Setting up a new experimental system to studying the link between functional histone PTMs and double strand break repair






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In eukaryotic cells the DNA is packed to a protein backbone which is called chromatin. Specific regions of this compacted structure open and close when different process like information transmission of the DNA to RNA (transcription) takes place. Opened or closed chromatin structure affects the probability of DNA damage and has also impact on the repair of these DNA breaks as well. During the DNA repair processes histone post-translational modifications are important to open chromatin structure. Thus, alterations of chromatin structure are thought to influence not only the efficiency and kinetics of repair but also the choice of the repair pathway in cells. Slower repair speed or improper repair can also increase the probability of chromosome rearrangement by causing genome instability which is one of the early steps of cancer development. This underlines the importance of DDR studies in the context of chromatin for the identification of novel potential therapeutic targets in cancer treatment. With the help of engineered histone cluster and modified histone genes, our experimental system in *Drosophila* allows a unique setup to study the changes in chromatin marks during and after the repair. A powerful model for studying these aspects of chromatin structural changes has not been established in any other experimental systems until now.

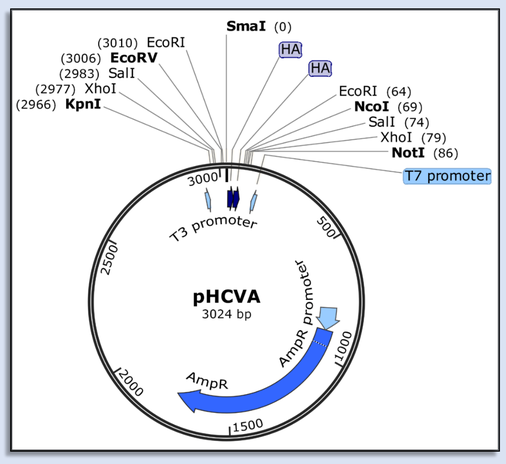


Figure 1: Schematic representation of the Acceptor Histone Cloning Vector (pHCVA) and Donor Histone Cloning Vector (pHCVD)
The pHCVA plasmid contains a 95 bp synthetic oligonucleotide, called 2AB, which contains the cleavage sites of six restriction endonucleases (*SmaI, EcoRI, NcoI, SalI, XhoI, NotI*) and the coding region of hemagglutinin epitope (HA). In addition to the acceptor plasmid, pHCVD includes the 31 bp length synthetic oligonucleotide, called oligo 1. The oligo 1 consists of six restriction endonucleases (*SmaI, EcoRI, NcoI, SalI, XhoI, KpnI*) restriction sites.

Name of mutation	Function of amino acid exchange	Known role of modification
K5R	Erase acetylation/ubiquitilation	Repression of transcription
K5Q	Mimic acetylation	Activation of transcription
K12R	Erase acetylation/ubiquitilation	Repression of transcription
K12Q	Mimic acetylation	Activation of transcription
K119R	Erase acetylation/ubiquitilation	Repression of transcription
K119Q	Mimic acetylation	Activation of transcription

Name of mutation	Function of amino acid exchange
S1A	Erase phosphorylation
S1E	Mimic phosphorylation
S18A	Erase phosphorylation
S18E	Mimic phosphorylation
K36R	Erase acetylation
K36Q	Mimic acetylation

Figure 2: Position and function of the H2A mutations
The figure shows the 14 mutated amino acid positions on histone H2A these mutations are generated by *in vitro* mutagenesis. The megaprimer technique allows to change the target triplet in the H2A gene, and it results in a single amino acid replacement which could mimic or erase specific PTMs.
We substituted the triplet coding the 5th, 12th, 14th, 36th and 119th lysine amino acids to arginine and glutamine. These lysins (K) can be acetylated and ubiquitylated during the DNA repair processes. Furthermore we exchanged the triplet encoding serine (S) at positions 1 and 18 to alanin (A) and glutamate (E) respectively.

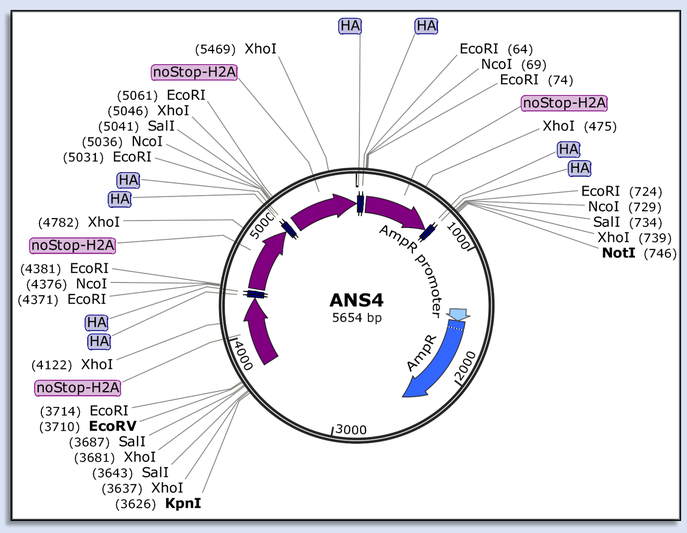


Figure 3: The ultimate structure of pHCVA (ANS4)
According to our cloning strategy, the final version of pHCVA contains a single PTM mutant H2A in four copies. In this process the pHCVD and pHCVA help to each other. First, the given histone gene is cleaved from the pHCVD, then it has two compatible ends, so its ends could be ligated with the pHCVA, which is digested by the same restriction endonuclease. For this, the pHCVD contains two copies of the restriction sites in tandem orientation. The histone genes tagged with hemagglutinin epitope will be expressed as a fusion protein in the animals.

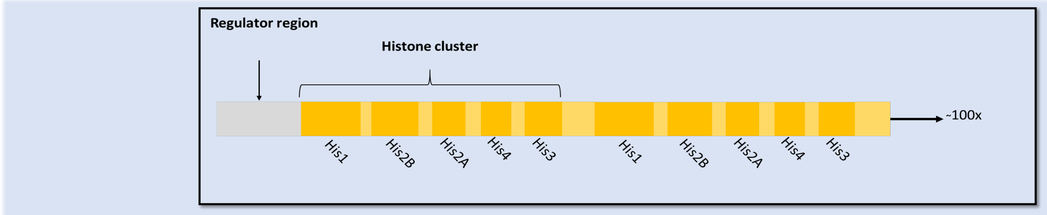


Figure 4: Histone cluster in Drosophila melanogaster's genome
In *Drosophila melanogaster* the histone genes (H1, H2A, H2B, H3 and H4) are located at a single genomic locus and approximately 100 copies of the histone cluster are presented in the genome. To generate a full copy of histone cluster we amplified this region by the help of polymerase chain reaction. To amplify the histone cluster, we used several strategies. First, we tried to amplify the entire locus in one part but despite all of these optimizing steps the histone cluster was not amplified in one PCR product. A new plan has been prepared considering the length of the repetitive sequence before the first copy of the cluster.

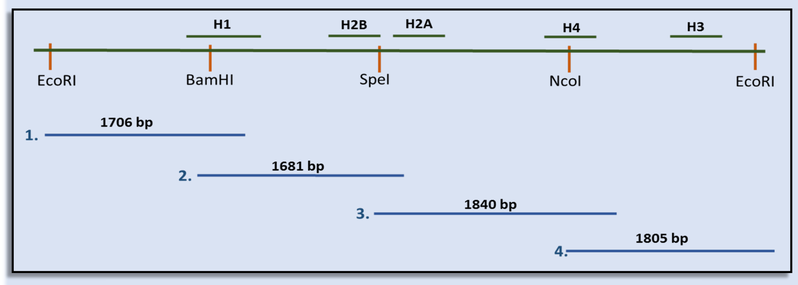


Figure 5: Amplification of the histone cluster in 4 fragments

Four primer pairs were designed to produce overlapping PCR products containing all the histone genes. In that way, the histone cluster can be reconstituted from the PCR products by *BamHI, SpeI, NcoI and EcoRI*, considering the recognition sites of specific restriction endonucleases. After the polymerase chain reaction, the appropriate fragments were isolated from the agarose gel and ligated in pJET 1.2 cloning vector. DH5α *E. coli* cells were used for the transformation. After the plasmid preparation, *BglII* endonuclease was used for the test digestion.

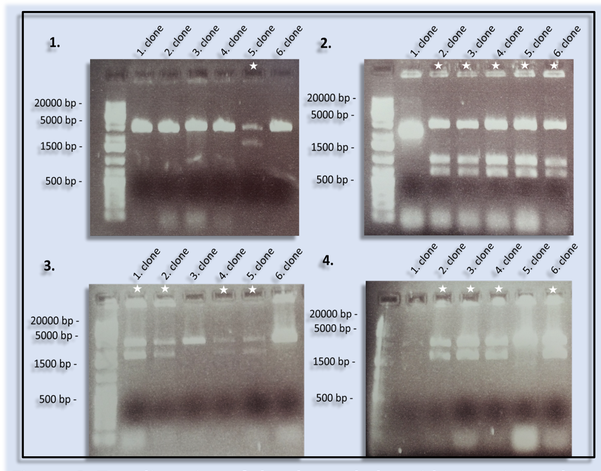


Figure 6: Test digesting of the cleaned plasmids containing the 4 fragments of the histone cluster.
By using this strategy, we could amplify the four overlapping DNA fragments. (Figure 6, white stars). To make sure that there is no amino acid change or frame shift mutation in the insert sequences the DNA fragments are sequenced and proved to be error free.

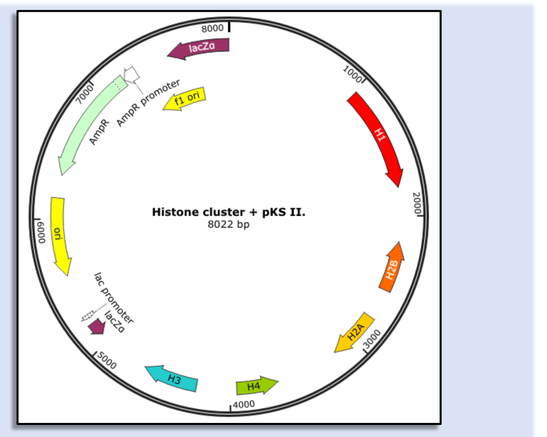


Figure 7: Map of the engineered histone cluster in pKS II. cloning vector
During the reconstitution of the histone culster pBluescript II KS(+) cloning vector was applied. All the fragments were cut out from pJET 1.2 cloning vector using appropriate restriction endonucleases (Fig. 5). Each overlapping fragment has been inserted one by one in a base accuracy way.

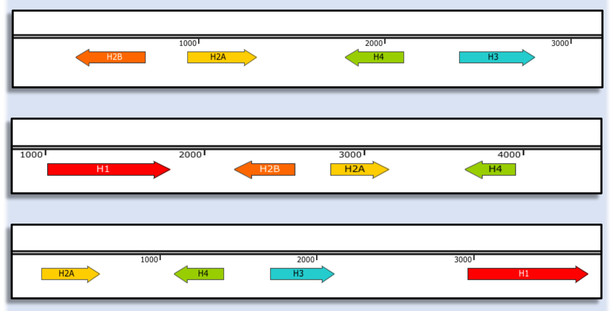


Figure 8: Deletion of H1, H2B and H3 from the Drosphila histone cluster

To set up the experimental system, deletion clusters are required. In order to obtain H1, H2B and H3 histone gene deletions we used restriction endonucleases. Because of the different strategies and the limitations of the endonuclease recongition sequences found in the histone cluster, H3 is partially, H1 and H2B are completely deleted from the histone cluster.

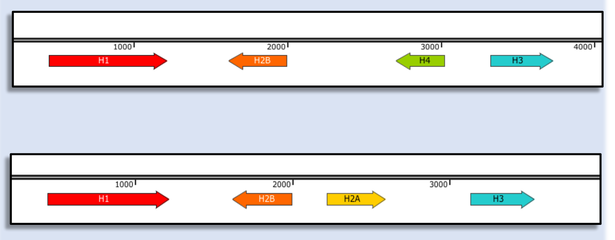


Figure 9: Deletion of H2A and H4 from the Drosophila histone cluster

In case of H2A and H4 histone gene deletion we applied a different strategy due to the lack of suitable restriction endonuclease recognition sequence at the targeted DNA region. To create those deletions the histone cluster was linearized with specific endonucleases at the mentioned genes above then *Bal-31* exonuclease was used for deleting genes from the cluster.



Figure 10: Schematic presentation of preparation strategy in transgenic animals
To reconstitute the histone PTM containing histone cluster, we are deleting the endogenous histones. As a following step, we reconstitute the histones by using the acceptor vector. It will allow the integration of the PTM mutant histone genes into the genome. Thus, the mutant histone proteins appear in the *Drosophila*'s nucleosomes.

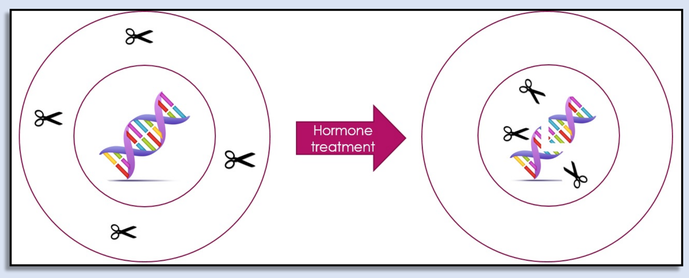


Figure 11: Experimental system: inducing DNA double strand breaks with ER-I-PpoI
For DSB induction we plan to use the CRISPR-CAS9 and I-PpoI based system. In our case the I-PpoI restriction endonuclease is fused to the hormon binding domain of estrogen receptor and tagged by hemagglutinin epitope. By adding 4OHT, (estrogen analog) the nuclear translocation of I-PpoI-ER can be observed. Then, the I-PpoI could cleave the DNA at the nucleus.

As a future step we try to setup an experimental system to study chromatin changes during DSB repair. We plan to take advantage of a *Drosophila* model system, which allows generating targeted single point mutations at the N-terminal tail of a histone. As a further step, the data obtained from the *Drosophila* screen will be tested in mammalian cells, as well. Thus, this will allow us to study whether additional histone modifications in eukaryotic cells have indeed a causative role in the process. Any of the positive results obtained from the screen will be tested by live cell imaging by using GFP labelled repair proteins to check the repair kinetics in time of the different histone mutations by chromatin immunoprecipitation (ChIP).

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