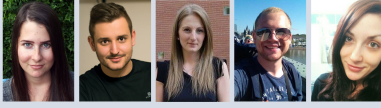


Drosophila as a New Tool to Study the Chromatin Structural Changes Activated by DNA Damages

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In eukaryotic cells DNA is tightly-wound around histone proteins creating a stable but dynamic structure, the chromatin. The dynamic exchanges between packaged or open states of the chromatin are important to maintain genome integrity. The histones can be post-translationally modified (PTM) by multiple histone-modifying enzymes and these PTMs trigger chromatin structural changes during transcription, replication and DNA repair. After the damage has occurred, an opened chromatin structure is formed around the DNA double-strand breaks (DSBs) in order to allow access for repair factors to the break sites, then the original chromatin structure is restored. Both of these actions are mediated by different histone PTMs, but some studies declare that histone PTMs could be important for the regulation of post-repair processes, as well. Finally failure in formation of histone PTMs could delay the repair kinetics leading to genome instability, which results in tumorigenesis. Our focus is to understand how the chromatin structure changes around the breaks and how it is regulated by different histone modifications. We have started to set up human cell culture-based and *Drosophila* *in vivo* experimental systems by which we could study how unique histone post-translational modifications could affect the DNA repair. Our results would allow to reveal new regulators of DNA repair and the final goal of our project is to identify potential biomarkers which could be used in anti-cancer therapies.

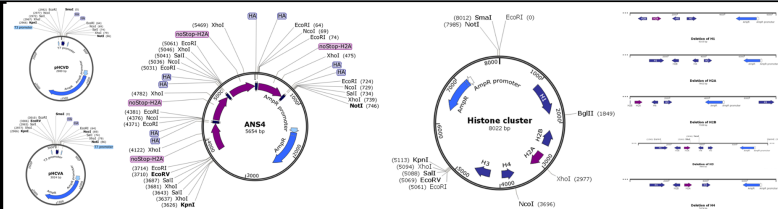


Figure 1: Schematic representation of histone cloning vectors and the engineered histone clusters

We have created two histone cloning vectors (pHCA, pHCD), which include synthetic oligonucleotides with the recognition sites of specified restriction endonucleases and two copies of the coding region of haemagglutinin epitope, and the final version of pHCA contains a single point-mutant histone gene in four copies. On the other hand, we have cloned the *Drosophila* histone cluster in a pBscript KS II vector to prepare engineered histone clusters by using exo- and endonucleases. In these modified clusters a single histone gene is deleted.

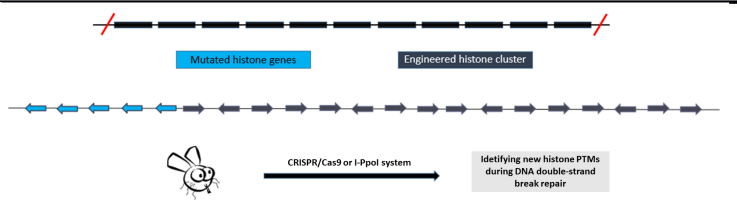


Figure 2: Preparation of transgenic animals with the mutated histones

To generate transgenic animals, we use the *Drosophila* model system, where we delete the endogenous histone clusters, which are presented approximately in 100 copies at a single genomic locus. The endogenous histone clusters are substituted with 12 copies of engineered histone clusters and point-mutant histone genes, which are integrated in three specified positions in the genome. The mutant histones mimic or erase unique histone PTMs, thus we could reveal the role of single histone PTMs during DNA repair. We induce double-strand DNA breaks with the help of CRISPR/Cas9 and I-Pol endonucleases, in order to examine the impact of mutations on the DNA repair kinetics.

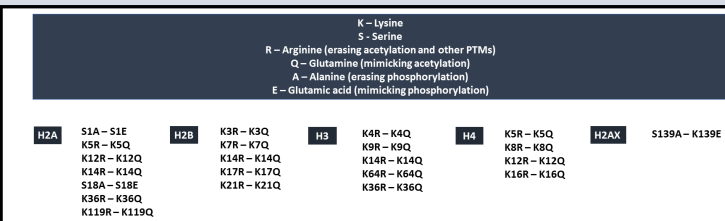


Figure 3: Single amino acid exchange in histone genes via *in vitro* mutagenesis

We have prepared approximately 50 different histone mutations via *in vitro* mutagenesis. Using the Megaprimer technique a single triplet was changed at a specified position, thus resulting a single amino acid exchange in the histone proteins. The mutations inhibit the formation of the given histone PTM, or mimic their presence on histones. Therefore, we could examine the features of unique histone PTMs and we are able to distinguish the function of each modification during DNA repair.

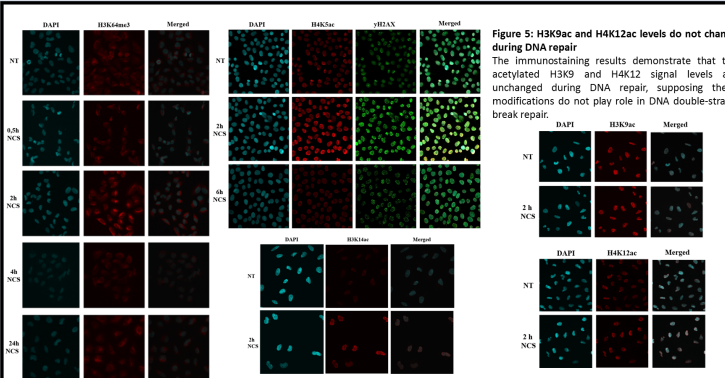


Figure 4: Kinetics of H4K5 and H4K12 acetylation and H3K64 trimethylation levels in U2OS cell line during DNA repair

We observed increased acetylation level of lysine 5 of histone H4 (H4K5ac) and lysine 12 of histone H4 (H4K12ac) upon DNA damage induction (NCS). The immunostaining results show that the acetylation level of H4K5 and H4K12 is increased 2 hours after the NCS treatment [10 ng/ml]. The same phenomenon can be observed in the case of trimethylated lysine 64 of histone H3 (H3K64me3) levels. From this data we can conclude that, the acetylation of H4K5 and H4K12 and trimethylation of H3K64 might be required for DNA double-strand break repair. The modifications appear in a short time after the breaks occurred, therefore we could assume that they facilitates the opening of chromatin structure, thus promoting the proper DNA repair.

We generated a novel experimental system where we could identify novel histone PTMs and we could validate whether they appear at the break site at single cell level by using STORM technique. By using this method we could find unique histone PTMs, which could be used as biomarkers the characterization of tumor stages as well as the early detection of cancer progression.

Our system based on six different screening and validation techniques:

- Screening histone PTMs in *Drosophila* *in vivo* experimental system
- and validating the candidates in human cell-culture based system
- Check the distribution of the identified PTMs by using CSK immunostainings
- Using I-Pol based ChIP system to check the distribution of histone modifications around the break site
- Silencing histone modifiers by siRNA to identify which complexes are responsible for the histone PTM changes following DSB
- Using STORM microscopy to visualize the repair process in a single cell level

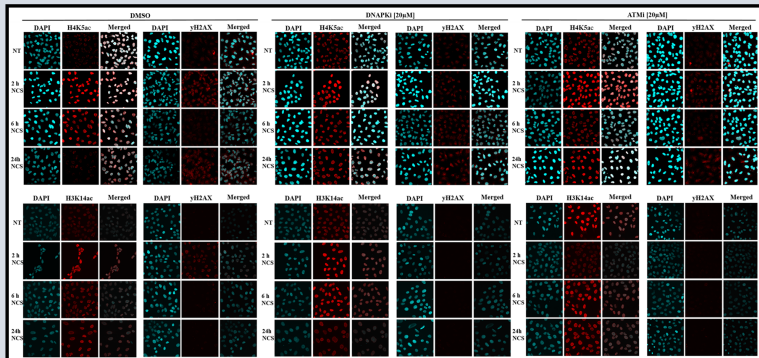


Figure 5: H3K9ac and H4K12ac levels do not change during DNA repair
The immunostaining results demonstrate that the acetylated H3K9 and H4K12 signal levels are unchanged during DNA repair, suggesting these modifications do not play a role in DNA double-strand break repair.

The H4K5 and H3K14 acetylation changes depend on DNAPK and ATM kinases
In order to examine the regulatory role of PI3K kinase in the acetylation of H4K5 and H3K14, we have treated the cells with ATM - (KU5933) and DNAPK - inhibitor (Nu7026) [20μM] during neocarzinostatin treatment [10 ng/ml]. The inhibition of DNAPK decrease H4K5ac level compare to DMSO treated samples, while ATM inhibition does not affect the level of acetylated H4K5. In contrast, the H3K14ac signal shows decrease only after ATM inhibition, assuming that ATM somehow regulates the acetylation of H3K14.

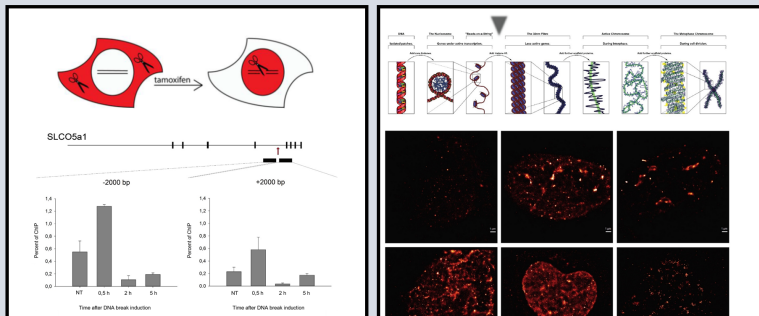


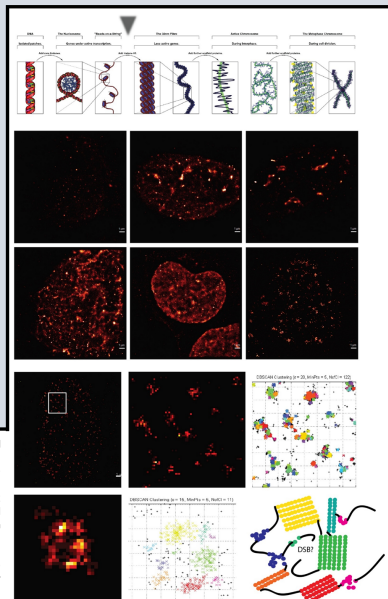
Figure 6: Chromatin immunoprecipitation of H3K64me3

Using an I-Pol endonuclease based system we could investigate the distribution of histone PTMs around the break by using chromatin immunoprecipitation (ChIP) technique. By using this method we already demonstrated that the H3K64me3 level is increased around the DNA breaks. This data confirms that H3K64me3 has an important role in DNA repair.

Figure 7: Checking the distribution of histone PTMs at the single cell level by using super resolution STORM microscopy

In order to see the spatial distribution of the histone modifications at the break site we optimized a specific immunostaining method. By this we could dissolve a single DNA damage spot into several subdomain (see the upper six figures) reaching 20 nm resolution. By the help of STORM microscopy and the clustering algorithm we could estimate the number of nucleosomes in each subcluster which could allow us to calculate the possible chromatin structures around the break site.

As a following step we will use DNA FISH technique in order to see the distance of the histone PTMs spreading and also we will compare our super resolution models to ChIP qPCR and ChIP sequencing data.



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Collaborators
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STORM microscopy - Miklós Erdélyi (SZTE)

