Doctoral School of Multidisciplinary Medical Sciences

Biochemistry, Biophysics, Molecular and Cell Biology Programme

Faculty of Medicine

UNIVERSITY OF SZEGED

Ph.D. Thesis

The biological significance of nuclear actin

Péter Borkúti

Supervisor: Péter Vilmos Ph.D.

2022.

Publications related to the subject of the Ph.D. thesis

I. Borkúti, P., Kristó, I., Szabó, A., Bajusz, C., Kovács, Z., Réthi-Nagy, Z., Lipinszki, Z., Lukácsovich, T., Bogdan, S., & Vilmos, P. (2022). Parallel import mechanisms ensure the robust nuclear localization of actin in *Drosophila*. *Frontiers in Molecular Biosciences*. https://doi.org/10.3389/FMOLB.2022.963635
IF: 6.113 (Q1)

II. Bajusz, C., Kristó, I., Abonyi, C., Venit, T., Vedelek, V., Lukácsovich, T., Farkas, A., Borkúti, P., Kovács, Z., Bajusz, I., Marton, A., Vizler, C., Lipinszki, Z., Sinka, R., Percipalle, P., & Vilmos, P. (2021) The nuclear activity of the actin-binding Moesin protein is necessary for gene expression in *Drosophila*. *The FEBS Journal*, 288(16), 4812–4832. https://doi.org/10.1111/FEBS.15779
IF: 5.62 (Q1)

III. Borkúti, P., Bajusz, I., Bajusz, C., Kristó, I., Kovács, Z., & Vilmos, P. (2019) Testing the biological significance of the nuclear localization of actin. *Biopolymers and Cell*, 35(3), 204–204. https://doi.org/10.7124/BC.000A06
IF: 0.218 (Q4)

Cumulative impact factors of papers directly related to the subject of the thesis: 11.951

Publications not directly related to the Ph.D. thesis

I. Bajusz, C., Borkúti, P., Kristó, I., Kovács, Z., Abonyi, C., & Vilmos, P. (2018) Nuclear actin: ancient clue to evolution in eukaryotes? *Histochemistry and Cell Biology*, 150(3), 235–244. https://doi.org/10.1007/s00418-018-1693-6
IF: 2.64 (Q1)

List of abbreviations

ABPs – actin binding proteins
BDSC – Bloomington Drosophila Stock Center
BiFC – bimolecular fluorescence complementation
DGRC – Drosophila Genomics Resource Center
GFP – Green fluorescent protein
IVTT – *in vitro* transcription and translation
LMB – leptomycin B
NES – nuclear export signal
NLS – nuclear localization signal
NPC – nuclear pore complex
Pi – phosphate
P-TEFb – Positive transcription elongation factor b
PTMs – posttranslational modifications
RNAPII – RNA polymerase II
SUMO – Small ubiquitin-like modifier
YFP – Yellow fluorescent protein

1. INTRODUCTION

1.1. Actin, one of the most ancient proteins

Actin was first isolated by Brúnó F. Straub in 1942, in Szeged. It is one of the most abundant proteins in eukaryotic organisms, with evolutionarily highly conserved structure, and ancient origin. Actin's earliest form appeared in the common ancestors of bacteria, archaea and eukarya. In regard of their structure, prokaryotic actin-like proteins (e.g. MreB, FtsA and ParM), eukaryotic actin-like proteins (e.g. ARPs and HSPs) and eukaryotic actin all show globular tertiary structure, composed of four domains. The structurally formed lobes are divided by an ATP binding, deep cleft in all these proteins. The common functional and structural properties of these proteins unequivocally outline an ancient and divergent actin superfamily.

Prokaryotes utilize the 'one-filament-one-function' system to maintain and organize their cytoskeleton. For example, MreB functions as cell shape maintainer and it also recruits the key machineries for cell wall synthesis. Another notable actin-like protein in prokaryotes is FtsA, which is essential for the proper assembly of cell division machinery. During cell division, bacterial plasmids are segregated by the actin-like protein ParM.

On the contrary, eukaryotes use actin as the single protein that is responsible for fundamental processes like cytoskeleton organization, cell motility, and intracellular trafficking, just to mention a few. Eukaryotic cells don't need to maintain high levels of actin-like proteins, and without the many function-specific actin variants, actin can be involved in a wider range of biological processes.

Although, the number of actin genes varies widely, the evolution of eukaryotic actin is in a "frozen state", the protein of different eukaryotic organisms is highly conserved. The emergence of this universal-actin-pool system is the result of the evolution of a more complex level of regulation that allows the maintenance of a large number of individual functions of actin. This higher level of regulation is provided by a large number of actin binding proteins. Interestingly, the sequence homology they share with conventional actin varies between 15-70%.

1.2. Actin is a cytoskeletal protein

1.2.1. Structure of G- and F-actin forms

Actin is present in eukaryotic cells either in monomeric, G-actin form or in polymeric filamentous form, F-actin. In human, monomeric actin has 3 isoforms: α-actin (present in muscle cells), β-actin (present in non-muscle cells) and γ-actin (present in both muscle and non-muscle cells. These isoforms differ only in a few amino acids on their N-terminal end, that most certainly is responsible for functional differences of actin isoforms, resulting in

morphologically and cytoarchitecturally different cells, in vertebrates. However, in the case of *Drosophila melanogaster*, differences in the amino acid sequence of the six actin isoforms do not have any functional role.

The functions of actin are also altered by posttranslational modifications. Such modifications include sumoylation, acetylation, methylation or ribosylation. Upon sumoylation, small ubiquitin-like modifier (SUMO) protein targets and covalently binds to specific lysines in the conserved sumoylation motif of proteins. This leads to alterations in gene transcription, protein-protein interaction or cellular translocation. In human, lysines in position 68 and 284 are necessary for actin sumoylation. The former is responsible for the stabilization of actin-SUMO interaction, while the latter blocks the accessibility of actin's nuclear export signal (NES), therefore directly affecting the nuclear-cytoplasmic translocation of actin.

Since the initial mapping of actin structure, it is well known that the 375 amino acid long polypeptide chain forms 4 subdomain that forms the globular structure of actin. Actin can also be divided into a small and a large subunit based on the size of the subdomains. The polypeptide chain between these two domains forms two clefts. The upper cleft of actin is able to bind ATP and Mg^{2+} while the lower cleft serves as the binding site for a variety of actin binding proteins (ABPs).

As mentioned before, actin is present in cells in either G- or F-actin form. The forming of F-actin originates from an actin nucleus, with the assembly of 3-4 G-actin molecules. The growing filament has structural polarity, which comes from the different speed of monomer incorporation into the filament. The incorporation is energy dependent, where the bound ATP is slowly hydrolysed into ADP and phosphate (Pi). The hydrolization and release of ADP and Pi is much slower than the assembly kinetics of the filament, ultimately creating ATP or ADP+Pi enriched and ADP bound filament ends. The fast-growing end is called barbed end, while the opposite end of the filament is the slow-growing pointy end. This process is especially energy demanding and slow, and the fast growing actin filament formation is only achievable with the help of ABPs, *in vivo.*

1.2.2. Cytoplasmic functions of actin

Beside the organization of the cytoskeleton, actin is involved in numerous cellular processes like for example plasma membrane internalization, cytokinesis or muscle contraction. The involvement of actin in these processes is aided and tightly regulated by the different actin binding proteins. With the help of different ABPs, actin patches are formed at the site of membrane internalization, and the mechanical forces coming from the growing actin filaments

results in the formation of endocytic vesicles. In the case of cytokinesis and muscle contractions, actin working together with different type of myosin proteins, the length of actin filaments is shortened, resulting in either the separation of dividing cells, or in macroscopic movement of muscle contractions. These are just a few simple examples to give a glimpse of the diverse cellular functions that actin takes part in with ABPs.

1.3. Nuclear actin

1.3.1. Discovery and forms of nuclear actin

Early observations on nuclear actin were widely controversial. It was first discovered in nuclear subcellular fractions of calf thymus cells, and later, with the help of electron microscopy, researchers successfully observed fibrillary actin bodies inside the nucleus which are formed in response of cellular stress. Today it is undisputed, actin is not only present in the nucleus, but also has a number of nuclear functions inside the compartment.

Within the nucleus, three forms of actin are known in the nucleus. Nuclear monomeric actin is considered to be identical to cytoplasmic G-actin, but the exact posttranslational modifications and binding partners of nuclear actin are still largely unknown. Nuclear actin oligomers lack an obvious filamentous structure, but there are some reports of functionally active nuclear actin filaments as well. The third known form is nuclear actin rod, which, unlike cytoplasmic F-actin, is not detectable with phalloidin. This is likely due to the fact that nuclear actin rods are highly decorated with Cofilin, thus preventing phalloidin staining, which suggests the structural distinction from cytoplasmic actin filaments.

1.3.2. The nucleocytoplasmic transport of actin

The presence of actin both in the cytoplasm and the nucleus argues for the existence of a regulated, active nuclear transport that maintains stable actin levels in the cell compartments. However, experimental data suggest that, even though actin's 42 kDa mass falls within the range of passive diffusion barrier of NPCs (40-60 kDa), the protein is actively transported via NPCs. Actin lacks canonical nuclear localization signals (NLS), but one of its binding partner, Cofilin contains a bipartite SV40 type NLS. Dopie et al. demonstrated that actin is imported into the nucleus in complex with Cofilin. Their work also revealed that the Cofilin-actin complex does not translocate alone into the nucleus, but in complex with Importin 9.

In parallel, it is obvious that the nuclear export mechanism of actin also has to be a regulated active process. Actin has two putative NES signals that is rich in leucine and hydrophobic amino acids. This type of NES is recognized by CRM1, one of the major nuclear

exportin. Upon leptomycin B (LMB) treatment, CRM1 is inhibited by the drug and nuclear actin accumulation and rod formation is observed. This led to the conclusion that CRM1 is the main nuclear export factor of actin, but was met with criticism. LMB treatment causes cellular stress, and nuclear accumulation of actin is not directly linked to the actin exporting nature of CRM1. It was also experimentally proved, that human CRM1 does not recognize actin as a cargo.

Stüven et al. experimentally proved, that Exportin 6 forms a stable complex with the profilin-actin complex in the nucleus. The absence of Exportin 6 leads to the nuclear accumulation of actin, which CRM1 cannot prevent. These findings suggest that the Exportin 6-profilin-actin interaction forms the main export competent complex in vertebrates.

1.3.3. The functions of nuclear actin

The list of nuclear actin functions is rapidly growing since nuclear actin was found to be involved in almost all basic nuclear activities. The most known nuclear mechanisms that actin is implicated in, are transcription regulation, chromatin remodeling, DNA-damage repair, mRNA export, maintenance of nuclear structure and size, and pronucleus formation.

Actin always co-purifies with RNA polymerase II (RNAPII) complex. G-actin recruits Positive Transcription Elongation Factor b (P-TEFb) to activate RNAPII through phosphorylation. In polymeric form, actin interacts with nuclear myosin, and together they contribute to transcription.

Actin also directly interacts with transcription factors. G-actin can bind Myocardin-related transcription factor A (MRTF-A) in the cytoplasm. This masks the NLS of MRTF-A, thus preventing its translocation to the nucleus, and also inhibits the interaction with Serum Response factor (SRF). Upon serum induction, G-actin level rapidly decreases, and MRTF-A is released from the regulation of monomeric actin and can carry out its transcriptional activating task together with SRF.

Extensive research of the past decade shed light to actin's involvement in the modulation of chromatin organization. The Ino80-Arp4/Actin/Arp8 module has a linker DNA sensing activity and actin ATP hydrolysis is crucial for the movement of the DNA remodeling complex along the DNA. These three examples above do not summarize the complete activity of nuclear actin, they only give an idea on how diverse are the functions of actin in the nucleus.

1.4. *Drosophila melanogaster* is an appropriate model system to study nuclear actin

The model organism of our research was *Drosophila melanogaster*. *Actin* genes show redundancy and high evolutionary conservation also in the fly. From the six actin coding genes two are widely expressed, so called cytoplasmic forms (*Act5C* and *Act42A*), and four are muscle-specific isoforms (*Act57B*, *Act76B*, *Act87E*, and *Act88F*). The intracellular actin level is tightly regulated most likely through autoregulation. High G-actin level results in the degradation of actin mRNA, while low monomeric actin level increases actin mRNA synthesis.

The two cytoplasmic actins, Act5C and Act42A, differ only in two amino acids, which difference does not bear any functionality, the protein coding region of the two isoforms are substitutional. However, Wagner et al. showed that mutations in the *Act5C* gene are lethal, demonstrating non-redundant functions. The tissue specificity and relative levels of *Act5C* and *Act42A* transcripts are different. The *Act5C* mRNA level is particularly high in the developing brain, while *Act42A* mRNA is abundant in the gonad and in certain sections of the intestine. This suggests that the unique functions of cytoplasmic actins of *Drosophila* are not caused by the slight variation in the primary structure, but are rather due to differences in gene regulation.

As in eukaryotes, actin is present in the nucleus of *Drosophila melanogaster* cells, and our laboratory demonstrated that all six *Drosophila* actin isoforms localize to the nucleus. Act5C was the perfect candidate for our studies, since the structure and regulation of the fly *Act5C* gene is well described, its relatively small size makes the genetic and molecular manipulations easy, and the absence of the Act5C protein is lethal to the animal.

2. AIMS

Even today, the essentiality of nuclear actin remains poorly understood. In this work we aimed to investigate the biological significance of nuclear actin through testing the robustness of its nuclear localization. To this aim, we designed a genetic system in *Drosophila melanogaster* which would enable us to investigate this question at the level of the whole organism. With our genetic system we sought to answer the following specific questions:

1. Does an extra NES sequence decrease nuclear actin levels?
2. If yes, to what extent this affects the viability of the animal?
3. Can the absence of the previously described sumoylation of actin also decrease actin levels in the Drosophila cell nucleus?

4. Can the complete lack of RanBP9, the already known importin for actin, eliminate actin from the nucleus?
5. What is the effect of the complete lack of RanBP9 on the viability of the animal?
6. What is the consequence of the combination of these effects in terms of viability?
7. Are there actin import factors other than RanBP9 in *Drosophila*?

3. MATERIALS AND METHODS

3.1. Fly stocks and husbandry

All *Drosophila* stocks were maintained and crosses were carried out on standard cornmeal, yeast, sucrose *Drosophila* medium at 25°C unless otherwise noted. *Drosophila* stocks were obtained from Bloomington Drosophila Stock Center (BDSC) or Kyoto Stock Center. Stocks expressing transposase were kindly provided by Rita Sinka (Univ. of Szeged). Injection of *Drosophila* embryos were carried out by the BRC Drosophila Injection Facility (BRC, Szeged).

To investigated embryonic and larval lethal phenotypes, *Act5C* null mutant flies left to lay eggy on black agar medium. Egg laying was synchronized, and 200 individual eggs were collected. The development of animals was monitored for 72 hours.

3.2. Generation of null mutations

To create the *Act5C* null mutation, the stocks, crossing schemes, and instructions described by the DrosDel project were applied.

In order to delete the *RanBP9* gene, P-element carrying stock No. 205564 was crossed with flies carrying transposase source on their second chromosome balancer. The mutagenesis was performed using the standard *Drosophila* P-element-mediated mutagenesis method.

For the molecular characterization of mutant lines, genomic DNA was prepared according to the protocol of Cold Spring Harbor Protocols.

Mutant lines were validated by PCR amplification. Primers Act5CFup2 (5'-CCAGTTGCGGAGGAAATTCTC), Act5CRev2 (5'-ATGATGCGATTAAAGTGCCGT), and Pry4 (5'- CAATCATATCGCTGTCTCACTCA) were used to amplify the mutant *Act5C* gene. The primers RanBP9_Fw1 (5'-TTGTACTGAGCAGGCTTAACA) and RanBP9_Rev2 (5'-GGTTTGCATTCTAAAAGCCTCG) were used to determine the break points of the deletion in *RanBP9*. PCRs were performed according to the standard protocol for DreamTaq polymerase and amplicons were Sanger sequenced by Eurofins Genomics TubeSeq Service.

3.3. Creating Act5C expressing transgenic lines

For the generation of the transgenes expressing modified Act5C proteins under the regulation of the endogenous promoter, the 7.2 Kbp genomic region around Act5C was PCR amplified with the 5'for(Gateway) and 3'rev(Gateway) reverse primers using Q5 High-Fidelity DNA polymerase. The sequences encoding the NES and V5 epitope tags were cloned after the START codon of *Act5C* using overlapping PCR reactions and the following primers: 5'rev, 3'for, Actin-seq1, Actin-seq2, Bam-Mlu-Not-for, Bam-Mlu-Not-rev, Stop-for, Stop-rev, NESfor, NESrev, V5for, and V5rev (primer sequences can be found in the dissertation).

The Act5C genomic regions expressing modified Act5C proteins were inserted into pBluescript II SK vector, then subcloned with the BamHI + XhoI enzymes into the pAttB *Drosophila* transformation vector. The FLAG encoding dsDNA oligo was inserted downstream from the NES and V5 tags with the help of a unique MluI restriction site. The constructs were sequence verified and injected into *Drosophila* embryos carrying an attP integration site on their second chromosome at cytological location 25C6.

3.4. Rescue experiments

All rescue experiments were performed on normal Drosophila food media at 25^{o}C. Crossing schemes were planned and carried out by standard *Drosophila* mating methods. Virgin females were always collected from null mutant stocks, while males were collected from the transgenic "rescue stocks". Male progeny of the different genotypes was categorized by phenotypic markers.

3.5. Molecular cloning and DNA constructs

3.5.1. Cloning of Act5C-K285R expressing constructs

To acquire the *Act5C* gene at a manageable size for mutagenic PCR, a region of the previously amplified *Act5C* genomic fragment was excised from the Actin5C-pAttB construct with FastDigest HindIII restriction enzyme. Then fragment was gel-extracted and subcloned into pBSK+ vector using HindIII and T4 DNA ligase enzymes. The nucleotide substitution at K285 of Act5C was carried out on this construct with QuickChange II Site-Directed Mutagenesis Kit using Act5C_K285R_Fw and Act5C_K285R_Rev designed with the QuickChange Primer Design online tool of Agilent. All plasmid isolation was performed with High-Speed Plasmid Mini Kit. After sequencing, the Act5C-K285R-pBSK+ construct was digested with the HindIII enzyme. The fragments of the correct size were extracted from agarose gel, and the Act5C-K285R-pAttB construct was created by T4 DNA ligase. The

validated Act5C-K285R-pAttB construct was injected by the BRC Drosophila Injection Facility (BRC, Szeged) into embryos carrying an AttP integration site at cytological location 25C6 (BDSC stock #25709). Three transgenic fly stocks were generated via standard *Drosophila* mating methods.

For the experiments in cultured *Drosophila* cells, the mutagenesis reaction was performed on the Act5C-pAGW construct with the aforementioned mutagen primers, and the construct was validated by sequencing.

3.5.2. Gateway cloning of importin CDS for BiFC experiments and *Act5C* for *in vitro* pull-down experiments

The cDNAs of importin candidates obtained from the Drosophila Genomics Resource Center (DGRC) Gold Collection were PCR amplified with Q5 High-Fidelity DNA polymerase using gateway cloning primers.

Entry clones were generated by recombining the PCR products with Gateway BP Clonase II Enzyme mix into the pDONR221 plasmid. After sequencing the clones, the CDSs were subcloned with Gateway LR Clonase II Enzyme into split YFP tagging vectors (Gohl et al. 2010). Actin was labelled at the amino-terminus with the N-terminal fragment of YFP, while bait proteins were tagged both at the N- and C-terminal ends with the C-terminal fragment of YFP. To generate His-tagged actin, the CDS of non-polymerizable $Act5C^{R63D}$was cloned into pET16b vector with LR reaction.

3.6. Cell culturing

The S2R+ *Drosophila* cell line was maintained at 25°C in Schneider's *Drosophila* medium complemented with 10% Fetal Bovine Serum and 1% antibiotics (Penicillin-Streptomycin). The Effectene Transfection Reagent Kit was used to transfect cells according to the manufacturer's instructions. For the live imaging of cells, $8x10^6$ cells/35 mm glass bottom petri dishes were used.

For the importin screen, prey and bait expressing constructs were co-transfected with pMT-Gal4 vector expressing Gal4 under the control of an inducible metallothionein promoter. Two days after transfection, $CuSO_4$ was added to the cells in 1 mM final concentration to induce protein expression. After two hours, the $CuSO_4$ containing medium was replaced with 3 ml of fresh medium and the interaction was visualized with a Zeiss LSM800 confocal microscope.

3.7. Immunohistochemistry

All steps of the immunostaining experiments were carried out at RT. For larval salivary gland dissections, laid eggs were allowed to develop for 3 days, then L3 stage larvae were dissected in 4% paraformaldehyde-PBS followed by a washing step with PBS. Glands were permeabilized with PBT for 20 minutes. Non-specific reactions were blocked with PBT-N solution for 1 hour. Next, samples were incubated overnight (O/N) with anti-FLAG primary antibody at 4°C. After washing three times with PBT for 10 minutes, samples were incubated in PBS containing Alexa488 coupled anti-mouse secondary antibody and DAPI (0.2 μg/ml) for 1 hour in the dark. After a final wash with PBS, stained glands were transferred to microscope slides, mounted in 20 μl Fluoromount-G medium, and imaged with a confocal microscope (Olympus Fluoview FV1000).

3.8. Database search, quantification of pixel intensities and statistical analysis of data

The nuclear-cytoplasmic fluorescence intensity ratio was measured with Image J software. ROIs in the nucleus and the cytoplasm were drawn by hand. In every experiment 3-6 larval salivary glands, with a minimum of 5 cells/gland, were measured. Mean value and standard deviation (SD) were calculated with Microsoft Excel, while unpaired t-tests for statistical analysis and graph creation were performed with GraphPad Prism 9.

3.9. *In vitro* pull-down experiments

3.9.1. Recombinant protein purification

For protein purification, the Actin5C^{R63D}-pET16b construct was transformed into *E. coli* SixPack competent cells. A single transformed colony was grown in 50 ml of standard liquid growth medium until the culture's density reached 0.6-0.8 OD. Protein expression was induced O/N with the addition of 0.5 mM IPTG. Induced bacteria were washed and lysed by sonication in Lysis Buffer 10 ml + 1,5 ml EDTA free 7X PIC. The lysate was centrifuged for 15 minutes with 10,000 x *g* at 4°C. HisPur Cobalt Resin beads were washed with 2ml of Lysis Buffer + 0,5 mg/ml BSA, for 5 minutes, at 4°C, two times. Beads were centrifuged at 500 x *g* for minutes, at 4°C. Following the centrifuge step, 10 ml of lysate, supplemented with 50 μl BSA (100 mg/ml) was added to the cobalt beads and the mixture was incubated for 2 hours at 4°C, on rotating platform. After incubation, the beads were washed 3 times, for 20 minutes with 5 ml of Wash Buffer and centrifuged at 500 x *g* for 5 minutes at 4°C and. Finally, His-Act5C^{R63D} protein (bait) immobilized to beads was stored in 250 μl storage buffer at -20°C.

3.9.2. *In vitro* transcription and translation (IVTT) of prey proteins

Coupled *in vitro* transcription and translation of ^{35}S-labelled importin candidates was carried out using the TNT Quick Transcription/Translation System as described earlier. Purified His-Act5C^{R63D} was used as bait while His-GFP-FLAG served as negative control. Briefly, for each IVTT reaction, 90 ng of T7-promoter-regulated cDNA template (Tnpo-pOT2, Tnpo-SR-pOT2 RanBP9-pOT2, RanBP11-pOT2, Cadmus-pOT2, Ketel-pOT2, Msk-pHY22, Artemis-pHY22) was used in 15 µl reaction volume containing 11 µl TNT master mix, 0,33 µl PCR enhancer, 0.54 µl MBq ^{35}S-methionine, 0,33 µl U RNAsin and 0,33 µl 50x EDTA-free protease inhibitor cocktail. The reaction was carried out at 30°C for 1 h followed by centrifugation at 4°C, for 5 min at 17,000 x *g*. From the supernatant 0.25 µl was used as input, while 7,3 µl was used for each *in vitro* pull down assay.

3.9.3. *In* vitro His-pull down assay and autoradiography

The *in vitro* pull-down assay was based on the method described earlier. His-FLAG-EGFP or His-Act5C^{R63D} immobilized onto Cobalt beads were washed with Pull-down Wash Buffer 1 and mixed with the IVTT-produced ^{35}S-labeled prey proteins in Binding Buffer for 1.5 hours at 4°C. Beads were washed three times with PDWB1, followed by 3 washes with Pull-Down Wash Buffer 2. Finally, beads were collected in 1x SDS Sample Buffer and boiled for 5 minutes. Proteins were separated in 10% SDS-PAGE and blotted onto PVDF membrane. The membrane was dried and directly used for autoradiography. Exposure to autoradiography film (Kodak, Biomax MS) was carried out at -80°C using Low energy transcreen (Kodak Biomax LE).

4. RESULTS

4.1. The basic concept of the genetic system that was developed to investigate the functions of nuclear actin

The complete lack of *Act5C* function causes lethal phenotype, and the gene itself is relatively small sized, making experimental manipulations easier. Since all actin coding genes are highly conserved in flies, the CRISPR/Cas9 gene editing system is not applicable to modify *Act5C in vivo.* Therefore, *Act5C* was edited *in vitro*, and afterwards reintroduced into the genome as a transgene. In parallel, the endogenous *Act5C* gene was deleted, thus eliminating wild type *Act5C*, while modified Act5C expressing transgenes helped to observe the effects of changes in nuclear actin levels.

4.2. Generation and molecular characterization of *Act5C* null mutant flies

Since *Act5C* null mutant stock is not available, we used the Drosdel P-element system to generate a *bona fide* null mutant. Using this method, we created two independent null mutant candidate lines, $Act5C^{RS1}$ and $Act5C^{RS2}$. Both lines showed lethality in homo- and hemizygous forms.

Deletions in *Act5C* were identified with an RS element-specific forward and *Act5C*-specific reverse PCR primer pair. The PCR product amplified from the $Act5C^{RS1}$ line was sequenced, which ultimately validated that $Act5C^{RS1}$ line carries a true amorphic mutation of *Act5C*, as the entire *Act5C* coding region and most of the 3' UTR region was removed. This mutant stock will be referred to as *Act5C* null.

4.3. Phenotypic characterization of *Act5C* null mutant flies

4.3.1. Embryonic lethality

The *Act5C* null mutant stocks showed strong lethality, so we determined in which developmental stage lethality occurs. We found that both *Act5C* null mutant animals show only mild embryonic lethality, therefore the strong lethality occurs later in development.

4.3.2. Larval lethality

The *Act5C* deletion was balanced with FM7-GFP balancer chromosome, in order to distinguish hemizygous, null mutant male larvae from their heterozygous female or wild type siblings. The experiment revealed that the development of all hemizygous larvae was arrested at the first developmental stage, and subsequently all (100%) died at this stage. In addition to lethality, we noticed a drastic reduction in their body size, and their movements were slow and disoriented compared to their heterozygous or wild type siblings.

4.4. Creation and validation of *Act5C* transgenic stocks

4.4.1. Constructing the transgenes and establishing transgenic stocks

Tags were inserted *in vitro* after the start codon of Act5C. The modified Act5C expressing transgenes were subcloned into pAttB vectors. Transforming vectors were injected into embryos that carry an attP docking site, and transgenic lines were established. The modifications included V5-, NES-, V5-FLAG-, NES-FLAG- tagging. The unmodified *Act5C* transgene served as wild type control. All transgenes are expressed under the native transcriptional regulation of the *Act5C* gene.

4.4.2. Validation of *Act5C* transgenic stocks

To validate the proper transgenic expression, larval salivary glands from each transgenic lines were dissected and immunostained against V5 or FLAG epitope tags. Results showed wild type production and localization of Actin5C, suggesting that protein expression and functions are normal in these animals.

To analyze the effect of the NES tag on nuclear actin levels, the nuclear-cytoplasmic ratio of NES-FLAG-Act5C fluorescence signal intensities was calculated and compared to that of V5-FLAG-Act5C expressing line. We concluded that the NES motif does not completely abolish, but significantly (25%) reduces the amount of protein in the nucleus.

4.5. Rescuing the lethality of Act5C null mutation with transgenes expressing tagged Act5C protein isoforms

In our first rescue experiment, lethal phenotype of *Act5C* null mutation was rescued with unmodified (control) and modified Act5C expressing transgenic stocks. We counted the number of male offspring with different phenotypes and calculated the ratio between them. Despite our expectations, we could not detect any significant difference between the proportion of males rescued with NES-Act5C, Act5C or V5-Act5C protein forms. The result of the experiment revealed that the 25% decrease in nuclear actin amount is not sufficient to affect its functions, which explain the full functionality of decreased nuclear actin levels. It was also concluded that the V5-tagged Act5C protein was as effective in rescuing the lethal phenotype of the *Act5C* mutation as the control, and therefore the V5-tagged form of actin was used as a control in subsequent experiments.

4.6. Decreasing the nuclear actin level via disruption of the K285 sumoylation site in Act5C

Next we sought to lower nuclear actin level by disrupting the Act5C sumoylation site. We generated GFP-tagged Act5C-K285R and NES-Act5C-K285R expressing constructs. *Drosophila* S2R+ cultured cells were transfected with the constructs and nuclear-cytoplasmic fluorescence signal ratio was calculated. Results showed no significant differences between wild type Act5C and the point mutant variants.

In parallel, Act5C-K285R expressing stocks were generated, and the lethal phenotype was rescued with these stocks. The result of rescue experiments showed no significant decrease in the number of surviving males rescued with the non-sumoylatable isoforms of Act5C. This

suggests that sumoylation of actin at K285 in *Drosophila* does not act as a robust nuclear retention signal.

4.7. Decreasing nuclear actin level by knocking out the actin importin, RanBP9

In order to further decrease the amount of actin in the nucleus, the *RanBP9* (Importin 9) gene was knocked out by P-element remobilization. Homozygous null mutant flies showed complete sterility, but otherwise no additional phenotype was observed. To investigate the effect of *RanBP9* null mutation on nuclear actin levels, the null mutation was introduced into the Act5C expressing transgenic stocks. Larval salivary glands of the stocks were stained for actin with FLAG antibody, and the ratio of nuclear vs. cytoplasmic fluorescence was calculated as previously described. We found that on heterozygous *RanBP9* background no significant change in nuclear actin levels was observed, but the homozygous *RanBP9* allele decreased the level of nuclear actin by 18%. We concluded, that the complete loss of RanBP9 decreases but does not fully eliminate actin from the nucleus.

Next, we investigated the effect of *RanBP9* null mutation in combination with the NES tagging of actin. For this aim we created an *Act5C; RanBP9* double null mutant stock, and rescued the lethal phenotype with different forms of actin. The experiment revealed, that homozygous null mutation of *RanBP9* decreases viability by only ~30%. In contrast, rescuing *RanBP9* homozygous null animals with NES-tagged Act5C drastically reduced viability by ~70%.

We concluded that forced export of nuclear actin by the NES tag, and decreased nuclear import caused by homozygous *RanBP9* null mutation exhibit a synergistic effect on lethality. But together they are still not able to decrease viabilty to zero which indicates that the nuclear actin pool is maintained in a manner that effectively overcomes perturbing mechanisms. Our main conclusion was that RanBP9 is not the exclusive nuclear importin of actin.

4.8. Identifying new importins of Act5C

4.8.1. *In Vitro* Transcription coupled Translation assay

In order to find novel importins that are responsible for the nuclear localization of actin, we investigated eight beta importins: Cadmus (*cdm*), Moleskin (*msk*), RanBP9 (*Importin 9*), Artemis (*Arts*), Ketel (*Fs(2)Ket*), RanBP11 (*Importin beta 11*), Transportin (*Tnpo*), and Transportin-Serine/Arginine rich (*Tnpo-SR*). First, we performed and *in vitro* IVTT assay with them. Importins were labelled with ^{35}S methionine, and non-polymerizable monomeric actin,

Act5C^{R63D} was His-tagged. The pull-down experiment revealed that Cadmus, Moleskin, RanBP11, Tnpo and Tnpo-SR can bind monomeric His-tagged actin *in vitro.*

4.8.2. Bimolecular fluorescence complementation (BiFC) assay

To confirm the interactions between the newly identified beta importins and actin, we performed a BiFC-based screen in cultured Drosophila S2R+ cells. The Act5C protein was tagged on its N-terminus with a truncated N-terminal part of the YFP protein. Importins were tagged at both amino-termini with the C-terminal portion of YFP. We observed positive fluorescent signal (indicating interaction between bait and prey) in the case of Moleskin, Artemis, Cadmus, Ketel and Tnpo-SR.

5. NEW RESULTS AND CONCLUSIONS

The validation of modified (NES-, V5-, and FLAG-tagged) Act5C expressing stocks, and the rescue of the *Act5C* null-mutant lethal phenotype with them confirmed, that NES-tagging of Act5C significantly reduces the nuclear level of actin. However, this decrease neither affected the viability of flies nor did lead to any other discernable phenotype. Because the disruption of actin's sumoylation motif failed to enhance its nuclear export, we concluded that sumoylation does not act as a major nuclear retention signal of actin in *Drosophila*. In contrast, the complete elimination of the RanBP9 importin significantly lowered nuclear actin levels, however viability decreased only if the rescue was carried out together with NES-tagging of actin. The reduction of viability was still not 100%, which suggests the existence of other nuclear import mechanisms acting in parallel to the RanBP9 pathway. With the help of *in vivo* and *in vitro* assays we identified four new β-importins that interact with actin.

We successfully developed a genetic system that is suitable for the investigation of nuclear actin functions at the level of the organism. Our results provide direct evidence that the nuclear presence of actin is greatly secured, which in turn argues for essential biological functions for nuclear actin.

6. FUNDING

This work was supported by NKFIH (National Research, Development and Innovation Office) through the National Laboratory for Biotechnology program, grant NKFIH-871-3/2020 (Péter Vilmos), PD127968 (Ildikó Kristó), and the Dr. Rollin D. Hotchkiss Foundation (Péter Borkúti).

7. ACKNOWLEDGEMENTS

I would like to express my deep and sincere gratitude to my tutor and supervisor Péter Vilmos PhD for allowing me to work in his *Drosophila* nuclear actin laboratory, for helping me to grow professionally, and for accompanying me with his expertise and enthusiasm.

I would like to thank my closest colleagues, Ildikó Kristó PhD, Anikó Szabó PhD, Csilla Abonyi, Csaba Bajusz PhD, and Zoltán Kovács, for helping me with their advices to develop throughout these years, and for creating a good atmosphere in the laboratory.

I would like to thank Miklós Erdélyi for providing me the opportunity to carry out my work in the Institute of Genetics, BRC, and the whole *„Drosophila* community*”* in the Institute of Genetics for their persistent support and selfless devotion.

I owe my deepest gratitude to my parents, my mother Dóra Podmaniczki and my father Zoltán Borkúti, and my family for their support in my entire life.

I am much obliged to my wife, Linda Szabó for her endless love and support that helped me reach my goals.

Last but not least, I want to thank all my friends for their support, especially Vivien Veronika Viziné Mező and Balázs Vizi.