Final Report on OTKA grant No.108538

The aim of the work

Our work was based on the idea that the functions of the evolutionarily conserved, cytoskeletal, actin-binding ERM protein of *Drosophila melanogaster*, Moesin, are not restricted to the cortical actin network of interphase cells but the protein plays role also in the formation of the so called spindle matrix during cell division, moreover, it is a functional component of the interphase nucleus.

Results

1. In the first year we aimed to demonstrate that Moesin directly participates in mitotic spindle function.

To explore the requirement of Moesin in spindle function, live video microscopy was applied in very early fly embryos with reduced levels of both maternal and zygotic Moesin protein. We recorded mitotic abnormalities that have not been reported before for Moesin and which cannot be explained by the lack of cortical stiffness and cell rounding or problems in anchoring astral microtubules to the cell cortex, as it was proposed in the literature before (Carreno et al.; Kunda et al.). Considering the localization pattern of Moesin and that at these developmental stages the Drosophila embryo is a syntitium in which nuclei divide without cell membranes, we concluded that – at least at this stage - Moesin plays a direct role in spindle activity.

To uncover if Moesin's activity is performed directly at the microtubule spindle or it is rather part of the actin-based, so-called spindle matrix, we investigated whether the spindle structure formed by Moesin is different from the microtubule spindle. We found that 1) the accumulation of Moesin precedes the construction of the microtubule spindle, 2) the fusiform structure formed by Moesin persists even after the disassembly of the microtubule spindle, 3) the formation of the fusiform structure by Moesin depends on F-actin. In addition to these, we have also shown that the C-terminal actin-binding domain of Moesin as well as its main binding partners, actin and Phosphatidylinositol 4,5-bisphosphate (PIP2) which mediates its conformational activation, are also present at the spindle, further confirming that Moesin directly associates to the mitotic spindle area.

Next, we analyzed in the early embryo the localization and activity of a Moesin variant that cannot be stabilized in its open, active conformation by phosphorylation at T559 (MoeTA). MoeTA was enriched at the site of the mitotic spindle at every developmental stage but unlike the wild type protein, it was found almost totally absent from the cytoskeletal island around the spindle in syncytial blastoderm embryos and from the cortex in cellularized embryos. The expression of MoeTA in wild type embryos resulted in an unusual mitotic spindle morphology and lethality. It was apparent that the microtubule spindle did not overlap with the protrusions providing further evidence that the two structures are different.

To confirm that Moesin is a new member of the spindle matrix, double mutant lines were created carrying one copy of *moe* mutation and one mutant allele of a spindle matrix gene (*Chromator, Megator, Skeletor, East*). Centrosomes, spindles and DNA were visualized in these double mutants to test for the interaction between them and *moe*. We found that although single mutants show mild or no mitotic phenotype and only slightly abnormal mitotic index alone, in heterozygous double mutants the mitotic phenotypes as well as the mitotic index was significantly elevated. The double mutants exhibited primarily pro- and prometaphase blocks (spindle matrix proteins are necessary for the assembly of the microtubule spindle in prophase) indicating further that Moesin and spindle matrix proteins function in the same process during mitosis and thus providing additional evidence for the direct involvement of Moesin in spindle function.

According to our proposal, we published these results in a scientific journal and presented them on two international and two domestic conferences.

2. In the second and third years we planned to focus on the nuclear localization and function of Moesin.

In the first set of experiments we aimed to unequivocally demonstrate that Moesin is present in the interphase nucleus. For this, we equipped the protein with various tags (GFP, mCherry, V5, HA and Myc) and expressed these forms both in cultured cells and live animals. All antibody stainings, including the one for the endogenous, untagged protein, were identical and clearly showed nuclear localization for Moe. The detection of Moesin in nuclear protein extract by western blot confirmed this finding. The sub-nuclear localization of Moesin was determined with the help of the giant, polytenic nuclei of Drosophila salivary gland cells. We found that both the tagged and the endogenous Moesin proteins localize to the sites of active transcription of the chromosomes. To demonstrate that this localization is biologically relevant, we monitored the nuclear localization of Moesin after heat shock or ecdysone hormone treatment and found that upon these stress conditions Moesin accumulates in the nucleus as well as at the transcriptionally hyperactive stress response regions, called chromosome puffs.

For the analysis of the nuclear import of Moesin we carried out FRAP experiments. We bleached the Moe-GFP signal in the entire nucleus with a short and strong laser pulse and followed the recovery of the fluorescent signal to measure the nuclear import rate of Moe-GFP. We could observe a slow and relatively low level of recovery of the fluorescent signal, which led us to conclude that during interphase the nuclear transport of Moesin is moderate. Next, we investigated the intranuclear binding of Moe-GFP by bleaching the fluorescent signal in the nuclei of salivary gland cells. We found that, after an intensive but short bleaching of a small nuclear area, the fluorescent signal was completely lost in the whole nucleus which suggests that, similarly to the cytoplasm, the binding of Moesin is very dynamic in the nucleus.

To examine the nuclear transport of Moesin, an RNAi screen was carried out in cultured S2R+ cells. The individual depletion of 21 nuclear import and export factors in the cells transiently expressing Moe-GFP revealed that the knockdown of Nup98 leads to the strong accumulation of Moesin in the nucleus. The silencing of Rae1, a known interacting partner of Nup98, also led to the enrichment of the Moe-GFP signal in the nucleus, confirming that the nuclear export of Moesin is dependent on Nup98/Rae1. The depletion of Nup98 in live animals further confirmed the idea that Nup98/Rae1 are either involved in the nuclear export of Moesin or they all play role in mRNA export.

Nuclear Moesin is involved in transcription

To confirm or exclude the possibility that Moesin is needed for the formation of transcriptionally active chromatin puff structures at the sites of transcription, we induced transcription at a specific chromosomal location without the formation of the puff structure. After heat shock, we found an extra Moesin-positive band at this chromosomal location suggesting that Moesin is not required for the formation of the transcriptionally active chromatin structure. The treatment of Drosophila salivary glands with a drug called Triptolide which causes the disassembly of the transcription complex resulted in the disappearance of both the RNA PolII and Moesin signals providing further evidence that in the nucleus Moesin participates in transcription or transcription linked processes rather than in the formation of the chromatin structure.

Next, we transfected cultured Drosophila S2R+ cells with hemagglutinin tagged Moesin (HA-Moesin) and analyzed its chromatin bound levels by using chromatin

immunoprecipitation (ChIP). In the experiments we measured the HA-Moesin occupancy at the *Hsp70* gene before and after heat shock treatment. We observed that heat shock resulted in elevated binding of Moesin at the *Hsp70* gene body but not at the *Act42A* gene or intergenic regions supporting the idea that nuclear Moesin plays role in transcription.

To determine the phase of transcription in which Moesin is involved, we directly and systematically compared the distribution of Moesin along the chromosomes to the localization pattern of the active forms of Pol2 by using antibodies that recognize either the initiation-specific (Pol2-PS5) or the elongation-specific (Pol2-PS2) forms of Pol2. The triple immuno-staining experiments for Moe+Pol2-PS2+Pol2-PS5 revealed that the loci with strong initiation activity showed weak or moderate signal for Moesin whereas at the sites of elongation the localization of Moesin was strong suggesting that the protein is not involved in initiation but plays a role in elongation and/or in the steps following elongation. To analyze the role of Moesin in the termination phase of transcription, we employed the well-characterized transcriptional program of the wandering third-instar larval stage (Capelson et al. 2010). We monitored the ecdysone-induced puffs at positions 74EF and 75B, whose expression is induced during this period of development, and the *Sgs3* intermolt gene at location 68C, which is turned off by ecdysone induction. The analysis of Moesin recruitment to gene loci 74EF/75B and 68C during development revealed an accumulation of Moesin in parallel to Pol2-PS2 enrichment (elongation) as well as a loss of Moesin signal as transcription terminated. These results together revealed that Moesin binds genes that are undergoing activation, and its function is coupled to the elongation phase of transcription at the target loci.

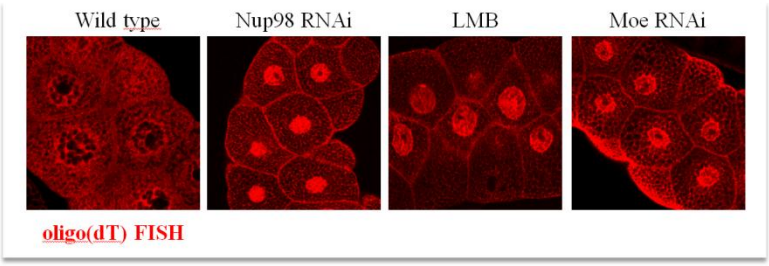
Moesin plays role in mRNA export

During these experiments, we noticed that in the chromosome preparations Moesin staining also gives a punctate pattern outside the chromatin. Therefore, since Moesin seemed to be involved in transcription elongation or in the steps following elongation we examined if these dots correspond to splicing speckles or the messenger ribonucleoprotein (mRNP) particles. The co-immuno staining for endogenous Moesin and the nuclear speckle marker protein, SC35 in salivary gland nuclei did not reveal any co-localization. In contrast, the double labeling of polytenic chromosome preparations for Moesin and Rae1 or Moesin and the poly-A Binding Protein confirmed that the spots Moesin localizes to are mRNP particles. This observation together with our previous finding that the depletion of the mRNA export proteins Nup98 or Rae1 leads to the nuclear accumulation of Moesin strongly support the idea that in the nucleus Moesin is involved in mRNA export.

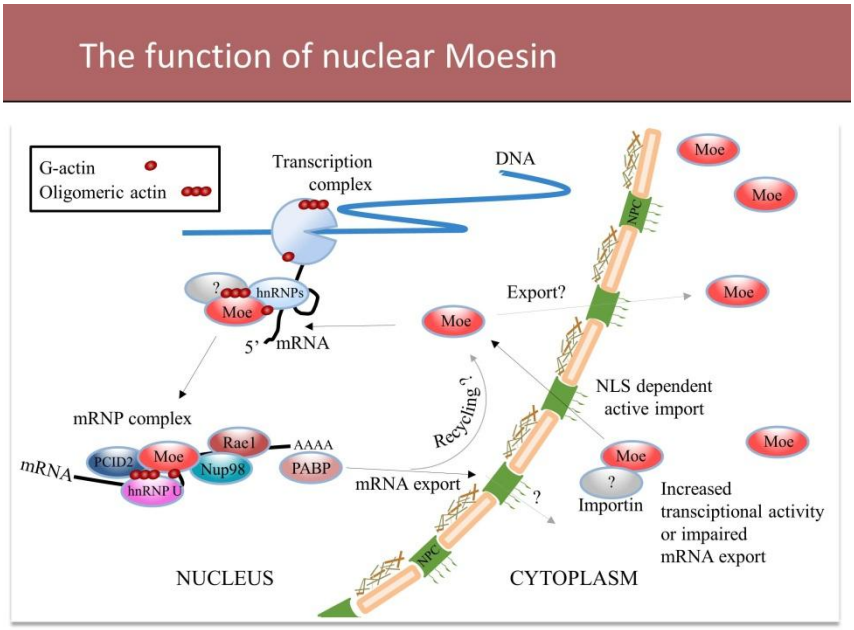
The analysis of genetic interaction and co-localization between Moesin and its predicted nuclear interacting partners did not lead to new results, we could not identify new interacting partners for Moesin in the interphase nucleus by these methods. As a next approach, we used the sensitive and relatively new DuoLink (PLA) technology to test for molecular interactions but the system did not work in our hands. Therefore, to confirm that Moesin is a member of mRNP complexes, we performed an immuno-precipitation screen with Drosophila mRNA export factors and Moesin. We optimized the isolation of nuclear protein fraction of cultured S2R+ Drosophila cells and used the GFP tagged versions of Nup98, Rae1 and the V5 epitope tagged PCID2, ZC3H3, l(1)10Bb, CG2063, CG2685, CG14701, CG31126 proteins. In this experiment the physical interaction between Rae1 and Nup98 reported before (Blevins et al.) has been confirmed, and the PCI domain-containing Protein 2 (PCID2) was successfully co-precipitated both with the full-length Moesin protein and the Moesin FERM domain alone. The PCID2 protein is part of the TREX-2 mRNA export complex and in *Drosophila* it was shown to associate with NXF1 and affect mRNA export from the nucleus (Farny et al.). All these data strongly suggest that through its FERM domain Moesin binds the mRNP complex

member PCID2 thus further supporting the view that in the nucleus Moesin participates in mRNA export.

To confirm the idea that Moesin is involved in nuclear mRNP export, we performed a functional test by monitoring the effect of Moesin knock down on the nuclear export of mRNAs. Similarly to the positive controls (Nup98 RNAi and LeptomycinB treatment), mRNA accumulated in the nucleus of Moe RNAi treated salivary gland cells, as revealed by the strong nuclear accumulation of the oligo(dT) FISH *in situ* signal (see figure).



Since our results suggested that Moesin participates in mRNA editing and/or transport and not directly in transcription, the Affimetrix chip experiment, originally planned for the second year to investigate the possible role of Moesin in transcription, has not been carried out. We applied more simple and less expensive approaches (co-localization and immuno precipitation) instead of the chip method. We think that this change did not hinder the successful completion of the project.



In sum, we demonstrated that the Drosophila ERM protein, Moesin is a functional component of the interphase nucleus where it participates in mRNA export. In accordance with the research plan, the results of the second and third years have been presented in the form of posters and oral presentations at four international and six domestic conferences. We published two review papers about this topic and submitted two research papers for which we are currently finishing the last experiments required by the reviewers.

References:

Blevins MB, Smith AM, Phillips EM, Powers MA. 2003. Complex formation among the RNA export proteins Nup98, Rae1/Gle2, and TAP. J Biol Chem 278(23):20979-88.

Capelson M, Liang Y, Schulte R, Mair W, Wagner U, Hetzer MW. 2010. Chromatin-bound nuclear pore components regulate gene expression in higher eukaryotes. Cell 140(3):372-83. doi: 10.1016/j.cell.2009.12.054

Carreno S, Kouranti I, Glusman ES, Fuller MT, Echard A, Payre F (2008) Moesin and its activating kinase Slik are required for cortical stability and microtubule organization in mitotic cells. J Cell Biol 180(4): 739–46. http://dx.doi.org/10.1083/jcb.200709161

Farny NG, Hurt JA, Silver PA. 2008. Definition of global and transcript-specific mRNA export pathways in metazoans. Genes Dev 22(1):66-78.

Kunda P, Pelling AE, Liu T, Baum B (2008) Moesin controls cortical rigidity, cell rounding, and spindle morphogenesis during mitosis. Curr Biol 18(2): 91–101. http://dx.doi.org/10.1016/j.cub.2007.12.051