Sarcomeres are the smallest structural and functional units of muscles. Whereas the structural properties of the sarcomeric complexes have been resolved in details over the past decades, much less is known as to how these filament systems assemble *in vivo*. In particular, the initial assembly of thin filaments, the regulation of actin dynamics and organization during myofibril formation and maintenance remained poorly understood. The major aim of our OTKA grant was to better understand the molecular mechanisms of myofibrillogenesis by focusing on the functional analysis of some key players, such as the formin DAAM (Dishevelled Associated activator of Morphogenesis) and the actin elongation promoting Sarcomere Length Short (SALS) protein, as well as that of Flightless-I (Fli-I), Tmod and Lmod2.

Below I will summarize our most important findings in 6 major points.

1. Studies on the muscle functions of DAAM, a formin type of actin assembly factor

By capitalizing on our preliminary data, we established that the Drosophila and mouse members of the DAAM formin subfamily are sarcomere associated actin assembly factors. Analysis of Drosophila DAAM mutants revealed a pivotal role in myofibrillogenesis of larval somatic muscles, indirect flight muscles and the heart. We found that loss of dDAAM function results in multiple defects in sarcomere development including thin and thick filament disorganization, Z-disc and M-band formation, and a near complete absence of the myofibrillar lattice. Our data suggested that besides a role at the barbed end of the thin filaments, dDAAM also functions at the pointed end where it antagonizes the capping protein Tropomodulin (Tmod). Based on these observations, we proposed that DAAM family formins are very good candidates for being the long sought-after muscle actin nucleators, that also promote filament elongation by assembling short actin polymers that anneal to the Z-disc anchored growing filament (**Molnar et al., 2014; PLOS Genetics**). At that time it was a novel model that we aimed to critically test during the course of our subsequent studies.

During the initial phase of our project, we teamed up with the laboratory of Rolf Bodmer (Sanford-Burnham Medical Research Institute, La Jolla, USA) to show that in the developing heart tube the expression and activity of DAAM is controlled by the Tinman/Nkx2-5 transcription factor and Cdc42 (a Rho GTPase), respectively. We found that DAAM, together with the non-muscle myosin Zipper, is required for proper heart lumen formation through the spatiotemporal regulation of the actomyosin network (**Vogler et al., 2014; Journal of Cell Biology**). Therefore, these results further strengthened our former conclusion that DAAM family formins are important regulators of myofibrillogenesis not only in striated muscles but also in the heart muscle.

In order to complete the biochemical characterization of DAAM, critical for sarcomere assembly, we studied the role of the C-terminal regions of Drosophila DAAM in its interaction with actin with *in vitro* biochemical and *in vivo* genetic approaches. We found that the DAD-CT region binds actin in vitro and that its main actin-binding element is the CT (C-tail) region, which does not influence actin dynamics on its own. However, we also found

that it can tune the nucleating activity and the filament end–interaction properties of DAAM in an FH2 domain-dependent manner. We also demonstrated that DAD-CT makes the FH2 domain more efficient in antagonizing with capping protein. On the basis of our findings, we proposed an alternative model of the structural and functional modes of formin:actin interaction (**Vig et al., 2017, JBC**). These data were helpful to better assess the potential molecular function of DAAM at the barbed and pointed end of the sarcomeric actin filaments.

We noticed that muscles express two DAAM isoforms, a shorter DAAM-PB and a longer DAAM-PD isoform, the latter is exhibiting a much higher expression level than the other. Thus, one of the major goals of our proposal was to characterize the biophysical and *in vivo* properties of the long isoform of DAAM (DAAM-PD). To this end, to study the muscle function of DAAM-PD, we created a PD isoform specific antibody, a transgene and a mutant allele. With the help of these tools we determined that the PD isoform is essential for viability as well as for trachea and muscle development. Intriguingly, the isoform specific antibody not only confirmed the trachea and muscle specific accumulation of the protein, but also allowed us to reveal that the PD isoform is highly enriched at the Z-disc (barbed end) whereas the PB isoform is enriched at the M-line (pointed end). By using biochemical assays, we found that the presence of the purified PD-specific domain (consisting of 310 aminoacids) has no significant influence on the actin assembly activity of DAAM. This suggested that the PD domain is not involved directly in the modulation of the actin assembly activity, instead it may play a role in the subcellular localization or targeting of DAAM to a specific area at the Z-disc. In agreement with this, we revealed that after muscle specific overexpression of the PD-specific domain, this truncated protein exhibits a specific localization pattern in sarcomeres even in the absence of the endogenous protein. Part of these protein localization data were further verified and refined with superresolution microscopy (see below in point 6.); and the manuscript describing that analysis is currently under preparation.

As the vertebrate DAAM orthologs are known to be expressed in muscles, we wanted to test for evolutionary conservation. By using the C2C12 myogenic cell line we examined the localization pattern of mDaam1 during myogenic differentiation. We revealed that mouse Daam1 (mDaam1) is expressed from the early stages of myogenesis (already 24 hours after induction) and the expression is maintained at least until 14 days. The protein accumulates into two broad bands along the M-line which is consistent with a role in actin regulation and which is highly similar to the localization pattern of DAAM in Drosophila larval muscles. Similar staining patterns were found in cardiomyocytes induced from embryonic stem cells. With regard to mDaam2 we found that the gene is also expressed in induced C2C12 cells and cardiomyocytes. Together, these results support for an evolutionary conserved role in muscle development (**Molnar et al., 2014; PLOS Genetics**), and they indicate the possibility of redundant functioning of mDaam1 and mDaam2 during myogenesis.

As a summary of this part, we identified an important and evolutionary highly conserved new player of sarcomeric thin filament formation. Functional characterization of this formin protein allowed us to propose a novel model for sarcomeric thin filament formation.

2. Functional analysis of the SALS protein.

Proper development of Drosophila muscles requires the activity of the SALS protein. In particular, SALS is required to promote thin filament elongation from the pointed end with an unknown mechanism. To begin to analyze how SALS interacts with actin, we first focused on its "canonical" actin binding domains, the two tandemly located WH2 domains. We produced recombinant SALS constructs containing the isolated WH2 domains and analyzed their interactions with actin monomers and filaments by using a combination of protein biochemical, fluorescence spectroscopy and microscopy approaches. We found that the isolated WH2 domains of SALS possess sequestration and depolymerizing activities. These functions support the accumulation of monomeric actin *in vitro*, which does not reconstitute the *in vivo* activity of the full-length protein, but it is in line with the dominant negative effect observed upon muscle specific expression of the WH2 containing truncated forms. Based on these findings we proposed that regions of SALS other than the WH2 domains and/or other sarcomeric proteins (such as tropomyosin) must play essential roles in adjusting and adapting the activity of SALS for its proper biological function (**Toth et al., 2016, JBC**).

To extend our *in vitro* studies on the effects of SALS on actin dynamics, we investigated the full length SALS protein, as well as its N-terminal and C-terminal regions (N-SALS and C-SALS, respectively) in pyrene labeled actin based fluorescence spectroscopy assays. We found that both SALS and C-SALS inhibit actin assembly, presumably by sequestration. In contrast, we have not detected any effect in the presence of N-SALS. To dissect the functional properties of SALS, a structure-function analysis was carried out with Drosophila transgenic lines that carry the same subfragments of SALS that were tested *in vitro*. These truncated constructs were assayed for protein localization and rescue abilities in a *sals* mutant genetic background (**Figure 1.**). We found that N-SALS mainly accumulates at the Z-disc (barbed end of the actin filaments), while a weak signal can also detected at the M-line (pointed end of the thin filaments), and the presence of N-SALS is not sufficient to rescue the lethality of *sals*. Conversely, C-SALS displays a predominantly M-line accumulation, and it is able to nearly fully rescue the lack of *sals*. In addition to these studies, we used an affinity purification assay followed by LC-MS/MS analysis to determine the molecular interaction partners of SALS in adult muscles. These experiments suggested that the pointed end capping Tmod protein is the main partner of SALS (also confirmed by co-IP experiments). Together, these findings suggested a new model for SALS function in pointed end elongation: the major function of SALS is to bind Tmod and prevent its pointed end capping activity. We plan to publish these results in near future.

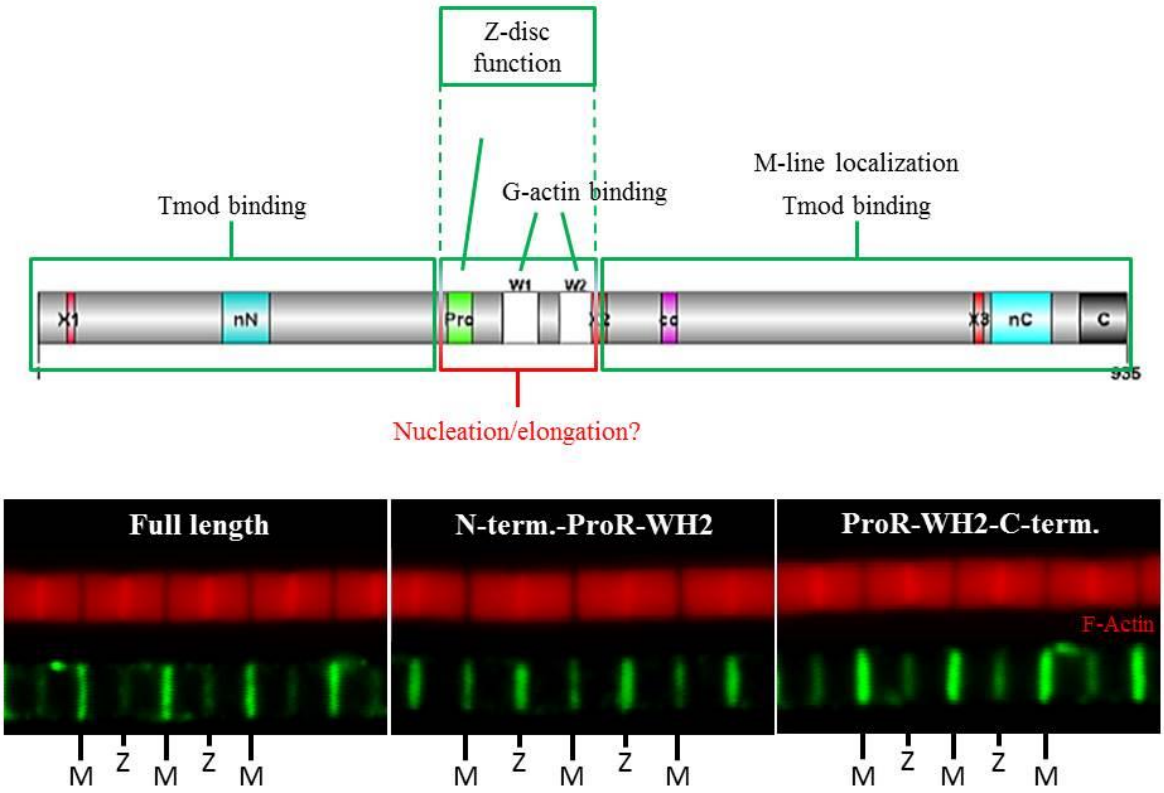

Figure 1. Upper panel: schematic structure of the SALS protein and the results of our structure-function analysis. **Lower panels**: sarcomeric localization of the full-length, N-SALS and C-SALS proteins.

3. Structural features and activities of the Lmod2 protein

To gain a more complete picture about the possible mechanisms of sarcomeric thin filament formation, we started to analyze the structural features and the activities of cardiac leiomodin2 (Lmod2) in actin dynamics as Lmod2 is considered to be the functional ortholog of Drosophila SALS. We found that Lmod2 accelerates actin assembly in an ionic strength dependent manner, which relies on its N-terminal regions. We also show that Lmod2 binds to the sides of actin filaments and induces structural alterations in actin filaments. Upon its interaction with the filaments Lmod2 decreases the actin-activated Mg2+-ATPase activity of skeletal muscle myosin. Our observations suggest that through its binding to the side of actin filaments and its effect on myosin activity, Lmod2 has more functions in muscle cells than it was indicated in previous studies (**Szatmari et al., 2017; PLOS One**).

4. Functional properties of the Flightless-I protein

Drosophila Flightless-I (Fli-I) is another protein that has been linked to thin filament formation, however, the molecular mechanisms of Fli-I are virtually unknown. To begin to address that issue we started to investigate the activities of the recombinantly produced gelsolin homology (GH) domains of Fli-I in *in vitro* experiments (pyrene-actin based

fluorescence kinetics, steady-state anisotropy, TIRF microscopy, sedimentation). Our data suggest that Fli-I is an actin filament barbed end capping protein and this activity relies on its GH1-3 domains, while the isolated GH4-6 and LRR regions do not influence actin dynamics in our experiments. We are currently summarizing these results in the form of a manuscript and plan to submit it in the near future.

Based on the analysis of the human proteins, the GH4-6 domains of Fli-I directly interact with the C-terminal DAD of DAAM, and Fli-I GH4-6 was proposed to enhance the GTP-RhoA-mediated dissociation of the DID domain from the DAD segment, thereby potentially regulating the inactive-active transformation of DAAM. Our fluorescence spectroscopy investigations showed that Drosophila Fli-I GH4-6 does not have any effect on spontaneous actin dynamics. In contrast, it inhibits DAAM catalyzed actin assembly, which is specific to the DAD-CT containing CDAAM construct but not to FH1-FH2 lacking the C-terminal regions. This observation suggests that Fli-I interacts with DAAM and it is mediated by the C-terminal regions of the protein, but instead of promoting the actin assembly activities of DAAM, Fli-I seems to restrict DAAM mediated actin filament formation in the *in vitro* context. In our subsequent studies we aim to investigate the underlying molecular mechanisms and the functional outcome of the concerted action of DAAM and Fli-I as two barbed end capping proteins in actin polymerization.

5. Comparative investigations of the gelsolin homologue domain proteins

To better understand how Fli-I could function in muscles at the molecular level, we initiated a comparative study of two gelsolin homology domain proteins: Fli-I and gelsolin (GSN). It is well established that the actin activities of gelsolin are regulated in a Ca-dependent manner and the structural rearrangements in the molecule upon Ca-binding are essential for its actin interactions. In fluorescence spectroscopy experiments (using Atto488 ATP) we showed that purified gelsolin binds ATP and as a potential functional consequence this interaction can have importance in the activation cycle of the protein. In contrast, no such interaction was detected in case of Fli-I. Consistent with this, the residues critical for ATP binding of gelsolin are not conserved in Fli-I. On the other hand, our investigations revealed that Fli-I interacts with actin and caps filament barbed ends in a Ca-independent fashion. Altogether the above observations suggest that the activities of gelsolin and Fli-I are regulated differently. We hypothesize that the structure of the two GH-domain proteins differ. To test this assumption fluorescence spectroscopy experiments were performed (quenching of fluorescence by acrylamide, steady-state anisotropy measurements). In support of our hypothesis, the values of both anisotropy and the Stern-Volmer constant indicate that the tryptophan residues of GSN are more exposed in response to Ca-addition as compared to that of measured in Ca-free environment. In contrast, no such change was observed for Fli-I. We plan to continue the comparative structural analysis of these GH proteins by differential scanning calorimetry and circular dichroism.

Within the framework of these studies, the biochemical properties of GSN was successfully investigated with regards to other interesting aspects, yielding to several

publications (**Szatmari et al., 2018; PLOS One; Kis-Bicskei et al., 2018; Biophysical Journal; Horváth-Szalai et al., 2017; CLINICAL BIOCHEMISTRY**)

6. Superresolution microscopic analysis of structural sarcomeric proteins.

Besides the functional studies on sarcomere assembly, one of our main goal was to deepen our knowledge about the structural organization of the myofibrils by using state-of-the-art microscopic methods. To this end, we compared EM and three major superresolution techniques (STED, SIM and STORM), and found that among these STORM provides far the best resolution on individual myofibrils. This approach led us to determine the sub-sarcomeric position of a large set of muscle proteins with a previously unprecedented 10 nm resolution. Most notably, we uncovered the position of nearly all actin regulatory proteins, such as DAAM, Fhos (another formin involved in sarcomere formation), SALS, Tmod, Tropomyosin, Profilin and Fli-I (**Figure 2.**). These observations provided a number of novel insights into the molecular architecture of the sarcomeres, and among others, revealed a close colocalization of Tmod and SALS further confirming the possibility of direct binding. Together these results will allow us to draw a novel model both for the mechanism of actin filament elongation and for molecular organization of the sarcomeres at the Z-disc and at the M-line.

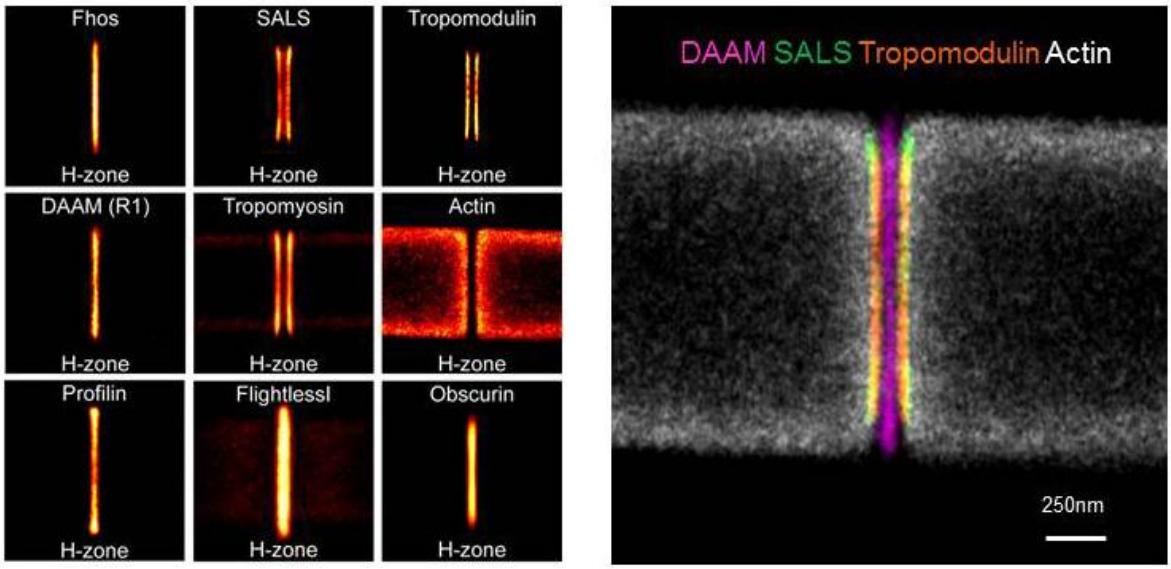

Figure 2. Sarcomeric localization of a set of actin regulatory proteins as revealed by STORM microscopy.

In summary, we applied a complex methodology to gain new insights into the structural and mechanistic aspects of myofibril formation. Our studies led to the publication of 7 papers directly linked to the main topic of our application, and helped the publication of 10 additional papers that are more loosely related to the muscle topic. In addition, currently we are preparing an important manuscript based on a large set of STORM data that allows

us to construct a novel structural model of myofibrils and that we hope to become a seminal paper in the field.