1. The goal of this project

Chromosomal breaks are usually associated with pathological disorders but a growing body of evidence point towards their role in a number of physiological processes, as meiotic recombination or class switch recombination. The main goal of this project was two-fold: first, we aimed to find associations between the occurrence of certain histone modifications / histone modifying enzymes, non-B DNA structures (e.g. RNA-DNA hybrids) and DNA damage formation (as DNA double-strand breaks (DSBs) and single-strand breaks (nicks). Second, we wished to understand how the molecular components of DNA breakage is embedded with higher-order chromatin architecture. Our investigations were performed in the model organism *S. cerevisiae* upon various metabolic settings and in mitotic and meiotic growth conditions.

2. Result summary

2.1 **Screening for chromosomal R-loops and hyper-recombinants**

It is well known that persistent R-loops predispose cells to chromosomal instability, therefore we decided to set up a high-throughput cytological screen for the quantification of endogenous R-loop levels in *S cerevisiae* cells. R-loops were immunofluorescently labeled by an RNA-DNA hybrid-specific monoclonal antibody (S9.6) and the signal was detected by laser scanning cytometry (LSC) on large cell populations (n≥1000; Figure 1.).

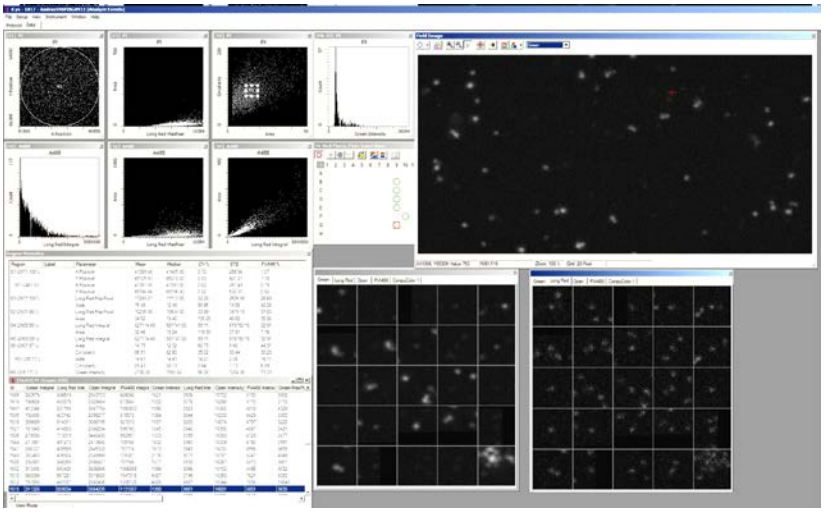

Figure 1. Screenshot of an LSC-based R-loop quantification screen

The analysis was performed on a collection of histone point-mutants (mimicking the presence or absence of the underlying histone modifications) – mainly focusing on post-translationally modifiable amino acid residues. Among the 50 strains tested so far we did not find any histone mutations that significantly affected the level of chromosomal R-loops. The R-loop screen is being continued until we test all amino acid residues on all core histone proteins.

As an alternative of our LSC screen, we set up a flow cytometry-based recombination screen to detect potential hyper-recombinants among the above and other mutants (Figure 2.). In this assay a plasmid (pGLG) was introduced by high-throughput transformation into the cells that contained a disrupted (non-fluorescent) EGFP cassette. If the level of DSBs was higher in a particular mutant the inserted region underwent recombination that restored the EGFP ORF - resulting in the emission of green fluorescence.

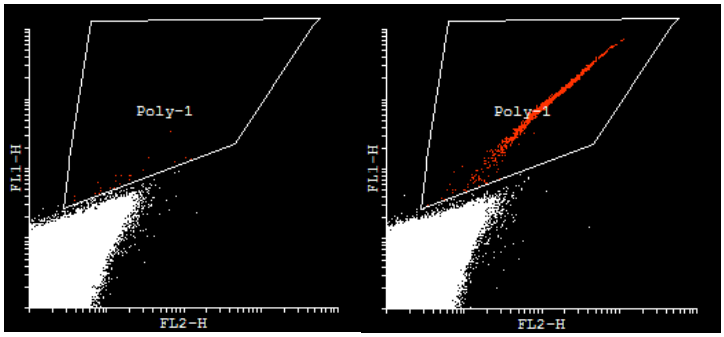

Figure 2. Flow-cytometric measurement of recombination. Left: wild-type cell; Right: *tho1Δ* mutant

By counting the percentage of green cells in the FL-1 channel we estimated the recombination frequency in each mutant. In the metabolic conditions tested (YPD, YPG, YPgal), beyond the *tho1Δ* deletion mutant (defective in mRNA splicing) we could not sort out any histone point mutants from our screen that significantly differed from the isogenic wild-type control in respect of recombination phenotype. To draw a final conclusion from our investigations the hyper-rec screen is also being continued until we test all amino acid residues on all core histone proteins.

2.2 **Structural characterization of a disease-causing histone point mutation: H3K27M**

Despite that our screens did not reveal so far any histone point-mutation associated to hyper-rec phenotype or elevated R-loop formation, we decided to focus on a particular side-chain (H3K27) as it has been described recently that the histone H3K27M substitution mutation drives the aggressive brain tumor glioblastoma multiforme (GBM) (Schwartcentuber *et al.* Nature 2012). Our aim was to understand how the K27 lysine to methionine substitution affects the nanostructure of nucleosome core particles. The mutation was introduced into a cloned H3 gene on a pPET3 expression vector, and the recombinant histone protein was expressed and purified from *E coli* (Figure 3.).

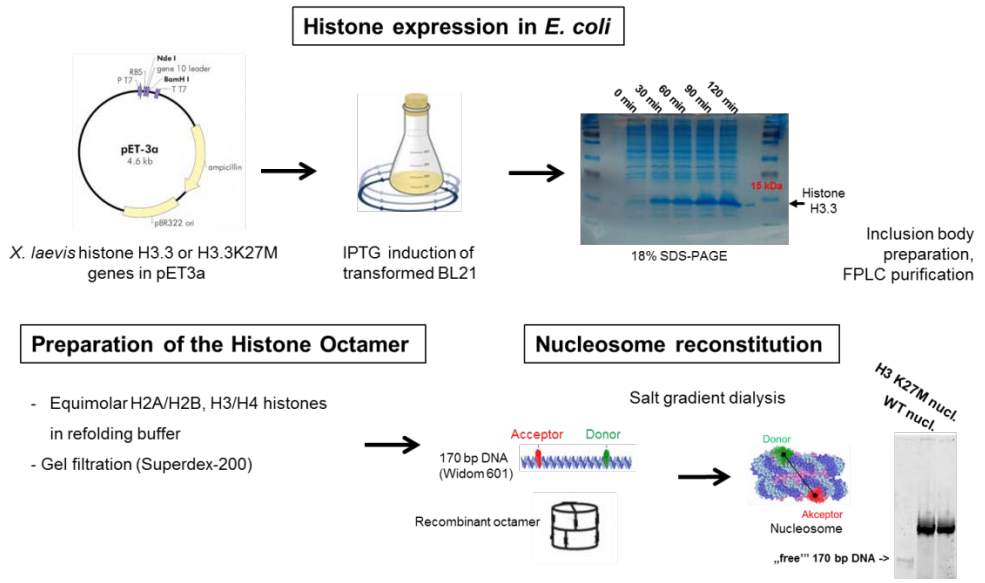

Figure 3. The flowchart of FRET measurement performed on *in vitro* reconstituted nucleosomes

Purified histone octamers were combined in equimolar amounts with the SELEX 601 nucleosome positioning DNA (Widom 601 sequence; gift from Katalin Tóth, Heidelberg) to give rise to nucleosome core particles. The positioning DNA sequence contained a FRET donor/acceptor fluorophore pair at their 5'- and 3' termini (e.g. 5'-Alexa 488 / 3'-Alexa 546) that enabled us to measure molecular-scale distances by FRET (performed in collaboration with Katalin Tóth, DKFZ, Heidelberg).

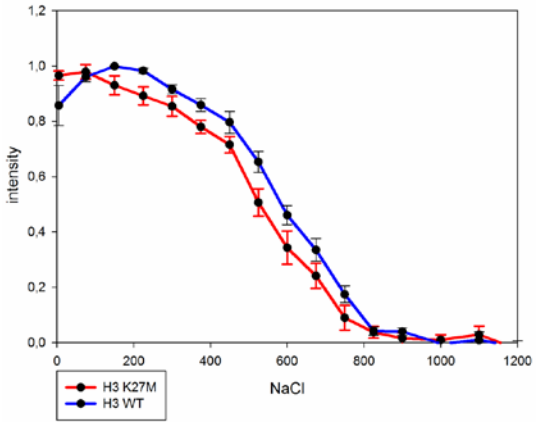

Figure 4. Salt-dependent stability of wild-type (WT) and H3K27M point mutant nucleosomes as measured by FRET

Based on these measurements we observed that the mutant nucleosomes have a more opened configuration, resulting in a significantly reduced salt-dependent stability compared to the wild type nucleosome core particle (Figure 4.). These measurements are being continued so that we explain how the altered nanostructures lead to disease formation.

2.3 **Genome-wide mapping of chromosomal R-loops and single-strand DNA breaks (nicks)**
Since our earlier data (Székvölgyi L *et al.* PNAS 2007) suggested that R-loops were associated to single-strand discontinuities in normal, non-apoptotic cells, we wanted to establish an unbiased genome-wide localization map of these structures so that we can correlate their genomic positions. We worked out a limited *in situ* nick translation-based DNA immunorpecpitation method (nick-DIP) that enabled the simultaneous capture of nick and R-loops in the same cells (Figure 5.).

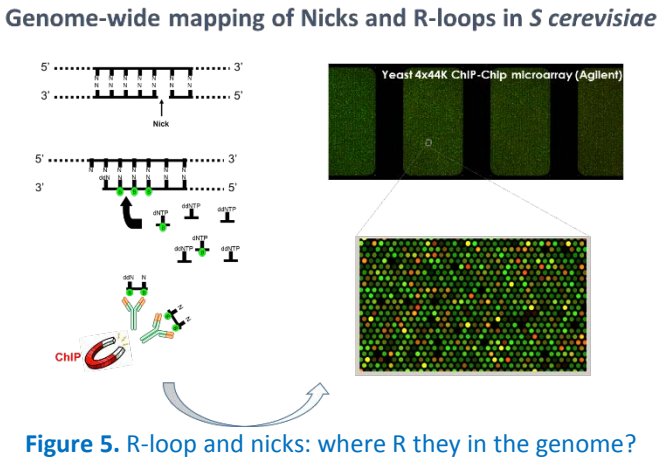

Figure 5. R-loop and nicks: where R they in the genome?
The R-DIP and nick-DIP approaches make it possible to map these structures on the genome-wide scale

Agarose embedded, deproteinized cells were nick translated in the presence of chain terminator ddNTPs + biotin-dUTP (nick tagging) and the nicks and R-loops were immunoprecipitated by anti-biotin and S9.6 antibodies, respectively. The IP and input samples were co-hybridized to whole-genomic microarrays (Agilent) and sites of significant enrichment (peaks) were determined by the *COCAS* ChIP-on-Chip analysis suite. From this analysis we could confirm that chromosomal R-loops are juxtaposed with ss nicks (Figure 6.) as nick are strongly enriched over R-loops, and *vice versa*, R-loops are enriched over the position of nicks. Importantly, this was the first detection of the physical association between

these two entities. We hypothesize that uncontrolled R-loop formation could give rise to persistent nicks that might be converted to a DSB during the next DNA replication – leading to genome instability.

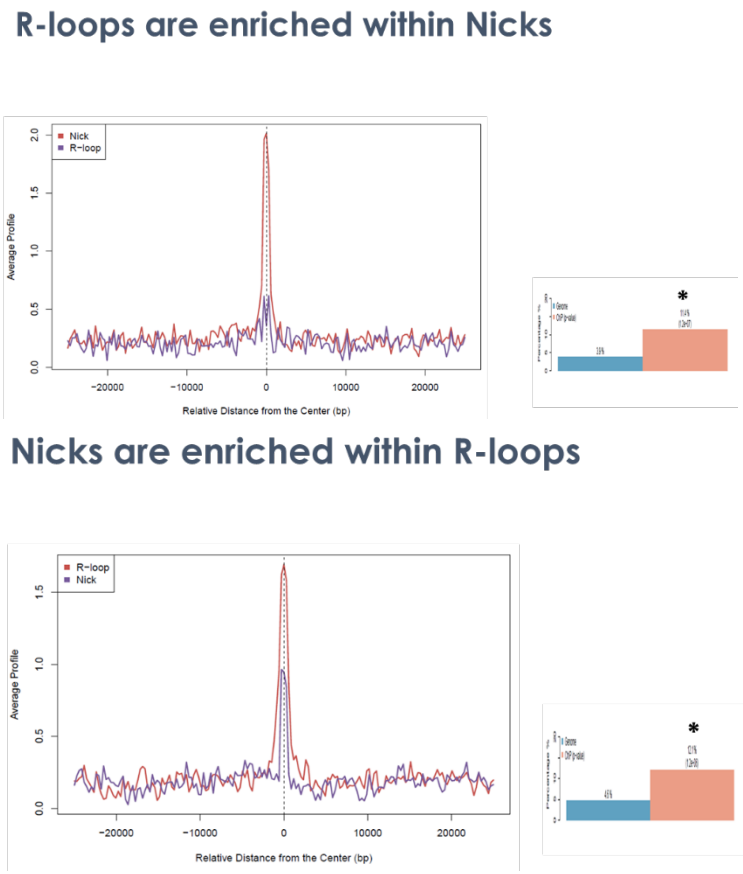

Figure 6. Anchor plots showing the enrichment of R-loop signal over nick peaks (upper panel) and *vice versa*, the nick signal over R-loop peaks (lower panel).

2.4 **Meiotic DSB formation and histone modifications**

Next, we decided to test if other metabolic conditions than mitotic growth (e.g. sporulation) could affect the recombination pattern in our selected mutants. The process of meiosis is accompanied by a ~1000-fold increased level of recombination activity and in yeast it is possible to induce a highly-synchronous meiosis enabling us to directly capture the recombination-initiating DNA lesion, i.e. a DNA double-strand break (DSB), by Southern blot. Since earlier data suggested that histone H3 methylation plays a role in meiotic recombination we focused on the modifiable H3 lysine 4 residue (H3K4R/Q histone point mutants) and the H3 lysin methyase complex COMPASS. We deleted the genes coding for the seven subunits of COMPASS (Figure 7.) and determined the level of DSB formation at the *YCR48W* hotspot. With the exception of one subunit (Shg1) all deletions significantly reduced the rate of recombination initiation.

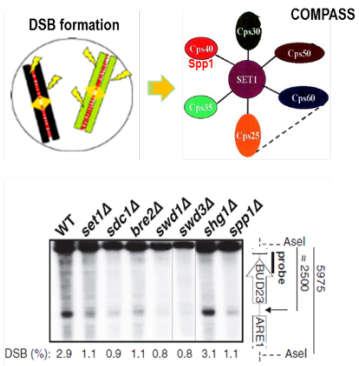

Figure 7. Deletion of the COMPASS subunits reduces the frequency of meiotic DSB formation

Based on this finding we initiated a collaboration with Laurent Acquaviva & Vincent Géli (CRCM, Marseille) and Alain Nicolas (Institut Curie, Paris) in order to build and test a number of COMPASS-based recombination targeting cassettes: the Gal4 DNA binding domain was fused with each COMPASS subunits so that the fusion protein could be tethered to UAS_{GAL4} binding sites. This unique tool enabled us to target histone modifying enzymes to predefined genomic sites in order to find causality between the presence of histone modifications and DSB formation.
When we targeted the catalytic subunit (Set1) to the *GAL2* region (which is normally a coldspot regions lacking DSBs) the GAL4BD-Set1 fusion protein could induce high levels of DSB formation within this naturally cold region (Figure 8.A.).

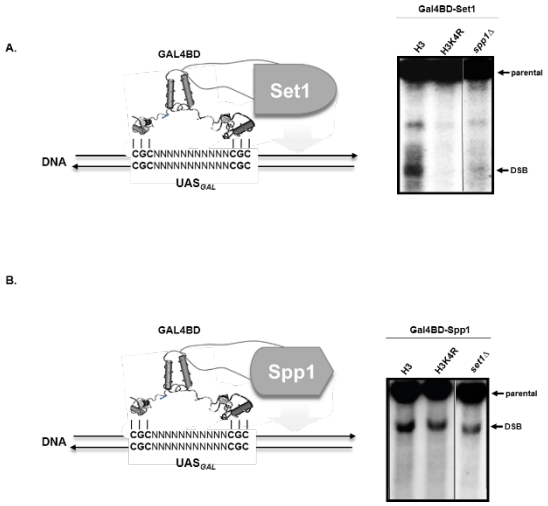

Figure 8. Deletion Modulating the DSB pattern by targeting COMPASS proteins to ectopic chromosomal regions. (A) Gal4BD-Set1 and (B) Gal4BD-Spp1 fusion proteins induce meiotic DSB formation within recombinationally cold regions. Gal4BD-Set1 targeted DSBs depend on the presence of the modifiable histone H3 lysine 4 residue (H3K4R mutant) as well as the presence of Spp1 (*spp1Δ* mutant), whereas Gal4BD-Spp1 targeted DSBs are independent of the H3 lysine 4 residue and Set1 (*set1Δ* mutant).

The GAL4BD-Set1-targeted DSBs were dependent on the modifiable lysine 4 residue (see the absence of DSBs in the K4R mutant) as well as histone H3K4 methylation. Surprisingly, when we targeted the GAL4BD-Spp1 fusion protein to the same site we observed an even higher level of DSB formation that was completely independent of Set1 and the lysine 4 residue, and H3K4me (Figure 8.B). It turned out that the presence of the PHD finger subunit Spp1 is crucial to induce meiotic DSBs.

3. Closing remarks

The ultimate goal of this project was to understand the biological role of histone modifications and non-B DNA conformations (as RNA-DNA hybrids) in the formation of single-strand and double-strand DNA breaks. We were following a rather broad approach to answer to our scientific question (from cytological/genetic screening to FRET-based and genome-wide techniques) that eventually led us to propose a working model for higher-order chromatin organization (Figure 9.) in one metabolic condition, i.e. in meiosis.

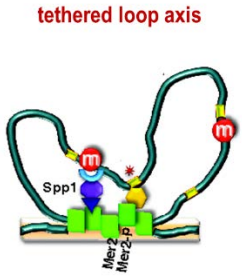

Figure 9. The working model of meiotic recombination initiation in the context of higher-order chromatin structure

The unexpected finding on the role of Spp1 in meiotic recombination initiation was supplemented with the result that Spp1 interacts with the chromosome axis protein Mer2: a yeast two-hybrid screen identified Mer2 – one of the 12 "DSB proteins" essential for recombination initiation - as a direct interactor of Spp1, and we also showed that Spp1 and Mer2 make a contact bridge during the selection of recombination hotspot regions. Collectively, we have revealed how higher-order chromosome structure and DSB regulation are interrelated and it attributes a pivotal role to Spp1 acting at recombination hotspots.

We published these results in the journal of Science as a joint-first author with Laurent Acquaviva. Also, a follow-up paper extending the primarily datasets has been published in the journal of CSH Perspect Biol as a joint-last author with Alain Nicolas.

A methodological strand of our work related to the genome-wide analyses of ss breaks and R-loops – has been assembled into a manuscript:

Zsolt Karányi, László Halász, Lóránt Székvölgyi

Metapeak: the peak-based enrichment profiling of chromatn immunoprecpitation sequencing (ChIP-Seq). Also, the genome-wide nick and R-loop mapping data are being completed and are expected to be published the near future.

In summary, our studies significantly improved our knowledge on how chromosomal break formation and recombination is controlled in relation to higher-order chromatin context and coupled to histone modifications.