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POINT OF VIEW

Does eIF3 promote reinitiation after translation of short upstream ORFs also in mammalian cells?

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ABSTRACT

Reinitiation after translation of short upstream ORFs (uORFs) represents one of the means of regulation of gene expression on the mRNA-specific level in response to changing environmental conditions. Over the years it has been shown-mainly in budding yeast-that its efficiency depends on *cis*-acting features occurring in sequences flanking reinitiation-permissive uORFs, the nature of their coding sequences, as well as protein factors acting *in trans*. We earlier demonstrated that the first two uORFs from the reinitiation-regulated yeast *GCN4* mRNA leader carry specific structural elements in their 5′ sequences that interact with the translation initiation factor eIF3 to prevent full ribosomal recycling post their translation. Actually, this interaction turned out to be instrumental in stabilizing the mRNA·40S post-termination complex, which is thus capable to eventually resume scanning and reinitiate on the next AUG start site downstream. Recently, we also provided important *in vivo* evidence strongly supporting the long-standing idea that to stimulate reinitiation, eIF3 has to remain bound to ribosomes elongating these uORFs until their stop codon has been reached. Here we examined the importance of eIF3 and sequences flanking uORF1 of the human functional homolog of yeast *GCN4, ATF4*, in stimulation of efficient reinitiation. We revealed that the molecular basis of the reinitiation mechanism is conserved between yeasts and humans.

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Introduction

Translation of mRNA has four stages: initiation, elongation, termination, and ribosome recycling. During recycling, the post-termination 80S ribosome is first split into the small 40S and large 60S subunits by the energy-depended action of ABCE1.[1] However, mRNA and deacylated tRNA remain bound to the small subunit and must be removed in the second step by a joint action of either canonical initiation factors eIF1, eIF1A and eIF3, or by eIF2D (also known as Ligatin) or by the heterodimer MCT1-DENR.[2-5] As such, ribosome recycling can be considered as the link between translation termination and initiation because termination, recycling and initiation use several factors in common, like for example eIF3.[6-8] Even though ribosome recycling naturally captures the translational cycle, there are specific exceptions where the completion of the full recycling step is undesirable or even detrimental, and the termination reaction is followed by reinitiation (REI) on the same mRNA molecule at a site downstream of the stop codon (reviewed in[6,9,10]). Translation reinitiation is a gene-specific regulatory mechanism where upon translation of the so-called REI-permissive short upstream ORF (uORF) only the large 60S subunit and deacylated tRNA are recycled, whereas the mRNA is retained on the post-termination 40S subunit to allow REI downstream. It has been well established that most of relatively widespread uORFs across all eukaryotic genomes in principle inhibits expression of the main ORF by preventing the fully recycled ribosome to reach its start site. Hence, existence of REI-permissive uORFs, which are often part of intricate regulatory circuits together with REI-non-permissive uORFs, is very critical as it enables-upon various stimuli-efficient expression of a main ORF. Importantly, various oncogenes, proteins involved in differentiation, development, cell cycle, stress response, learning and memory forming can be found on the list of REI-regulated mRNAs (see for example[11-13]).

Practically since the onset of this scientific direction, the textbook example of an mRNA regulated *via* REI has been the yeast *GCN4* gene encoding a very potent transcriptional activator.[14] The *GCN4* mRNA contains four short uORFs in its 5′ leader, out of which the first two (uORF1 and uORF2) are highly REI-permissive, while the remaining two (uORF3 and uORF4) allow only negligible levels of REI.[15,16] Their specific effects in combination with stress-induced changes in the level of one of the key initiating complexes composed of Met-tRNA$_i^{Met}$ and eIF2·GTP (the so-called ternary complex-TC) create a fail-safe mechanism that allows *GCN4* translation only under specific stresses.[15,17] The trick is that the distance between the REI-permissive vs. non-permissive uORFs is long enough that under non-stress conditions (characteristic of high TC levels)

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most of the post-termination 40S ribosomes scanning downstream from uORF1 or uORF2 stop codons will reacquire the TC before AUG of uORF3 or uORF4 has been reached-as a result the GCN4 protein cannot be made. At the same time, it is short enough to ensure that under specific stress conditions (characteristic of low TC levels, when longer time is needed to reacquire the TC), majority of these ribosomes will rebind the TC after bypassing the REI-non-permissive uORFs-as a result these will be skipped and the *GCN4* translation eventually initiated. Over the years it has been demonstrated that the REI potential of uORF1 and uORF2 is determined by: (i) the presence of the AU-rich motif in the 3′ sequence; (ii) their defined length and coding triplets composition; (iii) specific REI-promoting elements (RPEs) situated in their 5′ sequences; and (iv.) the functional interaction of some of the RPEs (namely RPE i. and iv. of uORF1 and RPE v. of uORF2) with the N-terminal domain (NTD) of the a/TIF32 subunit of the translation initiation factor 3 (eIF3) within the context of the post-termination mRNA·40S complex.[15,16,18-24] The favorable location of the a/TIF32-NTD on the 40S subunit next to the mRNA exit channel[25-27] led to an idea that while the eIF3-bound 40S ribosome scans through the region upstream of uORF1 (or uORF2) and translates it as the fully assembled 80S ribosome still bound by eIF3, the RPEs progressively fold into a specific secondary structure. Upon termination, eIF3 interacts with these RPEs to specifically stabilize only the small ribosomal subunit on the uORF1 (or uORF2) stop codon. Thanks to this incomplete ribosomal recycling, the post-termination 40S subunit can, upon acquisition of other essential eIFs, subsequently resume scanning for REI downstream.[21] Actually, continued presence of some eIFs on early elongating ribosomes as a prerequisite for efficient REI had been a long standing hypothesis[18] that was strongly supported by our most recent yeast work.[28] With the help of a newly developed *in vivo* RNA-protein Ni^{+2} pull down (Rap-Nip) assay we have clearly demonstrated that eIF3 does travel with early elongating ribosomes and interacts with RPEs *in vivo*, and this eIF3s ability is critical for stimulation of efficient reinitiation downstream of REI-promoting uORFs. Besides eIF3, the mRNA-delivery eIF4F complex, and particularly the central one-third fragment of eIF4G interacting with eIF3 and eIF4A, was also suggested to remain bound to early elongation ribosomes and promote efficient REI, at least in an *in vitro* reconstituted mammalian system.[29] However, firm experimental evidence is lacking in this case.

Here we set out to examine whether the just described molecular mechanism of REI relying on *cis*-acting features of REI-permissive uORFs and eIF3 is conserved between yeasts and humans. We used an extensively studied mRNA encoding transcriptional activator ATF4 (the mammalian functional homolog of yeast GCN4) that contains two uORFs as a reporter that we mutagenized. We also knocked down several eIF3 subunits, in particular eIF3a (implicated in REI in yeasts[21]) and eIF3h (shown to stimulate REI in plants[30,31]), and checked their effects on REI efficiency in human cells. Our analysis revealed that the *ATF4*s uORF1 is in analogy to uORF1 of *GCN4* also surrounded by *cis*-acting features, with those occurring in its 5′ leader specifically structured, that ensure its permissiveness for REI. Furthermore, we also show that human eIF3h (like its plant counterpart) enhances efficiency of REI.

Novel insights into the molecular mechanism of reinitiation in human cells

Sequences flanking uORF1 of ATF4 substantially increase its reinitiation potential

As mentioned above, mammalian *ATF4* mRNA contains only two uORFs in its leader in contrast to four uORFs of yeast *GCN4* (Fig. 1A). However, only the first uORF1 of the two fulfills the requirements of a typical short uORF with a REI-potential because it is composed of only three sense codons and the distance between its stop codon and AUG of uORF2 is in most species 87 nucleotides or close to it. (Based on Kozak 1987,[32] the optimal distance ensuring efficient REI in mammals is 80 nt and more.) This could enable a similar mode of regulation under stress vs. non-stress conditions like in the case of *GCN4* despite the fact that *ATF4*s uORF2 is markedly different from *GCN4*s uORFs 3 and 4. It is too long to be even considered as an uORF with some REI potential (59 amino acids residues) and, most importantly, its sequence partially overlaps the *ATF4* ORF in a different reading frame. Therefore, according to the current model, all ribosomes that reinitiate on uORF2 will under normal conditions terminate past the *ATF4* AUG and thus prevent its translation.[33,34] Nonetheless, taking into account the striking similarity between the *GCN4*s and *ATF4*s uORF1 with respect to their arrangement and proposed function, we were curious to examine what else they have in common. In other words, we asked whether *ATF4*s uORF1 utilizes an identical molecular strategy to that of *GCN4*s uORF1.

To answer this question, we first isolated total RNA from human HEK293T cells and using the 5′ RLM-RACE system from Ambion, generated cDNA carrying full-length 5′ UTR of human *ATF4* and precisely mapped its transcriptional start site (Fig. 1C). We then replaced the 5′ and 3′ sequences (either individually or in combination) of human *ATF4*s uORF1, which might hypothetically correspond to the 5′ RPEs and 3′ AU-rich motif of *GCN4*s uORF1, with stretches of supposedly linear (CAA)n triplets (Fig. 1A). In detail, we replaced 69 nts upstream of uORF1 (in "CAAup") and 25 nts downstream of uORF1 (in "CAAdown"); in addition we combined these mutations in a single construct "CAAup+down." The resulting mutant variants were introduced into the uORF1-only *ATF4-Luc* construct containing solitary uORF1 kindly provided by the Wek's laboratory[33] and the luciferase activity, as an indicator of the REI efficiency, was measured in HEK293T cells and normalized to mRNA levels of individual constructs. Please note that the inhibitory effect of uORF2 was neutralized by mutating its AUG to AGG implying that these constructs could be analyzed without using a stress inducer. As shown in Fig. 1B, both sequences flanking uORF1 are-in a striking analogy to the *GCN4*s uORF1-required for efficient REI. Replacement of the 5′ sequence ("CAAup") decreased the REI efficiency to a greater extent (down to ~47%) than the replacement of the 3′ sequence ("CAAdown;" down to ~71%), suggesting that its contribution is significantly greater. Interestingly, the opposite is true in case of the *GCN4*s uORF1[21]. The combination of both mutations ("CAA up+-down") produced a fully additive effect-downregulation to ~35% (Fig. 1B). Together these findings strongly indicate that both upstream and downstream sequences of uORF1

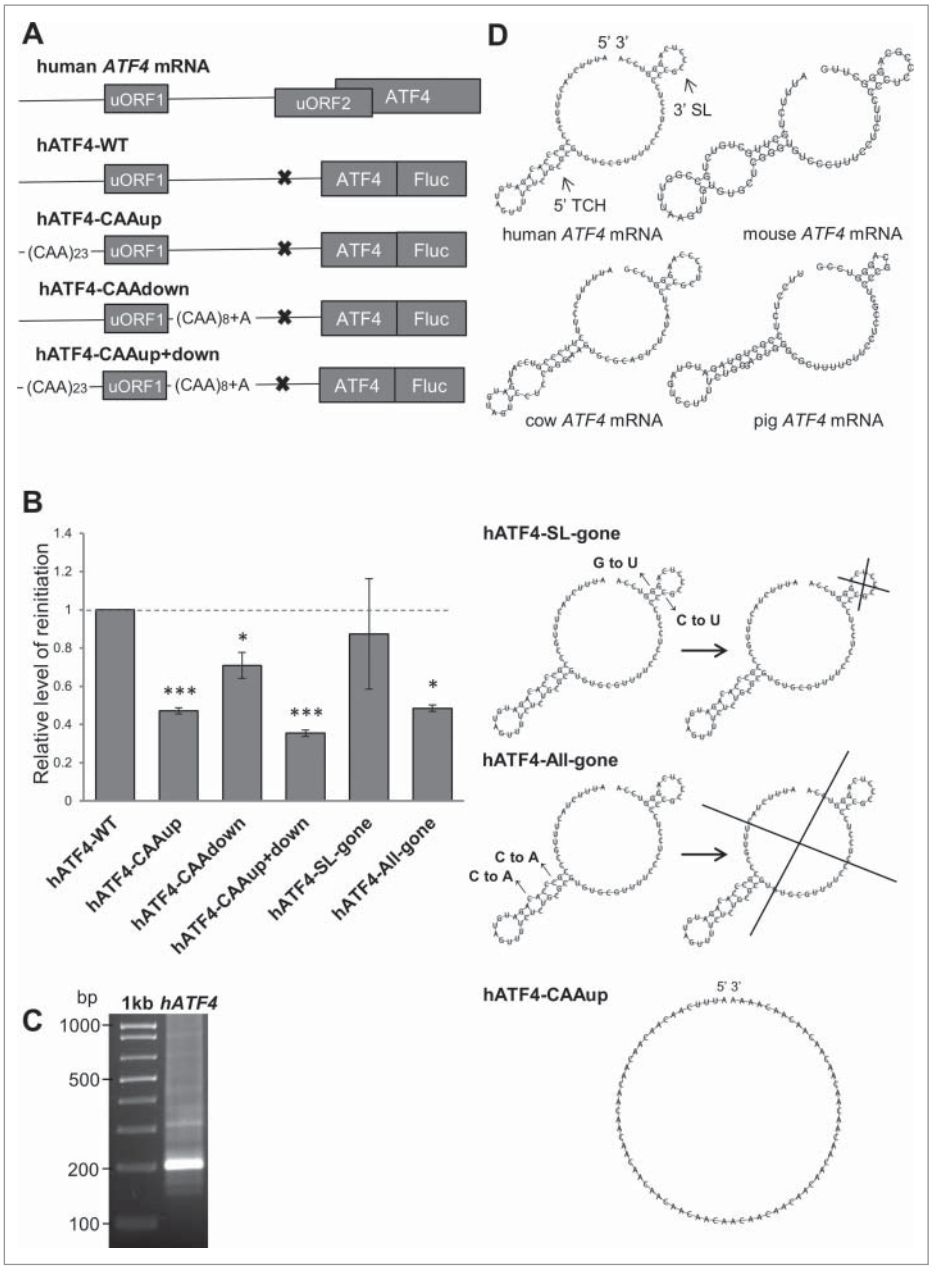


Figure 1. Flanking sequences of human *ATF4*s uORF1 individually contribute to ensure its high propensity for reinitiation. A) Schematics of the human *ATF4* mRNA and mutagenized constructs used in this study. Please note that the inhibitory effect of uORF2 was neutralized by mutating its AUG to AGG to simplify the analysis, because thus modified constructs could be analyzed without using a stress inducer. In "CAAup," a major part of the original sequence upstream of uORF1 was replaced by 23 CAA triplets (the transcriptional start site and 9 nts immediately preceding AUG of uORF1 that are buried in the mRNA binding channel of the ribosome terminating on uORF1 were left intact, as in case of *GCN4*s uORF1[21]); in "CAAdown," the original sequence encompassing 25 nts immediately following the stop codon of uORF1 was replaced by 7 CAA triplets followed by one CAAA tetranucleotide; in "CAAup+down," both of these substitutions were combined. B) All constructs shown in A) were transfected into HEK293T cells and subjected to Dual luciferase assay normalized to mRNA levels (Fluc/Rluc) as described in Materials and Methods. The statistical analysis was performed using One sample *t* test; statistical significance is indicated by stars (one star means $P \leq 0.05$, 3 stars $P \leq 0.001$). C) 5RLM-RACE of human *ATF4* cDNA prepared from total RNA derived from HEK293T cells. DNA sample was separated by gel electrophoresis in a 2% agarose gel, cut out and processed to be sequenced (the obtained sequence indeed corresponded to the 5′ UTR of human *ATF4* mRNA - NM_182810 in NCBI). Size markers in base pairs are indicated in the left. D) Secondary structure predictions of the entire 5′ sequence of *ATF4*s uORF1 in indicated mammals as determined by the RNA Vienna package software.[35] The bottom panels depict the "CAAup" mutation and 2 double-point substitutions engineered to disrupt either the individual structures or the 5′ UTR fold as whole. Please see the main text for further details.

independently contribute to its overall REI potential by more than 60%, suggesting that this important translational control mechanism is evolutionary conserved.

Given the fact that the 5′ sequence of the *GCN4*s uORF1, as well as the 5′ sequence of a single uORF of another yeast transcriptional activator YAP1, contain specific structural and sequence-specific REI-promoting *cis*-acting features, the RPEs,[21] we next investigated whether the 5′ sequence of *ATF4*s uORF1 also adopt some specific structure, and if so, whether it is also important for efficient REI. Therefore, we subjected the entire region preceding the *ATF4* uORF1 to *in silico* modeling by the RNA Vienna package software.[35] As in case of *GCN4*, our prediction was based on the fact that the 5′ sequence is not a standalone molecule with a rigid structure but its fold forms

and changes dynamically as the sequence emerges from the ribosomal mRNA exit pore.[21] Hence, we divided the 5′ UTR of uORF1 into two consecutive segments and first folded the extreme 5′ segment, which formed a stable triple-circle hairpin ("5′ TCH") (Fig. 1D). After that we added the other segment and continued with modeling of the entire 5′ sequence as it emerged from the mRNA exit pore with the initially identified 5′ triple-circle hairpin structure "pre-folded." As a result, a stem-loop formed proximal to the 3′ end ("3′ SL"), in addition to the 5′ triple-circle hairpin. These structures and their spacing not only resemble similar structures representing *GCN4*s RPEs ii. and iv.,[21] they also seem to be very well conserved at least among other mammalian species (Fig. 1D).

To examine the prospective physiologic importance of these structures, we further used *in silico* modeling to design and test minimal mutations disrupting one or the other structure using the same reporter system as described above. The first mutation, "SL-gone" (where C64 and G73 were both mutated to Us), was designed to disrupt the 3′ stem-loop; however, its effect on luciferase activity was very mild (∼13% reduction) indicating that this stem-loop contributes to REI only negligibly (Fig. 1B). We did not find any computational prediction that would disrupt selectively only the 5′ triple-circle hairpin, hence as the second mutation we chose "All-gone" (where C17 and C21 were both mutated to As), which disrupts both structures. Strikingly, the effect of this mutation showed the same dramatic drop in the luciferase activity as the "CAAup" construct (down to ∼49%) strongly suggesting that the 5′ triple-circle hairpin highly likely is what lies behind the REI-promoting effect of 5′ sequences of *ATF4* uORF1 (Fig. 1B). However, at present we cannot tell whether it is the entire specific structure or only some sequential motif within this structure, like for example the apical circle that is required for its function in promoting REI.

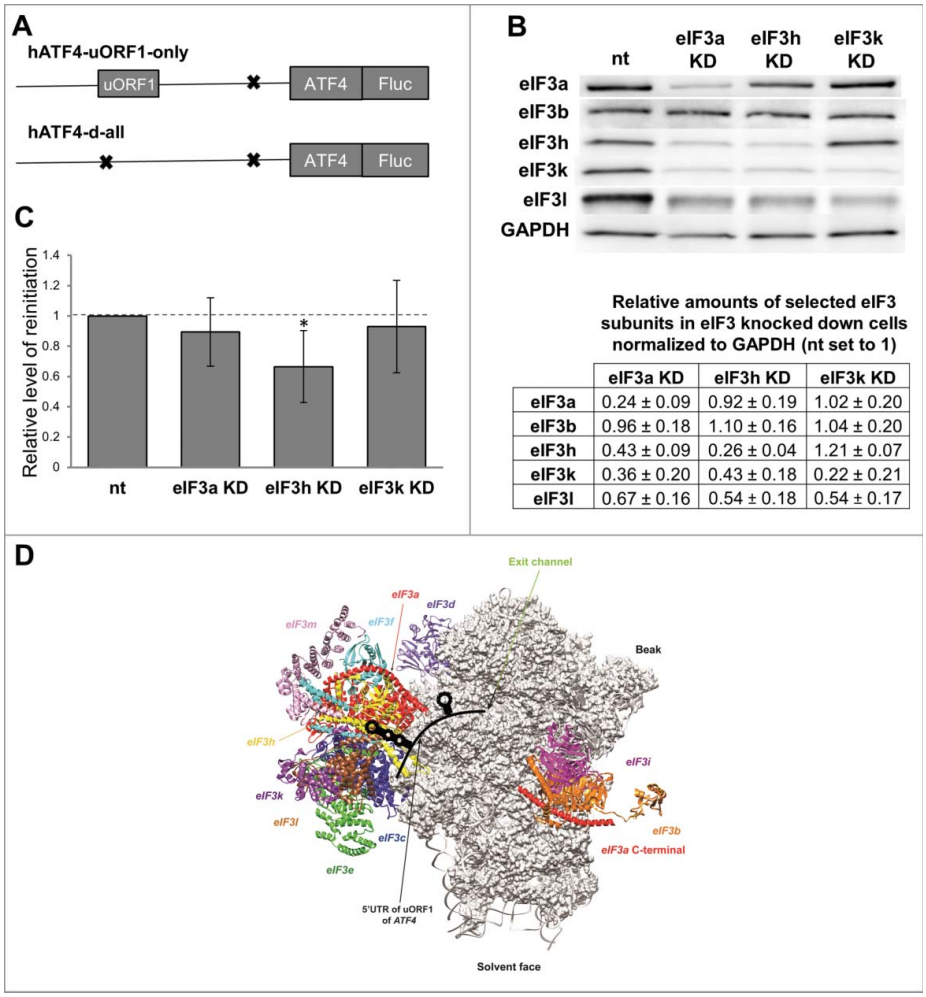
eIF3h promotes translation reinitiation in human cells

As mentioned above, RPE i. and iv. of the *GCN4*s uORF1 specifically interact with the N-terminal domain of the a/TIF32 subunit of eIF3 and this interaction is instrumental for stabilizing the 40S·mRNA post-termination complex. Hence the next obvious question we asked was whether human eIF3 also contributes to efficient REI on the *ATF4* mRNA. To our knowledge, the prospective role of eIF3a in REI has never been tested in the past; however, there are a few reports implicating the eIF3h subunit in REI in plants.[30,31] To address this question, we individually reduced the eIF3a and eIF3h expression by knocking them down with On-target plus siRNA system (Dharmacon) as described before,[36,37] and measured the luciferase activity in thus treated HeLa cells transfected with human *ATF4-Luc* constructs bearing either uORF1 alone ("uORF1-only") or none of the uORFs ("d-all") (Fig. 2A). The latter construct was used for normalization purposes. As a control we used cells treated with non-targeting siRNA, as well as cells knocked down for eIF3k. The knock down efficiency for all 3 eIF3 subunits was as observed before[37]-expression was reduced by ∼70–80% (Fig. 2B). Please note that we used HeLa instead of HEK293T cells owing to the fact that the efficiency of down-regulation of all eIF3 subunits is significantly greater in HeLa cells.[36] Also note that the eIF3k knock down results in the loss of only two non-essential subunits (eIF3k by itself and its interacting partner eIF3l) from the rest of human 12-subunit eIF3, the eIF3h knock down eliminates itself plus both eIF3kandl, whereas the eIF3a knock down pretty much destroys the entire eIF3 complex leaving intact only the "Yeast-Like-Core" assembly composed of the eIF3b–eIF3i–eIF3g subunits (Fig. 2B).[36,37] As shown in Fig. 2C, the eIF3k knock down displayed practically no impact on the efficiency of REI; similarly the eIF3a knock down produced only an insignificantly modest reduction (by ∼11%). However, the eIF3h knock down led to a statistically significant reduction by ∼34%. Importantly, the eIF3a knock down as the only knock down downregulated general translation initiation rates as judged from our measurements of the "d-all" construct; this is expected given the detrimental consequences of the eIF3a knock down on the overall integrity of the entire eIF3 complex and its function in general initiation.[36,37] Hence, we cannot conclude anything specific regarding its involvement in REI in mammals. However, the fact that the eIF3h knock down (co-downregulating also the expression of the eIF3kandl dimer) clearly impacted the efficiency of REI, whereas the eIF3k knock down (co-downregulating only the eIF3kandl dimer) showed no impact whatsoever, suggests that eIF3h does enhance efficiency of REI also in humans.

Concluding remarks

Two questions we asked in this article were: (1) Is there any mechanistic resemblance in the *modus operandi* between REI-permissive uORFs from mRNA leaders of functional homologs from two rather diverse eukaryotic organisms like yeasts (*GCN4*) and humans (*ATF4*)?; and (2) Does eIF3a and/or eIF3h promote reinitiation in mammals? The answer is yes to both questions. Flanking sequences of *ATF4*s uORF1 independently contribute to significantly boost the basic level of REI that this uORF allows. In addition, its 5′ sequence contains two well conserved structural features-the 5′ triple-circle hairpin and the 3′ stem-loop-that resemble the structural features of *GCN4*s uORF1 and the former of which seems to be fully responsible for the observed effect. Finally, whether or not eIF3a promotes REI as in budding yeast cannot be judged from our analysis; however, eIF3h does seem to be involved like in plants. In fact, it is interesting to note that human eIF3h seems to adopt a similar position on the ribosome to the REI-promoting N-terminal domain of yeast eIF3a/TIF32; i.e., right next to the mRNA exit channel (Fig. 2D), where it could interact with the 5′ triple-circle hairpin post uORF1 translation. This further supports the idea that in the 12-subunit eIF3 complex, eIF3h has a direct role in stimulating reinitiation.

According to recent reports, uORFs occur at a much higher frequency in mammalian (∼45%) mRNAs than in yeast (∼13%).[11,38-40] Yeast studies on *GCN4* and *YAP1* mRNA leaders,[16,21,41] as well as an early report examining REI efficiency of randomly generated uORFs[42] strongly suggest that majority of yeast uORFs are severely REI-non-permissive. Even though uORFs are prevalent translational repressors also in mammals,[43,44] there is a prevailing notion that uORFs in mammalian mRNAs (including randomly laboratory-designed uORFs)



Relative amounts of selected eIF3 subunits in eIF3 knocked down cells normalized to GAPDH (nt set to 1)

	eIF3a KD	eIF3h KD	eIF3k KD
eIF3a	0.24 ± 0.09	0.92 ± 0.19	1.02 ± 0.20
eIF3b	0.96 ± 0.18	1.10 ± 0.16	1.04 ± 0.20
eIF3h	0.43 ± 0.09	0.26 ± 0.04	1.21 ± 0.07
eIF3k	0.36 ± 0.20	0.43 ± 0.18	0.22 ± 0.21
eIF3l	0.67 ± 0.16	0.54 ± 0.18	0.54 ± 0.17

Figure 2. The eIF3h subunit of human eIF3 enhances efficiency of resumption of scanning from *ATF4*s uORF1 for reinitiation downstream. A) Schematics of *hATF4-Fluc* constructs used in C). B) Efficiency of siRNA-mediated downregulation and co-downregulation of protein levels of selected eIF3 subunits normalized to house-keeping GAPDH and Non-targeted (nt) control cells estimated by Western blotting. C) Relative *Firefly* luciferase signals obtained from HeLa cells knocked down for indicated eIF3 subunits transfected with either the "uORF1-only" or "d-all" constructs (the latter was used for normalization purposes), expressed as relative percentages of *Fluc* signals obtained from Non-targeted control (nt) cells. The *Firefly* luciferase signals were individually normalized to mRNA levels of each reporter, which were beforehand normalized to the spike RNA added before the RNA extraction. Statistical analysis was performed using One sample *t* test; statistical significance is indicated by stars (one star means $P \leq 0.05$). D) Graphical illustration of the proposed arrangement of the post-termination complex on *ATF4*s uORF1 with its secondary structures interacting with the eIF3h subunit of eIF3 to promote resumption of scanning for REI on the *ATF4* mRNA. Depicted is the exit channel view of the 48S PIC (adopted from[47]) illustrating 12 color-coded eIF3 subunits with eIF3a and eIF3h indicated by an arrow. The 5′ UTR of the *ATF4*s uORF1 highlighting its secondary structures is shown in black.

are in general less repressive for REI than in yeast, usually reducing protein expression by 30 to 80% (i.e., they allow at least some resumption of scanning and reinitiation),[11] which suggests that there might be a smaller requirement for specific sequences.[9] Our results from both yeast and humans seem to agree with this theory, because whereas eliminating all *cis*-acting sequences flanking uORF1 or uORF2 of yeast *GCN4* fully abolished their REI potential,[16] substituting the similar sequences flanking *ATF4*s uORF1 with unstructured stretches of CAA repeats reduced the efficiency of REI "only" by ~65% (Fig. 1B; hATF4-CAAupanddown). Hence it does seem likely that most of mammalian uORFs are less inhibitory than in yeast, and only if they form an integral part of some sophisticated stress-related regulatory system often containing more than one uORF, nature equipped some of them with specific *cis*-acing features that render them highly permissive. What lies behind this difference between budding yeast and humans (mammals)?

It could very well be the nature of the two initiation factors that have been implicated in stimulating REI in one and/or the other organism; i.e., eIF3 and to a lesser extent also eIF4G,[20,21,29] and that differ most dramatically between yeast and vertebrates in several aspects. (1) Human eIF3 has practically twice as many subunits than its yeast counterpart; (2) human eIF4G is markedly longer and has more direct interacting partners; and 3), perhaps the most important difference is that mammalian eIF3 does directly interact with eIF4G; however, in yeasts this contact is supposedly only bridged by eIF5 and eIF1 (reviewed in [6,9]).

If we assume that eIF4G and eIF3 are indeed capable to persist throughout uORF translation on the mammalian 80S ribosome to stabilize the post-termination mRNA·40S complex, their direct contact could substantially empower this stabilization process. This would set the basal level of permissiveness for REI in mammals higher than it is set in the budding yeast, where these two factors do not directly interact, which may weaken the eIF4G interaction with elongating ribosomes. In the light of the recent findings, the fact that the recycling factor ABCE1 interacts with the intersubunit face of the 40S subunit

even after ribosomal recycling and most likely also promotes the initiation phase in close co-operation with eIF3[45,46] further suggests that eIF4G could, *via* its direct connection with eIF3, modulate ribosomal recycling in a way that would favor dissociation of only the 60S subunit and deacylated tRNA, which would stimulate REI. Since reinitiation-as a molecular phenomenon-is also rather interesting from the medical point of view (there is a rapidly growing number of articles reporting contributions of defective uORF functions to various human diseases[10]), more work is certainly needed to fully understand the mechanistic aspects of this intriguing difference, as well as the reinitiation mechanism as a whole.

Material and methods

Dual luciferase reporter assays

HEK293T cells were grown at 37°C and 5% CO_2 in 6-well plates in DMEM (Sigma, cat # D6429) supplemented with 10% FBS (Sigma, cat # F7524). The cells were lysed directly on plate with 1x Glo Lysis Buffer (Promega, cat # E266A) exactly 24 hours after the *Firefly* and *Renilla* reporter plasmids transfection with TurboFect (Thermo Scientific, cat # R0531). The lysate was then transferred into a white flat-bottom 96-well plate and part of the lysate was stored for RNA isolation. The Dual-Glo® Luciferase Assay System (Promega, cat # E2940) was used according to the vendor's instructions. The *Renilla* luciferase signal was used for normalization purposes. Total RNA was isolated using the RNA Blue reagent (Top Bio, cat # R013) according to the manufacturer's instructions. After the Turbo DNase digestion (Ambion, cat # AM2238), cDNA was synthesized using the High-capacity cDNA reverse transcription kit (Applied Biosystems, # 4368813). qPCR was performed using 5 × HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne # 08–25–00020). The mRNA levels of *Firefly* luciferase were normalized to *Renilla* luciferase mRNA levels. The obtained qPCR data were used for normalization of measured luciferase activities. qPCR primers are listed in Supplementary Table S1.

siRNA treatment, whole cell extract preparation and Western blotting

HeLa cells were grown at 37°C and 5% CO_2 in 6-well plates in DMEM (Sigma, cat # D6429) supplemented with 10% FBS (Sigma, cat # F7524). 24 hours after seeding, cells were transfected with the ON-TARGETplus siRNA cocktail system from Dharmacon at a final concentration of 5 nM (human eIF3a cat # L-019534–00, eIF3h cat # L-003883–00, eIF3k cat # L-020216–02 and Non-Targeting siRNA cat # D-001810–10). INTERFERin (Polyplus, cat # 409) was used as a transfection reagent and transfection was performed according to the vendor's instructions.

For Western blotting, cells were harvested 3 d after siRNA transfection in lysis buffer containing 1M Tris-HCl pH6.8, 20% glycerol, 20% SDS, 2% β-merkaptoethanol and 5% bromphenolblue. All samples were resolved using SDS-PAGE followed by Western blotting. All primary antibodies used in this study are listed in Supplementary Table S2. The Western signals were developed using the SuperSignal West Femto Maximum Sensitivity Substrate from Thermo Scientific (cat # 34096) and detected in a G-Box imager from Syngene using a series of varying exposure times. Signals were processed with Quantity One (BioRad). The resulting values were normalized as indicated in the corresponding figure legend.

For siRNA treatments followed by *Firefly* luciferase reporter assays, transfection of *Firefly* reporter plasmids was performed 48 hours after the siRNA treatment and cells were harvested 24 hours later as described above. The *Firefly* luciferase signal was normalized to the reporter's mRNA level, which was beforehand normalized to the spike RNA (particularly yeast *RPL41a* mRNA) added before the RNA extraction. In detail, HeLa cells in 6-well plates were lysed in 200 μl of 1x Glo Lysis Buffer (Promega, cat # E266A). 70 μl of this lysate was directly used for the luciferase reporter assay, and another 70 μl was mixed with 2 μl of yeast spike *RPL41a* mRNA to the final amount of approx. 100 ng per sample, and subsequently also with 750 μl of RNA Blue reagent (Top Bio, cat # R013). The total RNA was isolated according to the manufacturer's instructions. After the Turbo DNase digestion (Ambion, cat # AM2238), cDNA was synthesized using the High-capacity cDNA reverse transcription kit (Applied Biosystems, # 4368813). qPCR was performed using 5 × HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne # 08–25–00020). The signal from *Firefly* luciferase reporter plasmid was normalized to its mRNA levels, which were already normalized to the spike *RPL41a* mRNA to correct for any loss during the RNA isolation. The obtained values with individual constructs were finally normalized to the nt siRNA and the "d-all" control construct, the latter of which corrects for defects in general translation initiation.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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