Investigation the nuclear connections and the role in mRNA export of the actin binding protein, Moesin (PD 127968)

The aim of the work

Previously we have demonstrated that the actin binding, cytoskeletal Moesin protein is present also in the cell nucleus, where it participates in mRNA export. Our aim was to identify and characterize the protein complex or complexes which contain Moesin, in order to reveal the exact molecular mechanism by which Moesin functions in the nucleus.

Results

1. In the first year we aimed to identify the nuclear interaction partners of Moesin by mass spectrometry analysis

To establish cultured Drosophila cell lines which stably express GFP and HA epitope tagged Moesin proteins for the mass spectrometry analysis, first we subcloned the Moesin cDNA into vectors suitable for Drosophila S2R+ cell transfection and constitutive protein expression. We verified the protein coding sequence and the GFP- and HA-tags by sequencing. Then we co-transfected the S2R+ cells with the Moesin expressing constructs and a hygromycin resistance plasmid, and selected for stably transfected cells by adding antibiotic to the cell culture media. After six weeks of selection, we tested the stable cell line for Moesin expression by fluorescence microscopy and western blot experiments, and found that Moesin is expressed with the epitope tags in the correct protein size.

From the stably transfected cells we isolated nuclear and cytoplasmic protein fractions, and carried out immunoprecipitation experiments with anti-GFP or anti-HA antibodies. The proteins anchored by Moesin were identified by mass spectrometry. The immunoprecipitation experiment performed with the cytoplasmic fraction recognized Actin as the strongest hit. Because Actin is the best known and most important interaction partner of Moesin, we concluded that the lysis buffer and the immunoprecipitation conditions are suitable to identify interaction partners for Moesin in the nucleus.

In the experiment using the nuclear protein fraction the most remarkable hits were proteins involved in transcription regulation and mRNA export. In fact, almost all the members of the Mediator complex were precipitated by Moesin. From the literature it is known that the Mediator complex beside functions in transcription regulation, it directly interacts with the Trex-2 complex, which is the main regulator of nuclear mRNA export. This is in good agreement with our previous data, because we showed earlier that Moesin participates in mRNA export. As members of the Trex-2 complex also showed interaction with Moesin, we hypothesize that Moesin could represent a link between the two mRNA export competent complexes.

Among the best hits of the LC-MS/MS experiment we also got several mRNA binding proteins and mRNA export receptors. Proteins involved in rRNA biogenesis, processing, and export from the nucleus were also significantly represented.

We repeated the immunoprecipitation and the mass spectrometry experiments six times.

2. In the second year we selected the potential interaction partners of Moesin and validated them with biochemical methods

The potential nuclear interaction partners of Moesin identified in the mass spectrometry experiments can be categorized into several groups based on their nuclear function. These groups are mRNA binding and mRNA export, Mediator complex members, rRNA transcription, ribosome assembly and export. We selected the candidates with the highest score from each functional subgroup to confirm their interaction with the Moesin protein in a series of co-immunoprecipitation (co-IP) assay.

For the co-IP tests the cDNA of each candidate gene was cloned into the pDONR221 vector, sequence verified, and then subcloned into an expression vector allowing FLAG epitope tagging (Gateway system). We co-transfected the constructs with a Moesin-GFP encoding expression vector into cultured S2R+ Drosophila cells. As a control experiment, we first checked the proper intracellular localization pattern of each expressed candidate protein by immunostaining with an anti-FLAG antibody. Next, we repeated the transfections, and on the third day we prepared total protein samples from the cells and performed the co-immunoprecipitation experiments with anti-FLAG or anti-GFP antibodies. The bait and the anchored proteins were detected by Western blotting.

We found that Moesin pulled down the Mediator complex members Med6 and Med15, which strongly suggests that Moesin interacts and shares function with this complex in the nucleus.

In the co-immunoprecipitation experiments Moesin also showed clear interaction with the RNA helicase protein Dbp45A and the Nucleolar Protein 16 (Nop16), indicating a possible new role for Moesin in ribosome biogenesis pathways.

3. In the third year (extended due to the covid pandemic) we verified the interactions with in vitro and in vivo methods

The applied mass spectrometry and co-immunoprecipitation techniques cannot distinguish between direct and indirect molecular interactions, therefore we turned to methods which are able to reveal direct protein interactions. For this aim, we subcloned the genes into plasmids that express proteins under the control of the T7 promoter, and are therefore suitable for the In Vitro Transcription and Translation (IVTT) procedure. In the IVTT experimental system [^{35}S]Methionine is incorporated into the newly synthesized proteins during translation, resulting in radiolabeled candidate proteins. We purified Glutathion S-transferase (GST) affinity tagged Moesin protein from *E. coli* bacteria as a bait, and used the proteins produced in the IVTT system as prey. In the GST pull-down experiments the bait protein was immobilized to glutathione beads, then the prey protein was applied. As a negative control we used the GST protein alone on the glutathione beads. After performing the binding reaction, we eluted the proteins from the beads and analyzed the results by autoradiography. For these assays we successfully produced 12 different members of the Mediator complex, those ones which had the highest score in our mass spectrometry analysis (Med6, Med7, Med8, Med11, Med15, Med16, Med17, Med19, Med22, Med23, Med27 and Med31). Then we tested their interactions with the GST-Moesin protein, and found that the Med7, Med15, Med17 and Med19 proteins showed interaction with Moesin *in vitro*, providing additional evidence that Moesin is a new member of the Mediator complex.

By this method we also identified the RNA helicase Dbp45A, and the PCID2 protein from the Trex-2 complex as interactors of Moesin. Both proteins are required for mRNA export to the cytoplasm further confirming Moesin's role in the process. The Nop16 protein involved in ribosome assembly also showed obvious binding to Moesin.

The protein interactions confirmed in the IVTT-coupled GST pull-down experiments were further validated in an *in vitro* direct binding assay. We subcloned the genes of the interaction partners into bacterial expression vectors containing a 6x-His affinity tag, and we purified the candidate proteins from *E. coli* bacteria. Next, we performed the GST pull-down experiments with the purified proteins and evaluated the results by western blot analysis. By this way we successfully verified that Moesin directly interacts with the Mediator complex members Med15, Med17 and Med19, and also with the RNA helicase Dbp45A, PCID2 and Nop16 proteins.

In the next set of experiments, we wanted to test that the direct protein interactions we identified in the previous *in vitro* binding assays, are present also in live cells *in vivo*. For this purpose we applied the Bimolecular Fluorescence Complementation (BiFC) technique which utilizes Split-YFP epitope tags (split YFP fragments can reconstitute a functional fluorescent protein when fused to interacting proteins). The appearance of the yellow fluorescent signal indicates not just the direct interaction between the proteins, but their subcellular localization as well. We created Drosophila gateway vectors for Split-YFP tagging, which enabled us the easy and quick tagging and testing of any protein in this assay. In the case of the Mediator complex subunit Med15, and RNA helicase Dbp45A we could observe a strong YFP signal restricted to the nucleus confirming their direct interaction with Moesin in the nucleus *in vivo*.

In the following experiments we focused on the interaction of Moesin with the Mediator complex. To unambiguously confirm that Moesin and the Mediator subunits are indeed components of the same protein complex in the nucleus we applied an additional semi-*in vivo* method, the Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE), which is a useful tool to determine the composition of multiprotein complexes. We isolated nuclear protein fraction from cultured Drosophila S2R+ cells transfected with Med7-FLAG and Med15-FLAG encoding constructs, and in the first dimensional running we separated the nuclear complexes under native conditions. Then we placed the gel slice on the top of an SDS containing denaturing gel to perform the second dimensional running, which separates the complexes into individual constituents. Proteins detected in the same lane come from the same complex. We detected Moesin, and the Med7 and Med15 subunits in the same lane, indicating that they are in the same nuclear protein complex *in vivo*.

As a final proof, we performed co-localization experiments on the polytenic chromosomes isolated from wandering *Drosophila* larvae. Moesin showed considerable co-localization with the Med15 protein along the chromosomes. They also colocalized at the puff regions, which are specific euchromatic regions with extreme high level of transcription induced at specific cytological locations by heat stress or ecdysone hormone.

In sum, we found that the actin-binding Moesin protein is a new member of the Mediator complex through directly binding the Med15 subunit. Our *in vivo* experiments also confirmed that Moesin interacts with Med15 in the nucleus and that they co-localize on the chromosomes. Mediator is a multi-subunit complex which, depending on its dynamic member composition, plays important role in transcription initiation and elongation, as well as in mRNA export. From the literature we know that Med15 binds to the regulatory regions of the heat shock genes, and thereby it participates in stress induced gene expression response. Our previous data demonstrated that upon heat stress Moesin accumulates on the promoter regions of heat shock genes (Kristó et al. 2017), and that it is required for the proper expression of these genes (Bajusz et al. 2021). We hypothesize that Moesin together with the Mediator complex could be involved both in the export and the transcription of the mRNA molecules. Currently we are performing chromatin immunoprecipitation experiments to investigate the possible mutual dependency of Moesin and Med15 proteins on the regulatory regions of heat

shock genes to reveal the functional and biological significance of the interaction of Moesin with the Mediator complex. After completing these experiments, we will publish our study in an international journal.

The Med7, Med17 and Med19 subunits also showed interaction with Moesin in the *in vitro* binding assays and we also found that Moesin directly binds the RNA helicase Dbp45A, and proteins involved in rRNA transcription and ribosome biogenesis. These findings indicate that Moesin plays role in different steps of RNA metabolism, and open possible new future directions in the research about the role of Moesin in the cell nucleus.

During the work I developed and described a method to detect Moesin's most important interaction partner, actin in the nuclear protein fraction of *Drosophila* ovaries by western blot. The manuscript has been accepted for publication as a book chapter in Methods in Molecular Biology.

I presented the results of this study at 5 international (1 oral and 4 poster presentations) and 3 domestic conference (poster presentations).