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THE CHANNEL-KINASE TRPM7 REGULATES PHOSPHORYLATION OF THE TRANSLATIONAL FACTOR eEF2 via eEF2-k

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Abstract

Protein translation is an essential but energetically expensive process, which is carefully regulated in accordance to the cellular nutritional and energy status. Eukaryotic elongation factor 2 (eEF2) is a central regulation point since it mediates ribosomal translocation, and can be inhibited by phosphorylation at Thr56. TRPM7 is the unique fusion of an ion channel with a functional Ser/Thr-kinase. While TRPM7's channel function has been implicated in regulating vertebrate Mg^{2+} -uptake required for cell growth, the function of its kinase domain remains unclear. Here, we show that under conditions where cell growth is limited by Mg^{2+} -availability, TRPM7 via its kinase mediates enhanced Thr56-phosphorylation of eEF2. TRPM7-kinase does not appear to directly phosphorylate eEF2, but rather to influence the amount of eEF2's cognate kinase eEF2-k, involving its phosphorylation at Ser77. These findings suggest that TRPM7's structural duality ensures ideal positioning of its kinase in close proximity to channel-mediated Mg^{2+} uptake, allowing for the adjustment of protein translational rates to the availability of Mg^{2+} .

Keywords

Hypomagnesia; Nutritional signalling; Protein translation; TRP ion channels; Ser-Thr kinase

1. INTRODUCTION

Nutritional signalling is at the core of cellular adaptive responses to changing environmental conditions. Given its high metabolic and energy requirements, protein translation is carefully adjusted to the level of cellular resources. The inhibitory phosphorylation of eukaryotic elongation factor 2 (eEF2) thereby plays a central role. The only known kinase of eEF2, eEF2-kinase (eEF2-k), phosphorylates eEF2 at Thr56, leading to its failure to bind ribosomes, and therefore its inactivation [1,2]. The activity of eEF2-k is regulated via

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phosphorylation at several positions through the mTOR pathway, which is a central signal integration system for cell growth control [3]. eEF2-k belongs to the small atypical family of α -kinases [4,5] that also includes the kinase portion of the TRPM7 “chanzyme” [6].

TRPM7 and its closest relative TRPM6 are the only known channel-kinase fusions, a structural feature unique to the vertebrate TRPM6/7 proteins. TRPM6-deficient patients suffer from hypomagnesemia with secondary hypocalcemia (HSH), leading to lethal seizures [7,8]. Affected individuals live a normal life if provided with a Mg^{2+} -rich diet. Similarly, we have found that a genetically engineered TRPM7^{-/-} DT40 B-cell line displays massive growth impairment and becomes Mg^{2+} -deficient. This phenotype can be completely reversed by supplementation of the media with 5–10 mM $MgCl_2$ or $MgSO_4$, but not with Mn^{2+} , Ca^{2+} , Zn^{2+} , or Ni^{2+} [9,10]. TRPM7 homologues are also involved in Mg^{2+} -homeostasis regulation in other organisms including *C. elegans* [11,12], and zebrafish [13]. The biophysical characterization of TRPM7 has shown that its pore is divalent cation selective, and permeable to Ca^{2+} and Mg^{2+} [9]. TRPM7-mediated currents are inhibited by intracellular Mg^{2+} or $MgATP$ [9,14], and native currents with properties resembling recombinant TRPM7 were thus named “Magnesium Inhibited Current” (MIC), also called MagNuM current (Magnesium Nucleotide Metal) [9,14]. Combined with the ubiquitous distribution of TRPM7’s gene expression, these findings collectively suggest that one crucial aspect of TRPM7-biology is to ensure adequate levels of Mg^{2+} in a wide variety of cell types. Although these functions might not be all solely related to Mg^{2+} -homeostasis, TRPM7 is essential in various biological contexts including cell proliferation [10,15], cytoskeletal organization and cell migration [16,17], as well as embryonic development in mice [18]. Studies with clinical significance have revealed a central role for TRPM7-mediated ion fluxes in the context of anoxic neuronal cell death [19], and that suppression of TRPM7 expression restricted to the neurons of the hippocampus protected these cells from ischemia-associated damage [20]. Furthermore, a genetic TRPM7 variant resulting in a channel with heightened sensitivity to intracellular Mg^{2+} appears to favor the occurrence of human degenerative disorders [21], and a recent genomics approach has shown that TRPM7 is one of only 18 genes that are dysregulated in three different animal disease models (multiple sclerosis, Alzheimer’s, and stroke) [22].

Based on the unique presence of a kinase domain covalently linked to the channel portion of TRPM7 in vertebrates, we hypothesized that TRPM7 might function as a master regulator of cellular Mg^{2+} -homeostasis, coordinating cellular functions with the availability of this essential growth factor. The phosphotransferase activity of TRPM7-kinase is neither required for channel activation, nor for its inhibition by intracellular Mg^{2+} [10,23,24]. The involvement of TRPM7 in signalling via phosphorylation of exogenous substrates, such as annexin I and myosin IIA has been suggested [17,25]. Because TRPM7-kinase is a relative of eEF2-k, and given that Mg^{2+} is an essential co-factor for protein translation, we investigated whether TRPM7 might be involved in adjusting the rate of protein synthesis to the availability of Mg^{2+} . Here, we show that the inhibitory Thr56-phosphorylation of eEF2 is increased under hypomagnesian conditions, and that this Mg^{2+} -dependent regulation requires TRPM7 with an active kinase. We further found that eEF2 does not appear to be a direct substrate of TRPM7-kinase, but rather, that TRPM7 is regulating the activity of eEF2’s cognate kinase eEF2-k. In vitro phosphorylation assays revealed that TRPM7 can phosphorylate eEF2-k on Ser77. Using a complementation approach with eEF2-k S77A and S77D mutants in a genetically engineered eEF2-k deficient DT40 cell line, we propose that phosphorylation of eEF2-k on Ser77 improves its protein stability, resulting in higher levels of phosphorylated eEF2, and consequently, reduced rates of protein synthesis. This is the first study supporting the idea that TRPM7 fulfils the role of an environmental sensor, adjusting the level of physiological processes, such as protein synthesis, to the availability of ions.

2. MATERIAL AND METHODS

2.1. Cloning and generation of cell lines stably overexpressing proteins of interest

HEK-293 T-Rex cells (Invitrogen) with stable and dox-inducible expression of human TRPM7 wt and kinase mutants have been previously described [10]. For use in *in vitro* phosphorylation assays, a C-Terminal TRPM7 fragment (TRPM7-kinase C3, aa position 1440 to 1865) and TRPM7-kinase (aa position 1562 to 1865) were cloned into the pcDNA4/TO-Flag plasmid to generate stably expressing HEK-293 cell lines. For expression of mouse eEF2 wt, its full length cDNA (GenBank™ accession number BC060707) was purchased as an I.M.A.G.E. clone (Invitrogen). Mouse eEF2-k cDNA [26] was cloned into the pcDNA4/TO-Flag and pcDNA5/TO-Flag plasmids, allowing for dox-inducible expression of N-terminally flag-tagged protein in HEK-293 and DT40 T-rex cells. Mouse eEF2-k cDNA was used to generate eEF2-k mutants S61A, S77A(D), S240A, S444A, and S61A/S240A using site directed mutagenesis (Stratagene). The predicted DNA sequences of all constructs were verified by sequencing.

2.2. Generation of an eEF2-k deficient DT40 B-cell line

Chicken *eEF2-k* genomic DNA and cDNA were obtained by screening NCBI chicken genome and cDNA libraries. The targeting vectors (*eEF2-k-histidinol* (His) and *eEF2-k-puromycin* (Puro) were constructed by replacing the genomic fragment containing exons 8 to 10, which corresponds to a highly conserved region of the catalytic domain, with histidinol or puromycin cassettes. These cassettes were flanked by 3950 bp and 1780 bp of chicken *eEF2-k* genomic sequence on the 5' and 3' sides, respectively. The predicted DNA sequences of all constructs were verified by sequencing. The linearised targeting constructs were sequentially introduced into DT40 cells stably expressing the Tet-repressor (similar to the HEK-293 cells described above) by electroporation. Cell clones were selected in the presence of puromycin and histidinol.

The integration of the histidinol targeting construct into the *eEF2-k* gene locus was analysed by Southern Blot. To this purpose, genomic DNA was digested with NcoI. Restriction enzyme sites (N, NcoI), probe for Southern blot analysis (white bar), and targeted exons are indicated in fig. 4A. Targeting of the second allele was indirectly analysed by detection of the *eEF2-k* mRNA using RT-PCR, and confirmed by Western blot to detect eEF2-phosphorylation levels. The first allele targeting using the *eEF2-k* histidinol construct resulted in homologous recombination with a frequency of 20%. The targeting of the second allele showed a similar success rate using the *eEF2-k* puromycin targeting vector.

The RT-PCR reaction to detect *eEF2-k* transcripts was performed using RNA from potential eEF2-k deficient DT40 cell clones, or from DT40 wt cells (positive control). The control PCR was performed with following chicken specific oligonucleotides for NudT9 (housekeeping enzyme):

ACGTGTCGACATGTTGGCCTTCGCCACTGTGCC and
TAGCTACAAACTGCAAGATGTTTTTGCCAGTA.

2.3. Cell culture

Transiently or stably transfected HEK-293 cells were maintained in DMEM medium supplemented with 5% fetal bovine serum, and Blasticidin S (5 µg/ml, Invivogen) to maintain stable expression of the Tet-repressor. Zeocin (400 µg/ml, Invitrogen) was added for HEK-293 cell lines transfected with pcDNA4TO constructs, and hygromycin (100 µg/ml, Calbiochem) when pcDNA5TO constructs were used. Protein overexpression was induced by adding dox (100–1000 ng/ml) to the growth medium.

The TRPM7-deficient DT40 cell lines complemented with stable overexpression of hTRPM7 wt (cWT TRPM7, also cWT M7) or mutant channels (cKR M7, and cΔK(inase) M7) in an inducible fashion were described previously [10]. These cell lines were cultured in complete, chemically defined media with added 10 mM MgCl₂ for normal maintenance. DT40 wt cells or eEF2-k^{-/-} DT40 cells were kept in the same medium without additional Mg²⁺. To study native eEF2 phosphorylation, complete, chemically defined HyQ CCM1 and customized Mg²⁺-free HyQ CCM1 media (Hyclone) supplemented with 1% chicken serum (Sigma) were used as indicated.

2.4. In vitro phosphorylation assays of hTRPM7 WT, mutant channels, eEF2 and eEF2-k

TRPM7 wt, TRPM7 mutant channels, a TRPM7-C-terminal fragment (TRPM7-C3), TRPM7-kinase, eEF2, or eEF2-k were immunoprecipitated with anti-flag or anti-HA coated beads. The beads were incubated 20–30 minutes at 32°C in a total volume of 40 μl reaction buffer (50mM Tris-HCl, 0.1 (v/v) β-mercaptoethanol, 10 nM Calyculin A, 10mM MgAc, 1 mM MnCl₂, 0.5 mM CaCl₂, 100 mM Mg-ATP and/or 10 μCi γ³²P-ATP (specific activity of 3000 Ci/mmol) as indicated. Phosphorylation was analysed following gel electrophoresis and blotting, either by film and phosphor-imaging (radiolabeling experiments), or anti-P-Thr-/P-Ser-immunoblotting. The pH was kept constant in all phosphorylation reactions at 7.2.

2.5. Immunoblotting

0.1–10 × 10⁶ (when specified the larger cell numbers per gel lane were used to detect eEF2 basal phosphorylation) HEK-293 cells, DT40 wt or mutant cells with dox inducible expression of the indicated molecules were plated, and expression induced by adding dox to the growth media for 24–48 hours. HEK-293 or DT40 cells were lysed using standard protocols, or 250 ng purified eEF2-k protein was used for (co-) immunoprecipitation experiments. Anti-flag, anti-HA, and anti-GST (all from Sigma) immunoprecipitations were performed from cell lysates; the phosphatase inhibitor Calyculin A (10 nM, Cell Signalling) was added to the lysis buffer for phosphorylation experiments. Proteins of whole cell lysates, or immunoprecipitated proteins were washed with lysis buffer, then separated by SDS/PAGE using 6% or 8% polyacrylamide gels, and transferred to a PVDF membrane. The membranes were analysed by anti-flag (Sigma), anti-HA (Roche), anti-V5 (Invitrogen), two motive specific anti-P-Ser (tyrosine (Y) and arginine (R) contexts), anti-P-Ser77 eEF2-k, anti-P-Ser359 eEF2-k (both from Santa Cruz), anti-P-Ser399 eEF2-k (kindly provided by Dr. C. Proud), anti-eEF2 or anti-P-eEF2 (both from CellSignalling) immunoblotting, as indicated – blots were sometimes reprobed after stripping. Densitometric evaluations of the obtained results were performed on a Macintosh computer using the public domain NIH Image program (<http://rsb.info.nih.gov/nih-image/>).

2.6. Statistical Analysis

Data are expressed as means ± SEM. Statistical significance was evaluated utilizing Prism 4.0 software to perform a Student's *t*-test (unpaired, two-tailed) for comparisons between mean values.

3. RESULTS

3.1. Growth-limiting hypomagnesic conditions elicit an increase in eEF2 Thr56-phosphorylation in DT40 B-cells

Although it is known that suboptimal levels of Mg²⁺ negatively impact rates of protein synthesis, potential molecular mechanisms underlying this observation remain undefined [27–29]. We aimed at determining whether withdrawal of Mg²⁺ from the medium would

elicit an increase in the inhibitory phosphorylation the translational elongation factor eEF2 at position Thr56 in DT40 wildtype (wt) cells, known to lead to a decreased rate of protein synthesis. Chemically defined media were used, allowing for modification of $[Mg^{2+}]_o$ without affecting other nutrients [30]. Using a phospho-Thr56-eEF2 specific antibody, we found P-eEF2 levels to be increased 1 and 2 h after resuspension of DT40 cells into Mg^{2+} -free medium (Fig. 1A). Furthermore, the cellular growth rate of DT40 cells positively correlated with the amounts of Mg^{2+} provided (Fig. 1B).

3.2. TRPM7 with an intact kinase mediates the phosphorylation of eEF2 under low Mg^{2+}

We next tested whether TRPM7-kinase might influence the level of eEF2-phosphorylation in relation to the availability of extracellular Mg^{2+} . We used the above mentioned TRPM7^{-/-} DT40 cells complemented with stable, doxycycline (dox)-inducible expression of either hTRPM7 wt (cWT TRPM7, Fig. 2A), or of a kinase-deleted version of TRPM7 (cΔkinase TRPM7), or of the phosphotransferase-deficient K1648R TRPM7 point mutant (cKR TRPM7). We have previously shown that expression of TRPM7 K1648R restores wt growth behavior and intracellular Mg^{2+} -levels without supplementary extracellular Mg^{2+} , whereas TRPM7 Δkinase only partially compensates for the loss of TRPM7, possibly because of an overall destabilization of the TRPM7 Δkinase channel structure or assembly [10]. When the medium was supplemented with 10 mM $MgCl_2$ to support the growth of the TRPM7^{-/-} cells, no difference in eEF2 basal phosphorylation could be seen in these three cell lines upon induction of expression of TRPM7 wt, K1648R or Δkinase (Fig. 2B). In contrast, when the cells were resuspended in Mg^{2+} -free medium for 1 or 2 h, eEF2 phosphorylation was clearly increased upon expression of wt hTRPM7 (Figs. 2C, D left two lanes). No change in eEF2 phosphorylation could be documented in cTRPM7Δkinase cells (Fig. 2C, right lanes). However, this does not demonstrate the requirement for TRPM7-kinase, since the lack of increased eEF2 phosphorylation could result from the poor complementation of the growth and Mg^{2+} -deficiency phenotype by TRPM7Δkinase. To unequivocally address the contribution of TRPM7-kinase in Mg^{2+} -dependent eEF2 phosphorylation, we analysed TRPM7^{-/-} cells complemented with the phosphotransferase-deficient K1648R TRPM7 mutant. We found that although TRPM7-K1648R rescues the growth defect and Mg^{2+} -deficiency of TRPM7^{-/-} cells, it does not restore eEF2 phosphorylation under hypomagnesian conditions (Fig. 2D, right lanes). This set of results indicates that the rapid modulation of eEF2 phosphorylation in response to changes in the availability of Mg^{2+} requires TRPM7 with its active kinase domain.

3.3. TRPM7 associates with, and phosphorylates mouse eEF2-k on Ser77

Using an *in vitro* assay with co-immunoprecipitated overexpressed TRPM7 and eEF2 proteins, we failed to detect any significant TRPM7-mediated increase in eEF2-phosphorylation beyond basal level (Fig. 2E). This absence of direct TRPM7-mediated eEF2-phosphorylation was also described in a recent publication using radionucleotide labeling [31]. It is conceivable that TRPM7 regulates P-eEF2 levels by modulating signalling components upstream of eEF2. Since the vast majority of reports describe eEF2-k as the only kinase promoting eEF2 Thr56-phosphorylation, we investigated whether TRPM7 might mediate eEF2 phosphorylation in concert with eEF2-k. We analysed the potential interaction between TRPM7-kinase and eEF2-k by using proteins purified from bacteria, reducing the probability of co-associated components mediating the association. We found that purified GST-tagged eEF2-k, but not the GST protein by itself, pulls down MBP-tagged TRPM7-kinase (Fig. 3A). We consequently hypothesized that eEF2-k might be a substrate of TRPM7. *In vitro* phosphorylation experiments conducted with immunoprecipitated epitope-tagged eEF2-k and TRPM7-k using radioactive phospholabeling, or a phosphoserine specific antibody, demonstrated TRPM7-dependent eEF2-k phosphorylation (Fig. 3B). Only the phosphoserine antibody raised against the KxY/FxpS motif detected TRPM7-

mediated eEF2-k phosphorylation, not the corresponding antibody in the arginine context (Fig. 3B, right panel). Based on the antibody recognition sequence, we surveyed the mouse eEF2-k sequence and found S61 and S240 to be preceded by phenylalanine or tyrosine. We generated the S61A and S240A eEF2-k mutants, but saw no change in the levels of TRPM7-mediated phosphorylation in these constructs, even in a double S61A/S240A mutant. We then decided to analyse known eEF2-k phosphorylation sites. We first tested two different phospho-eEF2-k specific antibodies, anti P-Ser366 (CellSignaling), and anti P-Ser399 (kindly provided by Dr. C. Proud), with no success. Finally, we tested the anti eEF2-k P-Ser78 antibody (Santa Cruz). We found that this antibody recognized TRPM7-phosphorylated eEF2-k (Fig. 3C, left panel). In contrast, the mouse eEF2-k S77A (human S78) mutant we generated was not detected by anti-eEF2-k-P-Ser78, confirming the antibody's specificity (Fig. 3C, right panel).

3.4. eEF2-k-deficient DT40 cells do not exhibit increased eEF2-phosphorylation under suboptimal Mg^{2+} -conditions

To substantiate the contribution of eEF2-k to the increase in eEF2-phosphorylation under hypomagnesian conditions, we took advantage of the genetic malleability of the DT40 avian B-cell system [32] to generate an eEF2-k deficient cellular model. We opted to delete three exons encoding the catalytic region of eEF2-k (Fig. 4A). No more transcript of *eEF2-k* could be detected by RT-PCR in several DT40 cell clones, indicating disruption of both *eEF2-k* alleles (Fig. 4B left panel). Because there is currently no available anti-eEF2-k antibody recognizing the DT40 chicken version, we analysed the levels of P-Thr56-eEF2 as a read-out of deficiency in eEF2-k enzymatic activity. We found the basal eEF2 phosphorylation seen in wt DT40 cells to be extremely weak in eEF2-k^{-/-} cells, even when ten times more cells than in previous experiments were applied per gel lane (Fig. 4B right panel). We obtained multiple eEF2-k deficient clones, all of which exhibited no obvious phenotype as compared to wt DT40 cells.

We subjected eEF2-k-deficient cells to the same Mg^{2+} -withdrawal protocol as previously, and found that eEF2 Thr56-phosphorylation cannot be detected nor increased under these conditions (Fig. 4C, right lanes), despite loading lysates obtained from up to ten times more cells per gel lane than in previous experiments (Figs. 1–3). By introducing flag-tagged mouse eEF2-k into eEF2-k^{-/-} DT40 cells (c eEF2-k WT), hypomagnesia-induced eEF2-Thr56-phosphorylation increase was recovered (Fig. 4C, left lanes). When combined with the finding that eEF2-phosphorylation upon Mg^{2+} -deprivation is strictly dependent on TRPM7 with an active kinase domain (Fig. 2), we conclude from these results that both TRPM7 and eEF2-k are required for the increase in eEF2-phosphorylation in response to suboptimal Mg^{2+} -conditions.

3.5. eEF2-k^{-/-} cells complemented with S77A or S77D eEF2-k mutants have lost their Mg^{2+} -sensitive regulation of eEF-2 phosphorylation levels

The eEF2-k^{-/-} cell line complemented with flag-tagged mouse eEF2-k enabled us to investigate the effect of varying Mg^{2+} -levels on eEF2-k itself. Mg^{2+} -deprivation results in consistently increased eEF2-k serine-phosphorylation. Unexpectedly, we found that additionally to the increase in phosphorylated eEF2-k, the total protein level of eEF2-k also seemed augmented (Fig. 5A). We thus compared the levels of eEF2-k under hypermagnesian (10 mM $MgCl_2$), physiologically normal (1mM), and hypomagnesian conditions, and confirmed that under conditions of suboptimal Mg^{2+} availability eEF2-k protein levels are increased (Fig. 5B).

Since observations described above indicate that TRPM7 can phosphorylate eEF2-k on Ser77, we generated two eEF2-k point mutants; S77A for disruption of the S77

phosphorylation site, and S77D to mimic phosphorylation. Both were used to complement eEF2-k^{-/-} DT40 cells. Despite similar amounts of eEF2 protein across all samples, we found that eEF2-k-S77A cell clones exhibited lower levels of eEF2-k than eEF2-k^{-/-} DT40 cells complemented with eEF2-k wildtype or S77D (representative clones shown in Fig. 5C). This further supports the idea that Ser77 phosphorylation might have a positive effect on eEF2-k protein levels. We found that eEF2-k-S77D complemented eEF2-k^{-/-} cell line show elevated eEF2 Thr56-phosphorylation already under standard growth conditions. This suggests that the phosphorylation-mimicking S to D mutation of Ser77 in eEF2-k leads to an overall increase in cellular eEF2-k activity. Importantly, as shown in Fig. 5D, eEF2 Thr56 phosphorylation cannot be further increased when [Mg²⁺]_o is suboptimal (Fig. 5D). In contrast, the S77A complemented eEF2-k^{-/-} cells exhibit lower overall eEF2 Thr56-phosphorylation, consistent with the decreased eEF2-k levels. [Mg²⁺]_o-sensitivity of eEF2 phosphorylation is also lost in the S77A mutant, further supporting the notion that Ser77 in eEF2-k is crucial in conferring the TRPM7- and Mg²⁺-dependent regulation of eEF2 Thr56 phosphorylation.

3.6. Phosphorylation of eEF2-k on Ser77 under hypomagnesic conditions requires active TRPM7-kinase

Finally, we wanted to investigate whether eEF2-k phosphorylation can be adjusted in a TRPM7-kinase-dependent manner. The data presented in Fig. 5A (bottom two panels) suggested that even when taking into account the increase in eEF2-k protein levels, there still is a clear increase in eEF2-k phosphorylation upon Mg²⁺-deprivation, but this does not prove the involvement of TRPM7-kinase. This question cannot be easily answered since the currently available antibodies do not immunoprecipitate native chicken eEF2-k from the TRPM7^{-/-} DT40 cells complemented with hTRPM7 wt, or the TRPM7 K1648R-mutant (cWT-M7, cKR TRPM7). We therefore generated cell lines with stable and dox-inducible expression of flag-tagged mouse eEF2-k in the cWT-M7 and cKR-M7 background (Fig. 5E, left panel). Using an antibody generally recognizing phosphorylated serine residues, we were able to document TRPM7-dependent overall Ser-phosphorylation of eEF2-k, which appears to be slightly increased following 1h under low-Mg²⁺-conditions (Fig. 5E right top panel). Importantly, specific phosphorylation of eEF2-k on Ser77 is clearly elevated under hypomagnesic conditions, but only in cWT-M7 cells, and not in cKR-M7 cells (Fig. 5E right panel). Together, these results suggest that although TRPM7 might phosphorylate more than one Serine residue in eEF2-k, phosphorylation of Ser77 is the crucial TRPM7-mediated regulatory event in response to Mg²⁺-deprivation, as further indicated by our finding that mutating Ser77 results in a loss in Mg²⁺-sensitivity of eEF2's Thr56 phosphorylation levels.

4. Discussion

Collectively, our findings support a model in which the channel-kinase TRPM7 participates in modulating rates of protein translation by mediating eEF-2 phosphorylation in response to the availability of Mg²⁺ (Fig. 6). This is from our review of the literature the first example of a TRPM7-kinase dependent signalling event in response to changing nutritional conditions. TRPM7 acts thereby as a sensor of extra- and/or intracellular Mg²⁺-availability. We show that when Mg²⁺-availability is not sufficient to sustain cellular functions, TRPM7-kinase phosphorylates eEF2-k on Ser77 (Ser78 in human eEF2-k), possibly contributing to the observed increased stability of eEF2-k, and consequently increased inhibitory eEF2 Thr56 phosphorylation. Ultimately, this unsuspected signalling pathway would lead to an increase in rates of translational elongation when Mg²⁺ is available, and conversely decreased translational efficiency under suboptimal nutritional availability of Mg²⁺.

There have been previous indications in the literature that not only the phosphorylation state of eEF2-k, but also the adjustment of its amount play a role in regulating eEF2 activity [33–

36]. One study describes that hypoxic conditions result in a 5–10 fold post-translational increase in eEF2-k protein levels that correlates with enhanced Thr56-eEF2 phosphorylation [37]. One sole study has begun to address the question of eEF2-k's stability and degradation via the ubiquitin-proteasome pathway [38], but does not investigate how this process might be regulated. We provide here the first clue that Ser77-phosphorylation in mouse eEF2-k increases its amount, hinting at the possibility that eEF2-k phosphorylation can affect its turnover. We differ in our characterization of the effect of Ser77-phosphorylation from previous work, which had found P-Ser78 to inhibit calmodulin binding, and therefore eEF2-k activity [39]. Because in our hands the eEF2-k quantity effect only becomes obvious under hypomagnesian conditions, or when using a complementation approach in eEF2-k^{-/-} DT40 cells, this discrepancy could originate from the different growth conditions and experimental systems.

A remaining question for future investigations is the mechanism by which the activity of TRPM7-kinase is modulated by Mg²⁺-availability. One possible model is that the unique covalent bond between the kinase and ionic pore of TRPM7 allows for the close proximity of the kinase domain to the ionic micro-environment surrounding the TRPM7-pore. This implies that the requirement for Mg²⁺/MgATP of the phosphotransferase activity of TRPM7-kinase itself is the Mg²⁺ sensing mechanism that mediates the ensuing phosphorylation of its substrates. This model might however be too simplistic, since it does not take into account the Mg²⁺-sensitivity and gating mechanism of the TRPM7 ion channel portion, which is known to not require its kinase domain. TRPM7-mediated ion flow is efficiently inhibited by intracellular Mg²⁺ and Mg-nucleotides, which is the reason for the designation of TRPM7-like native currents as MIC (Magnesium Inhibited Cation), or MagNuM (magnesium-nucleotide-regulated metal ion currents) [9,40]. As a result, most TRPM7 pores might actually be closed under conditions where Mg²⁺ is high, although it should be noted that Mg²⁺ first needs to permeate the pore to elicit channel closure. The opening probability of the channel is expected to be the highest under conditions of low Mg²⁺. It is therefore not certain that Mg²⁺-concentrations in close proximity to the pore positively correlate with extracellular [Mg²⁺]. As an alternative to the Mg²⁺-sensitivity of the kinase, a conformational coupling model seems attractive, where the physical linkage between the ion channel and its kinase allows for the kinase to be regulated by the actual opening status and gating of the channel. It will also be interesting to investigate whether other ions that permeate the TRPM7 pore such as Ca²⁺, but also other divalent cations such as Zn²⁺ or Ni²⁺ [41] can also affect TRPM7-kinase mediated signalling.

In sum, our results suggest that TRPM7's structural duality is reflective of its biological function as a Mg²⁺-sensor, which TRPM7 is ideally poised to assess since it includes a Mg²⁺-permeable pore, and as a signalling molecule transmitting this information into the cell via its intrinsic kinase. Mg²⁺ has been previously discussed as a key parameter and direct correlative of cell proliferation [28,42,43]. The present study further supports the notion that TRPM7 is a pivotal element of cell physiology and cell proliferation control.

5. Conclusions

1. Hypomagnesian conditions are sufficient to elicit an increase in the inhibitory phosphorylation of the translational elongation factor eEF2.
2. TRPM7, the fusion of a Mg²⁺-permeable/-regulated ion channel with an active Ser/Thr kinase, is required for the increase in Thr56-eEF2-phosphorylation following Mg²⁺-deprivation.
3. TRPM7 does not appear to directly phosphorylate eEF2 on residue Thr56.

4. The only known kinase of Thr56-eEF2, eEF2-k, is required for the TRPM7- and Mg^{2+} -dependent regulation of eEF2 phosphorylation, as demonstrated in an eEF2-k-deficient cell line generated for the present study.
5. TRPM7 can interact with, and phosphorylate eEF2-k on residue Ser77.
6. Suboptimal Mg^{2+} -concentrations not only result in elevated Ser77-eEF2-k levels, but also in an increase in the total eEF2-k protein amounts. Mutation of the Ser77 site in mouse eEF2-k leads to a loss of Mg^{2+} - and TRPM7-dependent modulation of the Thr56-eEF2 phosphorylation levels.

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Abbreviations

TRPM7	Transient Receptor Potential Melastatin 7
eEF2	eukaryotic elongation factor 2
eEF2-k	eEF2-kinase
WT	wildtype

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Figure 1. Endogenous eEF2 phosphorylation under growth-limiting Mg²⁺ deprivation in DT40 cells

(A) Analysis of eEF2 protein and P-Thr56 levels in wt DT40 cells by Western blot in response to reduced Mg²⁺-levels. DT40 cells were cultured in chemically defined medium with 10 mM MgCl₂, spun down, resuspended and cultured in Mg²⁺-free medium for 1 or 2 h. The blot was probed with anti eEF2-P-Thr56, and reprobed with anti-eEF2. Densitometric quantification is underneath the corresponding lanes (arbitrary unit); values of two independent experiments plotted on the right (**P* < 0.05). (B) Growth curves of DT40 wt cells cultured under the same set of conditions as in (A). *n*=3. Error bars represent the mean ± S.D.

Figure 2. Role of TRPM7 and its kinase domain in regulating native eEF2 phosphorylation upon Mg²⁺ deprivation

(A) Schematics of TRPM7 wt protein (TM=transmembrane span, CCR= Coiled Coil Region). (B) eEF2 protein and P-Thr56 levels in TRPM7^{-/-} cells complemented with dox-inducible expression of hTRPM7 wt (cWT), phosphotransferase-deficient (cKR), and kinase-deleted (cΔK) mutants with 10 mM Mg²⁺. Blot probed with anti eEF2-P-Thr56, reprobed with anti-eEF2. Densitometric quantification underneath the corresponding lanes. (C) Levels of eEF2 protein and P-Thr56 in cWT vs. cΔK DT40s. DT40 cells were cultured in chemically defined medium with 10 mM MgCl₂, spun down, and resuspended for 1 h in Mg²⁺-free medium. Western blot and densitometry as before. Normalized values of three independent experiments plotted. Mean value and SEM are indicated (**P* < 0.05). (D) Comparison of cWT with cKR DT40s, using same culturing and Mg²⁺-starvation protocol as before. Western blot, densitometry and statistics as above; n=3 (**P* < 0.05). (E) Flag-tagged eEF2 overexpressed in HEK-293 cells alone or with a C-terminal flag-tagged fragment of hTRPM7 containing the kinase (C3-M7, aa 1440-1865). *In vitro*

phosphorylation assays in the presence of 100 μ M Mg-ATP using anti-flag immunoprecipitates (25 min at 32°C). Analysis of eEF2-phosphorylation by blotting using anti P-Thr56 eEF2. Blot reprobed with anti-flag. Please note that the P-Thr56 eEF2 antibody recognizes phosphorylated C3-M7 fragment, which includes a Ser/Thr stretch found to be hyperphosphorylated on over 35 residues [44].

Figure 3. TRPM7 interacts with, and phosphorylates mouse eEF2-k at serine site 77

(A) Each 500 ng purified GST-eEF2-k (mouse) and MBP-hTRPM7-kinase (human) were mixed and immunoprecipitated with anti-eEF2-k. Co-associated TRPM7-kinase was detected by blotting with anti-MBP. n=2 (B) Left panel: Overexpression in HEK-293 cells of flag-eEF2-k alone, or with flag-TRPM7-kinase. *In vitro* phosphorylation assays using anti-flag immunoprecipitates with 10 mCi γ^{32} P-ATP. Protein phosphorylation represented by autoradiography. Right panel: eEF2-k expressed alone or with a kinase-containing fragment of hTRPM7 (C3-M7, aa 1440–1865) in HEK-293 cells. *In vitro* phosphorylation assays with 100 μ M Mg-ATP as above. Analysis of eEF2-k-phosphorylation by blotting using two different motif-specific anti phospho-serine antibodies. Protein expression levels verified with anti-flag. (C) Flag-eEF2-k wt expressed alone or with flag-TRPM7-C3 in HEK293 cells. Following flag-immunoprecipitation and *in vitro* phosphorylation, eEF2-k phosphorylation was analysed using anti P-Ser-77 eEF2-k (left panel). Similar experiment using the eEF2-k S77A mutant (right panel). Protein expression levels verified with anti-flag. The shown results are representative of three independent experiments.

Figure 4. The increase in eEF2 Thr56-phosphorylation under hypomagnesian conditions requires eEF2-k

(A). Linear protein domain organization of eEF2-k, with the deleted catalytic region represented as a hatched box, and the N-terminal calmodulin (CaM) binding site as a black rectangle (upper drawing). Below, schematics of wt and mutated DT-40 eEF2-k alleles show the genomic region replaced by the puromycin and histidinol resistance cassettes. (B). Validation of the eEF2-k^{-/-} DT40 cell line: RT-PCR experiments using cDNA obtained from the DT40 wt parental cell line, or from an eEF2-k^{-/-} DT40 mutant clone were performed. Chicken eEF2-k specific primers were used to amplify fragments of endogenous DT40 eEF2-k, and primers against the house-keeping cNudT9 gene were used as a positive control (left panel). Lysates from 10⁶ DT40 cells were generated in order to efficiently see basal eEF2-Thr56 phosphorylation by immunoblotting using anti-P-Thr56-eEF2 antibody; the blot membrane was subsequently stripped and reprobed with anti-eEF2 antibody to assess protein loading levels. The results shown are representative of two separate experiments. (C). Mg²⁺-starving experiment was conducted as described previously (see Fig. 1), only that lysates were generated from 10⁶ cells instead from 10⁵ cells, to ensure we would be able to detect low signal intensities. DT40 eEF2-k^{-/-} mutant cells and its complemented version (ceEF2-k) were cultured for 2 hours in complete, chemically defined media (serum-free) containing 0 or 10 mM MgCl₂, and immunoblotting analysis of protein lysates from these cells was performed using anti-PThr56-eEF2 antibody. The blot membrane was subsequently stripped and reprobed with anti-eEF2. When appropriate, densitometric quantification is indicated underneath the corresponding lanes (arbitrary unit). The results shown are representative of three separate experiments.

Figure 5. The increase in eEF2 Thr56-phosphorylation under hypomagnesium conditions correlates with the amount of eEF2-k protein, which requires an intact eEF2-k Ser77 site

(A) Cell lysates from DT40s (eEF2-k KO line and its complemented version ceEF2-k wt) analysed by immuno-blot to detect eEF-2 and PThr56-eEF2 (top two lanes), as well as by flag-IP and subsequent blotting with anti-flag and anti P-Ser to represent protein amounts and Ser-phosphorylation of flag-tagged eEF2-k under varying Mg^{2+} -conditions. (B) Cell lysates analysed by IP and blotting with anti-flag to represent protein amounts of flag-tagged eEF2-k in DT40 cells cultured for 2 hours in complete, chemically defined media with high Mg^{2+} (10mM), standard (1mM), or low (close to 0). Levels of eEF2 in the lysates assessed as control; representative of two separate experiments (C). Flag-eEF2-k protein levels and eEF2 basal phosphorylation in eEF2-k^{-/-} DT40 cells complemented with eEF2-k wt, or mutants S77A and S77D, under standard physiological Mg^{2+} (1mM). Equal numbers of ceEF2-k wt, cS77A and cS77D DT40s were cultured with dox and 1mM Mg^{2+} . Densitometric quantification is underneath the corresponding lanes. Representative of two separate experiments (D) Similar experiment as in (C), but under varying Mg^{2+} conditions (as indicated). (E) Left panel, stable, dox-inducible protein-expression of flag-tagged mouse eEF2-k wt in TRPM7^{-/-} DT40 cells complemented with hTRPM7 WT or KR-mutant. Right panel, Analysis of eEF2-k phosphorylation in these cell lines: Equal numbers of cells (2×10^7) stably co-expressing flag-eEF2-k and either TRPM7 WT or TRPM7 KR in the TRPM7^{-/-} background were cultured with dox under different Mg^{2+} conditions. Cell lysates analysed by flag-IP and blotting. Blot reprobbed with anti-flag. Densitometric quantification is underneath the corresponding lanes. Unless stated otherwise, shown experiments all representative of three independent experiments.

Figure 6. Model of TRPM7's involvement in eEF2-k/eEF2 regulation in response to suboptimal extracellular Mg²⁺-levels

A vertical slice through the plasma membrane is showing “half-a-pore” of the TRPM7 channel kinase, as the complete channel is thought to be tetrameric. Both TRPM7 protein termini, including the C-terminal kinase are cytosolic. “P” indicates phosphorylation.