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.

diphthamide | translation | −1 frameshifting | TOR signaling

The target of rapamycin (TOR) is a central hub for controlling cell growth according to nutrient levels, especially amino acids levels (1, 2). Mammalian TOR (mTOR) signaling is involved in metabolic disorders, cancer, and aging (1, 2). In yeast, TOR complex 1 (TORC1) activation induces expression of ribosomal proteins and proteins related to translation through phosphorylation of its downstream targets, including Sch9 and Sfp1 (3–8). Phosphorylated Sch9 can activate protein translation initiation (8) and at the same time phosphorylate and inactivate transcriptional repressors, Dot6/Tod6 and Stb3, to induce the expression of genes important for protein synthesis (3, 5, 9). On the other hand, TORC1 activation suppresses the catabolic pathways including autophagy through phosphorylation of Atg13 (10). Similarly, in humans, the activation of mTORC1 phosphorylates its downstream targets, including p70 S6 kinase (11), 4EBP1 (11), ULK1 (12–14), and ATG13 (12–14), to promote protein synthesis and suppress autophagy (2).

In nutrient-rich conditions, TORC1 or mTORC1 is activated following a series of upstream signaling events. The activation of TORC1 or mTROC1 is mostly conserved in yeast and humans (1, 15, 16). The activation of TORC1 or mTORC1 requires RAG family GTPases, encoded by Gtr1 and Gtr2 in yeast, and RAGA, RAGB, RAGC, and RAGD in humans (1, 16). The RAG family GTPase is activated when RAGA/B or Gtr1 is bound by GTP and RAGC/D or Gtr2 is bound by GDP in humans and yeast, respectively. The nucleotide-bound state for mammalian RAGs and yeast Gtr1/2 is tightly controlled by conserved GTPase activating proteins (GAP) and guanine nucleotide exchange factor (GEF). In yeast, Vam6 serves as a GEF to activate Gtr1, which in turn activates TORC1 (15). Seh1-associated subcomplex inhibiting TORC1 (SEACIT) serves as GAP for Gtr1 in yeast while the GATOR1 complex is the GAP for RAGA/B in humans (1, 15, 16). Seh1-associated subcomplex activating TORC1 (SECAT) and the GATOR2 complex bind and inhibit the function of SEACIT and GATOR1 in yeast and humans, respectively (1, 15, 16), thus activating TORC1 and mTORC1 (*SI Appendix*, Fig. S1).

Diphthamide is a posttranslational modification that is only found on eukaryotic elongation factor 2 (EF2), a GTPase essential in the elongation step of translation (17–20). Diphthamide biosynthesis requires four steps involving at least seven proteins, with the first step being chemically challenging and requiring a noncanonical radical SAM enzyme (20). Diphthamide modification and all the biosynthetic genes are conserved in all eukaryotes, suggesting that it has important biological functions. The importance of diphthamide is further confirmed by the finding that deletion of diphthamide biosynthesis genes is lethal in mice (21–25), and mutations in these genes cause developmental defects in humans (26, 27). However, the exact function of diphthamide is still unknown. It was proposed that diphthamide is essential for ensuring translation fidelity by preventing −1 frameshifting (21, 28). Recent structural studies suggested that diphthamide may bind to mRNA during translocation and prevent the fluctuation-caused slippage (29). However, the function of diphthamide in preventing −1 frameshifting was only demonstrated in an artificial system with HIV-programmed −1 frameshifting motif (21, 28). Before our study described here, no proteins in eukaryotic cells with physiological frameshifting motif have been identified to be affected by diphthamide.

Here, we computationally analyzed all yeast genes to identify mRNA sequences that contain a slippery sequence followed by a complex secondary structure. We hypothesized that such mRNA sequences have a higher probability of frameshifting without diphthamide. This analysis led us to identify several proteins crucial for activating TORC1 as potential targets affected by diphthamide. We then biochemically validated that the translation of these proteins is indeed affected by diphthamide deficiency and that diphthamide

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.

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Competing interest statement: H.L. is a founder and consultant for Sedec Therapeutics.

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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 the type X XXY YYZ, where X denotes any nucleotide, Y denotes A or U, and Z is A, U, or C (30). Diphthamide was shown to prevent −1 frameshifting in HIV-programmed −1 frameshifting motif (21, 28). Based on the HIV-programmed −1 frameshifting motif, a slippery sequence followed by a 46–base pair (bp) stable secondary structure without a spacer sequence, we adopted an energy calculation of the 46 bp following the potential slippery sequence using Vienna RNA (31, 32). We sort each protein with a slippery motif based on the minimum free energy (MFE) of the 46-bp mRNA following the slippery sequence. Some proteins were identified with multiple potential slippery sequences; the lowest MFE was used for ranking these proteins. We set a cutoff of the lowest MFE <−9.0 kcal/mol. With this condition, 850 out of 5,917 proteins in the yeast genome were identified with potential −1 frameshifting motifs (*SI Appendix*, Table S1).

To find out whether −1 frameshifting motif frequency correlates with the protein size, we plotted the protein size against the lowest MFE for all yeast mRNAs. We noticed that the stronger −1 frameshifting motif is more frequent in larger proteins (*SI Appendix*, Fig. S2*A*). We further calculated the median of protein size for proteins with the same lowest MFE. We plotted the median against the lowest MFE (*SI Appendix*, Fig. S2*B*). The plot indicates a moderate trend that stronger −1 frameshifting motif is more frequent in larger proteins.

To facilitate the choice of proteins for biochemical validation, we next performed KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database search using STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) functional protein association networks (Table 1). Nine pathways were annotated, including MAPK signaling, autophagy, and protein processing in the endoplasmic reticulum (ER). Among all the pathways annotated, we picked the autophagy pathway for biochemical validation as this pathway can be easily related to an observable phenotype.

Autophagy is triggered by nutrient starvation through TORC1 inhibition (12–14). After TORC1 inhibition, the autophagy process is performed by ATG family of proteins in yeast (12–14). Many proteins in the autophagy pathway with potential −1 frameshifting motifs in Table 1 affect TORC1 activity. Therefore, to find out whether the computational prediction reflects what happened in the cell, we tested whether diphthamide deficiency causes growth defects under rapamycin treatment. Rapamycin inhibits TORC1. If the translation of any of the TORC1 pathway proteins are affected by diphthamide deficiency, we expect that diphthamide deficiency would make the yeast cells more sensitive to rapamycin treatment.

We obtained yeast strains with deletion of each diphthamide biosynthesis gene (Dph1-7) and examined their growth phenotype with and without rapamycin treatment. The deletion of Dph genes did not cause a growth defect in the absence of rapamycin treatment (Fig. 1*A*). However, under rapamycin treatment, all the Dph deletion strains showed slower growth compared to the wild-type (WT) strain (Fig. 1*A*). The data provide initial support that the pathway annotation based on our computation analysis could provide useful insights.

TORC1 is also responsible for sensing amino acids and promoting growth. Unlike mammalian cells, yeast cells are able to synthesize all the proteinogenic amino acids. To test whether diphthamide deletion strains are compromised in sensing of amino acid, we utilized the fact that the laboratory strain BY4741 is histidine auxotroph. We starved BY4741 WT, ΔDph2, and ΔDph5 for histidine overnight. The growth was measured after addition of histidine to the starved yeast. ΔDph2 and ΔDph5 show much a slower growth rate compared with WT BY4741 (Fig. 1*B*). The same growth rate difference was also observed with the starvation of both methionine and cysteine or with the starvation of leucine (*SI Appendix*, Fig. S3). These results indicate that without diphthamide, the yeast cells cannot efficiently sense the presence of amino acids to grow, which is fully consistent with the hypothesis that the TORC1 signaling pathway is compromised in diphthamide-deficient cells.

To further verify that the TORC1 signaling is down-regulated in the ΔDph2 strain, we tested whether the phosphorylation of TORC1 substrate, Sch9, is suppressed (8). Since there is no antibody for phosphorylated Sch9, we utilized a chemical cleavage assay developed by Urban et al. (8). After chemical cleavage, the C-terminal of phosphorylated Sch9 appears on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as multiple bands with higher molecular weight than that of unphosphorylated Sch9 (8). The C-terminal of Sch9 from the WT strain indeed appeared as multiple higher molecular bands compared with Sch9 from the ΔDph2 strain (Fig. 2*A*), indicating that Sch9 in the ΔDph2 strain had lower phosphorylation levels. Consistent with this, without the chemical cleavage, Sch9 from the WT strain showed as a single band with higher molecular weight than Sch9 from the ΔDph2 strain (*SI Appendix*, Fig. S4). These data support that Sch9 had lower phosphorylation levels in ΔDph2, indicating a lower TORC1 activity.

TORC1 and mTORC1 activation pathways are mostly conserved between yeast and humans (1). To test whether diphthamide deletion also affects the mTORC1 pathway, we generated a Dph4 CRISPR knockout (KO) HEK293T cell. The mTORC1 signaling pathway was tested between the HEK293T cell with HEK293T Dph4 KO cells. Dph4 KO showed low 4EBP1 phosphorylation levels (Fig. 2*B*). To further confirm this, we also reintroduced Dph4 or control vector into Dph4 KO HEK293T cells. Compared to the introduction of control vector, the introduction of Dph4 expression vector in the cells restored diphthamide formation and increased phosphorylation of 4EBP1 (Fig. 2*C*). Thus, the deletion of diphthamide suppresses the mTORC1 signaling in human cells as well.

Interestingly, treating the cells with rapamycin led to a decrease in diphthamide level, indicating TORC1 suppression leads to suppression of diphthamide biosynthesis (Fig. 2*D*). On the other hand, activation of mTORC1 signaling with addition of amino acid promoted the formation of diphthamide (Fig. 2*E*). Thus, TORC1 activation promotes diphthamide biosynthesis, which in turn promotes TORC1 activation, forming a positive feedback loop.

The above phenotypic results support that our computational analysis for predicting biological pathways affected by diphthamide is effective. To find out whether the translation of the predicted proteins was indeed decreased in diphthamide deletion yeast cells, given the effect of diphthamide deficiency on TORC1 signaling, we focused on testing proteins involved in this pathway. In yeast, TORC1 is consisted of Tor1 or Tor2 along with Kog1 and Lst8 (33). The TORC1 complex can be activated by RAG GEF Vam6 (15). It can also be activated by the SEACAT complex, which consists of Sec13, Seh1, Mtc5, Rtc1, and Sea1 (16, 34) (*SI Appendix*, Fig. S1). Among these, Mtc5, Rtc1, Vam6, Tor1, and Tor2 were predicted to contain a −1 frameshifting motif (Table 2). Due to the lack of antibodies for these yeast proteins that can be used for Western blot analysis, we tagged the C-terminal of these gene with 3xFlag tag at the endogenous loci using homologous recombination (35). With the tagging strategy, we can then detect the endogenous protein levels by blotting for Flag. The C-terminal tagging of Tor2 were unsuccessful (possibly because Tor2 is an essential gene), but the tagging of other genes was successful (*SI Appendix*, Fig. S5). Among the four proteins tested, the levels of Rtc1 and Vam6 were down-regulated while levels of Mtc5 and Tor1 did not change in

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, GPI18, ROT2, POF1, MSS, URA10, ALD6, FAA2, RPA12, ADH7, PUT2, TGL3, POL3, HXK2, HMG2, MIP1, MET10, MCY1, ISN1, YLL08W, RPO31, ATP16, ERR2, DUR1,2, KGD1, FOL2, LYS4, RPB8, KGD2, SPE1, MNL2, TPS2, DH3, QRI1, PSD2, MNN10, RIB3, NRK1, POL12, ERG10, PCM1, TES1, ATP4, TRP3, GLC3, RPC10, RIB7, COX13, PNP1, ICL2, GDB1, MNN9, RPM15, ALG11, GWT1, PMT6, RPO21, MNN1, GAB1, GPI13, SEC59, SER2, KTR6, MET7, ERG27
MAPK signaling pathways	SST2, STE20, FKS1, FKS3, SKM1, HOG1, RSP, MSS4, MKK2, HKR1, BEM2, SWI4, PKH2, PKH1, PKH3, CLB6, RGA1, FAR1, SKO1, CDC24, SWI6, STE4, GSC2, STE7, TUS1, SPA2
Meiosis	SMC1, MCM, APC4, TOR2, NDT80, TPD3, ESP1, RED1, RAD24, MCM6, MCM7, HOP1, SWI4, CLB6, IRR1, PPH22, CDC, MCM, ZIP1, SWI6, RIM11, CYR1
Biosynthesis of antibiotics	STR2, MET2, ERR3, PDA1, ALD6, ADH7, HXK2, HMG2, MCY1, YLLo8W, ERR2, KGD1, LYS4, KGD2, SPE1, QRI1, ERG10, PCM1, TRP3, PRM15, SER2, ERG27
Cell cycle	SMC1, MCM5, APC4, TPD3, ESP1, RAD24, MCM6, SCC2, MCM7, SWI4, CLB6, CDC4, IRR1, FAR1, PPH22, CDC5, LTE1, YCS4, MCM3, BFA1, SWI6
Autophagy	TOR2, ATG7, VAM6, AVT3, MON1, GCN4, TOS3, TOR1, VPS41, VPS16, RAS1, SEC17, PPH22, ATG14, ATG16, GCN2, PEP3
90S preribosome	DIM1, MRD1, RRP, UTP7, UTP20, BUD21, TSR1, NOP14, CKA2, ENP1, UTP8, PRP43, UTP21, ECM16, UTP9, UTP13, NAN1
RNA transport	RPR2, NUP157, NMD2, NUP53, NUP188, CDC33, RPM2, NUP192, MSN5, TIF3, NUP85, NUP170, MLP2, STO1, TIF35
Protein processing in ER	CWH41, ROT2, SSM4, SSE2, SEC63, MNL2, SED4, DOA1, NPL4, LHS1, SCJ1, SFB2, GCN2, CDC48, PNG1

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. 3*G*) (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 in WT or ΔDph2 strains. The ratio between ΔDph2 and WT (ΔDph2/WT) yeast strains was then obtained and a ratio less than 1.0 would indicate a translation decrease in the ΔDph2 strain. Indeed, the translation of Rtc1 and Vam6 was suppressed in the ΔDph2 strain (Fig. 3 *H* and *I*).

To demonstrate that translation suppression is due to −1 frameshifting in ΔDph2 deletion strain, we decided to delve into the sequence of Rtc1 as Rtc1 shows a stronger translation suppression. It is interesting to notice that there are three predicted −1 frameshifting motifs located adjacent to each other forming a long frameshifting-prone sequence (Fig. 4*A*). 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. 4*B*) (37). Both frameshifting motif 1779 to 1831 and frameshifting motif 1836 to 1942 showed a frameshifting-inducing effect in ΔDph2 (Fig. 4*C*). 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. 3*G*). The Rtc1(1 to 1998) sequence contains all the predicted frameshifting motifs. After deleting the frameshifting motif, Rtc1(1836 to 1889), the translation efficiency of the Rtc1(1 to 1998) was restored to a level similar to that of control

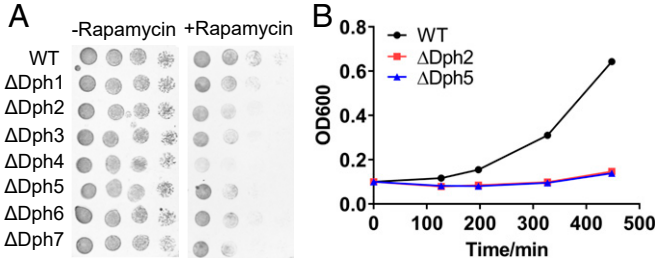

Fig. 1. Diphthamide deletion strains are more sensitive to rapamycin and grow slower after amino acid starvation followed by readdition of amino acid. (*A*) Diphthamide-deficient yeast shows slower growth in low-dose rapamycin treatment. All Dph deletion strains show similar growth to the WT strain (*Left*) 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.

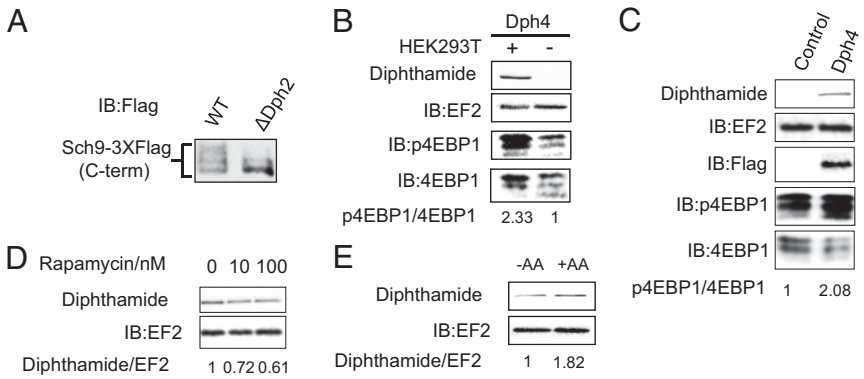

Fig. 2. Diphthamide promotes TORC1 and mTORC1 signaling. (*A*) Diphthamide promotes Sch9 phosphorylation in yeast. A chemical cleavage assay was used to detect the phosphorylation level of Sch9. Endogenous Sch9 was tagged with triple Flag tag (3xFlag) at the C-terminal using homologous recombination 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 diphtheria toxin-mediated ADP-ribosylation 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 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 (1836 to 1889) −1 frameshifting motif is important for the translation suppression in ΔDph2 (Fig. 4*D*). Furthermore, we overexpressed N-terminal Flag-tagged Rtc1 in WT and ΔDph2 strains. Upon treatment with a proteasome inhibitor, MG132, a band with Flag tag signal was detected around 67 to 69 kDa (Fig. 4*E*). This additional band was the same size as predicted if the −1 frameshifting happens within the long frameshifting-prone sequence (Fig. 4*A*). Collectively, these results demonstrate that −1 frameshifting within the long frameshifting-prone sequence of Rtc1 leads to its translation suppression in ΔDph2.

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 embryotic 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

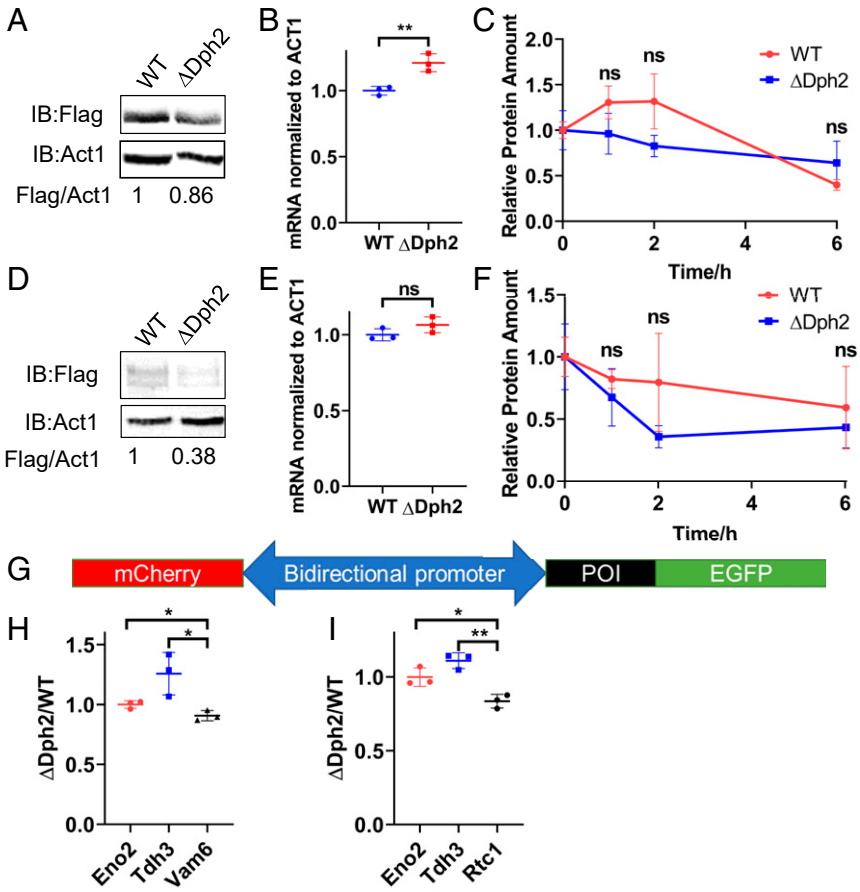

Fig. 3. Vam6 and Rtc1 are translationally down-regulated in diphthamide-deficient yeast. (*A*) Vam6 expression is down-regulated in ΔDph2. Endogenous Vam6 was tagged with a Flag tag using homologous recombination, and the level of Vam6 was detected using Western blot for Flag. (*B*) The mRNA level of 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. (*I*) Translation of Rtc1 monitored by the reporter system. ns, not significant; $*P < 0.05$; $**P < 0.01$.

Methods

Reagents, Antibodies, and Plasmids Used. Rapamycin was obtained from Cell Signaling Technology (CST 9904S). Flag–horseradish peroxidase (A4285 Sigma) was used for Western blot with 1:5,000 dilution. Antibodies for eEF2 (CST 2332S), 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 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).

Genome-Wide Search for Slippery Site. Yeast gene nucleotide sequences were obtained from the *Saccharomyces cerevisiae* database (https://www.yeastgenome.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 optical density $(OD)_{600} = 0.2$ the next day. After 6 h, yeast was washed three times with water. And the OD_{600} 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 OD_{600} of 0.1 in SC media. The cells were than cultured at 30 °C, and the OD_{600} 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 H_2O, 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

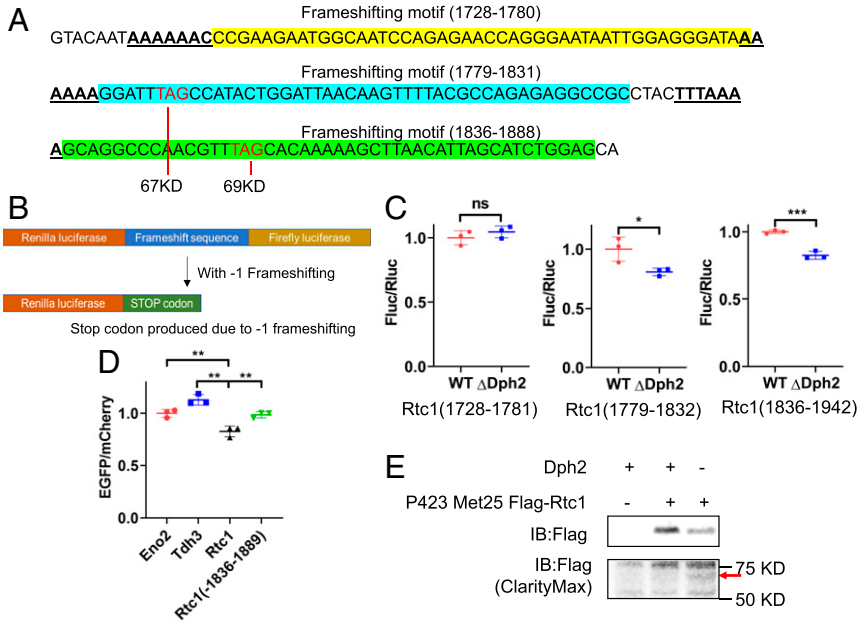

Fig. 4. Translation suppression of Rtc1 in ΔDph2 is due to the presence of −1 frameshifting motifs. (*A*) A long frameshifting-prone sequence formed by three predicted frameshifting motifs in Rtc1. The −1 frameshifting yields two premature proteins with stop codon (TAG) highlighted in the sequence. The sizes of these premature proteins are labeled. (*B*) The scheme of the luciferase assay to test frameshifting efficiency. Frameshifting motifs are inserted between Renilla luciferase and Firefly luciferase. If frameshifting happens, the expression of Firefly luciferase will decrease. The ratio between the activity of Renilla luciferase and Firefly luciferase determines the frameshifting efficiency. (*C*) Two out of three frameshifting motifs in the long frameshifting-prone sequence show frameshifting-inducing effect. (*D*) Deletion of the frameshifting motif Rtc1(1836 to 1889) restores the translation efficiency of Rtc1(1 to 1998) in the reporter assay. (*E*) A premature Rtc1 was detected in ΔDph2 strain with Western blot upon the treatment of protease inhibitor, MG132. The *Upper* was the Flag signal of full-length Rtc1. The *Lower* was obtained by exposing the lower section of the same gel with Clarity Max ECL substrate (Bio-Rad). The size of the premature protein is in line with the −1 frameshifting in the long frameshifting-prone sequence of Rtc1. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

quantification. The signal of a weaker band of premature protein of Rtc1 was obtained using Clarity Max ECL substrate (Bio-Rad).

Generation and Culture of 293T Dph4KO Cells. Dph4 KO was generated according to a published protocol (44). The 293T Dph4KO cells were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% (volume/volume) heat-inactivated fetal bovine serum (Invitrogen).

Rhodamine-NAD Labeling of EF2 to Detect the Levels of Diphthamide Modification. The labeling of EF2 with diphthamide modification was performed as previously described with the following modifications (45). Briefly, 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 fragment right 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′ agtcagGTCGACatggtgagcaagggcgaggag and YZ850_EGFP_HindIII_Flag_3′ agtcagAAGCTTttaCTTATCGTCGTCATCCTTGTAATCcttgtacagctcgtccatgcc and inserted into pTH644-CENBEVY with SalI and HindIII double digestion. mCherry was amplified with YZ847_mCherry_XmaI_5′ agtcagCCCGGGatggtgagcaagggcga YZ848_mCherry_EcoRI_HA_3′ agtcagGAATTCctaAGCGTAATCTGGAACATCGTATGGGTActtgtacagctcgtccat and inserted into pTH644-CENBEVY-EGFP with XmaI 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 OD_{600} around 1.0. Yeast cells were harvested by centrifugation at 500 × *g* for 4 min. 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 N-terminal insertion of Eno2 in the corresponding strains.

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 culture overnight. The next day, overnight culture was diluted and cultured until OD_{600} reached 0.6. Using glass beads, 5 mL of yeast culture was lysed. A total of 5 μL of protein extract was used for luciferase assay following the protocol of Dual-Luciferase Reporter Assay System (Promega).

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

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