

Activation of a GPCR leads to eIF4G phosphorylation at the 5' cap and to IRES-dependent translation

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Abstract

The control of mRNA translation has been mainly explored in response to activated tyrosine kinase receptors. In contrast, mechanistic details on the translational machinery are far less available in the case of ligand-bound G protein-coupled receptors (GPCRs). In this study, using the FSH receptor (FSH-R) as a model receptor, we demonstrate that part of the translational regulations occurs by phosphorylation of the translation pre-initiation complex scaffold protein, eukaryotic initiation factor 4G (eIF4G), in HEK293 cells stably expressing the FSH-R. This phosphorylation event occurred when eIF4G was bound to the mRNA 5' cap, and probably involves mammalian target of rapamycin. This regulation might contribute to cap-dependent translation in response to FSH. The cap-binding protein eIF4E also had its phosphorylation level enhanced upon FSH stimulation. We also show that FSH-induced signaling not only led to cap-dependent translation but also to internal ribosome entry site (IRES)-dependent translation of some mRNA. These data add detailed information on the molecular bases underlying the regulation of selective mRNA translation by a GPCR, and a topological model recapitulating these mechanisms is proposed.

Key Words

- ▶ GPCR
- ▶ FSH
- ▶ mRNA translation
- ▶ eIF4G
- ▶ IRES
- ▶ HEK293 cells

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Introduction

The regulation of protein synthesis at the level of translation is critical for cell growth and development (Proud 2007). Beside maintenance of cell homeostasis, changes in the rate of mRNA translation arise when environmental conditions vary, so that the cell can fine-tune its protein content by both increasing the rate of overall translation and/or of only a subset of selective,

adaptative mRNA. The signaling mechanisms that control the translational machinery involve changes in the phosphorylation of translation initiation and elongation factors as well as protein/protein interactions/dissociations occurring at the mRNA 5' cap. Several of these regulatory steps depend on the mammalian target of rapamycin (mTOR) C1 complex, including activation of the

ribosomal protein (rp) S6 kinases (S6K), which phosphorylate rpS6 and eukaryotic initiation factor 4B (eIF4B) (Cobb 1986, Tabarini *et al.* 1987, Jeno *et al.* 1988, Raught *et al.* 2004), among other proteins. Importantly, activation of mTOR leads to sequential hyperphosphorylation of the eIF4E-binding protein (4E-BP) and its release from eIF4E, allowing eIF4E to bind the scaffolding protein eIF4G that assembles active pre-initiation complexes (Brunn *et al.* 1997). Hence, the interaction of eIF4G and 4E-BP with eIF4E is mutually exclusive (Haghighat *et al.* 1995). eIF4G scaffold proteins such as eIF4A, an RNA helicase which unwinds secondary structures in mRNA 5'-UTRs that may otherwise impede scanning of the initiator codon by the 43S pre-initiation complex (Prevot *et al.* 2003). Phosphorylation of eIF4G by unidentified, mTOR-dependent kinases facilitates its activity (Raught *et al.* 2000).

G protein-coupled receptors (GPCRs) are the largest class of integral membrane receptors that transmit information from the extracellular environment inward. A hallmark of their cognate ligands is their diversity: they include glycoprotein hormones, chemokines, peptide neurotransmitters, and ions, as well as sensory signals such as light, odorants, or taste ligands. As can be guessed from this cornucopia of ligands, GPCRs are involved in most physiological processes, which explains why they are among the top biochemical targets of currently marketed pharmaceuticals (Drews 2000). In the last decade, it has been extensively reported that these receptors signal not only through G proteins but also through adaptor proteins, namely β -arrestins (Reiter & Lefkowitz 2006). Grossly, signaling events initiated by G proteins are assumed to regulate gene expression transcriptionally, whereas β -arrestins restrain some signaling pathways, such as the ERKs and MAPKs, inside the cytosol (DeFea *et al.* 2000, Reiter & Lefkowitz 2006). Presumably, some of these extra-nuclear signaling pathways could have an effect on the translational machinery (DeWire *et al.* 2008). The molecular mechanisms that regulate translation have been widely documented for tyrosine kinase receptors, but they are less understood for GPCRs. Several reports have proposed that the signaling mechanisms leading to translational regulation by both classes of receptors might be partly different, presumably leading to specific ultimate cell responses (Rozengurt 2007, Musnier *et al.* 2010).

The pituitary glycoprotein hormones interact with class A GPCR to regulate the growth, proliferation, differentiation, and survival of specialized target cells of the gonads (luteinizing hormone and follicle-stimulating hormone (FSH)) and of the thyroid (thyroid-stimulating hormone). As regulators of cell differentiation, they

control the local production of hormones and nutrients. For example, the FSH receptor (FSH-R), expressed in somatic cells of the gonads, indirectly controls male germ cell differentiation processes throughout life (Orth *et al.* 1988) and drives the ovarian follicle to the preovulatory stage in females (Richards *et al.* 1976). Once bound to their cognate receptors, glycoprotein hormones activate adenylyl cyclase to produce cAMP (Heindel *et al.* 1975). In Sertoli cells of the male gonad, we have reported that a subtle interplay between cAMP- and PtdIns-dependent signaling was required for FSH to stimulate p70S6K. A stimulatory effect of FSH on several proteins of the mTOR pathway, such as p70S6K, has been reported a while ago, in granulosa cells (Alam *et al.* 2004, Kayampilly & Menon 2007), as well as in Sertoli cells (Lécureuil *et al.* 2005, Musnier *et al.* 2009). However, the involvement of FSH in translation has been conclusively demonstrated only recently, in Sertoli cells, where our group has reported that FSH enhances the eIF4F assembly leading to the recruitment of selective mRNA to the polysomes (Musnier *et al.* 2012). In FSH-stimulated cells eIF4G is recruited to the mRNA 5' cap but unexpectedly, we failed to detect any dissociation of 4EBP, in contrast with the classical view.

Our group has observed that several properties of the FSH-induced signaling, such as cAMP production, ERK response (Kara *et al.* 2006, Wehbi *et al.* 2010), or p70S6K activation (León K, Boulo T, Musnier A, Gauthier C, Dupuy L, Poupon A, Reiter E, Crepieux P, 2014, unpublished observations) can be faithfully reproduced in HEK293 cells stably expressing the FSHR. Herein, our aim was to obtain mechanistic details of the control of mRNA translation by FSH in this generic cell model, and to address whether the regulation of the translational machinery is an intrinsic property of FSH-induced signaling or depends on the cell context.

Subjects and methods

Cells

The HEK293 cell line stably expressing the mouse FSH-R (HEK293-FSHR; 600 fmol/mg protein), as established previously (Wehbi *et al.* 2010), was grown in minimum essential medium (Klionsky *et al.* 2012) complemented with 10% heat-inactivated fetal bovine serum, 10 U/ml penicillin, 10 μ g/ml streptomycin, all purchased from PAA Laboratories GmbH (Pasching, Austria), and with 400 μ g/ml geneticin (Gibco Life Technologies, Inc., Invivogen Corporation, Paisley, UK). Before stimulation

with porcine FSH (100 ng/ml, unless otherwise mentioned), the cells were serum-starved overnight in MEM with 10 U/ml penicillin, 10 µg/ml streptomycin, 10% BSA, from Sigma–Aldrich, and 400 µg/ml geneticin and 1 M HEPES (PAA Laboratories GmbH). In some experiments, the cells were pre-incubated with 10 ng/ml rapamycin (Cell Signaling Technology, Inc., Beverly, MA, USA) for 1 h, before FSH stimulation.

Measurement of protein synthesis by ³⁵S-labeling

HEK293–FSHR cells were seeded at a density of 1250 cells/mm² the day before labeling and FSH stimulation. Each condition was assayed in triplicate. Then the cells were preincubated in MEM without methionine and cysteine (Sigma–Aldrich) for 30 min, before the addition of 25 µCi/ml L-[³⁵S]methionine/L-[³⁵S]cysteine (Perkin Elmer, Waltham, MA, USA) and 100 ng/ml FSH for 1.5 h. The cells were then washed three times with ice-cold PBS and lysed with TNET extraction buffer (20 mM Tris, pH 7.8, 50 mM NaCl, 5 mM EGTA, 1% v/v Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 4 mM Na₃VO₄, 5 µg/ml leupeptin, 5 µg/ml pepstatin, and 5 µg/ml aprotinin). One aliquot of each sample was precipitated with 10% (w/v) trichloroacetic acid on 3 MM GF/C glass microfiber filters (Whatman, GE Healthcare UK Ltd, Buckinghamshire, UK) and precipitated radiolabeled proteins were quantified by scintillation counting using a Packard Tri-Carb 1600 counter. An equivalent aliquot of each sample was directly placed into the scintillation liquid to quantify the total incorporation of L-[³⁵S]methionine/L-[³⁵S]cysteine within cells.

Luciferase activity analysis

HEK293–FSHR cells were transfected with 5 µg of plasmid in 21 µl of Fugene reagent in 8 cm² dishes. A plasmid encoding a bicistronic mRNA that includes the vascular endothelial growth factor A (VEGF-A) IRES_B has been reported by Huez *et al.* (1998). Forty-eight hours after transfection, the cells were split into 24-well plates, serum-starved for 2 h, and then stimulated with increasing concentrations of FSH for 24 h. Each condition was assayed in triplicate. The cells were then lysed in 100 µl of passive lysis buffer provided by the manufacturer, and the luminescence was quantitated in cell extracts following the manufacturer's guidelines (Dual luciferase Assay, Promega) using a Luminoskan Ascent 185 (Thermo Labsystems, Waltham, MA, USA) to estimate Renilla and firefly luciferase activities. The IRES activity was

determined by calculating the firefly luciferase:Renilla luciferase ratio, and its converse.

Western blot analysis

Cell lysates corresponding to an equal number of cells were resolved by 10 or 15% acrylamide SDS–PAGE, electrophoretically transferred to a nitrocellulose membrane (Whatman, Dassel, Germany) and probed with the appropriate primary antibody, generally diluted 1:1000. Rabbit polyclonal antibodies raised against phospho-eIF4E (S209), phospho-eIF4G (S1108), phospho-4E-BP1 (S65), (T70), (T37/46), and pan-4E-BP1 were purchased from Cell Signaling Technology, Inc. The mouse MAB raised against eIF4E was from BD Biosciences Pharmingen (San Jose, CA, USA). Rabbit polyclonal anti-eIF4G antibody was from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). HRP-coupled anti-rabbit and anti-mouse antibodies were purchased from GE Healthcare UK Ltd and diluted 1:3000 or 1:5000, respectively, to detect antigen–antibody interactions by West Pico chemiluminescence (Thermo Scientific Life Science, Rockford, IL, USA). The membranes were reprobed with an anti-GAPDH (Santa Cruz Biotechnology, Inc), or an anti-vinculin (Sigma–Aldrich) antibody, to monitor gel loading. All the films were scanned and the optical density of the signals was measured with the ImageMaster 1D Elite version 4 Software (Amersham Biosciences).

7-Methyl-guanosine pull-down assay

Twenty millions cells at 50–60% confluency were rinsed once with PBS and then resuspended in 300 µl of 2× lysis buffer (40 mM HEPES, pH 7.4, 0.2 mM Na₃VO₄, 100 mM NaCl, 0.4 mM EDTA, 20 mg/ml aprotinin, 20 mg/ml leupeptin, 1 mM Pefablock AEBSF, 2 mM dithiothreitol, 100 mM β-glycerophosphate, 20 mM pyrophosphate, and 100 mM NaF, all from Sigma Chemical Co.) and gently sonicated on ice. Cell debris was discarded by centrifugation for 15 min at 13 000 g at 4 °C and proteins were quantified in the supernatants by a Bradford assay (Bio-Rad). For each sample, 50 µl of 7-methyl-guanosine (m⁷GTP)-coupled Sepharose beads (GE Healthcare UK Ltd) were equilibrated twice in 1× lysis buffer and were saturated with 10% BSA. Then 100 µg of cell lysate were added to the beads in 500 µl 2× buffer, and the samples were tumbled for 1.5 h at 4 °C. After three washes in 1× lysis buffer complemented to 100 mM NaCl, samples were boiled in 50 µl of 2× Laemmli sample buffer and analyzed by western blotting.

Network graphical representation

CellDesigner is a structured diagram editor for modeling biochemical networks. Networks are drawn based on the graphical notation system (SBGN) proposed by Kitano *et al.* (2005). Models are stored using the Systems Biology Markup Language (SBML), a standard for representing models of biochemical and gene-regulatory networks. For the sake of clarity, the molecular species involved are represented as monomers only, the reactions are drawn as irreversible, although this does not reflect reality.

Statistical analysis

Data were analyzed using the GraphPad Prism Software (San Diego, CA, USA). The results shown are representative of at least three independent experiments, and are expressed as mean \pm s.e.m. One-way ANOVA was used to compare different samples with a control within a single group, or two-way ANOVA was used for multiple comparisons. ANOVA was followed by a Bonferroni's post-test. Differences among means were considered as significant at $P < 0.05$.

Results

FSH enhances cap-dependent translation in HEK293-FSHR cells

To investigate the influence of the FSH signaling pathway on the regulation of the translational machinery, we used a HEK293 cell line expressing the mouse FSH-R, the HEK293-FSHR cell line that we previously described (Wehbi *et al.* 2010). In agreement with our previous observations of Sertoli cells (Musnier *et al.* 2012), FSH increased protein neosynthesis in HEK293-FSHR cells (Supplementary Figure 1, see section on supplementary data given at the end of this article), only when cells were seeded at high density. On this basis, HEK293-FSHR cells appear to be an appropriate model to analyze the control of translation by FSH in details. Experiments were carried out at a cell density higher than 1250 HEK293-FSHR cells per mm^2 , with relatively short duration of FSH stimulation (90 min) in order to minimize regulatory effects at the transcriptional level. This procedure was chosen over actinomycin D treatment whose drawback is that it dampens the transcription of translational factors, such as eIF4E (Raught & Gingras 1999). In addition, with the exception of immediate early genes, in the mouse, the peak of transcriptional regulation by FSH occurs at 4 h (Sadate-Ngatchou *et al.* 2004).

As FSH stimulation enhanced overall levels of protein synthesis, we investigated whether this effect was due to an effect of FSH-induced signaling on cap-dependent translation, by assaying luciferase activity in cells transfected with a plasmid encoding a bicistronic mRNA. Expression of the first cistron, Renilla luciferase (rluc), is cap-dependent. The FSH stimulatory effect on cap-driven translation was significant and increased in a dose-dependent manner (Fig. 1). These observations indicate that FSH's stimulatory effect on protein neosynthesis could result, at least in part, from cap-dependent translation.

Uncoupling of 4E-BP1 phosphorylation and 4E-BP1 release from the 5' cap, in response to FSH

The eIF4E protein is made available by its dissociation from its inhibitor 4E-BP1. Sequential phosphorylation events of the latter (Brunn *et al.* 1997) culminate with phosphorylation of Ser65 before 4E-BP1/eIF4E dissociation (Gingras *et al.* 2001a). As expected, when the 5' cap drives mRNA translation, FSH significantly increased 4E-BP1 phosphorylation, first on Thr70 (Supplementary Figure 2A and B, see section on supplementary data given at the end of this article) and then on Thr37/46 (Supplementary Figure 2C and D), although the fold-changes were relatively tiny. Surprisingly, Ser65 was transiently dephosphorylated (Supplementary Figure 2E and F). However, $m^7\text{GTP}$ pull-down experiments carried out with extracts from cells

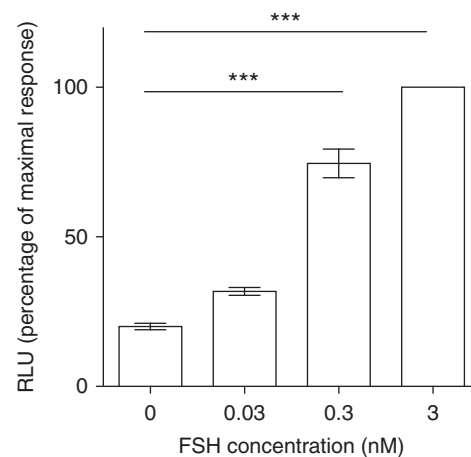
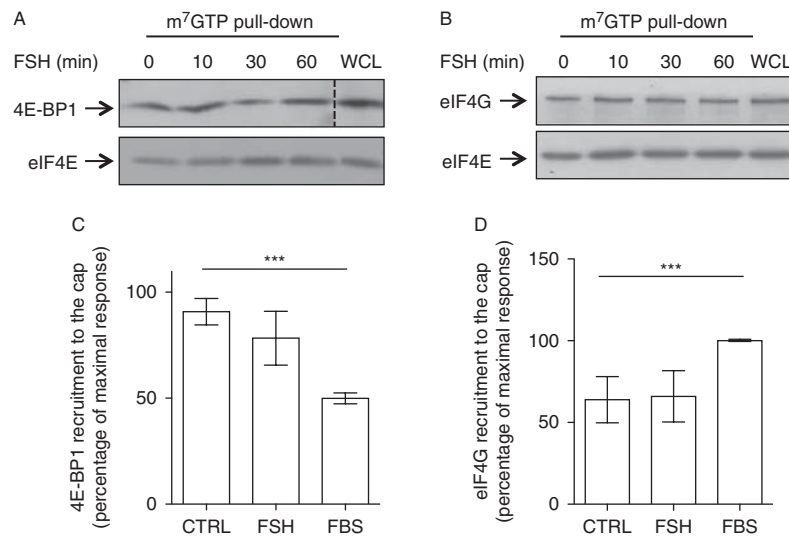


Figure 1 FSH enhances cap-dependent translation. HEK293-FSHR cells were transfected with bicistronic expression vector plasmids expressing cap-dependent Renilla luciferase. Forty-eight hours after transfection, cells were serum-starved for 2 h, prior to stimulation for 24 h with increasing concentrations of FSH, as indicated. Results are expressed as relative light units (RLU) of cap-driven luciferase activity. Values represent the mean \pm s.e.m. of three independent experiments, each done in triplicate. *** $P < 0.001$.

**Figure 2**

Cap-binding proteins in FSH-stimulated cells. (A and B) Cap-binding proteins were precipitated using m⁷GTP-Sepharose-coated beads and analyzed by western blot to detect 4E-BP1 and eIF4G association, as indicated. WCL, whole cell lysate. Western blots were reprobed with an anti-eIF4E antibody to detect eIF4E constitutively bound to the cap as

stimulated with FSH showed that 4E-BP1 was retained on the 5' cap (Fig. 2A). Hence, regardless of its phosphorylation status upon FSH stimulation, 4E-BP1 remains associated with the cap in HEK293-FSHR cells.

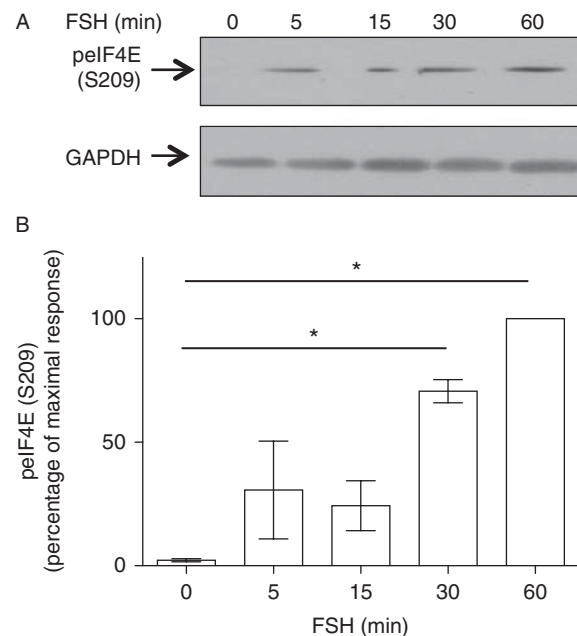
From these results, we anticipated that binding of 4E-BP1 to the cap-binding protein eIF4E would preclude further recruitment of other components of the translation initiation complex, such as eIF4G which competes with 4E-BP1 for a common docking site on eIF4E (Haghighat *et al.* 1995). This assumption was verified by cap-column affinity, since eIF4G appeared to be constitutively bound to the cap and was not further recruited upon FSH stimulation (Fig. 2B).

In contrast, in agreement with widely reported data, fetal bovine serum (FBS) stimulation led to both 4E-BP1 dissociation (Fig. 2C and Supplementary Figure 3A, see section on supplementary data given at the end of this article) and eIF4G recruitment to the 5' cap (Fig. 2D and Supplementary Figure 3B).

FSH input on the eIF4F complex

To gain insights into the mechanisms that could mediate FSH-regulated translation, we first analyzed eIF4E status. eIF4E was constitutively resident on the 5' cap (Fig. 2A and B), and its phosphorylation was enhanced upon hormonal stimulation (Fig. 3A and B).

an input control. Results for one representative experiment out of five are shown. In (C) and (D), respectively 4E-BP1 ($n=4$) and eIF4G ($n=3$) recruitment to the 5' cap was compared in FSH-stimulated vs 20% FBS-stimulated cells. One hundred percent was not reached in case the timepoint of the maximum response varied. *** $P<0.001$.

**Figure 3**

FSH increases eIF4E phosphorylation. Cells were treated with FSH for the indicated period of time before lysis. (A) Phosphorylation of eIF4E on Ser209 was analyzed by western blot. Membranes were reprobed with an anti-GAPDH antibody to monitor gel loading. (B) Densitometry analysis of western blots probed with antibodies raised against eIF4E phosphorylation on Ser209 ($n=3$). Values are expressed as mean \pm s.e.m. * $P<0.05$.

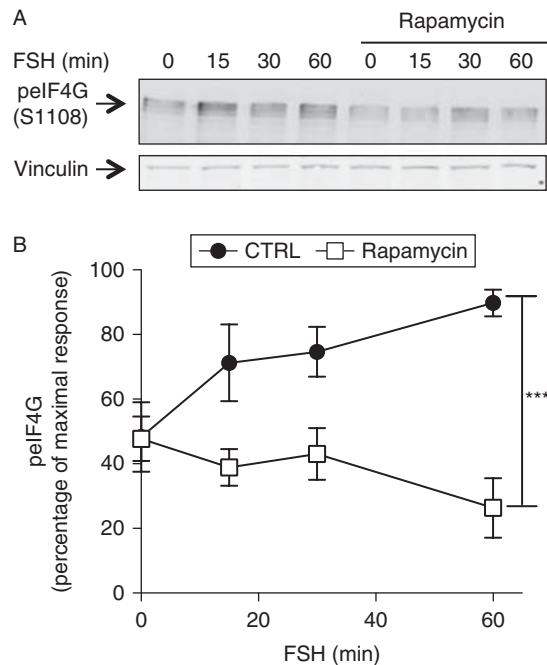


Figure 4 eIF4G phosphorylation is a rapamycin-sensitive step in FSH-regulated translation. (A) Western blot analysis of eIF4G phosphorylation on Ser1108, in HEK293-RFSH cells pre-treated or not with 10 nM rapamycin before FSH stimulation for the indicated period of time. Membranes were re-probed with an anti-vinculin antibody to monitor gel loading. (B) Densitometry analysis of western blots probed with antibodies raised against eIF4G phosphorylation on Ser1108 ($n=6$). Values are expressed as mean \pm S.E.M. One hundred percent was not reached in case the timepoint of the maximum response varied. *** $P<0.001$.

Next, in an attempt to reconcile the fact that FSH enhances cap-dependent translation (Fig. 1) but without enhancing the recruitment of eIF4G to the 5' cap (Fig. 2B), we explored eIF4G phosphorylation status in response to FSH. As shown in Fig. 4A and B, FSH stimulation led to a significant level of eIF4G phosphorylation. Interestingly, eIF4G phosphorylation was dramatically inhibited by rapamycin, an immunosuppressant microlide that binds to the cytosolic FK506-binding protein (FKBP12) to preclude mTOR and raptor interaction within the mTORC1 complex (Oshiro *et al.* 2004), indicating that mTOR could be involved in the regulation of eIF4G. Notably, our preliminary results indicate that FSH stimulation leads to the recruitment of mTOR to the cap (not shown). Cap-column experiments indicated that eIF4G phosphorylation actually occurred at the 5' cap, and was prevented by rapamycin treatment of cells (Fig. 5A and B). In contrast, constitutive eIF4G interaction with the 5' cap was not hampered by rapamycin, indicating that eIF4G is constitutively bound

to the 5' cap in HEK293-FSHR cells and that mTOR-dependent signaling leads to its FSH-dependent phosphorylation *in situ*.

FSH also enhances IRES-dependent translation of selective mRNA

As rapamycin hampered most (66 vs 49% in control conditions), but not all FSH-driven, protein neosynthesis (Supplementary Figure 4, see section on supplementary data given at the end of this article), we assumed that the rapamycin-resistant component was related to IRES-dependent translation that is not supposed to be regulated by mTOR. Several FSH-responsive genes encode mRNAs containing IRES in their 5'-UTR, such as c-myc, VEGF, or fibroblast growth factor 2 (FGF2). One of these natural FSH target genes in Sertoli as well as in granulosa cells encodes the VEGF-A mRNA (Sasson *et al.* 2003), whose 5'-UTR includes an IRES. Translation of the VEGF-A mRNA is initiated from this IRES exclusively, whereas cap-dependent translation is not involved (Huez *et al.* 1998).

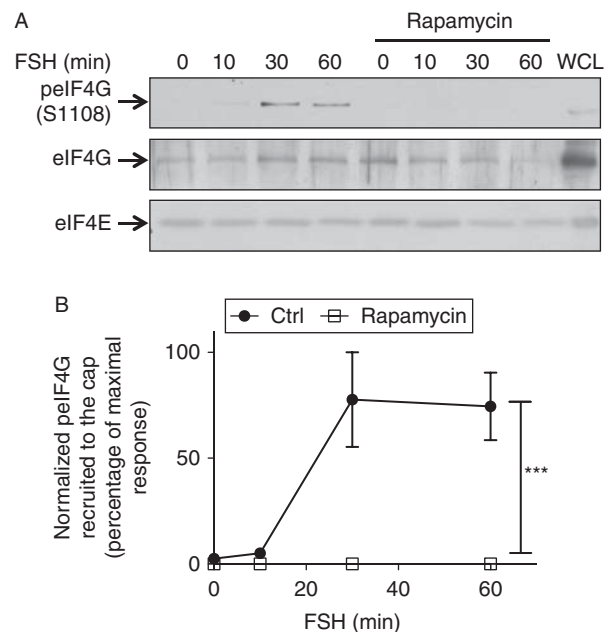


Figure 5 eIF4G is phosphorylated at the 5' cap, in a rapamycin-sensitive manner, upon FSH stimulation. (A) eIF4G phosphorylated on Ser1108 was precipitated using m⁷GTP-Sepharose-coated beads and analyzed by western blot, in a time-course experiment for FSH stimulation, as indicated. WCL, whole-cell lysate. Western blots were re-probed with anti-eIF4G, to normalize for eIF4G bound to the 5' cap, and with an anti-eIF4E antibody to detect eIF4E as an input control. (B) Densitometry analysis of western blots probed with antibodies raised against phosphorylated eIF4G ($n=3$). Values are expressed as mean \pm S.E.M. *** $P<0.001$.

Hence, HEK293–FSHR cells were transfected with a plasmid encoding a bicistronic mRNA, including the VEGF-A IRES_B-driven luciferase. FSH significantly enhanced the luciferase expression driven by the VEGF-A IRES_B in a dose-responsive manner (Fig. 6A). As expected from our above-mentioned data, 3 nM FSH also enhanced cap-dependent translation (Supplementary Figure 5, see section on supplementary data given at the end of this article). However, VEGF-A IRES_B-driven translation increased with a sharper slope than cap-dependent translation, as illustrated when the data were expressed as the ratio of IRES- vs cap-dependent translation (Fig. 6B). This stimulatory effect seemed to be IRES-specific because it was not observed with the FGF2 IRES (Fig. 6C), included in the bicistronic vector used in experiments shown in Fig. 1.

Discussion

This work brings detailed insights into the mechanisms involved in GPCR-dependent control of translation, and indicates that cap-dependent translation could occur even in the absence of exchange between 4E-BP1 and eIF4G as eIF4E binding partners. Previously, FSH has been shown to regulate selective mRNA translation in its natural target cells, Sertoli cells, which constitute the seminiferous tubules (Musnier *et al.* 2012). However, the mechanisms involved remained elusive, because eIF4G was recruited to the 5' cap upon FSH stimulation, whereas 4E-BP1 appeared to remain associated. Herein, in a convenient cell model, namely HEK293 cells stably expressing the FSHR, eIF4G appeared to be regulated by mTOR-mediated phosphorylation, once bound at the 5' cap. Previously, rapamycin-sensitive eIF4G phosphorylation has been shown to be involved in cap-dependant translation (Raught *et al.* 2000). Inducible phosphorylation of eIF4G on Ser1108 has been correlated with enhanced translation efficacy, and rapamycin-sensitivity has been observed in response to anabolic factors such as insulin-like growth factor 1 or insulin, acting through a tyrosine kinase receptor. Also, the GPCR for gonadotropin-releasing hormone mediates eIF4G phosphorylation in LβT2 cell lysates (Nguyen *et al.* 2004). But to our knowledge, rapamycin-sensitive eIF4G phosphorylation occurring at the 5' cap, in response to a ligand-bound GPCR, has never been reported before. Consistently, protein neosynthesis induced by FSH stimulation in HEK293–FSHR cells, as well as in Sertoli cells (Musnier *et al.* 2012), was sensitive to rapamycin, involving mTOR in this process. However, mTOR may not be the kinase that directly phosphorylates eIF4G, as indicated by the recent identification of

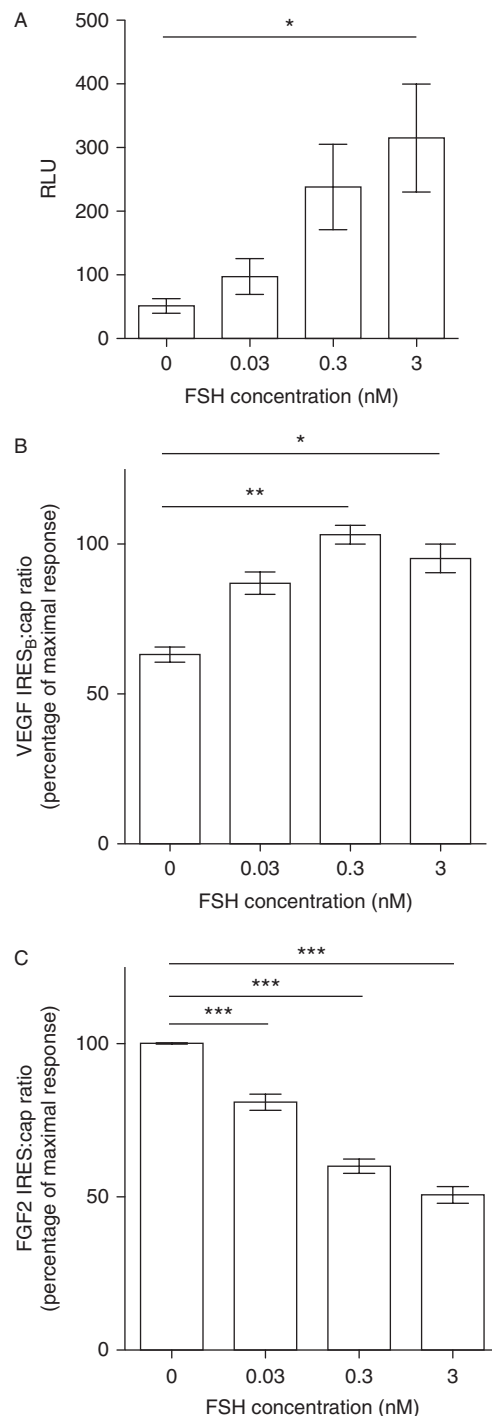


Figure 6

FSH also enhances IRES-dependent translation. HEK293–FSHR cells were transfected with plasmids expressing cap-dependent Renilla luciferase and VEGF IRES_B-dependent firefly luciferase. Forty-eight hours after transfection, cells were serum-starved for 2 h, prior to stimulation for 24 h with increasing concentrations of FSH. (A) Results are expressed as RLU. (B and C) Comparison of the ratio of VEGF IRES_B:cap-driven luciferase activity (B) vs the FGF2 IRES:cap ratio (C). Values are expressed as mean \pm s.e.m. ($n=3$). * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

rapamycin-sensitive enzymes of the SR-specific protein kinase (SRPK) family as eIF4G kinases, which have Ser1108 as a substrate (Hu *et al.* 2012).

Another prominent target of mTORC1 is 4E-BP1, whose phosphorylation is required for cap-dependent translation (Thoreen 2013). In this study, we observed phosphorylation events on Thr37/46 of 4E-BP1, which are mTOR targets but are not expected to lead to dissociation from eIF4E (Gingras *et al.* 1999). Rather, phosphorylation of these residues is supposed to prime the subsequent phosphorylation of serum-responsive sites such as Thr70 and Ser65, which are also more sensitive to rapamycin (Gingras *et al.* 2001b). Herein, we observed that FSH enhances phosphorylation of Thr70 but not of Ser65 that

was transiently dephosphorylated for an unclear reason. Phosphorylation of 4E-BP1 has been proven to be instrumental in driving its release from eIF4E (Kimball & Jefferson 2010), which is not observed here. Maybe the relatively small changes in phosphorylation of these regulatory sites might be responsible for the lack of dissociation of 4E-BP1 from the 5' cap that is observed here. To reconcile the detection of cap-dependent translation with the fact that 4E-BP1 was not dissociated from the 5' cap upon FSH stimulation, whereas eIF4G was not recruited, we postulate that the isoforms of eIF4E binding either to eIF4G or to 4EBP1 could be distinct, as already reported with *in vitro* translated proteins (Joshi *et al.* 2004; Fig. 7). In support of this assumption, our

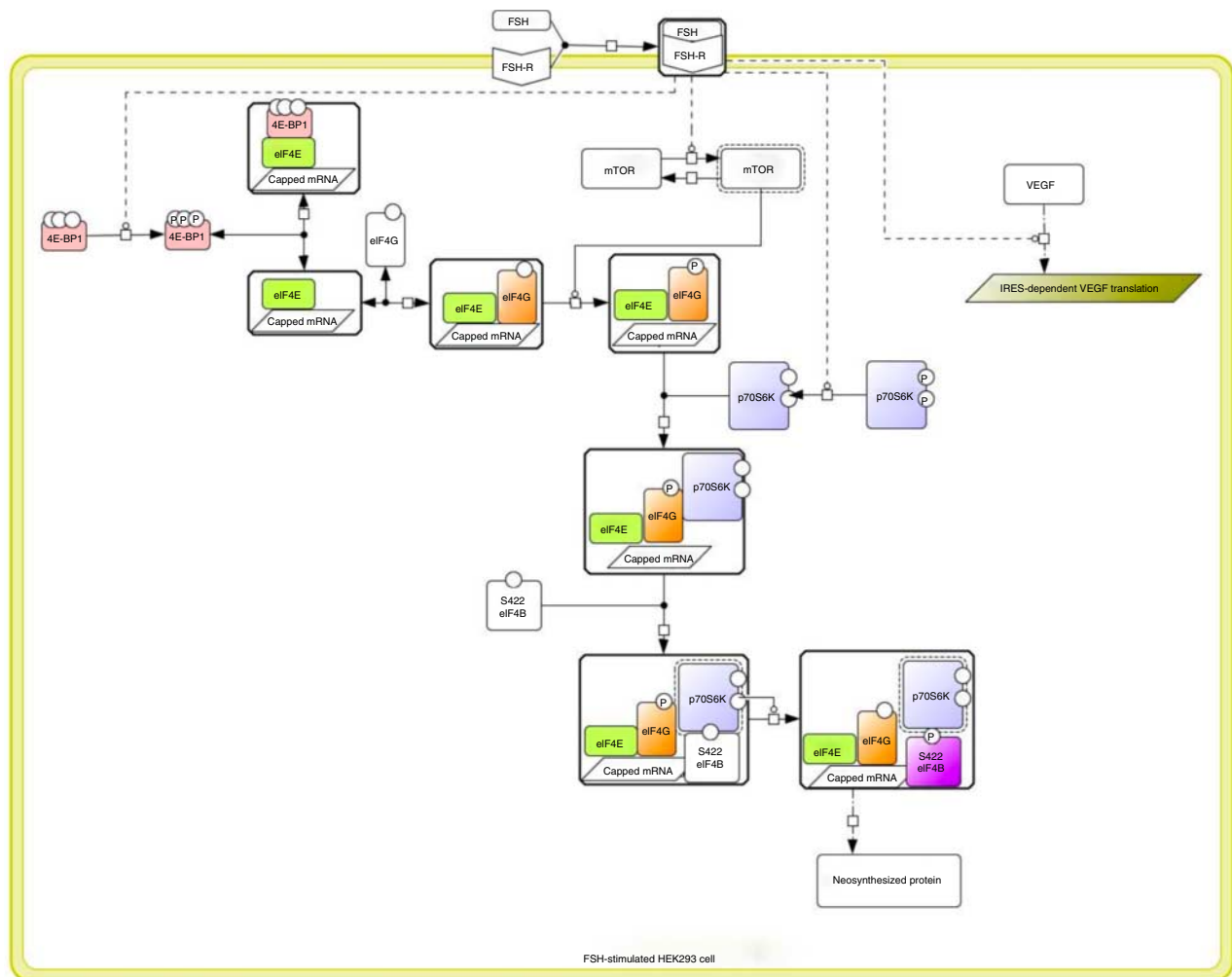


Figure 7

Molecular network triggered by FSH to control translation in HEK293–FSHR cells. The Cell Designer editor has been used. Only the molecular species analyzed in this study are shaded. Complexes are surrounded by black boxes. Dashed lines indicate indirect catalysis. The semantics of the diagram editor are as follows: protein, □; active protein, ◻; receptor, ◻; RNA, ≡; direct catalysis, →; phosphorylation, (P); association, >-; dissociation, <-; translation, →. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0009>.

preliminary data indicate that phosphorylated eIF4G preferentially interacts with the eIF4E3 isoform in the presence of FSH. Owing to the lack of one aromatic residue to sandwich the methyl-7-guanosine cap, eIF4E3 has been shown recently to exhibit unique interaction properties with the m7-GTP-cap (Osborne *et al.* 2013), indicating that it may compete with eIF4E1 for the same mRNA, but induce an opposite fate, such as tumor suppression.

Finally, considering the rapamycin sensitivity of the initiation complex components, the phosphorylation of eIF4E has recently been shown to regulate the translation of selective mRNA (Wendel *et al.* 2007), in an mTOR-dependent manner (Stead & Proud 2013). From our previous (Musnier *et al.* 2012) and present data, it is likely that FSH does not induce bulk general neosynthesis, as can be seen for example, in the hypertrophic response of cardiomyocytes stimulated by agonists of the α 1-adrenergic receptor (Wang & Proud 2002). Rather, FSH would selectively modulate translational regulation of a discrete subset of mRNA selectively. For example, the c-fos mRNA is selectively recruited to polysomes in FSH-stimulated Sertoli cells. Likewise, the VEGF_A mRNA is also recruited to polysomal fractions under these conditions. This mRNA appears to be exclusively regulated by IRES-dependent translation (Huez *et al.* 1998). Under these conditions, translational selectivity could also apply to IRES-containing mRNA because, as shown here, the FGF2 IRES is not regulated upon FSH stimulation.

In conclusion, in a generic cell model, new insights into the mechanisms whereby a GPCR controls translation have been gained. Rapamycin-sensitive eIF4G phosphorylation at the 5' cap may be a surrogate for the classical exchange between eIF4G and 4E-BP1.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0009>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

P Crepieux, E Reiter, J Morales, and P Cormier designed the project. P Crepieux, K León, and A Musnier wrote the manuscript. K León, T Boulo, A Musnier, C Gauthier, and L Dupuy did the experiments. A Poupon and J Morales proposed the theoretical model. S Heyne and R Backofen provided structural information on the VEGF IRES. All the authors discussed the data and commented on the manuscript.

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