Diphthamide promotes TOR signaling by increasing the translation of proteins in the TORC1 pathway

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Diphthamide, a modification found only on translation elongation factor 2 (EF2), was proposed to suppress −1 frameshifting in translation. Although diphthamide is conserved among all eukaryotes, exactly what proteins are affected by diphthamide deletion is not clear in cells. Through genome-wide profiling for a potential −1 frameshifting site, we identified that the target of rapamycin complex 1 (TORC1)/mammalian TORC1 (mTORC1) signaling pathway is affected by deletion of diphthamide. Diphthamide deficiency in yeast suppresses the translation of TORC1-activating proteins Vam6 and Rtc1. Interestingly, TORC1 signaling also promotes diphthamide biosynthesis, suggesting that diphthamide forms a positive feedback loop to promote translation under nutrient-rich conditions. Our results provide an explanation for why diphthamide is evolutionarily conserved and why diphthamide deletion can cause severe developmental defects.

Significance

Diphthamide is a posttranslational modification that has been known since the 1970s. It is conserved in all eukaryotic cells, and its biosynthesis requires at least seven proteins. However, its exact biological function has remained unclear. Our results demonstrate that diphthamide promotes target of rapamycin (TOR) signaling by promoting the translation of two proteins in the target of rapamycin complex 1 (TORC1) pathway that contain slippery sequences in their messenger RNA (mRNA). Our finding explained why diphthamide is evolutionarily conserved and why it is crucial in animal development. Our results also suggest that regulating the translation of slippery sequences by diphthamide could be a widely used mechanism to tune translation in eukaryotes, which is different from the recoding hypothesis.

Author contributions: Y.Z. and H.L. designed research; Y.Z., Z.L., J.Z., and M.W. performed experiments; Y.Z. and H.L. analyzed data; and Y.Z. and H.L. wrote the paper.

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can promote TORC1 signaling by promoting the translation of these proteins through preventing −1 frameshifting.

Results

Diphthamide was reported to maintain translation fidelity by suppressing −1 frameshifting. To identify proteins regulated by diphthamide, we set out to do a genome-wide profiling of all potential frameshifting sites. In the known programmed −1 frameshifting motifs, a common feature is a slippery sequence followed by a secondary structure that induces pause of translation elongation, mostly a pseudoknot (30). The slippery sequence is generally of secondary structure that induces pause of translation elongation, motifs, a common feature is a slippery sequence followed by a thamide, we set out to do a genome-wide profiling of all potential

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the ΔDph2 strain compared to the WT strain (Fig. 3 A and D and SI Appendix, Fig. S6). To further demonstrate the point, Eno2 and Tdh3 with no predicted frameshifting motifs were used as negative control. Eno2 and Tdh3 protein expression level did not decrease in ΔDph2 strain compared with WT strain (SI Appendix, Fig. S6). These results not only confirmed that the bioinformatics prediction is effective but also explained how deletion of diphthamide suppresses TORC1 activation.

To further confirm that the down-regulation of Rtc1 and Vam6 is due to translational suppression, we checked their mRNA levels and protein degradation. Compared to the WT strain, the mRNA level of Rtc1 and Vam6 did not decrease in ΔDph2 (Fig. 3 B and E). The protein degradation of Rtc1 and Vam6 did not show a significant difference between WT and ΔDph2 (Fig. 3 C and F). These results support that the Rtc1 and Vam6 down-regulation in ΔDph2 is through translation.

Furthermore, we constructed a reporter system based on a centromeric plasmid with a bidirectional promoter, pTH644 (Fig. 3G) (36). We put mCherry on one side of the bidirectional promoter and EGFP on the other side. We then insert Rtc1 and Vam6 at the N-terminal of the EGFP so that a fusion protein with EGFP would be translated. The fluorescence ratio between EGFP and mCherry would represent the relative translation efficiency of Rtc1 and Vam6. Full-length Eno2 and Tdh3 insertion at the N-terminal of EGFP was used as control. The EGFP/mCherry ratio obtained with Vam6 and Rtc1 was normalized to that obtained with Eno2 insertion (36). We put mCherry on one side of the bidirectional promoter, pTH644 (Fig. 3B) (37). Both frameshifting motif 1779 to 1831 and frameshifting motif 1836 to 1942 showed a frameshifting-inducing effect in ΔDph2 (Fig. 4C).

To further demonstrate that the −1 frameshifting motifs are responsible for translation suppression, we inserted Rtc1 N-terminal 1998 bp at the N-terminal of EGFP in the reporter system we generated (Fig. 3G). The Rtc1(1 to 1998) sequence contains all the predicted frameshifting motifs. After deleting the frameshifting motif, Rtc1(1836 to 1889) was restored to a level similar to that of control frameshifting motifs located adjacent to each other forming a long frameshifting-prone sequence (Fig. 4A). The sequence includes the predicted frameshifting motif with the lowest MFE in Rtc1. We tested the frameshifting efficiency of these three frameshifting motifs using a published luciferase assay (Fig. 4B) (37). Both frameshifting motif 1779 to 1831 and frameshifting motif 1836 to 1942 showed a frameshifting-inducing effect in ΔDph2 (Fig. 4C). (37).

In each experiment, we monitored the growth curve of WT strain and ΔDph deleted strain before and after readdition of amino acid. (A) Diphthamide deficient yeast shows slower growth in low-dose rapamycin treatment. All Dph deletion strains show similar growth to the WT strain (Left) without rapamycin but show slower growth than the WT strain with rapamycin. Genotype of each strain is indicated on the Left. Each row represents a serial dilution from Left to Right. (B) ΔDph2 and ΔDph5 grow slower compared with WT in SC media after overnight histidine starvation followed by readdition of histidine. The growth curve was monitored with reintroduction of histidine. Reintroduction of histidine was at T = 0.

Table 1. KEGG pathway annotation of proteins with potential −1 frameshifting motifs

| Annotated pathways | Proteins involved |
|--------------------|------------------|
| Metabolic pathways | STR2, HER2, URA, MET2, ANP1, GDH2, DAK1, GPI17, CWH41, STR3, PSA1, PLC1, ERR3, ALG8, UTR1, PDA1, AAH1, GPI13, ROT2, POP1, M55, URA10, ALD6, FAA2, RPA12, ADH7, PUT2, TGL3, POL3, HXX2, HMG2, MPI, MET10, MCM1, ISN1, YLL08W, RPO31, AT516, ERR2, DUR1, KGD1, FOL2, LYS4, RP88, KG2D, SPE1, MNL2, TP52, D3H, ORI1, PSD2, MNN10, RIB3, NKR1, POL12, ERG10, PCMI, TES1, ATP4, TRP3, GLC3, RPC10, RIB7, COX13, PNP1, ICL2, GBD1, MNN9, RPM15, ALG11, GWT1, PML6, RPO21, MNN1, GAB11, GPI13, SEC59, SER2, KTR6, MET7, ERG27 |
| MAPK signaling pathways | S2ST, STE20, FK51, FK53, SKM1, HOG1, RSP, M55, MKK2, HKR1, BEM2, SWI4, PKH2, PKH1, PKH3, CLB6, RGA1, FAR1, SKO1, CDC24, SWI6, STE4, GSC2, STE7, TUS1, SPA2 |
| Meiosis | SMCMC1, MCMC4, APCC4, TOR2, NDT80, TPDP3, ESP1, RED1, RAD24, MCM6, MCM7, HOP1, SWI4, CLB6, RRC1, PHH22, CDC, MCM, ZPIC1, SWI6, RIM11, CYR1 |
| Biosynthesis of antibiotics | STR2, MET2, ERR3, PDA1, ALD6, ADH7, HXX2, HMG2, MCM1, YLlo8W |
| Cell cycle | SMC1, MCM5, APCC4, TPDP3, ESP1, RAD24, MCM6, SCC2, MCM7, SWI4, CLB6, CDC4, IRI1, FAR1, PHH22, CDC5, LTE1, YCS4, MCM3, BFA1, SW16, TOR2, ATG7, VAM6, AV73, MON1, GCN4, TO53, TOR1, VPS41, VPS16, RAS1, SEC17, PHH22, ATG14, ATG16, GCN2, PEP3 |
| Autophagy | 90S preribosome | DIM1, MRD1, RRP, UTP7, UTP20, BUD21, TS1, NOP14, CAP2, ENU1, UTP8, PRP43, UTP21, ECM16, UTP9, UTP13, NAIN1 |
| RNA transport | RPR2, NUP157, NMD2, NUP53, NUP188, CDC33, RPM2, NUP192, MNS5, TIC3, NUP85, NUP170, ML2P, STO1, TIC35 |
| Protein processing in ER | CW4H1, ROT2, SSM4, S32E, SEC63, MNL2, SED4, DOA1, NPL4, LHS1, SC1, SFB2, GCN2, CDC48, PNG1 |

Fig. 1. Diphthamide deletion strains are more sensitive to rapamycin and grow slower after amino acid starvation followed by readdition of amino acid. (A) Diphthamide-deficient yeast shows slower growth in low-dose rapamycin treatment. All Dph deletion strains show similar growth to the WT strain (Left) without rapamycin but show slower growth than the WT strain with rapamycin. Genotype of each strain is indicated on the Left. Each row represents a serial dilution from Left to Right. (B) ΔDph2 and ΔDph5 grow slower compared with WT in SC media after overnight histidine starvation followed by readdition of histidine. The growth curve was monitored with reintroduction of histidine. Reintroduction of histidine was at T = 0.
Modification was detected with a diphtheria toxin-mediated ADP-ribosylation reaction with a fluorescent-labeled NAD+ analog. The level of EF2 protein was detected using Western blot. (Eno2 sequence), demonstrating the Rtc1 (ΔΔmeshifting motif is important for the translation suppression in using CRISPR. The phosphorylation level of 4EBP1 was detected by Western blot.

Discussion
Diphthamide was proposed to suppress –1 frameshifting (21, 28, 29). However, no endogenous cellular protein targets of diphthamide had been identified. A frequent cause of –1 frameshifting is a combination of slippery sequence with complex secondary structures that stalls the translation elongation (30). Using a computational approach, we identified potential proteins for which translation may be affected by diphthamide. We found that the TORC1 signaling pathway contains several proteins that are regulated by diphthamide. Diphthamide is not present in prokaryotes, is present but not essential in lower eukaryotes like yeast, and is essential in vertebrates, which have increased complexity of the proteome (21–28, 38). This is consistent with the fact that our computational analysis shows that longer proteins are more likely to contain slippery sequences (SI Appendix, Fig. S2) that will require diphthamide to prevent the –1 frameshifting.

Our results also provide an explanation why this posttranslational modification is retained through evolution. Cells without diphthamide have lower TORC1 activation and thus lower growth rate when nutrients become available. Although the biosynthesis of diphthamide involves at least seven proteins with multiple energy consumption steps, it gives yeast the advantage of fast growth when switched from low-nutrient to rich-nutrient conditions. Yeast cells can grow faster in a changing environment if they retain the diphthamide biosynthesis genes.

Our results can also explain why loss of diphthamide is detrimental to animal development. Since diphthamide deletion leads to a suppression of mTORC1 signaling, it is expected that cell growth will be affected. In a multicellular organism, the effect is most obvious during development where cell growth is essential. Our results are in line with the observation that mice with a diphthamide deletion show general delay in embryonic development (22–25) and that human patients with mutations in Dph1 show a delay in developments (26, 27).

Predicting –1 frameshifting motifs has been the subject of much previous research (39–42), which is driven by the hypothesis that many mRNA may contain programmed –1 frameshifting that allow “translational recoding” (43). Our finding here is extending these –1 frameshifting motifs into a different regulatory direction. Instead of being a programmed frameshifting for recoding purposes, these motifs are being utilized to control protein levels in response to TORC1 signaling to allow optimal cellular growth. Among the 850 yeast proteins that we predicted to contain the –1 frameshifting motif, we only picked 5 proteins in the TORC1 signaling pathway to biochemically validate. And two proteins were confirmed to be regulated by the diphthamide modification. While this success rate is modest due to difficulties in precise RNA three-dimensional structure prediction, the results may hint at a much broader regulation of translation by diphthamide and warrants future research to investigate whether the translation of other predicted proteins is also regulated by diphthamide. The clustering of multiple frameshifting motifs may increase the chance of frameshifting happening within the region, which is the case for the Rtc1 long frameshifting-prone sequence. It is likely that a single frameshifting motif may not lead to obvious protein expression decrease, but the additive effect of multiple frameshifting motifs in the protein can lead to a more profound decrease. The clustering and number of frameshifting motifs should be taken into consideration to identify protein affected by diphthamide deletion in the future.

Table 2. Proteins required for TORC1 activation with a potential slippery sequence

| Protein name | Number of slippery sequences | Lowest MFE (kcal/mol) |
|--------------|------------------------------|-----------------------|
| MTC5         | 11                           | −14.1                 |
| RTC1         | 7                            | −9.1                  |
| VAM6         | 6                            | −11.7                 |
| TOR1         | 6                            | −9.9                  |
| TOR2         | 10                           | −9.6                  |

Fig. 2. Diphthamide promotes TORC1 and mTORC1 signaling. (A) Diphthamide promotes Sch9 phosphorylation in yeast. A chemical cleavage assay was used to detect the phosphorylation level of Sch9. Endogenous Sch9 was tagged with triple Flag tag (3xFlag) at the C-terminal using homologous recombination to allow detection of Sch9 by Flag antibodies. In the WT strain, C-terminal of Sch9 was shown as multiple higher molecular weight bands after chemical cleavage, indicating a higher phosphorylation level of Sch9 in the WT strain. (B) Diphthamide promotes 4EBP1 phosphorylation in HEK293T cells. Dph4 was knocked out using CRISPR. The phosphorylation level of 4EBP1 was detected by Western blot. (C) Diphthamide promotes 4EBP1 phosphorylation in HEK293T cells. Either a control vector or Dph4 encoding construct was reintroduced into HEK293T Dph4 KO cells. The phosphorylation level of 4EBP1 was detected by Western blot. (D) Diphthamide level decreases under rapamycin treatment. HEK293T cells were treated with 0, 10, or 100 nM rapamycin, and the level of diphthamide modification was detected with a diphertheria toxin-mediated ADP-riboseylation reaction with a fluorescent-labeled NAD+ analog. The level of EF2 protein was detected using Western blot. (E) Diphthamide level increases with amino acid supplement. HEK293T cells were cultured without amino acid or with amino acid. The level of diphthamide modification was detected with a diphertheria toxin-mediated ADP-riboseylation reaction with a fluorescent-labeled NAD+ analog. The level of EF2 protein was detected using Western blot.
Saccharomyces cerevisiae obtained from the Genome-Wide Search for Slippery Site. (Addgene:20753; RRID: Addgene_20753).

University, New Haven, CT (Addgene plasmid no. 20753; http://n2t.net/addgene:20753).

Mark Hochstrasser, Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT (Addgene plasmid no. 29695; http://n2t.net/addgene:29695).

was a gift from Tobias von der Haar, School of Biosciences, University of Kent, Canterbury, Kent, UK (Addgene plasmid no. 29695; http://n2t.net/addgene:29695; RRID: Addgene_29695). pFA6a-6xGLY-3xFLAG-HIS3MX6 was a gift from Mark Hochstrasser, Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT (Addgene plasmid no. 20753; http://n2t.net/addgene:20753; RRID: Addgene_20753).

Methods

Reagents, Antibodies, and Plasmids Used. Rapamycin was obtained from Cell Signaling Technology (CST 99045). Flag-horseradish peroxidase (A4285 Sigma) was used for Western blot with 1:5,000 dilution. Antibodies for eEF2 (CST 23325), 4EBP1 (CST 9452S), phospho-4EBP1 (CST 2855S), and β-actin (SC-47778) were used for Western blot with 1:1,000 dilution. pTH644-CENBEVY was a gift from Mark Hochstrasser, Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT (Addgene plasmid no. 20753; http://n2t.net/addgene:20753; RRID: Addgene_20753).

Genome-Wide Search for Slippery Site. Yeast gene nucleotide sequences were obtained from the Saccharomyces cerevisiae database (https://www.yeast-genome.org/). Slippery sequence with X XXY YYZ was searched. X denotes any nucleotide, Y denotes A or U, and Z is A, U, or C. The folding energy of 46 bp after the slippery sequence was calculated using Vienna RNA (31, 32).

For each gene, if the minimum of MFE of each potential slippery sequence was less than −9.0 kcal/mol, it was identified as genes with potential slippery site. The protein set with potential slippery site was imported into string database (https://string-db.org/) online under the multiple protein section. After analysis, database search was selected under the viewer section.

Yeast Rapamycin Sensitivity Assay. Yeast was cultured in yeast extract peptone dextrose (YPD) media overnight. The overnight culture was diluted to adjusted OD600 of 0.1 in SC media. The cells were then cultured at 30 °C, and OD600 was monitored at different time points. After 6 h, yeast was washed three times with water. And the OD600 was adjusted to 0.2 with autoclaved water. The culture was diluted serially in fourfold increments. In total, 4 μL of each dilution was spotted on YPD plates with or without 10 nM rapamycin.

Yeast Growth Curve Measurement. Yeast was grown overnight in synthetic complete (SC) media. The overnight culture was washed three times with water and then transferred to SC media without histidine, or methionine and cysteine overnight, or to SC media without leucine for 6 h. The starved yeast was spun down, washed three times with autoclaved water, and then adjusted OD600 of 0.1 in SC media. The cells were then cultured at 30 °C, and the OD600 was monitored at different time points.

Sch9 Phosphorylation Blot. Sch9 phosphorylation was detected after cleavage with 2-Nitro-5-thiocyanatobenzoic acid (NTCB) following the same procedure reported by Urban et al. (8).

Yeast Culture and Lysis for Western Blot Quantification. Yeast cells were cultured to OD around 0.5 and harvested by centrifugation. Yeast cells were then resuspended in 200 μL H2O, to which 34 μL 1.85 M NaOH with 0.74% 2-mercaptoethanol was added. The mixture was incubated on ice for 10 min. Then, 16 μL trichloroacetic acid was added followed by incubation on ice for 10 min. The mixture was centrifuged for 15 min at 21,000 × g. The protein pellet was collected and washed with 500 μL acetone. Protein pellet was dried and resuspended in 200 μL 4% SDS with sodium phosphate buffer, pH 7.4. Protein concentration was quantified using Pierce BCA Protein Assay Kit (Thermo). A total of 40 μg protein extract was used for Western blot.

Zhang et al.

Diphthamide promotes TOR signaling by increasing the translation of proteins in the TORC1 pathway.

Fig. 3. Vam6 and Rtc1 are translationally down-regulated in diphthamide-deficient yeast. (A) Vam6 expression is down-regulated in ΔDph2. Endogenous Vam6 was tagged with a Flag tag using homologous recombination, and the level of Vam6 was detected using Western blot for Flag. (B) The mRNA level of Vam6 was measured using RT-PCR. (C) Vam6 protein degradation was monitored using cycloheximide chase. WT and ΔDph2 strains were treated with 35 μg/mL cycloheximide for indicated period of time. Vam6 was monitored using Western blot. (D) Rtc1 expression is down-regulated in ΔDph2. Endogenous Rtc1 was tagged with a Flag tag using homologous recombination, and the level of Rtc1 was detected using Western blot for Flag. (E) The mRNA level of Rtc1 was measured using RT-PCR. (F) Rtc1 protein degradation was monitored using cycloheximide chase. WT and ΔDph2 strains were treated with 35 μg/mL cycloheximide for indicated period of time. Rtc1 was monitored using Western blot. (G) The construction of the reporter plasmid. (H) Translation of Vam6 monitored by the reporter system. ns, not significant; *P < 0.05; **P < 0.01.
predicted frameshifting motifs in Rtc1. The 500 bp to the second 500 bp was PCR amplified, transformed into yeast, and before the stop codon was cloned into the plasmid pFA6a-6xGLY-3xFLAG-tations (46). The 500-bp fragment from the 3′ogous recombination as previously described with the following modifications (45). Briefly, the HEK293 cell was collected and lysed with lysis buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40. NAD+ in cell lysis was removed using Bio-Rad Micro Bio-spin 6 columns. The flow through was quantified. The signal of a weaker band of premature protein of Rtc1 was obtained using Clarity Max ECL substrate (Bio-Rad).

Modification.
The labeling of EF2 with diphthamide modification was performed as previously described (45). Briefly, the HEK293 cell was collected and lysed with lysis buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, and 1% Nonidet P-40. NAD+ in cell lysis was removed using Bio-Rad Micro Bio-spin 6 columns. The flow through was quantified using Pierce BCA protein assay kit, and 10 μg of the protein was used for labeling and quantification of diphthamide-modified EF2.

Labeling of Endogenous Yeast Proteins with 3xFlag Tag. The strains expressing endogenous 3xFLAG-tagged Vam6 and Rtc1 were generated using homologous recombination as previously described with the following modifications (46). The 500-bp fragment from the 3′ of the open reading frame (ORF) before the stop codon was cloned into the plasmid pFA6a-6xGLY-3xFLAG-HIS3MX6 (Addgene plasmid 20753) right before the 3xFlag tag. The 500-bp stop codon fragment after the stop codon of the ORF was cloned into the same vector after the His3MX6 selection marker. The fragment from the first 500 bp to the second 500 bp was PCR amplified, transformed into yeast, and plated on SC agar plates with histidine dropout for selection. Two positive colonies are used for Western blots.

Construction of the Reporter System. EGFP was amplified with YZ849_EGFP_SalI_5′-agtcagGAATTCctaAGCGTAATCTGGAACATCGTA-AGTCGACatggtgagcaagggcgaggag and YZ850_EGFP_HindIII_Flag_3′-agtcagGTCGACatggtgagcaagggcgaggag and inserted into pTH644-CENBEVY with Sall and HindIII double digestion. mCherry was amplified with YZ847_mCherry_Xmal_5′-agtcagCCCGGGatggtgagcaagggcgaggag and YZ848_mCherry_EcoRI_HA_3′-agtcagGAATTCtctaaCCGTAATCTGGAACATCGTA-TGGGTActtgtacagctcgtccat and inserted into pTH644-CENBEVY-E0GP with Xmal and EcoRI double digestion. The protein of interest was inserted at the N-terminal of EGFP.

Fluorescent Quantification of N-terminal Translation Efficiency. Yeast was cultured to OD600 around 1.0. Yeast cells were harvested by centrifugation at 500 × g for 4 minutes. Yeast cells were washed three times with water. After the final wash, the cell pellet was resuspended in 700 μl water. Fluorescent reading of yeast suspension was obtained using BioTek Cytation 5 plate reader. The EGFP signal was excited at 479 nm and read at 520 nm. The mCherry signal was excited at 579 nm and read at 616 nm. The ratio of EGFP/mCherry was calculated and normalized to EGFP/mCherry signal with the reader. The EGFP signal was excited at 479 nm and read at 520 nm. The mCherry signal was excited at 579 nm and read at 616 nm. The ratio of EGFP/mCherry was calculated and normalized to EGFP/mCherry signal with the reader. The EGFP signal was excited at 479 nm and read at 520 nm. The mCherry signal was excited at 579 nm and read at 616 nm. The ratio of EGFP/mCherry was calculated and normalized to EGFP/mCherry signal with the reader. The EGFP signal was excited at 479 nm and read at 520 nm. The mCherry signal was excited at 579 nm and read at 616 nm. The ratio of EGFP/mCherry was calculated and normalized to EGFP/mCherry signal with the reader. The EGFP signal was excited at 479 nm and read at 520 nm. The mCherry signal was excited at 579 nm and read at 616 nm. The ratio of EGFP/mCherry was calculated and normalized to EGFP/mCherry signal with the reader. The EGFP signal was excited at 479 nm and read at 520 nm. The mCherry signal was excited at 579 nm and read at 616 nm.

Luciferase Assay for Determination of Frameshifting Efficiency. Frameshifting efficiency was determined as previously described (37). Briefly, yeast cells overexpressing the frameshifting motif-containing sequence were cultured until OD600 reached 0.6. Using glass beads, 5 mL of yeast culture was lysed. Yeast cells were washed three times with water. After the final wash, the cell pellet was resuspended in 700 μl water. Luciferase reading of yeast suspension was obtained using BioTek Cytation 5 plate reader. The luciferase signal was excited with a dual luciferase reporter assay using luciferase reagents from Promega.

Data Availability. All study data are included in the article and/or SI Appendix.

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Diphthamide promotes TOR signaling by increasing the translation of proteins in the TORC1 pathway