THE FUNCTION OF THE *DROSOPHILA* SPEKTRAPLAKIN DURING EPITHELIAL CLOSURE PROCESSES

Main points of the Ph.D.thesis

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Introduction

In several naturally occurring morphogenetic events two tissues move towards each other until they meet and fuse. These closure events happen mostly during embryogenesis at the midline of the developing body. Defects of these closure events result in dramatic consequences, such as cleft lip or palate or neural tube defects. Our goal is to understand the strategies animals apply to close epithelial openings. *Drosophila melanogaster* has in the past provided a powerful experimental system for the genetic dissection of developmental processes. Dorsal closure represents the last major morphogenetic movement during embryogenesis, when two opposed epithelial sheets converge toward the midline where they meet, sealing a hole at the dorsal surface of the embryo.

Efficient dorsal closure requires the dynamic rearrangement of the cytoskeleton in epithelial cells. DME cells form a leading edge facing towards the dorsal opening, where they accumulate an actomyosin cable. In addition, DME cells extend actin-rich cellular protrusions, such as filopodia and lamellipodia, mediating initial contacting of the opposing DME cells.

At the onset of closure, DME cells display an irregularly distributed network of MTs. During closure, MTs reorganize to form acentrosomal bundles that are aligned along the dorsal-ventral cell axis. Although the bundles are stable, individual MTs remain highly dynamic, and at the leading edge they grow into cell protrusions.

Genetic screening, biochemical and cell biological approaches have uncovered a large number of structural and signalling molecules required for these closure events. Several studies have highlighted the importance of reorganization of actin-based structures, such as filopodia and lamellipodia, but the function of the microtubule (MT) network is very poorly understood. Our primary goal was the identification of genes required for MT network structure and function during dorsal closure and the *in vivo* analysis of these genes by determining their exact role in epithelial closure.

Aims

The aim of our study was to perform an RNAi-based genetic screening method combined with automated in vivo video microscopy to uncover novel components required for cytoskeletal reorganization and function during dorsal closure.

We aimed to inactivate many *Drosophila* genes encoding for microtubule-associated proteins and detect resulting phenotypic defects by automated *in vivo* image analysis at three hierarchic organizational levels: dynamic instability parameters of the microtubules, morphology of the microtubule network and dynamic properties of the closure process.

We also aimed at giving a detailed genetic analysis of a promising candidate gene which will provide insights into the mechanisms how individual components of a complex system contribute to a fundamental biological process at the molecular, cellular and organism level.

Methods

- RNAi-based genetic screening coupled with in vivo fluorescent video microscopy
  - We designed and synthesised gene silencing double-stranded RNAs (dsRNAs) by *in vitro* transcription.
  - Early Drosophila embryos were microinjected with dsRNAs.
  - Treated embryos were then subjected to live cell imaging and the whole closure process was recorded.

- CRISPR (clustered regularly interspaced short palindromic repeats) genom editing method was used to generate the novel $shot^{\Delta EGC}$ allele.
  - Target site- specific sequences for the *shot* gene were ligated into the expression vector.
  - Early *Drosophila* embryos were microinjected with mixture of plasmids.
  - Novel *shot* mutations were selected by their lethal phenotype in a complementation analysis.
  - Deletion was verified by sequencing.

- We examined MT distribution in wild and *shot* mutant epithelial cells via immunohistochemical labeling.

- FRAP (Fluorescence recovery after photobleaching) assays were applied to analyze the dynamic properties of the MTs.

- We analyzed the dynamic instability parameters of MTs and the protrusion dynamics of DME cells by live imaging.

- A detailed structure-function analysis of the *shot* gene was performed by tissue specific rescue experiments.

Results

1. To uncover novel genes required for microtubule reorganization and function, we have applied an RNAi-based screening method combined with automated *in vivo* video microscopy and we identified the *short stop (shot)* gene. The silencing of *shot* results in abnormal dorsal closure. Quantification of closure dynamics in *shot* mutant revealed that *shot* function is essential for efficient zippering.

2. Drosophila Shot protein is involved in other epithelial closure processes such as embryonic wound healing or closure of the adult thorax during metamorphosis, demonstrating its general role in epithelial closure processes.

3. CRISPR-based genome editing was applied to generate a novel *shot* mutant allele specifically impairing its binding to MTs. The resulting truncated protein, which we designated $Shot^{\Delta EGC}$, lacks the EF-hand, the Gas2 and the C-tail domains, leading to complete loss of the MT-binding activity.

4. In order to understand the role of the individual protein domains of Shot in dorsal closure, we investigated the mutant phenotypes of various *shot* mutant alleles abolishing distinct Shot activities. In addition, we carried out a detailed structure-function analysis of Shot using a series of *shot* transgenes in rescue experiments. Our results demonstrate that both the actin and the MT-binding activities of Shot are required for dorsal closure and suggest that Shot acts as an actin/MT cross-linker in mediating the zippering step of dorsal closure.

5. FRAP assays were applied to analyze the turnover of tubulin, which reflects the dynamic properties of the MTs. A qualitative analysis of the FRAP curves revealed that Shot regulates the dynamic pool of MTs. In mutant cells, the growth rate of MTs increased significantly, but Shot is not required for the regulation of MT growth direction along the actin filaments. These results demonstrate that Shot regulates the morphology of the microtubule network by stabilizing the dynamic microtubules in the DME cells.

6. We investigated the MT network of epithelial cells in isoform-specific shot mutants via immunohistochemical labelling. In addition we expressed various truncated versions of Shot in $shot^{sf20}$ null mutant embryos. In summary, we conclude that the MT- binding activity

of Shot is required but is not sufficient for MT stabilization and the actin-binding activity of Shot is also required for MT stabilization. Actin- and MT-binding activities of Shot are simultaneously required in the same molecule for proper MT organization in DME cells. In summary, Shot functions as an actin-MT crosslinker to ensure proper MT regulation at the leading edge of DME cells during dorsal closure.

7. Our previous experiments revealed that both the actin and the MT regulatory activities of Shot function in DME cells to ensure proper MT organization and dorsal closure. Restoration of wild-type MT organization in *shot* mutants, however, was not sufficient for proper zippering, suggesting that an additional activity of Shot might be required for dorsal closure. Therefore, we investigated the actin network of DME cells by analyzing actin accumulation and protrusion formation at their leading edge. Our results demonstrate that Shot promotes filopodia formation at the leading edge of the epithelial cells.

Summary

Dorsal closure of the *Drosophila* embryonic epithelium provides an excellent model system for the *in vivo* analysis of molecular mechanisms regulating cytoskeletal rearrangements. We investigated the function of the *Drosophila* spectraplakin Short stop (Shot), a conserved cytoskeletal structural protein, during closure of the dorsal embryonic epithelium. We demonstrated that Shot functions as an actin-microtubule cross-linker in mediating zippering. At the leading edge of epithelial cells, Shot regulates protrusion dynamics by promoting filopodia formation. Detailed cell biological analysis revealed that *shot* controls the morphology of the MT network in the epithelial cells by regulating dynamic properties of microtubule growth. FRAP analysis and *in vivo* imaging of microtubule growth revealed that Shot stabilizes dynamic microtubules. We propose that Shot-mediated interactions between microtubules and actin filaments facilitate filopodia formation which promotes zippering by initiating contacting of opposing epithelial cells.

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List of publications

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Drosophila small ovary gene is required for transposon silencing and heterochromatin organization, and ensures germline stem cell maintenance and differentiation.
Development, 2018 Dec 4;145(23).
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