in a manner generally dictated by the presence of particular penultimate codons that, in 40S ribosome profiling data, were found to confer heightened dependence on DENR for bulk 40S recycling at stop codons throughout the translome¹³. Indeed, the degree of DENR-dependence often inversely correlates with the propensity of the deacylated tRNA decoding the penultimate codon to dissociate spontaneously from post-termination 40S complexes in reconstituted termination/recycling systems^{13,19}. Accordingly, it was proposed that only those uORFs harboring penultimate codons with low rates of spontaneous dissociation of the cognate tRNAs require DENR/MCTS1 to accelerate release of the deacylated tRNA and allow the post-termination 40S to resume traversing downstream and reacquire the TC at the now-empty P site, enabling REI. The DENR-requirement for REI included the regulatory short uORF that stimulates REI on *ATF4* mRNA (uORF1), allowing scanning ribosomes to bypass a more distally positioned uORF (uORF2) that inhibits initiation at the main CDS, both in mammalian cells¹³ and fruit flies²⁰.

To reconcile the apparent discrepancy between yeast Tma factors and their mammalian counterparts, it was suggested¹³ that release of the deacylated tRNA by MCTS1/DENR does not lead to dissociation of post-termination 40S subunits at relatively short uORFs in mammalian cells owing to retention of eIF3, eIF4G1, or eIF4E by 80S ribosomes translating

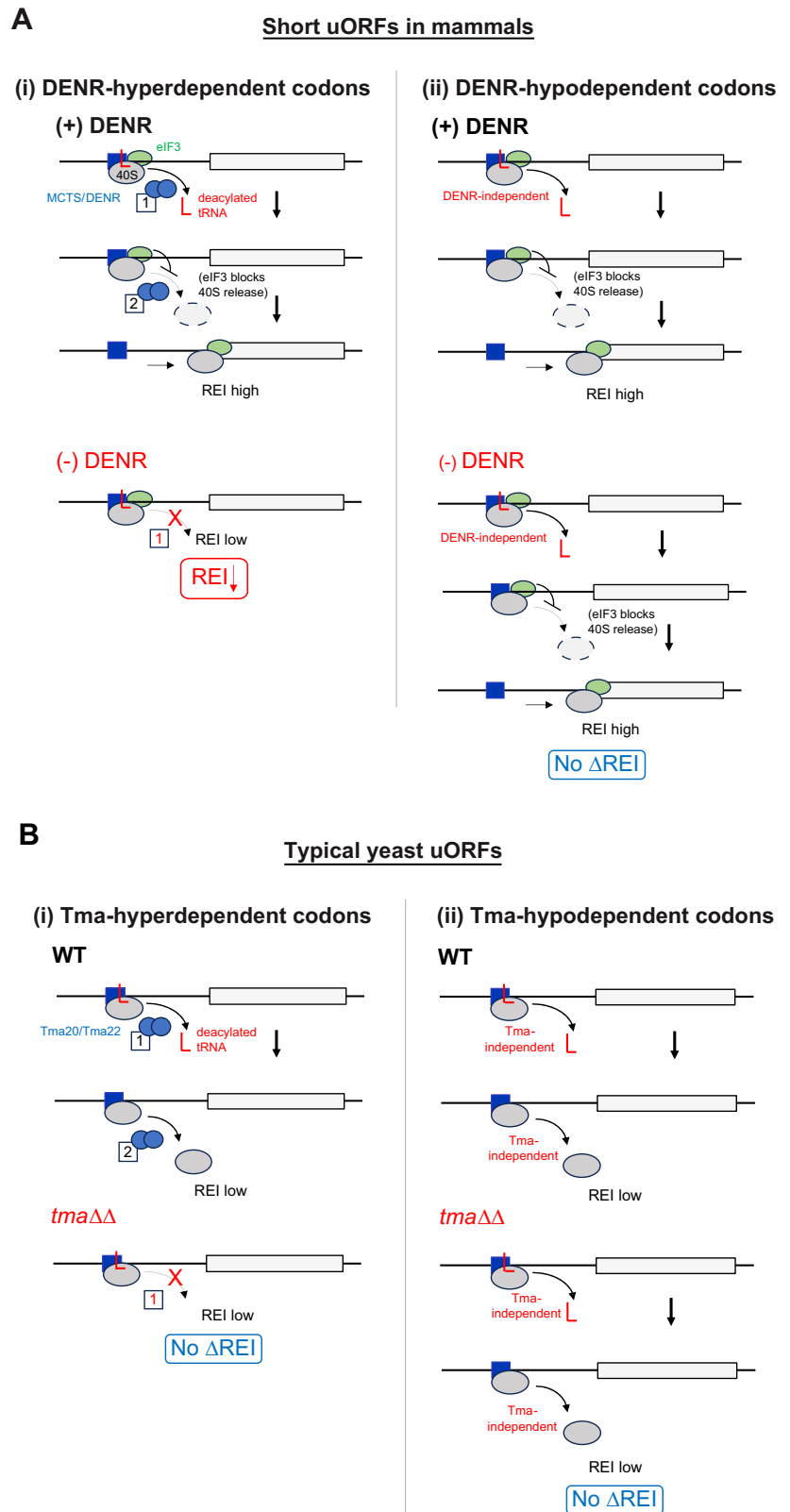
the uORFs and continued occupancy of these factors on the post-termination 40S subunits^{21–23}. These retained eIFs presumably impede the 40S dissociation function of MCTS1/DENR, making the only observable consequence of depleting DENR a reduction in REI at the subset of uORFs requiring DENR for efficient release of the deacylated tRNA, whose continued presence in the P site in the absence of DENR then prevents the 40S post-termination complexes from traversing downstream or recruiting TC to the still-occupied P site. Depleting DENR has no effect on REI at the short uORFs where deacylated tRNA dissociates independently of MCTS1/DENR. These alternative outcomes are depicted schematically in Fig. 1A(i)–(ii). At typical uORFs in yeast, by contrast, release of the deacylated tRNA, whether or not it is highly dependent on the Tma proteins, would lead to subsequent dissociation of post-termination 40S subunits from mRNA and low-level REI following most uORFs in WT cells, because eIFs are generally not retained by 80S ribosomes translating yeast uORFs^{24–27}. As such, a further reduction in REI in cells lacking Tma factors is not expected at typical yeast uORFs even when they harbor penultimate codons highly dependent on the Tma factors to stimulate release of deacylated tRNAs from the post-termination 40S subunits. These expected outcomes for typical uORFs in yeast are shown in Fig. 1B(i)–(ii).

A different outcome would be predicted for a handful of atypical uORFs in yeast, where 40S dissociation is counteracted by initiation factors that remain associated with post-termination 40S complexes. The 5'-proximal AUG-initiated uORF in *GCN4* mRNA, uORF1, is the best characterized such uORF in yeast. It is optimized for eIF3 and eIF4G binding and retention of post-termination 40S subunits by the presence of cis-acting REI promoting elements (RPEs) rendering it highly permissive for REI downstream (Fig. 2)^{5,27,28}. Accordingly, the 40S post-termination complexes at the uORF1 stop codon should not spontaneously dissociate from mRNA on release of the deacylated tRNA, as described above for short uORFs in mammalian cells (Fig. 1A). This in turn should enable us to determine whether eliminating the Tma proteins confers reduced REI at uORF1 variants equipped with penultimate triplets with heightened dependence on Tma factors for release of deacylated tRNA in bulk 40S recycling. At the same time, we should observe little or no effect at uORF1 variants containing penultimate codons that are less dependent on the Tma proteins in bulk 40S recycling, in the manner depicted in Fig. 1A(i)–(ii) for DENR-depletion in mammalian cells.

Importantly, *GCN4* uORF1 is functionally equivalent to uORF1 in *ATF4* mRNA, and *GCN4* and *ATF4* translation are governed by very similar REI mechanisms⁵. However, whereas *ATF4* uORF1 is DENR-dependent for efficient REI^{13,20}, we determined previously that translational control of WT *GCN4* mRNA occurs normally in the *tma20 Δ tma64 Δ* mutant²⁹. This finding implies that the Tma20/Tma22 heterodimer is not required to dissociate deacylated tRNA^{Cys} from the native penultimate UGC codon of WT *GCN4* uORF1. Consistently, UGC was judged to be hypodependent on DENR in mammalian cells¹³ and one of the least Tma-dependent codons for bulk 40S recycling in yeast¹⁶. There is also evidence that the cognate tRNA^{Cys} is weakly associated with mammalian 40S post-termination complexes¹⁹. Here, using uORF1 as a genetic tool, we examined whether substituting the native UGC codon in *GCN4* uORF1 with different codons judged to be Tma-hyperdependent for bulk 40S recycling will reduce REI in *tma $\Delta\Delta$* cells in the manner observed for DENR-dependent short uORFs in DENR-depleted mammalian cells (Fig. 1A(i)–(ii)).

In contrast to REI-permissive uORF1, the fourth AUG-initiated uORF in *GCN4* mRNA (uORF4) does not allow retention of eIFs on post-termination 40S subunits, owing to multiple, surrounding cis-acting sequences, thus making it non-permissive for REI and repressive to *GCN4* translation in WT cells^{26,27,30–33}. We reasoned that uORF4 is a typical example of short uORFs in yeast that do not allow REI owing to efficient dissociation of 40S post-termination complexes regardless of the requirement for Tma factors in releasing tRNA (Fig. 1B(i)–(ii)). uORF4 contains a penultimate CCG proline codon, whose dependence on DENR for recycling and REI in human cells is ambiguous¹³, and was neither unusually dependent nor independent of the Tma proteins for

Fig. 1 | Predicted mechanisms governing MCTS1/DENR mediated recycling and REI at mammalian short uORFs and expected functions of yeast Tma20/Tma22 at typical REI-non-permissive yeast uORFs. MCTS1/DENR or Tma20/Tma22 are depicted as linked blue ovals whose distinct functions in releasing the penultimate deacylated tRNA (red “L”) and subsequently dissociating the empty 40S from the mRNA are labeled with boxes 1 and 2, respectively, and solid or dashed arrows. **A** DENR-mediated recycling at short mammalian uORFs depending on the presence of (i) DENR-hyperdependent penultimate codons requiring the recycling factors for efficient tRNA release, or (ii) DENR-hypodependent codons where tRNA can be released at high levels spontaneously. (i) DENR-hyperdependent uORFs: in WT cells, “(+) DENR”, MCTS1/DENR efficiently releases the penultimate-codon tRNA (function 1) but its second function in 40S dissociation is impeded by eIF3 (green oval) retained on post-termination 40S subunits to enable high-level REI at the main CDS. In cells depleted of DENR, “(-) DENR”, diminished release of the penultimate-codon tRNA lowers REI. (ii) DENR-hypodependent uORFs: efficient release of the tRNA and high-level REI occur both in the presence (upper) or absence (lower) of DENR, yielding no decrease in REI on DENR depletion. **B** Tma-mediated recycling at typical yeast short uORFs depending on the presence of (i) Tma-hyperdependent or (ii) Tma-hypodependent penultimate codons. (i) Tma-hyperdependent uORFs: in WT cells (upper), Tma factors efficiently release the penultimate-codon tRNA (function 1) but also dissociate the empty 40S subunit from mRNA (function 2), conferring low-level REI. In *tmaΔΔ* cells (lower), the absence of Tma-mediated tRNA release helps to ensure low-level REI, yielding no change in REI in *tmaΔΔ* vs. WT cells. (ii) Tma-hypodependent uORFs: Tma-independent tRNA release occurs efficiently in both WT and *tmaΔΔ* cells, but REI is low in both cases because, lacking eIF3, the empty 40S subunits dissociate from the mRNA independently of Tma factors, for no change in REI in *tmaΔΔ* vs. WT cells.



bulk 40S recycling in yeast¹⁶. Since elimination of the Tma20/Tma22 heterodimer did not impact translational control of WT GCN4 mRNA²⁹, as mentioned above, it seems unlikely that CCG contributes to low-level REI at WT uORF4 by imposing a heightened requirement for Tma factors for tRNA release. Here, using uORF4 as another genetic tool, we tested the expectation that replacing the native CCG codon with other codons known to be hyperdependent on Tma proteins for bulk recycling

will not reduce REI in *tmaΔΔ* cells because, lacking eIF3, the 40S subunits will dissociate and fail to reinitiate regardless of the tRNA release kinetics (Fig. 1B(i), (ii)). We also examined whether converting the native 3-codon uORF1 and uORF4 to start-stop elements by eliminating their second and third coding triplets confers a dependence on the Tma proteins for REI in the manner observed for DENR with these specialized uORFs in mammalian cells^{17,18}.

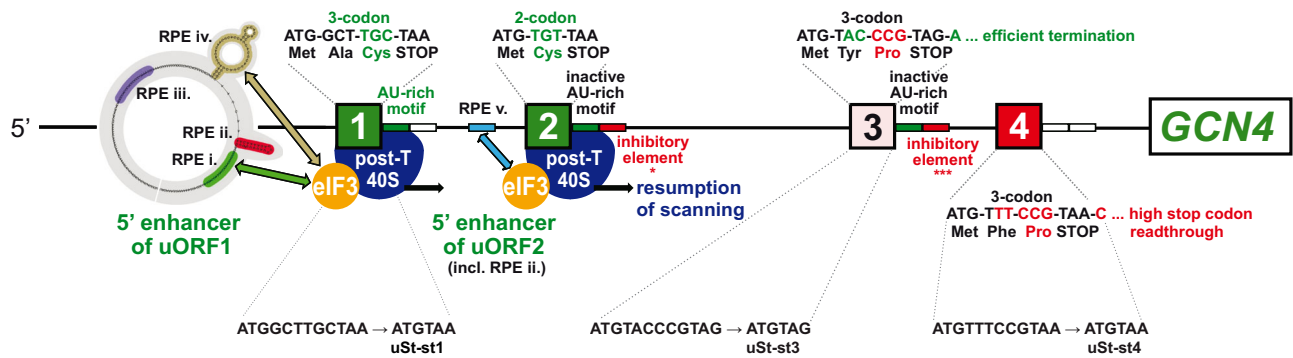


Fig. 2 | Summary of all *cis*-determinants that either promote or inhibit REI on *GCN4* mRNA after translation of its four short uORFs. Schematics of the 5' enhancers of uORF1 and 2 containing their respective RPEs, some of which functionally interact with eIF3 to promote resumption of scanning. Green color-coding generally indicates stimulatory effects of the corresponding *cis*-factors on efficiency of REI, whereas red color-coding indicates inhibitory effects (with the exception of

RPE ii. of uORF1, which is also stimulatory); the number of asterisks below the inhibitory elements of the uORF2 and uORF3 3' sequences depicts the degree of their inhibition as determined experimentally. Mutations converting uORFs 1, 3, or 4 to Start-stop elements uSt-st1, uSt-st3, or uSt-st4, described later in RESULTS, are given below the respective WT uORF schematics. Reprinted and modified with permission from ref. 33.

Our results depart from the expected outcomes for *GCN4* uORF4 shown in Fig. 1B and suggest instead a role for Tma factors at this uORF in dissociating 40S post-termination complexes from mRNA following release of deacylated tRNA, regardless of whether the penultimate triplets are Tma-hyperdependent in bulk recycling. Our findings for *GCN4* uORF1 also differ from those obtained for short, REI-permissive uORFs in mammalian cells¹³ (Fig. 1A(i), (ii)) in revealing that uORF1 generally allows efficient REI independently of Tma factors even when equipped with Tma-hyperdependent penultimate codons. Overall, the effects of Tma factors on REI at the functionally distinct *GCN4* regulatory uORFs equipped with different penultimate codons are not principally dictated by the Tma-dependence of these codons in bulk recycling. We also found that, unlike in mammalian cells, REI following start-stop uORFs is Tma-independent; and we further show that Tma64 plays a supportive role to that of Tma20/Tma22 in impeding REI at uORF4, consistent with its auxiliary role in bulk 40S recycling at most stop codons in yeast.

Results

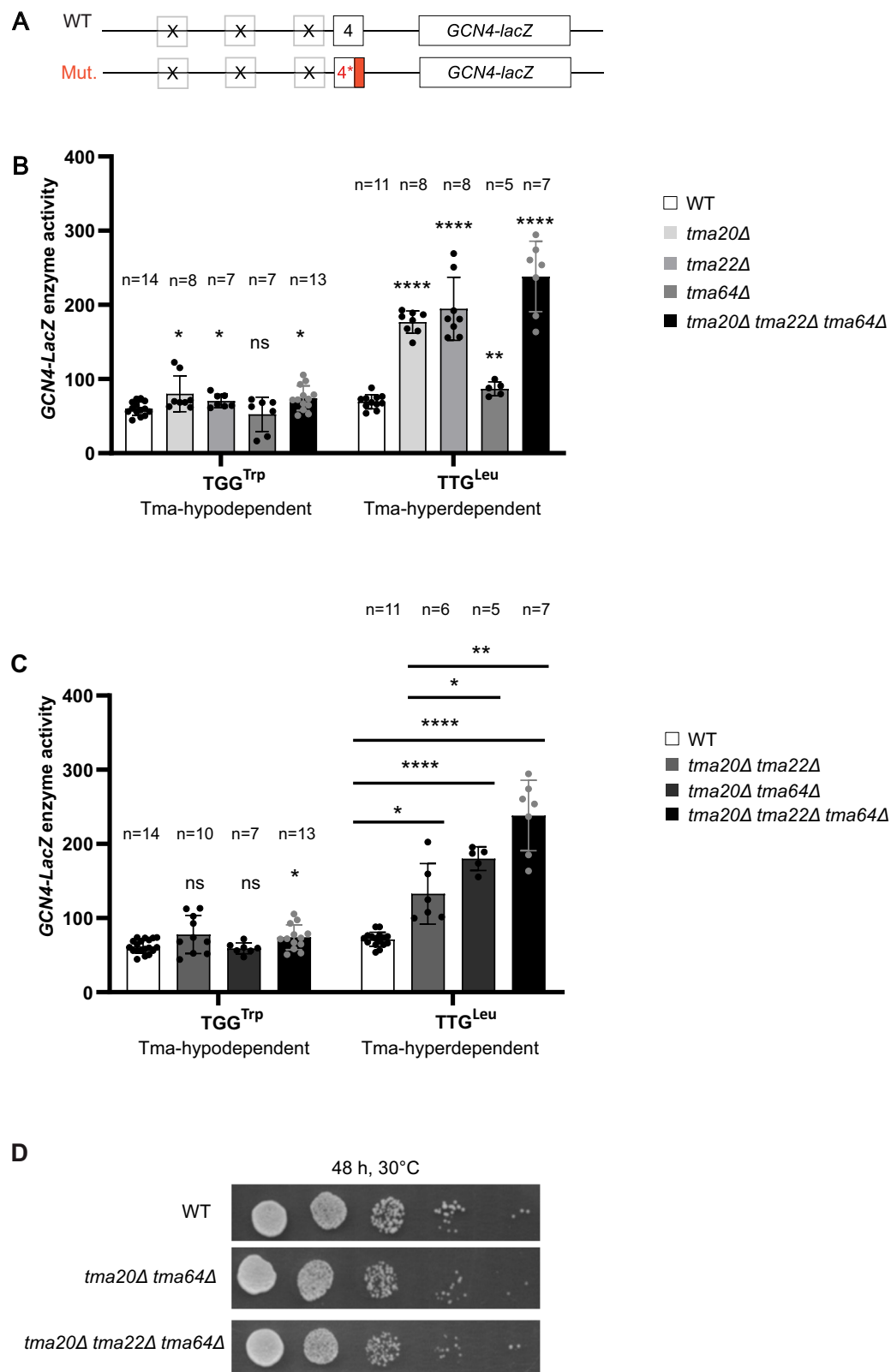
Tma64 partially substitutes for the Tma20/Tma22 heterodimer in suppressing REI after translation of the *GCN4* uORF4 variant containing the Tma-hyperdependent TTG^{Leu} codon

To examine the effects of replacing the penultimate codons of uORF1 or uORF4 with triplets that differ in their dependence on Tma factors in bulk 40S recycling, we employed the well-established *GCN4-lacZ* reporter system, which faithfully recapitulates the functions of the *cis* and *trans* regulatory elements/factors involved in *GCN4* translational control^{24,25,30–37}. This reporter contains the entire *GCN4* transcription unit, including the ~600 nt leader harboring the four uORFs (Fig. 2), with the *lacZ* coding sequences inserted in-frame with the *GCN4* main ORF. To study uORF1, we employed the “uORF1-only” reporter lacking the AUGs of uORFs 2–4. As it was shown previously that leaky-scanning of the WT uORF1 AUG codon is extremely infrequent³⁶, expression of β -galactosidase from this reporter provides a read-out of REI following termination at uORF1. Similarly, uORF4 variants were examined in the “uORF4-only” reporter lacking the AUGs of uORFs 1–3, which produces low levels of β -galactosidase owing to a lack of leaky-scanning of the uORF4 AUG codon in the absence of the other uORFs and highly inefficient REI downstream following uORF4 translation³⁴. Both uORF1 and uORF4 are present at their normal positions in the leader of these reporters.

We first examined the relative contribution of the Tma20/Tma22 heterodimer and Tma64 to the efficiency of REI following uORF4 translation, which we regard as an exemplar of typical REI-non-permissive uORFs in budding yeast^{3,13} (Fig. 1B(i), (ii)). To this end, uORF4-only reporters were generated (Fig. 3A) with the native penultimate CCG^{Pro}

codon replaced by either the TTG^{Leu} codon or TGG^{Trp} codon, judged to be the most or least dependent on Tma factors, respectively, for bulk 40S recycling in yeast¹⁶. (Tma-dependence for each triplet was assigned previously as the ratio of 40S occupancies in *tma20Δtma64Δ* vs. WT cells averaged across the stop codons of all genes. Here, we designate penultimate codons as being “hyperdependent” or “hypodependent” on Tma factors for recycling if they showed, respectively, greater or less than average *tmaΔΔ*/WT 40S ratios at a 99% confidence level; with all other triplets regarded as displaying “average” Tma-dependency¹⁶.) Assaying the reporters in the three single deletion strains lacking *TMA20*, *TMA22*, or *TMA64*, the *tma20Δtma22Δtma64Δ* triple deletion mutant, and the isogenic WT revealed that the *tma20Δ* and *tma22Δ* single deletion strains, and the triple deletion mutant (*tmaΔΔΔ*) produced a very modest, albeit significant change in expression of the reporter with the Tma-hypodependent TGG^{Trp} codon (Fig. 3B). This result matches the expected outcome depicted in Fig. 1B(ii). At odds with the expectations in Fig. 1B(i), however, the single deletions of *TMA20* or *TMA22* conferred significant ≥ 2 -fold derepression of the reporter containing the Tma-hyperdependent TTG^{Leu} codon. Although the single deletion of *TMA64* had only a small effect of lower significance, the triple mutant showed even higher expression of the TTG^{Leu} reporter compared to the *tma20Δ* and *tma22Δ* single mutants, at >3 -fold above the level in WT (Fig. 3B). One way to explain these unexpected findings on the TTG^{Leu} reporter is to propose that eliminating Tma20/Tma22's second function in dissociating post-termination 40S subunits from mRNA allows the retained subunits to traverse the leader and reinitiate downstream at the *GCN4* AUG codon, even though the first function of the heterodimer in releasing the deacylated leucyl tRNA is absent in *tmaΔ* mutant cells. As elaborated below, this in turn implies the occurrence of Tma-independent release of the deacylated tRNA at the TTG^{Leu} codon, allowing increased REI to occur when dissociation of the post-termination 40S subunit is impaired by *tma20Δ* or *tma22Δ* mutations. This proposal of both Tma-dependent and -independent mechanisms of tRNA release at uORF4 variants harboring Tma-hyperdependent penultimate codons is supported by additional evidence presented below.

The results on the TTG^{Leu} reporter in Fig. 3B further suggest that although Tma64 cannot fully substitute for the Tma20/Tma22 heterodimer it still contributes to inhibiting REI in cells lacking the heterodimer, as eliminating all three Tma proteins in the triple mutant conferred significantly greater derepression of the TTG^{Leu} reporter compared to the *tma20Δ* or *tma22Δ* single mutants (Fig. 3B). This inference was confirmed by our finding that the triple mutant and *tma20Δtma64Δ* double mutant, in which both the heterodimer and Tma64 are missing, exhibit significantly greater derepression of the TTG^{Leu} reporter compared to the *tma20Δtma22Δ* double mutant still containing Tma64 (Fig. 3C). Consistent



with this, no discernible difference in growth rate was observed between the *tma20Δtma64Δ* double mutant and the triple mutant, both of which grow slightly more slowly than the WT (Fig. 3D). Accordingly, in subsequent experiments, we considered the *tma20Δtma64Δ* and *tma20Δtma22Δtma64Δ* strains to be equivalent in completely lacking the functions of both the heterodimer and Tma64 in regulating REI.

Tma proteins suppress REI at uORF4 variants containing Tma-hypodependent as well as Tma-hyperdependent penultimate codons

To extend our analysis of the impact of different penultimate codons on Tma-dependent 40S recycling and REI, we examined uORF4-only reporters carrying the three classes of penultimate codons listed in Table 1 that are

Fig. 3 | Differential effects of various TMA deletions on expression of uORF4-only GCN4-lacZ reporters equipped with Tma-hypodependent or Tma-hyperdependent penultimate codons. **A** Schematic of the uORF4-only GCN4-lacZ reporters harboring either WT or mutant penultimate codons. **B** Yeast strains YSG181 (*tma20Δ*), YSG184 (*tma22Δ*), YSG178 (*tma64Δ*), or YKJ3 (*tma20Δtma22Δtma64Δ*), deleted for one or all three TMA genes and the corresponding WT strain YSG142 (WT BY4741) were transformed with uORF4-only reporters containing WT uORF4 (p226) or uORF4 variants with the penultimate Tma-average codon CCG exchanged for Tma-hyperdependent TTG^{Leu} (pKJ34) or Tma-hypodependent TGG^{Tyr} triplet (pKJ36). Reporter activity was assayed in whole cell

extracts for at least five independent transformants (with the specific *n* denoted in each graph) and activities in the *tmaΔ* transformants were normalized as described in “Methods”. Data are represented as ratios of normalized mean values $\pm$ SD of β -galactosidase activities in *tmaΔ* strains/WT strain. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, ns not significant; for details of statistical analysis, see “Materials and methods”. The exact *p* values can be found in Supplementary Data 1. **C** Same analysis as in (B) except comparing transformants of the double mutants YKJ6 (*tma20Δtma22Δ*) and YSG196 (*tma20Δtma64Δ*) to the triple mutant YKJ3 (*tma20Δtma22Δtma64Δ*). **D** Serial dilutions of strains of the indicated genotypes from (C) were spotted on minimal SD medium and grown for 48 h at 30 °C.

Table 1 | Fold-changes in uORF4-only and uORF1-only reporter expression in *tma* mutant vs. WT strains^a

Penultimate codon	<i>tma20Δ tma22Δ</i> / WT ratio (uORF4) $\pm$ SD	<i>tmaΔΔΔ</i> /WT ratio (uORF4) $\pm$ SD	<i>tma20Δ tma64Δ</i> / WT ratio (uORF1) $\pm$ SEM
Tma-hypodependent			
TAC ^{Tyr}	2.33 $\pm$ 0.64	1.47 $\pm$ 0.19	0.73 $\pm$ 0.24
TGG ^{Tyr}	1.30 $\pm$ 0.43	1.23 $\pm$ 0.28	1.15 $\pm$ 0.58
CAA ^{Gln}	1.42 $\pm$ 0.07	2.48 $\pm$ 0.45	1.06 $\pm$ 0.21
GCT ^{Ala}	2.10 $\pm$ 0.23	2.83 $\pm$ 0.57	1.04 $\pm$ 0.12
GAC ^{Asp}	N/A	N/A	1.03 $\pm$ 0.13
GAG ^{Glu}	N/A	N/A	0.92 $\pm$ 0.02
CAC ^{His}	N/A	N/A	0.84 $\pm$ 0.17
TTT ^{Phe}	N/A	N/A	0.94 $\pm$ 0.32
TGT ^{Cys}	N/A	N/A	0.85 $\pm$ 0.10
Tma-average			
CCG ^{Pro} (uORF4 WT)	1.50 $\pm$ 0.53	1.67 $\pm$ 0.53	0.92 $\pm$ 0.11
GCG ^{Ala}	2.92 $\pm$ 0.07	1.55 $\pm$ 0.19	0.79 $\pm$ 0.03
CTG ^{Leu}	4.13 $\pm$ 1.42	1.59 $\pm$ 0.34	0.76 $\pm$ 0.09
ATG ^{Met}	2.55 $\pm$ 0.33	2.73 $\pm$ 0.39	0.40 $\pm$ 0.03
CCA ^{Pro}	2.46 $\pm$ 0.11	3.13 $\pm$ 0.55	0.44 $\pm$ 0.16
TGC ^{Cys} (uORF1 WT)	N/A	N/A	0.78 $\pm$ 0.17
GAA ^{Glu}	N/A	N/A	0.87 $\pm$ 0.07
GTC ^{Val}	N/A	N/A	0.69 $\pm$ 0.04
GGG ^{Gly}	N/A	N/A	1.01 $\pm$ 0.13
CGG ^{Arg}	N/A	N/A	0.65 $\pm$ 0.19
CCC ^{Pro}	N/A	N/A	0.91 $\pm$ 0.18
TCG ^{Ser}	N/A	N/A	0.84 $\pm$ 0.05
AGG ^{Arg}	N/A	N/A	1.01 $\pm$ 0.12
CGC ^{Arg}	N/A	N/A	0.67 $\pm$ 0.20
Tma-hyperdependent			
TTG ^{Leu}	1.91 $\pm$ 0.58	3.43 $\pm$ 0.47	0.59 $\pm$ 0.06
ATT ^{Ile}	3.36 $\pm$ 1.26	2.40 $\pm$ 0.55	0.85 $\pm$ 0.33
TAT ^{Tyr}	1.55 $\pm$ 0.33	2.92 $\pm$ 0.36	0.96 $\pm$ 0.05
AAT ^{Asn}	2.26 $\pm$ 0.70	4.56 $\pm$ 0.78	0.83 $\pm$ 0.13
AAA ^{Lys}	N/A	N/A	0.76 $\pm$ 0.11
AAG ^{Lys}	N/A	N/A	0.86 $\pm$ 0.07

N/A not available.

^aList of the used constructs classified according to the Tma-dependency of their penultimate codons according to Young et al.¹⁶. This table contains average fold-change $\pm$ SD in reporter expression from biological replicates in either *tma20Δ tma22Δ tma64Δ* strain (YKJ3) and *tma20Δ tma22Δ* strain (YKJ6) (uORF4) or *tma20Δ tma64Δ* (H4520) (uORF1) vs. corresponding WT.

Tma-hypodependent (Fig. 4B), Tma-average (Fig. 4C), or Tma-hyperdependent (Fig. 4D) for 40S recycling in the translatome¹⁶. To this end, we measured their expression in both the *tma20Δtma22Δ* double mutant and the triple mutant to provide insights into the codon dependence of Tma64 vs. the Tma20/Tma22 heterodimer in suppressing REI. Considering first results for the *tmaΔΔΔ* triple mutant, we observed that all of the reporters with Tma-hypodependent codons (Fig. 4B, black bars) or Tma-average codons (Fig. 4C, black bars) showed statistically significant derepression in the triple mutant, ranging from 1.2-fold to 3.1-fold for different codons (Table 1, Tma-hypodependent & Tma-average data in col. 3). Derepression was also observed for the four reporters with Tma-hyperdependent codons (Fig. 4D; black bars), ranging from 2.4-fold to 4.6-fold (Table 1, Tma-hyperdependent data in col. 3). Thus, strong derepression in the triple mutant was observed for multiple reporters representing Tma-hypodependent codons (CAA^{Gln}, GCT^{Ala}) and Tma-average codons (ATG^{Met}, CCA^{Pro}) in addition to the four Tma-hyperdependent codons we examined.

Despite this apparent discrepancy with the previously published data on the Tma-dependency of different penultimate codons¹⁶, plotting the expression changes for Tma-hyperdependent, Tma-hypodependent or Tma-average penultimate codons measured in the triple mutant vs. WT (Fig. 5A) revealed a significant tendency for the four Tma-hyperdependent codons to exhibit greater derepression in the triple mutant compared to the four Tma-hypodependent codons tested (*p* = 0.0404). Nonetheless, since the Tma-average reporters containing ATG^{Met} or CCA^{Pro} and the Tma-hypodependent GCT^{Ala} and CAA^{Gln} reporters did not exhibit statistically smaller derepression ratios in the triple mutant compared to the Tma-hyperdependent reporters for codons TTG^{Leu}, ATT^{Ile}, and TAT^{Tyr} (Table 1, col. 3), we conclude that the Tma-dependency of penultimate codons determined previously¹⁶ is not a strong indicator of Tma-mediated suppression of REI following uORF4 translation.

Considering next results obtained in the *tma20Δtma22Δ* double mutant, where Tma64 can potentially substitute for the heterodimer, we found that five of the uORF4-only reporters that showed marked derepression in the triple mutant displayed substantially less derepression in the *tma20Δtma22Δ* double mutant, including reporters with CAA^{Gln} and GCT^{Ala} (Fig. 4B), CCA^{Pro} (Fig. 4C), TTG^{Leu}, TAT^{Tyr}, and AAT^{Asn} (Fig. 4D) penultimate codons (summarized in Table 1, cf. cols. 2–3). For these six reporters, containing Tma-hypodependent, -average, or -hyperdependent codons, it appears that Tma64 can partially substitute for heterodimer function in suppressing REI. The Tma-hypodependent CAA^{Gln} (Fig. 4B) and Tma-hyperdependent TAT^{Tyr} (Fig. 4D) codons illustrate the most complete functional substitution by Tma64, with only slight derepression in the *tma20Δtma22Δ* double mutant despite marked derepression in the triple mutant. Reporters with TAC^{Tyr} (Tma-hypodependent; Fig. 4B), ATG^{Met}, CTG^{Leu} and GCG^{Ala} (Tma-average; Fig. 4C), and ATT^{Ile} (Tma-hyperdependent; Fig. 4D) codons appear to represent the other extreme, wherein Tma64 cannot functionally compensate at all and eliminating the heterodimer alone in the double mutant confers derepression equal to or even greater than that found in the triple mutant also lacking Tma64. Finally, the comparatively low-level derepression observed in both mutants for the native uORF4 CCG^{Pro} (Fig. 4C) and TGG^{Tyr} reporters (Fig. 4B, black bars) makes it difficult to determine whether Tma64 can partially substitute

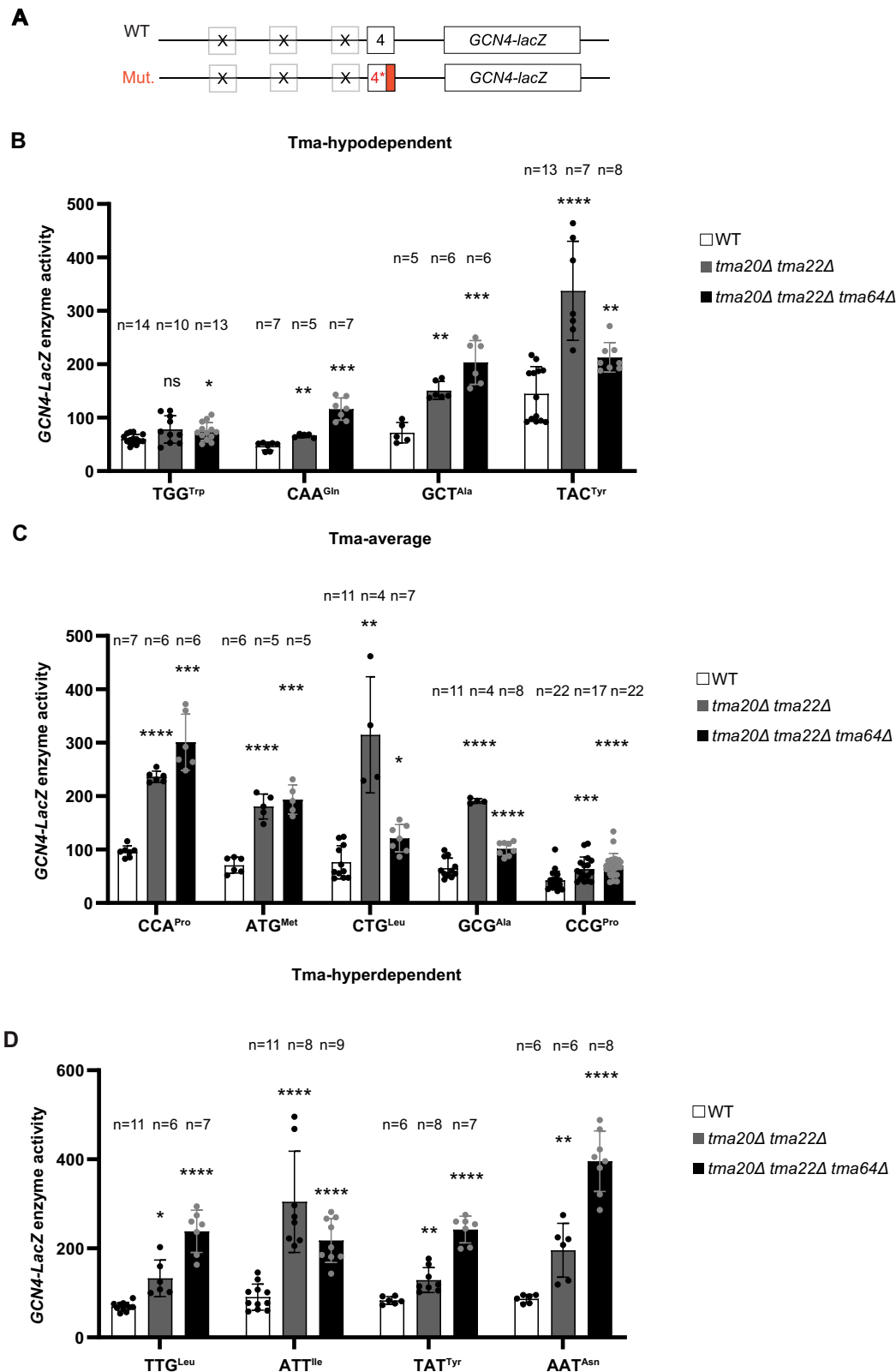
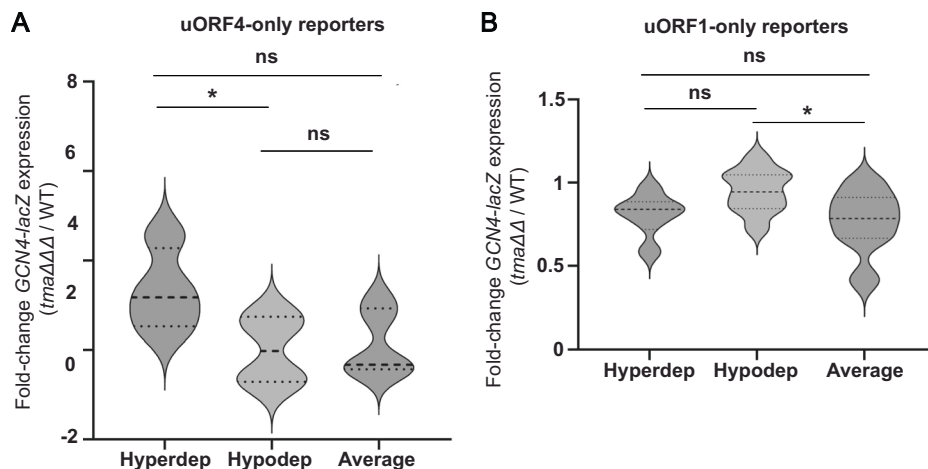


Fig. 4 | Differential effects of various TMA double and triple deletions on expression of uORF4-only GCN4-lacZ reporters equipped with Tma-hypodependent, Tma-average or Tma-hyperdependent penultimate codons. A Schematic of the uORF4-only GCN4-lacZ reporters harboring either WT or mutant penultimate codons. B–D Yeast strains YKJ6 (*tma20Δtma22Δ*) and YKJ3 (*tma20Δtma22Δtma64Δ*) and the corresponding WT strain were transformed with the WT uORF4-only GCN4-lacZ reporter construct (p226) and variants with the WT uORF4 penultimate codon (CCG) exchanged for the indicated

Tma-hypodependent, Tma-average or Tma-hyperdependent codons. Reporter activity was assayed in whole cell extracts for at least four independent transformants (with the specific *n* denoted in each graph) and activities in the *tmaΔ* transformants were normalized as described in “Methods”. Data are represented as GCN4-LacZ reporter enzyme activities in WT strain and *tmaΔ* strains. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, ns not significant; for details of statistical analysis, see “Materials and methods”. The exact *p* values can be found in Supplementary Data 1.

Fig. 5 | Changes in reporter expression for groups of Tma-hyperdependent, Tma-hypodependent or Tma-average penultimate codons in *tma* mutant vs. WT cells. A Violin plot of the average fold-changes in expression of uORF4-only *GCN4-lacZ* reporters harboring the four Tma-hyperdependent (Hyperdep), four Tma-hypodependent (Hypodep) or five Tma-average (Average) penultimate codons in the triple deletant yeast strain YKJ3 (*tma20Δtma22Δtma64Δ*) vs. isogenic WT strain. The average fold-change of expression determined from biological replicates only was calculated for each construct from results plotted in Fig. 4. Statistical analysis was performed using two-tailed unpaired *t*-test **p* < 0.05. **B** Violin plot of fold changes in expression of uORF1-only *GCN4-lacZ* reporters harboring the six Tma-hyperdependent, nine Tma-hypodependent and 14 Tma-average penultimate codons in *tma20Δtma64Δ* strain H4520 vs. WT strain BY4741. The average fold-change of expression determined from biological replicates was calculated for each construct from the results plotted in Fig. 6. Statistical analysis was performed using the two-tailed unpaired *t*-test. **p* < 0.05. The exact *p* values can be found in Supplementary Data 1.



for the heterodimer at these uORFs. Nevertheless, these findings overall raise the interesting possibility that the ability of Tma64 to partially substitute for the heterodimer in suppressing REI depends on the penultimate codon at uORF4, and this codon variability applies to both Tma-hyperdependent and Tma-average codons. If so, then the ability of Tma64 to suppress REI in cells lacking the heterodimer would not appear to be governed by the codon-dependence of Tma factors for bulk 40S recycling¹⁶.

Very few penultimate codons confer Tma-dependence at REI-permissive *GCN4* uORF1

The mammalian DENR/MCT51 heterodimer was shown to promote REI after certain short uORFs in mammalian cells, including *ATF4* uORF1¹³, whereas the yeast Tma20/Tma22 counterpart functioned oppositely to inhibit REI following uORF translation in yeast extracts¹⁶. As mentioned above, it was suggested that the discrepancy between mammals and yeast arises from the inability of post-termination 40S complexes at typical yeast uORFs to remain associated with the mRNA and migrate to downstream start codons following release of the tRNA decoding the penultimate codon¹³ (Fig. 1B(i)-(ii) vs. 1A(i)-(ii)). To test this hypothesis, we analyzed the effects of the *tma* mutations on REI at *GCN4* uORF1. As mentioned, this atypical uORF is surrounded by *cis*-acting RPEs, some of which interact with eIF3 subunits and promote retention of post-termination 40S complexes on the mRNA, enabling high-level REI (Fig. 2). Indeed, REI after uORF1 translation is ~20–25-fold higher than after uORF4 translation^{25,26,32,33}. The retention of post-termination 40S subunits at uORF1 makes the uORF1-only *GCN4-lacZ* reporter (Fig. 6A) ideally suited to examine whether Tma factors are required to dissociate deacylated tRNA and enable REI at the *GCN4* start codon in a manner limited to penultimate codons with heightened dependence on the Tma factors for bulk 40S recycling in yeast¹⁶. If so, then uORF1-only reporters equipped with Tma-hyperdependent codons should exhibit reduced REI and *GCN4-lacZ* expression in *tmaΔΔ* vs. WT cells in the manner observed for short uORFs in mammalian cells depleted of DENR (Fig. 1A(i)).

We exchanged the native uORF1 penultimate codon TGC^{Cys} in the uORF1-only reporter with other penultimate codons of all three classes defined above, including six Tma-hyperdependent, nine Tma-hypodependent, and 13 Tma-average codons. The native TGC^{Cys} codon is Tma-average, and the native TGT^{Cys} codon of the REI-permissive uORF2 of

*GCN4*³² was chosen as one of the Tma-hypodependent codons. Expression of the resulting reporters was assayed in the *tma20Δtma64Δ* double mutant and WT strains (Fig. 6 and Table 1, col. 4). At odds with the prediction that REI would be reduced in the mutant for reporters with Tma-hyperdependent codons, only one of the six reporters of this class, that containing the TTG^{Leu} codon, showed significantly reduced expression in the mutant (Fig. 6B, hyperdependent constructs). While TTG^{Leu} was judged to be highly Tma-hyperdependent for bulk 40S recycling, ATT^{Ile} and TAT^{Tyr} were equally so¹⁶, yet the reporters for the latter two codons showed unchanged expression in the mutant strain. Moreover, the constructs containing the Tma-average ATG^{Met} or CCA^{Pro} codons were the only reporters besides the TTG^{Leu} construct showing significantly reduced expression in *tma20Δtma64Δ* cells (Fig. 6B, Tma-average constructs). Fold changes in expression between WT and *tmaΔΔ* strains can be found summarized in Table 1, col. 4. Plotting the expression changes for the entire set of 29 codons examined at uORF1 (Fig. 5B) revealed a general tendency for reduced expression in the *tmaΔΔ* mutant vs. WT cells, but no significant difference in median expression ratios between Tma-hyperdependent reporters and either Tma-hypodependent or Tma-average reporters.

Figure 5B does reveal a small but significant difference between the median expression ratios between the Tma-hypodependent and Tma-average groups (*p* = 0.0394), which reflects the slightly increased REI observed in *tmaΔΔ* cells for the Tma-hypodependent group. None of the uORF1-only reporters, however, showed the ≥2-fold increased REI conferred by the *tmaΔΔΔ* mutation for most of the uORF4-only reporters (Fig. 5A), even for the ten cases where uORF1-only and uORF4-only reporters share the same penultimate codons (ATT, TTG, AAT, TAT, ATG, GCG, CTG, CAA, GCT, CCA). This is consistent with the prediction that the specialized RPEs at uORF1 allow 40S post-termination complexes to resist ribosome dissociation by the Tma factors that suppress REI at uORF4 lacking RPEs (Fig. 2). In summary, our findings indicate that uORF1 differs from mammalian short uORFs, including the REI-permissive uORF1 of *ATF4*, in lacking a requirement for Tma factors in dissociating deacylated tRNA from post-termination complexes at penultimate codons that impose a heightened Tma-requirement in bulk 40S recycling. A lack of Tma-dependence on REI also applies to eight of the nine DENR-dependent codons¹³ represented among our uORF1-only reporters (ATT, GCG, CTG, CCG, CGG, CCC, AGG, and GCT). In fact, ATG is the sole DENR-dependent codon we tested that confers a dependence on Tma factors for

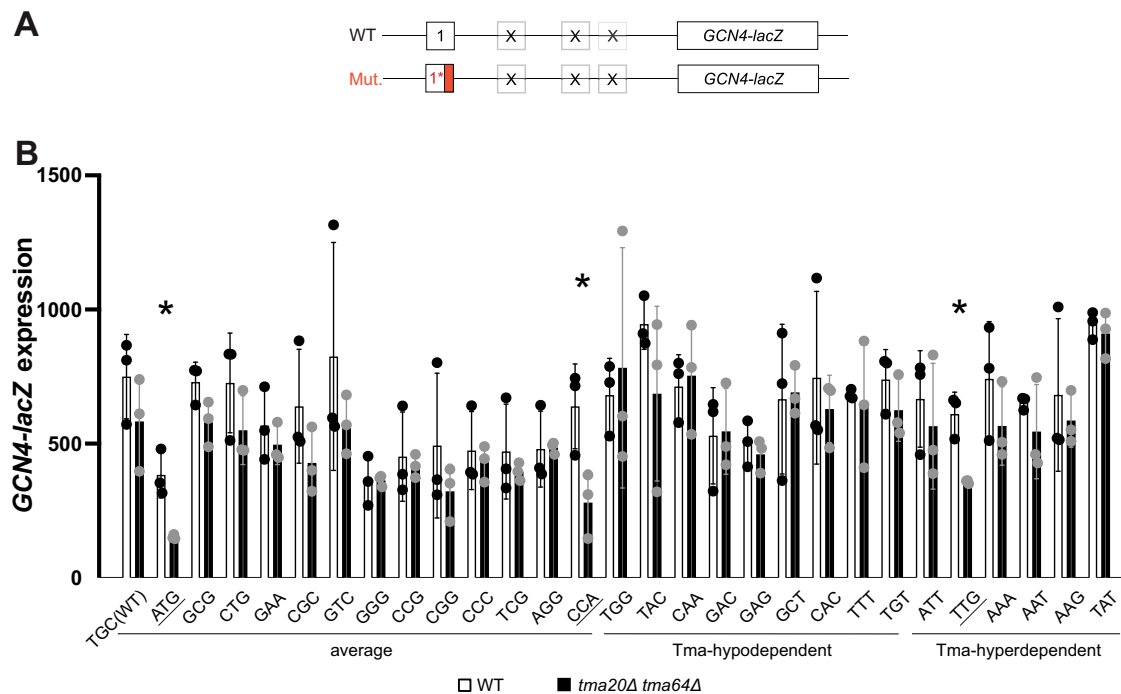


Fig. 6 | Differential effects of deleting both *TMA20* and *TMA64* on expression of uORF1-only *GCN4-lacZ* reporters equipped with Tma-average, Tma-hypodependent or Tma-hyperdependent penultimate codons. A Schematic of the uORF1-only *GCN4-lacZ* reporters harboring either WT or mutant penultimate codons. **B** Yeast strains H4520 (*tma20Δtma64Δ*) and WT strain BY4741 were transformed with uORF1-only *GCN4-lacZ* reporter constructs with the WT uORF1 penultimate codon (TGC) exchanged for the indicated Tma-average, Tma-hypodependent or Tma-hyperdependent codons. β -galactosidase activities

determined for each of three independent transformants ($n = 3$) of the *tma20Δtma64Δ* strain were divided by a factor of 1.316 prior to calculating the mean activities and S.E.M. values plotted here to normalize for differences in reporter expression between the two strains that are independent of the uORF1 variants and observed for an uORF-less reporter (as described in “Methods”). The mean values between mutant and WT were compared for each reporter in a two-tailed, unpaired Student’s *t* test to assign *p* values (* $p < 0.05$). The exact *p* values can be found in Supplementary Data 1.

REI at uORF1, but as noted above, ATG is Tma-average for 40S recycling in yeast.

In summary, neither Tma-dependence nor DENR-dependence of the penultimate codon is an accurate predictor of Tma-dependence for REI at *GCN4* uORF1 variants, despite the functional similarity between *GCN4* uORF1 and *ATF4* uORF1.

Function of yeast “start-stop” elements is not Tma-dependent

As mentioned earlier, mammalian DENR was shown to be required for REI after start-stop uORFs consisting of ATG followed by a stop codon^{13,18}. To test the Tma-dependence of start-stops for downstream REI in yeast, we deleted the 2nd and 3rd codons from native uORF1-, uORF3- and uORF4-only constructs, without altering any other sequences, producing uStart-stop-only constructs uSt-st1, uSt-st3 and uSt-st4, respectively. (See bottom of Fig. 2 for sequences of the three uSt-st uORFs.)

First, we noticed that all three start-stop elements are more inhibitory to REI than their native uORF counterparts, significantly reducing *GCN4-lacZ* expression in WT cells by ~50-fold, ~3-fold, and ~0.6-fold compared to the levels observed for native uORF1-only, uORF3-only, and uORF4-only reporters, respectively (Fig. 7A). Based on findings in mammalian cells, we reasoned that converting native uORF1 to uSt-st1 would confer a dependence on REI by the Tma proteins not observed for native uORF1 with its penultimate codon TGC^{Cys} (Fig. 6, TGC(WT)), reducing expression in *tmaΔΔ* cells. This was not observed, however (Fig. 7B, cols. 1–4), even though we found above that ATG^{Met} as the penultimate codon for 3-codon uORF1 conferred Tma-dependence for efficient REI (Fig. 6, ATG). Interestingly, converting uORF4 to uSt-st4 eliminated the significant derepression observed in *tmaΔΔ* cells for the native uORF4-only reporter (Fig. 7B, cols. 9–12). This suggests that conversion to a start-stop element eliminates the requirement for Tma factors for highly efficient 40S recycling at uORF4 and repression of REI downstream. The Tma proteins appear to make no

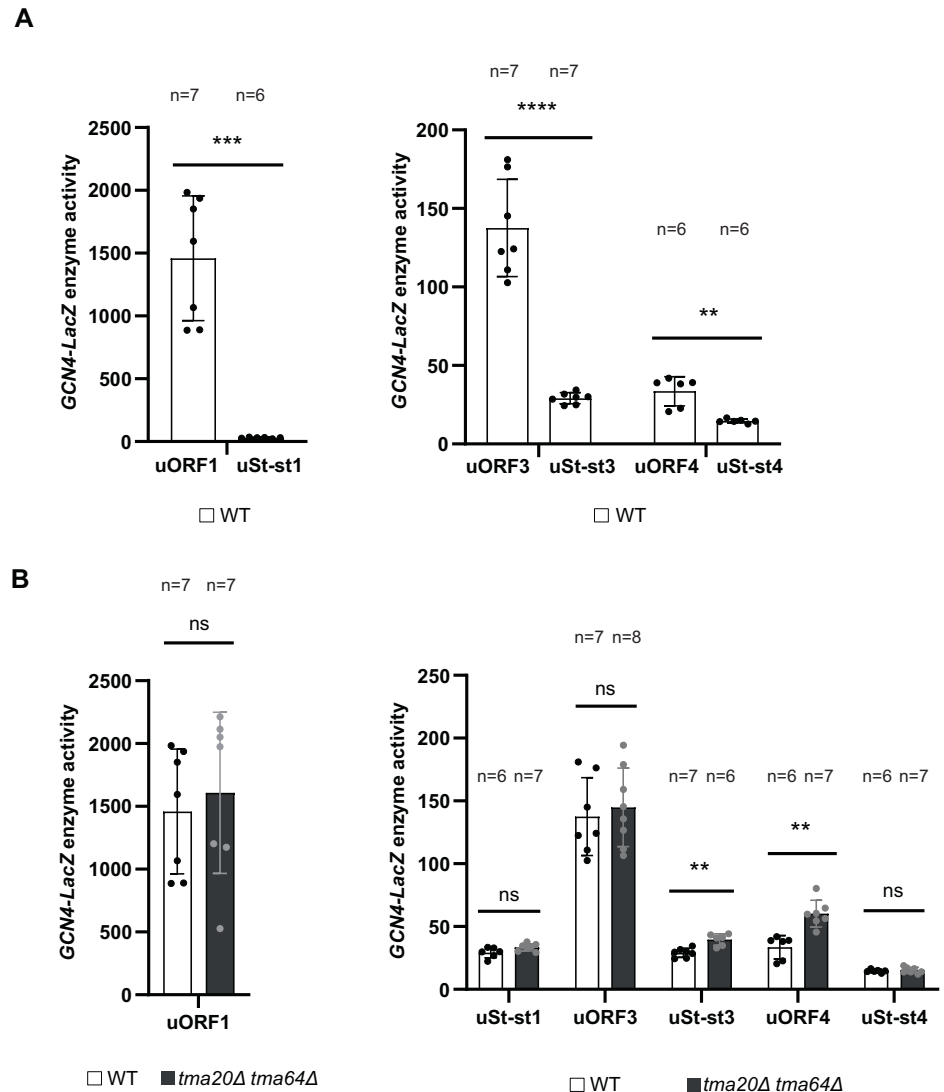
contribution to recycling and REI at native uORF3, as the uORF3-only reporter expression is unaffected by the *tma20Δtma64Δ* mutations; whereas its conversion to uSt-st3 confers a modestly significant Tma-dependence for recycling and suppression of REI (Fig. 7B, cols. 5–8). Overall, our findings suggest that, unlike their mammalian counterparts, the Tma proteins play little role in controlling REI after start-stop elements in yeast.

Discussion

As yeast Tma20/Tma22 and, to a lesser degree, Tma64 have been implicated in 40S ribosome recycling *in vivo*^{15,16}, we used an established reporter system based on *GCN4* translational control to examine the influence of yeast Tma20/MCTS1, Tma22/DENR and Tma64/eIF2D on REI downstream of short uORFs optimized for either REI (uORF1) or for recycling and suppression of REI (uORF4)⁵. In addition, we dissected the functional interplay between the Tma factors in regulating these alternative post-termination outcomes. To do so, we employed variants of uORF1 or uORF4 containing various penultimate codons that conferred either unusually strong dependence, average, or lower than average dependence on Tma proteins for bulk 40S recycling at most stop codons in yeast as genetic tools.

Using the uORF4-only *GCN4-lacZ* reporter, we first demonstrated that inserting Tma-hyperdependent codon TTG^{Leu} substantially increased REI in single mutants lacking *TMA20* or *TMA22* but only slightly in the mutant lacking *TMA64* (Fig. 3A). Deleting *TMA64* in the *tma20Δtma22Δ* mutant significantly enhanced this defect (Fig. 3C), suggesting that Tma64 can partially compensate for the absence of Tma20/Tma22 in suppressing REI. This conclusion agrees with previous 40S profiling data¹⁶, wherein a single deletion of *TMA20* or *TMA22*, but not *TMA64*, led to accumulation of unrecycled 40S subunits at stop codons translatome-wide, and this phenotype was more pronounced in the *tma20Δtma64Δ* deletion strain lacking both the heterodimer and Tma64. Consistent with this, we observed significantly increased REI for the TTG^{Leu} uORF4 variant in the

Fig. 7 | Effects of start-stop elements on downstream REI in the *GCN4-lacZ* reporter system and differential effects of *TMA20* and *TMA64* deletion on re-initiation downstream of start-stop elements. **A WT strain BY4741 was transformed with *GCN4-lacZ* reporter plasmids containing the following single WT uORFs: uORF1-only (p209), uORF3-only (pSG61_2), or uORF4-only (p226); or containing single Start-stop elements uSt-st1 (pKP78), uSt-st3 (pKP76), or uSt-st4 (pKP77). β -galactosidase activities were assayed in at least four biological replicates (with the specific *n* denoted in each graph) and data are presented as *GCN4-LacZ* reporter enzyme activities. **B** Strains YSG196 (*tma20Δ tma64Δ*) and the corresponding WT were transformed with the same set of *GCN4-lacZ* reporter plasmids described in (A) and reporter expression was measured as described there. Reporter activity values in the *tmaΔΔ* strain were normalized as described in “Methods”. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, ns not significant; for details of statistical analysis, see “Materials and methods”. The exact *p* values can be found in Supplementary Data 1.**



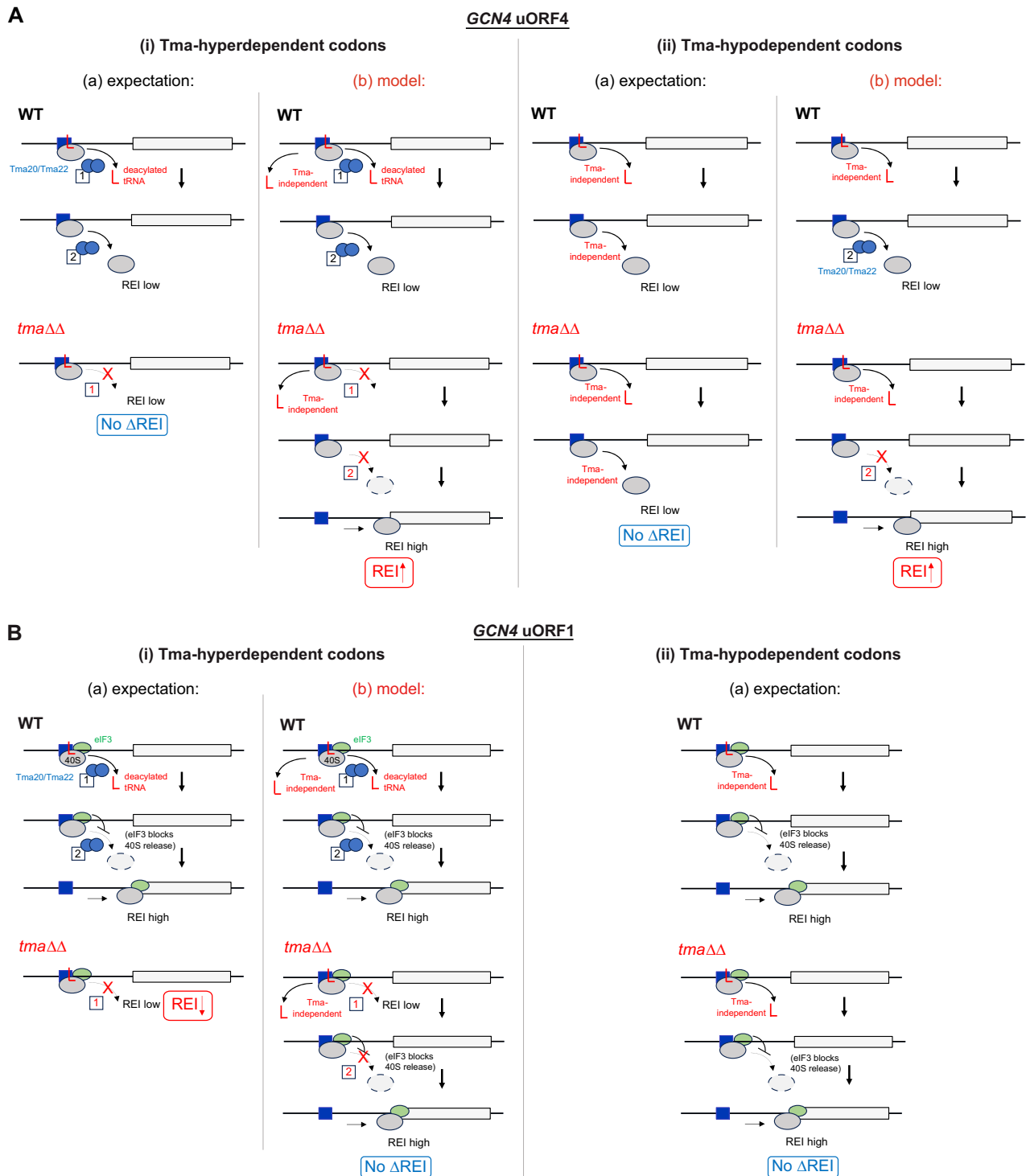
tma20Δtma64Δ double mutant and *tma20Δtma22Δtma64Δ* triple mutant compared to the *tma20Δtma22Δ* double mutant (Fig. 3C). We concluded that intact Tma20/Tma22 heterodimer is indispensable for WT 40S recycling and suppression of REI but receives functional support from Tma64 at the stop codon of this uORF4 variant.

Examining a total of 12 different uORF4 variants with different penultimate codons, plus native uORF4, revealed that the absence of all three Tma proteins in the *tmaΔΔΔ* mutant increased REI by less than 2-fold for native uORF4 containing CCG^{Pro}, and for the TAC^{Tyr}, CTG^{Leu}, GCG^{Ala} and TGG^{Tyr} uORF4 variants, but by more than 2-fold for the CAA^{Gln}, GCT^{Ala}, CCA^{Pro} and ATG^{Met} variants (Fig. 4B, C and Table 1). Since all these codons showed average or hypodependency on Tma factors in bulk recycling¹⁶, these mixed outcomes indicate that the Tma-dependency in bulk 40S recycling determined previously¹⁶ is generally a weak predictor of Tma-dependence in promoting 40S recycling and suppressing REI for different penultimate codons at uORF4. On the other hand, our finding that the group of Tma-hyperdependent reporters shows a significantly greater increase in median expression compared to the Tma-hypodependent and Tma-average groups (Figs. 4D, 5A and Table 1) suggests that Tma-hyperdependence in bulk recycling at least partly contributes to the impact of Tma proteins in blocking REI observed for uORF4 variants encompassing a range of Tma-dependencies.

Our finding that REI is elevated in *tmaΔΔΔ* cells at uORF4 variants containing Tma-average or Tma-hypodependent codons is difficult to

explain if dissociation of the empty 40S subunit is believed to occur spontaneously at these penultimate codons, which should maintain low-level REI in the presence or absence of Tma factors (Fig. 8A(ii)-(a)). Hence, in the model proposed in Fig. 8A(ii)-(b), we assume that Tma factors must figure prominently in dissociating the empty 40S subunits at Tma-hypodependent codons (recycling step 2 in WT), such that retention of a fraction of 40S subunits in their absence in *tmaΔΔΔ* cells allows increased REI downstream when release of the deacylated tRNA is achieved by a Tma-independent pathway. Our observation that the Tma proteins also inhibit REI at uORF4 variants containing Tma-hyperdependent codons is likewise difficult to explain. Specifically, the failure to release the deacylated tRNA in the *tmaΔΔΔ* mutant should block REI even though Tma-catalyzed dissociation of the 40S subunit from mRNA is absent, thus keeping REI low in *tmaΔΔΔ* cells (Fig. 8A(i)-(a)). Therefore, it seems necessary to propose that the deacylated tRNA eventually dissociates spontaneously or by another pathway in the absence of Tma factors, so that loss of Tma-catalyzed dissociation of the empty 40S subunit can enable REI and confer the increased REI we observed in *tmaΔΔΔ* cells (Fig. 8A(i)-(b)).

The models we propose in (b) panels of Fig. 8A(i)-(ii) assume that Tma-independent recycling can operate at all stop codons and that recycling at Tma-hyperdependent penultimate codons simply relies more heavily on Tma factors than at Tma-hypodependent codons. This possibility is consistent with the fact that eliminating all three Tma proteins confers only a



small effect on cell growth, implying the frequent occurrence of Tma-independent recycling throughout the translome.

We also investigated whether the Tma factors function similarly to MCTS1/DENR in promoting, rather than impeding, REI at short uORFs that are permissive for REI owing to retention of eIFs during uORF translation. It was proposed that the persistence of eIFs on 40S post-termination complexes prevents dissociation of the 40S subunits following MCTS1/DENR-catalyzed release of deacylated tRNA, allowing resumption of 40S migration and rebinding of TC for REI downstream. This explained why REI was impaired on depletion of MCTS1/DENR only for uORFs containing DENR-dependent penultimate codons¹³ (Fig. 1A(i)-(ii)). To

investigate this mechanism in yeast, we examined the *GCN4* uORF1-only reporter in which RPEs surrounding uORF1 increase eIF3 and eIF4F occupancy of the post-termination 40S subunits and confer high-frequency REI at *GCN4* (Fig. 2)^{25,26,32,33}. Consistent with results for MCTS1/DENR, REI was not reduced in the *tmaΔΔ* mutant for native uORF1 nor for 21 other uORF1 variants equipped with Tma-average or Tma-hypodependent penultimate codons but REI was reduced when uORF1 contained the Tma-hyperdependent TTG^{Leu} codon. However, at odds with results for MCTS1/DENR, REI was not reduced in *tmaΔΔ* cells at five other uORF1 variants with Tma-hyperdependent codons. This included the TAT codon present in uORF1 of *ATF4* mRNA—functionally equivalent to *GCN4* uORF1³⁸—

Fig. 8 | Expected vs. proposed functions for yeast Tma20/Tma22 at REI-permissive GCN4 uORF1 and REI-non-permissive GCN4 uORF4. Tma20/Tma22 (blue ovals), penultimate deacylated tRNA (red “L”), and the two functions of Tma factors (boxes 1 and 2) in releasing tRNA and dissociating empty 40S subunits, respectively (solid or dashed arrows), are depicted as in Fig. 1. **A** Tma-mediated recycling at GCN4 uORF4 depending on the presence of (i) Tma-hyperdependent or (ii) Tma-hypodependent penultimate codons. (i) Tma-hyperdependent uORFs. (a) expectation: as depicted exactly as in Fig. 1B(i) (presented again for comparison), in WT cells (upper), Tma factors efficiently release the penultimate-codon tRNA and also dissociate the empty 40S subunits, conferring low-level REI. In *tmaΔΔ* cells (lower), the absence of Tma-mediated tRNA release helps to ensure low-level REI, yielding no change in REI in *tmaΔΔ* vs. WT cells. (b) model: in WT (upper), deacylated tRNA can be released by Tma-hyperdependent or Tma-independent mechanisms, but Tma-mediated 40S dissociation maintains low-level REI. In *tmaΔΔ* cells (lower), Tma-independent tRNA release coupled with absence of Tma-mediated 40S dissociation confers the increased REI we observed in *tmaΔΔ* vs. WT cells. (ii) Tma-hypodependent uORFs. (a) expectation: as depicted exactly in Fig. 1B(ii), Tma-independent tRNA release occurs in both WT and *tmaΔΔ* cells, but REI is low in both cases because the empty 40S subunits dissociate from the mRNA

independently of Tma factors, for no change in REI vs. WT. (b) model: in WT (upper), 40S dissociation is catalyzed by Tma factors even at these Tma-hypodependent uORFs, and absence of this function in *tmaΔΔ* cells, coupled with Tma-independent tRNA release, confers the observed increased REI in *tmaΔΔ* vs. WT cells. **B** Tma-mediated recycling at GCN4 uORF1 depending on the presence of Tma-hyperdependent (i) or Tma-hypodependent (ii) codons. (i) Tma-hyperdependent uORFs. (a) expectation: as depicted for MCTS1/DENR in Fig. 1A(i), in WT cells (upper), Tma factors efficiently release the penultimate-codon tRNA but its second function in 40S dissociation is impeded by eIF3 (green oval) to enable high-level REI. In *tmaΔΔ* cells (lower), diminished release of the penultimate-codon tRNA lowers REI. (b) model: deacylated tRNA can be released from uORF1 variants by both Tma-hyperdependent and Tma-independent mechanisms in WT yeast (upper) and Tma-independent tRNA release maintains high-level REI in *tmaΔΔ* cells (lower), as 40S dissociation is impeded by eIF3 in both WT and *tmaΔΔ* cells, thus accounting for the unchanged REI we observed in *tmaΔΔ* vs. WT cells. (ii) Tma-hypodependent uORFs. (a) expectation: as depicted for MCTS1/DENR in Fig. 1A(ii), Tma-independent release of the tRNA and subsequent high-level REI occurs in the presence (top) or absence (bottom) of Tma factors, yielding no decrease in REI in *tmaΔΔ* vs. WT cells, as we observed.

that confers DENR-dependent REI on *ATF4* mRNA¹³. Moreover, REI was markedly reduced in *tmaΔΔ* cells when uORF1 contained the Tma-average penultimate codons ATG and CCA. Thus, dependence on the Tma factors for REI was conferred on uORF1 by only three of 29 penultimate codons examined, only one of which (TTG) is Tma-hyperdependent for bulk 40S recycling¹⁶. Moreover, there was no significant difference in median *tmaΔΔ* derepression ratios between uORF1-only reporters containing Tma-hyperdependent vs. Tma-average or Tma-hypodependent codons (Fig. 5B). While there is a general tendency for REI to be diminished at uORF1 variants in *tmaΔΔ* vs. WT cells (Fig. 5B), the Tma factors do not stimulate REI preferentially at uORF1 variants whose penultimate codons are Tma-hyperdependent in bulk recycling in the manner described for MCTS1/DENR at REI-permissive uORFs in mammalian cells.

One possibility is that the specialized interaction of GCN4 uORF1 RPEs with eIF3 generally overrides the requirement for Tma factors in dissociating the deacylated tRNA at Tma-hyperdependent penultimate codons. By preventing 40S dissociation from the mRNA, eIF3 might provide sufficient time for dissociation of the deacylated tRNA, either spontaneously or by a Tma-independent pathway, to prevent reduced REI in *tmaΔΔ* cells. This scenario is depicted in the model proposed in Fig. 8B(i)-(b) to explain our unexpected findings on uORF1 variants with Tma-hyperdependent codons. In support, numerous tRNAs were found to readily dissociate from the 40S subunit without the help of any dedicated factor¹⁹. Furthermore, structural analysis of MCTS1/DENR bound to the 40S subunit indicates that MCTS1 might occupy the same binding site on the ribosome as the eIF3a, b, and c subunits of mammalian eIF3³⁹. In addition, the ribosome binding interface in MCTS1 seems to be well conserved from yeast to higher eukaryotes (Fig. 9A), as we also observed when comparing the MCTS1 structure with the Tma20 AlphaFold structure prediction (Fig. 9B). This might indicate competitive ribosome binding between eIF3 and Tma20/Tma22, such that the uORF1 RPEs would promote binding of eIF3a vs. the Tma heterodimer and thereby prevent the latter from influencing REI appreciably at uORF1.

Presumably, interaction of deacylated leucyl tRNA with the TTG^{Leu} codon is too stable to overcome the requirement for Tma-mediated release, leading to the observed reduced REI for the TTG^{Leu} uORF1 variant in *tmaΔΔ* cells, in the manner predicted for DENR-hyperdependent codons (Fig. 1A(ii)). The same might be true for interaction of elongator methionyl tRNA and prolyl tRNA with their cognate ATG^{Met} and CCA^{Pro} codons, which also displayed Tma-dependence for REI at uORF1, even though ATG and CCA were not identified as being Tma-hyperdependent for bulk 40S recycling¹⁶. Supporting this idea, ATG was identified as a DENR-hyperdependent penultimate codon in mammalian cells both in bulk 40S recycling and reporter REI assays¹³.

Another consequence of introducing ATG as the penultimate codon of GCN4 uORF1 not yet mentioned is a significant reduction in REI in WT cells ($p = 0.04$), which is further exacerbated in the *tmaΔΔ* mutant (Fig. 6). A reduction in WT cells was also found for GGG^{Gly} ($p = 0.03$), CCG^{Pro}, CCC^{Pro}, AGG^{Arg} and GAG^{Glu} codon replacements ($p = 0.09$) (Fig. 6B). CCG^{Pro} is the evolutionarily conserved penultimate codon at uORFs 3 and 4 and was shown previously to inhibit REI when introduced at uORF1³³, which might result from delaying decoding or termination at the adjacent stop codon³³.

In addition to *ATF4* uORF1 and other REI-permissive uORFs, the MCTS1/DENR heterodimer was found to promote REI after translation of the *ATF4* start-stop element preceding uORF1¹³ and at various other start-stop elements in optimum Kozak context^{13,17,18,20}. In contrast, we found no evidence for Tma-dependent recycling and REI at the start-stop elements we derived from GCN4 uORFs 1, 3, or 4, all of which contain the optimum A-rich sequence context for initiation in yeast. Converting uORF3 and uORF4 to start-stop elements decreased REI in WT cells and diminished the increased REI observed for native uORF4 in *tmaΔΔ* cells. It appears that uSt-st4 exhibits an elevated level of spontaneous 40S recycling that bypasses the contribution of Tma factors to recycling seen at native uORF4. Converting uORF1 to start-stop element uSt-st1 also dramatically decreased REI in WT cells, apparently disabling the RPEs that enable high-level REI at native uORF1. Although uSt-st1 behaves like a typical inhibitory uORF, 40S recycling appears to occur without any contribution from the Tma factors at this element. The fact that uSt-st1, uSt-st4, and uSt-st3 do not show decreased REI in *tmaΔΔ* cells in the manner we observed for the GCN4 uORF1 variant with ATG^{Met} as penultimate codon (Fig. 6B) is not surprising considering that, similar to uORF4, the St-st elements appear to lack the capacity for eIF3-mediated retention of 40S post-termination complexes (Fig. 2).

In summary, we found that most replacements of the uORF4 penultimate codon confer increased REI in mutants lacking both Tma64 and the Tma20/Tma22 heterodimer compared to the native CCG codon, suggesting a codon-dependent role for the Tma factors in dissociating 40S post-termination complexes at this uORF, even for those penultimate triplets that were shown previously to be Tma-hypodependent for recycling at other stop codons throughout the translome. These findings imply that the deacylated tRNA can dissociate in the absence of Tma factors at both Tma-hyperdependent and Tma-hypodependent penultimate codons, so that loss of Tma-catalyzed recycling of the empty 40S subunit permits elevated REI at uORF4 in the mutant cells (models in (b) panels of Fig. 8A(i)-(ii)). In contrast to findings on short REI-permissive uORFs in mammalian cells, and *ATF4* uORF1 in particular¹³, we found that only one of six Tma-hyperdependent penultimate codons introduced at GCN4 uORF1 conferred reduced REI in *tma20Δtma64Δ* cells. These last findings indicate that

complete list of primers). Subsequently, the amplified DNA was used to transform yeast to confer resistance to antibiotics Geneticin G418 (BioConcept), Nourseothricin (Jena Bioscience), or Hygromycin B (Carl Roth GmbH), respectively, on YPD agar medium. YSG178 (*tma64Δ*) was derived from WT BY4741 (YSG142) by transformation with a *TMA64*-specific *kanMX4* deletion cassette. YSG196 (*tma20Δtma64Δ*) was generated from YSG181 (*tma20Δ*) by transformation with a *TMA64*-specific *natNT2* deletion cassette. YKJ3 (*tma20Δtma22Δtma64Δ*) was generated from YSG196 (*tma20Δtma64Δ*) by transformation with a *TMA22*-specific *hphNT1* cassette. YKJ6 (*tma20Δtma22Δ*) was generated from YSG181 (*tma20Δ*) by transformation with a *TMA22*-specific *natNT2* cassette. Deletion of WT alleles and replacement with the appropriate deletion cassettes was verified by colony PCR⁴² using gene-specific primers SG295 (for *TMA20/YER007C-A*), SG294 (for *TMA64/YDR117C* deletions) and SG296 (for *TMA22/YJR014W* deletions) in combination with universal primer PB238 complementary to all deletion cassettes. Strains YSG181 (*tma20Δ*) and YSG184 (*tma22Δ*) were acquired from the Euroscarf yeast deletion collection.

All plasmids employed in this study are listed in Supplementary Data 2. All uORF4-only reporters were created by replacing the *Sall*-*BstEII* fragment of uORF4-only *GCN4-lacZ* construct p226⁴³ with the appropriate *Sall*-*BstEII* fragments generated by either PCR primer mutagenesis or DNA synthesis. Fragments generated by PCR primer mutagenesis were amplified using common forward primer KJ27 (mapping near the *Sall* cloning site) and the following mutagenic reverse primers (mapping near the *BstEII* site) for constructing the indicated plasmids: primer KJ25 for plasmid pKJ34, primer KJ26 for pKJ35, primer KJ24 for pKJ36, primer KJ78 for pKJ57, primer KJ80 for pKJ58, primer KJ76 for pKJ59, primer KJ74 for pKJ61, primer KJ77 for pKJ62. DNA fragments for constructing pKJ17, pKJ23, pKJ20 and pKJ21 were synthesized by GeneArt Gene Synthesis (Thermo Fisher). All fragment inserts in the final constructs were verified by Sanger sequencing, and the DNA sequences are listed in Supplementary Data 3.

The start-stop constructs pKP76, pKP77 and pKP78 were created by replacing the *Sall*-*BstEII* fragment of the *GCN4-lacZ* construct pSG194³³; a uORF1-only construct created by replacing the coding sequence of uORF1 by that of uORF2) by the appropriate *Sall*-*BstEII* fragments generated by GeneArt Gene Synthesis (Thermo Fisher). These fragments contain point mutations in start codons of uORFs (point mutations in uORFs 1, 2 and 4 in pKP76, point mutations in uORFs 1, 2 and 3 in pKP77 and point mutations in uORFs 2, 3 and 4 in pKP78) and deletions of sense codons between start and stop codons in uORF3 (pKP76), uORF4 (pKP77) and uORF1 (pKP78), respectively, which creates start-stop elements uSt-st3, uSt-st4 and uSt-st1. The mutation scheme of these constructs is also shown in Fig. 2. All fragment inserts in the final constructs were verified by Sanger sequencing, and the DNA sequences are listed in Supplementary Data 3.

All uORF1-only reporters were created by replacing the *Sall*-*BstEII* fragment of WT *GCN4-lacZ* construct p180⁴⁴ with the appropriate *Sall*-*BstEII* fragments, generated by DNA synthesis by LifeSct LLC, containing point mutations in the start codons of uORFs 2–4 (*uORF2* ATG to CTG, *uORF3* ATG to AGG, and *uORF4* ATG to AGG) and either WT uORF1 (plasmid pSG61) or uORF1 variants with altered third codons (pSG62–pSG89). The inserts in the resulting plasmids were sequenced in their entirety to confirm the desired sequences. The sequence of the *Sall*-*BstEII* fragment of pSG61 (with WT uORF1) is listed in Supplementary Data 3; sequences of all other constructs differ only by the replacements of the third uORF1 codon with the triplet indicated in Table S2 for the corresponding plasmids.

GCN4-lacZ reporter assays

β-galactosidase specific activities (in units of nmol of ONPG cleaved per min per mg of protein) were assayed in whole cell extracts as described previously³³ in yeast transformants of uORF4-only and uSt-st *GCN4-lacZ* reporter plasmids, and as previously described⁴⁵ for transformants of uORF1-only reporter plasmids and the “uORF-less” *GCN4-lacZ* reporter p227³⁰. Briefly, cells were transformed with plasmids bearing *GCN4-lacZ*

fusion reporters and grown in synthetic SD media to mid-log phase. Cells were centrifuged, washed with deionized water and broken using glass beads in Breaking Buffer (100 mM Tris 8.0, 4% Glycerol, 1 mM β-mercaptoethanol), then clarified by centrifugation (18,400 × g, 30 min, 4 °C). β-galactosidase activity (measured as nanomoles of o-nitrophenyl-3-Dgalactosidase cleaved per minute per milligram of protein) was normalized to protein concentration of the whole cell extract, which was measured by Bradford assay with BSA as standard.

For the uORF4-only and uSt-st-only reporter assays, the normalized β-galactosidase activity for each reporter construct in transformants of *tmaΔ* strains was determined by dividing the unnormalized β-galactosidase activities by a normalization factor. This factor was calculated separately for each individual experiment by assaying the activity of the “uORF-less” reporter (p227) in WT and *tmaΔ* strains in four transformants under the same conditions as uORF4-only/uSt-st-only reporters. Then, the mean of *tmaΔ* “uORF-less” activities was divided by the mean of WT “uORF-less” activities, determining the normalization factor.

For the uORF1-only reporter assays, β-galactosidase activities determined for each replicate transformant of the *tma20Δtma64Δ* strain were divided by a factor of 1.316 prior to calculating the mean activities to be compared to the mean unnormalized activities for the replicate transformants of the WT strain in a two-tailed, unpaired Student's *t* test to assign *p* values to differences between the means in mutant vs. WT cells. The normalization factor was determined by assaying six transformants each of the same *tma20Δtma64Δ* and WT strains harboring the “uORF-less” reporter (p227) under the same conditions employed for uORF1-only reporters. The *tma20Δtma64Δ*/WT ratio of mean expression values was 1.316, which differed significantly from unity in a two-tailed *t*-test with a *p*-value of 0.003.

Yeast spotting assay

Yeast strains were spotted onto minimal synthetic defined (SD) medium plates in five serial 10-fold dilutions (starting with OD₆₀₀ 0.5) and grown for 48 h at 30 °C.

Multiple sequence alignment and protein structure visualization

Multiple sequence alignment of *S. cerevisiae* Tma20 (UniProt accession P89886), *D. melanogaster* MCTS1 (UniProt accession Q9W445), *C. elegans* C11D2.7 (UniProt accession Q8MXH7) and *H. sapiens* MCTS1 (UniProt accession Q9ULC4) protein sequences was performed using Clustal Omega⁴⁶ with default settings through European Bioinformatics Institute Tools services⁴⁷. Similarity scores and identity percentages for the sequences were determined using the UniProt BLAST tool.

Tma20 protein structure was predicted using the AlphaFold3 algorithm⁴⁸ through AlphaFold Server (Google DeepMind) services. MCTS1/DENR complex structure³⁹ was obtained through PDB accession 6MS4. PyMOL 3.0 was used for visualization of the structures.

Statistics and reproducibility

Statistical significance was determined as follows. For all uORF4-only and Start-stop reporter assays, all data were tested for normality by the Shapiro–Wilk test. Based on the normality test, data were compared either by the parametric, unpaired, two-tailed Welch's *t* test, or by the non-parametric Mann–Whitney test; **** indicates *p* < 0.0001; ****p* < 0.001; ***p* < 0.01; **p* < 0.05; ns non-significant. All individual data points are shown as part of bar plots and the number of replicates is shown above each bar. The SD was calculated from all biological replicates for each construct in each strain. For uORF1-only reporter assays, an unpaired, two-tailed Student's *t* test was employed. Statistical analysis and visualization were performed in GraphPad Prism, version 9.4.1 (GraphPad Software). All replicates shown in the reporter assays graphs were independent transformants—biological replicates.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

All data from this study are available within this paper and its Supplementary Information. All plasmids and strains used in this study are available upon request from the authors. Source data for graphs presented in the main figures can be found at Supplementary Data 1.

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Author contributions

St.Gu., L.S.V. and A.G.H. conceived and designed the project. K.J. and Sw.Ga. carried out majority of experiments and performed the data analysis; they were assisted by K.P. at the onset of this study. K.J., Sw.Ga., L.S.V. and A.G.H. interpreted the results. K.J., L.S.V. and A.G.H. wrote the paper with input from Sw.Ga.

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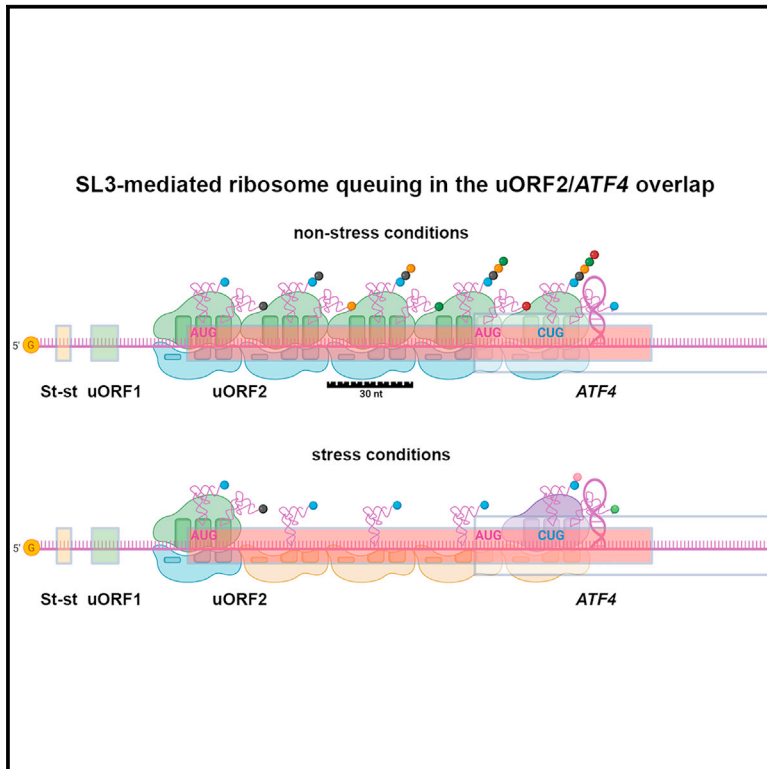
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Stem-loop-induced ribosome queuing in the uORF2/*ATF4* overlap fine-tunes stress-induced human *ATF4* translational control

Graphical abstract



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In brief

Traditionally, *ATF4* translational control was thought to comprise only delayed reinitiation. Smirnova et al. describe additional layers of *ATF4* translational regulation, including substantial leaky scanning of *ATF4*'s AUG1 and ribosome queuing implemented by a stem-loop (SL3). SL3 co-operates with an upstream near-cognate start codon and localizes to the uORF2/*ATF4* overlap.

Highlights

- A highly conserved stem-loop (SL3) occurs in the uORF2/*ATF4* overlap
- SL3 enables ribosome queuing to provide another layer of *ATF4* translational control
- SL3 is preceded by a near-cognate CUG in the *ATF4* frame with which it genetically interacts
- The canonical *ATF4* translation start site is a subject of substantial leaky scanning



Article

Stem-loop-induced ribosome queuing in the uORF2/ATF4 overlap fine-tunes stress-induced human ATF4 translational control

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SUMMARY

Activating transcription factor 4 (ATF4) is a master transcriptional regulator of the integrated stress response, leading cells toward adaptation or death. ATF4's induction under stress was thought to be due to delayed translation reinitiation, where the reinitiation-permissive upstream open reading frame 1 (uORF1) plays a key role. Accumulating evidence challenging this mechanism as the sole source of ATF4 translation control prompted us to investigate additional regulatory routes. We identified a highly conserved stem-loop in the uORF2/ATF4 overlap, immediately preceded by a near-cognate CUG, which introduces another layer of regulation in the form of ribosome queuing. These elements explain how the inhibitory uORF2 can be translated under stress, confirming prior observations but contradicting the original regulatory model. We also identified two highly conserved, potentially modified adenines performing antagonistic roles. Finally, we demonstrated that the canonical ATF4 translation start site is substantially leaky scanned. Thus, ATF4's translational control is more complex than originally described, underpinning its key role in diverse biological processes.

INTRODUCTION

Eukaryotic cells have evolved several mechanisms to cope with various environmental stressors. These include a complex signaling pathway referred to as the integrated stress response (ISR).^{1,2} Whereas its external triggers are oxygen or nutrient deprivation or viral infection, the main internal stressor is the accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (ER). Importantly, the ISR can also be induced by activation of oncogenes.³ The response is "integrated," as all stress signals are transduced by a family of four serine/threonine kinases and converge into a single event, which is the phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2 α).⁴

eIF2 assembles into a ternary complex (TC) together with guanosine triphosphate (GTP) and the initiator Met-tRNA^{Met}. This complex, together with other eIFs, facilitates Met-tRNA^{Met}

recruitment to the ribosomal P site so that the pre-initiation complex (PIC) can recognize the translation start site and initiate translation.⁵ After the AUG selection process, eIF2 with hydrolyzed guanosine diphosphate (GDP) is ejected from the initiation complex. To participate in the next translational cycle, it must be regenerated by the eIF2B-mediated exchange of GDP for GTP.⁶ The ISR-transduced phosphorylation of the α subunit of eIF2 prevents this regeneration step, leading to a significant decrease in TC levels and a general translational shutdown (reviewed in Gunišová et al.⁷ and Dever et al.⁸).

A handful of specific mRNAs escape this shutdown and become efficiently translated to begin adaptation to the acute stress.⁹ One of them encodes the transcription factor ATF4, a master regulator of the ISR.^{10,11} Upon relief from the acute stress, eIF2 α is dephosphorylated, the ISR ceases, and general protein synthesis resumes.¹² However, if the stress is too intense or persists for too long, then it becomes chronic,¹³ the adaptive



response capacity is exhausted, and programmed cell death is triggered. Thus, the final outcome of the ISR depends largely on the level and duration of eIF2 α phosphorylation and, consequently, on the extent of upregulation of factors escaping the shutdown.²

Therefore, the timing and level of ATF4 expression are critical parameters determining cell fate. Even minor disturbances of ATF4 function contribute to serious pathologies, such as the neurodegenerative Parkinson's, Alzheimer's, and Huntington's diseases; prion diseases, and various types of retinal degeneration.¹⁴ Furthermore, persistent overactivation of ATF4, which does not induce apoptosis, is linked to many cancers because of the continuous expression of adaptive genes that sustain the stress response.¹⁵

Under normal conditions, human *ATF4* mRNA is constitutively transcribed at low levels, but the protein is undetectable. Induction of its expression is mediated by the *ATF4* 5' mRNA leader containing two upstream open reading frames (uORFs): a short 4-codon uORF1 followed by a longer 60-codon uORF2, which overlaps with the *ATF4* reading frame in a -1 frame. In analogy with its extensively studied yeast functional homolog *GCN4*,^{7,8,16–18} it has been proposed that the primary route of ATF4 translational control occurs via the so-called delayed translational reinitiation (REI).^{10,11} This model is underpinned by the stress-induced decrease in TC levels and the opposing properties of the two *ATF4* uORFs (Figure 1A).^{7,8}

Specifically, after translation of the REI-permissive uORF1, only the large ribosomal subunit is recycled, while the small subunit remains associated with the mRNA and, upon regaining scanning ability, can reinitiate downstream. This process is enabled by the short length of uORF1 and the presence of stimulatory flanking sequences interacting with the initiation factor eIF3.¹⁹ This interaction allows eIF3 to remain transiently associated with elongating and terminating ribosomes on short uORFs to allow REI.^{20–22}

A key aspect of this delayed REI mechanism is that reacquisition of the scanning competence of the post-termination 40S is limited by its binding of a new TC and that the time required for TC acquisition increases significantly with decreasing TC levels.^{10,11} The original model posits that, in the absence of stress, the 40S acquires the TC relatively quickly, recognizes the uORF2 AUG, and, due to its overlap with *ATF4*, no *ATF4* protein is produced. Conversely, the stress-induced decrease in TC levels delays the TC acquisition by the majority of 40Ss traversing downstream of uORF1. This allows them to skip the AUG of uORF2 and acquire the TC while *en route* to the *ATF4* start codon to induce its synthesis.

Despite the depth of the original studies,^{10,11} a growing body of evidence suggests the existence of additional regulatory modes beyond the delayed REI mechanism. For example, three independent studies have reported substantial translation of uORF2 even under stress.^{23–25} In addition, leaky scanning over uORF2 has been shown to contribute to ATF4 induction.²³ It has been proposed that an N⁶-methyladenosine (m⁶A) post-transcriptional modification in the non-overlapping region of uORF2 of mouse *ATF4* mRNA functions as a barrier to ribosomes scanning downstream of uORF1 and that stress triggers demethylation of m⁶A, removing this block.²⁶ Quantitative translation initiation sequencing (QTI-seq) performed in the same study

also implicitly questioned the true initiation start site of the *ATF4* coding sequence, which begins with three nearly consecutive AUGs. (Note that the *ATF4* transcript in the mouse is largely conserved with the human transcript [see also Figure S1A] and was examined in the original studies.¹⁰) Employing the Selective Translation Complex Profiling (Sel-TCP-seq) technique,²⁷ we showed that the 5' most proximal and so far the least studied uORF0 (hereafter referred to as Start-stop [St-st] because it consists only of an initiation and termination codon), which precedes uORF1 (Figure 1A), represents an additional barrier that may inhibit ATF4 expression in the absence of stress.²² All of these findings suggest a more complex regulatory system exceeding the relatively straightforward mode of delayed REI, whose full understanding may help to explain all aspects of the ATF4 contribution to ISR as its master regulator.

Here, we subjected the human *ATF4* mRNA leader to complex bioinformatics analysis and uncovered several previously unknown *cis*-acting features. Following detailed mutational analysis using our newly developed and well-controlled reporter assay revealed that (1) a highly conserved stem-loop in the uORF2/*ATF4* overlap causes ribosome queuing, and stalled or slowed-down ribosomes may initiate at a near-cognate CUG codon upstream of this stem-loop; (2) the inhibitory uORF2 has an unexpected stress-inducible character and is relatively highly translated even under stress, which could be explained by the ribosome queuing; (3) the first two AUGs of *ATF4* are substantially leaky scanned; and (4) two prospective, highly conserved adenine modification sites contribute to overall regulation in opposing ways, one of them antagonizing the role of the stem-loop. Overall, our data suggest that translational control of ATF4 comprises a multilayered regulatory circuit of diverse sequence and structural elements that fine-tune its expression under different stimuli.

RESULTS

Revisiting translational control of human ATF4

Published analyses of ATF4's translational control were carried out using a mouse *ATF4* mRNA reporter (mouse and possum *ATF4* lack St-st, which is otherwise highly conserved among vertebrates; Figure S1A),²⁸ and luciferase was fused with *ATF4* only two codons downstream of its AUG.¹⁰ The wild type (WT) and specific mutation-carrying reporters were individually co-transfected with a control *Renilla* plasmid into mouse embryonic fibroblast (MEF) cells and luciferase activity was measured. We perceived two potential bottlenecks in this arrangement. (1) In addition to lacking St-st, the original ATF4 reporter also lacked much of the uORF2/*ATF4* overlap (as well as the entire *ATF4* ORF), which limited investigation of its role. (2) Its activity was not normalized to *ATF4* mRNA levels, which are known to change,²⁹ but to *Renilla* activity, which, by definition, drops rapidly upon stress-induced translational shutdown. To resolve these discrepancies, we designed an entirely new ATF4-based reporter, this time using the human sequence, and an experimental workflow that avoided normalizing ATF4 expression levels to any reference genes whose expression would be translationally shut down under stress conditions.

This CMV promoter-driven reporter preserves the entire V2 transcript leader sequence and the entire coding region of

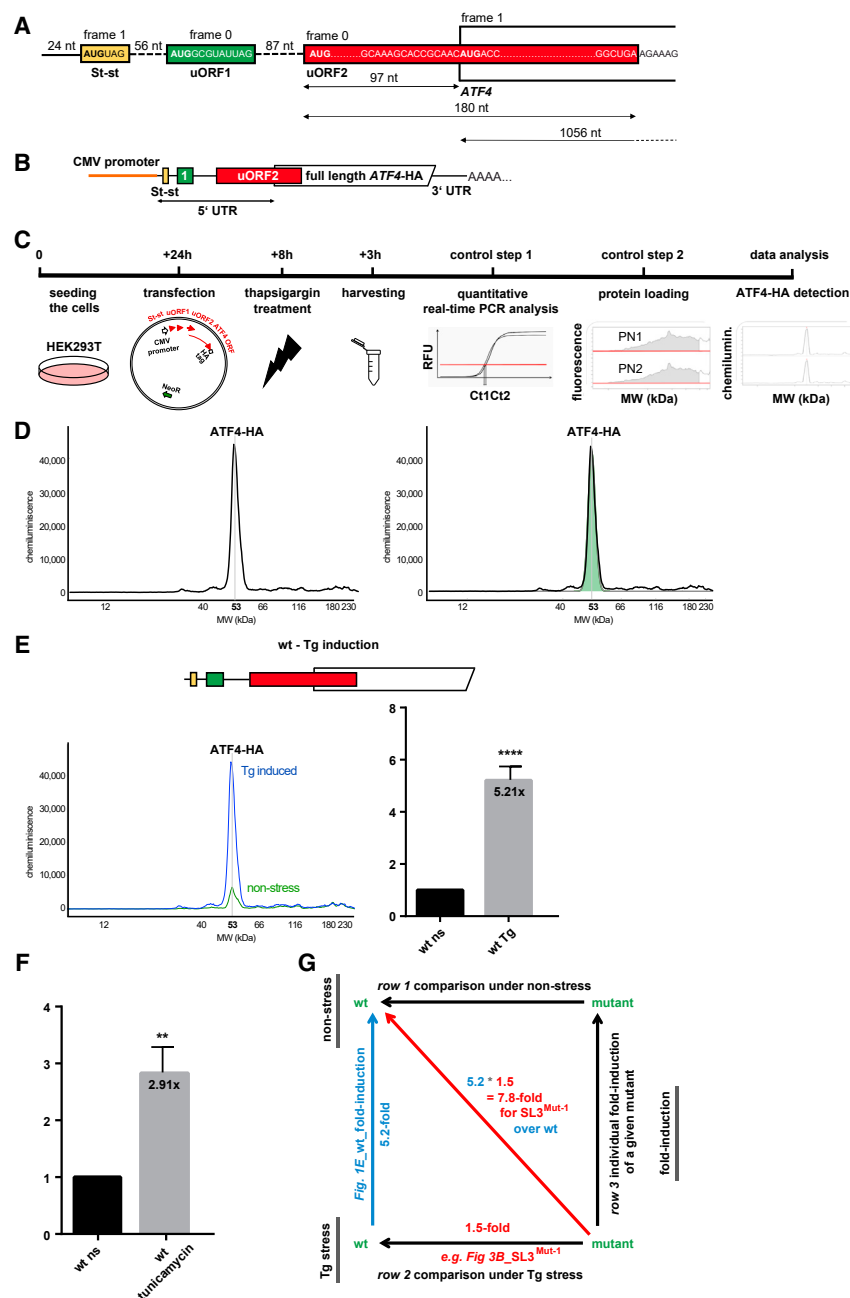


Figure 1. Revisiting translational control of human ATF4: Reporters and experimental setup

(A) Schematic showing the 5' end of the mRNA encoding human activating transcription factor 4 (ATF4; NM_182810.2, transcript variant 2), featuring the color-coded Start-stop (St-st) element (yellow), REI-permissive (green) 3-codon uORF1, and inhibitory uORF2 (red) overlapping with the beginning of the main ATF4 ORF, frames 0 and 1. Distances are given in nucleotides.

(B) Schematic of the WT CMV-driven ATF4-HA-tagged construct; the HA tag was placed immediately upstream of the ATF4 stop codon.

(C) Experimental workflow described in the form of a timeline diagram. PN, Jess protein normalization detection module.

(D) All HEK293T cell lysates were subjected to protein separation and immunodetection using the Jess system. The signal, detected in the capillary, is represented as an electropherogram (a single peak of the ATF4-HA tag full-length protein, size of 53 kDa, left) and was automatically quantified (right). Expression of the WT construct under 3 h of Tg stress conditions 8 h post transfection, detected by anti-mouse HA tag antibodies, is shown.

(E) Stress-induced upregulation of ATF4-HA protein expression under Tg stress (blue) compared with non-stress conditions (green). Quantified “fold induction” data were plotted (n = 17, right). The differences between experimental groups were tested by a t test. Variables are presented as mean ± SD, and p < 0.05 was considered statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

(F) Stress-induced upregulation of the ATF4-HA protein expression from the WT reporter under tunicamycin stress compared with non-stress conditions (set to 1). Quantified “fold induction” data were plotted (n = 3) and analyzed as in (E).

(G) Experimental setup illustrating determination of fold change values when comparing (1) each mutant reporter construct vs. the WT construct separately under non-stress (top horizontal arrow) and Tg stress conditions (bottom horizontal arrow) and (2) the fold induction expression of a mutant under stress with the same mutant under non-stress (right vertical arrow) or the WT under stress vs. no stress (blue left vertical arrow). The red diagonal arrow indicates calculated fold induction changes of a given mutant under stress vs. WT under non-stress conditions; i.e., how much each mutant increases or decreases the ~5.2-fold induction of the WT reporter.

ATF4, which is extended with a hemagglutinin (HA) tag at its C terminus (Figure 1B). Twenty-four hours after seeding, HEK293T cells were individually transfected with the same amount of a control WT reporter or its mutant derivatives, and after 8 h, one-half of the cultures was treated with thapsigargin (Tg; induces the unfolded protein response (UPR)) and the other half with DMSO (control) (Figure 1C). Therefore, the WT control was used as a reference in every single experiment. Exactly 3 h after Tg treatment, cells were harvested using a standard Glo lysis buffer supplemented with protease inhibitors by the vendor (Promega), and the lysates were subjected to a fully quantitative,

automated, and capillary-based high-throughput immunoassay (“western blotting”) using the JessTM Protein Simple instrument, as described in detail in the STAR Methods (Figure 1C).

Figure 1D shows an example of the output data in the form of an electropherogram, which displays the intensity detected along the length of the capillaries; the generated peaks can be quantified by calculating the area under the curve. In case of ATF4-HA, the highly specific signal detected by the anti-HA tag antibodies is displayed as a single peak at 53 kDa and shows ~5.2-fold induction (p < 0.0001) upon Tg treatment 8 h post-transfection (Figure 1E; see Table S1 for average values of fold induction for

the WT construct and Table S2 for individual values from each experiment). Tunicamycin-stressed cells (treated the same way only exposed to stress for 4 h) generated nearly 3-fold induction (Figure 1F; Tables S1 and S2). For statistical analysis, see STAR Methods. Please note that we routinely recorded a slightly increased activity of our reporter even under non-stress conditions (Figure 1E, green peak), the source of which is explained in STAR Methods. This feature of our reporter system proved to be very useful throughout the study because it served as a “background” value used to compare the effects of the mutations on both basal ATF4 expression and its inducibility under Tg stress; this would have been impossible with a background value of zero. For experiments validating that our newly developed reporter system faithfully mimics endogenous ATF4 regulation, please see STAR Methods and Figures S1B–S1D and S2A.

St-st modestly inhibits ATF4 expression, uORF1 allows for 50% downstream REI, and a solitary, inhibitory uORF2 allows for high ATF4 stress inducibility

We began our analysis by testing the effect of the St-st and re-testing the effects of uORFs (on their own) on ATF4 expression by mutating the AUGs of the respective other two elements in the otherwise WT construct (Figures S3A and S3B). First, we measured the control construct (d-all), where AUGs of all three elements were mutated. This construct determined the maximal expression level of ATF4, which was ~22.7-fold and ~3.3-fold higher under “non-stress” and “Tg stress” conditions, respectively, compared with the values of the WT construct that were set to 1 for each of these two conditions individually (Figures S3A and S3B; Tables S3, S4). The last plot in the d-all column (“fold induction”) with the d-all non-stress value set to 1 indicates that this construct is expectedly not stress inducible, in contrast to the WT (Figures 1E and S3C).

Note that all “fold induction” experiments throughout the study (e.g., row 3 of Figure S3A) were conducted separately from the “non-stress” and “Tg stress” experiments (e.g., rows 1 and 2, Figure S3A). To further clarify our setup, in the “non-stress” and “Tg stress” experiments, we compared individual mutants with the WT separately under the respective conditions, whereas in the “fold induction” experiments, we compared expression of a given mutant under stress with that of the same mutant under no stress (DMSO) (Figure 1G). This means that both sets of experiments were individually controlled for the equal amount of reporter mRNA and protein levels, as described above and in STAR Methods.

Our results, presented in detail in STAR Methods and Figure S3, confirmed major aspects of the original model and supported the idea that St-st acts as a general repressive element (i.e., a roadblock), as observed before.^{22,28} Unexpectedly, they also revealed a stress induction capability of the uORF2-only reporter of an unknown mechanism (see below).

Sequence analysis of the 5' UTR of the human ATF4 mRNA reveals additional elements potentially contributing to ATF4 translational control

Next, we carefully screened the ATF4's 5' UTR and the uORF2/ATF4 overlap in the coding region for sequence and structure features that might suggest additional modes of regulation (Fig-

ure 2A). Their detailed description is provided in STAR Methods. The most notable element, predicted by our analysis, is a stem-loop (SL3), with $\Delta G = -15.40$ kcal/mol, that is highly conserved among vertebrates (Figure S1A) and could be inhibitory. It is located roughly in the middle of the uORF2/ATF4 overlap (Figure 2A) and has a hairpin structure with the highest free energy predicted for this particular sequence region. Our analysis also predicted a total of four potential sites of m⁶A methylation within well-defined motifs (Figure 2A). Interestingly, the ATF4 gene begins with two nearly consecutive AUGs followed by a third in proximity, all in frame (Figure 2A, dark green). Both the canonical AUG1 and AUG2 have a medium Kozak initiation context; AUG1 overlaps with DRACH2, and AUG2 is located two codons downstream. AUG3 has a weak Kozak initiation context and is exposed in the open loop of SL3. It represents the 17th codon downstream of AUG1. Both AUG2 and AUG3 are highly conserved among vertebrates (Figure S1A).

Given this knowledge, we created an array of mutants for all of these predicted elements (Figure 2A) and first evaluated them in a large-scale screen. Subsequently, we focused our analysis on mutations in elements that had observable effects, like SL3.

SL3 delays the flow of ribosomes in the uORF2/ATF4 overlap and genetically interacts with the upstream near-cognate CUG codon

Next, we unfolded SL3 by either quadruple C-to-A (in SL3^{Mut-1}) or GGG to AUC (in SL3^{Mut-2}) substitutions (Figure 2A) and observed ATF4 expression to increase by ~1.3- and ~1.7-fold under non-stress and by ~1.5- and ~1.8-fold under Tg stress conditions, respectively, over the WT construct set to 1 (Figure 3B, rows 1 and 2; Tables S5 and S6). This means that SL3 mutations add a further ~50%–80% increase (Figure 3B, row 2) to the ~5.2-fold induction of the WT reporter (Figure 3A) to reach the robust ~7.8- to 9.4-fold induction (5.2 multiplied by 1.5 or 1.8) under stress conditions. Figure 1G illustrates how these fold induction changes were calculated. Accordingly, loosening SL3 also increased the inducibility of both of these mutant constructs, measured and compared separately under stress vs. non-stress conditions, from ~5.2-fold to ~5.7- to 5.9-fold (Figure 3A vs. Figure 3B). These results suggest that SL3 forms and acts as physical barrier, delaying scanning/translating ribosomes under both conditions.

Eliminating SL3 in the d2 construct (in d2-SL3^{Mut-1}) showed a similar increase over the d2 construct alone as in the case of the wt-SL3^{Mut-1} mutant over the WT (Figure 3B, rows 1 and 2; Tables S5 and S6). Note that the overall induction (d2-SL3^{Mut-1} over the WT) under Tg stress was robustly increased by ~17.5-fold (Figure 3B, row 2). These results strongly suggest that both uORF2 and SL3 act together to reduce ATF4 translation.

Initiating 48S PIC with the start site placed in the P site covers ~12 nt from the mRNA entry site up to the P site (excluding it) and ~15–25 nt downstream of the P site (including it) to the mRNA exit site (Figure 2B).^{22,27} We noticed a near-cognate CUG codon in weak Kozak context positioned 20 nt upstream of SL3 (Figure 2A), which is highly conserved among vertebrates (Figure S1A). This position is ideal to force a scanning 48S PIC stalled at or slowed by SL3 to initiate at CUG (Figure 2B). To test this hypothesis, we replaced CUG with CUA (in CUG^{Mut})

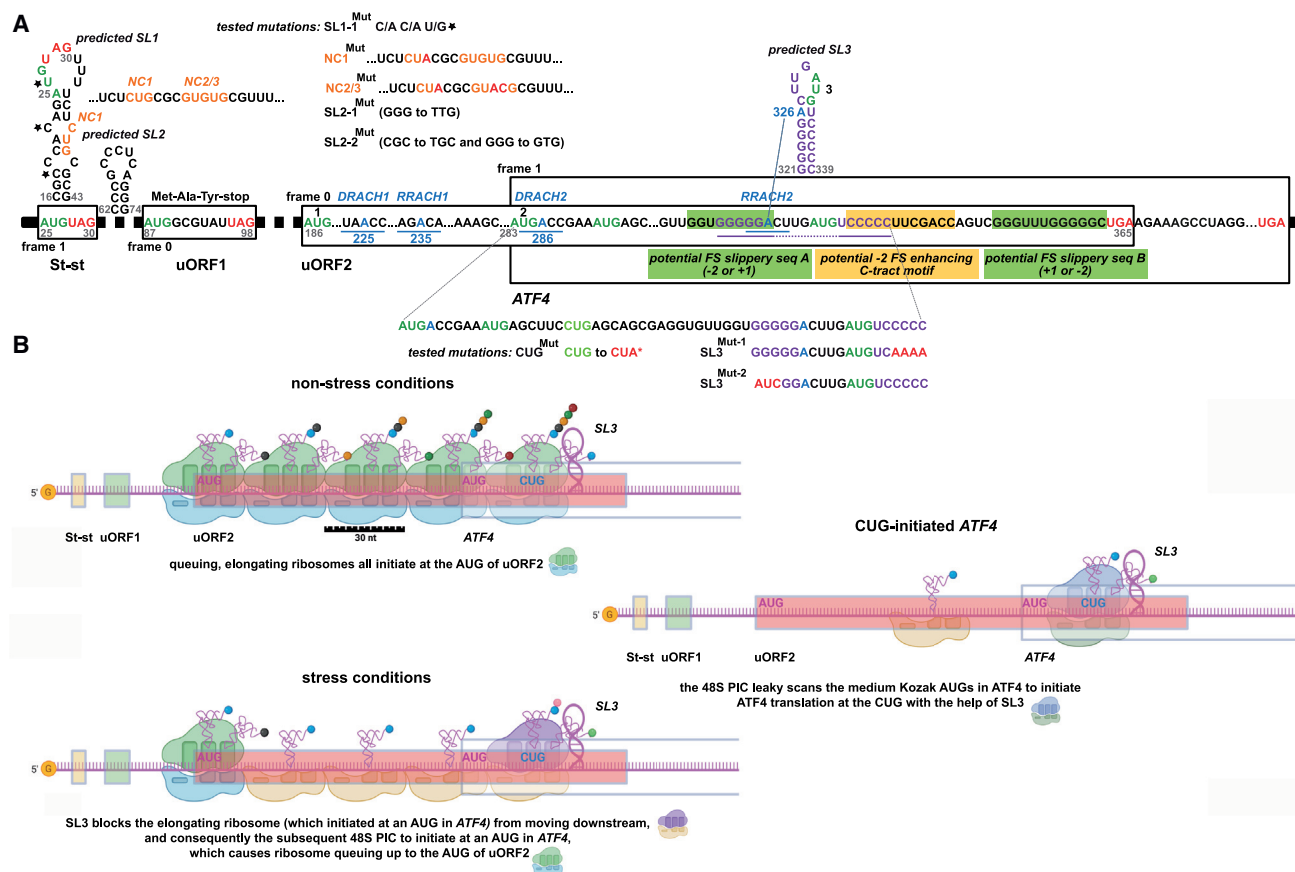


Figure 2. Sequence analysis of the 5' UTR of the human *ATF4* mRNA and ribosome queuing model expanding the mode of *ATF4* translational control

(A) Schematic of previously unknown, bioinformatically predicted, potential regulatory features within the 5' UTR of the *ATF4* mRNA and beginning of the *ATF4* main ORF; point mutations are depicted. For details, please see the main text.
 (B) Model of the ribosome queuing mechanism under non-stress vs. stress condition employing SL3 and near-cognate CUG as an additional layer of *ATF4* translational control. For details, please see the main text. Created with [BioRender](#).

and observed that the mutation significantly reduced *ATF4* expression by ~20% under both conditions tested, which corresponds to a 4.2-fold reduction (5.2 of the WT construct multiplied by 0.8) of “fold induction” under stress (Figure 4B, center, row 2; Tables S7 and S8).

These findings suggest that a small proportion of the *ATF4* protein might be several amino acids shorter due to this CUG-initiated synthesis downstream of the main initiation site. Interestingly, combining CUG and SL3 mutations in a single SL3^{Mut-1}-CUG^{Mut} construct nullified the opposing effects of the two individual mutations, suggesting a functional interaction between the two elements (Figure 4B, right; Tables S7 and S8). Specifically, when the translation-inhibitory SL3 was removed, the translation-enhancing CUG was no longer needed, perhaps because a flux of ribosomes initiating at the *ATF4* was released to maximize the synthesis of the full-length *ATF4* protein. It remains to be investigated whether the CUG-initiated *ATF4* protein is stably produced in WT cells.

To further investigate the functional interplay between SL3 and CUG, we analyzed three publicly available ribosome sequencing

(ribo-seq) libraries generated from cells exposed to various ER stressors; namely, two sets of HEK293T cells treated with arsenite^{24,30} and HeLa cells subjected to tunicamycin-induced stress.³¹ We compared the total number of footprints (FPs) in the *ATF4* mRNA leader under stressed vs. non-stressed cells (Figure S4) as well as the distribution of FPs with ribosomes positioned with their P site to a specific codon (Figure S5). In addition to the pile-ups of FPs on the St-st and AUGs of uORF1 and uORF2 (the latter two are observed in only two datasets; Figures S4A–S4D), all three datasets clearly revealed a pile-up of FPs ~20 nt upstream of SL3, with a visible reduction under stress (Figure S5). In contrast, there were virtually no FPs on the AUG1 of *ATF4*, even under stress, while the remainder of the coding region was correctly stress induced in all datasets. A closer look at the FPs indicated a strong preference of the P site locating to the CUG in all three datasets (Figure S5). We argue that, even though the FP coverage in the uORF2/*ATF4* overlap upstream of SL3 was relatively low, the repeated occurrence of this prominent “CUG” peak in 3 independent datasets did not occur by chance. Therefore, the existence and

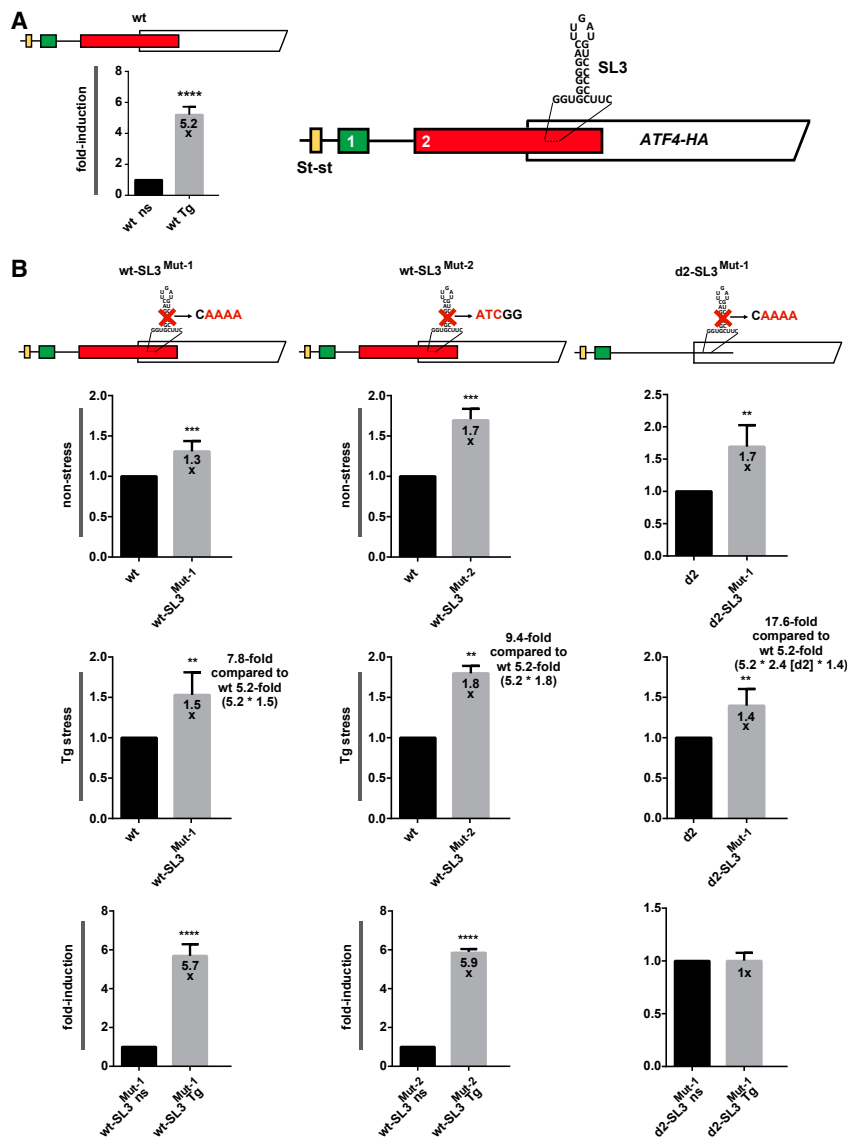


Figure 3. SL3 delays the flow of ribosomes in the uORF2/ATF4 overlap

(A) Same as Figure 1E for better comparison.

(B) Same as Figure 1E except that the SL3 ATF4 mutant constructs depicted at the top of the corresponding panels were subjected to Jess analyses. Relative ATF4-HA protein expression levels were plotted as ratios of values obtained with an indicated mutant construct vs. the WT set to 1 under “non-stress” (row 1) and “Tg stress” (row 2) conditions; “fold-induction” plots (row 3) depict ratios of Tg stress vs. non-stress values obtained with a given mutant construct. The differences between experimental groups were tested by the t test, except wt-SL3^{Mut-1} Tg stress and d2-SL3^{Mut-1} non-stress, where a Mann-Whitney test was used ($n \geq 3$).

tioned with its P site on the AUG of uORF2. The ribosome queue would not be resolved until the barrier posed by SL3 was breached (Figure 2B).

According to this model, under stress, the first 80S ribosome initiating at the AUG of ATF4 would soon be halted or at least slowed down by SL3, preventing the incoming 48S PIC from moving forward to reach the ATF4’s AUG due to a spatial constraint (Figure 2B). This constraint would then be extended into a queue of 48S PICs that would span up to the AUG of uORF2. Consequently, the most 5’ 48S PIC could potentially initiate at uORF2, triggering its translation under stress, although the length of such a mixed queue of 48S PICs and 80S ribosomes is hard to predict due to the variable FP length of scanning 48S PICs. In any case, this model could explain that, contrary to what the “delayed REI” implies, uORF2 is translated even under stress at substantial levels,

which has been observed by us (see below) and others.^{23–25}

Ribosome queuing contributes to the overall translational control of ATF4

Our model would also imply that, thanks to SL3, CUG could be utilized as an alternative start site for all 40S ribosomes that leaky scan the ATF4’s AUG (Figure 2B), and below we demonstrate that this AUG is indeed rather “leaky.” Such an intricate system would ensure well-balanced induction of ATF4, which is highly desirable given its key role.¹⁴

The existence of SL3 and its interplay with the upstream CUG intrigued us because it could trigger (1) uORF2-to-ATF4 frame-shifting (STAR Methods) or (2) ribosome queuing; i.e., the phenomenon implicated, for example, in translational control of the antizyme inhibitor mRNA.³² Specifically, under non-stress conditions, SL3 could form a queue of 80S ribosomes elongating from the AUG of uORF2, reducing its translation rate and, thus, completely eliminating any leaky scanning. This, in turn, would result in even tighter suppression of ATF4 translation under normal conditions. Indeed, based on an 80S ribosome’s average FP length of 30 nt,^{33,34} altogether five 80S ribosomes could be accommodated in this queue, with the most 5’ ribosome posi-

To test this model, we extended the coding sequence of ATF4 by inserting a 6 × c-Myc tag (30 nt each) just after AUG1 (Figure 5; Tables S9 and S10); i.e., two codons upstream of AUG2 (Figure 5A). If our logic was correct, then this extension would increase ATF4 expression in the otherwise WT construct under both stress and non-stress conditions while having no effect in the absence of SL3 because extending the spacing between SL3 and the AUGs of uORF2 and ATF4 should eliminate SL3’s negative effect. This is exactly what we observed (Figure 5B). Further supporting this model, unfolding SL3 in the absence of

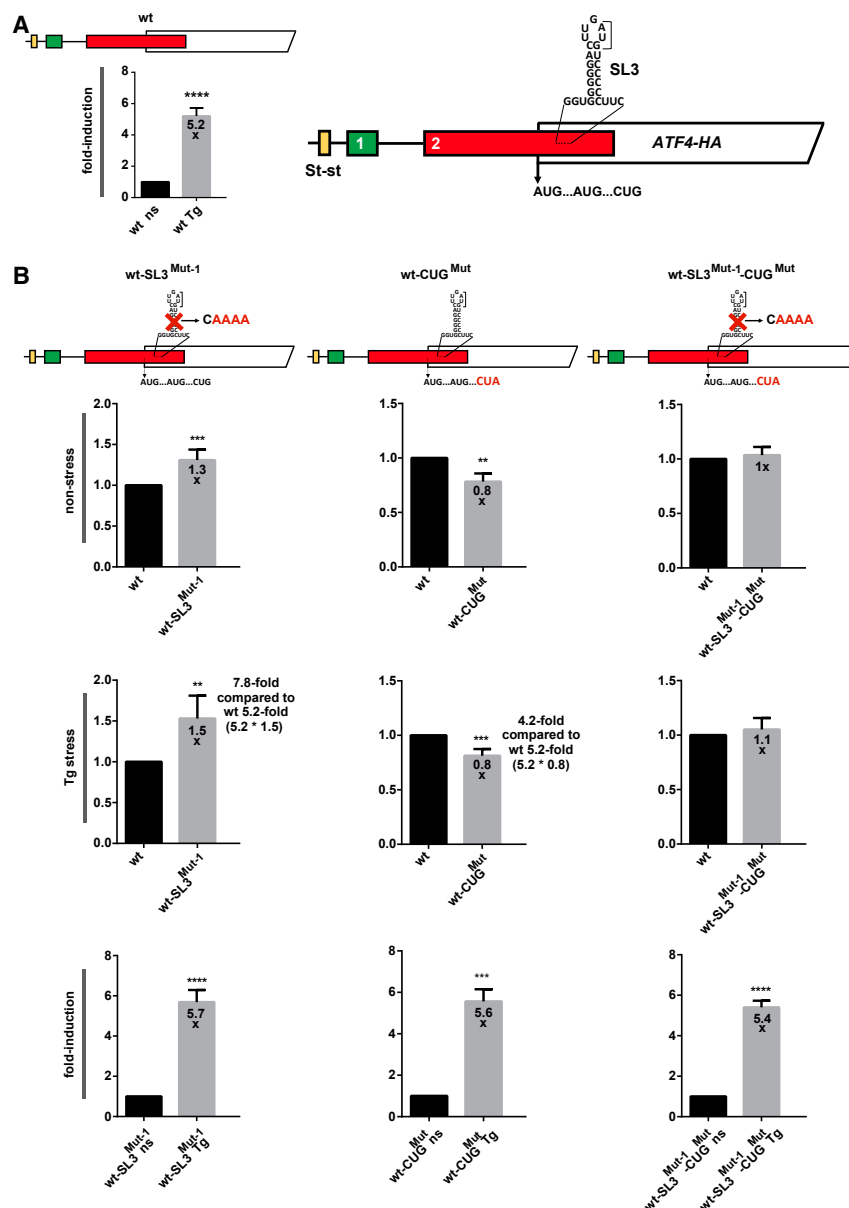


Figure 4. SL3 genetically interacts with the upstream CUG near-cognate codon

(A) Same as Figure 1E for better comparison.

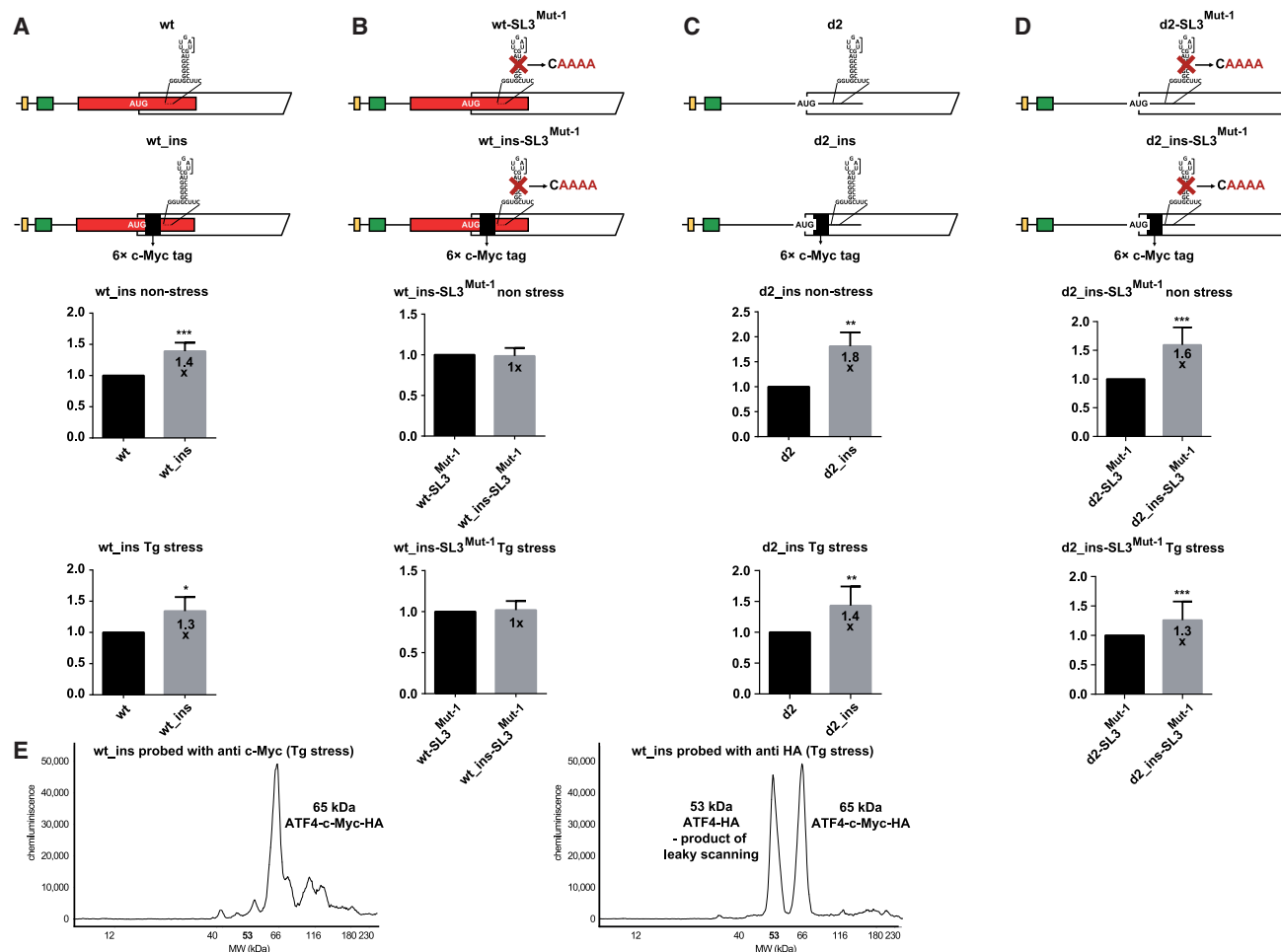
(B) Same as Figure 3B except that the CUG to CUA and SL3^{Mut-1} ATF4 mutant constructs depicted at the top of the corresponding panels were subjected to Jess analyses (n ≥ 3).

To further support the ribosome queuing hypothesis, we treated the non-stressed and stressed HEK293T cells (without any reporter) with the cross-linking agent formaldehyde (HCHO) or control (non-cross-linking) cycloheximide, lysed the cells, applied RNase I, isolated total RNA, and subjected the resulting RNA sample to RT-qPCR. For this ribosome protection assay, three sets of primers were used: A1 (for amplicon 1), covering almost the entire putative queuing region (132 nt of a total of ~150 nt, beginning with uORF2's AUG and ending right in front of SL3); A2 of a similar length, covering the region immediately downstream of SL3; and A3 of a similar length, covering the region in the middle of the ATF4 CDS (Figure 6A). Primer sets A2 and A3 were used independently for normalization and were designed based on the publicly available ribo-seq data used in this study (Figures S6B, S4, and S5) in the regions with detectable but low ribosome coverage. All 3 primer pairs had comparable efficiency ranging from 1.95 to 2.05 and generated fragments of the expected length upon RNase I treatment, as also verified by sequencing (data not shown). We reasoned that, if ribosomes were queued, then they should protect the putative queuing region (A1) from the RNase I digestion more so than the other two control regions (A2 and A3). Accordingly, we found a robust enrichment of A1 over A2 in stressed (~42-fold)

and also to a lesser but still very high extent (~11-fold) in non-stressed cells in the RNase I-digested HCHO samples compared with undigested samples (Figure 6B; for raw data, see Data S1). Consistently, the cycloheximide non-cross-linking control, which, by definition, should protect queued 80S ribosomes with lower efficiency, showed a much smaller difference (~3-fold); moreover, regardless of stress (Figure 6C). Importantly, given that only HCHO, but not cycloheximide, can protect 40S-bound mRNAs, the clear difference in fragment protection under stress vs. non-stress conditions observed in RNase I-digested HCHO-cross-linked samples suggests the existence of a mixed queue of 48S PICs and 80S ribosomes under stress. Collectively, these data further support our model proposing the formation of the 80S queue under non-stress conditions and a

uORF2 (d2_ins-SL3^{Mut-1}) did not eliminate the stimulatory effect of the 6× c-Myc tag extension (Figures 5C and 5D). These results further underscore the observed interplay between uORF2 and SL3 by showing that the SL3 effect relies largely on initiation at the AUG of uORF2 (see also below). Importantly, while the c-myc detection revealed only a single peak of 65 kDa generated by the wt_ins construct, as expected given the 6× c-Myc tag insertion, the HA probing detected an additional peak just above, running at the size of the original ATF4 protein (53 kDa; Figure 5E). This peak could only be explained by a shorter ATF4 variant(s) produced by initiation at AUG2 and/or the CUG and/or AUG3, in further support of substantial leaky scanning at AUG1, as inferred above.

and also to a lesser but still very high extent (~11-fold) in non-stressed cells in the RNase I-digested HCHO samples compared with undigested samples (Figure 6B; for raw data, see Data S1). Consistently, the cycloheximide non-cross-linking control, which, by definition, should protect queued 80S ribosomes with lower efficiency, showed a much smaller difference (~3-fold); moreover, regardless of stress (Figure 6C). Importantly, given that only HCHO, but not cycloheximide, can protect 40S-bound mRNAs, the clear difference in fragment protection under stress vs. non-stress conditions observed in RNase I-digested HCHO-cross-linked samples suggests the existence of a mixed queue of 48S PICs and 80S ribosomes under stress. Collectively, these data further support our model proposing the formation of the 80S queue under non-stress conditions and a



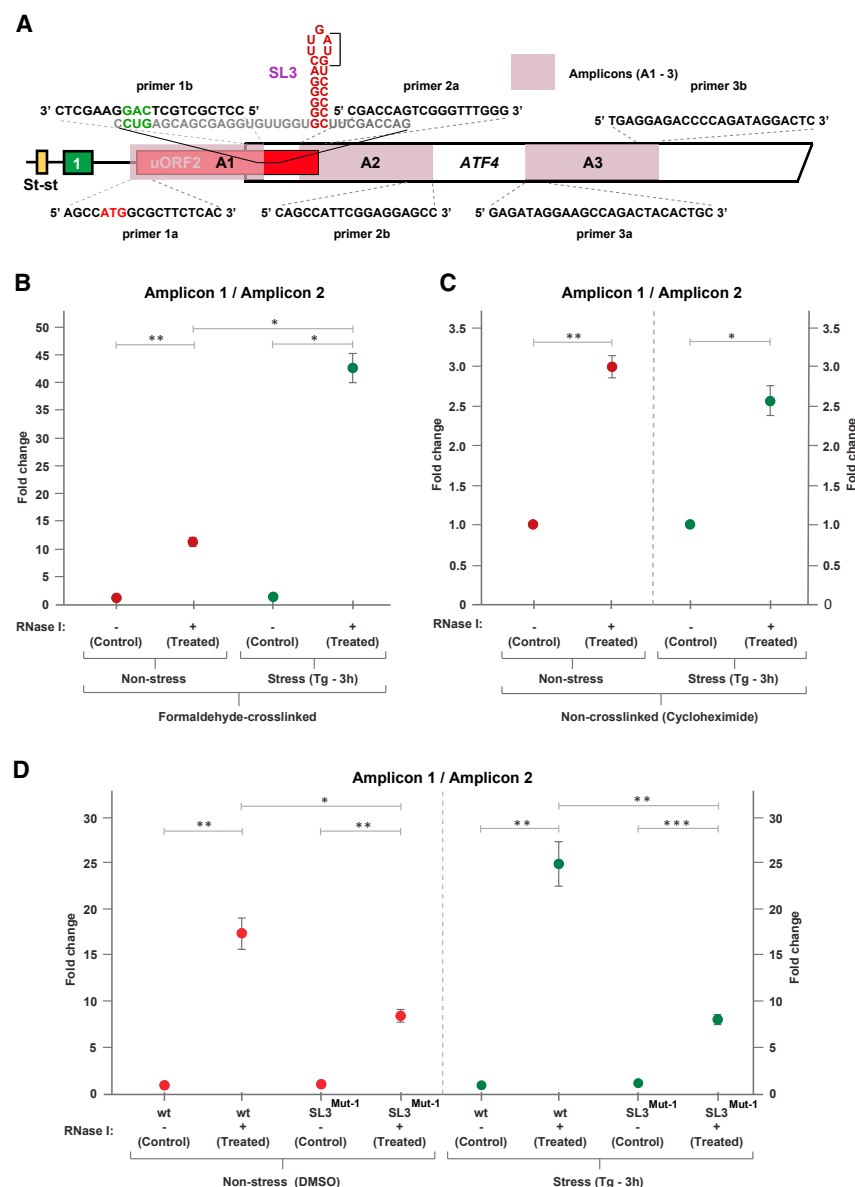
mixed queue under stress conditions (Figure 2B), the latter of which seems to be more prevalent (Figure 6B). Note that similar results were also obtained when A1 was compared with A3 (Figures S6A and S6B; Data S1).

Importantly, to demonstrate that it is the SL3 that prompts ribosome queuing, we repeated the ribosome-protection assay, but instead of non-transfected cells, we employed HEK293T cells transiently transfected with plasmids carrying either WT or SL3-mutated (SL3^{Mut-1}) *ATF4* reporters. As shown in Figures 6D and S6C (Data S1), the SL3 elimination significantly reduced the difference in fragment protection efficiency of A1 over A2 and A3 under both non-stress (by >2-fold) and stress conditions (by >3-fold). The fact that the fragment protection was not abolished completely by the SL3^{Mut-1} mutation is naturally caused by the presence of the endogenous *ATF4* mRNA with the fully preserved SL3.

Finally, we employed the recently developed RiboCrypt tool (<https://ribocrypt.org>) to analyze the disome sequencing (dis-

ome-seq) data (mRNA fragments protected by two stacked ribosomes) in a study where eIF5A, generally recognized as a ribosome rescue factor at polyproline or proline-proline-glycine stretches,^{35,36} was knocked down.³⁷ We observed by far the most prominent disome peak downstream of the uORF2 start codon (5' ends of the reads were mapped), followed by a much smaller and less sharp peak about 60 nt downstream and another smaller peak upstream of SL3; i.e., another 60 nt downstream, in WT cells (Figure S6E).

Thus, compared with the rest of the leader sequence, except for the extreme 5' disome peak of unknown origin, the uORF2/*ATF4* overlap region showed considerable disome coverage, with the two peaks set apart by 60 nt (the expected size of a disome). This fits well with our model predicting a queue of five ribosomes, with the first one in the queue located with its P site on the AUG of uORF2, together covering the region of roughly 150 nt. Interestingly, in the eIF5A knockdown cells,



the major peak downstream of uORF2 practically disappeared, as did the extreme 5' disome peak, while the coverage of the entire stretch of the following 60 nt downstream of AUG of uORF2 increased substantially. Although we cannot explain the loss of the two major peaks, we propose that the increased coverage of this relatively broad region suggests defective collision clearance due to the lack of eIF5A, further supporting our queuing hypothesis.

uORF2 translation under stress is more prevalent than expected

To further investigate the characteristics of uORF2 translation under stress, we next tested for evidence of uORF2-to-ATF4 frameshifting. As presented in detail in STAR Methods, multiple approaches completely ruled out this possibility but clearly sug-

Figure 6. Ribosome protection assay demonstrating that SL3 pauses ribosomes and prompts their queuing under both non-stress and stress conditions

(A) Schematic showing the ATF4 mRNA with the sequences of three primer pairs amplifying three different amplicons (A1–A3; also indicated in Figure S6E): 1a–1b (the latter shown in the 3' to 5' direction for better illustrative purposes) for the putative queuing fragment and 2a–2b and 3a–3b for two control fragments downstream of SL3 used in the ribosome protection assay.

(B) HEK293T cells were cross-linked with formaldehyde (HCHO) and then subjected to the ribosome protection assay as described in STAR Methods. qPCR product levels of the recovered putative queuing region (A1) are normalized to the region immediately downstream of SL3 (A2) as well as to the internal RNA isolation control (SPIKE) with the non-stress values set to 1. Results are representative of three independent replicates, and values are expressed as mean $\pm$ SD. Statistical significance was assessed using unpaired two-sided t test (* $p < 0.01$, ** $p < 0.001$) with Bonferroni correction.

(C) HEK293T cells were treated with cycloheximide (a non-cross-linking agent) and then subjected to the ribosome-protection assay as described in STAR Methods. Results from three independent replicates were analyzed as described in (B) with non-stress values set to 1 (* $p < 0.01$, ** $p < 0.001$).

(D) HEK293T cells were transiently transfected with plasmids carrying either WT or SL3-mutated (in SL3^{Mut-1}) ATF4 reporters and treated as described in (B). Results from three independent replicates were analyzed as described in (B) with the WT values set to 1 (* $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$).

gested that ATF4 might be translated from several alternative start sites, and all of the resulting variants are induced by uORF2 (Figures S7–S9; Tables S11–S14).

Noteworthy, the final piece of evidence for the absence of frameshifting arose from an insertion of a 10 $\times$ c-Myc tag into the uORF2 frame exactly 21 nt downstream of its AUG (Figure S7B,

uORF2_ins). Strikingly, using this construct, we also observed that the 10 $\times$ c-Myc tag insertion in uORF2 behaved similarly as the 6 $\times$ c-Myc insertion past the AUG1 of ATF4 with respect to the role of SL3. In contrast to the WT, where mutating inhibitory SL3 increased the ATF4 expression (Figure 4A), unfolding SL3 in the 10 $\times$ c-Myc tag insertion in uORF2 had no effect (Figure S10A, left column; Tables S15 and S16) under non-stress conditions, the same as in the case of the 6 $\times$ c-Myc insertion past the AUG1 of ATF4 (Figure S10B; Tables S17 and S18), and even decreased ATF4 expression under stress. The uORF2 levels (probed with c-myc) seemed to be unchanged with or without SL3 under both conditions (Figure S10A, right column; Tables S15 and S16), as would be expected. Importantly, even with this SL3 mutant and uORF2-extended construct, we reproduced the unexpectedly small reduction

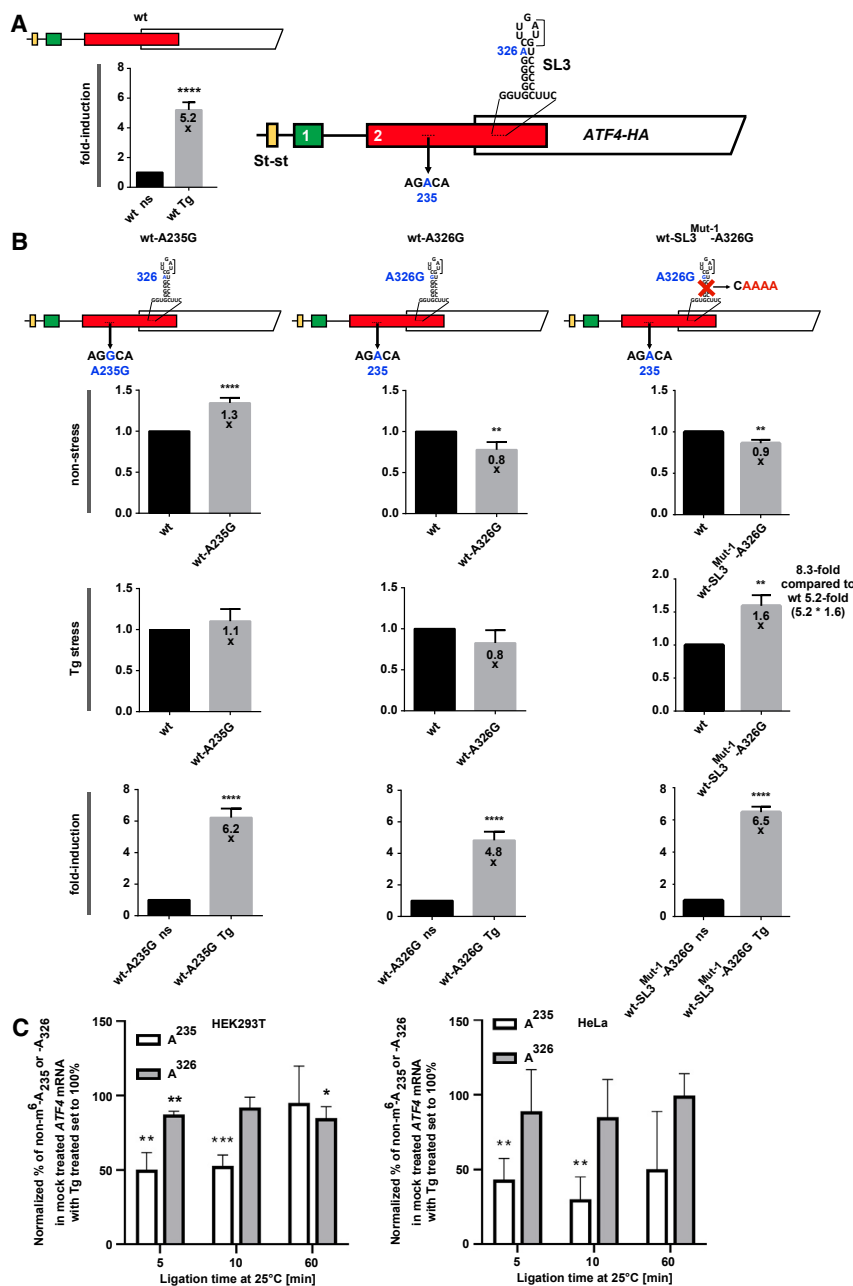


Figure 7. mRNA methylation further fine-tunes ATF4 translation

(A) Same as Figure 1E for better comparison.

(B) Same as Figure 3B except that A235G (left) and A326G either alone (center) or in combination with SL3^{Mut-1} (right) ATF4 mutant constructs depicted at the top of the corresponding panels were subjected to Jess analyses ($n \geq 3$).

(C) mRNA fragments prepared from either HEK293T (left) or HeLa (right) cells carrying the A235G and A326G mutations were subjected to the T3 ligation assay as described in the main text. The normalized percentage of unmodified A₂₃₅ or A₃₂₆ bases of the ATF4 mRNA expressed in mock-treated vs. Tg-treated cells, with the latter set to 100%, was plotted as shown ($n \geq 4$).

mRNA methylation further fine-tunes ATF4 translation

It has been proposed that, under non-stress conditions, A225 in the non-overlapping region of uORF2 in mouse ATF4 mRNA is modified by m⁶A, which functions as a barrier, blocking access of ribosomes scanning downstream of uORF1 toward the ATF4 ORF.²⁶ (Please note that in the NCBI reference sequence NM_001287180.1 used here, mouse A225 corresponds to mouse A258; however, for clarity, we refer to it as it was originally described: A225.) Upon stress, this m⁶A is supposedly demethylated, thereby unblocking access. Of the four potential methylation sites we identified computationally, we chose to investigate the effect of human A235 (NCBI reference sequence NM_182810.2), as it seemed to match the mouse A225 best with respect to position (not sequence), and human A326, which is located within SL3 and could thus modify its function.

Both sites, highly conserved among vertebrates (Figure S1A), were substituted with unmodifiable Gs. While A235G significantly increased (by 30% compared with the WT), the basal level of ATF4 expression under non-stress conditions, as originally

(by ~40%) in the uORF2-c-Myc expression level under Tg stress (compare Figure S10A, right column, “fold induction”, and Figure S7B), further strengthening the interpretation that uORF2 is translated at a much higher level even under stress than could be expected based on the original model. Collectively, our data suggests that the exact placement of SL3 within the uORF2 coding sequence with a defined length represents a precise molecular design that expands the existing delayed REI model to a more complex model that includes ribosome queuing to ensure well-tuned stimulation of ATF4 expression.

reported,²⁶ A326G reduced expression by ~20% under both conditions (Figure 7B, left and center; Table S19; Table S20), indicating that they may have opposing effects. To support these findings, we performed a T3 ligation assay^{38,39} and investigated changes in the modification (possibly methylation) status of A235 and A326 in native ATF4 transcripts isolated from a pool of poly(A) mRNAs from HEK293T and HeLa cells under non-stress and stress conditions. The T3 ligase is sensitive to modification sites like m⁶A during the ligation reaction in qPCR reactions.^{38,39} We first designed specific L (left) and R (right) probes based on the sequences flanking the two prospective m⁶A sites of

interest: *ATF4*-A235 (probes L1 and R1) and *ATF4*-A326 (probes L2 and R2). After ligation, the resulting fragment was amplified by qPCR using universal primers that were complementary to the same adapter sequences attached to all L and R probes.³⁸ Because the presence of m⁶A significantly reduces the T3 ligase efficiency, less effective qPCR amplification determines under which conditions (non-stress vs. stressed) the particular A is modified (possibly methylated). For control and normalization purposes, we also designed two additional probe sets complementary to two regions bearing A residues but not present in any DRACH motifs: *ATF4*-A267 (probes L3 and R3) and *ATF4*-A311 (probes L4 and R4). Any changes in the amplification patterns of qPCR fragments resulting from their ligation therefore served as a quantitative measure reflecting changes in the abundance of *ATF4* transcripts in total poly(A) mRNAs isolates under either non-stress or stress conditions.

Ligation reaction time intervals of 5 and 10 min revealed that the *ATF4* mRNA region containing the A235 but not the A326 residue had an ~30%–50% reduction in ligation efficiency under non-stressed conditions (Figures 7C; Table S21). This result suggests that modification (possibly methylation) of A235 might indeed contribute to inhibition of *ATF4* translation under normal conditions. To the best of our knowledge, modification at A235 has not yet been described for human *ATF4* mRNA. Therefore, it is difficult to predict whether A235 is modified with m⁶A, m⁶Am, m¹A or something else. Nonetheless, the direct detection of m⁶A in the homologous region of the mouse *ATF4* transcript by Zhou et al.²⁶ with a similar phenotype when mutated strongly suggests that human *ATF4* A235 is also m⁶A-modified and serves as a barrier to ribosome progression, like SL3, but only under non-stress conditions.

Although our T3 ligation assay did not confirm that A326 is also modified, the fact that it may stimulate basal *ATF4* expression, at least based on our reporter assays (Figure 7B), and that it is situated in the open loop of inhibitory SL3, prompted us to test the effect of the A326G SL3^{Mut-1} and A235G SL3^{Mut-1} (as a control) double mutations. Whereas the latter phenocopied the effect of SL3^{Mut-1} alone under both conditions tested (data not shown), the former double mutation abolished the stimulatory effect of the SL3 elimination on *ATF4* expression, the same as the CUG^{Mut}, but only under non-stress conditions (Figure 7B, right; Tables S22 and S23).

These findings indicate that the residue A326 plays a stimulatory role under non-stress conditions and acts in concert with SL3, of which it is a part. Overall, our data suggest the importance of the A326 residue specifically under non-stress conditions, as its mutation to G renders SL3 even more inhibitory. At the same time, it neutralizes a stimulatory effect of the SL3 elimination. To reconcile these ostensibly contradictory observations, we propose that, under normal conditions, potentially modified A326 destabilizes the inhibitory SL3 to balance its effect but requires SL3 to be unfolded to fully unleash its stimulatory potential. The precise molecular mechanism is unknown, nor is it not known why it does not work the same way under stress conditions.

DISCUSSION

The work presented capitalizes on our newly developed human *ATF4*-based reporter using an experimental workflow that

avoided normalizing *ATF4* expression levels to any reference genes whose expression would be translationally shut down under stress conditions. It extends the original model of delayed reinitiation to control *ATF4* translation and explains some newer evidence that has challenged some of its paradigms. In particular, we uncovered an additional layer of regulation of *ATF4* expression implemented by stable SL3 immediately downstream of a substantially leaky AUG1 of *ATF4*, co-operating with a near-cognate start codon (CUG), all inside the region overlapping with uORF2. Detailed analysis of this uORF2/*ATF4* overlap region suggests ribosome queuing upstream of SL3, which may result in initiation at CUG and unexpectedly high translation of uORF2 even under stress, which the original model did not consider. Furthermore, our results confirmed, for human *ATF4*, a translation-inhibitory role of m⁶A modification of A235 observed in the mouse²⁶ and identified a previously unknown, potential modification site (A326) antagonizing the SL3 function under normal conditions. Therefore, we propose that the *ATF4* regulatory “armamentarium” is much more complex than previously thought.

Unexpectedly high uORF2 translation under stress was initially reported by Starck et al.,²³ revealing a high degree of peptide expression from both uORF1 and uORF2 during both normal growth and under stress conditions. The results were obtained from constructs fusing the uORF with a tracer peptide to the respective sequence. The observation was supported by results from ribosome profiling experiments by different groups.^{24,25} To explain this discrepancy with the original model, it was suggested that translation of the *ATF4* coding region under stress is a combination of reinitiation and leaky scanning past both uORFs. Given the complexity of the *ATF4* leader, it is conceivable that the tracer peptide employed by Starck et al.²³ could have unintentionally affected the results; e.g., by separating and/or breaking the additional *ATF4* regulatory elements we identified here. However, our reporter system not only confirmed the reported high levels of uORF2 expression under stress conditions, it additionally proposed a mechanistic explanation for this phenomenon based on ribosome queuing ahead of SL3.

Ribosome queuing in the uORF2/*ATF4* overlap region

The mechanism of ribosome queuing is known from other systems, such as translation of antizyme inhibitor 1 (AZIN1).³² The mRNA leader of *AZIN1* contains a relatively long, inhibitory, and non-AUG-initiated upstream conserved coding (uCC) region, with a conserved, polyamine-dependent Pro-Pro-Trp (PPW) motif pausing elongating ribosomes. At low polyamine levels, the majority of scanning ribosomes skip this uCC and translate AZIN1; those that infrequently initiate at its AUU start site will translate the uCC, terminate, and become recycled. High polyamine levels, however, interfere with eIF5A function and cause those very few ribosomes translating the uCC to stall at PPW. Subsequently scanning ribosomes, which again mostly skip the AUU start codon, as well as the occasional elongating ribosomes form a queue upstream of the PPW-stalled ribosome. This queue then stimulates uCC initiation at the AUU as scanning ribosomes are jammed all the way up to this site, reinforcing the PPW-imposed elongation stall to efficiently suppress AZIN1 synthesis.

The additional layer of ATF4 translation control that we propose is at least in part analogous to the AZIN1 mechanism. Specifically, the scenario with 80S ribosomes elongating from the AUG of uORF2 and subsequently queuing upstream of SL3 under non-stress conditions (Figure 2B), which prevents translation of ATF4 and ultimately also that of uORF2, is similar to the AZIN1 system at high polyamine levels. This way, the queuing would reinforce uORF2 initiation and very efficiently limit skipping of its AUG. It would also minimize the leakiness of the whole system to the lowest possible level: at zero demand, there is zero ATF4 synthesis. Upon stress, SL3 could slow down 80S ribosomes elongating from the AUG of ATF4, allowing for the formation of a queue consisting of both scanning 48S PICs and elongating 80S ribosomes (Figure 2B).

Interestingly, and somewhat paradoxically, we also found that removal of a potential modification of A326 upon stress would further stabilize SL3. We suggest that the SL3-imposed queue could then (1) enhance selection of alternative start codon(s) in ATF4 and (2) prevent ATF4 overinduction. This process would involve reduced but still substantial translation of uORF2 even under stress, as has been observed. We further speculate that the near-cognate CUG could serve as another warrant licensing ATF4 synthesis, overcoming the leakiness of the ATF4 start site(s) (Figure 2B). It seems that the ATF4 queuing system is well optimized for its complex function, as our data imply that it depends strongly on the precise spacing between SL3 and upstream start codons (either uORF2 or ATF4). When we increased this spacing, either through the uORF2 or ATF4 reading frame, the tight ATF4 control was relaxed.

Importantly, in addition to the reporter analysis, several other observations supported the ribosome queuing hypothesis: (1) the published disome-seq data suggested formation of roughly two disomes (80S couples) in the region spanning the uORF2 AUG and SL3 whose distribution changes upon eIF5A knock-down (Figure S6E); (2) our ribosome protection assays indicated a specific protection of a 132-nt-long fragment in the same region depending on intact SL3 (Figures 6 and S6A–S6C); and (3) analysis of publicly available ribo-seq libraries generated from cells exposed to various ER stressors revealed prominent FP peaks with a strong preference for the CUG placement in the P site (Figures S4 and S5). All of these results are consistent with a queue of 4–5 ribosomal species (either 80S couples or a mixture of 80Ss and 48S PICs) accumulated between uORF2 AUG and SL3.

It is understandable that, given the variable length of mRNA FPs of 48S PICs compared with the relatively well-defined FP length of elongating 80S,^{22,27,33,34} it is difficult to predict the exact position of the most 5' ribosomal species. This is made even more difficult because, as suggested by Ivanov et al.,³² ribosome queuing forces an initiating ribosome to spend more time near the start codon, where it can migrate back and forth by ~15 nt, as shown previously.⁴⁰ Therefore, the operational space for the last 48S PIC in the queue to find the translation initiation site is relatively wide, increasing the probability for initiation even if the start codon is not in an optimal context. Concordantly, altering the spacing between uORF2 and SL3 cancels their functional interaction, indicating that the defined length of the queue for exerting its role is critical.

Based on mounting evidence from the literature, it is now well established that the precise mode of ISR induction, whether through UPR activation, starvation, or inhibition of tRNA synthetases, likely has very distinct downstream effects that manifest in highly nuanced ATF4 translational control followed by unique modes of ATF4-mediated transcriptional responses.⁴¹ In light of this high degree of plasticity both in the control of ATF4 expression and its subsequent regulatory effect on its own transcriptional “regulon,” it seems plausible that a complex network of translation regulatory modes, as described here, is required to adjust ATF4 expression to a variety of conditions and tissues. Given the involvement of deregulated ATF4 expression in various pathologies and cancers, the complexity of its translational control should be kept in mind when considering therapies directed against this potent regulator of cell life or death.

Limitations of the study

One of the limitations of this study is that data obtained with our newly developed reporter system, which we consider to be otherwise robust, are not supported by insertion of key mutations directly into the endogenous ATF4 locus by, for example, CRISPR-based gene editing. Although they were not feasible given the scale of this study, this will be addressed by the ongoing experiments. Another issue to consider is that, owing to the fact that our reporter mRNAs were highly overexpressed in cells (STAR Methods), we recorded slightly increased ATF4 expression under non-stress conditions even with the WT reporter. This was, however, highly desirable because at least minimal ATF4 expression under non-stress conditions was instrumental for monitoring and comparing effects of our mutations also on the basal level of ATF4 expression. Finally, more experiments are needed to demonstrate that A235 and A326 are indeed modified, possibly by m⁶A, in human cells and that ATF4 can be stably expressed from the near-cognate CUG codon upstream of SL3.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2024.113976>.

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AUTHOR CONTRIBUTIONS

L.S.V. conceived and designed the project. A.M.S., V.H., M.P.M., S.G., D.P., P.H., M.S., A.H., P.B., J.R., S.C., and K.J. carried out all experiments and analyzed the data. L.S.V. interpreted results and wrote the paper with input from A.M.S., S.G., and C.V. and partly from M.P.M., D.P., P.H., J.R., M.S., and M.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-HA tag [HA.C5]	abcam	Cat# ab18181; RRID:AB_444303
Rabbit monoclonal anti-ATF-4 (D4B8)	Cell Signaling	Cat# 11815S; RRID:AB_2616025
Mouse monoclonal anti-ATF4 (B-3)	Santa Cruz	Cat# sc-390063; RRID:AB_2810998
Mouse monoclonal anti-c-Myc tag [9E10]	Dr. Tomás Vomastek	N/A
Bacterial and virus strains		
DH5alpha	Invitrogen	Cat# EC0112
DH10B	Invitrogen	Cat# 18290015
Chemicals, peptides, and recombinant proteins		
Dimethyl sulfoxide (DMSO)	Sigma	Cat# D2438-5X
Thapsigargin	Invitrogen	Cat# T7458
Tunicamycin	Sigma-Aldrich	Cat# T7765-1MG
Glo Lysis Buffer	Promega	Cat# E2661
TurboFect	Thermo Fisher	Cat# R0532
RNA blue	Top-Bio	Cat# R013
UltraPure Distilled Water	Thermo Fisher	Cat# 109977-035
Chloroform	VWR BDH Chemicals	Cat# 22711.290
2-Propanol	VWR BDH Chemicals	Cat# 20842.312
Ethanol 96% vol	VWR BDH Chemicals	Cat# 20822.290
Glycogen	Thermo Fisher	Cat# AM5910
High-capacity cDNA reverse Transcription Kit	Applied Biosystems	Cat# 4368814
RNase Inhibitor	Applied Biosystems	Cat# N8080119
TURBO DNase	Invitrogen	Cat# 2238G
10×TURBO DNase Buffer	Invitrogen	Cat# 8167G
DNase Inactivation Reagent	Invitrogen	Cat# 8174G
HOT FIREPol EvaGreen qPCR Mix Plus 5×	Solis BioDyne	Cat# 08-25-00020
10×ThermoPol Reaction Buffer	NEB	Cat# B9004S
Vent DNA Polymerase	NEB	Cat# M0254L
1x T3 ligation buffer	NEB	Cat# B0535S
T3 DNA ligase	NEB	Cat# M0317L
Plasmid Buffer P1	QIAGEN	Cat# 19051
RNase A	QIAGEN	Cat# 19101
Plasmid Buffer P2	QIAGEN	Cat# 19052
Plasmid Buffer N3	QIAGEN	Cat# 19064
Plasmid Buffer PE	QIAGEN	Cat# 19065
Plasmid Buffer PB	QIAGEN	Cat# 19066
Buffer QG Solubilization Buffer	QIAGEN	Cat# 1014876
T4 DNA Ligase	Roche	Cat# 10716359001
Ligation Buffer, 10×	Roche	Cat# 11243292001
Quick CIP	NEB	Cat# M0525S
Midori Green Direct	NIPPON Genetics EUROPE	Cat# MG06
BstBI	NEB	Cat# R0519S
HindIII-HF	NEB	Cat# R3104S
HpaI	NEB	Cat# R0105S

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
EcoRV-HF	NEB	Cat# R3195S
PstI-HF	NEB	Cat# R3140S
rCutSmart Buffer	NEB	Cat# B6004S
Criterion TGX Precast Gels 4–20%	BIO-RAD	Cat# 5671093
SuperSignal West Femto Maximum Sensitivity Substrate	Thermo Fisher	Cat# 34096
Bio-Rad Protein Assay Dye Reagent Concentrate	BIO-RAD	Cat# 5000006
GeneArt Strings DNA Fragments	Invitrogen	N/A
EcoSpin Spin Columns for DNA	Epoch Life Science	Cat# 1910-25
SUPERaseIn RNase inhibitor	Invitrogen	Cat# AM2694
Ambion™ RNase I	Invitrogen	Cat# AM2294
Tissue culture test plate, 6 wells	Techno Plastic Products	Cat# 92406
Critical commercial assays		
12–230 kDa Fluorescence Separation Module	Protein Simple bio-technie	SM-FL004-1
8×EZ Standard Pack 1	Protein Simple bio-technie	PS-ST01EZ-8
Anti-Mouse Detection Module	Protein Simple bio-technie	DM-002
Protein Normalization Module	Protein Simple bio-technie	DM-PN02
Deposited data		
Analyzed data	Ichihara, et al., 2021 ³⁰	GSE174329
Analyzed data	Rendleman et al., 2018 ³¹	GSE113171
Analyzed data	Andreev, et al., 2015 ²⁴	GSE55195
Experimental models: Cell lines		
HEK293T	ATCC	CRL-3216; RRID: CVCL_0063
HeLa	ATCC	CCL-2; RRID: CVCL_0030
Oligonucleotides		
See Data S3 for a list of oligonucleotides		N/A
Recombinant DNA		
pCMV-EGFP-N2	Roithová et al., 2020 ⁴²	N/A
WT ATF4-HA Tag	This paper	N/A
dSt-st	This paper	N/A
d1	This paper	N/A
d2	This paper	N/A
d-all	This paper	N/A
St-st-only	This paper	N/A
uORF1-only	This paper	N/A
uORF2-only	This paper	N/A
dSt-st-NC1	This paper	N/A
St-st-only-NC1	This paper	N/A
St-st-only-NC2/3	This paper	N/A
dSt-st-SL1	This paper	N/A
St-st-only-SL2-1	This paper	N/A
St-st-only-SL2-2	This paper	N/A
dSt-st-SL2-1	This paper	N/A
dSt-st-SL2-2	This paper	N/A
d-all-SL2-2	This paper	N/A
wt-A235G	This paper	N/A
wt-SL3 ^{Mut-1}	This paper	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
wt-SL3 ^{Mut-2}	This paper	N/A
dSt-st-SL3 ^{Mut-1}	This paper	N/A
d-all-SL3 ^{Mut-1}	This paper	N/A
uORF2-only-SL3 ^{Mut-1}	This paper	N/A
wt-AUG3 ^{Mut} (3 rd ATF4 AUG)	This paper	N/A
dSt-st-AUG3 ^{Mut} (3 rd ATF4 AUG)	This paper	N/A
wt-UUG ^{Mut} -AUG3	This paper	N/A
wt-SL3 ^{Mut-1} -AUG3 ^{Mut}	This paper	N/A
wt-A326G	This paper	N/A
wt-SL3 ^{Mut-1} -A326G ^{Mut}	This paper	N/A
wt-3AUG ^{Mut}	This paper	N/A
d2-3AUG ^{Mut}	This paper	N/A
3AUG ^{Mut} -stop	This paper	N/A
3AUG ^{Mut} -short	This paper	N/A
3AUG ^{Mut} -short-stop	This paper	N/A
uORF2_ins	This paper	N/A
d2_ins (uORF2_ins => AUG to AGG)	This paper	N/A
uORF2_ins_3AUG ^{Mut}	This paper	N/A
wt-CUG ^{Mut}	This paper	N/A
wt-SL3 ^{Mut-1} -CUG ^{Mut}	This paper	N/A
wt_ins	This paper	N/A
wt_ins-SL3 ^{Mut-1}	This paper	N/A
d2_ins	This paper	N/A
d2_ins-SL3 ^{Mut-1}	This paper	N/A
uORF2_ins-SL3 ^{Mut-1}	This paper	N/A
uORF2-ATF4-HA fusion	This paper	N/A

Software and algorithms

Compass for SW ver. 6.3.0	ProteinSimple	https://www.bio-technie.com/resources/instrument-software-download-center
GraphPad Prism ver. 10.1.2	GraphPad Software, San Diego, CA, USA	RRID:SCR_002798
SciPy ver. 1.10.1	Virtanen et al., 2020 ⁴³	RRID:SCR_008058
Python 3.11.3 ver. 8.1.1	Rossum and Drake, 2009 ⁴⁴	https://www.python.org
Matplotlib ver. 3.7.0	Hunter, 2007 ⁴⁵	RRID:SCR_008624
R ver. 4.2.2: 'pheatmap' package ver. 1.0.12	Kolde and Kolde, 2015 ⁴⁶	N/A
R ver. 4.2.2: 'RiboWaltz' package	Lauria et al., 2018 ⁴⁷	N/A

Other

Jess TM Automated Western Blot System	Protein Simple Bio-Techne	004-650
RiboCrypt tool	https://ribocrypt.org	N/A
G-Box imager	Syngene	N/A
Mendeley data	http://www.mendeley.com/	RRID:SCR_002750; https://doi.org/10.17632/zmdvhwggg8.1
Quantity One Software	Bio-Rad	RRID:SCR_014280

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Leoš Shiva Valášek (valasekl@biomed.cas.cz).

Materials availability

Plasmid constructs generated in this study are available upon request.

Data and code availability

- The data generated in this paper and used for the preparation of main Figures is available in [Tables S1–S23](#), as well as from the [lead contact](#) upon request. Additionally, this paper analyzes existing, publicly available data. These accession numbers for the datasets are listed in the [key resources table](#). Data have been deposited at Mendeley and are publicly available as of the date of publication. Accession numbers are listed in the [key resources table](#).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human cell line

The HEK293T cell line (ATCC) was used for all experiments in this study; the HeLa cell line (ATCC) was used only for the T3 ligation assay. The source for commercial HEK293T as well as HeLa cell line is reported to be female. Cells were grown in Dulbecco's modified Eagle's Medium (DMEM) high glucose medium supplemented with 10% fetal bovine serum (FBS) at 37.0°C with 5.0% CO₂ concentration. Cells were seeded into 6-well plates (Techno Plastic Products) to 2.5 mL of medium per well and grown 24 h prior to transfection at approximately 40–50% confluency.

Bacterial strains

Bacterial strains DH5 α and DH10B (Invitrogen) were used in this study.

METHOD DETAILS

Validation of the newly developed ATF4-HA reporter system

To understand what lies behind the “background” ATF4 expression under non-stress conditions and to validate that our newly developed reporter system faithfully mimics endogenous ATF4 translational regulation, we used traditional western blot analysis. We reasoned that the non-stress peak could originate from the DNA transfection which is known to be stressful to cells⁴⁸ and/or from CMV promoter-driven transcription generating a high level of the *ATF4-HA* mRNA, which could magnify the normally negligible leakiness of ATF4 repression under non-stress conditions. As shown in [Figure S1B](#), transfection of our WT reporter using turbofect, but not turbofect or DMEM alone, resulted in a mild ATF4 induction even without Tg treatment (lanes 2 and 8 vs. lanes 3, 4 and 9, 10). This result could suggest that DNA transfections trigger mild stress as reported before, however, control transfections of an empty vector did not trigger such a response ([Figure S1C](#)). Therefore, we concluded that a high level of our CMV-driven reporter mRNA (>100 times higher than endogenous *ATF4* mRNA levels, as demonstrated below) resulted in detectable basal levels of ATF4 protein in otherwise unstressed cells, as also observed by others using the CMV-driven ATF4 expression.^{49,50} Nonetheless, clear Tg-mediated induction of the endogenous ATF4 in lanes 6 and 7 in the “ATF4 (anti-ATF4)” panel of [Figure S1B](#) (compared to lanes 3 and 4), and of our ATF4-HA reporter in lane 5 in the “ATF4-HA (anti-HA)” panel (compared to lane 2), demonstrate that both ATF4 variants respond to Tg treatment in the same manner. Due to the absence of signal in lanes 3 and 4 we could not quantify the level of induction of the endogenous ATF4 alone. Nonetheless, the level of induction of ATF4-HA calculated from Jess (5.2 ± 0.5) or western blots (3.9 ± 1.3) and the level of induction of combined endogenous ATF4 and ATF4-HA (lanes 5 versus 2 in the “ATF4 (anti-ATF4)” panel showing the cumulative amounts of both HA-tagged and endogenous proteins that anti-ATF4 antibodies recognize) calculated from Jess (5.5 ± 0.6) or western blots (3.4 ± 0.2) correspond nicely to what we expected ([Figure S1D](#)). Given the fully quantitative nature of Jess measurements, we consider them to be the most reliable.

Importantly, the observed induction pattern is solely attributable to ATF4 translational control, as the mRNA levels of both *ATF4-HA* and endogenous *ATF4* (measured separately using highly specific reverse primers matching the *ATF4* stop codon region, by which these two alleles differ due to the HA tag sequence) remained virtually unchanged under non-stress vs. stress conditions ([Figure S2A](#)). Collectively, these results document that our reporter mirrors the behavior of endogenous ATF4 regulation.

As aforementioned, when we compared *ATF4* mRNA levels under non-stress conditions using primer sets A1 and A2 downstream of SL3 (Amplicons 2 and 3 defined in the results chapter “Ribosome queuing contributes to the overall translational control of ATF4” and [Figure S6](#)), we revealed over 100-fold more *ATF4* mRNA in transfected (expressing both endo and exogenous *ATF4* mRNA) than in non-transfected (expressing endogenous *ATF4* mRNA alone) ([Figure S6D](#); [Data S1](#)).

St-st modestly inhibits ATF4 expression, uORF1 allows for 50% downstream REI, and a solitary, inhibitory uORF2 allows for high ATF4 stress-inducibility

Keeping St-st as the only element in the *ATF4* leader resulted in ~20.6-fold increase in ATF4 expression under non-stress conditions and ~3.1-fold increase under Tg stress over WT ([Figures S3A and S3B](#); [Tables S3 and S4](#)). The former value is only modestly but reproducibly lower than that of d-all (reduced by ~10%) supporting the idea that St-st acts as a general repressive element, i.e., a roadblock, as observed before.^{22,28}

The uORF1-only construct, where only the AUG of uORF1 is preserved, led to 12.2-fold and 2.7-fold upregulation in non-stress and Tg stress conditions, respectively, while also showing no stress inducibility compared to WT (Figures S3A–S3; Tables S3 and S4). These results clearly confirm the stress-independent, REI-permissive nature of this uORF and indicate that more than 50% of ribosomes that translate uORF1 (~12-fold induction of uORF1-only over WT [Figure S3A - row 1] divided by ~23-fold induction of d-all [Figure S3A - row 1]) undergo partial recycling and reinitiate downstream upon reacquisition the TC.

According to the original delayed reinitiation model, uORF2 serves as a barrier capturing most, if of not all, 40S ribosomes that resumed scanning after termination at uORF1 under non-stress conditions, thus preventing ATF4 expression because its sequence extends into the *ATF4* CDS out of frame. Under stress conditions, characterized by low TC levels, its expression should be dramatically reduced, allowing ATF4 expression. In the WT set-up, uORF2 might still be expressed to some degree because not 100% of ribosomes skip its AUG even under decreased TC levels, but the amount of those that do should be substantially higher when compared to non-stress conditions. In any case, in the uORF2-only set-up, this strong barrier should theoretically not allow any significant inducibility of the reporter under stress conditions, because once the ribosome initiates at uORF2 as the first and only upstream uORF, the effect on ATF4 is independent of the TC levels.

Accordingly, we found that the uORF2-only construct showed an ~80% drop in reporter expression compared to the WT under both normal and Tg stress conditions indicating dramatically reduced initiation at *ATF4* (Figures S3A and S3B – rows 1 and 2; Tables S3 and S4). However, in stark contrast to the above logic, comparing stress with non-stress values of uORF2-only (“fold-induction” – row 3) revealed an unexpected ~3.2-fold increase in ATF4 translation under stress that could not be explained by the original model. We argue that this large increase cannot be explained purely by the proposed increased leaky scanning over uORF2 under stress.²³

Next, we constructed mutants in which we eliminated one upstream element at a time and compared the resulting constructs with the WT set to 1 (Figures S3B and S3D; Tables S3 and S4). Eliminating St-st (in dSt-st) showed no significant effect under both conditions. Nonetheless, we repeatedly observed higher, but not significant, stress induction of dSt-st compared to WT (increased by ~10% from WT ~5.2-fold–~5.8-fold; Figures S3C vs. S3D – row3; Tables S3 and S4), which argues for the St-st’s role as a stress-independent roadblock reducing processivity of the scanning ribosome. These results, and the fact that mouse and possum *ATF4* lacks the St-st, indicate that uORFs 1 and 2 are largely sufficient to ensure stress-induced translation of ATF4.

While removal of the REI-permissive uORF1 (in d1) significantly reduced ATF4 expression by ~70% under both non-stress and Tg stress, eliminating the inhibitory uORF2 (in d2) dramatically increased ATF4 expression (~12.1-fold) under non-stress conditions and slightly (~2.4-fold) under stress (Figures S3B and S3D rows 1 and 2; Tables S3 and S4), all as expected from the original model. The latter result nicely corroborates that ~50% of ribosomes terminating at uORF1 reinitiate downstream (~12-fold induction of d2 over WT [Figure S3D - row 1] divided by ~23-fold induction of d-all [Figure S3A - row 1]), as we observed with the uORF1-only construct (Figure S3A).

Comparison of the stress induction potentials of each construct showed that d2 is very similar to uORF1-only and d1 to uORF2-only (Figures S3D and S3A, “fold-induction” plots in the respective columns), except that the presence of the St-st further magnified the uORF2 inducibility (from ~3.2-fold to ~5.8-fold; “fold-induction” plots in Figure S3A – uORF2-only versus Figure S3D – d1). This result is again in line with St-st serving as a ribosome barrier to maintain low basal levels of ATF4 expression.

Taken together, our results confirmed major aspects of the original model but raised the question as to the mechanism behind the unexpected stress induction of the uORF2-only reporter.

Sequence analysis of the 5' UTR of the human *ATF4* mRNA reveals additional elements potentially contributing to ATF4 translational control

Here we asked if there are some so-far unidentified regulatory elements in the *ATF4* mRNA leader besides St-st and both uORFs. We had previously predicted and experimentally validated the existence of two stem loops (SL1 and 2) preceding uORF1 (Figure 2A), the first of which cooperates with eIF3 to unleash the full REI potential of uORF1.¹⁹ SL1 also contains one near-cognate CUG start site (NC1) that is immediately followed by two overlapping GUG near-cognates (NC2/3) that are no longer part of SL1.

Notably, the SL1 and its NC1, and NC2/3 are not universally conserved in eukaryotes (Figure S1A). The St-st is conserved in most mammals examined, except mouse a few others. The SL2 is conserved in primates and, interestingly, its formation is even slightly more favorable in mouse and rat than in human. Indeed, the presence of uORF1 and uORF2 is well conserved.

The next element, predicted by our analysis, is another stem-loop (SL3), with $\Delta G = -15.40$ kcal/mol, which could potentially be inhibitory and is highly conserved among vertebrates (Figure S1A). It is located roughly in the middle of the uORF2/*ATF4* overlap (Figure 2A) and has a hairpin structure with the highest free energy predicted for this particular sequence region.

Further, our analysis predicted a total of four individual sites of m6A methylation within well-defined motifs (Figure 2A); DRACH1 (with A225) is located in the non-overlapping, likely unstructured region of uORF2, followed by RRACH1 (A235) in the same region, which is homologous to the predicted methylated A in mouse mRNA²⁶; RRACH (R = G or A; H = A, C or U), DRACH (D = A, G or U). The DRACH2 (A286) motif occurs in the uORF2/*ATF4* overlap and covers the *ATF4* main initiation codon with a modifiable adenine located just downstream of the AUG. Thus, its modification could interfere with AUG recognition during start site selection. Finally, RRACH2 (A326) locates to the stem of SL3; therefore, it could potentially affect its stability. Although the DRACH1 and 2 motifs are not universally conserved in eukaryotes, RRACH1 and 2 motifs are highly conserved in mammals (Figure S1A) and could therefore play an important role in ATF4 regulation.

The *ATF4* gene begins with two nearly consecutive AUGs followed by a third in close proximity, all in frame (Figure 2A, in dark green). Both the canonical AUG1 and AUG2 have a medium Kozak initiation context; AUG1 overlaps with DRACH2, and AUG2 is located two codons downstream. AUG3 has a weak Kozak initiation context and is exposed in the open loop of SL3. It represents the 17th codon downstream of AUG1. Both AUG2 and AUG3 are highly conserved among vertebrates (Figure S1A).

The uORF2/*ATF4* overlap also contains two putative conserved sliding sequences (FS-A and -B) and a so-called C-tract motif,⁵¹ which could potentially prompt the elongating ribosome to switch the reading frame from uORF2 to *ATF4*. We investigated this hypothesis by examining the frame of ribosome footprints from a dataset collected from HeLa cells subjected to stress.³¹ Specifically, we mapped ribosome protected fragments in the region of the uORF2/*ATF4* overlap and found that, during acute ER stress, reads continued to locate to the uORF2 reading frame (frame 0) and perhaps even increased, while there was no coverage of the *ATF4*'s frame (frame 1) (Figure S2B). In fact, *ATF4*'s translation appeared to initiate downstream of the predicted SL3 after 4 h of Tunicamycin stress.

Sliding region FS-A involves 3 consecutive glycine codons, and because of its overlap with SL3, it can only function if SL3 is not formed. Switching from uORF2 to the *ATF4* reading frame could be mediated by either a +1 or –2 programmed ribosomal frameshifting (PRF). Of these two possibilities, the latter is much more likely because both types of glycine tRNAs, with GGG in the P-site and GGA in the A-site, are well positioned for re-pairing after a –2 shift.⁵¹ In support of this hypothesis, 8 nucleotides downstream of FS-A occurs the C-nucleotide-rich C-tract motif (CCCCCUUCGACC) with high similarity to the inhibitory C-motif promoting –2 PRF in arboviruses.² Since 8 nucleotides is considered the optimal distance for the C-motif to act as the inhibitory element that can cooperate with the upstream sliding sequence to promote –2 PRF, we designed constructs to examine this option (see below).

FS-B is located just upstream of the uORF2 stop codon, thereby partially fulfilling the +1 PRF criteria.⁵¹ Yet, neither of its glycine codons had the potential to slow or stop ribosomes, as they belong to the high codon usage category. Since FS-B also did not meet the –2 PRF criteria, it likely has no role in *ATF4* translational control.

Given this knowledge, we created an array of mutants for all these predicted elements (Figure 2A), and first evaluated them in a large-scale screen. It resulted in no observable effect for mutations in NC1 to NC3 in SL1, both DRACH motifs, mutations unfolding SL1 or SL2 (specifically with respect to the St-st function), and mutations of AUG3 inside the *ATF4* coding region (data not shown). Therefore, we focused our analysis on mutations in other elements that had observable effects.

uORF2 translation under stress is more prevalent than expected

To investigate characteristics of uORF2 translation further, we next tested for evidence of uORF2 to *ATF4* frameshifting. To do so, we mutated the first 3 AUGs of *ATF4* (Figure S7A, in 3AUG^{Mut}), which would eliminate all *ATF4* variants except for the potential frame-shifted fusion protein. However, we observed that the peak corresponding to the 53 kDa WT protein did not vanish but shifted to 52 kDa (Figure S7A) and its relative expression level decreased by ~5.5-fold under both conditions when the 3 AUGs were mutated (Figure S7A, bottom electropherogram; Tables S11 and S12). This 52 kDa peak in 3AUG^{Mut} remained inducible under Tg stress, indicating that despite the lack of these three *ATF4* AUGs, the regulatory system remained fully responsive. Further, we observed two additional, clearly discernible peaks at 44 and 34 kDa, which were consistently also inducible upon stress. Careful inspection of the *ATF4* sequence suggested that the 52 kDa peak may correspond to the almost full-length protein initiated either on the CUG or on five other nearby near-cognate codons (Figure S8A), whereas the 44 and 34 peaks corresponded to shorter *ATF4* variants initiated on internal AUGs further downstream.

These results clearly suggest that *ATF4* might be translated from several alternative start sites, and the variants are induced by uORF2. However, since none of these peaks occurred at a higher molecular weight than the original *ATF4* protein, the idea of frameshifting resulting in a longer *ATF4* variant was not supported. Concordantly, shortening the *ATF4* sequence by removing 103 amino acid residues from the C-terminal segment of its CDS shifted all three peaks upward, exactly according to the corresponding loss in molecular weight (Figure S8B – 3AUG^{Mut} versus Figure S8C – 3AUG^{Mut}-short). Furthermore, inserting 2 consecutive stops immediately downstream of the uORF2/*ATF4* overlap (in the *ATF4* reading frame) eliminated only the heaviest peak but not the other two peaks, in both the full-length and shortened 3AUG^{Mut} constructs (Figure S8D – 3AUG^{Mut}-stop; Figure S8E – 3AUG^{Mut}-short-stop), strongly suggesting that the initiation codon for the 52-kDa protein lied within the overlap region, but not in the uORF2 frame. Consistently, combining 3AUG^{Mut} with an AUG to AGG mutation of uORF2 had no effect on the size and distribution of the aforementioned 3 protein products (Figure S9A, in d2-3AUG^{Mut}). Indeed, inserting 2 consecutive stops into the WT construct at the same place as in case of the 3AUG^{Mut} construct completely eliminated the signal (data not shown).

The final piece of evidence for the absence of frameshifting arose from an insertion of a 10x c-Myc tag into the uORF2 frame exactly 21 nt downstream of its AUG (Figure S7B, in uORF2_{ins}). This mutant resulted in only one *ATF4*-HA peak corresponding to ~53 kDa, with inducibility unchanged compared to the WT (Figure S7B, upper electropherogram). Note that the expected size of the fusion protein is ~62 kDa but it runs at ~57 kDa, as verified experimentally (Figure S9B; Tables S13 and S14). Probing with c-Myc antibodies revealed a single peak at ~40 kDa corresponding to the uORF2-c-Myc fusion protein, whose presence was strictly depended on the presence of the AUG of uORF2 (Figure S7B; bottom panel, uORF2_{ins} => AUG to AGG in red) and whose intensity remained unexpectedly high (reduced only by ~40%) even under Tg stress (Figure S7B, bottom electropherogram; see Tables S11 and S12). Consistently, combining uORF2_{ins} with the triple AUG mutation of *ATF4* produced three Tg-inducible peaks of the same size as observed with the 3AUG^{Mut} alone (Figure S9C [uORF2_{ins}_3AUG^{Mut}] versus Figure S8B [3AUG^{Mut}]). Finally, mutations of the computer-predicted slippery sequences (Figure 2A) showed no effect (data not shown). Altogether, these findings clearly ruled out

possible uORF2 to *ATF4* frameshifting and strongly supported the idea that uORF2 was translated even under stress and at a much higher level than could be expected based on the original model.

Construction of the ATF4-HA tagged plasmids

To create the ATF4-HA tagged reporter plasmids, the hATF4-wt-HA-Tag-3'UTR (NM_182810.2) ordered as GeneArt Strings DNA Fragment (Invitrogen) was cloned into the empty Clontech EGFP-N2 high copy number vector,⁴² while removing the GFP insert. All hATF4-HA tagged constructs contain the CMV promoter, the full 5'UTR of *ATF4*, the full CDS of *ATF4*, and the natural 3'UTR of *ATF4*. The HA tag is placed at the very C terminus of the *ATF4* CDS just upstream of its stop codon; all these plasmids have the kanamycin resistance. Mutant constructs were generated using either GeneArt Strings (Invitrogen) or PCR using 10×ThermoPol Reaction Buffer (NEB), Vent DNA Polymerase (NEB) and specific primers. Cloning details of all plasmids are described below; for the list of all plasmids, primers and GeneArt strings, please see [Data S2–S4](#). Plasmid isolation was performed using QIAGEN Plasmid Mini Kit and EconoSpin DNA Spin Columns (Epoch Life Science). QIAGEN QIAquick Gel Extraction Kit and QIAGEN QIAquick PCR Purification Kit (QIAGEN) were used for insert isolation.

WT ATF4-HA Tag was created by inserting *HindIII*-*NotI* digested hATF4-wt-HA-Tag (GeneArt String DNA Fragment; Invitrogen) into *HindIII*-*NotI* digested *pCMV-EGFP-N2* high copy vector.

dSt-st was created by inserting the *HindIII*-*PstI* digested fusion PCR product obtained with primers hATF4-St-st_AGG-F and hATF4-St-st_AGG-R using wt ATF4-HA Tag as a template into *HindIII*-*PstI* digested wt ATF4-HA Tag.

d1 was created by inserting the *HindIII*-*PstI* digested fusion PCR product obtained with primers hATF4-uORF1_AGG-F and hATF4-uORF1_AGG-R primers using wt ATF4-HA Tag as a template into *HindIII*-*PstI* digested wt ATF4-HA Tag.

d2 was created by inserting the *PstI*-*EcoRV* digested fusion PCR product obtained with primers hATF4-uORF2_AGG and SW ATF4 d120 SphI R using wt ATF4-HA Tag as a template into *PstI*-*EcoRV* digested wt ATF4-HA Tag.

d-all was created by inserting *HindIII*-*NotI* digested hATF4-d-all-HA-Tag (GeneArt String DNA Fragment; Invitrogen) into *HindIII*-*NotI* digested *pCMV-EGFP-N2* high copy vector.

St-st-only was created using fusion PCR with wt ATF4-HA Tag serving as a template and the following combination of primers: (i) hATF4-uORF1_AGG-F + hATF4-uORF1_AGG-R and (ii) hATF4-uORF2_AGG and SW ATF4 d120 SphI R. The resulting PCR product was digested with *HindIII* and *EcoRV* and inserted into *HindIII*-*EcoRV* digested wt ATF4-HA Tag.

uORF1-only was created using fusion PCR with wt ATF4-HA Tag serving as a template and the following combination of primers: (i) hATF4-St-st_AGG-F + hATF4-St-st_AGG-R and (ii) hATF4-uORF2_AGG and SW ATF4 d120 SphI R. The resulting PCR product was digested with *HindIII* and *EcoRV* and inserted into *HindIII*-*EcoRV* digested wt ATF4-HA Tag.

uORF2-only was created using fusion PCR with wt ATF4-HA Tag serving as a template and the following combination of primers: (i) hATF4-St-st_AGG-F + hATF4-St-st_AGG-R and (ii) hATF4-uORF1_AGG-F + hATF4-uORF1_AGG-R. The resulting PCR product was digested with *HindIII* and *PstI* and inserted into *HindIII*-*PstI* digested wt ATF4-HA Tag.

dSt-st-NC1 was created by inserting the *HindIII*-*PstI* digested PCR product obtained with primers hATF4-dSt-st_NC1 and hATF4-PstI-R using dSt-st as a template into *HindIII*-*PstI* digested dSt-st.

St-st-only-NC1 was created by inserting the *HindIII*-*PstI* digested PCR product obtained with primers hATF4-St-st-only_NC1 and hATF4-PstI R using St-st-only as a template into *HindIII*-*PstI* digested St-st-only.

St-st-only-NC2/3 was created by inserting the *HindIII*-*PstI* digested PCR product obtained with primers hATF4-St-st-only_NC2/3 and hATF4-PstI-R using St-st-only as a template into *HindIII*-*PstI* digested St-st-only.

dSt-st-SL1 was created by inserting the *HindIII*-*PstI* digested PCR product obtained with primers hATF4-dSt-st-SL1 and hATF4-PstI-R using dSt-st as a template into *HindIII*-*PstI* digested dSt-st.

St-st-only-SL2-1 was created by inserting the *HindIII*-*PstI* digested fusion PCR product obtained with primers hATF4-SL2-1-F and hATF4-SL2-1-R using St-st-only as a template into *HindIII*-*PstI* digested St-st-only.

St-st-only-SL2-2 was created by inserting the *HindIII*-*PstI* digested fusion PCR product obtained with primers hATF4-SL2-2-F and hATF4-SL2-2-R using St-st-only as a template into *HindIII*-*PstI* digested St-st-only.

dSt-st-SL2-1 was created by inserting the *HindIII*-*PstI* digested fusion PCR product obtained with primers hATF4-SL2-1-F and hATF4-SL2-1-R using dSt-st as a template into *HindIII*-*PstI* digested dSt-st.

dSt-st-SL2-2 was created by inserting the *HindIII*-*PstI* digested fusion PCR product obtained with primers hATF4-SL2-2-F and hATF4-SL2-2-R using dSt-st as a template into *HindIII*-*PstI* digested dSt-st.

d-all-SL2-2 was created by inserting the *HindIII*-*PstI* digested fusion PCR product obtained with primers hATF4-SL2-2-F and hATF4-SL2-2-R using d-all as a template into *HindIII*-*PstI* digested d-all.

wt-A235G was created by inserting the *PstI*-*EcoRV* digested fusion PCR product obtained with primers hATF4-A235G-F and hATF4-A235G-R using wt ATF4-HA Tag as a template into *PstI*-*EcoRV* digested wt ATF4-HA Tag.

wt-SL3^{Mut-1} was created by inserting the *PstI*-*EcoRV* digested fusion PCR product obtained with primers hATF4-SL3_CAAAA-F and hATF4-SL3_CAAAA-R using wt ATF4-HA Tag as a template into *PstI*-*EcoRV* digested wt ATF4-HA Tag.

wt-SL3^{Mut-2} was created by *PstI*-*EcoRV* digested hATF4-wt-SL3^{Mut-2} (GeneArt String DNA Fragment; Invitrogen) into *PstI*-*EcoRV* digested wt ATF4-HA Tag.

dSt-st-SL3^{Mut-1} was created by inserting the *PstI*-*EcoRV* digested fusion PCR product obtained with primers hATF4-SL3_CAAAA-F and hATF4-SL3_CAAAA-R using dSt-st as a template into *PstI*-*EcoRV* digested dSt-st.

d-all-SL3^{Mut-1} was created by inserting the *PstI-EcoRV* digested fusion PCR product obtained with primers hATF4-SL3_CAAAA-F and hATF4-SL3_CAAAA-R using d-all as a template into *PstI-EcoRV* digested d-all.

uORF2-only-SL3^{Mut-1} was created by inserting the *PstI-EcoRV* digested fusion PCR product obtained with primers hATF4-SL3_CAAAA-F and hATF4-SL3_CAAAA-R using uORF2-only as a template into *PstI-EcoRV* digested uORF2-only.

wt-AUG3^{Mut} (3rd ATF4 AUG) was created by inserting the *PstI-EcoRV* digested fusion PCR product obtained with primers hATF4-3rdAGG-F and hATF4-3rdAGG-R wt ATF4-HA Tag as a template into *PstI-EcoRV* digested wt ATF4-HA Tag.

dSt-st-AUG3^{Mut} (3rd ATF4 AUG) was created by inserting the *PstI-EcoRV* digested fusion PCR product obtained with primers hATF4-3rdAGG-F and hATF4-3rdAGG-R dSt-st as a template into *PstI-EcoRV* digested dSt-st.

wt-UUG^{Mut}-AUG3 was created by inserting *PstI-EcoRV* digested hATF4-wt-UUG^{Mut}-AUG3 (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

wt-SL3^{Mut-1}-AUG3^{Mut} was created by inserting *PstI-EcoRV* digested hATF4-wt-SL3^{Mut-1}-AUG3^{Mut} (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

wt-A326G was created by inserting *PstI-EcoRV* digested hATF4-wt-A326G (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

wt-SL3^{Mut-1}-A326G was created by *PstI-EcoRV* digested hATF4-wt-SL3^{Mut-1}-A326G (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

wt-3AUG^{Mut} was created by inserting *PstI-EcoRV* digested hATF4-wt-3AUG^{Mut} of ATF4 (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

d2-3AUG^{Mut} was created by inserting *PstI-EcoRV* digested hATF4-d2-3AUG^{Mut} of ATF4 (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

3AUG^{Mut}-stop was created by inserting *PstI-EcoRV* digested hATF4-3AUG^{Mut}-stop (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

3AUG^{Mut}-short was created by inserting *EcoRV-NotI* digested hATF4-3AUG^{Mut}-short (GeneArt String DNA Fragment; Invitrogen) into *EcoRV-NotI* digested wt-3AUG^{Mut}.

3AUG^{Mut}-short-stop was created by inserting *EcoRV-NotI* digested hATF4-3AUG^{Mut}-short (GeneArt String DNA Fragment; Invitrogen) into *EcoRV-NotI* digested 3AUG^{Mut}-stop.

uORF2_ins was created by inserting *PstI-EcoRV* digested hATF4-uORF2_ins (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

d2_ins (uORF2_ins => AUG to AGG) was created by inserting *PstI-EcoRV* digested hATF4-d2_ins (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

uORF2_ins_3AUG^{Mut} was created by inserting *PstI-EcoRV* digested hATF4-uORF2_ins_3AUG^{Mut} (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

wt-CUG^{Mut} was created by inserting *PstI-EcoRV* digested hATF4-wt-CUG^{Mut} (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

wt-SL3^{Mut-1}-CUG^{Mut} was created by inserting *PstI-EcoRV* digested hATF4-wt-SL3^{Mut-1}-CUG^{Mut} (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

wt_ins was created by inserting *PstI-EcoRV* digested hATF4-wt_ins (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

wt_ins-SL3^{Mut-1} was created by inserting *PstI-EcoRV* digested hATF4-wt_ins-SL3^{Mut-1} (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

d2_ins was created by inserting *BstBI* digested d2 into *BstBI* digested wt_ins.

d2_ins-SL3^{Mut-1} was created by inserting *BstBI* digested d2 into *BstBI* digested wt_ins-SL3^{Mut-1}.

uORF2_ins-SL3^{Mut-1} was created by inserting *BstBI* digested uORF2_ins into *BstBI* digested wt-SL3^{Mut-1}.

uORF2-ATF4-HA fusion was created by inserting *PstI-EcoRV* digested hATF4-uORF2-ATF4-HA fusion (GeneArt String DNA Fragment; Invitrogen) into *PstI-EcoRV* digested wt ATF4-HA Tag.

Preparation of whole cell extracts

HEK293T cells pre-grown for 24 h as described in the Human cell lines section were transfected with ATF4-HA reporter constructs using TurboFect transfection reagent (Thermo Fisher); 2.5 µg of plasmid DNA was used per one well in the 6-well plate in all experiments. Exactly 8 h after transfection, cells were treated with either 1 µM thapsigargin (Invitrogen) for 3 h or Dimethyl sulfoxide (DMSO) (Sigma) as a control. Cells were lysed directly on the plate using 200 µL Glo Lysis Buffer (Promega), which is recommended to be used with the JessTM Automated Western Blot System (Protein Simple Bio-Techne). One-half of the lysate was saved for RNA isolation followed by a qPCR control step and the other half of the lysate was subjected to JessTM SW assay according to the supplier's instructions. In the experiments with tunicamycin (Sigma-Aldrich), 8 h after transfection, HEK293T cells were treated with either 0.5 µM tunicamycin for 4 h or DMSO as a control. Harvesting of the cell lysates was performed essentially the same as for thapsigargin.

RT-qPCR control step

Total RNA was isolated using RNA blue reagent (Top-Bio) according to the manufacturer's instructions. Subsequently, a TURBO DNase (Invitrogen) cleavage step was performed. For cDNA synthesis, 0.5 μ g of total RNA was used for each sample using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). As described before,^{52,53} at least three 10-fold serial dilutions of cDNA for each mutant with the WT pair, examined in each individual experiment, were compared using RT-qPCR, and the maximum difference criterion of 1 cycle had to be met for all sample dilutions in all relevant controls (including control for the transfection efficiency) at the time of lysis, otherwise samples were discarded. To compare the reporter levels for all mutants, the reverse primer was designed to be in match with HA tag (to avoid interference with endogenous *ATF4*). Primers matching the plasmid region carrying neomycin/kanamycin resistance were created (to ensure the same level of transfection efficiency for each pair of samples). Internal controls for RNA isolation efficiency and cDNA synthesis were used as well (SPIKE RNA and the corresponding primers). The qPCR primers are listed in [Data S3](#). For qPCR reactions, HOT FIREPol EvaGreen qPCR Mix Plus 5x (Solis BioDyne) was mixed with 0.8 μ M primers and cDNA and run using the following program: 95°C for 15 s, followed by 43 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s.

JessTM Simple western (SW) assay (Protein Simple)

JessTM SW Assay was used according to the manufacturer's protocol. In brief, the entire process in the Jess machine can be described as follows: proteins are separated by their molecular weight in a capillary pre-aspirated with separation and stacking matrices. Once the separation is complete, UV light immobilizes the proteins to the capillary wall. After immobilization and clearing of the matrix from the capillary, the immunoprobings process is initiated, first by incubation with the primary antibody, then with the secondary HRP conjugate, and finally with the chemiluminescent substrate. The emitted chemiluminescent light is then recorded by a CCD camera and automatically quantified. For the experiments conducted as part of this study, the lysate was mixed with 1 $\times$ Sample Buffer in accordance with the amount calculated in SW Sample Calculator for each sample, and subsequently Fluorescent 5 $\times$ Master Mix containing DTT solution and 10 $\times$ Sample Buffer was added (Protein Simple bio-technique). Twenty-four samples were tested in each Jess run. Samples were mixed by vortexing, boiled at 95°C for 5 min and spun down using a benchtop microcentrifuge. Together with the tested samples, biotinylated ladder was pipetted into 12–230 kDa Pre-filled Plates from Fluorescence Separation Module (Protein Simple bio-technique). Mouse monoclonal anti-HA tag (abcam) or anti-c-Myc antibodies (provided by Dr. Tomáš Vomastek) were pipetted along with Anti-Mouse Detection Module (Protein Simple bio-technique). The plate was centrifuged for 5 min at 686 x g at room temperature and inserted into the Jess machine along with Fluorescent Capillary Cartridges. Data analysis and control of the results obtained by the Jess instrument were performed in Compass for SW software (version 6.3.0) (ProteinSimple).

The key point to mention is that the same amount of total protein loaded in all capillaries was pre-estimated using Bio-Rad Protein Assay Dye Reagent Concentrate (BIO-RAD) and BSA titration curve was performed before each Jess run with serial dilutions of BSA in PBS. Afterward, Protein Normalization Module (Protein Simple bio-technique) was applied for a thorough comparison and finally only up to 20% difference between samples was tolerated using PN Module, otherwise samples were discarded. Please note that the 180 kDa peak, which occurs occasionally in electropherograms, represents (based on the manufacturer's instructions) a non-specific peak arising from cross-reactions between fluorescence (PN kit) and chemiluminescence (target antibody) reagents during capillary detection. According to the manufacturer, the only occasionally nature of this peak is not known.

Human *ATF4* mRNA transcript sequence analysis

The V2 mRNA transcript of human *ATF4* was analyzed using freely available bioinformatics tools designed to predict RNA sequence and structural motifs, such as vsfold5, Hotknot, Pknot, pKiss, RECODE, PRFdb, SCRAM, and BERMP.

Western blot

All samples were resolved using Criterion TGX Precast Gels 4–20% (BIO-RAD) followed by western blotting. All primary antibodies used in this study are listed in [key resources table](#). The signal was developed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher) and detected in a G-Box imager (Syngene) using series of varying exposure time. Data were analyzed using Quantity One Software (Bio-Rad) and deposited at Mendeley; accession numbers are listed in the [key resources table](#).

Ribosome protection assay

HEK293T cells were seeded in 20 mL of media in the 15 cm dish and grown to a confluency of 60–70% before being treated with either DMSO (control) or thapsigargin (1 μ M) for 3h. For experiments involving either WT or SL3^{Mut-1} *ATF4* reporters ([Figures 7D](#) and [S5C](#)), cells were independently transfected with either reporter plasmid at approximately 40–50% cell confluency and – 8 h after transfection – treated with DMSO (Control) or thapsigargin (1 μ M) for additional 3h. Cells were then treated with formaldehyde (“cross-linked”, incubated with HCHO 0.8% for 5 min at 4°C and the reactions were quenched with 75 mM glycine for 5 min, as described^{22,27}) or cycloheximide (“non-crosslinked”; incubated with 100 μ g/mL cycloheximide for 1 min at 37°C, as shown before,⁵⁴ following which cell lysates were prepared. Concentration of cell lysates were measured on NanoDrop at OD₂₆₀ and all samples were divided into two-halves. One-half (5 AU OD₂₆₀ per 1 mL of a lysate) was treated with Ambion RNase I (4U) (Invitrogen) at 30°C for 10 min at 500 rpm to digest all enzyme-accessible RNA. The other half was processed the same way but without the RNase I treatment. Nuclease reactions were stopped with SUPERaseIn RNase inhibitor (8U) (Invitrogen). All samples (RNase I treated and

non-treated) were supplemented with 1 mL of RNA blue (Top Bio) and mixed vigorously by inverting the tubes. To reverse crosslinking, formaldehyde-crosslinked samples were incubated at 65°C for 20 min with an intermittent shaking by inverting the tubes. Subsequently, all the samples were cooled down at RT for 5 min and 5 μ L of SPIKE RNA (*in vitro* transcribed yeast RPL41a) was added as an internal control of RNA isolation to all the samples and RNA was isolated according to the manufacturer's (Top Bio s.r.o) instructions. The resulting RNA pellets were dissolved in 40 μ L of nuclease free water. RNA concentrations were measured using NanoDrop. To remove any DNA contaminants, 2.5 μ g of RNA samples were treated with TURBO DNase (Invitrogen) according to the manufacturer's instructions. For cDNA synthesis, 1 μ g of DNase-treated RNA was used for each sample using a High-Capacity cDNA Reverse Transcription kit. For qPCR reactions, HOT FIREPol EvaGreen qPCR Mix Plus 5x was mixed with 0.4 μ M primers (for the SPIKE primer set) and 0.25 μ M (for all Amplicon 1–3 primer sets) primers and cDNA, and run using the following program: 95°C for 15 s, followed by 40 cycles of 95°C for 15 s, 57.4°C (for SPIKE primer set) or 62°C (for all Amplicon 1–3 primer sets) for 20 s, and 72°C for 20 s. Melting curve was measured between 60°C and 90°C to assess amplification of the single specific product. qPCR data was analyzed as described in the main text and corresponding figure legends.

Total and poly(A) RNA isolation

Total RNA for T3 ligation assay was isolated using the RNA Blue reagent (Top-Bio) according to the manufacturer's instructions, 5 mL of the reagent was used *per* one 15 cm dish containing HEK293T or HeLa cells grown to approximately 80% confluency. The resulting RNA pellets were resuspended in RNase-free water (Thermo-Fisher) and the concentration was quantified by NanoDrop. Subsequently, poly(A) RNA for T3-ligation assay was purified using the Poly(A)Purist MAG kit (Thermo Fisher) according to the manufacturer's instructions. The poly(A) RNA was stored in 70% ethanol at –80°C and the subsequent steps of precipitation and precipitate resuspension in RNase-free water were finished just before proceeding to the T3-ligation assay.

T3 ligation assay and qPCR

The T3 ligation assay was adopted from.³⁸ Briefly, each ligation reaction mixture A consisted of 20 nM of corresponding probe SGL (Data S3), 20 nM of corresponding probe SGR, 1x T3 ligation buffer (NEB), and ~200–500 ng of poly(A) RNA. The ligation reaction mixture B consisted of T3 DNA ligase (NEB) diluted to 10U/ μ L with ligation buffer. Mixtures A were heated at 85°C for 3 min and then incubated at 35°C for 10 min, and then the ligation reaction mixtures B were added. The final volumes of the ligation reaction mixtures were 10 μ L and contained 10U of T3 DNA ligase. These resultant mixtures were then incubated at 25°C for 5, 15 or 60 min and chilled on ice immediately. qPCR was carried out according to vendor's instructions (Solis BioDyne) using Bio-Rad CFX384 Real-Time PCR System. The 10 μ L qPCR reactions contained 1x HOTFIREPol EvaGreen qPCR Mix Plus, 200 nM of each adapter primer (SGadapterFOR and SGadapterREV) and 1 μ L of individual ligation reactions and were run using the following program: 95°C for 15 min followed by 44 cycles of 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s. Melting curves were analyzed between 65°C and 90°C. Results were analyzed using Bio-Rad CFX Manager. Cycle differences between thapsigargin-treated and mock-treated samples were calculated for each individually analyzed region and the resultant ratios were then used for the normalization of each of the investigated regions (containing either *ATF4* A₂₃₅ or A₃₂₆) to both control regions (containing *ATF4* A₂₆₇ and A₃₁₁).

Ribosome profiling datasets analysis

Riboseq fastq files of untreated or sodium arsenite (GSE17432, GSE55195) or tunicamycin (GSE113171) treated samples from previously published datasets^{24,30,31} were used for analysis in this study. Reads devoid of rRNA and tRNA sequences were aligned to the human reference genome and transcriptome (GRCh38.p13, annotation release 109) by STAR aligner (2.7.10a)⁵⁵ using local alignment with 10% mismatches, and with indels and soft clipping allowed for transcriptomic alignment. Transcriptomic alignments were further filtered to obtain unique alignments using custom programs.⁵⁶ The Unique transcriptomic alignments and MANE project (v. 1.0) annotation were used to generate ribosome foot-print coverage and P-site analysis plots of *ATF4* transcript by RiboWaltz package.⁴⁷

QUANTIFICATION AND STATISTICAL ANALYSIS

Variables were tested for normality using the Shapiro-Wilk normality test. Based on the normality test, the differences between experimental groups were tested by the t test or by Mann-Whitney test (stated in the Figure's legend). Variables are presented as mean $\pm$ SD and p values of <0.05 were considered statistically significant. GraphPad Prism statistical software (ver. 10.1.2, GraphPad Software, San Diego, CA, USA, RRID:SCR_002798) and SciPy (ver. 1.10.1)⁴³, Matplotlib (ver. 3.7.0)⁴⁵ libraries in Python (ver. 3.11.3)⁴⁴ were used for statistical analyses and visualization. The heatmaps showing the differences in the relative expression of mutant versus WT constructs were created in R (ver. 4.2.2) using the 'pheatmap' package (ver. 1.0.12).⁴⁶ For the ribosome protection assay, statistical significance was assessed using unpaired, two-sided, t test with Bonferroni correction.

ADDITIONAL RESOURCES

This work does not use additional resources.