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PhD Thesis

Comparative analysis of the role of formins in *Drosophila* embryonic dorsal closure

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ABBREVIATIONS

Arm	Armadillo
AS	amnioserosa
Capu	Cappuccino
Cas9	CRISPR-associated protein 9
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CyO	Curly of Oster
DAAM	Dishevelled associated activator of morphogenesis
DC	dorsal closure
Dia	Diaphanous
DNA	Deoxyribonucleic Acid
Dpp	Decapentaplegic
EGFP	Green fluorescent protein
en	engrailed
F-actin	Filamentous actin
FH1	formin homology 1
FH2	formin homology 2
FL	full length
Form3	Formin3
Frl	formin-related proteins in leukocytes
GFSTF	EGFP-FlAsH-StrepII-TEV-3xFlag
GTP	guanosine triphosphate
HRP	Horseradish peroxidase
JNK	Jun N-terminal kinase
LE	leading edge
LOF	loss-of-function
LSM	laser point scanning confocal microscope
Moe	Moesin
ns	not significant
PCR	Polymerase chain reaction
Rho	Ras homologous
RNA	Ribonucleic Acid
TM3	Third Multiple 3
twi	twist
UAS	Upstream Activation Sequence
ZASP52	Z band alternatively spliced PDZ-motif protein 52

PUBLICATIONS

1. Publications related to the thesis

I. **Krisztina Tóth**, István Földi, József Mihály. A Comparative Study of the Role of Formins in *Drosophila* Embryonic Dorsal Closure. CELLS 11 : 9 Paper: 1539 , 18 p. (2022)
IF: 6.6 (Q1)

II. Szilárd Szikora, István Földi, **Krisztina Tóth**, Ede Migh, Andrea Vig, Beáta Bugyi, József Maléth, Péter Hegyi, Péter Kaltenecker, Natalia Sanchez-Soriano, József Mihály. The formin DAAM is required for coordination of the actin and microtubule cytoskeleton in axonal growth cones. JOURNAL OF CELL SCIENCE 130 : 15 pp. 2506-2519. , 14 p. (2017)
IF: 4.401 (Q1)

Cumulative impact factors of papers directly related to the subject of the thesis: 10.001

1. Publications not directly related to the thesis

I. István Földi*, **Krisztina Tóth***, Rita Gombos, Péter Gaszler, Péter Görög, Ioannis Zygouras, Beáta Bugyi, József Mihály. Molecular Dissection of DAAM Function during Axon Growth in Drosophila Embryonic Neurons. CELLS 11 : 9 Paper: 1487 , 20 p. (2022)
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INTRODUCTION

Drosophila embryonic dorsal closure (DC) is an excellent model to study cell shape changes and cytoskeleton rearrangements. During this late morphogenetic process, a dorsal hole, filled with the extraembryonic amnioserosa (AS) tissue, closes through the elongation of the lateral epidermal cell sheets. The initiation of DC is at stage 12 of embryogenesis, right after the germ band retracts and its termination is at stage 15. Between these two stages, the front of the epithelial cells, called leading edge (LE) cells, elongate dorsoventrally, and produce an actin cable around the dorsal hole, the AS cells begin to contract, filopodia and lamellipodia protrude from the LE cells and finally they pull together the two opposite epithelial sheets forming a seamless contact along the midline.

DC is a rather complex process in which at least three different forces contribute at the same time to succeed: contraction of the AS cells, the stretching effect of the actin cable in the LE cells, and zipping of the two epithelial sheets. One of their main regulatory elements is the actomyosin network consisting of F-actin and non-muscle Myosin II. In AS cells, it is located in the medioapical region and in a cortical ring at the adherens junctions, while in the LE cells it is present at the anterior part of the cells, at the junction of the epidermal and AS cells. The main force generated by the actomyosin network is the pulsing apical contractions of the AS cells which gradually pull the surrounding epidermis dorsally. During these contractions, transiently appearing F-actin/non-muscle Myosin II foci are present in the medioapical region of the AS cells, in addition, the level of junctional myosin changes. The other important force generator is the supracellular actomyosin cable around the dorsal gap which is produced by the LE cells. Besides actomyosin, another essential building block of this cable is the Z band alternatively spliced PDZ-motif protein 52 (ZASP52). In addition, it was shown that the heavy and light chain components of non-muscle Myosin II are essential to build the actomyosin cable. The main role of this cable is to provide line tension that straightens the epidermis front and restricts the protrusive activity of the LE cells, in addition it is responsible for stabilization of the cellular interactions and preventing scarring of the embryonic tissue. The other dominant structures during DC are the actin and microtubule bundles in the LE filopodia, which guarantee the seamless zipping of the epithelium. During this process, the opposing LE cells with identical parasegmental information recognize each other and form adhesion sites while they push the AS tissue down inside the embryo, which ultimately forms a tube below the AS-LE contacts.

Research over the last few decades has identified a plethora of proteins that contribute to the formation and regulation of the actomyosin systems in DC. For example, the JNK and Dpp

signaling pathways promote the formation of the supracellular actomyosin cable around the dorsal gap, the contractility of which is regulated by Rho GTPases. In addition, two members of the Diaphanous-related formins, actin assembly factors representing another class of the Rho effectors, have also been shown to play a role in the process of DC.

The formin protein family belongs to the major cytoskeleton regulators as they act as *de novo* actin nucleators and support filament elongation. Beyond actin regulation, some formins were shown to be able to interact with microtubules as well and they play a role in crosslinking of the actin and microtubule cytoskeleton. Whereas the vertebrate genomes encode 15 formin genes, there are only 6 formins in *Drosophila*: Diaphanous (Dia), formin-related proteins in leukocytes (Frl), Dishevelled associated activator of morphogenesis (DAAM), Formin homology 2 domain-containing (Fhos), Cappuccino (Capu) and Formin3 (Form3). Of these, Dia, thought to be indispensable during cytokinesis, is involved in multiple aspects of DC, such as stabilization of the adherens junctions and regulation of non-muscle Myosin II during the closure process and it affects the formation of filopodia in both the LE and AS cells. Frl, the other formin linked to DC, promotes the assembly of a medioapical actin subpopulation in AS cells.

AIMS OF THE STUDY

Drosophila embryonic dorsal closure is a fairly complex process involving different tissues and forces which are based on dynamic cell shape changes and cytoskeletal rearrangements. To better understand how this process takes place, we need to understand how the different cytoskeletal elements are regulated in it. The main cytoskeletal component involved is the actin cytoskeleton which can be regulated by numerous proteins. One of these proteins is the well-characterized formin protein family, two members of which, Dia and Frl, have already been shown to be involved in the regulation of DC. Dia has a role in adherens junction stabilization in the LE and AS cells and it is also responsible for the formation of filopodia and lamellipodia in these two cell types. Frl promotes the assembly of a specific medioapical actin-subpopulation in the AS cells. Interestingly, LOF analysis of these formins revealed that none of them is essential for the closure process, rather they affect the cellular dynamics of DC. These results highlight the robustness of the process and suggest that the different actin-regulatory proteins may contribute to the regulation of different actin subpopulations. Thus, it is possible that additional formin-type of actin regulators may play a role in DC and possibly affect other actin networks than the ones regulated by Dia and Frl. To address this question, we decided to perform a comprehensive analysis of the 6 *Drosophila* formins during embryonic DC. We wanted to study the morphological changes as well as that of the dynamics of DC in all relevant formin mutants. To this end, we had to create a formin null mutant strain for *form3* and develop a new method to measure the required dynamic parameters.

MATERIALS AND METHODS

1. Drosophila stocks and genetics

Flies were raised at 25°C under standard conditions. The following mutant strains were used: *w*^{1118} (BL *#3605*), *Form3-GFSTF* (BL #65385), *Capu-GFSTF* (BL #66507), *Arm::GFP* (BL #8556) and *69B-Gal4* (BL #1774) provided by the Bloomington Drosophila Stock Center, *dDAAM*^{Ex4} (Gombos et al., 2015), *en-Gal4,UAS-Moe::mCherry* (Jankovics et al., 2011), *frl*^{59} (Dehapoit et al., 2020); *dia*^{1}*/CyO* (Castrillon et al., 1993) and *form3*^{1} (see below). Where necessary, zygotic mutants were selected by using a *CyO, twi-Gal4, UAS-EGFP* or *TM3, twi-Gal4, UAS-EGFP* balancer chromosome; protein and mRNA expression data were retrieved through FlyBase, the Drosophila databank. The *UAS-FL-Form3* and *UAS-FL-Form3 I450A* transgenic lines were generated with standard cloning techniques by using the pTWF-attB vector.

The *form3*^{1} mutant was generated by the CRISPR/Cas9 technique. Two 20 bp long gRNAs (TCGCCACCTGTCCTCCGGA and TGGGTCGCATGAAGCTGCT), were designed with homology to the first intron and the last coding exon of *form3 and* cloned into the pCFD4 vector. To facilitate the identification of the expected deletions, we used an insertional mutant that carried a GFP marker in the gene (BL #23411). After co-injection of the guide RNA expressing plasmid with Cas9 into this stock, we selected for loss of the GFP marker in the larval progeny, candidates picked up this way were subsequently validated by PCR and sequencing. Based on the sequencing data, the expected 13,559 bp deletion was detected from the genomic DNA of the mutant strain. Because in this stock only 37 bps remain from the coding region, we consider *form3*^{1} as a protein null allele, which is homozygous viable and fertile, although it exhibits reduced viability and fertility when compared to wild type.

2. Antibody generation

Frl antibody was generated in rat after immunization with a purified recombinant protein containing the amino acid residues 687-1183 of Frl. The sera were collected with standard methods, and the specificity of the antibody was confirmed by Western blot.

3. Immunohistochemistry

Fixation and immunostaining of *Drosophila* embryos were performed as described in Jankovics and Brunner (Jankovics and Brunner 2008). The following primary antibodies were used: rabbit anti-Zipper (Larkin et al 2020) 1:100, rabbit anti-Dia (a kind gift from S. Wasserman, University of California, San Diego, La Jolla, CA) 1:200, rat anti-Frl (described above) 1:500, rabbit anti-DAAM-R4 (Gombos et al., 2015) 1:500, rat anti-FHOS (Chougule et

al., 2016) 1:200, chicken anti-GFP (Abcam) 1:1000, mouse anti-Flag (Sigma) 1:500, mouse 2A12 (Developmental Studies Hybridoma Bank) 1:40. As secondary antibodies, we used the appropriate Alexa-488 or Alexa-546 coupled antibodies (ThermoFisher Scientific) 1:600. Actin was labeled with Alexa-488, Alexa-546 or Alexa-647 coupled phalloidin (ThermoFisher Scientific) 1:80. The embryos were mounted in the ProLong Gold antifade reagent (Life Technologies).

Imaging was performed on a Zeiss LSM880 confocal microscope with an Airyscan detector, using 40x/NA 1.3 oil or 63x/NA 1.4 oil objectives. Images were restored using the Huygens Professional (Scientific Volume Imaging) and Fiji (Schwartz et al., 2016) software.

4. Western blot analysis

Western blots were performed by using standard procedures. Rat anti-actin (1:10.000, MAC 237, Abcam), rabbit anti-Dia (1:2000, a gift from S. Wasserman, University of California), rat anti-Frl (1:1000, described above), rabbit anti-DAAM-R4 (1:5000, Gombos et al., 2015) and mouse anti-Flag (1:1000, M2, Sigma-Aldrich) were used as primary antibodies. Anti-Rabbit-HRP (1:10.000; Jackson) and anti-mouse IgG-HRP (1:5000, Dako) were used as secondary antibodies, proteins were visualized with the chemiluminescent Millipore Immobilon kit.

5. Live imaging and image analysis

Embryos were dechorionated in 50% bleach, mounted in water onto a glass-bottom cell culture dish (MatTek), and imaged with Zeiss LSM880 confocal laser scanning microscope using 40x/NA 1.3 oil or 20x/NA 0.8 dry objectives. Every video was acquired at 25 ℃. For the analysis of dorsal closure (acquired with 20x objective), we performed Z-series of 14 planes separated by 1.2 µm and acquired every 4 minutes. For AS dynamics (acquired with 40x objective), we filmed Z-series of 11 planes separated by 0.9 µm and acquired every 30 seconds.

All image processing and data analysis were performed using Fiji (Schwarz et al., 2016) and Microsoft Excel 2016.

6. Trachea analysis

We determined the number of discontinuous fusion points from the nine anastomosis sites in the dorsal trunk.

7. AS cell dynamics

To measure AS cell contractility, Z-series were maximum-projected and were segmented using Fiji plugin Tissue Analyzer. If required, we manually corrected the segmentation results. Using the wand tool in Fiji, we measured the apical AS cell surface area of the six most central

AS cells per embryo in a 15 min time window which began when the dorsal gap was 50 μm wide. The selected time window roughly corresponding to the middle of the closure process, and it is suitable for standardization as well as for high quality live imaging. The relative apical area change was normalized as described in Pasakarnis et al. (Pasakarnis et al., 2016). Using "IF" and "AND" equitation in Excel, we calculated the height and the number of the amplitude from the normalized relative apical area change.

8. AS cell shape

To measure the regularity of AS cell shape we divided the actual cell shape area ($AS_{area}(t_x)$) when the dorsal gap was 50 μm wide by the surface of the fitted convex hull ($Ch_{area}(t_x)$), and we continued it until the 15 min time window ended. This way, we obtained a ratio that indicates the convolution level of the segmented cell shape: $N(t_x) = AS_{area}(t_x)/Ch_{area}(t_x)$

The measured AS cells were the same as the ones analyzed for AS cell contractility.

9. AS cell area

We used the wand tool to measure the AS cell area from the segmented Z-series when the dorsal gap was 50 μm wide. The measured AS cells were the same we used for AS cell shape determination.

10. Filopodia number and length

Filopodia number and length were calculated with the line tool within six *en-Gal4* stripes per embryo when the LEs were 30-50 μm apart from each other.

11. Dorsal closure parameters

The width and height of the dorsal hole were measured on maximum-intensity Z-projections using the line selection tool in Fiji. Timepoint zero was defined right after the germ band was retracted and head involution began. The convergence speed of the LE was measured as described in Pasakarnis et al. (Pasakarnis et al 2016). The zipping speed was calculated as follows: $v(t_x) = \big((length(t_x) - length(t_{x+1})\big)/\Delta t$, where $v(t_x)$ is the zipping speed, $length(t_x)$ is the length of the dorsal hole in a given frame, $length(t_{x+1})$ is the length of the dorsal hole in the following frame and Δt is the time interval between the frames.

12. Statistics and figures

Statistical analysis was carried out using Prism 8 (GraphPad Software Inc.). The D'Agostino-Pearson omnibus test was used to assess the normality of the data. Significance levels: ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. At least three independent experiments were used in the statistical analysis. Figures and drawings were created in Illustrator CS6 (Adobe).

RESULTS

Dia, Frl, DAAM and Form3 localize to AS and epidermal cells during the dorsal closure

According to immunostaining experiments, consistent with previous reports we detected Dia in the cell cortex of both the epidermal and the AS cells, as well as at the apical adherens junctional region of LE cells. FRL is mostly present in the cytoplasm of the AS cells, although a weaker staining is present in the cortex of the AS cells and that of the epidermal cells as well. In addition, the Frl signal was found in the nuclei of AS cells and along the LE actin cable. Similar to the findings of Flores-Benitez and Knust (Flores-Benitez and Knust, 2015), DAAM showed a significant accumulation in the cortex of the epidermal cells and it was also present in the AS cells, while Formin3 exhibited a similar localization pattern as DAAM, although it is stronger in the cytoplasm of the AS cells. In the cases of Fhos and Capu, we detected only background staining. Thus, the embryonic protein expression pattern of the *Drosophila* formins indicates that 4 of the 6 formin proteins are present in the tissues contributing to DC, and the different localization of these four formins may indicate different functions during the process.

The formin LOF mutations used during our studies

To investigate the contribution of these four formins to DC, we used loss-of-function approaches to reduce each protein level. Since there was no suitable *form3* allele that could be used as a null mutant, we used the CRISPR/Cas9 technique to generate a novel null allele, $form3^{1}$ (Methods), that is a 13.5 kb deletion in *form3* removing 99% of the coding region, including the functionally indispensable two formin homology domains (FH1 and FH2). Based on previous work, point mutant alleles of *form3* affect the fusion of the main tracheal airways. Thus, to verify our new allele, we investigated the tracheal system in $form3^{1}$ homozygous embryos. We found that a significant amount of $form3^{1}$ embryos exhibit a discontinuous lumen in the dorsal trunk of their tracheal system. The rescue experiments further confirmed that this phenomenon was due to the lack of *form3*. Furthermore, the expression of an actin processing mutant transgene, UAS-Form3 I450A, in $form3^{1}$ embryos revealed that, in contrast to the wild type, the actin-processing point mutant is unable to restore the continuity of the main airways, suggesting that Formin3 affects the development of the trachea through regulation of the actin cytoskeleton.

Formins influence the process of DC

The morphological analysis of DC revealed that all four formins affect the appearance of the dorsal hole. The effects of *dia* and *form3* are much alike, while *frl* and *DAAM* contribute to different phenotypes. Despite the different defects in gap morphology, most of the embryos can still successfully complete DC so we analyzed the length of the closure. Already in *DAAM* and *frl* mutant embryos we observed an increased closure time, while in *form3* and *dia* mutants this time was further increased. As an alternative way to analyse this, we measured the convergence of the two opposite epithelial sheets. The decrease of the dorsal hole width followed a sigmoidal curve, except in the case of *frl* mutants, where the curve appears rather linear and the maximum convergence speed was strongly reduced, which might explain this linear change in gap width. Taken together, these observations suggest that the lack of *DAAM* has the weakest effect on the duration of the closure process and the convergence of the LE. Similar to the morphological phenotypes, the lack of *form3* and *dia* has a very similar effect on these dynamic parameters while *frl* seems to display a different temporal contribution to this.

Form3, DAAM and Frl mediate the zipping of the epithelial sheets

The abnormally narrow dorsal hole and the altered zipping speed in the case of *frl* mutant embryos indicate its role in the zipping process. However, the zipping speed is decreased by the other three formin mutants as well, which means that all four formins have an effect on the zipping. The analysis of the number and length of filopodia involved in the process revealed that except *dia*, the contribution to zipping of the other three formins is independent of filopodia, and implicit to this scenario is that they affect another actin population than the one present in filopodia.

Formins differently regulate the shape of the AS cells

For the quantification of the AS cell shape we created a method which was able to determine the convolution level of the cell shape. According to our results, except for *frl* mutant, the AS cell membranes were clearly more straight and less wavy than in controls. Since it is known that the AS can autonomously drive DC, changes in cell shape can be an important factor in the regulation of the process.

Formins are crucial for proper contraction of the AS cells

The measurement of the AS cell contraction revealed a decrease in the amplitude of the cell contractions in the case of all four formin mutants examined. Based on the fact that the

contraction behavior of the AS cells is significantly altered by all mutants, but cell shape changes are not affected by the *frl* mutant, we conclude that these formins have a different contribution to AS cell motility and AS cell shape alone not define the dynamics of the contractions.

SUMMARY

Formins are well characterized actin nucleators and elongators. These proteins participate in actin cytoskeleton regulation in many different tissues. Two formins, Dia and Frl, are proven to be involved in actin cytoskeleton regulation during the dorsal closure as well. The contribution of formins in this process is not surprising, as the forces generated during the course of DC are mainly based on F-actin and actomyosin, and microtubule based cytoskeletal mechanisms. Whereas the contribution of branched actin networks is not reported, linear actin cables are required for pulsatile contraction of the AS cells, LE actomyosin cable formation and LE protrusions. Considering that most of the unbranched actin filaments are assembled by formin proteins, other formins than Dia and Frl are likely to be involved in the regulation of DC. At the cellular level, Dia is required for filopodia formation in the LE and AS cells, while Frl is implicated in the formation of a persistent, medioapical actin subpopulation in the AS cells promoting the propagation of Myosin II-induced contractile forces. These observations suggested that Dia and Frl are required in two different cell types, and consequently, they regulate at least two different actin populations.

To clarify whether additional formins are involved, we performed a comprehensive analysis of all six *Drosophila* formins. These studies established that, in addition to Dia and Frl, Form3 and DAAM are also expressed in the LE and AS cells during DC. The different localization of these formins indicates that they might regulate the DC in a different manner.

LOF experiments showed that in the absence of the four formins various phenotypic defects happened during DC, including morphological alterations and changes in the dynamic parameters of the process. Our data indicate that the effects of *form3*, *dia* and *DAAM* are similar to each other both with regard to the morphological phenotypes and to DC dynamics, whereas *frl* has distinct effects in both respects. These differences are best explained by different temporal, and presumably, spatial requirements for the two classes of formins, i.e. Frl is required from the early phases of DC, while Form3, Dia and DAAM provide an important contribution only during the final phases. Curiously, however, we found that zipping is slowed

down in all formin mutants, including *frl*, and conversely, AS cell dynamics is not only affected by *frl*, but also by the other three formins. As to zipping, unlike Dia, Form3, DAAM and Frl are not required for filopodia formation, and unless they regulate filopodia dynamics, they may contribute to zipping by regulating a non-filopodial actin subpopulation. Regarding the behavior of the AS cells, our results indicate that each formin is required for the efficient contraction of these cells. Nevertheless, while *DAAM* and *frl* reduce, *form3* and *dia* slightly increase the frequency of AS cell pulsation, revealing a differential contribution. Collectively, the analysis of DC dynamics in the formin mutants strongly suggests that each of the four formins is involved in zipping and AS cell contraction as well.

Overall, we have shown in this study that four DC-related formins regulate the dynamics of embryonic dorsal closure differently. Although they play a significant role, none of the formins alone is essential to the success of the process, further confirming that this key morphogenetic event is particularly robust and secured by a variety of cytoskeletal mechanisms. The known molecular function of the formin protein family suggests that Dia, Form3, DAAM and Frl are required for the assembly of various F-actin subpopulations critical to proper force generation in the LE and AS cells during DC. So far, only one such Frl-dependent actin subpopulation was identified in the AS cells, and although LE filopodia formation is known to be Dia-dependent, identification of the additional formin dependent actin structures awaits future elucidations.

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