Video Article

Xenopus laevis as a Model to Identify Translation Impairment

Amélie de Broucker[1], Pierre Semaille[1], Katia Cailliau[2], Alain Martoriati[2], Thomas Comptdaer[1], Jean-François Bodart[2], Alain Destée[1], Marie-Christine Chartier-Harlin[1]

[1]Team "Early Stages of Parkinson's Disease" of the Jean-Pierre Aubert Research Center, INSERM UMR-S1172, CHRU Lille, University of Lille 1 and Lille 2

[2]Team "Signal Division Regulation", CNRS UMR 8576, University of Lille 1

Correspondence to: Marie-Christine Chartier-Harlin at marie-christine.chartier-harlin@inserm.fr

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Abstract

Protein synthesis is a fundamental process to gene expression impacting diverse biological processes notably adaptation to environmental conditions. The initiation step, which involves the assembly of the ribosomal subunits at the mRNA initiation codon, involved initiation factor including eIF4G1. Defects in this rate limiting step of translation are linked to diverse disorders. To study the potential consequences of such deregulations, *Xenopus laevis* oocytes constitute an attractive model with high degrees of conservation of essential cellular and molecular mechanisms with human. In addition, during meiotic maturation, oocytes are transcriptionally repressed and all necessary proteins are translated from preexisting, maternally derived mRNAs. This inexpensive model enables exogenous mRNA to become perfectly integrated with an effective translation. Here is described a protocol for assessing translation with a factor of interest (here eIF4G1) using stored maternal mRNA that are the first to be polyadenylated and translated during oocyte maturation as a physiological readout. At first, mRNA synthetized by *in vitro* transcription of plasmids of interest (here eIF4G1) are injected in oocytes and kinetics of oocyte maturation by Germinal Vesicle Breakdown detection is determined. The studied maternal mRNA target is the serine/threonine-protein-kinase mos. Its polyadenylation and its subsequent translation are investigated together with the expression and phosphorylation of proteins of the mos signaling cascade involved in oocyte maturation. Variations of the current protocol to put forward translational defects are also proposed to emphasize its general applicability. In light of emerging evidence that aberrant protein synthesis may be involved in the pathogenesis of neurological disorders, such a model provides the opportunity to easily assess this impairment and identify new targets.

Video Link

The video component of this article can be found at http://www.jove.com/video/52724/

Introduction

Proteins are essential elements of cellular life and thus at larger-scale of the organism. They ensure the majority of cellular functions including structure, transport, reaction catalysis, regulation, gene expression etc. Their expression is the result of a complex mechanism of translation allowing the conversion of an mRNA into protein. The translation is subjected to various controls to adapt and to regulate gene expression according to the cell needs, during development and differentiation, aging, physiological stresses or pathological manifestations.

Translation is divided into 3 phases (initiation, elongation and termination) and presents 3 initiation translation systems in order to respond to these needs: cap-dependent, cap-independent *via* Internal Ribosome Entry Segment (IRES) structures and cap-Independent Translation Enhancers (CITE).

Most eukaryotic mRNA are translated in a cap-dependent manner *via* the 7-methylguanosine 5'-triphosphate cap that serves as a recognition feature during protein synthesis. This cap binds to eIF4E, a component of eIF4F complex with eIF4G1 and eIF4A. Associated with other partners like poly(A) Binding Protein (PABP), eIF2-GTP-Met-tRNA^{Met}, these translation initiation factors allow to circularize mRNA and improve its accessibility to forms 43S complex until the AUG initiation codon recognition[1]. This event corresponds to the end of the translation initiation *i.e.,* the first step of translation.

Cap-independent translation is used by mRNA encoding for essential proteins under stressed conditions that induce for instance cell proliferation and apoptosis. This mechanism involves secondary structures in mRNA 5'- untranslated region (UTR) called IRES, the carboxy-terminal end of eIF4G1 associated with eIF4A and the 43S complex. The binding of this 43S pre-initiation complex to IRES initiates the cap independent translation without the need for eIF4E factor[2,3].

Finally, another translation mechanism still not well understood supports this cap-independent translation activity under stressed conditions *via* CITE structures located within mRNA UTR [4].

Through these various modes of translation differing by their initiation steps, translation plays a critical role in cellular homeostasis and any change in one of these processes would thus impact the organism with small to large scale effects. Indeed, the initiation is a rate limiting step governing the correct translation processes of mRNA into proteins and is thus the target of numerous controls and regulation points[5]. Whether it is for the latter or for components of these processes, if one turns out to be defective, it will perturb the established balance in the cell and thus could lead to pathologic conditions. In this context, mutations in translation factors have been involved in several disorders including neurodegenerative disorders such as `leukoencephalopathy with vanishing white matter´ (eIF2B1-5 subunit)[6], in Walcott-Rallison syndrome (EIF2AK3 gene encoding for PERK)[7], potentially in Parkinson's disease (eIF4G1 p.R1205H)[8]. It is therefore important to conduct cellular and molecular studies of these mutant proteins to increase our knowledge on disease development and on the general process of translation initiation.

To carry out these studies, it is essential to choose the most adequate models to observe the consequences of these mutations. *Xenopus laevis* oocytes are particularly well adapted due to their physiological and biochemical properties: physiological synchronicity (blocked in phase G2 of the cell cycle), high capacity of protein synthesis (200-400 ng/day/oocyte), high number of extracted oocytes from a same animal (800-1,000 oocytes/female) and cell size (1.2-1.4 mm in diameter) which facilitates their manipulation. Microinjection of *Xenopus* oocytes with synthesized mRNA can easily be performed to dissect translation steps. In this view it presents other advantages. Given the speed of meiosis progression and of translation after mRNA microinjection (~24 hr), *Xenopus* oocyte represents a fast system compared to reconstituted cellular systems (extracted from *E.coli*, wheat germs or rabbit reticulocyte...) in which an mRNA is translated with a reduced translation rate and at a lower speed. So, the effects of a mutation introduced in an mRNA will be quickly observable and easily studied in several oocytes. Another advantage of *Xenopus* oocytes is that maternal mRNAs are latent and protein translation is blocked before progesterone stimulation. Addition of progesterone is thus a good means of controlling the translation induction. Cytoplasmic polyadenylation does not occur during oogenesis. It begins during oocyte maturation in progesterone-stimulated oocytes in a temporal order and continues throughout early development and could be used to study the different steps of translation.

The polyadenylation of mos mRNA is among the first to occur and it belongs with Aurora A/Eg2, Histone-Like B4 mRNA to the class of “early maturation” genes as defined in Charlesworth *et al.* (2004)[9]. The translational induction of “late” mRNA such as Cyclin A1 and Cyclin B1 occurs around the time of germinal vesicle breakdown (GVBD). Mos mRNA encodes a serine/threonine-protein kinase. Its translation is crucial since it induces the MAP kinase cascade that indirectly activates the oocyte maturation. Indeed, in response to progesterone, polyadenylation of mos mRNA is enhanced *via* a process involving Aurora A/Eg2 regulatory proteins and other RNA binding proteins with the 3’UTR of mos mRNA. This increased polyadenylation of mos mRNA leads to an increase of mos protein level, which in turn activates MEK1. This process mediates the activation of the extracellular signaling-regulated kinase 2 (ERK2) (**Figure 1**). This signaling cascade can then trigger the maturation M-phase promoting factors, a complex formed by Cyclin B and Cdc2 kinase, and eventually results in meiotic resumption.

Therefore in *Xenopus laevis* oocytes, the study of maternal mRNA such as mos could easily be used to test their translatability with several endpoints from their efficient polyadenylation to translation of several mos signaling components, including also the determination of the GVBD rate. This system is therefore interesting to evaluate the first consequences of mutations in translation initiation factors without interference of newly transcribed mRNA or of transfection efficiency, problems often occurring with eukaryotic cell studies.

Here, a protocol is established where mutant eIF4G1 mRNAs are microinjected in *Xenopus laevis* oocytes and the translation of maternal mRNA is tested. In the presence of a defect in GVBD progression, mos mRNA polyadenylation which is essential for progression through the oocyte meiotic cell cycle and for the subsequent translation of early and late class mRNAs is ascertained. The phosphorylation Aurora A/Eg2 and ERK is also studied to confirm the consequence of mos deregulation.Thus, *Xenopus* oocytes represent a simple way to analyse different steps of mRNA translation.

Protocol

All *Xenopus* experiments were performed at the animal facility of the Lille 1 University according to the rules of the European Community Council guidelines (86/609/EEC) for laboratory animal experimentation. The animal protocol was approved by the local institutional review board (Comité d’Ethique en Experimentation Animale Nord-Pas-De-Calais, CEEA 07/2010).

1. Oocyte Handling

1. Prepare the anesthetic solution: dissolve 1 g of tricaine methane sulphonate powder in a 1 L of sterile water.
2. Plunge female *Xenopus laevis* into this solution, cover the beaker to avoid escape and wait approximately 45 min for the animal to be completely sedated (without any reaction to a leg pinch).
3. Wash the frog with soap and rinse with tap water then place the animal on its back on clean aluminum foil.
4. Clamp the skin of the lateral part of the abdomen and at the ovaries level with forceps. Make an incision of approximately 1 cm with scissors cleaned before use with ethanol 70%. Make sure that the section is deep enough and to reach the underlying abdominal wall to allow excision of ovarian tissue in which several oocytes in various stages of development are found.
5. Dissect the ovary lobes, wash them 4 times in a Petri dish (50 mm) with ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES adjusted to pH 7.4 with NaOH, supplemented with 50 µg/ml streptomycin/penicillin, 225 µg/ml sodium pyruvate, 30 µg/ml soybean trypsin inhibitor, 1 µl/ml tetracycline) to remove all traces of blood and debris.
   Note: Tetracycline allows an optimal conservation and a good recovery after microinjection treatment.
6. Store them in a covered Petri dish submerged in the medium at 14 °C, allowing their preservation for one week.
7. Stitch the abdominal wall and the skin with veterinarian absorbable thread and a suturing needle (3 or 4 stitches will be necessary).
8. Place the animal in a beaker without water and moister the skin with tap water until the frog is moving again. Cover the beaker to prevent escape.
9. Separate carefully the ovary lobes in the medium in groups of 5 to 10 oocytes by using 2 pairs of forceps under a stereomicroscope with a 10-fold magnification. Select oocytes at stage VI. They can be recognized by i) their shape and color with a brown pigmented animal pole and yellow vegetative pole separated by a clear belt ii) their size superior to 1.2 mm in diameter[10].

10. Incubate the selected oocytes in a collagenase solution (1 mg/ml collagenase A from *Clostridium histolyticum*, dissolved in ND96 without tetracycline) in a Petri dish under gentle agitation during 45 min to facilitate the oocyte defolliculation (collagenase digests oocyte/follicular cell connections). Rinse the oocytes 3 or 4 times with ND96 medium kept at 14 °C.
    Note: Do not incubate the oocytes less or more than 45 min due to the risk of destroying oocyte surface proteins and of causing poor viability. Collagenase treatment can induce spontaneous GVBD.
11. Incubate oocytes in the medium for 3-4 hr. Remove follicular cells with fine tweezers under binocular magnifying glass and keep them at 19 °C in ND96 medium.

2. Preparation of mRNA Synthesis

1. Linearize 5 µg of the following plasmids by enzymatic digestion using *Pme*I enzyme.
    NOTE: pcDNA6.2/V5-DEST containing a 1599 amino-acids eIF4G1 cDNA wild type (eIF4G1-WT; NM_198241), pcDNA6.2/V5-DEST containing an eIF4G1 dominant negative cDNA (eIF4G1-DN) with mutations *c.2105T>G c.2106A>C, c.2120->G, c.2122T>A, 2125T>-* and *c.2126T>G* in the region of interaction between eIF4G1 and eIF4E at position 612 to 618 of the corresponding protein disturbing the interaction between eIF4G1 and eIF4E[8], pcDNA6.2/C-EmGFP containing Chloramphenicol Acetyl Transferase (GFP). Prepare 2 mixtures for each of the 3 plasmids: the first including *Pme*I enzyme and the second without enzyme as control.
2. Precipitate plasmid DNA using a classical ethanol procedure and resuspend it in 20 µl of Nuclease-free H_2O.
3. Determine its concentration using a spectrophotometer. The concentration should be superior to 150 ng/µl to transcribe cRNA.
4. Run 1 µl of samples and their controls with 5 µl of loading buffer on a 0.8% agarose gel to verify the plasmid linearization.
5. Use a kit for *in vitro* transcription and cRNA purification according to manufacturer's instructions. The transcribed cRNA are resuspended in 20 µl of Nuclease-free H_2O. Samples can be stored at -80 °C if needed.
6. Prepare the MOPS 10X migration buffer containing 0.2 M MOPS (pH 7.0), 20 mM sodium acetate and 10 mM EDTA (pH 8.0). Sterilize solution with a 0.45 µm filter. Stock the solution protected from the light at RT to avoid solution oxidation.
7. Clean the electrophoresis tank successively with NaOH, HCl and thoroughly rinsed with double-filtered water for an O/N to avoid RNase and prevent RNA degradation.
8. Cast a 1.5% agarose gel containing MOPS and formaldehyde. Warm until complete agarose dissolution and let it cool down to 55 °C. Under fume hood, add 0.1 volume of MOPS 10X, 6.6% of formaldehyde and 4 µg of ethidium bromide (10 mg/ml). Pour the gel under the fume hood and wait approximately 1 hr for the gel to harden.
    Note: Formaldehyde and Ethidium Bromide are toxic.
9. Prepare samples for migration in a 0.2 ml tube with 1 µl of cRNA, 8.8% of formaldehyde, 60% of formamide and 0.1 volume of MOPS 10X. Incubate for 3 min at 70 °C and put the tubes on ice for 10 min. Centrifuge samples few seconds at 5,000 x g. Add 2 µl of gel loading buffer.
    Note: Formamide is toxic.
10. Run samples for 20 min at 90V. Soak the gel in Nuclease-free H_2O O/N to destain the gel before analyzing it to check the cRNA quality.
11. Determine cRNA concentration using a spectrophotometer and store them at -80 °C.

3. Microinjection of Synthesized RNA and Oocytes Maturation Stimulation

1. Prepare the necessary equipment for oocytes microinjection. Use a micropipette puller to pull small glass capillary. Under stereomicroscope, break off the extremity of the capillary with the tweezers to create a blunt end. Fill the capillary micropipette with mineral oil using a 1 ml syringe with a millipore 0.45 µm filter at the opposite extremity. Mount the micropipette onto the microinjection pipette. Choose an accurate micropipette calibrated by the manufacturer to give good accuracy when used with appropriate glass capillary.
2. Perform microinjection 1-2 hr after defolliculation, necessary delay for oocyte recovery on oocytes kept at 14 °C. Use Petri dishes with the bottom scraped with forceps to create an adhesive surface for oocytes and fill them with ND96 medium.
3. Arrange oocytes along a scraped lane in a Petri dish allowing a successive injection of oocytes with the capillary micropipette at an angle of 45 °C.
4. Inject in the oocyte equatorial zone, below the pigmented animal area for an optimal diffusion of samples in the cytoplasm. Insert only the thinnest part of capillary tip on approximately 150-200 µm of depth.
5. Inject 30 ng of cRNA obtained respectively from the different plasmids in a volume of 60 nl in a first oocyte. After injection, wait for 5-10 sec before removing the capillary tip to avoid sample escape (**Figure 2A**).
    Note: Do not exceed 120 nl. Inject as slowly as you can. A control with distilled water is recommended.
6. Move manually the Petri dish to inject the next oocyte.
    Note: Make sure that the tip is not clogged by generating a small pulse out of the bath solution after 2 to 3 microinjections.
7. Transfer injected oocytes in 24 wells culture plates (10 oocytes /well) filled with 3 ml of fresh ND96 medium and leave them at 19 °C.
8. Incubate the oocytes in ND96 with progesterone (PG) (2 µg/ml) to trigger meiotic maturation (GVBD) at 19°C, 15 hr or 4 hr after microinjection of polyadenylated cRNAs obtained respectively from pcDNA6.2/V5-DESTeIF4G1 and pcDNA6.2/C-EmGFP used as a control (**Figure 2A**).
    Note: Co-injection of fluorescent marker with the cRNA is a nice tool to ensure that the cRNA was injected and remained in the oocyte. Perform such preliminary experiments using a cRNA of interest with a GFP tag.
9. Microinject as in #3.5 different ratios (1:3; 2:2; 3:1) of polyadenylated eIF4G1-WT and eIF4G1-DN cRNAs in order to test the translation effect of eIF4G1-WT cRNAs on the mutant phenotype (**Figure 2D**).

4. Germinal Vesicle Breakdown Determination

1. Count the number of mature oocytes after PG stimulation by observing the presence of a white spot at the black animal pole with a stereomicroscope. Repeat the counting every hour until the twenty-fourth hour (**Figure 2B, 2C**).

5. Western Blot (WB) Analysis

1. Homogenize the group of 10 oocytes by back and forth movements with a micropipette tip, at 4 °C in 200 µl in the following buffer: 50 mM Hepes, pH 7.4, 500 mM NaCl, 0.05% SDS, 5 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 10 mg/ml soybean trypsin inhibitor, 10 mg/ml benzamidine, 1 mM PMSF, 1 mM sodium vanadate.
2. Centrifuge samples for 15 min at 10,000 x g to remove the lipid (upper phase) and the membrane (bottom phase) fractions. Collect the cytoplasmic fraction, store an aliquot to determine protein concentration and complete the remaining with Laemmli or a Bis-Tris sample buffer (1:1).
   Note : Bis-Tris gels and loading buffer have a more neutral pH that protects proteins from hydrolysis
3. Heat 20 µg of sample at 95 °C for 10 min. Load each sample into the wells of the acrylamide gel. Place the gel in a tank with appropriate buffer.
4. Perform an electrophoresis for 1 h at 200 V.
5. Realize a WB in TBS pH 8.0 (Tris HCl 15 mM, NaCl 150 mM, Tween 0.1%, containing 10% bovine serum albumin) with a goat anti-Aurora A/Eg2 (1:3,000, 2 hr) or with a rabbit anti-Aurora A/Eg2-P (1:1,000, O/N at 4 °C), mouse anti-ERK2 (1:3,000, 2 hr), goat anti-ERK2-P (Tyr204) (1:3,000, 2 hr), rabbit anti-mos (1:5,000, 4 hr), rabbit anti-GFP (1:3,000, 2 hr) antibodies.Use antibodies mouse anti-V5 (1:10,000, 2 hr) and rabbit anti-Rsk (1:1,000, 2 hr) (as loading controls).
6. Wash the membrane 3 times for 10 min in TBS-Tween and incubate 1 hr with either an anti-mouse or anti-rabbit or anti-goat (IgM) horseradish peroxidase-labeled secondary antibody at dilutions of 1:5,000, 1:7,500 and 1:5,000 respectively.
7. Perform 3 washes of 10 min in TBS-Tween and detect the antigen-antibodies complexes with the Advanced ECL Detection system.

6. Polyadenylation Assay

1. Sample 5 oocytes per condition in a 1.5 ml. Wash them with 1 ml of Nuclease-free PBS 1X (pH 7.4). The following steps will be made under fume hood.
2. Extract RNA using a classical organic procedure (see manufacturer instructions).
3. Recover RNA by centrifugation (10,000 x g) for 5 min at 4 °C. Remove supernatant and air dry RNA for 10 min.
4. Resuspend RNA pellet in 30 µl of Nuclease-free H_2O.
5. Take 15 µl of samples in a new tube and add 85 µl of Nuclease-free H_2O. Clean up these samples to improve their quality on silica's column according to the manufacturer's instructions. Elute the RNA using 30 µl of Nuclease-free H_2O
6. Run 1 µl of samples with 5 µl of loading buffer on a 0.8% agarose gel to check the RNA integrity.
7. Determine the RNA concentration using a spectrophotometer.
8. Prepare 2 ligation mixtures per condition (*i.e.,* eIF4G1-WT, eIF4G1-DN and the H_2O control) with the following reagents: 10 U of RNA ligase, 0.1 volume of 10X buffer, 1 mM ATP, 10% of PEG8000 50%, 0.1 µg of primer P1 (5'-P-GGTCACCTTGATCTGAAGC-NH2-3')[11] and bring the final volume to 10 µl with Nuclease-free H_2O. Add in the first mixture RNA obtained from oocytes without PG stimulation and in the second mixture RNA obtained from PG stimulated oocytes.
9. Incubate for 1 hr at 37 °C then for 20 min at 65 °C to inactivate the enzyme.
10. Use a cDNA Reverse Transcription (RT) kit. Prepare the RT mixture, per tube: 0.1 volume of 10X RT buffer, 0.1 µg of primer P2 (5'-GCTTCAGATCAAGGTGACCTTTTT)[10], 4 mM dNTP mixture, 50 U of reverse transcriptase and complete with Nuclease-free H_2O to final volume of 10 µl. Add 10 µl of ligation reaction obtained previously. Perform the RT under conditions described by the manufacturer.
11. Prepare Polymerase Chain Reaction (PCR) mixture, per tube: 0.1 volume of buffer 10X, 1.5 mM $MgCl_2$, 133 µM dNTP mixture, 0.2 µM specific primer, 0.2 µM primer P2, 0.025 U *Taq* polymerase, 1 µl of cDNA and complete with Nuclease-free H_2O to final volume of 50 µl. Perform the PCR under following conditions: 50 °C for 2 min, 95 °C for 10 min, [95 °C for 1 min, 56 °C for 30 sec, 72 °C for 30 sec]*40 cycles, 72 °C for 10 min[9]. The specific primers are as follows: mos, GTTGCATTGCTGTTTAAGTGGTAA Histone-like B4, AGTGACAAACTAGGCTGATATACT; Cyclin A1, CATTGAACTGCTTCATTTTCCCAG; Cyclin B1: GTGGCATTCCAATTGTGTATTGTT[9].
12. Prepare a 3% agarose gel. Add in each PCR products 10 µl of loading buffer. Run the gel with 10 µl of samples at 110 V for an optimal migration. Analyse the gel after 10 and 20 min to observe a change of size reflecting the length of the tail poly(A) and thus the RNA maturation which is a prerequisite to their translation.

Representative Results

Kinetic maturation of *Xenopus* oocytes and determination of the percentage oocyte GVBD after 24 hr of PG stimulation (**Figures 2B, 2C**):

In order to study the translational consequences of the eIF4G1-DN mutation, the response to PG in *Xenopus laevis* oocytes microinjected with cRNA eIF4G1-DN is compared to eIF4G1-WT and to other control conditions (H_2O, GFP). The controls enable to assess the incidence of oocyte microinjection regardless of the nature of injected cRNA or of eIF4G1 overexpression.

The kinetic maturations of 10 oocytes characterized by microinjected control conditions (H_2O and GFP) are similar to WT and the number of undergoing GVBD are very close to each other at 12 and 24 hr after the PG stimulation (**Figure 2B**). After 24 hr, maturation of oocytes reaches 95.7% for WT, 97.1% for H_2O and 97.5% for GFP (**Figure 2C**). Thus, microinjections with H_2O or control cRNAs or eIF4G1 cRNAs have little effect on oocyte meiosis release. Conversely 8 hr after PG stimulation, the number of oocytes microinjected with eIF4G1-DN cRNAs undergoing GVBD is delayed compared to eIF4G1-WT cRNAs (**Figure 2B**).The meiosis release of eIF4G1-DN *versus* eIF4G1-WT oocytes significantly decreases by 85.8% and 77.4% at 12 hr and 24 hr after PG stimulation respectively *(***Figure 2C**). It is also noteworthy that 13 hr after PG stimulation, the number of mature oocytes reaches a maximum for all conditions except for eIF4G1-DN for which the maximum is achieved only 20 hr after PG stimulation. Thus, the presence of eIF4G1-DN mutation leads to poorer oocyte meiosis release compared to eIF4G1-WT.

Restoration of oocyte maturation in the presence of eIF4G1-DN thanks to the eIF4G1-WT (**Figure 2D**):

To determine whether the eIF4G1-DN cRNA microinjection impairs or blocks the oocyte maturation, different ratios of eIF4G1-WT and eIF4G1-DN cRNAs are co-microinjected. In these conditions, an increase of oocyte maturation is observed as the levels of eIF4G1-WT cRNA raise and those of eIF4G1-DN cRNA decrease.While maturation is observed in 17.5% of oocytes with one dose of eIF4G1-DN cRNAs, this percentage reaches 76.7% with co-microinjection of 3 doses of eIF4G1-WT cRNAs and the presence of one dose of eIF4G1-WT cRNAs leads to 95% of oocyte maturation. This experiment shows that the maturation defect of *Xenopus* oocytes microinjected with eIF4G1-DN is not irreversible and can be partially restored.

Expression mos signaling proteins observed on WB as an indicator of translation ability before and after PG stimulation (**Figure 2E**):

The protein level of V5-tag in oocytes expressing eIF4G1-WT and eIF4G1-DN are similar reflecting similar microinjection efficiency. The level of Rsk protein used as protein loading control is also similar in all conditions before and after PG stimulation. The expression of the GFP protein is also present in oocytes microinjected with GFP cRNA stimulated or not with PG. These data indicates that the microinjection and the overexpression of eF4G1 do not perturb the translation of GFP. However, no GFP protein expression is detectable before and after PG stimulation in oocytes expressing the eIF4G1-DN. As expected, no endogenous mos expression is detectable in the absence of PG stimulation. In addition, mos expression is observed in the eIF4G1-WT and H_2O control conditions after PG stimulation in agreement with previous results showing that overexpression of eIF4G1-WT has little consequences on endogenous mos[16]. Conversely, a considerable decrease of mos expression is noticeable after PG stimulation.

The protein expression of some components of the mos signaling cascade is also tested. For instance, Aurora A/Eg2 protein is acting upstream to mos induction and no detection of its protein deregulation or of its phosphorylated form induced after PG stimulation is observed. Conversely, a deregulation of ERK2 phosphorylation induced by mos is observed in the presence of eIF4G1-DN.

These results suggested that the endogenous expression of mos RNA into protein and mos dependent ERK2 activation are affected by the expression of eIF4G1-DN.

mRNA Polyadenylation (**Figure 2F**):

Polyadenylation of several mRNAs (mos, Cyclin A1, Cyclin B1 and Histone-like B4) necessary for *Xenopus* oocytes meiosis recovery was tested. The performed assay enables to define whether an increase in the amplimer size corresponding to the length of the poly(A) tail of mRNA is present after PG stimulation. Indeed, with PG stimulation the addition of a poly(A) tail is observed in all conditions including the eIF4G1-DN mutation.

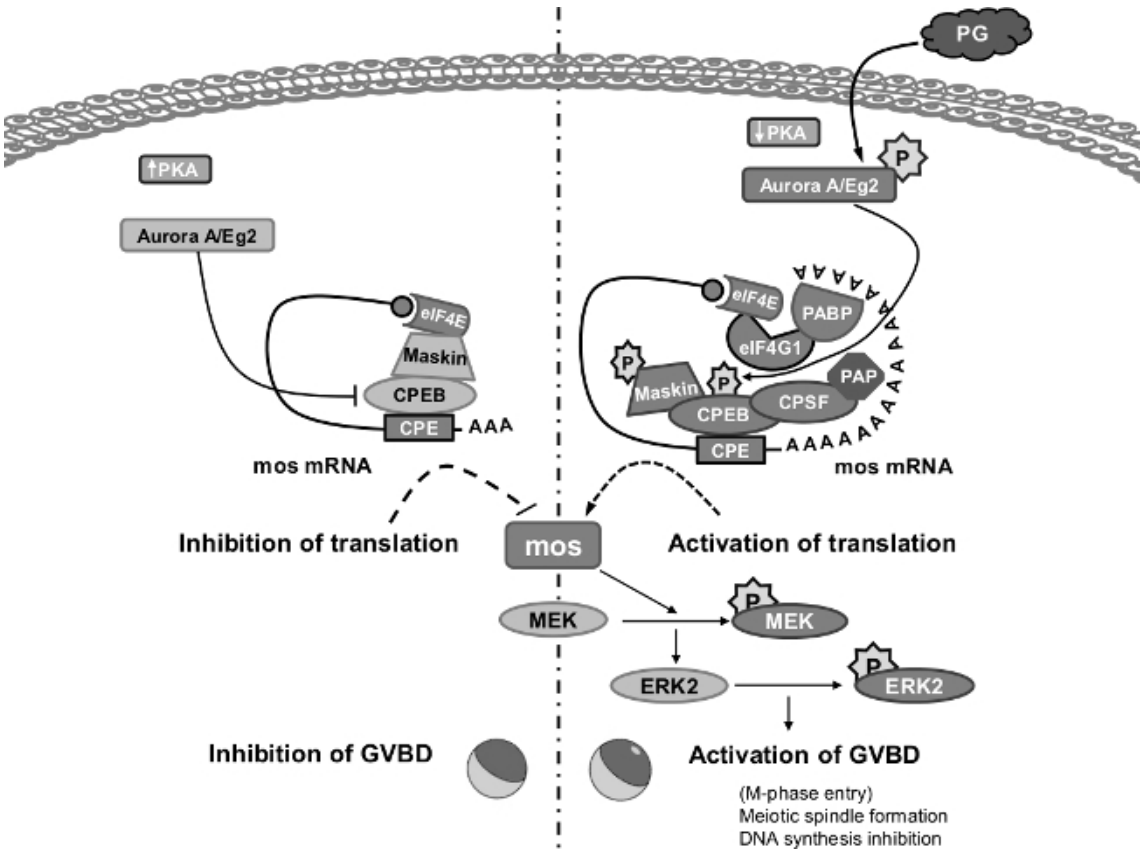

Figure 1. Schematic overview of MAPK cascade activation. Upon hormonal stimulation by PG rapid changes in the activities of several enzymes occur including those of the Aurora A/Eg2 and CPEB pathway. Hyperphosphorylation of Aurora A/Eg2 leads to CPEB (Cytoplasmic Polyadenylation Element Binding protein) phosphorylation. Phosphorylated CPEB recognizes the CPE (Cytoplasmic polyadenylation Element) on 3'UTR of mos mRNA, recruits CPSF (Cleavage and Polyadenylation Specificity Factor) and activates mRNA polyadenylation by PAP (Poly(A) Polymerase). PG stimulation also promotes successively Maskin phosphorylation *via* both action of PKA and cdk1, Maskin/eIF4E dissociation

and eIF4E/eIF4G1 association. eIF4G1 recruits translation initiation partners including PABP which interacts with eIF4G1 and recognizes the poly(A) tail. Once synthesized, mos activates MEK/MAPKK by phosphorylation, which in turns, activates ERK2/MAPK by phosphorylation. This so-called MAPK cascade is involved in the meiotic processes by controlling M-phase entry kinetic, spindle morphogenesis and inhibition of DNA synthesis. The cascade is embedded in a positive feed-back loop that sustains protein synthesis. One has to note that mos is not the only protein requested to be synthesized for GVBD activation; *i.e.,* Cyclin B are required to be translated for maturation achievement.

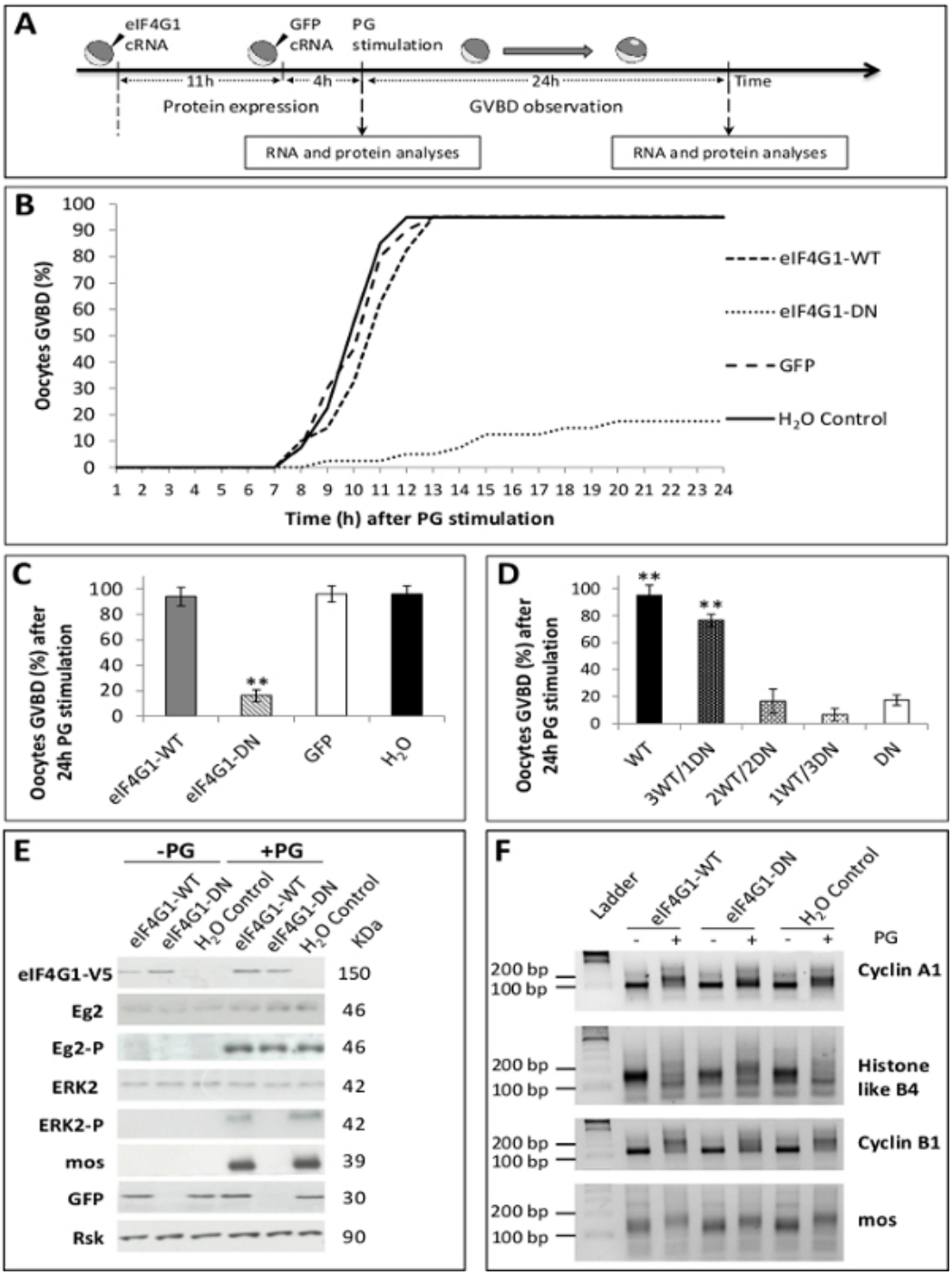

Figure 2. The eIF4G1-DN mutant affects oocyte maturation and translation in *Xenopus laevis*. RNA derived from plasmids encoding V5-tagged eIF4G1 proteins (WT and DN) are microinjected in *Xenopus laevis* oocytes. After 15 hr, maturation of oocytes is stimulated by progesterone (PG) (Experiments were performed at least 3 times and representative results are shown). **(A)** Schematicoverview of experimentation protocol. The injections of eIF4G1 and/or GFP cRNAs are represented by arrow-heads before PG stimulation. Samples for RNA and proteins analyses have been obtained at 2 time points (PG stimulation and at the end of the GVBD kinetic). **(B)** Kinetic maturation of 10 oocytes characterized by GVBD is tested during 24 hr after PG stimulation. A delay in oocyte maturation is observed in oocytes microinjected with eIF4G1-DN cRNAs compared to those microinjected with eIF4G1-WT cRNAs. **(C)** Percentage of mature oocytes 24 h after PG stimulation (n=4; *p<0.05). A decrease in oocyte maturation is observed in those microinjected with eIF4G1-DN cRNAs compared to eIF4G1-WT cRNAs. **(D)** Reversion phenotype assessment of oocytes microinjected with eIF4G1-DN mutant cRNAs and eIF4G1-WT cRNAs showing that the translation machinery is still functional when eIF4G1-WT is added to the mutant. **(E)** Proteins are extracted from oocytes and their levels determined by immunoblotting analysis. Perturbations of ERK2 phosphorylation, mos and GFP expression in the eIF4G1-DN conditions are observed. **(F)** Polyadenylation of mos, Cyclin A1, Cyclin B1 and Histone-like B4 mRNAs from oocytes stimulated or not with PG observed after PCR amplification and migration on 3% agarose gel. After PG stimulation, an increase in size >100 polyadenylation is detected for all conditions. The first lane corresponds to the ladder.

Discussion

Translation is a mechanism involved in the physiopathology of numerous human disorders including several neurodegenerative diseases. For instance in Parkinson's disease several reports suggested the impairment in translation associated with hereditary mutations[8,12,13].

Several cellular models are available to study translation. Here, in order to study the translational consequences of a mutation in eIF4G1 that acts as dominant negative mutation reducing the interaction between eIF4G1 and eIF4E partners[8], the *Xenopus laevis* oocyte is used. This model has the advantages of simplicity since it is composed of only one single giant cell facilitating the microinjection of synthetic mRNA, leading to a considerable amount of material for biochemical experiments and facilitating macroscopic observations of the different phases of oocyte maturation. This process is also time efficient compared to stable cell line generation. Moreover, several protocols of oocyte preparation and RNA microinjections have already been described in particular for studying cell cycle or nucleocytoplasmic transport[14,15]. Regarding the limits of such protocols to study translation, a particular attention should be taken regarding the concentration of mRNA. This concentration should not be to high (not higher than 30 ng in 120 nl maximum) in order not to saturate the translation process. Taking into account this element, *Xenopus* oocyte is an attractive model to study protein translation as attested by data presented in **Figure 2** with a mutant form of *EIF4G1* transcript.

Indeed, in the presence of the mutated eIF4G1, impairment in the translation of mos protein is observed and correlated with a decrease of 90% of GVBD compared to WT and to control conditions. Conversely, overexpression of eIF4G1-WT did not result in modification in the level of mos translation in agreement with literature data[16,17].

Several biological modifications might explain these results. Knowing that mos mRNA are of maternal origin and already transcribed before meiosis activation by PG, the perturbed mechanisms should occur between the transcription and translation steps. Noteworthy, mos translation is the result of a cascade of molecular events that start from a latent mRNA without poly(A) tail to the addition of the tail after PG stimulation to its translation[18,19]. Thus, several scenarios are possible including: defects in translation initiation might be suspected as the cause of this failure, or defects of phosphorylation of the factor leading to mos mRNA polyadenylation, or default of mRNA polyadenylation of the mos transcripts by itself could lead to a translational decrease.

To assess these last 2 mechanisms, the expression of these factors by WB is examined. Expression levels of phosphorylated Aurora A/Eg2, responsible for CPEB phosphorylation showed no disturbance in the presence of the mutated eIF4G1. In the same vein, mos mRNA polyadenylation or other mRNA polyadenylation is observed in the presence of the mutated eIF4G1 after PG stimulation (**Figure 2F**). To further confirm the polyadenylation profile Northern blot experiments would be recommended as well as performing sucrose gradient isolation of polysomes in order to establish whether the recruitment of mRNA to ribosome can occur[16].

So, by studying the first and last elements of the cascade, the perturbed stage was suspected to be downstream of the occurrence of polyadenylation. It is also important to test whether the mos expression defect gave the expected impact on the phosphorylation of ERK2. In the presence of the mutation, the decrease of mos expression led to a decrease of phosphorylated ERK2 level and consequently to a defect in the GVBD progression (**Figure 1 and 2E**). Thus, the eIF4G1 mutation is associated to an expected decrease of mos translation. The deletion of the eIF4E binding domain in eIF4G1 sequence in the eiF4G1-DN is probably responsible of a decrease in the eIF4G1/eIF4E interactions as previously suggested by a co-immunoprecipitation study[8] that might perturb the eIF4F complex formation.

This protocol has several interesting applications in cellular biology. This set-up is ideal as preliminary investigations for a number of fundamental questions in cellular biology such as: to better understand the role of specific domains of initiation factors and to define those that are essential to *in vivo* translation or oocyte maturation. In this context, Wakiyama *et al*. (2000) showed the importance in oocyte maturation of the amino-terminal region of eIF4G1 containing the binding domains for eIF4E and PABP[17]; to study splicing of initiation factors[20]; to decipher the mechanisms used by viruses hijacking host translation machinery for their purposes *via* eIF4G1 cleavage for instance with inhibition of cap-dependent translation and IRES structures of their mRNA, translated in cap-independent manner[21]; to study the role of mutation in translation factors on cell cycle since oocytes are models of choice for such study[22]; to study the main mechanisms governing initiation of translation *i.e.,* cap-dependent and cap-independent to define whether one or several systems are involved in a protein synthesis disturbance. Several variations of the current protocol could easily be applied to reach these aims, including determination of the translation efficiency by the SUnSET method23 or reporter mRNA microinjection. For instance, the use of a bicistronic reporter mRNA containing a first cistron in which *Renilla luciferase* will be cap-dependent translated whereas the second cistron containing *Firefly luciferase* will be translated *via* IRES structures should enable to define the mode of initiation to put forward fine defects and/or compensations to maintain translation homeostasis. In addition, such luciferase reporter RNA experiments offer the advantage to not rely on potential disrupting specialized developmental mechanisms due to incomplete conservation mechanisms with human.

In parallel to these fundamental questions, such protocol could be applied as a first step to address detrimental conditions that could contribute to neurological disorders. In physiological conditions, translation initiation steps are the privileged targets of regulations under stressed conditions such as oxidation, hypoxia, temperature change, irradiation and nutrient deprivation. In such conditions, a selective translation of transcripts encoding proteins that are essential against stresses and cell survival occurs. When this process is overwhelmed, stresses become pathologic and actively participate in the development of several neurological affections. Cellular stressors are involved in numerous neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis or prion disease (Creutzfeldt-Jakob)[24] and these stresses induce the activation of Unfolded Protein Response (UPR). One of these UPR mechanisms leads to a decreased translation *via* PERK activation and the phosphorylation of eIF2α subunit with the consequences of stopping translation by preventing binding between eIF4F and 43S complexes[25]. In *Xenopus* oocytes, the addition of oxidative agents and/or mutated genes known to be associated to such pathologies would mimic these stresses and establish whether the mutated genes can impact the translation processes. In Parkinson's disease for instance, the introduction of eIF4G1 p.R1205H in *Xenopus* oocyte associated or not to oxidative stressors or genome-wide analysis of mRNA polysomal profiles could address the question of the translation involvement in the physiopathology[26].

All these applications applied to the oocyte thus represent a large field of possibilities to study translation disturbances that are now known to be associated to several affections including neurodegenerative disorders.

Disclosures

The authors have nothing to disclose.

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