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The Levels of a Universally Conserved tRNA Modification Regulate Cell Growth*

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Background: Post-transcriptional modification of N^6-threonylcarbamoyl-adenosine (t^6A) is required for decoding function of tRNAs that pair A-starting codons.
Results: t^6A-modified tRNAs are required for growth, to modulate TOR activity and translation efficiency.
Conclusion: Levels of t^6A-modified tRNAs establish growth potential in eukaryotes.
Significance: Recognition in eukaryotes of a conserved mechanism of growth control that relies on tRNA post-transcriptional modification.

N^6-Threonylcarbamoyl-adenosine (t^6A) is a universal modification occurring at position 37 in nearly all tRNAs that decode A-starting codons, including the eukaryotic initiator tRNA ($tRNA_i^{Met}$). Yeast lacking central components of the t^6A synthesis machinery, such as Tcs3p (Kae1p) or Tcs5p (Bud32p), show slow-growth phenotypes. In the present work, we show that loss of the *Drosophila tcs3* homolog also leads to a severe reduction in size and demonstrate, for the first time in a non-microbe, that Tcs3 is required for t^6A synthesis. In *Drosophila* and in mammals, $tRNA_i^{Met}$ is a limiting factor for cell and animal growth. We report that the t^6A-modified form of $tRNA_i^{Met}$ is the actual limiting factor. We show that changing the proportion of t^6A-modified $tRNA_i^{Met}$, by expression of an un-modifiable $tRNA_i^{Met}$ or changing the levels of Tcs3, regulate target of rapamycin (TOR) kinase activity and influences cell and animal growth *in vivo*. These findings reveal an unprecedented relationship between the translation machinery and TOR, where translation efficiency, limited by the availability of t^6A-modified tRNA, determines growth potential in eukaryotic cells.

Transfer RNAs (tRNAs) are an essential part of protein synthesis machinery, decoding the linear information contained in mRNA into the three-dimensional information of proteins. Upon their discovery in the 1950s, tRNAs were considered to be simple adaptors that did not contain regulatory potential (1). Since then, tRNAs have been linked to diverse regulatory processes (2): uncharged tRNAs regulate gene expression in response to nutrient availability (3); tRNAs directly bind cytochrome *c* inhibiting apoptosis (4); reduced levels of the initiator tRNA ($tRNA_i^{Met}$) represses cell growth in yeast (5) and it is a limiting factor for growth in mammalian cells (6) and whole organism growth in *Drosophila* (7). Additionally, cytoplasmic tRNA availability has been shown to influence TORC1[2] (*target of rapamycin complex 1*) activity (8). Altogether these results reveal that in eukaryotic cells tRNAs are not only passive decoders of genetic information but also act as key regulators. The concentrations and charged levels of tRNAs are only one aspect of their potential as regulators. tRNAs can harbor a subset of the more than 100 known modifications (9). These range from simple methylations inserted by a single methyltransferase to complex modifications like wybutosine (yW) and queuosine (Q) that are synthesized in multistep pathways (10). tRNA modifications occur both in the body of the tRNA affecting its stability and folding, and in the anticodon stem loop (ASL) influencing mRNA decoding (11). To date, 15 tRNA modifications have been associated with human diseases, including Type 2 diabetes and Familial dysautonomia (12).

One of the few universally conserved modifications found in tRNA is N^6-threonylcarbamoyl-adenosine (t^6A) occurring at position 37 in tRNAs that decode A-starting codons (ANN) (10), including $tRNA_i^{Met}$ (Fig. 1*A*). It stabilizes codon-anticodon interaction (13), suggesting that t^6A might have a role regulating protein synthesis initiation. The modification was discovered over 40 years ago (14), but the enzymes involved in t^6A synthesis were only identified and characterized in all domains of life in the last few years (15–21) and recently renamed (22). In yeast, components of the threonyl-carbamoyl transferase complex (TCTC, previously named KEOPS/EKC (kinase, endopeptidase and other proteins of small size/endopeptidase-like and kinase associated to transcribed chromatin) are required for t^6A synthesis. Tcs3p (Kae1p) and Tcs5p (Bud32p) are part of the TCTC complex. Mutation of either gene eliminates t^6A in tRNA and cause strong slow-growth phenotypes (15). Recently, we showed that Prpk (p53-related protein kinase), the *Drosoph-*

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[2] The abbreviations used are: TORC1, target of rapamycin complex 1; ASL, anticodon stem loop; t^6A, N^6-threonylcarbamoyl-adenosine; TCTC, threonyl-carbamoyl transferase complex; KEOPS/EKC, kinase, endopeptidase and other proteins of small size/endopeptidase-like and kinase associated to transcribed chromatin; Prpk, p53-related protein kinase; Tsc, tuberous sclerosis complex; TRITC, tetramethylrhodamine isothiocyanate; $PHAt^6A$, positive hybridization in the absence of t^6A.

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ila homolog of Tcs5p, is required for TORC1 activity and cell growth (23), suggesting t^6A could be required for growth in metazoans.

Insulin and its downstream signaling (PI3K/TOR) play a paramount role in organ and cell growth in invertebrates and vertebrates. This signaling pathway allows transduction of hormonal and nutritional cues into the protein synthesis machinery (24–26). After activation, insulin receptor recruits Chico/IRS allowing PI3K activation, which increases the levels of phosphatidylinositol (3,4,5)-triphosphate causing Pdk1 to translocate and activate Akt1/Pdk1 at the plasma membrane. Akt1 influences protein synthesis in two major ways. First, it restricts 4E-BP transcription, an inhibitory factor of the elongation factor 4E, through inhibition of FOXO transcription factor. Second, Akt1 inhibits tuberous sclerosis complex (Tsc1–Tsc2), allowing the accumulation of GTP-Rheb and the subsequent activation of TOR (27). TOR activity is also regulated by the nutrient and energy status of the cell, as amino acid levels regulate the Rag GTPases and ATP/AMP ratio the AMP-activated protein kinase (28, 29). TOR ultimately enhances protein synthesis through an activating phosphorylation on S6K (which phosphorylates S6 ribosomal protein) and inhibiting 4E-BP, also by phosphorylation. In addition, TOR ensures a capable translational machinery controlling ribosome biogenesis and tRNA transcription (30).

Absence of t^6A leads to an increase in +1 and −1 frameshifts and increases non-AUG start sites (16), which could explain its requirement for growth in eukaryotes. In addition, considering the particular limiting nature of the initiator tRNA for cell and animal growth (5, 7) and as this tRNA is modified by t^6A, we were prompted to investigate the impact of t^6A in whole animal context using *Drosophila*. All previous studies on t^6A function and synthesis have been performed in unicellular organisms or cells in culture (15–21). In this study, we address for the first time the role of t^6A in a metazoan.

Experimental Procedures

Husbandry, Fly Stocks, and Morphological Analysis—Animals were raised at low density at 25 °C on standard meal containing wheat flour (50 g/liter), fresh yeast (100 g/liter), agar-agar (11 g/liter), dextrose monohydrate (80 g/liter), propionic acid (6 ml/liter), and Nipagin (1.56 g/liter). Stocks were obtained from Bloomington Stock Center, Exelixis Collection, and Vienna Drosophila Resource Center. *tcs3* coding sequence was amplified using primers Tcs3f-F, 5′-ggatccatggtttgcgctttgggtattg, and Tcs3f-R, 5′-ggatccttagtcatcccgccagctgacc, cloned into TOPO-TA vector (Life Technologies), sequenced (Macrogen), and subcloned in the pUAST vector using a BamHI restriction site to later develop transgenic animals following the standard germ line transformation protocol (31). *Drosophila* wings were mounted in a 1:1 mixture of lactic acid: ethanol as described in Ref. 23 and photographed under a Olympus BX51 microscope using a Moticam 2500 digital camera (Motic).

Staining, Western Blot, and RT-PCR Analysis—Nuclei were stained with TO-PRO-3 (1:200, Invitrogen) and F-actin with TRITC-labeled phalloidin (1 μg/ml, Sigma). Larvae were dissected and fixed as described by de Celis and co-workers (32). Confocal images were captured using a Zeiss LSM 510 Meta confocal microscope. For Western blot, rabbit polyclonal phospho-S6K, S6K, phospho-Akt, Akt, and phospho-eIF2α (all 1:1000 from Cell Signaling) and mouse anti-actin (1:1000 from Santa Cruz Biotechnology) were used. Blotting was performed as described in Ref. 33. For RT-PCR, total RNA was extracted from third instar larvae using TRIzol reagent (Invitrogen). cDNAs were synthesized with the Improm-II kit (Promega). Primers used were Tsc3-F, 5′-ATGGTTTGCGCTTTGGGTATTG, and Tsc3-R, 5′-TTAGTCATCCCGCCAGCTGACC. As a loading control actin cDNA was amplified using actin-F, 5′-GCGTCGGTCAATTCAATCTT, and actin-R, 5′-AAGCTGCAACCTCTTCGTCA. The PCR protocol was: denaturation 30 s at 95 °C, and 25 cycles of denaturation 95 °C s, annealing 55 °C, and elongation for 45 s at 72 °C. The final elongation was 5 min at 72 °C. Non-conventional splicing of Xbp1 mRNA was analyzed as described in Ref. 34.

tRNA Extraction and Detection by HPLC—For yeast and *Drosophila* tRNA extraction the method by El Yacoubi *et al.* (16) was used. HPLC analysis was performed as described in Ref. 35. The retention time of t^6A was determined using a t^6A standard synthesized by Darrell Davis at the University of Utah.

Positive Hybridization in Absence of t^6A-($PHAt^6A$) Assays—tRNAs were mixed with 3 volumes of incubation solution (65% formamide, 0.08% formaldehyde, 1.3× MOPS) and incubated for 5 min at 65 °C for denaturation, mixed with a volume of ice-cold ×20 SSC, and kept on ice until used. This mixture was spotted on Biodyne-A (Thermo Scientific) nylon membrane and cross-linked by exposing it for 3 min to UV radiation. Afterward, pre-hybridization was done using DIG Easy Hyb (Roche) for 1 h at 42 °C with constant shaking. Biotinylated probes were designed to complement the *Drosophila* $tRNA_i^{Met}$ (*tRNA:M-i:61D*, *CR32482*) and synthetized by Integrated DNA Technologies (IA). Both ACL (/5Bios(G/T)C TGG GTT ATG GGC CCA GC) and TΨL (/5Bios(G/G)A GCA AGG TTT CGA TCC TCG) were prepared 1:10,000 in DIG Easy Hyb and incubated overnight at 42 °C with constant shaking. The membranes were rinsed three times for 10 min with 2× SSC, 0.2% SDS, followed by two extra washes, the first one a room temperature and the second at 55 °C. Detection was carried our using BrightStar kit from Ambion (TX) following the manufacturer's instructions.

FLP-out Clonal Analysis and Mitotic Clones—All the fly stocks employed were generated by standard crosses. To generate FLP-out clones in fat body cells, offspring was subjected to heat shock (37 °C) at 36 ± 12 h after egg laying for 2 min. To generate mitotic clones in fat body cells, the offspring was heat shocked (37 °C) from 0 to 6 h after egg laying for 1 h. Third instar larvae with clones were processed and tissues were visualized by confocal microscopy.

Yeast Strains and Growth Conditions—Yeast strains were grown on YPD (DIFCO Laboratories) at 30 °C. Synthetic minimal, with or without agar, S.D. base or S.D. base Gal/raf with or without dropout (-uracil, -ura; -leucine, -leu; -histidine, -his) were purchased from Clontech and prepared as recommended by the manufacturer. Transformations were carried out as described in the pYES-DEST52 manual (Invitrogen). Growth curves were obtained using a Bioscreen C MBR (Oy Growth Curves AB Ltd., Finland) at 30 °C and at maximum shaking. 300

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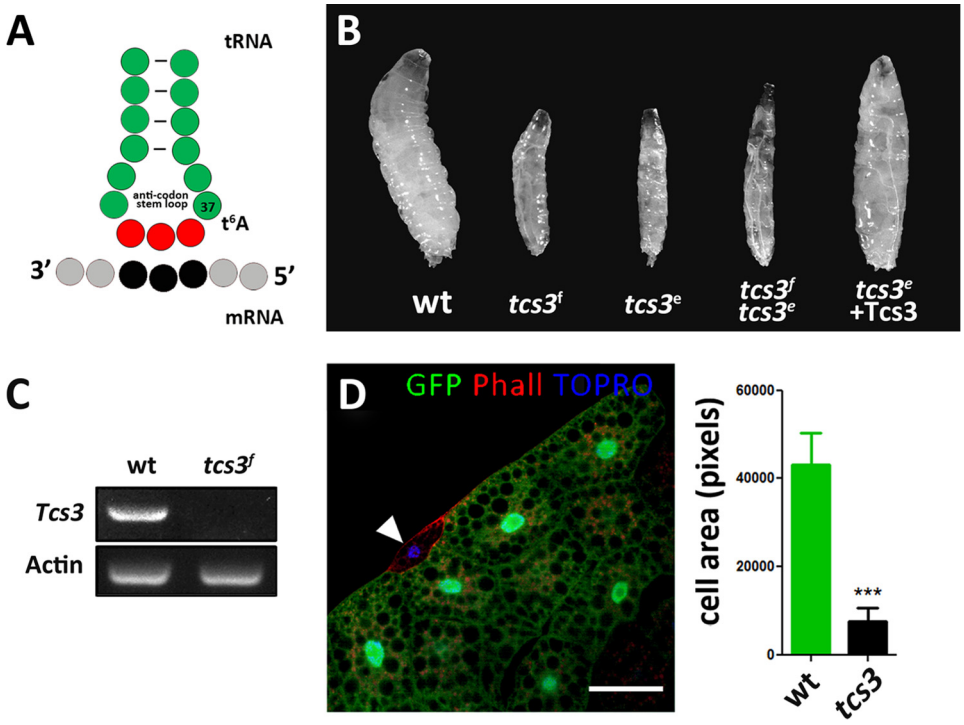

FIGURE 1. ***tcs3* mutants exhibit severe growth phenotypes.** *A,* schematic representation of anti-codon stem loop of tRNAs that pair A-starting codons, indicating position 37 where t^6A is present. Anti-codon (*red*) and codon (*black*) are also depicted. *B,* comparison between larvae from wild-type (*wt*), homozygous mutants for *tcs3* (*tcs3*^{f} and *tcs3*^{e}) and a trans heterozygous genetic background (*tcs3*^{f}/*tcs3*^{e}). Ubiquitous Tcs3 expression using the *armadillo-Gal4* driver (*tcs3*^{e}+*Tcs3*) rescued the growth phenotype. *C,* Tcs3 mRNA was not detected by RT-PCR in *tcs3*^{f}. Actin was amplified as loading control. *D,* mosaic fat body generated by FRT/FLP-mediated recombination shows that *tcs3* is required for growth cell autonomously. Cell area quantification shows that mutant cells (*GFP*−, *white arrowhead*) are significantly smaller than wild-type cells (*GFP*+) ($n = 50$ cells).

μl of culture was used in each well, and 10 replicates were used for each condition. Yeast cultures were grown on S.D. Gal/Raf −ura to saturation and diluted 500 times in S.D. Gal/Raf −ura before loading on the Bioscreen. The growth curves presented are the average of 10 independent replications.

Morphometric and Statistical Analysis—Wing area and hairs were quantified with Adobe Photoshop CS5 Extended using an analysis tool from at least 30 samples from female flies. Cell area was measured from fat body cells using ImageJ software (36). All data presented are mean ± S.D. and were subjected to Student's two-tailed *t* test. *p* values lower than 0.01 were considered to be significant, unless otherwise indicated. Flow cytometry was analyzed using FlowJo program.

Polysome Profiles—Gradients and ultracentrifugation were carried out as in Ref. 7. Polysome profiles were constructed by measuring 260 nm absorbance in samples from wild-type and *tcs3* mutants. The polysome fraction (indicated with a black line) was identified in divalent cation-free extraction buffer supplemented with chelating agents.

Bioinformatics—The Blast tools and resources at NCBI were used (37). Multiple protein alignments were performed with the ClustalW tool (38). Structure-based alignment was performed using the ESPript platform online (39). For domain recognition we used InterPro Scan (40).

Results

Drosophila Tcs3 Is Required for Organismal and Cell Autonomous Growth—BLASTP analysis using the *Saccharomyces cerevisiae* Tcs3p (Kae1p) sequence (NP_012964.2 YKR038C) as query, on the *Drosophila* proteome identified the CG4933-PA (95% coverage, $E = 5 \times 10^{-154}$) as best match. The *CG4933 locus* corresponds to an unnamed protein-coding sequence located in the left arm of the third chromosome at cytological position 72E2. The *locus* is predicted to produce a single transcript with 3 exons encoding a 347-amino acid protein. InterPro identified CG4933-PA as a member of Kae1/YgjD family, which is involved in the biosynthesis of t^6A (15, 41). Sequence alignment revealed conservation in several domains, particularly for amino acids that constitute the active center (data not shown). In FlyBase, CG4933-PA has been predicted to be part of TCTC (formerly KEOPS/EKC) and have threonylcarbamoyl transferase activity (42). Two insertional mutant alleles are available for *CG4933*; one has a PiggyBac transposon inserted in the 5′-UTR (f01978, BDSC) and the other in the coding sequence (e01173, Exelixis). Homozygous mutant larvae for these alleles exhibit a smaller size compared with wild-type animals (Fig. 1*B*). Mutants do not pupariate and die at larvae stages. These results are coherent with our previous reports on deficiency in another TCTC subunit, Tcs5 (Prpk) in the fly (23). We propose to name *CG4933* as *tcs3* (to be consistent with the new nomenclature and to reflect the ubiquitous role of this protein family). The two insertion alleles do not complement each other (Fig. 1*B*), confirming that both are mutant alleles of the same gene. Tcs3 mRNA was not detected by RT-PCR in *tcs3*^{e} mutant larvae (Fig. 1*C*). In addition, we could rescue the mutant phenotype by ubiquitously expressing the Tcs3 cDNA using the Gal4/UAS system (Fig. 1*B*, *right side*). To elucidate if

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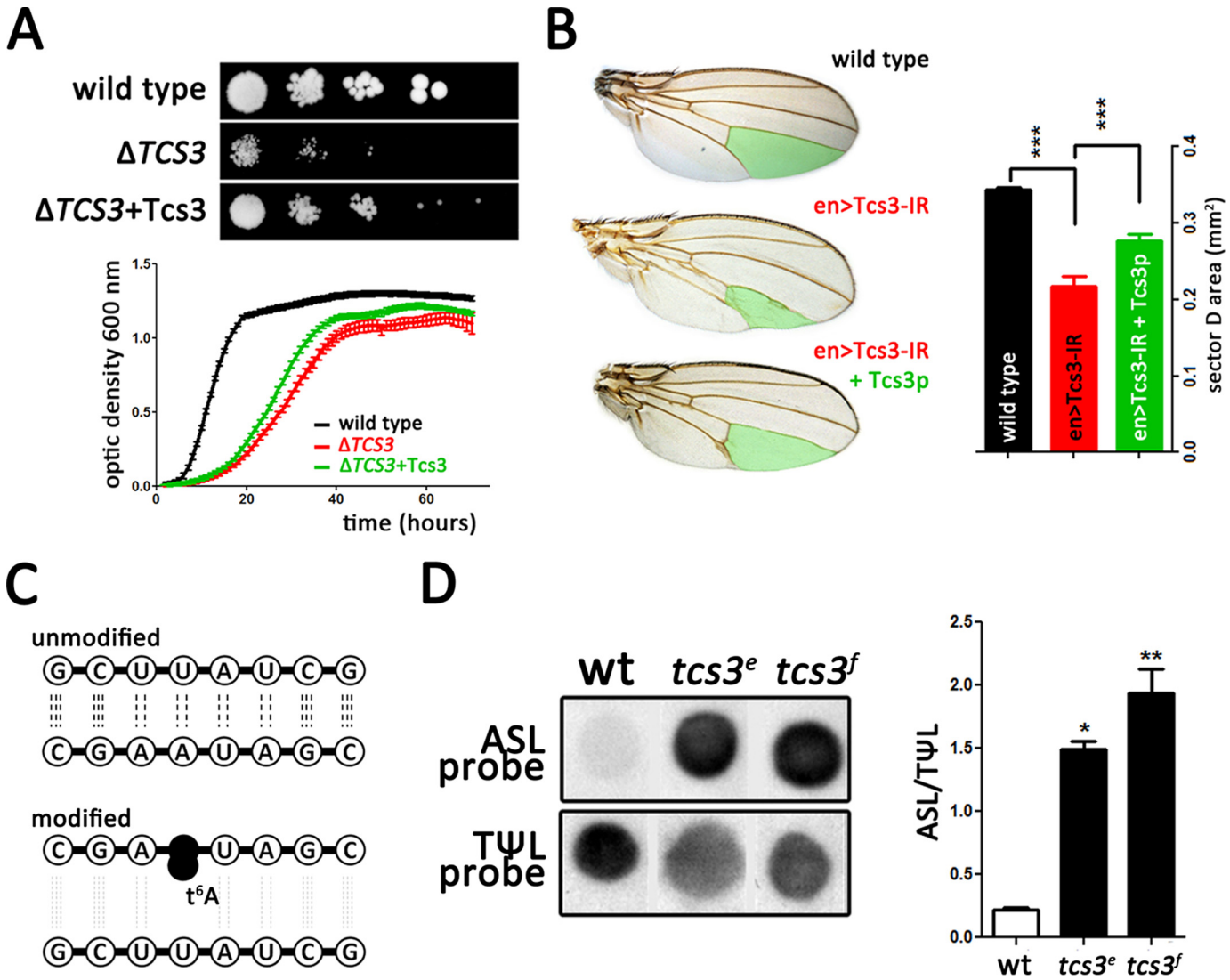

FIGURE 2. **Tcs3 is required for t^{6}A synthesis in *Drosophila*.** *A,* yeasts were plated in solid media in serial dilutions of 1:10 factor from left to right. Growth differences of wild-type, *TCS3* mutant cells (Δ*tcs3*), and mutant yeasts expressing the *Drosophila* Tcs3 homolog (Δ*tcs3*+*Tcs3*). Growth parameter of each strain was also analyzed in liquid media. Each strain has a color code ($n = 10$, $p < 0.005$). *B*, in *Drosophila*, Tcs3 was knocked-down using a specific inverted repeat construct expressed in the posterior wing compartment with the Gal4/UAS system (Tcs3-IR) and the growth phenotype evoked was rescued by yeast Tcs3p co-expression (*en*> Tcs3-IR+Tcs3). The area of sector D (colored in *green*) was measured and plotted following the same color code used in wing image labels ($n = 50$, mean ± S.D., *t* test $p < 0.005$). *C,* PHAt^{6}A. In brief, probes designed against different regions of the initiator tRNA evidenced the presence of t^{6}A modification. The strength of ASL probe (anti-codon stem loop) hybridization depends on the presence of t^{6}A, whereas the TΨL probe hybridizes an unmodified base stretch and serves as an internal loading control. When t^{6}A is absent, the tRNA-probe interaction is maximal, whereas its existence weakens the interaction. *D,* tRNAs from wild-type (*wt*) and mutant *tcs3* animals (*tcs3*^{e} and *tcs3*^{f}) were probed with ASL and TΨL probes. Dot blots were merged to make a better composition. Also a plot depicting the change in the ASL/TΨL signal ratio is shown. ASL signals obtained from *tcs3* mutants are significantly stronger than control counterparts ($n = 6$ samples).

the reduction in size phenotype is caused by a systemic failure or if it is a cell autonomous phenomenon, we generated mosaic animals by mitotic recombination and measured the cell area in fat body clones. Homozygous mutant cells presented a significantly smaller size than controls (Fig. 1*D*) indicating the cellular requirement of this gene.

Functional Complementation between Yeast and Drosophila Genes: Tcs3 Is Required to Modify the Initiator tRNA ($tRNA_i^{Met}$)—To establish the functional conservation between these two proteins, we carried out inter-species complementation experiments. Yeast *tcs3* mutants present a slow-growth phenotype (15) that can be partially, but significantly rescued, by ectopic expression of *Drosophila tsc3* (Fig. 2*A*). Consistently, the phenotype caused by Tcs3 knockdown in *Drosophila*, using an inverted repeated construct, was rescued in a similar extent by co-expressing yeast Tcs3p in the posterior compartment of the wing primordia using the *engrailed*-Gal4 driver (Fig. 2*B*). Altogether these results support the notion that *tcs3* is the *Drosophila TCS3* homolog and implicate its encoded protein in t^{6}A synthesis, which is required cell autonomously to sustain growth. Our *in silico* analysis and the interspecies complementation tests suggest that *Drosophila* Tcs3 is implicated in t^{6}A synthesis. This modification was detected using an adaptation of a hybridization method used to detect the presence of N^6-isopentenyl-adenosine (i^{6}A), another modification found at position 37 of specific tRNAs (43). Briefly, when the threonylcarbamoyl moiety is present in position Ala-37, it impairs Watson-Crick pairing between the tRNA and the specific DNA

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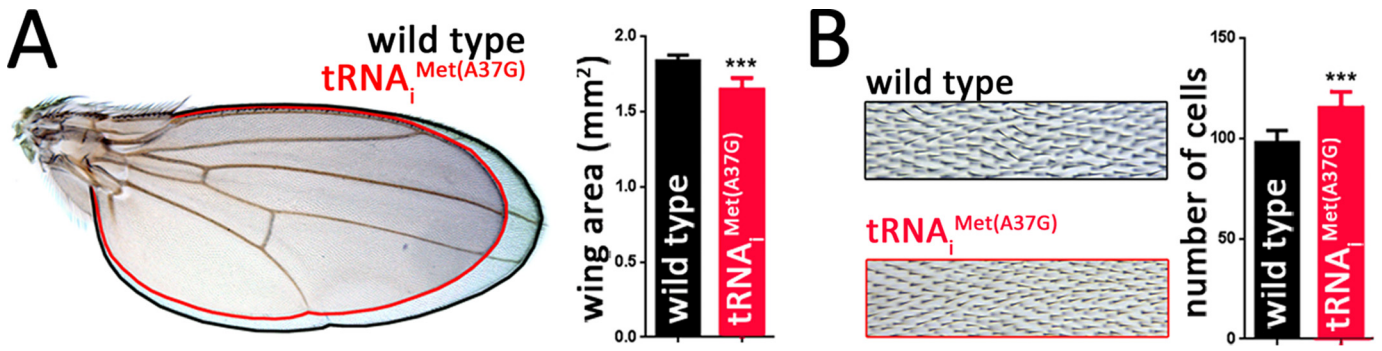

FIGURE 3. **Expression of an Ala-37 mutant initiator tRNA reduces growth.** *A*, comparison between wings from wild-type and from tRNA$_i^{Met(A37G)}$ homozygous transgenic animals. A significant reduction in wing area, together with a higher cell density (*B*) is detected in transgenic animals compared with control wings ($n = 50$ wings).

probe designed to complement the ASL. In the absence of t^{6}A, no interference occurs and base pairing can be complete. Consequently, a dimmer signal reveals a higher level of t^{6}A modification (Fig. 2*C*). We have named this technique PHAt^{6}A (positive hybridization in the absence of t^{6}A), and it correlates with the presence of t^{6}A in tRNAs.[3] Signal intensity obtained from tRNAs extracted from *tcs3* mutants was significantly stronger than control, suggesting a reduction in t^{6}A modification on tRNA$_i^{Met}$ (Fig. 2*D*). Our results indicate that Tcs3 is required for the specific modification of the tRNA$_i^{Met}$, however, this likely extends to the other tRNAs that recognize A-starting codons.

t^{6}A-modified tRNAs Are a Limiting Factor for Growth—tRNA$_i^{Met}$ is a limiting factor for growth in *Drosophila* (7) and is, in this organism, modified by t^{6}A (9). Hence, the t^{6}A-modified tRNA$_i^{Met}$ could be the actual limiting factor. To address this, a synthetic mutant tRNA$_i^{Met}$ that cannot be conjugated with t^{6}A was inserted in the *Drosophila* genome. In this construct, the Ala at position 37 was mutated to Gly (tRNA$_i^{Met(A37G)}$) thus interfering with the consensus sequence required for t^{6}A modification (44). Transcriptional regulatory sequences of this synthetic initiator tRNA, as well as 5′ and 3′ regions were taken from the endogenous sequences present in one of the four *Drosophila* initiator tRNA *locus* (*tRNA:M-i:61D, CR32482*) (45). Animals homozygous for the tRNA$_i^{Met(A37G)}$ insertion had smaller wings than control (Fig. 3*A*). Likewise, counting the wing hairs in an area showed that wings of transgenic animals have more cells by unit of area, indicating that transgenic wings are composed of smaller cells than wild-type animals (Fig. 3*B*). These results not only support the notion that initiator tRNA is limiting for growth (7), but suggests that the proportion of t^{6}A-modified initiator tRNA is the actual limiting factor. We then sought to increase the levels of t^{6}A-modified tRNA by overexpressing *tcs3* and asked whether this was sufficient to promote growth. Ubiquitous overexpression of *tcs3* correlated with a significant increase in t^{6}A-modified tRNA$_i^{Met}$ (Fig. 4*A*) and with larger wings (Fig. 4*B*). To confirm that growth enhancement was specifically caused by *tcs3* overexpression and not an indirect consequence of the insertion site of the UAS construct, we tested three different UAS insertions in combination with *daughterless*>Gal4 driver. Overexpression of *tcs3* with all transgenes promoted similar increased growth, indicating that overgrowth was caused specifically by *tcs3* overexpression (Fig. 4*B*). Also, overgrowth caused by *tcs3* was comparable with the effect caused by *maf1* (Pol III repressor) knockdown or the addition of an extra initiator tRNA *locus* (Fig. 4*B*), two experimental conditions that were previously shown to promote growth in *Drosophila* (7). *tcs3*-overexpressing animals were larger and composed of larger cells than control siblings, as flow cytometry analysis showed when control wing progenitor cells were compared with *tcs3*-overexpressing cells (Fig. 4*C*). Importantly, *tcs3*-overexpressing clones grew larger than controls, showing that the overgrowth response is a cell autonomous phenomenon (Fig. 4*D*). Altogether, these results show that t^{6}A-modified tRNAs are a cell autonomous limiting factor for growth. An equivalent result was obtained overexpressing *TCS3* in *S. cerevisiae*, as these cells grew faster than the control and contained more t^{6}A (Fig. 4, *E* and *F*). Overall our results indicate t^{6}A-modified tRNA$_i^{Met}$ is a limiting factor for growth and not only supports the hypothesis that the proportion of t^{6}A-modified tRNA$_i^{Met}$ determine the potential for cell growth, but also reveals the conserved nature of this feature among eukaryotic cells.

Levels of t^{6}A-modified tRNAs Alter TOR Kinase Activity—Because *tcs3* overexpression promotes growth (Fig. 4) and the phenotypes observed in *tcs3* mutants (Fig. 1, *B* and *D*) resemble deficiencies in positive regulators of the insulin/TOR pathway (46, 47), we examined if changes in t^{6}A-modified tRNA levels could modulate TOR activity. TOR is present in two structural and functionally different complexes (TORC1–2). TORC1 is related to growth control and one of its phosphorylation targets is S6K at Thr-398 (48). S6K phosphorylation was strongly diminished in *tcs3* mutants (Fig. 5*A*), suggesting the lack of TORC1 activity as a potential underlying cause of the growth deficiency. To directly activate TORC1, independently of t^{6}A levels, we overexpressed Rheb in homozygous *tsc3* mutants (49). Expression of Rheb in a *tcs3* mutant background was unable to rescue animal growth (Fig. 5*C*), even though TORC1 activity was enhanced in these animals (Fig. 5*D*). Also TORC2 activity was strongly reduced in *tcs3* mutants (Fig. 5*A*). These results indicate that tRNAs lacking t^{6}A caused a failure in the translation apparatus, probably due to poor or inaccurate codon recognition that disable it to sustain growth. To get insights about the protein synthesis status we performed polysome-profiling experiments and observed that *tcs3* mutants

[3] P. Thiaville, J. Bacusmo, D. Rojas-Benítez, A. Glavic, and V. de Crecy-Lagard, manuscript in preparation.

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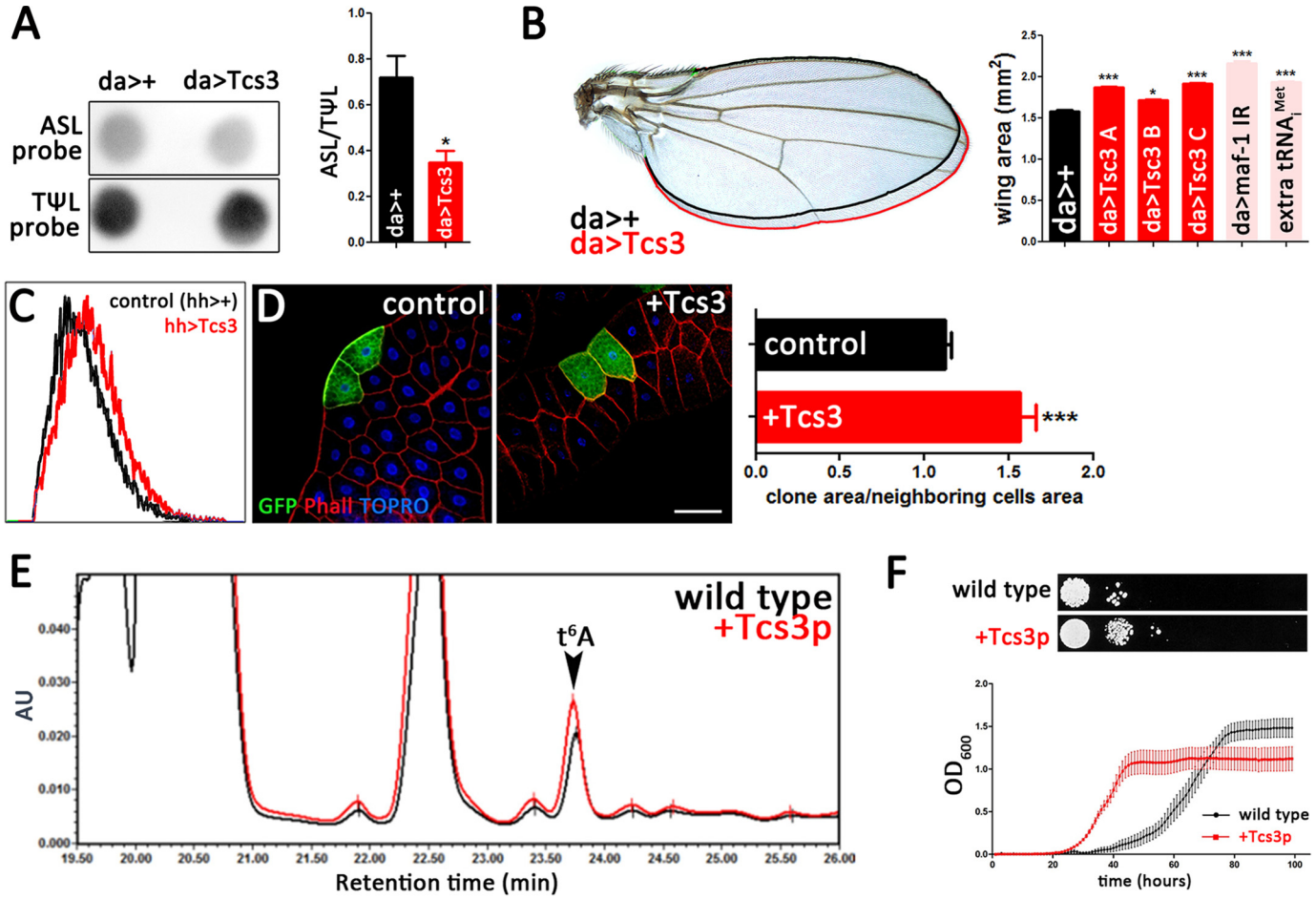

FIGURE 4. **An increment of t^{6}A-modified tRNAs promotes growth cell-autonomously.** *A,* representative image of PHAt^{6}A assay in samples from control and Tcs3-overexpressing animals. Signal intensity was quantified and plotted as ASL/TΨL ratio ($n = 4$ samples). *B,* a comparison between control (*black line*) and Tcs3-overexpressing wings (*red line*). Wing area was plotted showing that Tcs3 significantly promotes growth ($n = 50$ wings). As controls we used two other UAS insertions located in the second and third chromosome (Tcs3(B) and Tcs3(C)) were able to promote animal growth. Likewise other interventions with known effects on animal growth are: Maf1 knockdown and the addition of an extra *locus* of tRNA$_i^{Met}$. Measurements of wing area from control (da>+) and experimental animals show that these manipulations favor animal growth in a comparable range ($n = 50$, $p < 0.005$ to control). *C,* flow cytometry was used to compare cell size in Tcs3-overexpressing and control cells. *hedgehog*-Gal4 driver (*hh*>Gal4) was used to express Tcs3 and GFP in the wing posterior compartment, whereas control cells were obtained from the anterior compartment of the same imaginal discs (no GFP). Histogram are representative of 3 independent experiments. *D,* FLP-out Tcs3-overexpressing mosaic analysis (*bar* = 100 μm). Control clones express GFP only, whereas Tcs3-overexpressing clones express Tcs3 and GFP. Clone area was measured and normalized by the area of its neighbor cells; these ratios are presented in the chart ($n = 50$ cells). Wild-type yeast strain (BY4741) was transformed with empty pDEST52 vector (control) or with a pDEST52 construct to overexpress the *D. melanogaster* Tcs3p coding sequence (+Tcs3p). *E,* plot representing an archetypal HPLC elution profile of nucleosides from control (*black line*) or Tsc3p-overexpressing yeast (*red line*). *AU,* arbitrary units. *F,* serial dilution assays (1:10) were made from a suspension of cells with $A_{600} = 0.6$. Growth assay in liquid media were made measuring simultaneously the A_{600} of 10 independent wells and the plot is representative of 3 independent experiments.

have a strongly reduced polysome fraction (Fig. 5*B*), further supporting the notion of insufficient protein synthesis in *tcs3* mutants. This reduction of protein synthesis was not dependent on eIF2α phosphorylation, because it did not change in *tcs3* mutant larvae (Fig. 5*A*). On the other hand, increasing the proportion of t^{6}A-modified tRNAs by overexpressing *tcs3* stimulates TORC1 activity, as shown by the augmented phosphorylation of S6K (Fig. 5*E*). These findings expose a regulatory relationship between TOR activity and the availability of t^{6}A-modified tRNAs.

Discussion

tRNA molecules have a paramount role in protein synthesis and prior studies have centered on structural and biochemical features, overlooking their regulatory properties and functional interplay with cellular physiology. However, recent technology advances and shifts in research paradigms have opened new venues to investigate the non-canonical, ancestral, and divergent functions of tRNAs. The results presented here suggest a previously unexpected causal relationship by which changes in tRNA isoacceptors or their degree of modifications could not only be the result of cell differentiation processes or homeostatic responses to cellular stress, but also could channel an ontology trajectory that modulates the mode cells respond to environmental cues. Specifically, our results show a clear relationship between the level of t^{6}A modification, particularly of the initiator tRNA, and the growth potential of eukaryotic cells.

The machinery that synthetizes t^{6}A is conserved in all domains of life. In accordance, we identified *tcs3*, the *Drosophila* homolog of yeast *TCS3*, and established that *tcs3* is involved in t^{6}A synthesis. Furthermore, and in agreement with the severe slow-growth phenotype reported in yeast (15), we have deter-

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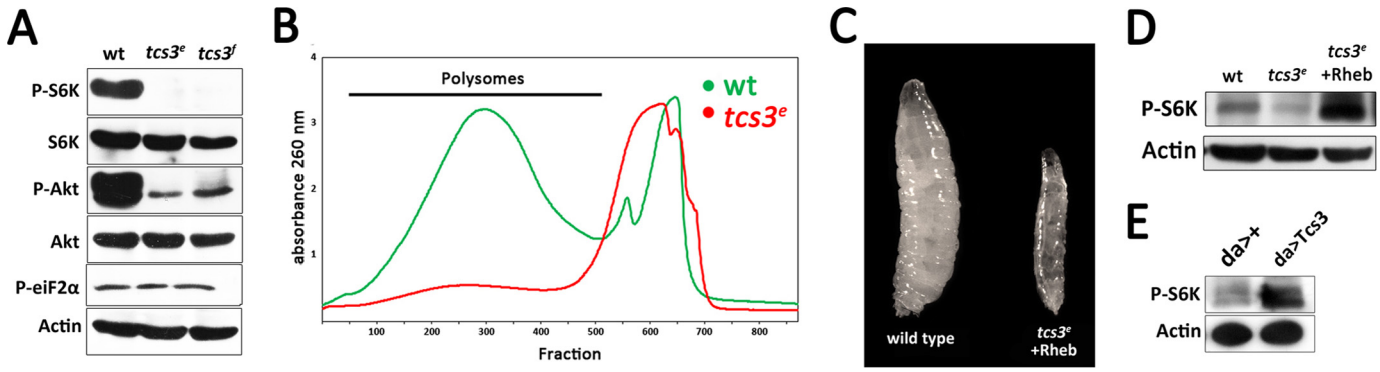

FIGURE 5. **Variations of TOR activity in animals with different levels of t^{6}A-modified tRNAs.** *A,* Western blot of S6K and Akt phosphorylation performed by TORC1 and TORC2, respectively, which in *tcs3* mutants (*tcs3^e* and *tcs^f*) was strongly reduced in comparison to wild-type animals. Total S6K, Akt, and actin were used as loading controls. Also we detected eIF2α phosphorylation at Ser-51 in these genetic backgrounds; actin was used as loading control. *B,* polysome profiles were constructed measuring 260 nm absorbance in samples obtained after ultracentrifugation in sucrose gradient. The polysome fraction (indicated with a *black line*) was identified in divalent cation-free extraction buffer and in the presence of chelating agents, wild-type (*green*) and *tcs3^e* mutant (*red*) larvae. *C,* comparison between wild-type and *tcs3^e* mutants expressing Rheb (*tcs3^e*+Rheb) using the *armadillo*>Gal4 driver. *D,* Western blot detection of S6K phosphorylation in mutant (*tcs3^e*) and mutant overexpressing Rheb (*tcs3^e*+Rheb). *E,* Western blot detecting S6K phosphorylation in control (da>+) and Tcs3-overexpressing animals (da>Tcs3). Western blot images are representative of 3 independent experiments in each case.

mined that this enzyme is necessary for cell autonomous growth in *Drosophila*, as we previously showed for Tcs5 (Prpk) (23). Metazoans are composed of different cell types, therefore making the phenotype observed in *tcs3* mutants even more complex than the ones observed in yeast and other unicellular organisms (15, 16, 18, 21, 50, 51). However, in every case analyzed, the deficiency of these enzymes strongly impairs cellular growth and proliferation. Interestingly, the possibility that fast growing or proliferating cells could have special requirements for t^{6}A-modified tRNAs is further hinted by the fact that human PRPK was originally identified in a transcriptional screen performed in IL-2-activated lymphocytes (52). In *Drosophila*, *tcs3* is differentially expressed in different anatomical structures and developmental times (53). Also OSGEP, the human homolog of *TCS3*, is deferentially expressed in human tissues (The Human Protein Atlas) (54). From a wider perspective, this indicates that t^{6}A-modified tRNAs have a relevant role modulating protein expression in different cellular contexts and in consequence, the study of the relationship of t^{6}A modification with protein synthesis regulation and growth control in physiological and pathological conditions, as in cancer, may emerge as a fertile area of research. In accordance with this, t^{6}A had been proposed in the past as a prognosis marker for breast cancer (55).

Changes in tRNA levels are well documented in physiological (56) and pathological (57) processes, as well as a cellular homeostatic response to face stress conditions (58). These variations allow cells to adapt to determined conditions regulating protein synthesis, a process termed adaptive translation (59). Also, changes in isoacceptors can promote or decrease translation of specific mRNAs. This type of behavior has been recently documented; proliferating cells present a different tRNA transcriptome compared with differentiating ones, and this is correlated with epigenetic landmarks in specific tRNAs *loci* (60). Thus, isoacceptor profiles would permit the cell to fine-tune gene expression to establish a particular proteome to face differentiation programs. In this regard, changes in the levels of tRNA post-transcriptional modification have not been considered enough and our findings invite to think that tRNA modifications, or at least t^{6}A-modified tRNAs, may have an important role in this regard. Changes in tRNA modification profiles have been reported to help cells cope with stressful conditions (58). Modification levels may have a direct role over cellular processes by conditioning, among others, the growth potential of a determined cell population.

Experiments published by the Grewal group (7) showed that the addition of an extra initiator tRNA *locus*, but no other tRNA, is sufficient to promote organismal growth in *Drosophila*. Our results showed that apparently the actual limiting factor for cell growth is the proportion of t^{6}A-modified initiator tRNA, as the addition of an extra *locus* that codes for a mutant initiator tRNA, which cannot be modified with t^{6}A, in fact inhibits growth. Thus, our results suggest that variations in the proportion of t^{6}A-modified initiator tRNA is able to condition the ability of cells to grow. Likewise *tcs3* overexpression consistently enhanced the levels of t^{6}A-modified initiator tRNA and promoted autonomous cell growth in accordance with our proposal.

It has been described, and it is widely accepted, that TORC1 is the central regulator of cell growth in eukaryotes. Because the phenotypes observed in *tcs3* mutants are remarkably similar to *tor* mutants (61) or some of its positive regulators (49, 62), an inter-relationship between the function of these elements would be expected. It has been shown that reductions in cytoplasmic tRNAs reduce TORC1 activity (8), suggesting the existence of an uncharacterized molecular feedback mechanism between the upstream protein synthesis controller, TORC1, and the canonical structural decoding blocks, the tRNAs. Furthermore, we found that *tcs3* mutants have a severe decrease in S6K phosphorylation, a direct target of TORC1, whereas *tcs3* overexpression enhanced it. Hence, levels of t^{6}A-modified tRNAs strongly influence TORC1 activity. This implies that the translation machinery, possibly through initiator complex assembly, is tightly linked with TORC1 and the activation of its downstream targets. Also *tcs3* mutants present a strong reduction of polysome fraction supporting a general failure in protein synthesis initiation caused by deficient ribosome assembly. In this respect, it has been described that the interaction of

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TORC2 with ribosome is required for its activation (63) and consequently, TORC2 activity was also reduced in *tcs3* mutants. However, neither the specific phosphorylation of Akt1 catalyzed by TORC2, nor the phenotypes of mutants in TORC2 components support a functional relationship that could explain our observations. Mistranslation and unfolded protein response activation have been reported in yeast *TCS3* mutants (18) and in *tcs3* and *tcs5* knockdown conditions in *Drosophila* (64), respectively. The results obtained with *tcs3* mutants indicate that the growth phenotype evidenced is complex and most likely based on the poor translation initiation, not induced by eIF2α activation, but due to a fundamental structural defect produced by the absence of t^6A-modified tRNAs.

Recently, Scheidt *et al.* (65) showed in yeast that absence of another tRNA modification altered TOR activity as well. Methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) is a modification present at position 34 and is required for the efficient decoding by $tRNA^{Lys}_{UUU}$ (10). The absence of mcm^5s^2U mislocalizes Gln3p due to reductions in TOR activity (65). Together with our results, these observations suggest that TOR activity is probably affected by the aberrant translation caused by deficiencies in either modification. The molecular nature of the components that connect translation and TOR are currently unknown. However, our findings indicate a tight association between the availability of t^6A-modified tRNAs and TOR to establish the general capability of the cell to grow.

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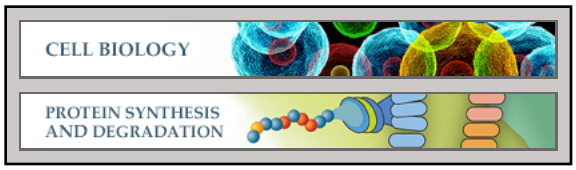

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