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NATURAL OCCURRENCE AND PHYSIOLOGICAL ROLE OF A TRUNCATED EIF4E IN THE PORCINE ENDOMETRIUM DURING IMPLANTATION*

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Synopsis

This is the first report providing evidence for a physiological role of a truncated form of the mRNA-cap binding protein eIF4E1. Our initial observation was that eIF4E, which mediates the mRNA cap function by recruiting the eIF4F complex (composed of eIF4E, 4G and 4A), occurs in two forms in porcine endometrial tissue in a strictly temporally restricted fashion. The ubiquitous prototypical 25 kDa form was found in ovaryectomized and cyclic animals. A new stable 23 kDa variant, however is predominant during early pregnancy at time of implantation. Northern blotting, cDNA sequence analysis, *in vitro* protease assay and mass spectrometry showed that the 23 kDa form does not belong to a new class of eIF4E proteins. It represents a proteolytically processed variant of eIF4E1, lacking not more than 21 amino acids at the N-terminus. Steroid replacements indicated that progesterone in combination with estradiol 17β induces the formation of the 23 kDa eIF4E. Modified cell free translation systems mimicking the situation in the endometrium revealed that, besides eIF4E, eIF4G was also truncated, but not eIF4A or PABP. The 23 kDa form of eIF4E reduces the repressive function of 4E-BP1 and the truncated eIF4G lacks the PABP binding site. Thus, we suggest that the truncated eIF4E provides an alternative regulation mechanism by an altered dynamic of eIF4E/4E-BP1 binding under conditions where 4E-BP1 is hypophosphorylated. Together with the impaired eIF4G/PABP interaction, the modified translational initiation might particularly regulate protein synthesis during conceptus attachment at the time of implantation.

INTRODUCTION

Protein synthesis is a major determinant of cell growth and differentiation. In the context of conceptus implantation, maternal ovarian steroids and embryonic signals like estrogens [1, 2] were found to influence proliferation and differentiation of the endometrium by modulating those signaling cascades regulating mRNA translation. This view is supported by results showing that signal transduction pathways involving the mitogene activated protein kinases (MAPK) and the serine/threonine protein kinase AKT can be activated by estradiol-17β [3, 4] or progesterone [5]. Among other processes, these kinases modulate protein synthesis at the translational level [6-8]. The main targets are translational initiation factors (eIFs) such as the mRNA-cap binding protein eIF4E and its repressors 4E-BP1 and 2.

In eukaryotes, more than 95% of proteins are synthesized via cap-dependent translation. In this context, eIF4E plays a key role in the recruitment of mRNAs to ribosomes [9]. The first detected, prototypical eIF4E is found in all eukaryotes. It has an apparent molecular mass of approximately 25 kDa and might be the rate limiting factor in translational initiation [10, 11]. This process is influenced by the secondary and tertiary structure of the 5`untranslated region (5´ UTR) of the mRNA and by the cap structure (m^7GpppN) of the mRNA molecules. The functional cap binding complex eIF4F is composed of the mRNA-cap-binding protein eIF4E, eIF4A, an RNA helicase responsible for the unwinding of secondary structures of mRNAs, and eIF4G serving as a scaffold protein for the assembly of the complex [11]. eIF4F mediates the cap function during translation initiation. However, today the most sophisticated model of translational stimulation is the so called “closed loop” model [12, 13]. Simultaneous interactions between the 5´cap and the 3´poly(A)-sequence of the mRNA are mediated by the protein factors, whereby eIF4G provides binding sites for eIF4E, eIF4A, eIF3 (which bridges eIF4E to the small ribosomal subunit), for the poly(A)-binding protein (PABP), MNK, the kinase which directly

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phosphorylates eIF4E and also for RNA. This complex facilitates ribosome binding and stimulates translation efficiency 8-10 fold [14].

The biological activity of the cap binding protein eIF4E can be regulated at different levels: First, eIF4E is phosphorylated at Ser-209 in response to a variety of stimuli such as growth factors, cytokines or amino acids [15]. It was shown that eIF4E phosphorylation reduces its affinity to the cap structure. eIF4E released from the cap, can probably bind to a different subset of mRNAs and enhance the translation rate [16].

Secondly, eIF4E-binding proteins can work as repressors for the eIF4E function. Specific proteins called 4E-BP1, 2 and 3 were shown to act as competitors for eIF4E/4G binding. When hypophosphorylated they form complexes with eIF4E and prevent the formation of the functional eIF4F complex [17]. 4E-BP1 and 4E-BP2 have at least six potential phosphorylation sites, and hierarchical phosphorylation reduces their affinity to eIF4E as well as suppressing their inhibitory effect on the translational initiation [7, 18].

Thirdly, subclasses of eIF4E encoded by different genes (eIF4E classes 1-3) or splicing variants were detected in many organisms from different species [19, 20]. Generally, some eIF4E related proteins may act as translation factors and stimulate either global mRNA recruitment or the recruitment of a subset of mRNAs. Others may exhibit only partial activity and thus act as inhibitors of translation. However, the complexity of the biological function of the different members of the eIF4E family is not fully understood and remains to be investigated in detail, especially in the light of recently detected new eIF4E binding partners [20].

Proteolytic processing resulting in stable variants was never detected for eIF4E in a physiological context, in contrast to other initiation factors such as eIF4G [21] or PABP [14]. However, structural analyses with recombinant, N-terminally truncated mammalian eIF4E forms have been performed [22, 23]. The N-terminal region of eIF4E, from amino acids 1 up to Gln 40 appeared to form a unique, flexible, unstructured segment. It was suggested that this segment is dispensable for cap dependent translation. A truncation of 33 amino acids from the N-terminus yielded a protein binding to the cap structure with the same affinity as the full length eIF4E [22]. More recently however, a regulatory impact of the N-terminal segment was suggested [24]. Deletions in this area result in different binding of the repressors 4E-BP1 and 2. These alterations depend on the length of the deleted segment and on the interaction state of both molecules [23-25].

In respect to eIF4G binding, deletions of up to 20 amino acids from the N-terminus of eIF4E from yeast did not result in a reduced affinity to eIF4G compared to the wild type. In contrast, deletions of larger segments (up to 35 amino acids) result in a significantly reduced affinity [26].

In the present study, we analyzed the expression of eIF4E in the porcine endometrium during implantation. At this time, as a consequence of embryonic/maternal cross-talk, structural and functional alterations occur in the endometrial epithelium, allowing attachment of blastocysts and their further development [27]. Surprisingly, we found a naturally occurring proteolytically processed eIF4E variant. The truncated 23 kDa form persists as a stable variant in the porcine endometrium during implantation. Altogether, our results suggest that the proteolytic cleavage of eIF4E represents an alternative regulatory mechanism of translational initiation, which is distinct from the well known regulation through phosphorylation of eIFs. An altered dynamic of eIF4E/4E-BP1 binding, together with the also observed impaired eIF4G/PABP interaction, might be important to regulate endometrial protein synthesis, particularly during non invasive implantation in pigs.

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EXPERIMENTAL PROCEDURES

Materials and those parts of the methods used for RNA analysis, Northern blotting and cDNA analysis are provided in the supplement.

Animals, steroid replacements and unilateral pregnant uterine horn

For all the animal studies, ethical guidelines were followed and the research has been authorized by German authorities (VI-522a-7221.31-1-036/00).

Mature German Landrace gilts (*Sus scrofa domestica;* 8.5 months, 120-125 kg) were estrus and ovulation synchronized as described [28]. Gilts were hysterectomized on day 13 (n = 7) of the estrus cycle and on days 13 (n = 10), 15 (n = 15; including 5 animals used for the model unilateral pregnant uterine horn; see below) and 30 (n = 7) of pregnancy (gilts were bred by artificial insemination 24 and 38 h after hCG). Additionally, on day 10 of the estrus cycle, 24 gilts were ovariectomized (OVX). Steroid replacements with EB and P_4 were performed as described [28]. In further experiments, one uterine horn from each of five pigs was endoscopically closed with a staple line of titanium clips before insemination. This treatment results in pregnancy in one uterine horn only.

Preparation of endometrial tissue extracts

For analysis of initiation factors, 1.5 g of endometrial tissue was used and the homogenate was prepared in the presence of protease inhibitors as described [28], except that the EDTA concentration was 10 mM. Supernatants were used for immunoblots and protein-protein interaction assays. For *in vitro* protease assays, the tissue was extracted with a buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 7.2 without any protease inhibitors. Protein concentration was determined as described [29]. Granulosa cells, *in vitro* matured porcine oocytes and blastocysts were handled as described [30, 31]. Furthermore, tissues from other organs (muscle, heart, kidney, brain, ovary, spleen, and liver) were homogenized as described above.

Protein analysis

For the analysis of eIF4F components, PABP and 4E-BP1 and 4E-BP2 by Western blotting, the standard SDS-PAGE and transfer method were used [32]. The blots were developed with the ECL-Plus detection system and digitalized using the Camilla Camera System (raytest, Straubenhardt; Germany). The optical density of the bands was measured and evaluated by Aida software (raytest, Straubenhardt; Germany). Binding assays with the different eIF4E form to m^7-GTP Sepharose was performed as described [31]. For the *in vitro* protease assay 50 ng of N-terminally GST-tagged full length eIF4E (51 kDa) served as an external substrate to be cleaved by endometrial lysates (20 µl) collected after OVX and at day P15. Incubation was performed for 0, 30, 60 or 120 minutes at 37°C. In parallel assays, the samples were treated with 5 mM EDTA, or EGTA either supplemented or not with 10 mM $CaCl_2$. The reaction was stopped by adding triply concentrated SDS-PAGE sample buffer. Samples were analyzed by Western blotting and probed for eIF4E and GST respectively. For semi-preparative eIF4E cleavage and subsequent MS analysis, 1 µg of GST- eIF4E fusion protein was used as an external substrate. To avoid high contamination with endometrial proteins and to achieve maximal cleavage, the cleavage reaction was carried out sequentially by serial addition of OVX or P15 endometrial lysates, diluted 1:20, for 30 min at 37°C. The addition of diluted lysate was repeated 4 times. The cleavage reaction was monitored on Coomassie stained SDS gels and by Western blotting. For MS analysis, tryptic

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in-gel digestion was performed and the MALDI-TOF-TOF measurement was carried out on the 4800 MALDI-TOF-TOF Analyzer (Applied Biosystems, Foster City, CA, USA) as described [33]. For the data base search the Mascot search engine Version: 2.104 (Matrix Science Ltd, London, UK) with a specific NCBI sequence data base was used.

In vitro translation and protein-protein interaction analysis

For *in vitro* translation, nuclease-treated rabbit reticulocyte lysate (RRL) from Promega was used to synthesize biotinylated proteins. To evaluate the eIF4E dependency of translation or to mimick the situation in the endometrium, the RRL was modified as follows: In the first case, eIF4E was depleted from the RRL by m^7-GTP-Sepharose as described [34] and the RRL was subsequently supplemented either with recombinant eIF4E (0.75 µl/50 µl assay volume) or unphosphorylated His-tagged 4E-BP1 (0.75 µg/50 µl assay volume). In the second case, the complete RRL was subjected to limited proteolysis by supplementation with endometrial lysates obtained after OVX or from pregnancy day 15. Titration studies were carried out in advance to achieve optimal cleavage of eIF4E and 4G while avoiding exorbitant contamination of the RRLs with endometrial proteins. Lysates containing 1.5 µg protein were added to 50 µl RRL. In additional assays, the treated RRLs were supplemented with recombinant 4E-BP1 (1 µg/50 µl RRL). All treatments of the RRLs were monitored by Western blotting. The *in vitro* translation reactions were carried out in a final volume of 50 µl with 60% RRL, 140 mM K-acetate, 0.5 mM Mg-acetate, 10 mM ceratin phosphate, 1 mM amino acid mix and 1 µl transcend tRNA. The RNA was stabilized by addition of 1 µg of RNasin. The reaction was started by adding 50 ng of capped, polyadenylated Xef1 mRNA. Samples were taken at time 0, and after 15 and 30 min. Biotinylated Xef1 translation products were analyzed by Western blotting and visualized by Streptavidin-HRP detection as described [35]. Directly after the *in vitro* translation reaction, the RRLs were analyzed by immunoprecipitation with immobilized anti-eIF4E as described [32] and probed for eIF4E, eIF4G, eIF4A, PABP1 and 4E-BP1. To analyze the binding of the eIF4E variants to 4E-BP1, recombinant N-terminally His-tagged full length 4E-BP1 was bound to Ni-NTA magnetic agarose beads according to the manufactures instruction. His-tagged 4E-BP1 (15 µl slurry, 10 µg His-tagged 4E-BP1) bound to the beads was incubated with endometrial lysates after OVX and from P15 for 2 hours at 4°C under permanent agitation. As negative controls, lysates were incubated with the beads without His-tagged 4E-BP1 under the same conditions. The beads were washed three times with buffer containing 50 mM NaH_2P0_4 pH 8.0, 150 mM NaCl, 20 mM imidazole, 0.005% Tween 20. Proteins were eluted from the resin with a buffer of the same composition supplemented with 250 mM imidazole. Bound fractions and precipitated supernatants were analyzed by Western blotting and probed for eIF4E and His respectively.

RESULTS AND DISCUSSION

A 23 kDa eIF4E form is preferentially expressed in the endometrium during implantation

A key result of our study is the detection of a 23 kDa small size variant of the mRNA-cap binding protein eIF4E in the porcine endometrium during early pregnancy at the time of implantation.

Western Blot analysis for eIF4E (Fig 1A) of the endometrial cytosol of pigs after ovariectomy (OVX), on day 13 of the cycle (C13) and on pregnancy days 13, 15 and 30 (P13, 15, 30) revealed a single band with a molecular mass of 25 kDa in the endometrium after OVX. The same band was predominantly detected on C13. On P13 and P15 the 25 kDa band disappeared almost

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completely and a band of approximately 23 kDa emerged. Both bands appeared on P30. A similar pattern was obtained when a phospho-specific antibody was used. The abundance of the truncated eIF4E significantly increased on day 15 of pregnancy, visible in the bar chart diagram.

To demonstrate that the eIF4E cleavage is not an artifact of the extract preparation, samples after OVX and P15 were homogenized either separately as controls or after mixing both tissues. Clearly, extraction of the mixed samples did not reduce the abundance of the prototypical 25 kDa eIF4E, nor did additional cleaveage products appear (Fig. 1B). This indicates that the eIF4E truncation in P15 samples is not an extraction artifact.

Analysis of various other porcine organs (muscle, heart, kidney, brain, ovary, spleen, and liver) and cells (oocytes, blastocysts, fibroblasts, and granulosa cells) did not reveal a truncated form of eIF4E (Fig 1C). However, traces of smaller proteins were found in kidney and liver samples. They differ in size compared with the endometrial 23 kDa eIF4E form and could not be detected with the phospho-specific antibody. We conclude that these proteins are not identical with the endometrial 23 kDa eIF4E (see below) and they were not further investigated. Additionally, both the 23 and 25 kDa eIF4E could be precipitated by m^7-GTP Sepharose (Fig. 1D).

Further investigations revealed that in the endometrial lysates from P15, eIF4G was affected by extensive proteolytic cleavage generating discrete fragments (Fig. 1E). However, other factors like eIF4A and PABP were not cleaved. Additionally, this assay revealed a reduced abundance of 4E-BP1 on P15. Moreover, 4E-BP1 appeared in the α-band which was previously shown to be the hypophosphorylated form [31]. In contrast, abundance of 4E-BP2 was not significantly reduced on P15, but also a shift to higher electrophoretic mobility was observed. We speculate that 4E-BP2 is dephosphorylated on P15 as well. However, the phosporylation of this protein has not been as extensively studied, thus we can not exclude that the observed faster migration of 4E-BP2 results even from truncation. Nevertheless, these different events, namely eIF4G fragmentation, 4E-BP1 dephosphorylation and partial degradation and 4E-BP2 dephosphorylation/truncation suggest that these modifications are regulated processes specific for early pregnancy.

We envisage that the generation of the truncated eIF4E is a regulated process as well. eIF4E- and eIF4G fragments are stably expressed and not an intermediate product arising through general degradation. No signs of apoptosis or general proteolysis were observed in P15 samples. For example, no difference in active Caspase 3 after OVX or at P15 was detected. Moreover, cultures of endometrial luminal epithelial cells from P15 remained vital. However, the 23 kDa eIF4E gradually disappeared in these cells and was replaced by the 25 kDa form after at least 24 hours (data not shown).

The truncated eIF4E is a proteolytically processed product of the prototypical 25 kDa eIF4E1 protein

Northern blotting and cDNA sequence analysis showed no indication for the occurrence of an alternatively spliced mRNA or an alternative gene encoding the truncated factor (see supplementary Fig 1 and 2). Therefore, we analyzed if the 23 kDa eIF4E is generated from the 25 kDa form by proteolytic cleavage (Fig 2). The *in vitro* protease assay with a GST-eIF4E fusion protein (51 kDa) as an external substrate revealed that the cell lysate after OVX does not generate any cleaved eIF4E, even when Ca^{2+} is added. In contrast, the P15 lysate generates a distinct fragment of ~23 kDa (Fig 2A). Here, the amount of the recombinant eIF4E decreased with the duration of incubation, while the truncated form increased. This reflects the sum of the 23 kDa form present in the P15 lysate and the newly processed 23 kDa eIF4E generated from the

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recombinant external substrate. Additionally, the GST blot showed a ~28 kDa fragment which must be composed of the 26 kDa GST tag linked to an approximately 2 kDa N-terminal eIF4E fragment. The results also revealed that the eIF4E cleavage is strongly inhibited by EDTA which can be revoked by additionally applied $CaCl_2$, suggesting the action of a Ca^{2+} dependent protease. In an additional experiment, the more Ca^{2+} specific chelator EGTA was used to analyzed eIF4E cleavage (Fig 2B). The result showed an identical effect as obtained by EDTA treatment. The quantification of the GST blot is presented in Fig. 2C as bar charts.

In general, maximal cleavage was already reached after 30 min of incubation. The truncated eIF4E remained stable for at least 20 hours. No further degradation of the truncated eIF4E could be observed, even after that long duration of incubation (data not shown).

Lysates from kidney or liver cells, which also reveal traces of a fast migrating band (Fig. 1C), could not cleave the GST-tagged eIF4E (data not shown). These results support the notion that these proteins are not identical with the endometrial 23 kDa form of eIF4E.

Generation of discrete fragments through proteolytic cleavage has been reported to affect different initiation factors in distinct physiological situations [21]. For instance, eIF4G, eIF3S1 and eIF4B were shown to be targets of Caspase 3 in the preapoptotic phase. Additionally, PABP is degraded during apoptosis [14]. In this case, proteolytic cleavage generally reduces the overall net rate of protein synthesis. In contrast, proteolytic processing of eIF4E has to date not been described. Thus, we localized the cleavage site in the eIF4E molecule.

The 23 kDa eIF4E form lacks at most 21 amino acids N-proximal

It was not possible to enrich large amounts of truncated eIF4E from native samples. Therefore, in an alternative *in vitro* protease assay, we used a larger amount of the GST-eIF4E fusion protein to obtain Coomassie stainable fragments for a localization of the cleavage site. Edman degradation was not successful, probably through N-terminal blockage of the truncated eIF4E. We thus performed MS/MS analysis to identify the cleavage site. A Coomassie stained gel is shown in Fig. 3A. The external substrate (band 1) which is overlaid by endometrial proteins in the P15 and OVX lane and two specific bands (bands 2, 3) which only were found in protease assays from P15 lysates are marked. The identity of these bands was ensured by Western blotting (Fig. 3B). Band 2 represents GST probably linked to the N-terminal fragment of eIF4E and band 3 was the truncated eIF4E. These three bands were recovered and subjected to MS analysis. The results of the MALDI-TOF-TOF MS are summarized in Fig 3C. In the GST-eIF4E fusion protein, the GST cloning vector and 7 fragments of eIF4E were identified (line 1). Fragments 1-4 comprise entirely the N-terminus, up to amino acid 61. Band 2 was identified as the GST cloning vector. Unfortunately, an N-proximal eIF4E fragment was not found in this assay (line 2). However, the protein sequence of the truncated eIF4E (line 3), could be established and found as beginning with amino acid 22 (see supplementary Fig. 1). This result suggests that the truncated eIF4E lacks N-proximal 21 amino acids and the proteolytic cleavage site is located between the residues at position K_{21} and T_{22}. However, K_{21} is targeted by trypsin in the MS-analysis. Therefore, we can not exclude the possibility that the eIF4E specific protease cleaves 2-3 amino acids further N-terminally. Hence, so far, it is highly speculative to appoint a protease for eIF4E cleavage. For example, it is not clear if a specific sequence motif or rather tertiary structural elements are responsible to direct cleavage. Nevertheless, the observed Ca^{2+} dependency and cytosolic localization of the proteolytic action might argue for a tissue specific calpain related protein. However, the identification of the eIF4E specific protease awaits further investigations.

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EB, P_4 and embryonic signals stimulate the proteolytic processing of eIF4E

Next we intended to determine if embryonic signals and/or maternal steroid hormones are involved in the induction of the proteolytic cleavage of eIF4E. We failed to establish an endometrial cell culture system in which formation of the truncated eIF4E could be induced. We thus used an animal model for this purpose. The influence of embryonic signals on the expression of the 23 kDa eIF4E was determined in animals, where one uterine horn was endoscopically closed before insemination. On P15, the abundance of the 23 kDa eIF4E was high in the endometrium of the embryo harboring uterine horn (Fig. 4A). The uterine horn without embryos showed only traces of the truncated factor. This observation suggested that embryonic estrogens stimulate the expression of the eIF4E processing protease in the progesterone primed endometrium. Therefore we performed steroid replacement experiments. Ovariectomized animals were treated with EB, P_4 or with both hormones (Fig 4B). This approach revealed a single band with a molecular mass of 25 kDa in the endometrium after OVX, and predominantly also after EB- or P_4 treatment. However, traces of the truncated eIF4E were found in some of the animals treated with EB or P_4, probably reflecting the animal-specific hormonal status. In contrast, the consecutive substitution of both steroid compounds resulted in a strong expression of the truncated eIF4E. Furthermore, only lysates from the animals with double substitution significantly stimulated the cleavage of the 25 kDa eIF4E when incubated for 90 min. These results are supported by an additional experiment. Only in samples from animals having been substituted with both, P_4 and EB, could the formation of the truncated eIF4E be reduced by EDTA. This reduction was abolished when Ca^{2+} was added (Fig 4C). All other lysates did not significantly stimulate any eIF4E processing even if $CaCl_2$ was added.

In conclusion, our results indicate that at the time of implantation, when the concentration of P_4 is high [28], the generation of the truncated factor is induced by embryonic signals, probably embryonic estradiol-17β.

The truncated eIF4E reduces the repressive function of 4E-BP1 in vitro

We suggest that the truncated eIF4E may play an important physiological role at the time of implantation of the conceptus. Therefore, we developed a cell-free translation assay which mimicked the situation in P15 samples to examine the potential regulatory impact of the truncated eIF4E in the translation process.

To this end, an *in vitro* translation assay with nuclease treated RRL was performed. The abundance of eIF4G, eIF4A, eIF4E and of the recombinant eIF4E and 4E-BP1 in the complete and eIF4E-depleted RRLs is presented in Fig. 5A. The complete RRL did not contain endogenous 4E-BP1 or 4E-BP2. Furthermore, the abundance of eIF4G and eIF4A is not strongly influenced by our eIF4E depletion method. The *in vitro* translation assays performed with these lysates showed that the translation of the capped Xef1 mRNA was not stimulated in the eIF4E depleted and in the 4E-BP1 substituted RRL (Fig 5B). Therefore, translational stimulation was strictly eIF4E dependent in our assay.

Next we modified the RRL to achieve conditions comparable to the situation in the endometrium. Therefore, the RRL was treated with endometrial lysates from animals after OVX as a control and from P15 to obtain cleaved eIF4E and eIF4G factors. At time 0, truncated eIF4E or eIF4G were absent or only detected in traces (Fig. 5C). Thus, the RRL was not heavily contaminated with endometrial factors. As expected, after 60 min of treatment only truncated forms of eIF4E and eIF4G were observed in the P15 treated RRLs (Fig. 5C). Interestingly, eIF4G was cleaved into several fragments with molecular masses from approximately 100 to 160 kDa.

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However, eIF4A and PABP were not affected. OVX treatment did not modify any of these factors and OVX treated RRL stimulated translation comparable to the control with the complete, unmodified RRL (Fig. 5D vs. 5B). A weaker stimulation was observed in the treated RRL of P15. In contrast, OVX treatment with 4E-BP1 substitution did not stimulate translation at all. This inhibitory effect of 4E-BP1 was slightly but noticeably reduced in the RRL treated with endometrial lysate of P15. The quantification of these results shows (Fig. 5E) the eIF4E dependency of translation (left panel), the inhibitory effect of 4E-BP1 and the partial relief of 4E-BP1 inhibition in the P15 treated RRL (right panel).

We did not, however, observe a complete repression of translation in P15 treated RRL. Only a reduction (~40 %) of translation efficiency compared with the OVX treated samples was observed. This is in accordance with results showing that only RRLs containing endogenous competitor mRNA or which were partially depleted of ribosomes show a cap-poly(A) mediated synergetic effect, whereas in other cases this effect was only additive [36].

Truncated eIF4E and eIF4G forms alter the dynamic of eIF4E/4E-BP1 binding and impair eIF4G/PABP interaction

To analyze the formation of the eIF4F complex and PABP binding in the cell-free translation assay, immunoprecipitations with immobilized anti-eIF4E antibody were performed directly after the *in vitro* translation reaction. In this experiment, we compared the input and the precipitates with the corresponding supernatant in OVX, P15 and 4E-BP1 substituted RRLs (Fig. 6A). This approach confirmed the stable abundance of the truncated eIF4E and eIF4G in the P15 treated RRL. In the OVX treated control, eIF4F was distinctly formed, as proven by the fact that eIF4G, and eIF4A was highly present in the precipitates and only traces remained in the supernatant. Moreover, PABP bound to the complex. As expected, in the 4E-BP1 substituted OVX treated RRL, the formation of eIF4G and PABP binding was significantly reduced. A different observation was made in the P15 treated RRL. Here, eIF4F was formed, but PABP did not bind to the complex. This indicates that the truncated eIF4G had lost the N-terminal located PABP binding site. A different behavior was also observed in the 4E-BP1 substituted P15 assay. No significantly reduced eIF4F formation was observed in comparison to non substituted P15 RRL. This points to a restricted 4E-BP1 function. Indeed, we observed reduced binding of 4E-BP1 to the truncated eIF4E. Most of the 4E-BP1 molecules remained in the supernatant. Likewise, PABP binding was impaired in this assay.

To analyze the altered eIF4E/4E-BP1 interaction in more detail, we performed a protein interaction assay with recombinant His-tagged 4E-BP1 bound to Ni-NTA beads (Fig. 6B). This approach showed that, whereas the 25 kDa form of eIF4E binds to 4E-BP1 (43%±2,3), the binding of the truncated factor is remarkably reduced (14,6%±4,3). As a control, the blot was reprobed with a His-antibody to ensure equal 4E-BP1 binding to the Ni-NTA resin (Fig. 6B lower panel).

Regarding the regulatory function of eIF4E in translational initiation, it is known that binding of apo-eIF4E to the eIF4G enhances its affinity to the cap [37-39]. Moreover, cap binding enhances the affinity for eIF4G and 4E-BP1 and 2 [38, 40, 41]. eIF4G and its competitors 4E-BP1 and 2 bind to the dorsal surface of eIF4E, near the N-terminal region which is located distal to the cap binding pocket [42]. The N-terminal segment comprises Gln 40 and is a unique flexible domain which is strongly conserved between human, mouse, rat and rabbit [23]. Considering the regulatory impact of the N-proximal segment of eIF4E, interaction analysis with recombinant N-terminal truncated eIF4E (Δ33 residues) revealed that the binding dynamic eIF4E to 4E-BP1 and 2 depends on the interaction state [24]. Kinetic-state SPR analysis revealed an

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approximately 2-fold increase of the apparent association rate constant and a ~20-fold decrease of the dissociation rate constant when the truncated eIF4E was compared with the full length counterpart. In contrast, in steady-state fluorescence analysis, the association constant was found to decrease by the factor four. Hence Tomoo et al., [24] discuss positive and negative contributions of the N-terminal region in 4E-BP1 and 2 binding. They describe the N-terminal region of eIF4E as a repressor for the association/dissociation with 4E-BP1 and 2. It stabilizes the complex in an equilibrium state and blocks 4E-BP binding in a non-steady kinetic state. However, more recent SPR analysis by this group [25] revealed that the m^7GTP/Δ16 eIF4E binary complex has more or less the same binding affinity for 4E-BP1 and 2 compared with the full length eIF4E. In contrast, the complex with Δ26 eIF4E showed a reduced association rate of 4E-BP1 and 2, whereas with Δ33 eIF4E the dissociation rate was reduced. These results indicate that deletions between 16 and 26 amino acid residues reduce the affinity of the m^7GTP/eIF4E binary complex for 4E-BP1, which is in accordance with our observations.

Little information is available comparing the interaction of the full length factor with truncated forms of eIF4E and eIF4G. Studies on yeast eIF4E, which has a ~30 % identity to mammalian eIF4E, showed that the N-terminus of eIF4E is required for tight binding to eIF4G and maintaining a long term eIF4F complex. Deletion of 20 residues did not influence binding whereas longer deletions (30-35 residues) significantly reduced eIF4E binding to the eIF4G peptide [26, 38].

Regarding the scaffold protein eIF4G, binding sites for components of eIF4F and for PABP have been identified to be located in different domains [43]. In the human eIF4G factor the PABP and eIF4E binding sites are located in the N-terminal segment (PABP aa 172-200; eIF4E aa 557-646). The eIF4A binding sites were found in the central- and C-terminal regions (aa 712-916 and 1241-1356). We detected the N-terminally truncated forms of eIF4G in the P15 treated RRL. Our results additionally showed that, in contrast to OVX, PABP binding to eIF4G was impaired in RRL treated with P15 endometrial lysates. This indicates a decoupling of the PABP from the eIF4E binding site. That means the PABP site is not present in the N-terminal truncated eIF4G which can be precipitated with immobilized eIF4E antibody. The estimated apparent size of the stable C-terminal fragments of eIF4G was 100 to 160 kDa, but the sequence of the N-terminal segment of rabbit eIF4G is not well established. A data base search indicates that the N-terminus of the rabbit factor coincides with position 196 of the human eIF4G factor. However, we suggest that, in RRL, the N-terminal region of eIF4G is quite similar to the human counterpart. For instance, eIF4G in RRL was shown to have an apparent molecular mass of ~220 kDa [44] and our N-terminal eIF4G antibody maps around Gly 188, which is located within the PABP binding site. Additionally, eIF4G/PABP interaction has been described in unmodified RRL [45, 36].

Truncated eIF4E and eIF4G forms provide an alternative regulation mechanism of translational initiation during implantatiom

It was shown that either cap or poly(A) stimulate translational initiation *in vivo* alone or synergistically [46]. Thus, the truncated eIF4E and eIF4G described in our study allow three different scenarios of regulation of translational initiation in P15 samples. These are: (a) reduced inhibitory impact of 4E-BP1 and 2, (b) impaired eIF4G/PABP interaction and (c) altered interaction between the truncated eIF4E and 4G.

The different interactions of the factors are summarized in Fig 7. Depicted is a highly schematic representation of eIF4E and eIF4G with the presumed cleavage sites marked by arrows (Fig 7A). Although the investigations were performed with recombinant 4E-BP1, the results might also apply for 4E-BP2 due to its similar binding property [25, 41]. Formation of the eIF4F

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complex and PABP binding is dependent on 4E-BP1 abundance after OVX (Fig 7B), whereas such a pronounced effect of 4E-BP1 was not observed during the interaction of the truncated factors on P15. Moreover, PABP1 binding is impaired at this developmental stage. These different interactions have different impacts on translational initiation. On the one hand a general reduction might be suggested by impaired "closed loop" formation of the mRNA. On the other hand, initiation could be stimulated by a reduced efficacy of the repressor 4E-BP1 even in situations where this protein is hypophosporylated.

Given the tight spatial restriction of the occurrence of the truncated eIF4E only at the site of implantation, we propose that the truncated factor contributes to establish the non invasive placentation which is found in pigs. Such an assumption is supported by the fact that especially the truncated form of eIF4E was not found in ongoing pilot experiments in species with invasive placentation like the mouse, and these endometrial lysates did not cleave exogenous substrates. Furthermore, when porcine embryos are transferred to ectopic sites, the trophoblast becomes invasive [47, 48]. This results in erosion of the adjacent epithelium. Therefore, the truncation eIF4E and eIF4G might be crucial for the expression of factors favoring non-invasive over invasive implantation. Thus, we envisage that the truncated factors differentially regulate a subset of mRNA moieties, even under conditions of persistent hypophosphorylation of 4E-BP1 and 4E-BP2. Mechanistically, these mRNAs might share peculiar properties in their 5´ UTR and quite possibly their activation might not strictly depend on poly(A) [13, 49, 50].

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FOOTNOTES

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Abbreviations

EB, estradiol benzoate; eIF, eukariotic initiation factor; MALDI –TOF MS, matrix assisted laser desorption/ionisation-time of flight mass spectrometry; MS, mass spectrometry; OVX, ovary ectomy; P15, pregnancy day 15; P_4 progesterone; PABP, Poly(A)-binding protein; RRL, rabbit reticulocyte lysate; SPR, surface plasmon resonance

Figure Legends

FIGURE 1. **Expression of eIF4E in the porcine endometrium and various other organs and cells and analysis of eIF4G, eIF4A, PABP and 4E-BP1, 2 at P15.** (**A**) Expression and phosphorylation of eIF4E. Two biological replicates from endometrium after OVX, on day 13 of the estrus cycle (C13) and on pregnancy day 13, 15 and 30 (P13, P15, P30) are shown. The blots

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were reprobed for ACTB as a loading control. The bar charts show the 25 kDa eIF4E (black) and the 23 kDa form (white) in relation to the abundance of the loading control ACTB. (OVX: n = 6; C13: n = 7; P13: n = 10; P15: n = 15; P30: n = 7). Bars represent means ± SEM. * indicates significant difference (*P < 0.05)*. (**B**) Extracts from OVX and P15 tissue were homogenized in protease inhibitory conditions, alone or together (mix) as indicated. (**C**) The expression of eIF4E in various porcine tissues and cells. (**D**) The ability of both eIF4E forms to bind to m^7-GTP Sepharose. C is the control after OVX; the input (in) and the bound fraction (b) after OVX from cyclic gilts (C13) and from P13, P30 is depicted. (**E**) Analysis of the expression of eIF4G, eIF4A, PABP, 4E-BP1 and 4E-BP2 in the endometrium on P15 compared to the situation after OVX. (C-E) representative blots from three biological replicates are shown.

FIGURE 2. ***In vitro* protease assay with recombinant GST-eIF4E fusion protein as an external substrate in endometrial lysates after OVX and from P15.** (**A**) Endometrial extracts after OVX and from P15 and the external substrate was incubated for 0, 0.5, 1 and 2 hours, also in the presence of EDTA/$CaCl_2$ and EDTA alone as indicated. Western blots of the assays for eIF4E (upper panels) and GST (lower panels) after OVX (left) and from P15 (right) are depicted. Controls (C) were cell extracts from porcine liver (eIF4E blot after OVX) and GST-tagged eIF4E protein (GST blot after OVX); cell extracts from porcine liver (eIF4E blot on P15) and GST protein (GST blot on P15). (**B**) Comparison of the EDTA and EGTA effect on eIF4E-GST cleavage in endometrial P15 samples. Samples were substituted with EDTA, EGTA and $CaCl_2$ as indicated and incubated for 90 min or not incubate (0) as a control. Shown are the Western blots for eIF4E and GST. (**C**) Evaluation of the GST blots from Fig. 2B as indicated.

FIGURE 3. **MS analysis of proteolytically cleaved recombinant GST-eIF4E fusion protein.** (**A**) Coomassie stained gel of a semi-quantitative cleavage of GST-eIF4E (band 2 and 3). As a control, the GST-eIF4E fusion protein was incubated without any lysate (band 1). (**B**) The reactivity of the bands 1, 2 and 3 with anti-eIF4E and anti-GST was confirmed by Western blotting. (**C**) The eIF4E comprising bands were analyzed by MALDI-TOF-TOF MS. Depicted are the identified peptide sequences with their relative location in the molecule.

FIGURE 4. **Analysis of the influence of embryonic signals and steroid replacements on the expression of the truncated eIF4E**. (**A**). Depicted is the abundance eIF4E in the embryo containing (P) and non containing (NP) uterine horn on P15 of the same animal (**B**) OVX animals were treated with EB and P_4 as indicated and samples were probed for the abundance of internal eIF4E directly (time 0) or after 90 min of incubation (time 90). The bar charts show the ratio of the 25 kDa eIF4E (black) and the 23 kDa form (white). Bars represent means ± SEM (n=6). * indicates significant difference (*P < 0. 5)*. (**C**) Analysis of Ca^{2+} dependent proteolytic cleavage of internal eIF4E after steroid replacement and incubation for 90 min at 37°C with Ca^{2+}, EDTA or both compounds..

FIGURE 5. **Analysis of modified RRL and *in vitro* translation assay.** (**A**) The RRL was analyzed for the abundance of eIF4G, eIF4A, eIF4E and 4E-BP1 and 4E-BP2. Shown is the complete RRL (RL), the m^7-GTP-Sepharose treated, eIF4E depleted RRL (-4E), the corresponding supernatant (P), the eIF4E depleted RRL substituted with recombinant eIF4E (+4E) and the complete RRL substituted with 4E-BP1 (+BP1). (**B**) Depicted are the translation products after 0, 15 and 30 min as obtained from complete, eIF4E depleted, eIF4E substituted and 4E-BP1 substituted RRL with Xef1 mRNA as a template as indicated. (**C**). The abundance

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and cleavage of eIF4G, eIF4E eIF4A and PABP of the OVX and P15 modified RRL was monitored after 0, 30 and 60 min. (**D**) After 60 min treatment the lysates were used for *in vitro* translation (IVT) of capped, polyadenylated Xef1 mRNA. Depicted are the translation products in complete RRL treated with endometrial lystes after OVX or from P15 and substituted with recombinant 4E-BP1 as indicated. (**E**) Quantification of the analysis of eIF4E dependency from Fig. 5 B and endometrial effects from Fig 5 D. Measured was the optical density of the bands of the translation products obtain by *in vitro* translation in RRLs treated as indicated.

FIGURE 6. **Analysis of eIF4F formation and PABP binding in the modified RRLs after *in vitro* translation and analysis of eIF4E/4E-BP1 interaction.** (**A**) The *in vitro* translation was terminated after 30 min and the RRLs were directly subjected to immunoprecipitation with immobilized eIF4E antibody. Compared is the input with the precipitated fractions (eIF4E-IP) and the corresponding supernatant. The samples were probed for eIF4E, eIF4G, eIF4A, PABP and His-BP1. (**B**) Binding of the truncated eIF4E to 4E-BP1. His-tagged 4E-BP1 bound to Ni-NTA magnetic agarose beads was incubated with endometrial lysates after OVX and from P15, and eIF4E binding was monitored. Equal His-4E-BP1 binding to the beads was also confirmed by Western blotting. Depicted is the input (in) the bound (B) and unbound (S) fractions from beads saturated with (+) or without (-) His-tagged 4E-BP1.

FIGURE 7. **Model for eIF4E function in the endometrium of gilts after OVX and on P15.** (**A**) A schematic representation of eIF4E and eIF4G is shown. The bold type arrows indicate the position of the cleavage site in eIF4E and the main cleavage sites in eIF4G utilized by the endometrial protease on P15. The N-proximal flexible region of eIF4E is highlighted (amino acid 1-40). The binding sites of rabbit eIF4G for PABP, eIF4E, eIF4A, eIF3 and MNK were deduced from human eIF4G by sequence homology. (**B**) The formation of eIF4F and PABP binding can be impaired by hypophosphorylated 4E-BP1 in samples after OVX. In contrast, eIF4F formation is not completely impaired by 4E-BP1 on P15 when eIF4E and eIF4G persist in truncated form. However, PABP binding to eIF4F and therefore closed loop formation is impaired as a result of the deletion of the binding site in the truncated eIF4G.

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Fig. 1

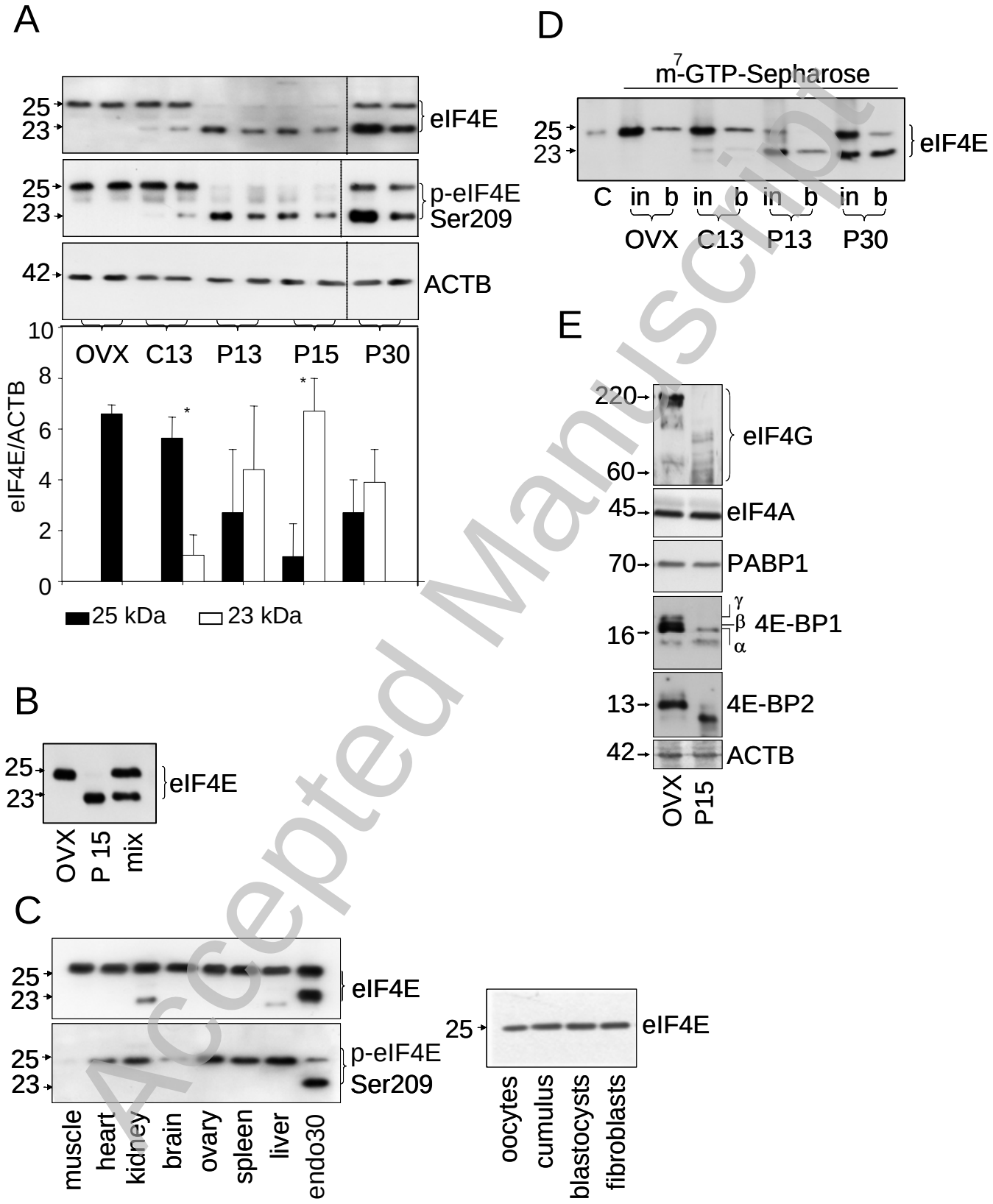

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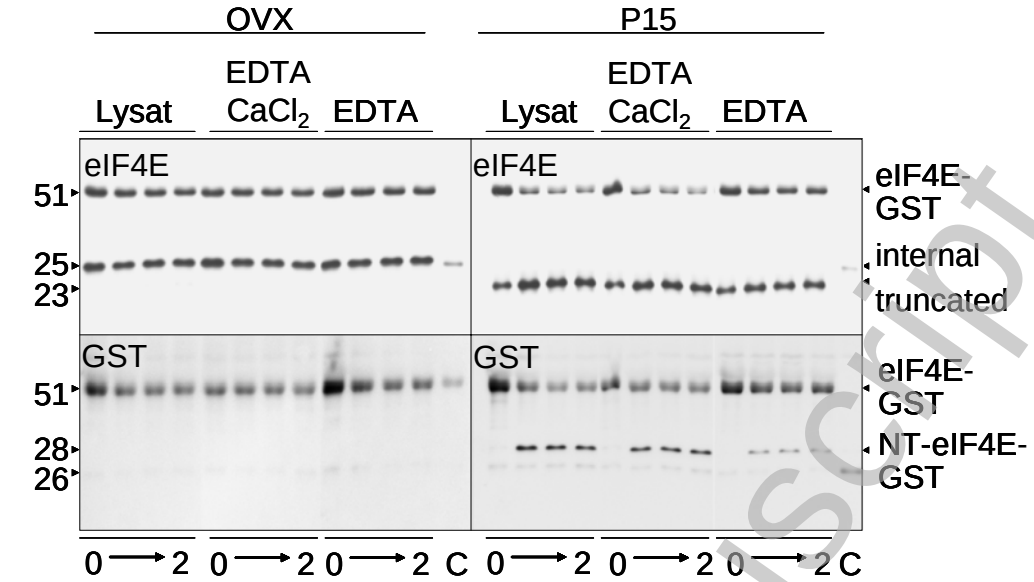
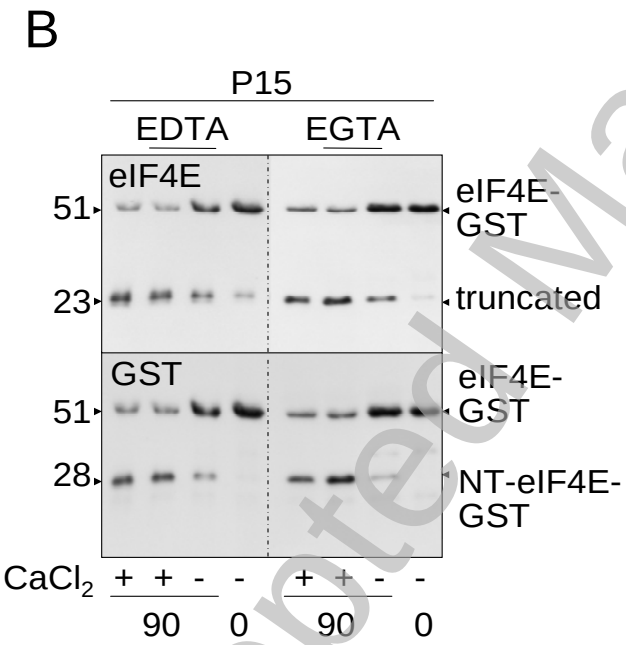
Fig. 2





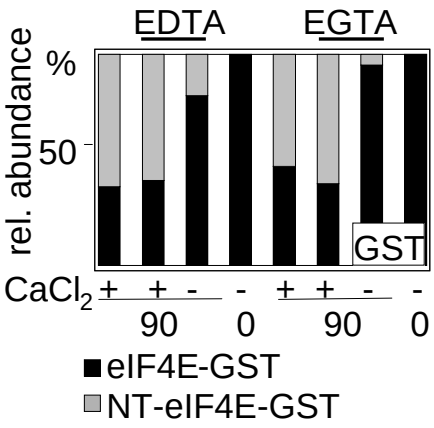


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Fig. 3

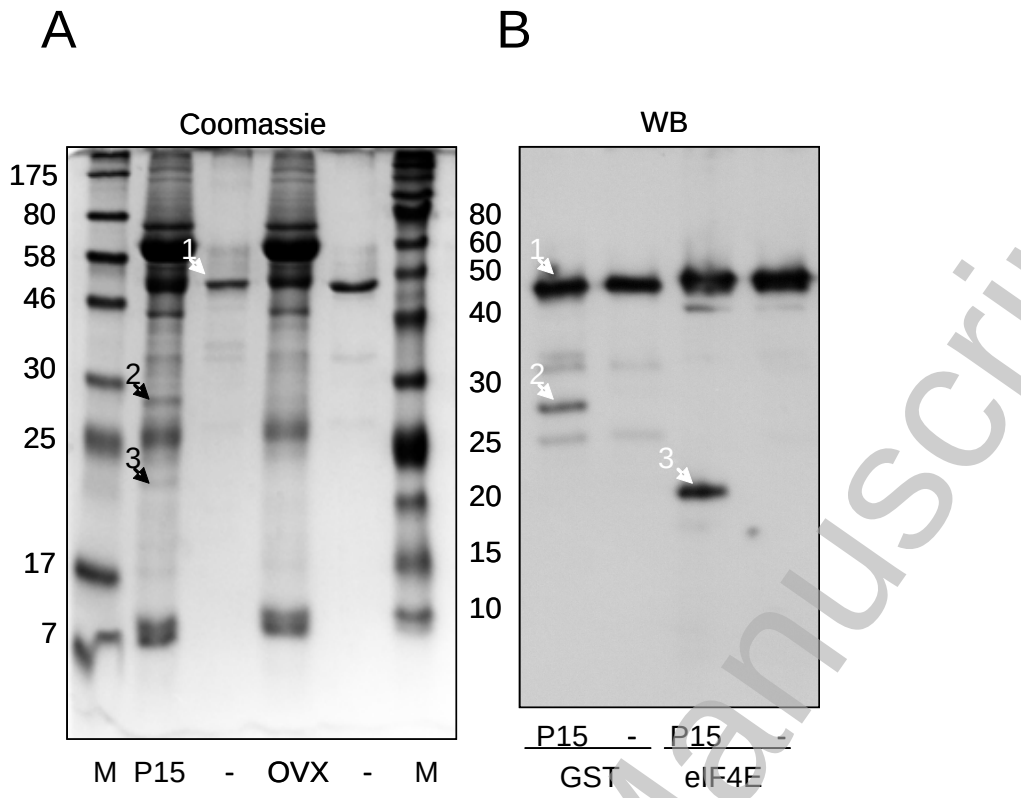

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Fig. 4

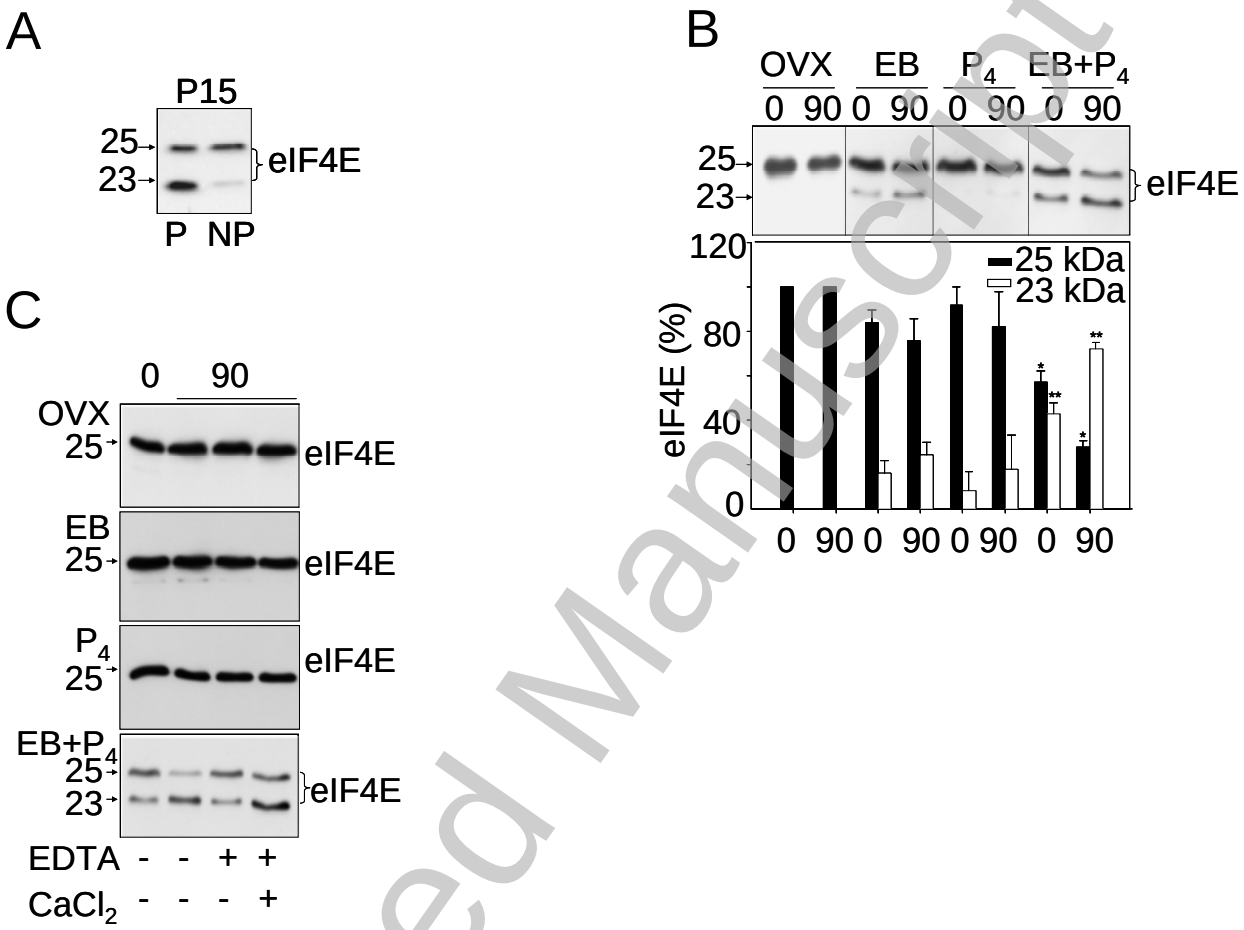

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Fig. 5

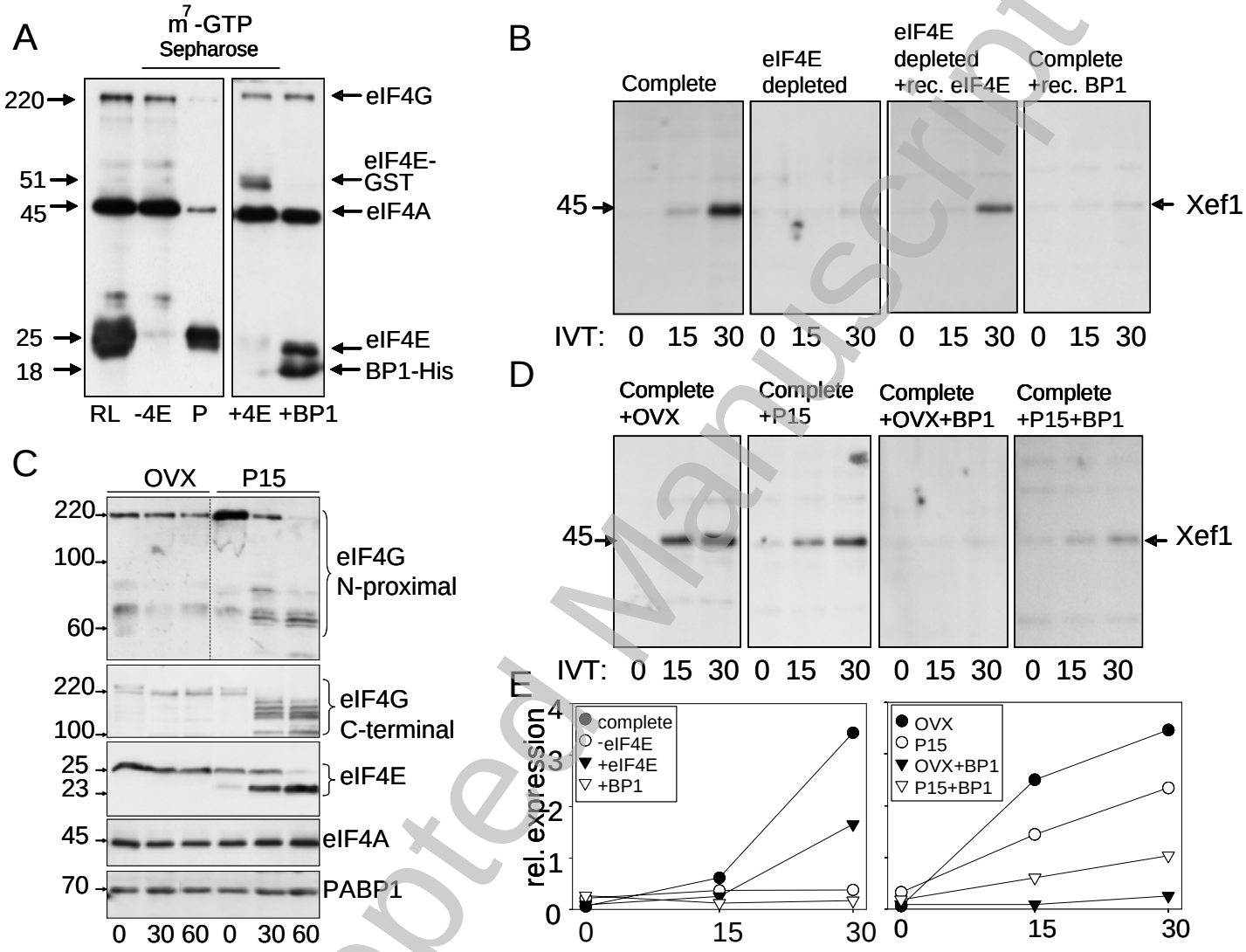

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Fig. 6

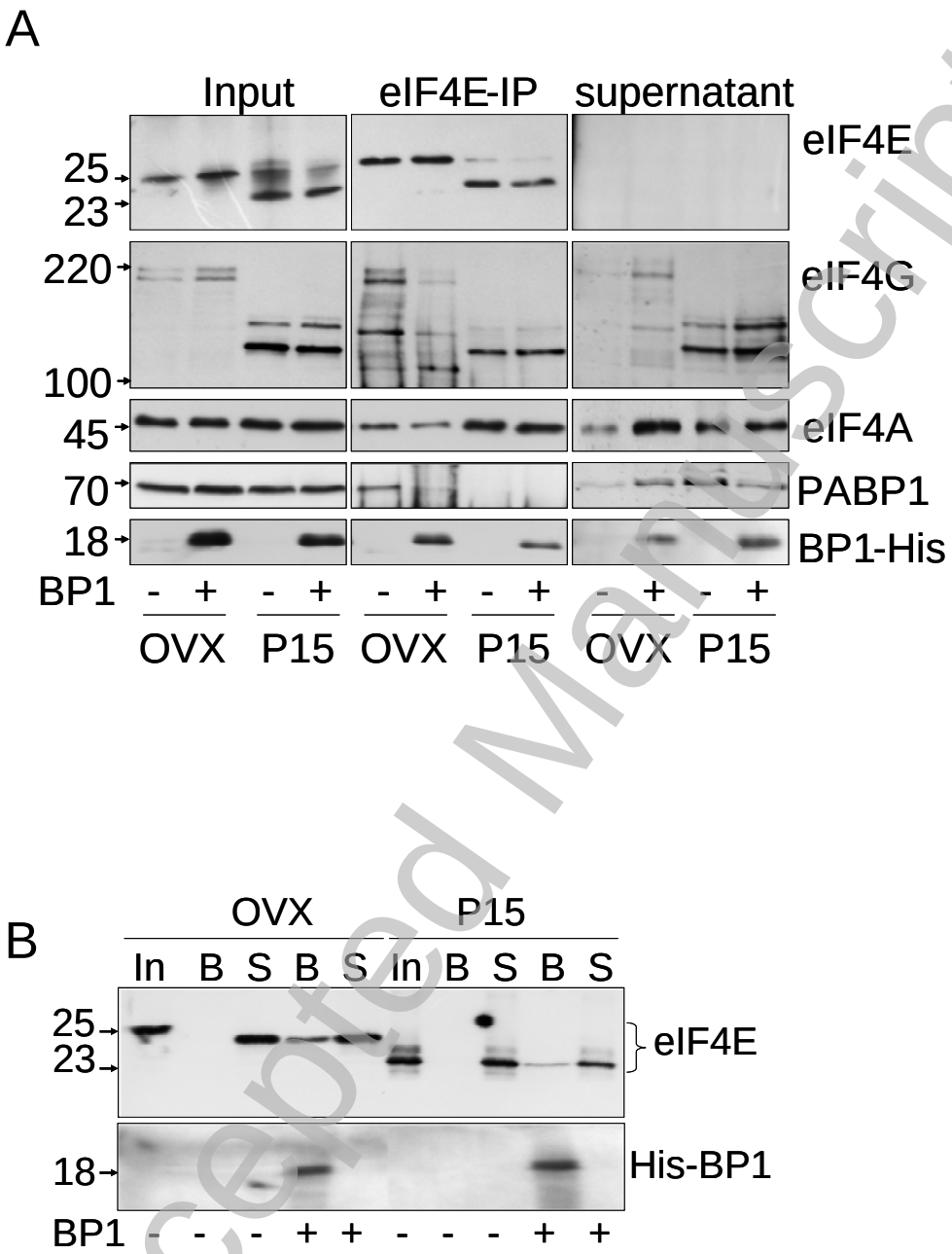

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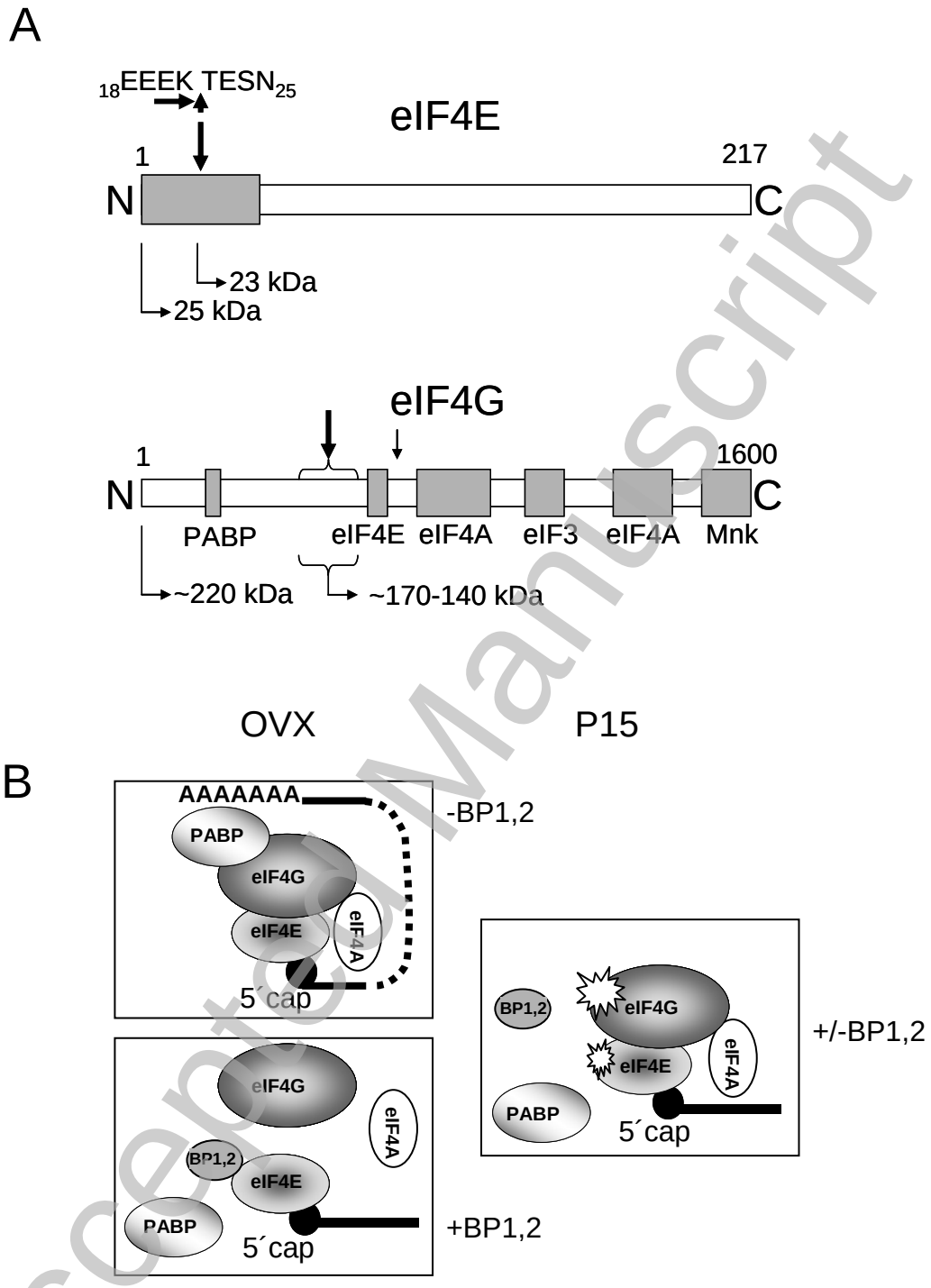
Fig. 7



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