Investigation of the function of the *Histone H4 replacement (H4r)* gene in *Drosophila melanogaster*

Ph.D. thesis summary

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Szeged

2022

Introduction

In eukaryotic cells, the packaging of DNA into the nucleus is performed by histone proteins. Through the condensation of chromatin, which is formed as a complex of histones and DNA, these proteins are responsible for genome stability and the regulation of gene function. The replication dependent canonical histones are found in all cells and are involved in the regulation of affected regions in a manner dependent on their post-translational modifications. In contrast, replication-independent histone variants have specific functions in regulating gene expression and may also show cell type-specific expression.

The canonical histone genes of *Drosophila melanogaster* are located in a large cluster on the 2nd chromosome, containing large number of gene units. In contrast to canonical histones, histone variants are located outside the canonical histone cluster in the genome, in one or two copies, and are generally slightly different in structure from their canonical counterparts, and their specific regulation allows them to perform tissue or differentiation specific functions.

Unlike other alternative histones, the amino acid sequence of the protein product of the *H4 variant (H4r)* gene is the same as that of canonical *H4.* In order to distinguish H4r from canonical H4 and to obtain information about its role, we epitope-tagged H4r, studied its spatial and temporal expression, and revealed its localization in chromatin at the nucleosomal level. Based on immunohistochemical assays, H4r is generally expressed in embryonic and larval developmental states, but in the developing nervous system, slight differences in H4r expression are observed between cell types, a smaller population of the brain cells showing H4r accumulation that is not restricted to cell-type or differentiation stadium. ChIP-seq experiments have shown that the localization of H4r is preferentially associated with regulatory regions, especially for genes involved in cellular stress responses. Taking together, the experimental data indicate that H4r has a specific variant histone function that differs from the function of the canonical H4, with which it might contribute to the formation of transcriptional memory in non-dividing cells.

Research methods

Sample collection:

- collection and decoration of embryos of different ages for immunostaining
- dissection of organs of L3 stage larvae and 1-day-old adults for immunostaining
- collection and treatment of 0-5 day old adult animals (heat shock or heat shock and regeneration), freezing, separation of heads for DNA, RNA and protein preparation

Molecular biological and genetic methods:

- construction of a plasmid encoding guide-RNAs targeting *H4r* gene by ligation-independent cloning (SLIC)
- construction of a plasmid carrying 3xFlag epitope-tagged H4r and the dsRed marker by classical cloning
- generation of transgenic *Drosophila* line expressing epitope-tagged H4r, lines expressing epitope-tagged H4r and cell type-specific Lamin B-GFP additionally, by plasmid injections and crossings; generation of a transgenic line expressing epitope-tagged H3.3 by crossing
- immunohistochemical assays and microscopy
- preparation of chromatin, RNA and protein
- conventional endpoint PCR, reverse transcription, qPCR
- Tricine-SDS-PAGE and Western blot
- chromatin immunoprecipitation, library construction and sequencing

Bioinformatical methods:

- using online databases and tools
- design of plasmid constructs and primers (SnapGene)

- analysis of microscopic images (ImageJ and Leica LAS AF)
- analysis of sequencing data (https://usegalaxy.eu/)
- statistical analysis (Excel and GraphPad Prism)

Results and discussion

1. Generation of *Drosophila* lines for monitoring and measuring H4r expression

Since the amino acid sequence of H4r is completely identical to that of canonical H4, and the two proteins differ only in their expression, for the functional examination of H4r it was necessary to distinguish the two H4 proteins from each other. For this purpuse, a plasmid containing the H4r gene with the extended genomic region was constructed, and the gene was fused to the sequence encoding 3xFlag epitope tag. For easy identification of animals producing Flag-tagged H4r, the *dsRed* marker gene flanked by two loxP recombination sites was inserted downstream to the *3xFlag-H4r* gene. Using the loxP recombination sites, we were able to remove the dsRed gene by Cre-mediated recombination, creating another *Drosophila* line that could be used for double immunostaining. The red fluorescent color of dsRed thus did not affect the results of immunohistochemical experiments. The resulting strain was used for crosses that resulted in cell type-specific production of GFP-labeled Lamin B by 3xFlag-H4r-producing animals. These transgenic animals were created to monitor cell-specific expression of H4r.

2. H4r is transferred the embryo as a maternal gene product, is incorporated into the chromatin of pronuclei, and is generally expressed in all cells during embryogenesis

A series of immunohistochemical studies have shown that H4r is highly expressed in the somatic cells of the ovaries and testes, however, due to the weaker expression it shows in the germline cells it can enter the embryo as a maternally derived protein product, is found in both maternal and paternal pronuclei. After the fusion of the pronuclei, it is incorporated into chromatin during nuclear divisions,

for which the cell uses a large amount of maternal H4r present in the cytoplasm. Following zygotic genomic activation, H4r is generally expressed in all cells during embryogenesis.

3. A smaller population of the brain cells show H4r accumulation during larval neuronal differentiation

Certain cells in the brain showed a large accumulation of H4r compared to the surrounding cells. Immunostaining of animals co-producing 3xFlag-H4r and cell type-specific Lamin B-GFP revealed that the vast majority of H4r-producing cells are cholinergic neurons, but GABAergic and glutaminergic neurons, as well as neuroblasts were also found among them, indicating that the accumulation of H4r is not restrected to certain cell-types or differentiation stadium.

4. The expression of *H4r* does not change significantly by heat shock and regeneration.

As the literature suggests that H4r may play a role in the regulation of chromatin around heat shock genes, we examined how the expression of the *H4r* gene changes at mRNA and protein levels, and how the amount of H4r incorporated into the chromatin changes following heat shock and regeneration. Based on the results of qPCR and western blot analyzes, neither mRNA nor protein production changed significantly for the environmental effects studied, and the amount of H4r incorporated into chromatin also remained constant. Based on these findings, the low but continuous expression of H4r may provide sufficient H4 protein for chromatin rearrangements upon environmental changes.

5. H4r binds preferentially to specific chromatin regions

Because H4r shows accumulation in certain cells of the brain that does not result in increased compaction of chromatin from cells that accumulate H4r, and the expression of H4r is much higher in the non-dividing cells than in the dividing ones,

we examined the genome-wide localization of H4r in the brain that mostly contains differentiated cells. In order to determine whether it has a distribution similar to that of canonical histones in accordance with the amino acid sequence identical to canonical H4, or whether it shows variant-specific distribution due to the expression of histone, in accordance with its replication independent expression, the genome-wide localization of H4r was compared to the canonical histone H3 and to another alternative histon H3.3. For this purpose we used animals that produce 3xFlag epitope-tagged H3.3 in the brain. Thus, based on ChIP-seq data with Flag and H3-specific antibodies, we found that the distribution of H4r is highly similar to H3.3 and differs sharply from canonical H3. While canonical H3 was abundant in the distal intergenic regions, H4r and H3.3 were localized mostly at the promoters of genes. This raised the possibility that H4r may play a specific role in transcriptional activation or repression. Examining the average amounts of H4r and H3.3 per nucleosome in each gene, we found that these two histone variants occur in significantly higher amounts on active and inducible genes than on inactive genes. These results further support the hypothesis that H4r may play a role in transcriptional activation.

6. The amount of H4r and H3.3 is increased in the heat shock genes, however, during transcriptional repression coming with regeneration, only H4r remains on the promoters of the affected genes

On heat shock genes, heat shock increases the amount of H4r and H3.3 per nucleosome, confirming the role of the two alternative histones in activation of transcription. However, during regeneration, heat shock genes are silenced, leading to an increase in the number of nucleosomes, and accordingly, the absolute amounts of the two proteins also increase significantly, more for H4r than for H3.3. Although the change per nucleosome is not significant for any of the variants, H4r increases slightly, but H3.3 decreases, i.e., nucleosomes inbuild during regeneration contain more H4r instead of canonical H4 and more canonical H3 instead of H3.3. This finding leads to the hypothesis that although the localization of the two variants

overlaps greatly and both are involved in transcriptional activation, H4r may also function independently of H3.3, remaining on repressed genes and thus it might play a role in the formation of transcriptional memory.

Summary

The results presented in the dissertation are integral parts of the epigenetic research conducted in our group. We have shown that despite the amino acid sequence of H4r identical to that of the canonical H4, H4r has variant functions: its genome-wide distribution shows high similarity to another histone variant H3.3, it is involved in the exchange of protamine to histones in the paternal pronucleus, it plays role in the transcription activation and the regulation of expression of inducible genes, and might play a role in the formation of transcriptional memory.

List of publication

MTMT ID: 10064691

Publication which the dissertation is based on:

Ábrahám A., Villányi Z., Zsindely N. et al. Despite its sequence identity with canonical H4, Drosophila H4r product is enriched at specific chromatin regions. Sci Rep 12, 5007 (2022). https://doi.org/10.1038/s41598-022-09026-x

IF: 4,379

Other publications:

Henn L, Szabó A, Imre L, Román Á, **Ábrahám A**, Vedelek B, et al. Alternative linker histone permits fast paced nuclear divisions in early Drosophila embryo. Nucleic Acids Research. 2020 Sep 18;48(16):9007–18. https://doi.org/10.1093/nar/gkaa624

IF: 11,501

SUPERVISOR'S DECLARATION

I, **Prof. Dr. Imre Miklós Boros, Dr. Dániel László Henn**, the undersigned, hereby declare as the supervisor of the candidate (Andrea Ábrahám) that the doctoral dissertation is her own work completed under my supervision and it conforms to the formal and content requirements of the Doctoral School of Biology (Univesity of Szeged).

Szeged, March 21st 2022.

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Prof. Dr. Imre Miklós Boros

professor

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Dr. László Dániel Henn

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CO-AUTHOR'S DECLARATION

I, the undersigned Prof. Dr. Imre Boros, Dr. László Henn, Dr. Zoltán Villányi, Dr. Nóra Zsindely, Dr. László Bodai, Dr. Áron Szabó and Gábor Nagy, declare that "Despite its sequence identity with canonical H4, Drosophila H4r product is enriched at specific chromatin regions" (Scientific Reports, 2022) and in the doctoral dissertation "Investigation of the function of the Histone H4 replacement (H4r) gene in Drosophila melanogaster" the role of the candidate, Andrea Ábrahám, is decisive. I agree that the results of our publication will be used by the candidate in her dissertation submitted to the Doctoral School of Biology at the University of Szeged Faculty of Science and Informatics, and I declare that I did not use these results in obtaining a scientific degree and will not do so in the future.

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