The role of histone acetylation and ubiquitylation in Drosphila neurons

The primary aim of this study was to collect new information on the histone homeostasis of cells, with particular emphasis on the role of specific histone modifications and histone turnover in non-dividing cells, furthermore to gain information on the interplay between histone modification and ubiquitin homeostasis.

In the first phase of the project our main activity was centered on establishing suitable experimental systems to study histone functions, and the role of specific enzymes involved in ubiquitin turnover. We attempted three different strategies to replace the wild type version of selected canonical histone(s) with modified version(s). These were: replacement of the whole chromosomal histone gene cluster by deletion and introducing 6-12 modified histone gene units (HGU) into the Drosophila genome (1), recombination of modified transgenic HGUs in Drosophila by the use of Flp-FRT and Cre-loxP recombination system (2), and over-expression of a modified histone on RNAi background that permits silencing the chromosomal canonical histone genes (3).

The first of these approaches is based on the observation that 12 histone gene units can rescue animals from which the whole histone gene cluster is deleted. The second approach relies on suitable specific recombination sites by which HGUs can be substituted with artificial ones. Both of these approaches require cloning of several copies of histone gene units in order to generate multiple copies of HGUs to be inserted into the genome. In trying to accomplish this we have repeatedly encountered difficulties which made us conclude that these approaches are unsuitable. The frequent rearrangements and instability of tandem repeated HGU copies in bacterial cells precluded gene manipulations to generate site specific histone modifications. As a consequence we had to use the third proposed approach: overexpression of modified histone(s) on RNAi silenced background. For this we established RNAi lines to silence H1 and H2A genes in Drosophila tissues. In parallel, RNAi insensitive wild type and mutant, GFP and epitope tagged H1 and H2A expressing transgenes were introduced into the Drosophila genome to overexpress modified histones on RNAi background. Our observation was that down-regulation of both H1 and H2A expression from the canonical genes was achieved, however, the rescue with the modified transgene(s) was partial and tissue dependent. As it turned out an inherent difficulty of this approach is that because of the required very high level histone protein production the drivers used for ensuring the transgene expression have unpredictable and unspecific secondary effects. These facts limited the possibility of drawing unambiguous conclusions from the experiments.
During the course of the project a significant technical development in gene manipulation has been achieved by development of the CRISPER/Cas 9 platform. We applied this technique extensively in the second part of the project to epitope mark histone genes, change domains between canonical and alternative histone forms and to introduce alteration into histone genes in order to make possible gene specific silencing. Finally these approaches proved the most useful in reaching the results summarized below.

We applied the CRIPR/Cas9 system also to generate null mutants of all DUB genes in Drosophila in a uniform genetic background. We have established transgenic gRNA lines for the majority of 45 DUB lines. In most of the cases, we were able to obtain mutation in founder animals that

expressed an active Cas9-gRNA complex. However, in some cases we had to face with the difficulty, that founder animals were sterile.

Below is a summary of the results we produced by the use of the mentioned experimental approaches on the specific roles of three different histone types and several DUB enzymes.

The role of specific deubiquitylases and H2A modifications in maintaining ubiquitin homeostasis

We established a Western blot based method to quantify changes in the ubiquitin equilibrium resulting from the loss of function of genes affecting the ubiquitin system. With the use of the developed method of ubiquitin detection we described the ubiquitin profile and the dynamically changing free and total ubiquitin levels during *Drosophila* development. We also monitored the changes in the cellular concentration of different ubiquitin forms resulting from the loss of function of two DUB genes. One of them, Rpn10/p54 encodes a proteasome subunit, while the other one encodes the DUB enzyme Usp5. The loss of function of both genes resulted an elevated intracellular ubiquitin level. We also elaborated our ubiquitin detection system by using statistical methods for data analysis and demonstrating effects of the experimental conditions. These results have been published (Nagy et al 2018).

In order to explore how the lack of ubiquitin PTMs of H2A influence cellular functions and in particular the cellular pool of free ubiquitin, we depleted H2A by RNAi and attempted to rescue the flies by expression of mutant H2A from transgenes. For this we generated constructs so that the expression of H2A from the transgene could be maintained in the presence of the inhibitor RNA that down-regulated the expression from the canonical H2A genes. This was achieved by introducing silent mutations which did not change coding but altered siRNA targets. Using this techniques we demonstrated that silencing of H2A expression in different tissues results in general lethality. By overexpression of the wild type H2A from siRNA resistant transgene a low level of rescue was observed in most cases. The lack of effective rescue might by a consequence of low level expression of H2A from the transgene. In the nervous tissue specific expression of the H2A transgene suppressed the lethality resulting in a good level of rescue that permitted testing the effects of specific H2A mutations. Among these we tested H2A K119R that prevents monoubiquitylation. Surprisingly, this mutant form of histone H2A was as effective in rescue as the wild type form of the gene. Consequently, according these data H2A ubiquitylation has no essential role in Drosophila.

Recently a connection was reported between longevity and the level of ubiquitylation of histone H2A on its K119 residue. We examined the effect of the lack of this post-translational mutation on the lifespan of animals. We found that flies expressing H2A under the control of the ubiquitously active *daughterless* Gal4 driver showed higher longevity than the control flies. However, the non-mutant histone H2A-expressing flies had even higher longevity than that of the K119R-mutant histone H2A-expressing flies. From these results we concluded that the higher longevity is a result of the overexpression of H2A and not that of the point-mutation that changes the ubiquitin target site (Fig 1.)

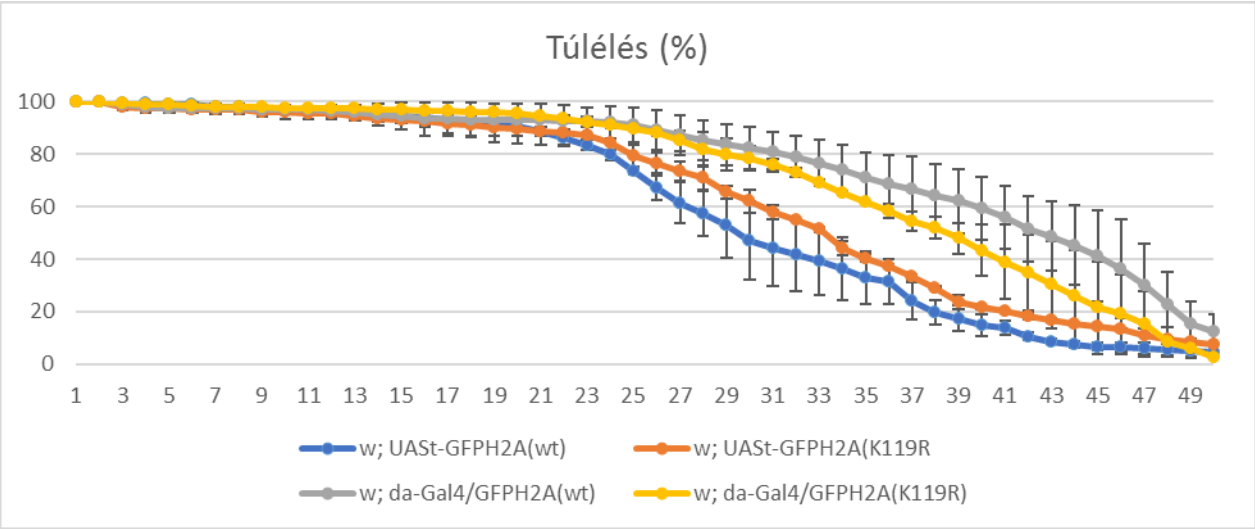


Fig.1. Survival of animals overexpressing wild type and ubiquitylation target mutant H2A

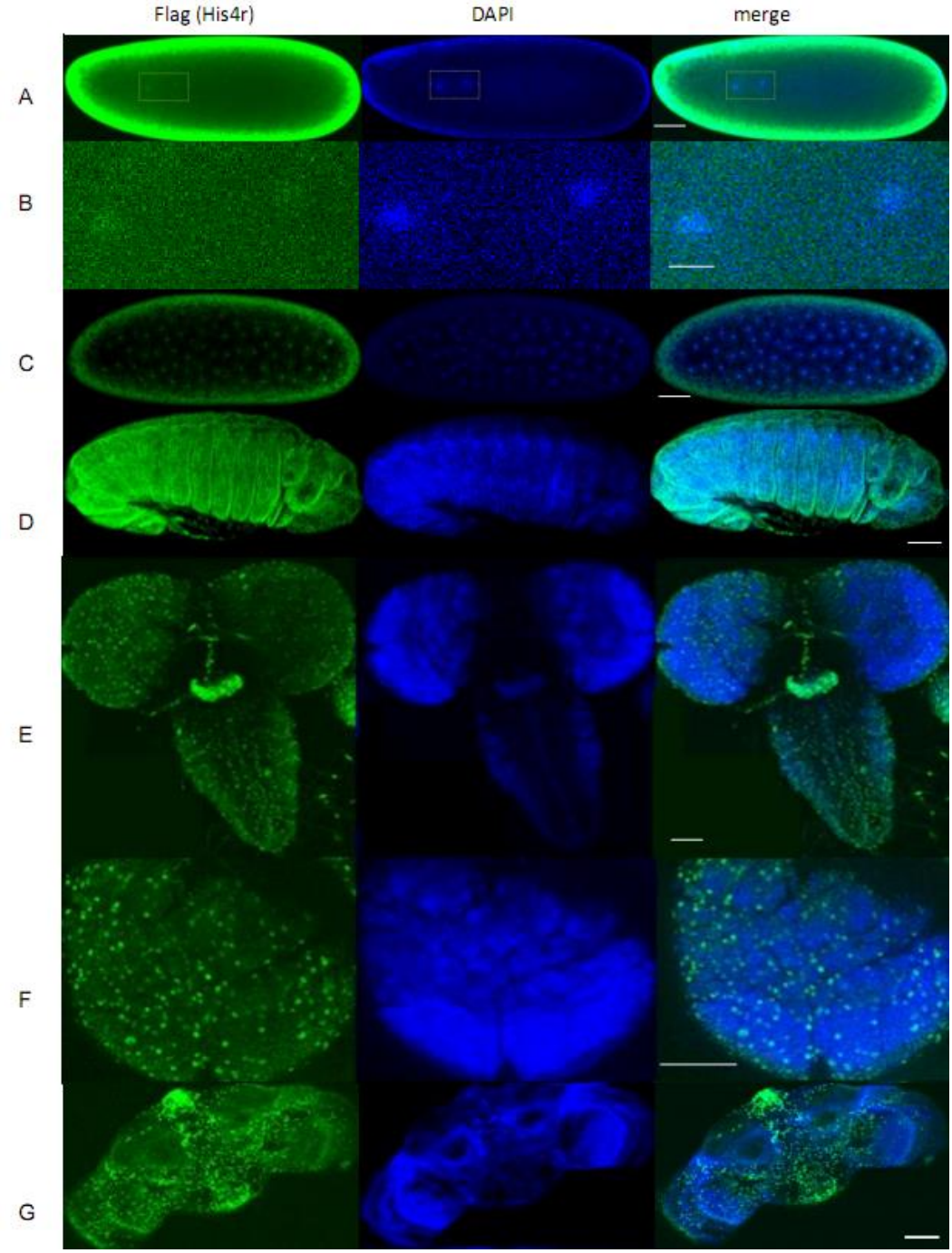
We also attempted to determine the effect of H2A K119R point-mutation on the free ubiquitin pool in L3 larvae. However, we found unexpected difficulties and inconsistency in ubiquitin determination in repeated experiments. This might result at least partly from the quality alterations of available antibodies. As a result, while we demonstrated that the level of H2A overexpression is similar in the point-mutant and non-mutant H2A expressing flies, we could not prove that the changes in the ubiquitin pool caused by the histone H2A K119R point-mutation effected the ubiquitin-mediated proteosomal degradation of other proteins in the cells. Our results on H2A mutation have been reported on several meetings in forms of posters and lectures and are awaiting for publication.

For the analysis of specific role of a de-ubiqulyating enzymes we performed genetic analyses of mutant phenotypes in Drosophila melanogaster and demonstrate that loss of Usp14 function results in male sterility, with defects in spermatid individualization and reduced testicular free monoubiquitin levels. These phenotypes were rescued by germline-specific overexpression of wild-type Usp14. Synergistic genetic interactions with Ubi-p63E and cycloheximide sensitivity suggest that ubiquitin shortage is a primary cause of male sterility. In addition, Usp14 is predominantly expressed in testes in Drosophila, indicating a higher demand for this DUB in testes that is also reflected by testis-specific loss-of-function Usp14 phenotypes. Collectively, these results suggest a major role of Usp14 in maintaining normal steady state free mono ubiquitin levels during the later stages of Drosophila spermatogenesis. The decreased size of the free ubiquitin pool is probably the cause of the male sterility of these mutants. Results on the analysis of Usp14 DUB mutant have been published (Kovács et al 2020)

The role of H4 variant histone

Replacement histone genes are present in different organisms in variable numbers and are in general to provide tissue or/and development specific functions. H4 variants are however more less frequent than H3 variants are and very little is known on their function. In Drosophila the H4 variant gene, H4r encodes the same protein as canonical H4, but its expression pattern is different and independent from cell cycle. Although the deletion of H4r gene does not cause any obvious phenotype, the gene could have specialized role in post-mitotic cells where canonical H4 is not

expressed. To reveal its function, we modified the endogen H4r gene by CRISPR/Cas9 gene editing. We used flies which express Flag epitope tagged H4r to analyse the expression pattern of variant H4 gene. We showed that His4r protein is transferred to the oocytes maternally, and we could detect the presence of the protein in each developmental stages and tissues (Fig. 2). In the larval and adult brain, specific cell types showed differing level of His4r accumulation. With immunohistochemistry we revealed that the majority of cells showing H4r positivity are cholinergic neurons, although fractions of GABAergic and glutaminergic neurons also show high level H4r expression. (Fig.2 bottom part).



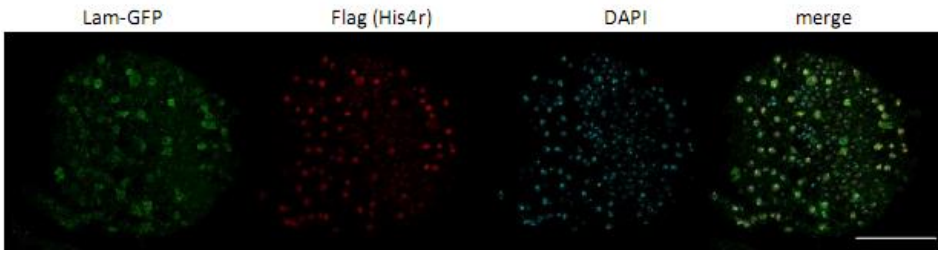


Fig. 2.
(A to G) H4r expression is detectable in Drosophila cells at different stages of development.
(Bottom) In cholinerg neurons indicated by LamGFP expression strong H4r expression is detectable.

We have also studied the chromosomal localisation of H4r by immunostaining of polythene chromosomes and performing ChIP-seq experiments under different conditions. While the former approach did not reveal a noticeable alteration in H4r localisation as compared to H4 expressed from canonical genes by chip-seq experiments we determined that the genome-wide localisation of H4r is dramaticly alter from that of canonical H3/H4 and resembles more to the localisation of H3,3 (Fig. 3)

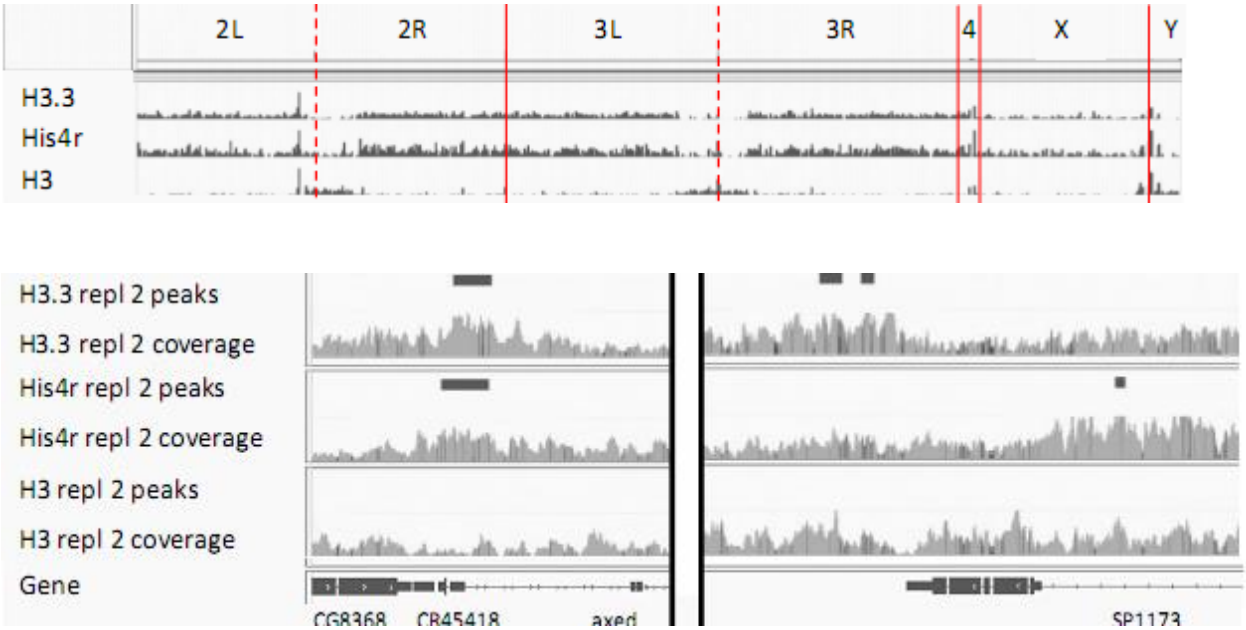
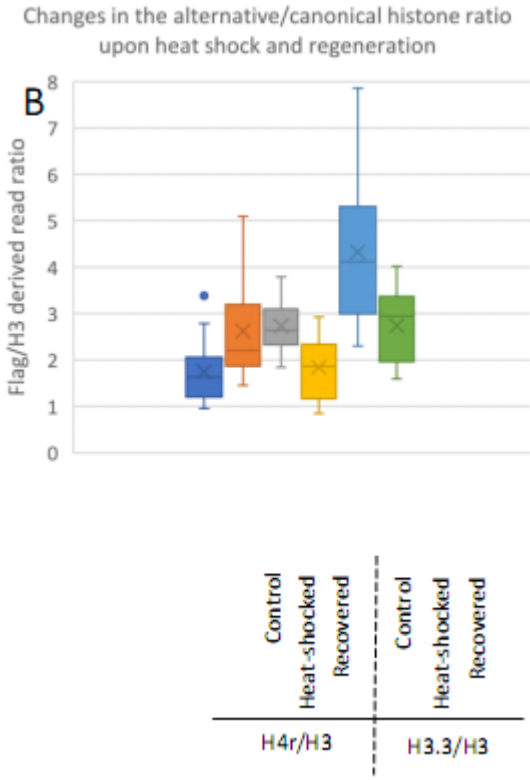


Fig.3.
Results of Chip-Seq experiments (top: genome wide, bottom: at specific regions) indicate similarity but not complete identity between the localisation of the two alternative histones and distinct differences in the localisations of H4r and H3.

Furthermore we found significant changes in the chromosomal location of His4r by ChIP-sequencing performed after heat shock and regeneration. Following regeneration after heat stress, His4r can be found preferentially at chromatin regions that had become active upon heat stress (Fig 4.) Moreover we also found a higher level of His4r on genes that have functions in responses

to environmental changes and in regulation of gene expression. All combined, results of ChIP-seq experiments support our proposal on the role of H4r in transcription memory. These data combined with others on H4, are summarised in a publication submitted for review.



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The role of alternative linker histone variant BigH1 during early embryogenesis

To study the role of Drosophila H1 PTMs, we generated H1 overexpressing line. H1 expressed from the transgene showed identical chromosomal localization with that expressed from the canonical genes and no obvious alterations resulting from H1 overexpression were observable in the structure of polythene chromosomes or heat shock puffs. Neither did H1 overexpressing flies show clear phenotypic changes, except reveiling H1 action as a position effect modifier. However, in analyzing the effects of H1 mutations we encountered a difficulty in finding proper RNAi lines which have phenotype strong enough to analyze the rescuing effect of modified H1 transgenes. Therefore we expanded our studies on the role of dBigH1, the single H1 variant in Drosophila. dBigH1 replaces canonical H1 in the Drosophila germ line and in the early, embryo and has and essential function in proper development. Use of alternative linker histone(s) during the beginning of embryonic development is not unique for Drosophila but a general characteristic in the animal word though we have no definite information on the significance of this. Therefore we found particularly interesting to decipher the special features of this protein and its differences from somatic H1. To this end we replaced domains of the endogen dBigH1 to H1 domains generating H1-dBigH1 chimeric proteins expressed under the control of BigH1 promoter. We

also replaced the entire BigH1 protein coding sequence to H1 (HHH mutants). These modifications were done by the use of CRISPR/Cas9 gene editing tool. We found that the interchange of the N- or C-terminal domain of the protein did not strongly influence the embryo viability and fly fertility under normal growth conditions. However, at low temperature embryo viability was strongly reduced in mutants in which the C-terminal or/and globular domains were H1 type compared to the wild type BigH1. Mutants displayed a nuclear fallout phenotype, indicating the importance of the central and C-terminal domains of BigH1 for proper pre-cellular nuclear cleavages. Immunostaining experiments revealed strong mitotic defects in these embryos and loss of synchronized divisions. We also observed a significant difference in the size of nuclei between *HHH* mutant and wild type syncytial embryos, and found that nuclei are consistently bigger in *HHH* embryos. Based on these observations we hypothesed that the chromatin structure of the *HHH* mutants is less compacted than it is in wild type animals. By MNase digestion assay however, we detected no obvious differences in the nucleosomal structure between the chromatin of *HHH* and BigH1 embryos. In contrast with that, we found alterations in the dynamics of chromatin structural changes during the rapid stages of nuclear cycles: Faster fluorescence recovery suggested that BigH1 facilitates a more dynamic nucleosome exchange than H1 during replication. Despite that, nucleosomes which were formed in the presence of alternative linker histone had a higher stability toward salt extraction.

Taken together, these results revealed that BigH1 allows formation of nucleosomes with stronger DNA-histone interactions and more dynamic exchange than somatic linker histone H1 during the rapid replications in pre-cellular *Drosophila* embryonic nuclei. We proposed that BigH1 allows the histone octamer to slide more freely along the DNA and to engage with DNA segments where positioning is the most favoured. On the contrary, nucleosomes formed in the presence of somatic H1 are constrained into positions which are energetically less optimal, but if appropriate regulators are present this nucleosome arrangement can support the gene expression program. This can explain the phenotype of chimeras where the central globular and C-terminal domains of BigH1 (responsible for nucleosome binding) are replaced with those of H1. The predicted difference between BigH1 and H1 in respect of their interaction with DNA supports our hypothesis on differing chromatosome formation by H1 and BigH1. We have published these results on the role of alternative linker histone in high quality publication (Henn et al 2020).

In addition to the results described above our study on the alternative linker histone also contributed to clarify an ongoing debate between Chinese and Spanish laboratories on the possible replacement of linker histone function. Results related to this question are partly published already and will be elaborated more in a forthcoming manuscript (Carbonel et al 2020). Similarly, our data are used to explain other aspects of alternative linker histone usage discussed in a paper published jointly with Spanish collaborators (Climent-Canto et al 2021).