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Final Report

Regulation of germ cell development and function
Az ivarsejtek kialakulásának és működésének vizsgálata

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The aim of the work

Insects have some of the longest sperm in the animal kingdom, although they contain the same components as the mammalian sperm, namely compact nucleus, acrosome, axoneme, mitochondria and plasma membrane. During *Drosophila melanogaster* spermatogenesis, following meiotic division, the 64 spermatids undergo a dramatic differentiation program that leads to formation of the highly elongated flagellated mature sperm. Elongation includes the reorganization of old organelles and the formation of new ones. The spermatids increase their length by 150-fold, producing 1.8 mm long spermatids. Elongation from round to elongated spermatids includes extensive membrane biosynthesis, remodeling and regulated vesicular transport. The 64 spermatids from each spermatogonium become polarized, and each spermatid has the nucleus at its apical end. Finally, the fully elongated spermatids undergo a synchronized individualization, which is facilitated by the apoptotic machinery, including proteasome-mediated degradation and caspases, but does not result in cell death. The gene products necessary for the post-meiotic stages are synthetized earlier. The transcriptional shut-off in post-meiotic stages, during spermatid elongation and individualization, makes these late stages of spermatogenesis particularly sensitive to even a small reduction in gene products.

Our aim was to identify new genes involved in the regulation of early and late stages of spermatogenesis and describe the precise molecular function of these components.

Results

To achieve our goal we screened for male sterility of the publicly available collections of Drosophila lines with precisely mapped transposon insertions (P element, *piggyBac* and Minos element) in the first year. We selected 59 lines with male and/or female sterility and did the basic genetic characterization of all of them. We identified and genetically characterized 7 male sterile alleles with post-meiotic elongation and individualization problem.

Main findings of our works during the course of this project are the following:

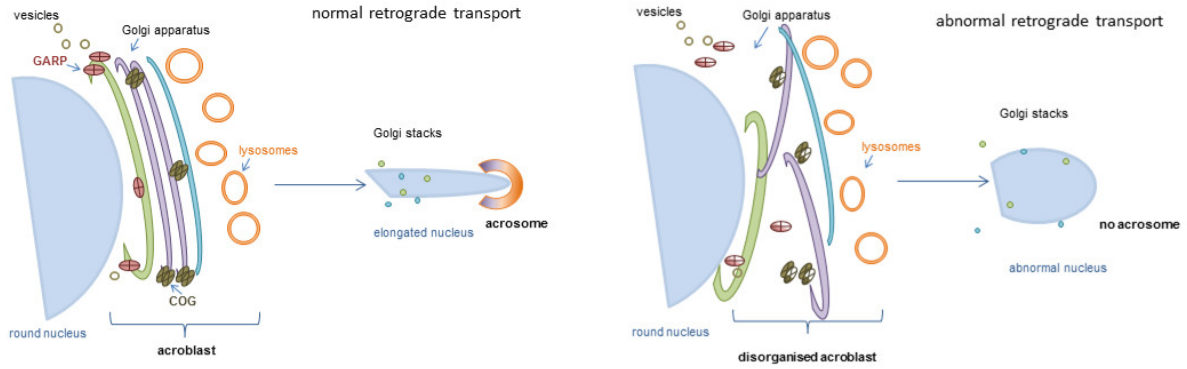
We found that the CdsA mutant shows defects in spermatid individualization and enlargement of mitochondria and the axonemal sheath of the spermatids, while somatic tissues are

unaffected. Mitochondrial asynchrony and overgrowth, as well as the enlargement of endomembranes of spermatids, are the most prominent aberrations seen in the CdsAms1 testes. We could genetically rescue the male sterile phenotype by overexpressing CdsA in a CdsA mutant background. Although CdsA function is not required for individualization complex formation, it may play an indirect role in its movement. Our testis-specific lipidomic analysis showed reduced PI and elevated PA level in $CdsA^{ms1}$ mutant testis. Although we measured a strong decrease in PI levels, we did not find changes in the localizations of PIPn, such as PI4P and PI(4,5)P2. Our results also highlights the importance of small signalling lipids, such as PA. We suggested that PA could be enriched at the axonemal sheath–mitochondria contact sites and induce the overgrowth of the ER and mitochondrial membranes in $CdsA^{ms1}$ testes. Overexpression of PI synthase in $CdsA^{ms1}$ testes partially suppresses male sterility. Our experiments showed that PI synthase somehow, probably indirectly, induces the reduction of PA without the upregulation of CdsA transcription itself. The results of lipidomic and genetic analyses of the CdsA mutant highlight the importance of correct lipid composition during sperm development and show that phosphatidic acid level is crucial in late stages of spermatogenesis. Our study demonstrates that a mutation in a single lipid biosynthetic gene may have pleiotropic effects on lipid metabolism, which together may lead to severe phenotypic alterations, such as male sterility. Lipidomic analysis suggests that the fruit fly testis is an ideal and sensitive system to study changes in lipid composition and their effects on development.

Laurinyecz B, Péter M, Vedelek V, Kovács AL, Juhász G, Maróy P, Vígh L, Balogh G, Sinka R. Reduced expression of CDP-DAG synthase changes lipid composition and leads to male sterility in Drosophila. Open Biol. 2016 Jan;6(1):50169. doi:10.1098/rsob.150169. PubMed PMID: 26791243

Golgi apparatus of developing Drosophila spermatids is in a perinuclear location just prior to and during the nuclear elongation phase of spermatogenesis. This specialized Golgi assemblage, known as the acroblast, is likely to be needed to organize the secretory pathway in this highly polarized cell type of the fruit fly. The Vps54 (Scat) protein, a subunit of the GARP tethering complex, is a central factor in the retrograde transport to the trans-Golgi. We found Drosophila $scat^1$ mutant is male sterile in Drosophila with individualization problems occurring during spermatogenesis. Another typically observed phenotype was the abnormal nuclear structure in elongated mutant cysts. When examining the structure and function of the Golgi, a failure in acrosome formation and endosome-Golgi vesicular transport were found in the $scat^1$ mutant. This

acrosome formation defect was due to a fault in the trans-Golgi side of the acroblast ribbon. When testing a mutation in a second retrograde transport protein, Fws, a subunit of the conserved oligomeric Golgi (COG) tethering complex, the acroblast structure, was again disrupted. fws^{P} caused a similar, albeit milder, acrosome and sperm individualization phenotype as the scat1 mutant. In the case of fws^{P} the cis side of the acroblast ribbon was dispersed, in-line with the intra-Golgi retrograde function of COG. Our results highlighted the importance of an intact acroblast for acrosome formation, nuclear elongation and therefore sperm maturation. Moreover, these results suggest the importance of retrograde tethering complexes in the formation of a functional Golgi ribbon.

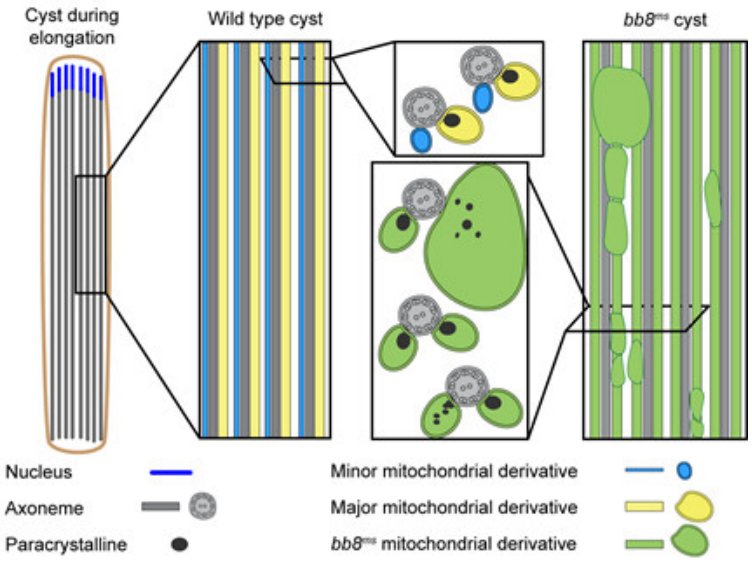


Schematics depicting the involvement of tethering complexes in acroblast integrity. When COG or GARP are non-functional, such as in the fws^{P} and $scat^{1}$ mutants, the Golgi ribbon spreads probably due to a lack of appropriate retrograde traffic.

Fári K, Takács S, Ungár D, Sinka R.
The role of acroblast formation during Drosophila spermatogenesis.
Biol Open. 2016 Aug 15;5(8):1102-10. doi:10.1242/bio.018275. PubMed PMID: 27481842

Mitochondria are essential organelles of developing spermatids in Drosophila, which undergo dramatic changes in size and shape after meiotic division, where mitochondria localized in the cytoplasm, migrate near to the nucleus, aggregate, fuse and create the Nebenkern. During spermatid elongation the two similar mitochondrial derivatives of the Nebenkern start to elongate parallel to the axoneme. One of the elongated mitochondrial derivatives starts to lose volume and becomes the minor mitochondrial derivative, while the other one accumulates paracrystalline and

becomes the major mitochondrial derivative. Proteins and intracellular environment that are responsible for cyst elongation and paracrystalline formation in the major mitochondrial derivative need to be identified. We investigated the function of the testis specific big bubble 8 (bb8) gene during spermatogenesis. We showed that a Minos element insertion in bb8 gene, a predicted glutamate dehydrogenase, caused recessive male sterility. We demonstrated bb8 mRNA enrichment in spermatids and the mitochondrial localization of Bb8 protein during spermatogenesis. We reported that megamitochondria develop in the homozygous mutant testes, in elongating spermatids. Ultrastructural analysis of the cross section of elongated spermatids showed the production of paracrystalline in both major and minor mitochondrial derivatives and megamitochondria. We showed the accumulation of glutamate in bb8 mutant testis biochemically, which was reduced back to wild type level by overexpression of the bb8 genomic region in bb8 mutant background. Our results suggest that the Bb8 protein and presumably glutamate metabolism has a crucial role in the normal development and establishment of the identity of the mitochondrial derivatives during spermatid elongation.



Schematics depicting the mitochondrial phenotypes of wild type and bb8^{ms} elongating spermatids

Vedelek V, Laurinyecz B, Kovács AL, Juhász G, Sinka R.
Testis-Specific Bb8 Is Essential in the Development of Spermatid Mitochondria.
PLoS One. 2016 Aug16;11(8):e0161289. doi: 10.1371/journal.pone.0161289. PubMed PMID: 27529784

The structure and the distribution of mitochondria during late spermatogenesis is known from decades from descriptive studies, but the molecular mechanism and components regulating the mitochondrial elongation and mitochondrial structure are poorly understood [1][2].

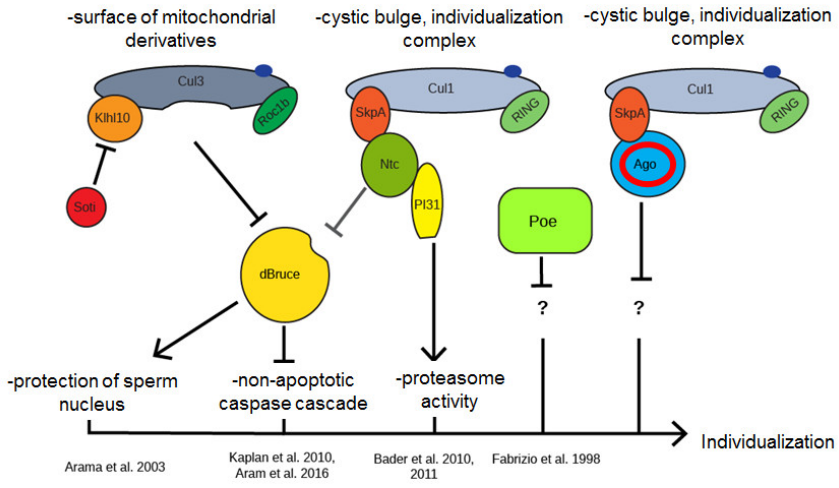
We found a male sterile allele of S-lap3 in our primary screen and started to investigate the function of the Sperm-Leucyl-aminopeptidase protein family (S-Laps) in Drosophila. S-Laps are metalloproteases from the M17 Merops family. There are 8 known S-Laps in *Drosophila*, all of which are testis specific. It is suggested that most of the genes encoding sperm leucyl-aminopeptidases were created by either gene duplication or retroposition. Proteomic studies revealed that the Sperm-leucyl-aminopeptidase (S-Lap) protein family, with all eight members of it, is an abundant constituent of the mature sperm, however their functions are unknown [3].

We measured the amount of the S-lap transcripts in different stages of spermatogenesis by qRT-PCR and by *in situ* hybridization and revealed that all 8 S-Laps were upregulated specifically during the meiotic and post-meiotic stages of late spermatogenesis. We have identified mutations in four S-Lap genes with male-sterile phenotype and characterized them genetically and morphologically. We used the CRISPR-Cas9 method to induce mutation in the remaining four S-Lap genes. In spite of their similarity, there is no redundancy between the investigated S-Lap genes, and they should have important independent role during spermatogenesis. We proved that the S-Lap proteins are localized to the post-meiotic elongated mitochondria and found that S-Lap mutants have mitochondrial structural abnormalities in the late stages, which leads to the disruption of individualization. We measured leucyl aminopeptidase activity and found that the lack of a single or double S-Lap proteins does not affect the testis-specific enzyme activity, what confirms the assumption that the diverged members of the protein family gained new function during spermatogenesis. We tried to express and purify Drosophila S-Laps from bacteria by overexpressing the coding region of the genes and we plan to characterize them biochemically.

We proved that S-Laps are biochemically behave as kinetically stable proteins, they are resistant to the detergent sodium dodecyl sulfate (SDS) and we identified them from the paracrystalline fraction by mass spectrometry. Transgenic lines with HA-tagged S-Laps were established to describe the more detailed subcellular localizations of the proteins by EM. Based on the phenotype, subcellular localization and the amount of the S-laps in the matured sperm, we propose that S-Laps could contribute to the formation of the mitochondrial paracrystalline material.

We have started to prepare the manuscript from the initial characterization of the S-Lap mutants and the function of the proteins

In Drosophila, non-apoptotic caspase activation and proteasomal degradation occur at the final stage of sperm differentiation, also called individualization, when cytoplasmic content is removed and degraded in the waste bag at the end of individualization to create a highly motile sperm. We identified and described a new component of the ubiquitin ligase complexes that regulates individualization. Ago^{ms} is a semi-lethal, male sterile allele of the *ago* gene, which encodes a protein that is an F-box subunit of an SCF E3 ligase complex. Testing the expression of the three annotated *ago* transcripts (ago-RA ago-RB and ago-RC) in wild type and mutant testes, we found that the ago-RB has a specific role during spermatogenesis. Characterizing the male sterile phenotype, we observed elongated cysts with spermatid individualization defects in *ago^{ms}* mutant testis. We showed that Caspase cascade is active in a*go^{ms}* mutants, however the actin cones are scattered, and unable to form cystic bulges and waste bags, probably the results of an incorrect protein degradation during individualization. We rescued of the *ago^{ms}* phenotype by overexpressing the mCherry-Ago protein in *ago^{ms}* mutant background and observed the accumulation of mCherry-Ago in the nuclei of the spermatocytes in early stages. In later stages, mCherry-Ago is localized to the base of investment cones in the cystic bulge, showing a similar pattern to the Cul1-SkpA-Ntc E3 ligase subunits [4]. Our results suggest that Ago has an independent or additional role in the known individualization specific SCF complexes in the degradation in the cystic bulge. We have started to find the biochemical partners of Ago during spermatogenesis.



We have started to prepare the manuscript from the initial characterization of the Ago male sterile mutant.

Next-generation sequencing methods, such as RNA-sequencing is a versatile tool to describe the developmental specific expression of genes in model organisms, such as *Drosophila melanogaster*. To gain a better understanding of cellular differentiation in Drosophila testis, we applied this method to analyze the stage specific transcriptome. We isolated the apical region, which contains stem cells and developing spermatocytes; the middle piece with meiotic cysts enrichment and the distal parts of the testis with elongated post-meiotic spermatids by dissecting and cutting wild type testis. Total RNA were isolated and analyzed by next generation sequencing, using Illumina MiSeq system. 17412 gene stage specific expression was described, including protein coding and non-coding RNA genes (lncRNAs). We quantified the significant stage specific transcripts by real-time quantitative polymerase chain reactions and by *in situ* hybridization. 5381 significant changes were identified, from which 808 genes were not tested before in previous microarray experiments. We identified the expression of 2061 lncRNAs and in 203 cases we found significant change between different parts of the testis, suggesting a stage specific accumulation and action of them. 165 lncRNAs have a testis specific enrichment according to general RNA sequencing databases and we found 135 post-meiotically enriched ones. The function of lncRNAs in many cellular developmental processes are known. lncRNAs participate with protein-coding genes in evolutionarily conserved coexpression networks during spermatogenesis [5]. To date, however, the functional significance of lncRNAs in spermatogenesis is unknown, with a few exceptions, such as polymorphic derived intron-containing (Pldi) RNA. The phenotype of the known testis-specific lncRNAs are mainly manifested in late Drosophila spermatogenesis [6].

This method allows us to identify testis specific isoforms of genes with pleiotropic expression and also, we were able to identify testis specific paralogues of central metabolic enzymes, cytoskeletal proteins and members of the protein degradation complexes, such as proteasome.

This new approach was able to identify several new, stage specific transcripts during spermatogenesis, which could help us to identify genes responsible for the organization of post-meiotic elongation and individualization of spermatids.

We have started to prepare the manuscript from the stage specific transcriptomic characterization of Drosophila testis by next generation sequencing.

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