Closing report on the

„Study of chromatin structural changes during Double Strand Break repair”

PD112118 NKFI project

The primary goal of this project was to understand how eukaryotic cells can recognize the appearance of a DSB, and how the chromatin structure is changed following DNA damage. During this period, we could set up an experimental system and we studied how the unique histone modifications could influence the process of DSB repair. Based on the original research plan, we generated 40 missense point mutations in histone genes and identified new post-translational modifications, which have not been reported to take part in DNA repair processes yet.

Project #1: DNA damage induced chromatin structural changes

During the NKFI fellowship period, we have generated missense point mutations resulted in alteration of histone post-translational modifications (PTMs), which could play a role in chromatin remodelling during DNA double strand break repair (DSBR). By using megaprimer-based *in vitro* mutagenesis, we could generate 20 unique mutations resulted in either the imitation or the inhibition of specific PTMs that were controlled by capillary sequencing. (Figure1). We have identified two PTMs, H3K14ac and H4K5ac, that could play important role during DSBR.

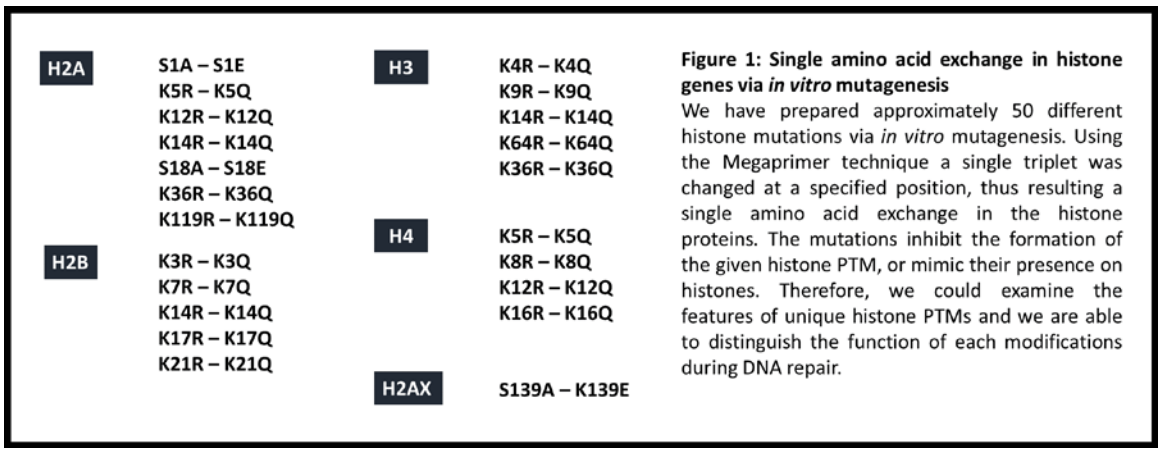


Figure 1: Single amino acid exchange in histone genes via *in vitro* mutagenesis
We have prepared approximately 50 different histone mutations via *in vitro* mutagenesis. Using the Megaprimer technique a single triplet was changed at a specified position, thus resulting a single amino acid exchange in 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 modifications during DNA repair.

To validate whether H3K14ac and H4K5ac also take part during DSBR in higher eukaryotes, we investigated the changes in the acetylation level of that by using DIvA cells derived from human U2OS cells. These cells are suitable for inducing DNA breaks in directed nuclear positions allowing us to follow the dynamic changes of these chromatin marks upon DSBs induction. We supported in human cells that the acetylation level of H3K14 and H4K5 were increased following DNA damage. These changes are shown on Figure 2 confirmed by both immunocytochemistry and Western blot experiments.

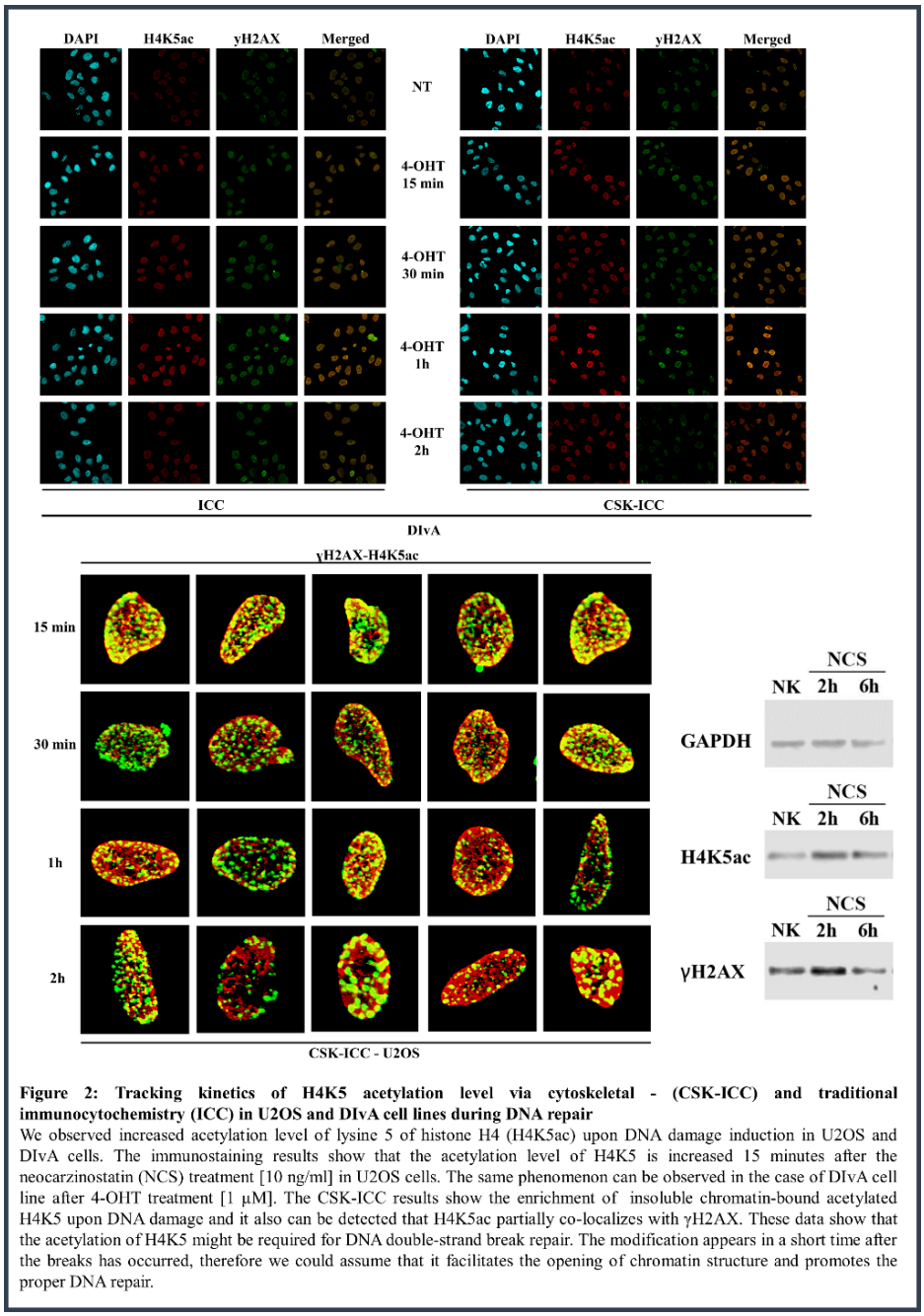


Figure 2: Tracking kinetics of H4K5 acetylation level via cytoskeletal - (CSK-ICC) and traditional immunocytochemistry (ICC) in U2OS and DIvA cell lines during DNA repair
We observed increased acetylation level of lysine 5 of histone H4 (H4K5ac) upon DNA damage induction in U2OS and DIvA cells. The immunostaining results show that the acetylation level of H4K5 is increased 15 minutes after the neocarzinostatin (NCS) treatment [10 ng/ml] in U2OS cells. The same phenomenon can be observed in the case of DIvA cell line after 4-OHT treatment [1 μM]. The CSK-ICC results show the enrichment of insoluble chromatin-bound acetylated H4K5 upon DNA damage and it also can be detected that H4K5ac partially co-localizes with γH2AX. These data show that the acetylation of H4K5 might be required for DNA double-strand break repair. The modification appears in a short time after the breaks has occurred, therefore we could assume that it facilitates the opening of chromatin structure and promotes the proper DNA repair.

By using specific HAT inhibitor against GCN5, we showed that the GCN5-containing histone acetyltransferase complexes could regulate the acetylation level of both H3K14 and H4K5. Additionally, we have also started to follow the distribution of these PTMs with chromatin immunoprecipitation-coupled NGS sequencing (ChIP-seq). In the following term, we plan to publish 2 scientific papers related to this part of the project.

In the second part of the NKFI project, we could optimize super-resolution STORM microscopy on U2OS cells and we started to investigate the distribution of histone PTMs, such as γH2AX, (H2AX S139P) within a single repair focus. With this setup, we could reach 20 nm resolution and resolve a single DNA damage spot. By using STORM microscopy, then the clustering algorithm of it, we could estimate the number of nucleosomes in each subcluster allowing us to reveal the possible state of chromatin structures around the break site.

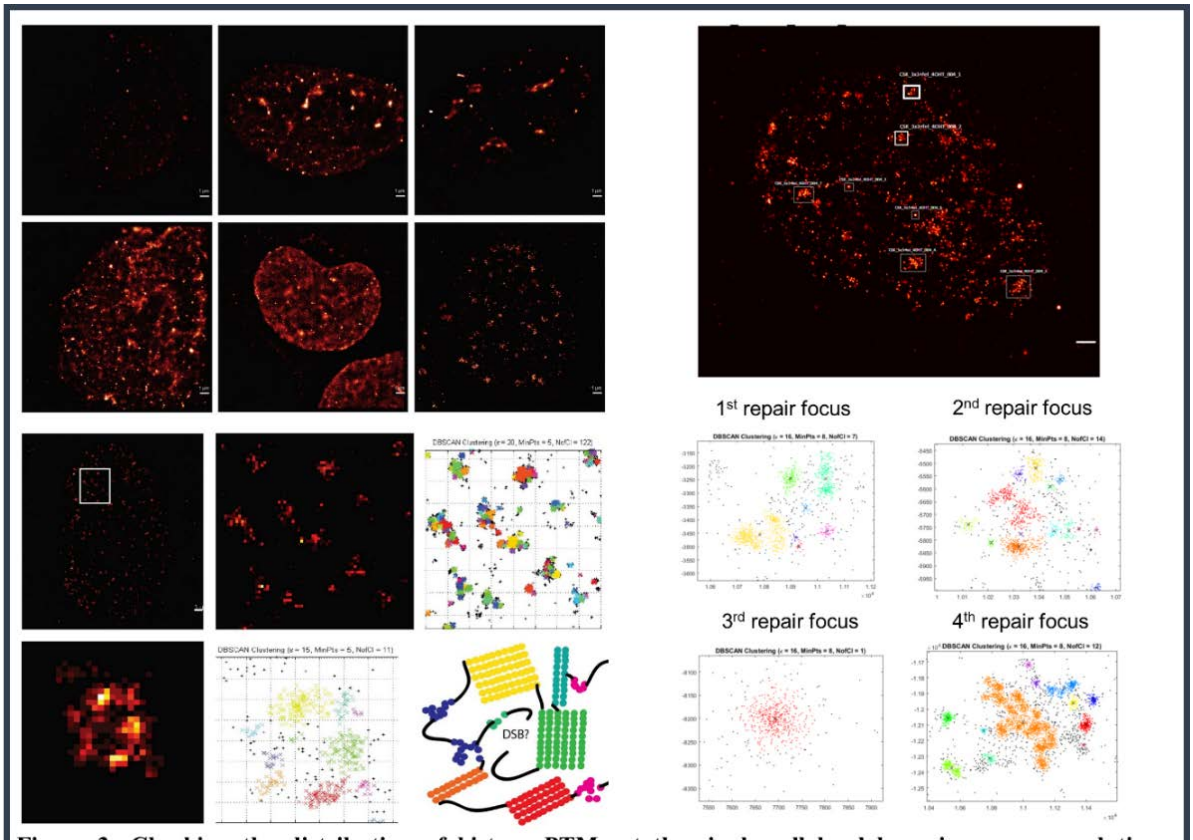


Figure 3: Checking the distribution of histone PTMs at the single cell level by using super resolution STORM microscopy

In order to elucidate 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 focus into several subdomains (see the upper six figures) reaching 20 nm resolution. By using STORM microscopy and a 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 determine the distance of the histone PTMs spreading.

With this technique, we could resolve a single DNA damage focus into several subdomains (Figure 3). We have unlimited access for STORM microscopy, since we had a collaboration with Miklós Erdélyi at the University of Szeged. Based on these results, we have been writing a manuscript, which will be submitted at the first part of 2018.

Project #2: DNA damage-induced transcriptional reprogramming regulated by histone post-translational modifications

It was reported that DNA damage can influence post-translational modifications of histone and non-histone proteins, as well. These modifications are important in damage induced cellular responses, such as DNA damage response, DNA repair, cell cycle arrest and transcriptional reprogramming. It has been known that ultraviolet (UV) B radiation is a dangerous environmental stressor, which can lead to photoaging, inflammation, immune suppression and tumour formation. We used a human keratinocyte model, HKerE6SFM, to demonstrate that UVB activates the transcription of most members of the 11q22.3 MMP gene cluster, including MMP13, MMP12, MMP3, MMP1 and MMP10. In accordance with the increased expression level of the MMP gene cluster upon UVB irradiation, RNA polymerase II showed increased occupancy at their promoters following UVB irradiation. These results also demonstrate increased histone H3K9 acetylation levels at the promoters of the MMP13, MMP12, MMP3, MMP1 and MMP10 genes. These findings suggest a coordinated transcriptional activation of genes in the MMP cluster at 11q22.3 and that acetylation of histone H3 at lysine 9 has an important role in the UVB-dependent enhancement of transcription of MMP genes in this region. These data are represented on Figure 4 and were published in January in the Scientific Reports.

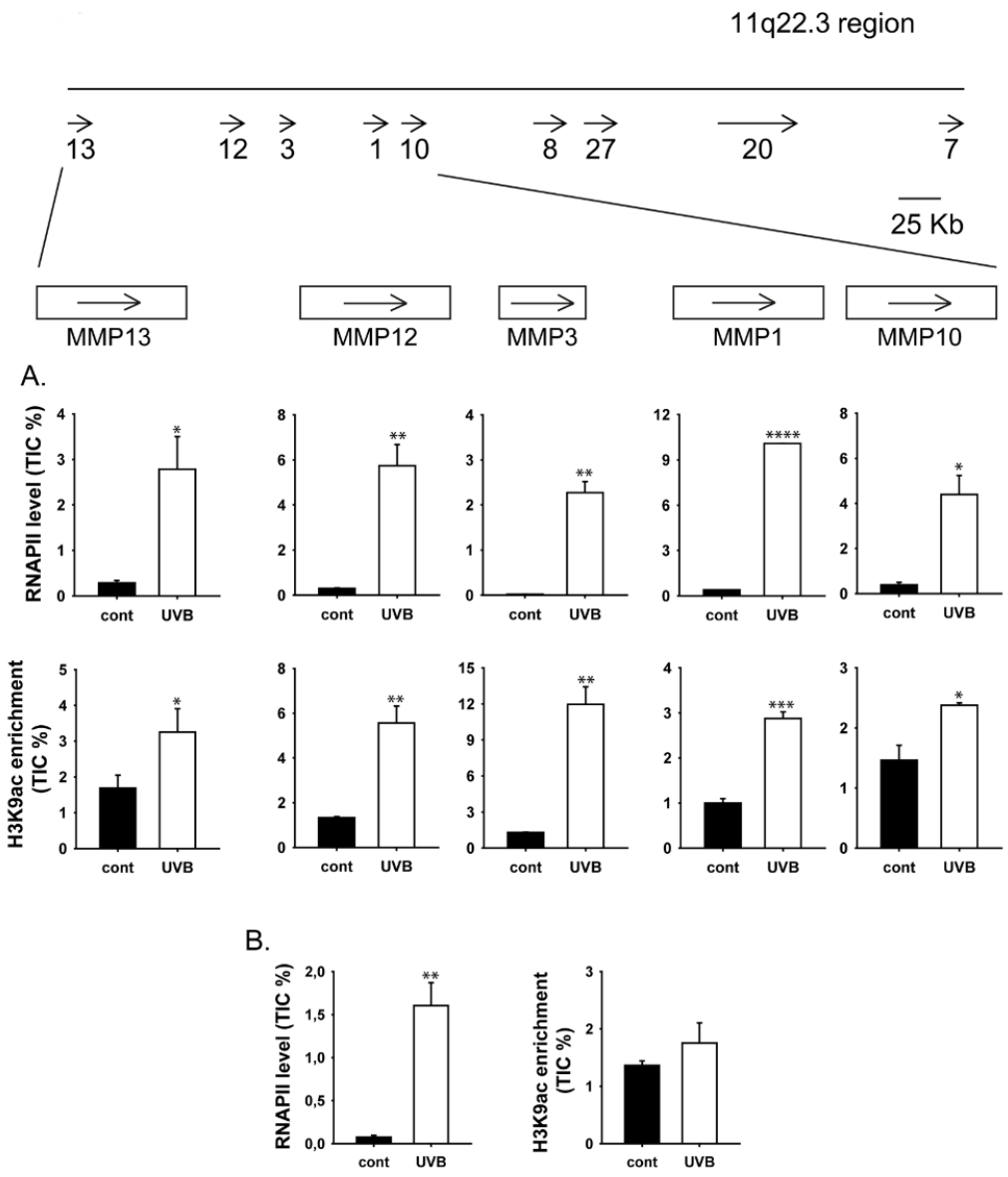
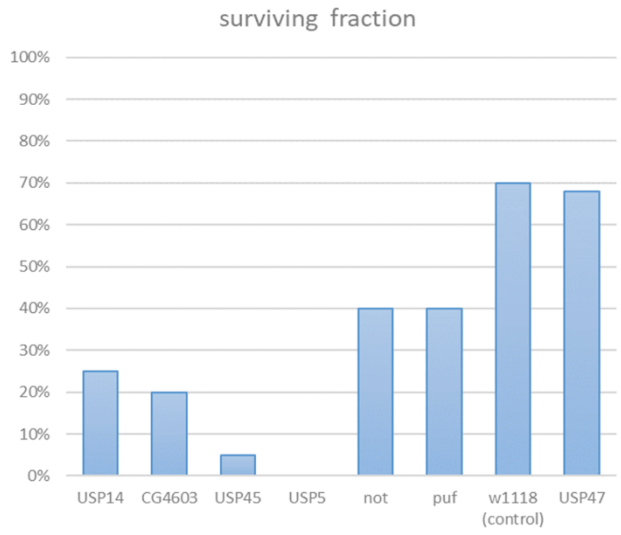


Figure 4. Recruitment of RNAPII and acetylated H3K9 on transcription initiation sites of MMP genes in human keratinocytes.
HKerE6SFM cells were exposed to 80 mJ/cm2of UVB irradiation or mock treated. Chromatin was prepared from each sample 24 hours after the treatment. Antibodies against RNAPII, H3 and acetylated H3K9 were used for the immunoprecipitation. Precipitated chromatin samples were analysed by qPCR. Enrichments of RNAPII, H3 and acetylated H3K9 were calculated as % of total inputs and normalized to the H3 content of control samples. The means, standard deviations and significance levels based on three independent experimental triplicates are indicated.

In addition, transcription blockage induced DNA damage could also activate the binding of non-histone proteins to DNA. The P53 tumour suppressor protein regulates the transcription initiation of its target genes by binding to specific DNA sequences at their promoter regions. In 2017, we reported the novel role of P53 in transcription elongation in human cells. Our data demonstrated that upon transcription elongation blockage, P53 is associated with genes that have not been reported as its direct targets. P53 could be co-immunoprecipitated with active forms of DNA-directed RNA polymerase II subunit 1 (RPB1), highlighting its association with the elongating RNA polymerase II. During a normal transcription cycle, P53 and RPB1 are localized at distinct regions of selected non-canonical P53 target genes and this pattern was changed upon transcription elongation blockage. Additionally, transcription elongation blockage-induced the proteasomal degradation of RPB1. Our results reveal a novel role of P53 in human cells during transcription elongation blockage that may facilitate the removal of RNA polymerase II from DNA. These data were published at Scientific Reports at the beginning of 2017.

Project #3: Identifying novel deubiquitylases implicated in DNA repair

In the last part of this fellowship, we performed a histone deubiquitylase (DUB) screen in collaboration with Péter Deák to identify new DUBs which might play a role in DNA repair. 40 different DUB mutants were X-ray and UV irradiated then their viability was validated following the irradiations. During this screen, we could identify 4 DUBs that could play role in DSBR. These DUBs have not been characterized in any DNA repair processes yet. Additionally, we started to characterize these DUBs and we successfully cloned their Drosophila and human homologues to expression vectors.



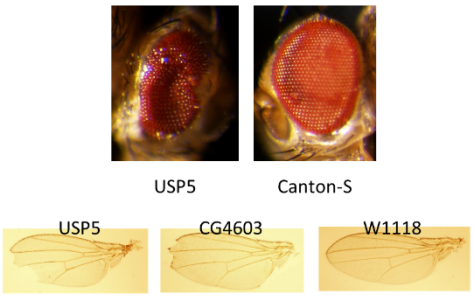


Figure 5. Deubiqitylases identified from the screen.
L3 larvae were irradiated with 80 mJ/cm2 of UVB or with 60G X-Ray respectively. The surviving animals were counted 12h after irradiations and shown on the graphs. The heterozygous siblings also show develomental defects.

In summary during the 3 years of NKFI funding, we published 6 papers in international journals, 4 of them related to the original proposal and 2 additional in collaboration to other groups tightly connected with the investigation of chromatin structural changes. Additionally, we published 3 additional research articles from the related project as it was indicated earlier in my report. We also published two short reports related to the project in Hungarian journals and we participated in 12 international conferences with posters or with presentations. Finally, 2 PhD students defended their thesis, and 3 MSc thesis were also obtained due to the NKFI funding.