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# Orc3, A Subunit of Drosophila Pre-Replication Complex Directly Binds mRNA and Interacts with ENY2 Subunit of the TREX-2 mRNA Export Complex

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## Abstract

ORC3 protein is a component of the *Drosophila* ORC pre-replication complex. As a component of ORC, *Drosophila* Orc3 interacts with the mRNP and with the TREX-2 mRNA export complex, and participates in the mRNP export from the nucleus to the cytoplasm. We found that Orc3 is associated with Xmas-2, the main structural protein of the TREX-2 complex, through its direct interaction with ENY2. Also, we detected that Orc3 directly binds mRNA with both the N-terminal AAA+-like domain and the C-terminal domain. Since we did not find a specific binding site for Orc3 within mRNA, it may indicate that Orc3 is responsible not for the specificity of the loading but for the maintaining of close contacts between the ORC, TREX-2, and mRNA.

**Keywords:** TREX-2; EMSA; RNP particle

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## Introduction

Origin recognition complex (ORC) was first discovered in yeast cells where its function is to recognize the replication initiation site and to guide the replication complex to the replication origins [1]. Later, homologs of the yeast ORC proteins have been found in all eukaryotic organisms [2-4]. It has also been demonstrated that unlike yeast ORC, the ORC proteins in higher eukaryotes have additional functions distinct from their role in replication initiation [5-8]. For instance, in our previous work we have demonstrated that in *Drosophila*, ORC interacts with the TREX-2/THSC/AMEX complex involved in the mRNA transport from the nucleus to the cytoplasm [9].

The latter complex was originally detected in yeast and includes Sac3 protein, two molecules of Sus1 protein, as well as Cdc31 and Sem1p proteins [10]. Orthologs of the TREX-2 complex have been found in higher eukaryotes [11,12]. Sac3 (Xmas-2 in *Drosophila*) within the complex, provides a platform for the interaction with other subunits, as well as with the complex-associated proteins. The N-terminal part of Sac3 is responsible for the mRNA binding and interacts with other proteins in the complex which are involved in the interactions with mRNA, namely, Thp1 and Sem1 [13-15]. The C-terminal part of Sac3 interacts with the two copies of Sus1 and with Cdc31, this sub-complex being responsible for the interactions with the nuclear pore [15,16].

In our laboratory, we have described the *Drosophila* TREX-2 complex and its individual subunits, Xmas-2 and ENY2. We have demonstrated that Xmas-2 and ENY2, the orthologs of the yeast Sac3 and Sus1 proteins, respectively, interact with each other and account for the mRNA export to the cytoplasm [16]. We have also shown that ENY2, as a part of the TREX-2 complex, is associated with the nuclear pore [16]. It was demonstrated further that ORC interacts with the TREX-2 complex. ORC subunits also interact with the mRNP complex and participate in mRNP export to the cytoplasm. Orc3 subunit is the strongest interacting subunit out of all ORC subunits, in the ORC association with the TREX-2 complex and mRNP particle [9]. In particular, this subunit most efficiently interacts with the Xmas-2 and ENY2 components of TREX-2 [9].

In the current work, we studied interactions of Orc3 with the Xmas-2 and ENY2 components of the TREX-2 complex. We found that Orc3 was associated with Xmas-2 through two molecules of ENY2. The EMSA with the labeled fragments of the *ras2* gene mRNA, demonstrated that *Drosophila* Orc3 directly interacts with mRNA with a low specificity. Our data suggest that Orc3 is most likely responsible for assembling the ORC, TREX-2, and mRNP complexes together.

# Methods

## Cloning of protein expression constructs

Coding sequences for the full-size Xmas-2 and ENY2 proteins with the C-terminally tagged HA epitope and His epitope, and coding gene regions, corresponding to the Orc3 N-terminal domain (1-333 aa), Orc3 C-terminal domain (334-721 aa), and full-size Orc3 N-terminally fused with three FLAG epitopes and GST epitope were cloned into pAc5.1/V5-His vector (Invitrogen) and pGEX-5X-1 (GE Healthcare) respectively. Vector constructs containing the coding regions of the Orc3 gene, corresponding to the protein's N-terminal and C-terminal domains fused with GST epitope, were generated.

## Transfection, expression, and purification of recombinant proteins

Transfection of Scheider-2 cells (S2) was performed using the Effectene Transfection Reagent as described in [17]. Expressions of the recombinant GST-tagged proteins were performed in *E. coli* BL21 cells at 20°C for 24 h. Cell were collected by centrifugation and stored at −70°C. Recombinant proteins with the GST epitope tag were purified from the cell lysates by binding to glutathione Sepharose and subsequently eluted by displacement with glutathione, according to the manufacturer's recommendations (GE Healthcare).

## GST pull-down assay

GST-fused protein immobilized on the glutathione Sepharose 4 fast flow resin (GE Healthcare) was washed extensively with LBST-100 buffer (50 mM Tris [pH-8.0], 100 mM NaCl, 5 mM $MgCl_2$, 20 % glycerol, 0.1% Triton X-100, and 0.5 mM DTT). His-E(y)2 or His-Xmas-2 proteins were added to the resin, and the interaction assay was carried out in a final volume of 600 mL. After incubation, beads were washed five times with increasing NaCl concentrations (LBST-100, LBST-300, and LBST-500) [18]. Western blotting and immunoprecipitation techniques were carried out using standard protocols.

## *Drosophila* cell culture extracts

*Drosophila* S2 cells were maintained at 25°C in the Schneider's insect medium (Sigma) containing 10% fetal bovine serum (HyClone, United States). To extract proteins, S2 cells were lysed. Cells were centrifuged at 2000 rpm at +4°C for 5 min and resuspended in 1 ml of 1x PBS with 25x PIC (Protease Inhibitor Cocktail, Roche). Cell were then centrifuged once more at 2000 rpm for 5 min at 4°C and resuspended in LB buffer, containing 10 mM Hepes pH-7.0, 0.4 M NaCl, 5 mM $MgCl_2$, 0.5% NP-40, 25x PIC, 1 mM DTT, and 0.3 µL of DNAse I, at the ratio of 1/10. Cells were incubated in LB buffer for 20 min on ice and the lysate was further centrifuged at 13000 rpm for 15 min at 4°C.

## *Drosophila* embryonic nuclear extract

The nuclear material was extracted from 0- to 12-h old *Drosophila* embryos with 0.42 M ammonium sulfate solution, as described [19].

## Immunoprecipitations

Antibodies were bound to protein A Sepharose (Sigma) following the manufacturer's recommendations. 100 µL of the protein extract in IP buffer (10 мM HEPES, pH 7.9, 5 мM $MgCl_2$, 0.1% NP-40, 0.15 M NaCl, 25x PIC, 1 mM DTT, RNase (Stratagene, 10 U/mL) 0.5 ng/µL of DNAse I) were incubated with 10 µL of Sepharose overnight at 4°C. Then, Sepharose was sedimented, supernatant was discarded, and the precipitate was washed three times with 100 µL of IP 500 buffer (10 мM HEPES, pH 7.9, 5 mM $MgCl_2$, 10% glycerol, 500 mM KCl, and 0.1% NP-40) for 10 min each. The equal volume of the electrophoresis loading buffer was further added to the precipitate, the obtained suspension was boiled, centrifuged, and supernatant was applied to the polyacrylamide gel for Western blotting.

## Antibodies

Polyclonal antibodies against Orc3 were described previously [9]. Anti-GST polyclonal antibodies were purchased from Santa Cruz Biotechnologies. Antibodies against Xmas-2 and ENY2 have been described previously [16].

## Synthesis of the radiolabeled RNA fragments

DNA templates for the α-$^{32}$P-RNA synthesis of the *ras2* mRNA fragments were prepared from pSK-ras plasmids by linearization of the cloned sequence from the 3'-end for the sense fragments. Radiolabeled RNAs were prepared using the RNA Labeling Mix and T7 and T3 polymerases (Roche Diagnostics), treated with RNase-free DNase I, and purified using the RNeasy Kit (Qiagen). Each radiolabeled RNA was analyzed by agarose gel electrophoresis and quantified by UV-spectrometry.

## Electrophoretic mobility shift assay (EMSA)

Purified recombinant proteins corresponding to the N-terminal and C-terminal parts of Orc3 were incubated with the radiolabeled RNA fragments in 20 µL of binding buffer, containing 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% Triton X-100, 5% glycerol, 1 mM EDTA, 1 mM DTT, 25x PIC, 40x RiboLock (ThermoScientific) for 1 h at 4°C. After binding was completed, PAGE loading buffer was added to the samples, and samples were applied to the 5% native polyacrylamide gel. Electrophoretic separation was carried out in 0.5x TBE buffer at 150 V for 80 min at 4°C. Radioactive signal was detected with the aid of the Cyclone StoragePhosphor Screen device. The signal in each line was quantitated using ImageJ program. Direct titration reactions were plotted as fraction of bound RNA versus the protein concentration and KD for each fragments were determined according to [20,21].

# Results

## Analysis of the Orc3 interaction with the TREX-2 complex subunits ENY2 and Xmas-2

Earlier, in the experiments with studying protein co-precipitations from the nuclear extracts, we have shown that Orc3 readily co-precipitates with both the Xmas-2 and the ENY2 subunits of the TREX-2 complex [9]. Xmas-2 (170 KDa) is the main subunit of the TREX-2 complex and it interacts via its C-end with two molecules of ENY2 (11 KDa). The direct interaction of *Drosophila* Xmas-2 and ENY2 was demonstrated [16]. In the current work, we have studied the interactions of the Orc3 protein with these two subunits in more detail. The domain organization of Orc3 is

highly conservative. It is tentatively subdivided into two parts, the N-terminal part which corresponds to an AAA+-like domain and contains motifs mediating protein interactions with nucleic acids, and the C-terminal part [22]. Earlier, some authors investigated interactions of Orc3 with other components of the ORC complex [22,23]. They have demonstrated that the N-terminal part of Orc3, representing the AAA+-like domain, interacts only with the C-terminal domain of Orc2 and with no other subunits of the complex [22]. In the C-terminal region of Orc3, there was identified an "Insertion" domain mediating an interaction with the Orc6 protein [23], as well as a WH domain involved in the interactions with Orc4, Orc5, and some other proteins **(Figure 1A)** [22].

To study interactions of Orc3 with the components of the TREX-2 complex, we split the Orc3 protein into two protein moieties, one of which (Orc3N) corresponded to the AAA+ domain and the other (Orc3C) contained the "Insertion" and the WD domains. This split was performed in order to determine which region of Orc3 contains the domain responsible for its interaction with the Xmas-2 and ENY2 subunits of the TREX-2 complex. We generated several expression constructs for the proteins representing the Orc3 N-terminal domain, or the Orc3 C-terminal domain, each fused with the FLAG epitope (Orc3N and Orc3C) in the *Drosophila* S2 cells. In these series of experiments, we also used expression constructs for Xmas-2 and ENY2 proteins fused with the HA epitope and the Orc3 protein fused with the FLAG epitope. To study interactions between Orc3 and Xmas-2, *Drosophila* S2 cells were double transfected with the following pairs of expression constructs: Orc3N-FLAG and Xmas-2-HA, Orc3C-FLAG and Xmas-2-HA, and Orc3-FLAG and Xmas-2-HA.

Protein co-immunoprecipitations from the S2 cell lysates **(Figure 1B)** did not reveal interactions between Orc3C or N-terminal part of Orc3 with Xmas-2. Weak interaction was observed between Orc3 and the Xmas-2 protein. Therefore, most likely, there are no significant interactions between these two proteins, under these conditions.

To study interactions between Orc3 and ENY2, S2 cell were double transfected with pairs of genetic constructs for the expression of Orc3N-FLAG and ENY2-HA, Orc3C-FLAG and ENY2-HA, as well as Orc3-FLAG and ENY2-HA. Co-immunoprecipitations with antibodies against the HA epitope and immunostainings of Western-blots with antibodies against FLAG **(Figure 1C)**, demonstrated that ENY2 interacted with both the N-terminal domain of Orc3 and its C-terminal region. However, it revealed a higher affinity for the C-terminal domain.

This indicates that each of the two Orc3 regions interacts with a single ENY2 molecule. Therefore, since we detected no significant interactions between Orc3 and Xmas-2, it is likely that one molecule of Orc3 interacts with two molecules of ENY2. Our data demonstrate that the interaction between Xmas-2 and Orc3 proteins, observed in the co-immunoprecipitation experiments of endogenous proteins from the nuclear extract, is not a direct one but is mediated by the ENY2 protein.

In line with this finding, antibodies against Orc3 efficiently co-precipitated ENY2 from the nuclear extract of *Drosophila* embryos, and vice versa **(Figure 1D)**. In addition, we investigated whether interaction of Orc3 and ENY2 was direct in the GST pull-down assay **(Figure 1E)**. The bacterially expressed GST-tagged Orc3 was immobilized on glutathione Sepharose. The His-tagged ENY2 was also expressed in bacteria, purified, and incubated with the glutathione Sepharose bound Orc3. Our data demonstrate that ENY2 strongly interacts with Orc3. In our experiments we did not observe a direct interaction of ORC3 with Xmas-2.

Xmas-2, the largest subunit of the *Drosophila* TREX-2 complex, forms the scaffold of the complex, while two ENY2 molecules bind with its C-terminal part **(Figure 1F)**. Orc3, presumably, interacts with the TREX-2 complex via binding to some motifs located in its N- and C-terminal parts, with the two ENY2 molecules present within the complex **(Figure 1F)**.

## Orc3 protein directly binds to the mRNA of the *ras2* gene

We have already demonstrated earlier that ORC subunits interact with the mRNP of the *ras2* gene [9], with the Orc3 protein demonstrating the highest affinity to *ras2* mRNP comparatively to all the other subunits. In the current work, we aimed to elucidate whether the *Drosophila* Orc3 protein directly interacts with mRNA. And if such an interaction takes place, which part of the protein, the N-terminal region corresponding to the AAA+-like domain, or the C-terminal region, is necessary for this interaction. Lastly, we wanted to determine which region of the *ras2* mRNA interacts with Orc3.

In this series of experiments, we used the polyacrylamide gel electrophoretic mobility shift assay (EMSA). The nucleotide sequences corresponding to the N-terminal and C-terminal regions of Orc3 were cloned into the pGEX vector in frame with the GST epitope. The target proteins were expressed in a bacterial system and were purified using glutathione Sepharose. The *ras2* mRNA sequences were subdivided into five fragments **(Figure 2)**. To synthesize sufficient amounts of the target mRNA fragments the corresponding DNA sequences were cloned into the pSK vector containing T3 and T7 promoters. For each DNA fragment, a radioactively-labeled RNA was synthesized using T3 or T7 RNA polymerase and α-32P UTP. The obtained radiolabeled RNAs were incubated in the binding buffer with the proteins corresponding either to the N-terminal or to the C-terminal domain of the Orc3 protein.

We observed that the Orc3 N-terminal region corresponding to the AAA+-like domain, interacted with all the fragments of the particular *ras2* mRNA **(Figure 3)**. At the same time, GST peptide did not interact with the RNA fragments by itself (lane 4), thus validating specificity of our experiments. Therefore, our experiments have demonstrated that Orc3 directly binds to mRNA.

The N-terminal region of Orc3 similarly interacted with all the RNA fragments, suggesting that there is no strong preference for interaction with a certain *ras2* mRNA region. However, this Orc3 protein region showed the highest affinity for the first and the fourth *ras2* gene fragments **(Figure 3B)**, comparatively to the other fragments. This may indicate that there is still some level of specificity in the interaction with these RNA fragments **(Figure 3)**.

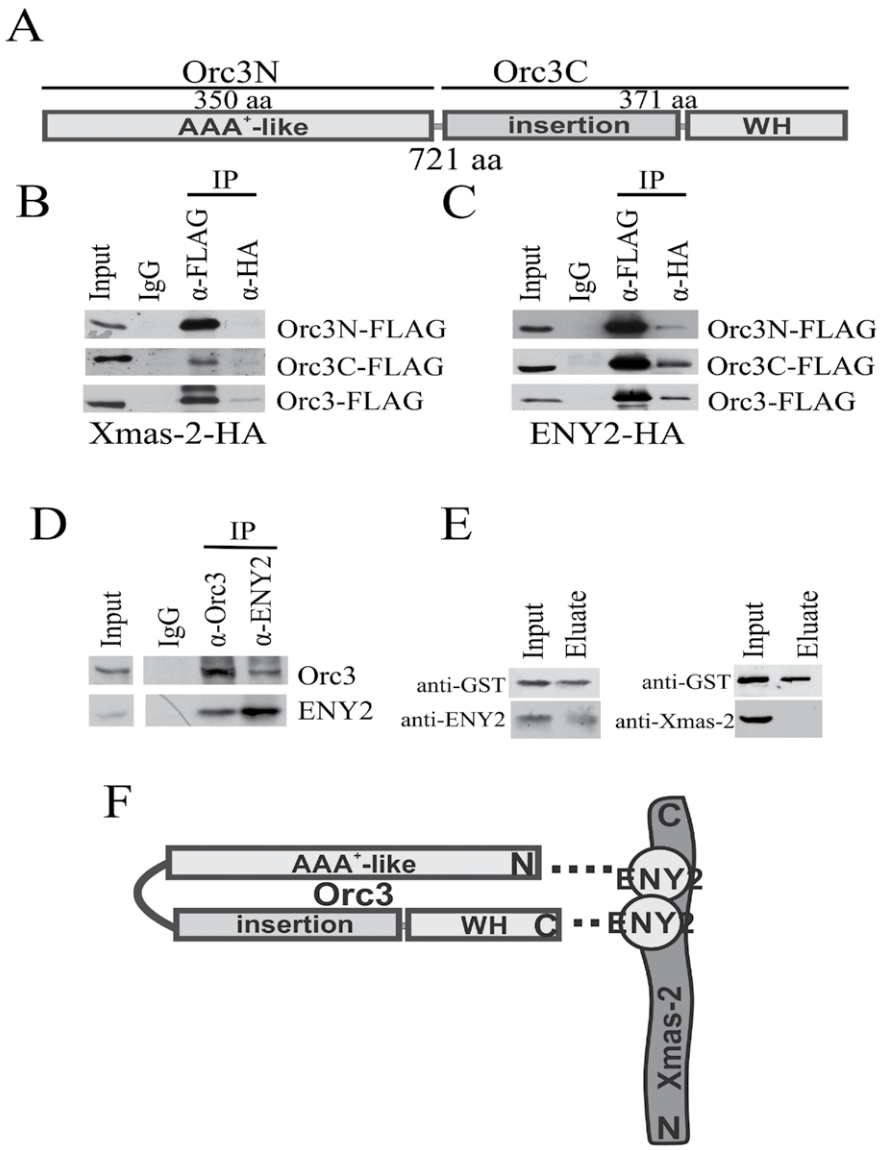


**Figure 1** Analysis of Orc3 interactions with the ENY2 and Xmas-2 proteins. A: Schematic representation of the *Drosophila* Orc3 protein domain organization. Orc3N -- N-terminal domain of Orc3 (350 aa); Orc3C -- C-terminal domain of Orc3 (371 aa); AAA+-like -- Orc3 domain responsible for binding with nucleic acids and the Orc2 protein; Insertion -- Orc3 domain responsible for binding with the Orc6 protein; WH -- Orc3 domain necessary for the interaction with different proteins [22, 23]. B and C: Western blot analysis of Orc3 interactions with the ENY2 and Xmas-2 proteins. B, Western blot analysis of the interactions between the Xmas-2 and Orc3N, Xmas-2 and Orc3C, and Xmas-2 and full-size Orc3 proteins over-expressed pairwise in the *Drosophila* S2 cells. C, Western blot analysis of the interactions between the ENY2 and Orc3N, ENY2 and Orc3C, and ENY2 and full-size Orc3 proteins over-expressed pairwise in the *Drosophila* S2 cells. D: The co-immunoprecipitation of Orc3 and ENY2 from the nuclear extract of *Drosophila* embryos and vice versa. E: The interaction of ORC3 and ENY2 or Xmas-2 in the GST pull-down assay. The bacterially expressed GST-tagged ORC3 was immobilized on glutathione Sepharose. The His-tagged ENY2 or His-tagged Xmas-2 were expressed in bacteria, purified, and mixed with the glutathione Sepharose4 bound ORC3 in the incubation buffer. 15 µL of this mixture was loaded onto the gel as Input. After the incubation glutathione Sepharose4 resin was washed and GST-bound proteins were eluted, 15 µL of eluates were loaded onto the gel (Eluate). Western blot was developed with antibodies against GST, ENY2, or Xmas-2. F: The schematic representation of presumable interactions between Orc3, ENY2, and Xmas-2.

Fragment 1 comprises the 5'-noncoding region of the gene, while fragment 4 corresponds to the proximal 3'-noncoding region.

The C-terminal domain of Orc3 also interacted with all the *ras2* mRNA fragments. The strongest interaction appeared to be with the third fragment, which corresponds to the distal part of the coding region of the gene **(Figures 4 A and 4B)**.

To summarize, both of the Orc3 regions were able to bind to the *ras2* RNA. Our data suggest that Orc3 possesses several RNA-binding domains localized in both parts of the protein.

## Discussion

Here, we studied interactions of the Orc3 protein, a subunit of

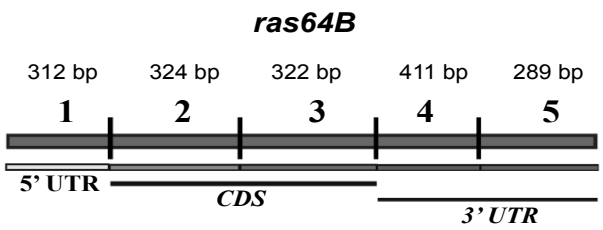


**Figure 2** Schematic subdivision of the *ras64B* mRNA into the fragments used in the RNA binding experiments. Indicated are the mRNA regions to which the fragments used in the experiment correspond.

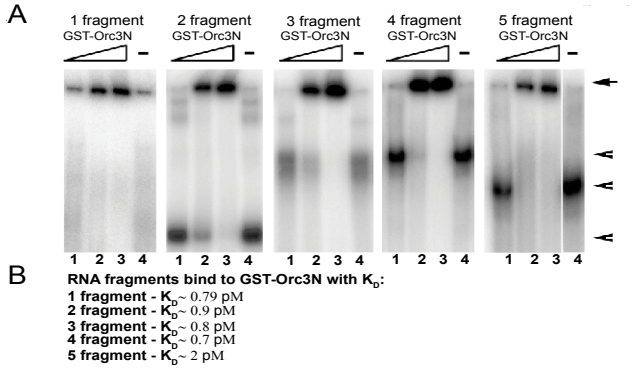


**Figure 3** EMSA analysis of the *Drosophila* Orc3N protein interactions with the *ras2* RNA fragments. A: Replicas from a 5% native polyacrylamide gel are shown. The arrow indicates the major high order gel shifted complex. Arrowheads show the migration of free RNAs. The concentration of each RNA fragment in the binding reaction was 1 pmol. Numbering of the RNA fragments is as shown on Figure 2. Orc3N-GST protein concentration was 0.5 pmol (first lane), 1 pmol (second lane), and 5 pmol (third lane). The concentration of GST peptide was 10 pmol (fourth lane). B: Calculated approximate KD for interactions of *ras2* RNA fragments with Orc3N protein.

ORC complex, with the Xmas-2 and ENY2 subunits of the TREX-2 complex and have demonstrated that Orc3 directly interacts with ENY2. These interactions were carried out by both the N-terminal domain and the C-terminal domain of Orc3.

Earlier, we have purified the *Drosophila* mRNA export complex, TREX-2, and found that it is associated with the ORC complex [9]. However, Orc2 was the only ORC subunit which did not co-purify with TREX-2. Orc2 interacts with the ORC complex via its Orc3 subunit. As was demonstrated previously, human Orc3 N-terminal domain interacts with Orc2, while its C-terminal domain is required for the interaction with Orc4 and Orc5 [22]. Here, we have found that the N-terminal domain of Orc3 could also interact with TREX-2. These data suggest that TREX-2 may replace Orc2 when it interacts with ORC and this may explain why Orc2 was not detected within the ORC-TREX-2 complex.

To study interactions of Orc3 with the Xmas-2 and ENY2 subunits of the TREX-2, we over-expressed two proteins, one corresponding to the N-terminal region of Orc3, and the other corresponding to

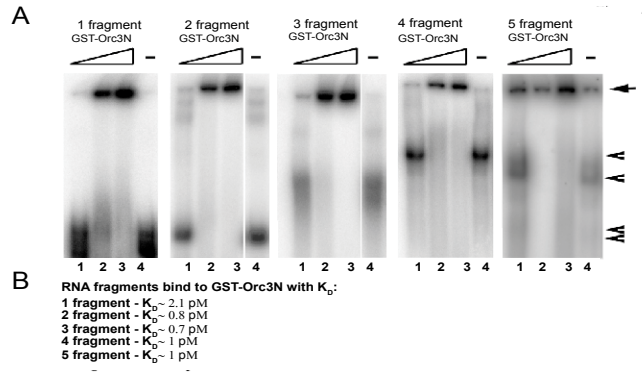


**Figure 4** EMSA analysis of the *Drosophila* Orc3C protein interactions with the *ras2* RNA fragments. A, Replicas from a 5% native polyacrylamide gel are shown. The arrow indicates the major high order gel shifted complex. Arrowheads show the migration of free RNAs. The concentration of each RNA fragment in the binding reaction was 1 pmol. Numbering of the RNA fragments is as shown on Figure 2. Orc3C-GST protein concentration was 0.5 pmol (first lane), 1 pmol (second lane), and 5 pmol (third lane). The concentration of GST peptide was 10 pmol (forth lane). B, Calculated apprximate KD for interations of *ras2* RNA fragments with Orc3C protein.

its C-terminal region. These proteins were co-expressed pairwise with the ENY2 and Xmas-2 proteins in the *Drosophila* S2 cells. Both fragments of the Orc3 protein, as well as the full size Orc3, interacted with ENY2. We suppose that the interactions between the three proteins, Xmas-2, ENY2, and Orc3, within the ORC-TREX-2 complex, are mediated by the interactions between Orc3 and ENY2, with both of the Orc3 domains being involved. It is well documented that TREX-2 complex contains two molecules of ENY2 [13,14]. It is thought that one of these molecules is involved in the interaction of TREX-2 with the nuclear pore complex [10,13,14,24,25]. It seems possible that Orc3 binds with both copies of the ENY2 protein and, if this were the case, it could also be involved in the interaction with the nuclear pore complex. However, this latter conjecture requires further study.

The Orc3 structure and function analysis revealed a domain potentially responsible for the interaction with nucleic acids. This domain encompasses two amino acid motifs capable of binding ATP or GTP. This nucleic acid interaction domain is localized in the N-terminal part of Orc3 and is called the AAA-like domain [26,27]. Traditionally, ORC proteins are known for their interaction with DNA and their initiation of the DNA replication [28]. Nevertheless, recently it has emerged that these proteins can also interact with RNA [29-33]. In our previous study using RNA immunoprecipitation, we have demonstrated that ORC proteins interact with the mRNP of several genes [9]. Here, we assessed the interactions of the Orc3 N-terminal and C-terminal domains with different fragments of the *ras2* mRNA. We found that both of the Orc3 termini are able to interact with the RNA fragments, suggesting that Orc3 has an RNA-binding ability. This also indicated that in the interactions between ORC and the mRNP particle, described by us previously [9], it is mRNA to which Orc3 binds directly, and not only the protein components of mRNP.

In the current work, we also studied which particular regions of the *ras2* mRNA interact with Orc3. We found that both Orc3 domains show no specificity in the interaction and bind to any of the RNA fragments. Interestingly, the N-terminal protein domain of Orc3 showed different affinity for RNA, than the C-terminal region. The N-terminus had the highest affinity to the RNA fragments 1 and 4, corresponding to the 5'-noncoding region and to the 3'-proximal part of the non-coding region, respectively. At the same time, the protein corresponding to the Orc3 C-terminal domain bound with the highest affinity to the different parts of the coding region of the *ras2* mRNA.

We hypothesize that Orc3 protein contains another, yet unidentified nucleic acid-binding domain which is located in its C-terminal part. Since the two truncated proteins show their maximum affinity to different mRNA fragments, it could be that the full-size native protein interacts with RNA by its both, N-terminal and C-terminal, domains which bind different mRNA regions to ensure a closer contact with the protein moiety of the mRNA-containing complex.

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## Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

## Author Contributions

VVP carried out first part of experiments, DVK – carried out EMSA experiments and wrote most of the paper. SGG conceived the idea for the project and wrote the paper with DVK.

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