The *TOR* signaling pathway regulates starvation-induced pseudouridylation of yeast U2 snRNA

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ABSTRACT

Pseudouridine (Ψ) has been identified in various types of RNAs, including mRNA, rRNA, tRNA, snRNA, and many other noncoding RNAs. We have previously shown that RNA pseudouridylation, like DNA and protein modifications, can be induced by stress. For instance, growing yeast cells to saturation induces the formation of Ψ93 in U2 snRNA. Here, we further investigate this inducible RNA modification. We show that switching yeast cells from nutrient-rich medium to different nutrient-deprived media (including water) results in the formation of Ψ93 in U2 snRNA. Using gene deletion/conditional depletion as well as rapamycin treatment, we further show that the *TOR* signaling pathway, which controls cell entry into stationary phase, regulates Ψ93 formation. The *RAS*/cAMP signaling pathway, which parallels the *TOR* pathway, plays no role in this inducible modification.

Keywords: *TOR* signaling pathway; U2 snRNA; inducible pseudouridylation; nutrient-deprivation stress

INTRODUCTION

Pseudouridylation (Ψ) is the most abundant post-transcriptional modification found in various types of RNAs, including mRNA, tRNA, rRNA, spliceosomal snRNA, and a number of other noncoding RNAs (Reddy and Busch 1988; Bjork 1995; Massenet et al. 1998; Ofengand and Fournier 1998; Yu et al. 2005; Grosjean 2009). For instance, there are ~100 and ~50 Ψs in mammalian and *Saccharomyces cerevisiae* rRNAs, respectively (Ofengand and Fournier 1998). Likewise, eukaryotic spliceosomal snRNAs contain a large number of Ψs, and many of them are conserved across species. For example, vertebrate U2 snRNA contains 13 Ψs (Reddy and Busch 1988; Massenet et al. 1998; Karijolich et al. 2009), three of which are also found in the *S. cerevisiae* U2 branch site recognition region (Massenet et al. 1998). Virtually all of these Ψs tested so far are functionally important (King et al. 2003; Liang et al. 2007; Piekna-Przybylska et al. 2008; Wu et al. 2016).

RNA pseudouridylation can be catalyzed by two distinct molecular mechanisms, which are either RNA-dependent or RNA-independent (Ganot et al. 1997; Ni et al. 1997; Massenet et al. 1999; Ma et al. 2003, 2005). RNA-independent pseudouridylation is catalyzed by stand-alone protein enzymes, which recognize the substrate and catalyze the U-to-Ψ conversion (Massenet et al. 1999; Hoang and Ferré-D'Amaré 2001; Ma et al. 2003). In contrast, a family of protein–RNA complexes, known as box H/ACA sno (small nucleolar) or sca (small Cajal body-specific) RNPs (Ganot et al. 1997; Ni et al. 1997; Huttenhofer et al. 2001; Darzacq et al. 2002; Zhao et al. 2002; Ma et al. 2005; Kiss et al. 2010), is responsible for RNA-dependent pseudouridylation. In each box H/ACA RNP, there is a unique box H/ACA RNA and four core proteins (Cbf5/Nap57/Dyskerin, Nhp2, Gar1, and Nop10) (Yu et al. 2005). The RNA component (box H/ACA RNA) forms a unique "hairpin-hinge-hairpin-tail" structure (Fig. 1). In each of the two hairpins, there is an internal loop (also called pseudouridylation pocket) that base pairs with the substrate RNA. Thus, the target uridine is identified and subsequently converted to Ψ by the catalytic component Cbf5 (Fig. 1). The three Ψs of *S. cerevisiae* U2, located at positions 35, 42, and 44, are introduced by either an RNA-dependent or RNA-independent mechanism. Specifically, snR81, a box H/ACA RNP, catalyzes Ψ42 formation (Ma et al. 2005), and stand-alone enzymes Pus7 and Pus1 are responsible for the formation of Ψ35 and Ψ44, respectively (Massenet et al. 1999; Ma et al. 2003). Pseudouridylation at these three sites occurs constitutively.

We previously showed that, in addition to the three conserved Ψs discussed above, additional pseudouridines can be introduced into *S. cerevisiae* U2 snRNA under stress conditions (Wu et al. 2011; Ge and Yu 2013). Specifically,

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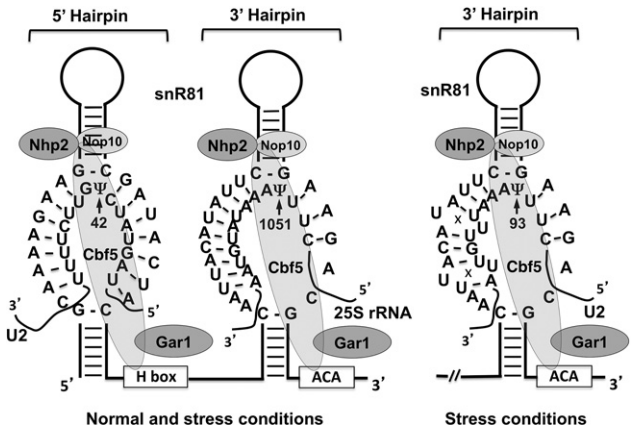


FIGURE 1. snR81 box H/ACA RNP-catalyzed pseudouridylation. The secondary structure (5′ hairpin-H box Hinge-3′-hairpin-ACA tail) and the sequences of two internal loops (pseudouridylation pockets) of snR81 box H/ACA RNA, along with their substrate sequences (paired with the internal loop sequences) are shown. The two sets of four core proteins (Cbf5, Nhp2, Nop10, and Gar1) that bind to box H/ACA RNA are also depicted. Under normal conditions, the 5′ pseudouridylation pocket within the 5′ hairpin of snR81 RNA guides (via base-pairing) the modification of U2 snRNA at position 42 (indicated), and the 3′ pseudouridylation pocket within the 3′ hairpin guides the modification of 25S rRNA at position 1051 (indicated). Under stress (nutrient-deprivation), the 3′ pseudouridylation pocket not only guides the modification of 25S rRNA at position 1051, but also directs the modification of U2 snRNA at position 93 (indicated at *far right*). Dash (-), base-pairing; cross (×), mismatch.

pseudouridylation occurs at position 93 (and to a lesser extent, at position 56 as well) when cells are grown to saturation; Ψ56 can also be induced by heat-shock. While the formation of Ψ56 is catalyzed by Pus7 in an RNA-independent manner, Ψ93 formation is catalyzed by snR81 (a box H/ACA RNP) in an RNA-dependent manner (Wu et al. 2011). The inducible nature of U2 pseudouridylation strongly suggests that this modification plays a regulatory role in pre-mRNA splicing, in which U2 snRNA participates, and indeed this proves to be true (Wu et al. 2011).

In the current work, we have studied inducible yeast U2 pseudouridylation at position 93 that is catalyzed by snR81 box H/ACA RNP, and demonstrated that Ψ93 formation is indeed induced by nutrient-deprivation and that the induction is regulated by the *TOR* pathway, a signaling pathway that plays an important role in determining how cells coordinate growth in response to changes in environmental conditions.

RESULTS

The formation of Ψ93 is induced by nutrient-deprivation

We previously showed that growing *S. cerevisiae* cells to saturation resulted in pseudouridylation in U2 snRNA at positions 56 and 93 (and to a lesser extent, position 91 as well) (Wu et al. 2011), suggesting that the formation of Ψ56 and Ψ93 (and Ψ91 as well) was induced by nutrient-deprivation. In order to confirm this nutrient-mediated induction, we decided to carry out further experiments, focusing on position 93 of U2, given that the formation of Ψ93 in *S. cerevisiae* U2 is much clearer and more efficient compared to the formation of Ψ56 and Ψ91. After growing yeast cells to log phase (nutrient-rich) in complete SD medium, we collected the cells and equally split them into four aliquots. Two of the aliquots were transferred to new complete SD medium; one was allowed to grow only to log phase (with dilution with new medium during growth) and the other to saturation phase (with no dilution). For the other two aliquots, one was transferred to a nutrient-deprived medium (synthetic medium lacking glucose) and the other to pure water. As expected, during the course of a 1-h continuous incubation, no cell growth was observed in SD medium lacking glucose or in water. Cells were subsequently collected, and pseudouridylation assay (CMC modification followed by primer extension) was performed.

As shown in Figure 2, Ψ93 was not detected in cells that were grown to log phase in complete SD medium (lanes 1 and 2). In contrast, Ψ93 was detected in cells grown to saturation, as expected (lanes 7 and 8). Importantly, Ψ93 (and to a lesser extent, Ψ91 as well) was also detected in cells incubated in synthetic medium lacking glucose (lanes 3 and 4)

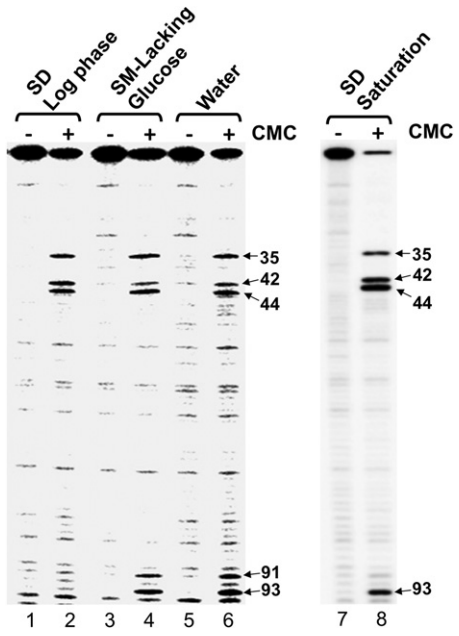


FIGURE 2. Induced formation of Ψ93 (as well as Ψ91) under different nutrient-deprivation conditions. Log-phase cells were either kept at log phase (lanes *1* and *2*), grown to saturation (lanes *7* and *8*), transferred to synthetic medium lacking glucose and incubated for another hour (lanes *3* and *4*), or transferred to pure water and incubated for another hour (lanes *5* and *6*). Cells were then collected and cellular RNAs recovered. Pseudouridylation assay (CMC-modification followed by primer extension) was subsequently performed. Signals corresponding to the three constitutively formed pseudouridines (Ψ35, Ψ42, and Ψ44) as well as inducibly formed Ψ93 and Ψ91 are indicated by arrows.

and in pure water (lanes 5 and 6). These results indicate that Ψ93 is indeed induced by nutrient-deprivation.

Blocking the *TOR* signaling pathway by rapamycin results in Ψ93 formation

The fact that pseudouridylation can be induced by nutrient-deprivation (Fig. 2) prompted us to carry out further investigations to determine what signaling pathway is involved.

It is well established that nutrient-starvation (e.g., cells grown to saturation) signals the shutdown of two parallel pathways, the *TOR* (target of rapamycin) and the *RAS*/cAMP pathways, thereby leading to the entry of cells into stationary phase (Broach 1991; Schmelzle and Hall 2000; Herman 2002; Schmelzle et al. 2004). It is thus likely that the formation of Ψ93 is controlled by either or both pathways. Since the *TOR* pathway can be readily blocked by rapamycin, we first tested the *TOR* pathway using this drug.

We treated yeast cells with rapamycin, and subsequently isolated total RNAs for pseudouridylation assay (CMC modification followed by primer extension). As shown in Figure 3, a clear Ψ93 signal was observed when rapamycin-treated cells were allowed to grow for 12 h to reach an OD_{600} of ~3 (far from saturation in YPD medium) (lane 3). We also carried out parallel control experiments where cells were treated with tunicamycin (an endoplasmic reticulum stress inducer) or DMSO (vehicle for rapamycin and tunicamycin) in exactly the same way (grown for 12 h); no or a very low level of Ψ93 was detected in tunicamycin-treated cells (OD_{600} of ~2; far from saturation), and a slightly higher level of Ψ93 was detected in cells cultured in DMSO medium (OD_{600} of ~8; close to but not at saturation) compared to the level of Ψ93 in rapamycin-treated cells (Fig. 3, cf. lanes 1 and 5 with lane 3). These results strongly suggest that the *TOR* signaling pathway is involved in induction of Ψ93 formation.

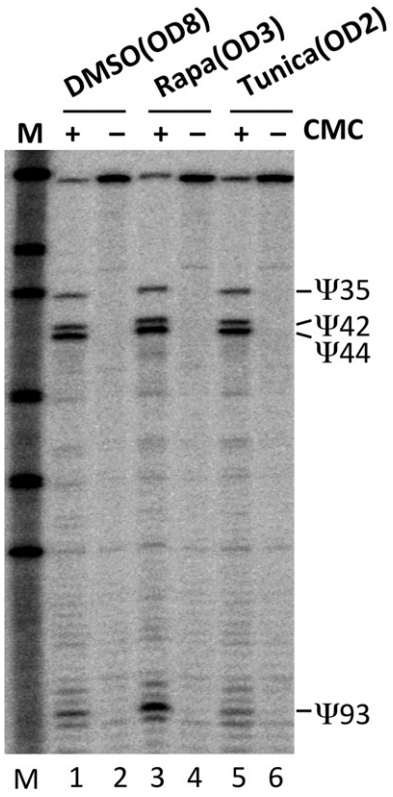


FIGURE 3. Ψ93 formation induced by rapamycin treatment. Yeast cells were treated with DMSO alone (lanes *1* and *2*), rapamycin dissolved in DMSO (lanes *3* and *4*), or tunicamycin dissolved in DMSO (lanes *5* and *6*) for ~12 h, and then harvested. Pseudouridylation assay was then performed. At the moment of cell harvest, DMSO-treated cells reached an OD_{600} of ~10, whereas rapamycin- and tunicamycin-treated cells reached an OD_{600} of only ~2–3.

Depletion of the Tor proteins results in Ψ93 formation even under nutrient-rich conditions

To further confirm the *TOR*-dependent Ψ93 formation, we used deletion strains coupled with conditional depletion to check for dependence of the *TOR* pathway.

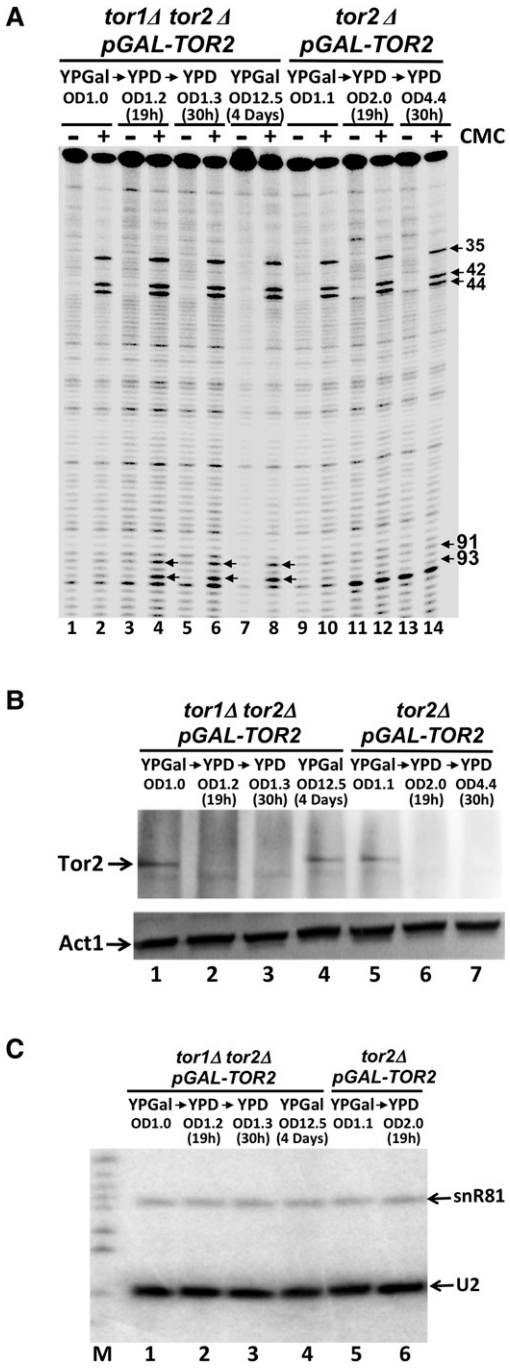
It is known that there are two complementary *TOR* genes (*TOR1* and *TOR2*) in *S. cerevisiae*. In order to check for dependence of the *TOR* signaling pathway, both *TOR1* and *TOR2* would need to be deleted. However, it is well established that, although tor1-deletion alone is viable, deletion of both *TOR1* and *TOR2* leads to lethality (Loewith et al. 2002). To overcome this problem, we took advantage of the availability of a yeast strain, JK350-21a, in which both chromosomal *TOR1* and chromosomal *TOR2* are deleted and a plasmid (pJK15)-borne *TOR2* is expressed when cultured in Gal medium (its expression is under the control of the Gal promoter) (Loewith et al. 2002). We grew the strain in Gal medium (YPGal) to log phase (OD 1.0), then transferred some cells to glucose medium (YPD, starting OD of 0.03), and then allowed the cells to grow for an additional 19 h, reaching an OD of 1.2, or 30 h to an OD of 1.3, thus depleting Tor2 while keeping the medium nutritionally rich. U2 RNA from these cells was then assayed for pseudouridylation. As shown in Figure 4A, while no Ψ93 was observed in U2 RNA isolated from log-phase cells (OD of 1) cultured in Gal-medium (lanes 1 and 2), a significant escalation of Ψ93 (and to a lesser extent, Ψ91 as well) was detected when cells were switched to glucose medium and grown to an OD of 1.2 or 1.3 (lanes 3–6). The level of Ψ93 (and Ψ91) in Tor2-depleted cells was comparable to that in Tor2-intact cells when they were grown in Gal-medium to saturation (OD 12) (lanes 7 and 8).

We also used another yeast strain, JK350-18a, in which only chromosomal *TOR2* is replaced by the plasmid-borne *TOR2* (pJK15), whose expression is controlled by the Gal promoter. We cultured this strain in exactly the same way as we did for strain JK350-21a, where both chromosomal *TOR1* and *TOR2* are replaced by the plasmid-borne *TOR2* (pJK15) (see above). As shown in Figure 4A, when the

medium is still rich in nutrients, depletion of Tor2 alone (wild-type *TOR1* background) was not sufficient to induce the formation of Ψ93 (lanes 11–14), just as seen in log-phase cells (lanes 9 and 10).

To ensure that Tor2 was indeed depleted after medium switch, we performed Western blotting using an anti-Tor2 antibody. As shown in Figure 4B, at the time of medium switch (lanes 1 and 5) or when cells were grown continuously in YPGal to saturation (lane 4), there was a clear Tor2 signal; 19 and 30 h after medium switch, no Tor2 was detected (lanes 2,3,6, and 7). In contrast, a similar level of actin, an internal loading control, was detected across all samples (lanes 1–7).

We also measured the level of snR81, a box H/ACA RNA responsible for directing the formation of Ψ93, in cells before and after Gal-to-Glu medium switch. Our primer-extension results showed that the snR81 level, relative to U2 (an internal loading control), was essentially the same under all culturing conditions (before and after medium switch) (Fig. 4C), suggesting that the *TOR* pathway regulates something else (rather than the level of the guide RNA) that is important for the induced pseudouridylation.



Depletion of the Ras proteins has no effect on induced formation of Ψ93

Since the *RAS*/cAMP signaling pathway, which is considered to function in parallel with the *TOR* pathway, is thought to also respond to nutrient-deprivation (Broach 1991; Schmelzle and Hall 2000; Herman 2002; Schmelzle et al. 2004; Ramachandran and Herman 2011), we next tested the possible interrelationship between the formation of Ψ93 and the shut-down of the *RAS*/cAMP pathway. Given that there are two interchangeable Ras proteins, Ras1 and Ras2, we created a conditional Ras-depletion strain where the chromosomal *RAS1* was deleted and the original *RAS2* promoter was replaced by a *GAL* promoter. Thus, in the new strain, only *RAS2* is expressed when cultured in galactose-containing medium.

As expected, when the new strain was grown to the log phase (OD 1.2) in galactose-containing medium (nutrient-rich), no Ψ93 formation was detected (Fig. 5, lanes 1 and 2); in contrast, when cells were further grown in the galactose-containing medium to saturation (OD 15 [nutrient-starvation]), Ψ93 was detected (Fig. 5, lanes 7 and 8).

FIGURE 4. The *TOR* signaling pathway controls Ψ93 formation. (*A*) Yeast strain (*tor1Δ tor2Δ pTOR2* [under the control of PGal]), where a Gal-controlled *TOR2* (plasmid borne) is present and both chromosomal *TOR1* and *TOR2* are deleted, was grown in galactose medium to OD 1.0 (lanes *1* and *2*). Cells were transferred to glucose medium and allowed to grow to OD of 1.2 (lanes *3* and *4*) or 1.3 (lanes *5* and *6*), or were allowed to continue to grow in galactose medium to OD 12 (lanes *7* and *8*). In addition, another strain (*tor2Δ pTOR2* [under the control of PGal]), where only chromosomal *TOR2* is replaced by a plasmid borne and Gal-controlled *TOR2*, was grown in exactly the same way as above. Lanes *9* and *10*, cells grown in galactose medium to OD 1.1; lanes *11* and *12*, cells grown in glucose medium to OD 2.0 after switch from galactose medium to glucose medium; lanes *13* and *14*, cells grown in glucose medium to OD 4.4 after switch from galactose medium to glucose medium. Upon completion of cell culture, total RNA was recovered and pseudouridylation assay conducted. Signals representing constitutively formed pseudouridines (Ψ35, Ψ42, and Ψ44) and inducibly formed Ψ93 and Ψ91 are indicated. (*B*) Upon completion of cell culture, total protein was also isolated and Western blot against Tor2 and Act1 (control) performed. (*C*) Total RNA described in *A* was also used for Northern analysis to monitor the level of snR81 box H/ACA RNA relative to the level of U2 snRNA (control).

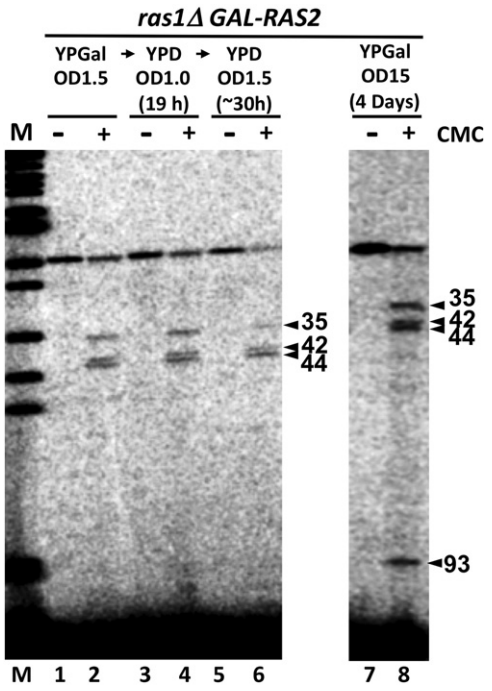


FIGURE 5. The *RAS*/cAMP signaling pathway is not involved in the regulation of Ψ93 formation. Yeast strain (*ras1Δ GAL-RAS2*), where the chromosomal *RAS1* was deleted and the chromosomal *RAS2* promoter was replaced by a *GAL* promoter, was cultured in exactly the same way as in Figure 4A. Lanes *1* and *2*, cells grown in galactose medium to OD 1.5; lanes *3* and *4*, cells grown in glucose medium to OD 1.0 after transferring some cells from galactose medium to glucose medium; lanes *5* and *6*, cells grown in glucose medium to OD 1.5 after transferring some cells from galactose medium to glucose medium; lanes *7* and *8*, cells grown in galactose medium to saturation (OD 15). Upon completion of cell culture, total RNA was isolated and pseudouridylation assay performed. Signals representing constitutively formed pseudouridines (Ψ35, Ψ42, and Ψ44) and inducibly formed Ψ93 are indicated.

However, when Ras2 was depleted in nutrient-rich medium, resulting from the change of medium (from galactose-containing medium to glucose-containing medium), no Ψ93 was detected (Fig. 5, lanes 3–6). These results indicate that, unlike the *TOR* pathway, the *RAS*/cAMP pathway is not involved in the regulation of Ψ93 formation.

DISCUSSION

We have shown that switching yeast cells from a nutrient-rich medium to a nutrient-deprived medium or water (thus leading to entry of cells into stationary phase) induces the formation of Ψ93 in U2 snRNA. We have further shown that upon depletion of both Tor1 and Tor2 or blocking the *TOR* pathway by introducing rapamycin, the *TOR* pathway inhibitor, pseudouridylation, is also induced at position 93 of U2 even under nutrient-rich conditions, indicating that the *TOR* signaling pathway, which controls cell entry into stationary phase (Broach 1991; Schmelzle and Hall 2000; Herman 2002; Schmelzle et al. 2004), also controls induced formation of Ψ93. Using a similar approach, we have also tested the interrelationship between the *RAS*/cAMP signaling pathway and the formation of Ψ93, and found that the *RAS*/cAMP pathway plays no role in Ψ93 formation.

It is well documented that there are two *TOR* complexes in the cell, TORC1 and TORC2, but only one, TORC1, is the target of rapamycin (Loewith et al. 2002). Thus, the fact that introduction of rapamycin into cells induces the formation of Ψ93 indicates that it is TORC1 (or inactivation of TORC1) that is responsible for this induced modification. It is also well established that there are two forms of TORC1 complex (containing either Tor1 or Tor2) that are functionally indistinguishable (Loewith et al. 2002). Consistently, we have shown that in order to induce the formation of Ψ93, both Tor1 and Tor2 have to be depleted, arguing for the functional redundancy of the two forms of TORC1 in regulating the formation of Ψ93. The fact that inactivation of TORC1 leads to Ψ93 formation also suggests that U2 pseudouridylation at position 93 (and perhaps at other starvation-inducible sites as well) is the default pathway that is inhibited or kept in check by the *TOR* signaling pathway in fast growing cells (nutrient-rich environment). This implies that pseudouridylation at the inducible sites disfavors pre-mRNA splicing (part of gene expression), an important process for growing cells. In this regard, we have shown that Ψ93 does indeed have an inhibitory effect on splicing (Wu et al. 2011).

It is known that the 3′ pseudouridylation pocket of snR81, which guides the induced formation of Ψ93, is also naturally responsible for the formation of Ψ1051 in 25S rRNA (a constitutive pseudouridylation site) under both saturation and nutrient-rich conditions (Fig. 1; Ma et al. 2005). Interestingly, the sequence flanking position 93 of U2 is not exactly the same as that of position 1051 of 25S rRNA (Fig. 1). In fact, while the 3′ pseudouridylation pocket of snR81 forms perfect base-pairing interactions with the sequence flanking position 1051 of 25S rRNA, it matches imperfectly with the sequence surrounding position 93 of U2. In the latter case, there are two mismatches in the middle of the 5′ base-pairing duplex (Fig. 1). Such an imperfect pairing will certainly weaken the interaction between the guide RNA (snR81) and the substrate (sequence surrounding position 93 of U2). According to what we know about the RNA-guided pseudouridylation mechanism (Xiao et al. 2009), position 93 would not be considered a snR81 substrate. Then how can snR81 direct pseudouridylation at position 93 under starvation conditions?

It is possible that the enzyme (snR81) is overexpressed under nutrient-deprivation conditions (triggered by inactivation of the *TOR* pathway). An elevated level of snR81 could allow the imperfect site (position 93 of U2) to be recognized and modified. However, our Northern analysis indicates that the level of snR81 does not change after switching from nutrient-rich to nutrient-deprived conditions (Fig.

4C), suggesting that the notion that overexpression of snR81 drives induced modification is not true. However, although the level of snR81 does not change, our result cannot rule out the possibility that the enzyme/substrate relocalizes upon induction (inactivation of the *TOR* pathway), such that snR81 (normally resides in or travels between nucleoli and Cajal bodies) becomes concentrated at the site of its substrate (U2), allowing position 93 to be modified. This possibility is yet to be tested. Finally, we also consider another possibility, where inactivation of the *TOR* pathway triggers the expression or redistribution of a protein(s) (or chaperone) that helps snR81 RNP (or substrate U2) restructure, thus increasing the binding affinity between the guide RNA and the imperfect substrate and permitting efficient pseudouridylation at the new site (position 93).

Our results have unambiguously demonstrated that the *TOR* signaling pathway regulates starvation-inducible pseudouridylation at position 93 of U2. Interestingly, besides Ψ93, there are a number of additional starvation-inducible pseudouridylation sites in U2 (e.g., position 56 and possibly position 91 as well, this study) and other spliceosomal snRNAs (G Wu and YT Yu, unpubl.), and even in mRNA (Carlile et al. 2014; Schwartz et al. 2014; Li et al. 2015). It is quite possible that pseudouridine formation at these positions is also regulated by the *TOR* pathway. Our current work opens the door to investigation into this possibility.

MATERIALS AND METHODS

Strains and plasmids

S. cerevisiae strains used here include BY4741 (*MATa his3 leu2 met15 ura3*), the *tor*Δ strains JK350-21a (*JK9-3da tor1::LEU2 tor2::ADE2*, pJK5-*TOR2*[*GAL CEN LEU2*]) and JK350-18a (*JK9-3da tor2:: ADE2, pJK5-TOR2*[*GAL CEN LEU2*]). The *tor*Δ strains (JK350-21a and JK350-18a) are kind gifts from Dr. Michael Hall (University of Basel) (Loewith et al. 2002). The *RAS*-altered strain *ras1Δ PGAL-RAS2* was derived from BY4741 (*MATa his3 leu2 met15 ura3 ras1:: kanMX Gal-RAS2* [*RAS2* -500*::URA3-GAL1*]).

Yeast culture

To examine inducible pseudouridine formation in a nutrient-deprivation state, a freshly streaked BY4741 colony was picked and inoculated into 100 mL of YPD or synthetic complete medium with dextrose (SD). When OD_{600} of the culture reaches 1.0, cells were spun down at 6000*g*. Cells were then transferred to a synthetic medium lacking dextrose (the same as SD except that there is no dextrose), or pure water at an OD of 1.0, and allowed to grow for another hour. For depletion of Tor2 (from a plasmid-borne PGal promoter) in the *tor1Δ tor2*Δ background, a colony of freshly streaked JK350-21a was inoculated into YPGal (synthetic complete medium with galactose) medium. When the culture reached an OD_{600} of 1.0, cells were spun down at 6000*g*, and then transferred to YPD medium at a starting OD of 0.03. Cells were harvested 19 h later at an OD of 1.2 or 30 h later at an OD of 1.3. For depleting Tor2 (from a plasmid-borne PGal promoter) in the *tor2*Δ background, a colony of JK350-18a was inoculated firstly into YPGal medium. Cells were spun down at an OD of 1.0 and transferred to YPD medium at a starting OD of 0.002. Cells were harvested 19 h later at an OD of 2.0, or 30 h later at an OD of 4.4. For depletion of Ras2 (from a PGal promoter) in ras1Δ background, strain *ras1Δ PGAL-RAS2* was inoculated into YPGal medium and grown to an OD of 1.5. Cells were spun down and transferred to YPD at an OD of 0.03, and harvested 19 h later at an OD of 1.0, or 30 h later at an OD of 1.5 (diluted with new YPD when necessary).

Drug treatment of yeast culture

A colony from freshly streaked BY4741 was inoculated into YPD medium and grown to early log phase. Cells were then used to inoculate new cultures at an OD of 0.5. DMSO, rapamycin (1 μg/mL), or tunicamycin (10 μg/mL) was added to the new cultures (YPD medium). Cells were allowed to grow for 12 h, and at that point, the cell cultures, which were treated with DMSO, rapamycin, or tunicamycin, reached an OD of 8.0, 3.0, or 2.0, respectively.

RNA extraction

RNA was isolated using TRIzol (Invitrogen). Briefly, ~30 OD cells were spun down, washed with cold PBS twice, and resuspended in 1 mL of TRIzol with ~100 μL of 0.5 mm glass beads. Yeast cells were then lyzed by vigorous vortexing. After centrifugation to remove cell debris and glass beads, the supernatant was mixed with 200 μL of chloroform and vortexed for 1 min. After centrifugation to separate the phenol and aqueous phases, the clear upper phase was transferred to a new tube and an equal volume of isopropanol was used to precipitate the RNA. The RNA was then washed with 70% ethanol and briefly air-dried. RNA was resuspended in ddH_2O and immediately used for subsequent experiments.

CMC-primer extension assay

Pseudouridines were detected by the CMC-primer extension assay, as previously described (Wu et al. 2011). Briefly, 10 μg of total cellular RNA was incubated with CMC (Sigma, cat. no. 29469) in reaction buffer (50 mM Bicine, 0.15 M CMC, 4 mM EDTA, and 7 M urea, pH 8.3) at 37°C for 20 min. RNA was then precipitated with 2.5 volume of ethanol and washed with 70% ethanol, and then resuspended in sodium carbonate (50 mM, pH 10.4) and incubated for 2 h at 37°C. RNA was then once again precipitated and cleaned up using ethanol. The resulting RNA pellet was suspended in annealing buffer (50 mM Tris–Cl pH 8.3, 60 mM NaCl, 10 mM DTT) and was heated at 90°C for 3 min in the presence of a 5′ ^{32}P-labeled primer. The primer used for mapping Ψ93 in U2 snRNA was 5′-CTCTTCCCGTCCATTTTATTATTTTG-3′. After heat-denaturation, the RNA-oligo mix was immediately chilled on ice, and 1.5 units of AMV reverse transcriptase and reaction buffer [9 mM Tris–HCl Ph 8.3, 10 mM NaCl, 1.8 mM DTT, 5 mM Mg $(OAc)_2$, and 0.3 mM of each dNTP] were added. The reaction was carried out at 42°C for 20 min. Reaction products were resolved on an 8% polyacrylamide, 8 M urea gel (acrylamide:bis = 19:1, EM science) and visualized via autoradiography.

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