ANTIBODY VALIDATION ARTICLE

Reactivity of vertebrate-directed phospho-eEF2 antibody against the *Caenorhabditis elegans* orthologue phospho-EEF-2 [version 1; peer review: 2 approved]

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Abstract

Eukaryotic protein translation is divided into three main stages: initiation, elongation and termination. Regulation of this process occurs at the initiation and elongation step. eEF2 kinase phosphorylates eEF2 factor, blocking its ribosome interaction and thus translation elongation. This kinase activity can be detected by measuring eEF2 phosphorylation status. Here I show that vertebrate-specific antibody against phospho-eEF2 has excellent reactivity against *C. elegans* orthologue protein phospho-EEF-2.

Keywords

EFK-1, EEF-2, translation regulation, *Caenorhabditis elegans*

This article is included in the **Antibody Validations** gateway.

Open Peer Review

Approval Status

	1	2
version 1		
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1. **Richard Silva**, Universidade Federal de São Paulo, São Paulo, Brazil
2. **Kevin N. Dalby**, University of Texas, Austin, USA

Any reports and responses or comments on the article can be found at the end of the article.

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Competing interests: The author declares no competing interests.

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Introduction

Protein synthesis determines the cellular proteome and its regulation is pivotal to maintaining cellular homeostasis. This process is divided into three main stages: initiation, elongation and termination¹. The most studied step in eukaryotes is translation initiation and its regulation is critical to cell survival under stress conditions. Elongation regulation is also important in modulating translation. During this process, eukaryotic elongation factor 2 (eEF2) catalyzes the translocation of peptidyl-tRNA from the A site to the P site on the ribosome. The phosphorylation of eEF2 at threonine 56 by eEF2 kinase (eEF2K) inhibits its binding to ribosome and thus its activity²⁻⁵ in stress- or starvation-related conditions⁶⁻⁸. eEF2 kinase is an atypical α -kinase, normally dependent on Ca^{2+} ions and calmodulin, and apparently has only one substrate, the elongation factor eEF2⁹. eEF2 phosphorylation by eEF2K results in a reduction in translation rate. *C. elegans* processes eEF2 and eEF2K orthologues, named as EEF-2 (94.8 kDa) and EFK-1 (87.8 kDa), respectively. As in other eukaryotes, the Thr56 residue and adjacent sequences in EEF-2 are conserved. The only described data about *efk-1* shows that it is important to nutrient deprivation resistance¹⁰. My lab studies the role of *efk-1* in various aspects of *C. elegans* survival and the simplest way to measure its activity is to determine the EEF-2 phosphorylation status. Here we show that phospho-eEF2 antibody from Cell Signaling Technology Inc., specific to vertebrates, has excellent reactivity against *C. elegans* phospho-EEF-2 and this property is preserved after freeze/thaw cycles.

Material and methods

C. elegans strains

The standard *C. elegans* strain N2 Bristol (Caenorhabditis Genetic Center – CGC- wild isolate) and knockout strain *efk-1* (CGC#RB2588), were maintained at 15°C and propagated on *E. coli* strain OP50 (CGC) using established procedures^{11,12}. Gene knockout was verified by Polymerase Chain Reaction (PCR) using GoTaq® DNA polymerase (Promega) and specific primers to *efk-1* (Fw- ATGACGATCGACACAACAAA/Rv- AGATCACCAACTC-CTTGAATATCG) and *act-1* (Fw-ACCATGTACCCAGGAATTGC/Rv- TGGGAAGGTGGAGAGGGAAG) (Figure 1).

Electrophoresis and western blot (WB)

Worms (n= ~10) were collected in M9 buffer¹² and washed three times by centrifugation at 1000 rpm for 1 min (RT) in M9 Buffer to remove bacterial cells. Worm pellets were heated (95°C) in 2X sodium dodecyl-sulphate (SDS) sample buffer (62.5mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 100mM DTT) for 10 min. Samples were loaded in a gradient gel (8–16% - GE Healthcare Lifesciences) using the ECL® gel box system (GE Healthcare Lifesciences) at 150V (as per manufacturer's protocol). Separated proteins were transferred to an ECL®-Hybond (GE Healthcare Lifesciences) membrane using semi-dry transfer system (Bio-Rad). The membrane was blocked with 5% bovine serum albumin (BSA) in Tris-Buffered Saline (TBS-50mM Tris, 150mM NaCl) containing 0.5% Tween 20 (TBS-T) for 1 h at room

Figure 1. *efk-1* knockout confirmation by PCR. An *efk-1* fragment (~790 bp) was amplified by PCR using specific primer pair to verify the knockout background (*efk-1* KO) using N2 worms as positive control and *act-1* amplification as load control.

temperature, then incubated overnight at 4°C with primary monoclonal antibodies raised against vertebrate phospho-eEF2 (Thr56) (1:1000 in TBS plus 2.5% BSA – 94.8 kDa - Cell signaling Technology Inc. #2331 (Danvers, MA – USA) – reactivity: human, mouse, rat, hamster, monkey, chicken) and a monoclonal anti- α -Tubulin produced in mouse (1:1000 – Sigma-Aldrich Co. LCC #T6047 - reactivity: human, chlamydomonas, African green monkey, chicken, mouse, bovine, rat, kangaroo rat, sea urchin) simultaneously. After washes, membrane was incubated with secondary anti-rabbit/anti-mouse IgG horseradish peroxidase (HRP) antibody for 40 minutes at room temperature, subject to new washes and incubated with anti-mouse IgG HRP antibody (HRP – 1:2000 – Sigma-Aldrich Co. LCC). Signal detection was performed with Luminata® forte HRP Western substrate (Millipore). Mixed primary antibodies were stored at -20°C until needed and thawed at room temperature when necessary. Reagents are listed in Table 1 and Table 2 and the WB protocol is given in Table 3.

Results

Using the described protocol, I could specifically detect the phosphorylated form of EEF-2, eEF-2 orthologue in *C. elegans* since in *efk-1* knockout worms, used as a negative control of *efk-1* activity, there is no equivalent signal in western blots (Figure 2 and Figure 3). Detection of α -tubulin was used as a western blot load control. Some differences can be observed in α -tubulin detection when comparing lanes (Figure 2 and Figure 3), despite the use of approximately the same number of nematodes. This can be explained by the fact that some worms remain attached to the pipette tip after washes, and this effect can be circumvented adding 0.01% Triton X-100

Table 1. Reagents for western blots.

Process	Reagent	Manufacturer	Catalogue number	Concentration/Composition
Sample preparation	Laemmli buffer 2X	Homemade		62.5mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 100mM DTT
Electrophoresis	ECL® gel box system gel	GE healthcare Lifesciences	28-9906-08	
	ECL gradient gel 8-16%		28-9901-58	
	ECL® gel running buffer 10X		28-9902-52	
Protein transfer	Semi-dry system	Bio-Rad		
	ECL® gel running buffer 1X plus 20% methanol	GE healthcare Lifesciences	28-9902-52	
Block and antibody incubation	TBS-T -BSA	Homemade		TBS-T: Tris-Buffered Saline (50mM Tris, 150mM NaCl) containing 0.5% Tween 20) plus 5% BSA (blocking), 2.5% BSA (primary antibodies) or 2.5% non-fat milk (secondary antibodies).
Washes	TBS-T	Homemade		
Target detection	Luminata® Forte Western HRP substrate	Millipore	WBLUF0500	
	ECL® Hyperfilm	GE Healthcare Lifesciences	28906836	
Reagents	BSA	Sigma-Aldrich	A7906	
	Tween 20		P1379	
	Non-fat milk	Itambe®		
	Methanol	Merck		

Table 2. Primary and secondary antibodies.

Antibody	Manufacturer	Catalog number	RRID	Concentration
Phospho-eEF2 (Thr56)	Cell Signalling Technology	#2331	RRID:AB_2277755	1:1000
monoclonal anti- α -tubulin	Sigma-Aldrich	T8203	RRID:AB_477579	1:1000
anti-rabbit IgG HRP antibody		A6154	RRID:AB_258284	1:2000
anti-mouse IgG HRP antibody		A4416	RRID:AB_258167	1:2000

Table 3. Western blot protocol.

Protocol steps	Reagent	Time	Temperature
Sample preparation	M9 washes (3X 1min each)	15 min	RT
	Laemmli buffer 1X	10	95
	Laemmli Buffer 1X	5	RT
Electrophoresis	ECL gel box (150V)	1h	RT
	Running buffer		
Transfer	Semi-dry transfer Bio-Rad (25V)	1h	RT
	Transfer buffer		
Blocking	TBS-T-BSA	1h	RT
Primary antibodies	TBS-T-BSA	ON	4°C
Washes (3 times)	TBS-T	5 min each	RT
1st secondary antibody	TBS-T-non-fat milk	40 min	RT
Washes (3 times)	TBS-T	5 min each	RT
2nd secondary antibody	TBS-T-non-fat milk	40 min	RT
Washes (5 times)	TBS-T	5 min each	RT
Detection	Luminata® Forte Western HRP substrate	1–5 sec (SE – short exposure) to 3 min (LE- long exposure)	RT

Figure 2. Phospho-eEF2 antibody specific to vertebrate eEF2 recognizes *C. elegans* phospho-EEF-2. Western blot showing that phospho-eEF2 antibody recognizes *C. elegans* (N2 Bristol) phospho-EEF-2, since protein detection signal is absent in *efk-1* knockout worms. **A)** phospho-EEF-2 detection at the first time antibody dilution in TBS-T-BSA. **B)** phospho-EEF-2 detection after five freeze/thaw cycles of the antibody in TBS-T-BSA. SE- short exposure (5 sec); LE-long exposure (1 min).

Figure 3. Phospho-eEF2 antibody and α -tubulin antibodies can be used simultaneously to *C. elegans* target detection. Western blot showing that phospho-eEF2 and α -tubulin antibodies can be incubated and developed simultaneously. *efk-1* knockout worms were used as negative control to phospho-EEF-2 detection showed in wild-type worms (N2). **A)** Western blot short exposure (1 min). **B)** Western blot short exposure (3 min). Indicated molecular weight based on Precision Plus Protein™ Dual Color Standard (Bio-Rad).

(Sigma Aldrich) to *C. elegans* wash buffer (M9 buffer). In addition, both antibodies (directed to target and load control) can be used and detected simultaneously, reducing analysis time (Figure 3).

EEF-2 phosphorylation is not observed in *efk-1* knockout *C. elegans*, indicating that EFK-1 is the sole EEF-2 kinase in my tested conditions. Surprisingly, primary antibodies diluted in TBS-T-BSA (either phospho-eEF2 and α -tubulin, together or individual dilutions), stored at -20°C , can be reutilized at least five times (by thawing at room temperature) without losing specificity/reactivity (Figure 2B).

Conclusion

In this work, I show that vertebrate-directed phospho-eEF2 antibody specifically recognizes *C. elegans* orthologue phospho-EEF-2. This finding is very useful to those who work with translation elongation regulation using *C. elegans* as a model and I recommend this antibody to detect EFK-1 activity in *C. elegans*.

Competing interests

The author declares no competing interests.

Grant information

This work was supported by CNPq – Centro Nacional de Desenvolvimento Científico e Tecnológico, Capes – Coordenação de Aperfeiçoamento de Pessoal de nível Superior, and PRPq-UFMG – Pro-reitoria de Pesquisa da Universidade Federal de Minas Gerais.

I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Kevin N. Dalby

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This article describes the use of a commercial vertebrate-specific antibody available from Cell Signaling Technologies to detect the site-specific (Thr56) phosphorylated form of the *C. elegans* ortholog of elongation factor 2 in lysates generated from the worms by western blotting.

The specificity of the antibody is demonstrated using efk-1 knock out worms.

Importantly, it is also established that no other kinase in *C. elegans* phosphorylates eEF2 on Thr56, thus, its phosphorylation can be used as a proxy for elk-1 (the eEF2K ortholog) activity.

It should be noted that this is a proxy however and does not directly measure elfk-1 activity. Changes in phospho-eEF2 can come about because of changes in phosphatase levels.

The technical aspects are well described.

This appears to be a very useful tool for analyzing phospho-eEF2 levels in *C. elegans*.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 05 October 2015

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Viviane Alves shows that a phospho antibody raised against mammalian eEF2 can specifically detect the phosphorylation status of its counterpart in *C. elegans*, EEF-2. This is conceptually a very important validation in the field, especially because antibodies produced to detect specific phosphorylation sites in mammalian cells very often do not cross-react with non-mammalian organisms. The challenge in making use of these antibodies is that successful detection may depend on the specificity and affinity of the antibody for the phospho-protein of interest and on the amino acids sequences surrounding the specific phospho site. To make matters worse, the sequence and size of phosphopeptides employed to raise these antibodies are not always provided by companies (including the anti phospho eEF2K antibody validated in this manuscript), making it hard to *in-silico* predict their cross reactivity before acquisition. Therefore, this manuscript will be highly valuable to prevent researchers interested in studying the regulation of elongation during protein synthesis in *C. elegans* to go through the laborious validation to detect the inhibitory activity of EEF2 in *C. elegans* under different cues. The author also provides the conditions and a very neat protocol for the simultaneous detection of phospho EEF2 with a loading control that will prove quite helpful for researchers, saving time and resources. The manuscript is clearly written and easy to read. The experiments are generally carefully performed with appropriate controls. Only minor suggestions and changes should be made (See below)

Consider revising or changing the following points (minor suggestions)

- Inhibitory phosphorylation of eEF2K is elicited in response to different cues. Therefore, it would be really informative to emphasize that the phosphorylation signal observed in figures 2 and 3 is basal. Maybe this could be included within the results section.
- In addition, basal phosphorylation signal is quite high and possibly out of linear range, even with short exposures, which could be troublesome for those aiming to detect subtle differences in phosphorylation among different samples submitted or not to different stress conditions. Perhaps, it would be helpful to add a suggestion in the manuscript for the use of a higher anti-phospho EF2K dilution or alternatively, to make use of a weaker chemiluminescent substrate solution.
- Conclusion could emphasize the important validation that the load control (tubulin) can be specifically and simultaneously probed in the same membrane.
- The confirmation of the knockout for efk-1KO *C. elegans* strains is important to prove the specificity of the antibody against EEF2. However, the PCR result (figure 1) was not addressed in the results section, which starts with figure 2. Consider including a simple paragraph with this result in this section.
- Although the author made use of a semi-dry transfer apparatus and membranes are only rinsed in the buffer when this system is employed, it has been previously shown that the composition of buffers may influence the semi-dry transfer efficiency (www.nature.com/protocolexchange/protocols/2925). Therefore, the author could consider including the recipe of the buffer in table 3.

- Maybe I missed the point, but why were membranes first incubated with both anti-rabbit/anti mouse IgG-HRP and then incubated a second time with anti IgG-HRP against mouse only? (See material and methods section).

- In figure 3B, substitute *short* for *long*

In the sentences:

- *C. elegans* processes eEF2 and eEF2K orthologues = substitute *processes* for *possess*.
- After washes, membrane was the incubated with secondary" = substitute *the* for *then*.
- Used as a negative control of efk-1 activity = Change efk-1 for EF2K (the activity refers to the enzyme not the gene itself).
- Antibody for 40 minutes at room temperature, subject to new washes and incubated = substitute *subject* for *subjected*.
- Can be circumvented adding = substitute with: *can be circumvented by adding*
- Membrane using semi-dry transfer system = substitute with: membrane using a semi-dry transfer system

Competing Interests: No competing interests were disclosed.

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