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Final report

We have developed a quantitative microscopy-based method, QINESIn, for the characterization of nucleosome stability *in situ*, in a histone composition-, PTM-, and cell cycle phase-specific manner (Imre et al., Sci. Rep. 2017). Making use of this method and a panel of monoclonal antibodies directed against different PTMs (obtained from H. Kimura, Yokohama, Japan), comparison of more than a dozen different PTMs has led us to conclude that there is one category of nucleosomes that stands out with its destabilized character, the +1 nucleosomes marked with H3K4me3 and H3K27ac. Since nicking of the DNA leads to nucleosome destabilization according to our published observations (Imre et al., Sci. Rep. 2017; Bosire et al., Plos One, 2019) we checked how the nicking induced DNA relaxation affects the stability parameters of the different nucleosomes. Intriguingly, the nucleosomes carrying these two marks were not further destabilized, in contrast with all the other nucleosomes. Based on these results we hypothesized that the vicinity of the +1 nucleosomes, specifically the promoters, may be already nicked, as opposed to all other nucleosomes tested. This hypothesis was based on our own break-mapping data and those of others (see below).

We have mapped endogenous DNA breaks by DIP-seq (as in Hegedus et al., NAR, 2018, by incorporating biotinylated nucleotides into the immediate vicinity of DNA breaks carrying free 3'OH, accessible in fixed cells) in human peripheral blood mononuclear cells (HPBs) in several independent biological experiments and also performed a similar experiment in mouse embryonic stem cells, before and after knocking out of three KDM4 genes (experimental system obtained via collaboration with Kristian Helin, Copenhagen, Denmark). The purpose of the latter experiment was to determine if the transient generation of promoter-proximal DNA breaks is the result of topoisomerase IIβ activity as suggested by the Rosenfeld group (Science 312(5781):1798, 2006), or to local, histone demethylation-related HCHO production by demethylases binding to H3K4me3 of the +1 nucleosome, recruiting Top2β secondarily (Perillo's group, Science, 319(5860):202, 2008). These two papers have been cited several hundred times since; however, leaving this crucial mechanistic question unanswered. The incidence of promoter-proximal nicks significantly decreased upon KDM4 KO both on the 5' and 3' side of the TSS, to about 500 bp in each direction. Based on mapping data available in databases for mES cells, accumulation of OGG1-related breaks has been observed 3' of the TSS. Thus, the endogenous, TSS-proximal DNA lesions reported earlier to be generated in the wake of histone demethylation at and around the +1 nucleosome may not be identical to those arising as a result of the endogenous topoisomerase II activity. Our nucleosome stability experiments (see below) have confirmed this hypothesis.

The +1 nucleosomes were significantly destabilized in our *in situ* assay also in mES cells and this state was reversed upon KDM4 KO. The intercalator-sensitivity of the H3K4me3 nucleosomes was shifted back to that of the control, showing that topoisomerase II was in place and potentially active, but rendered inactive in the absence of KDM4 activity. These results are deemed critical for our conclusions regarding the relationship between nucleosome stability and KDM4 status, and were reproduced in several independent biological experiments. We also made control

experiments with mES cells lacking the KO system but treated with tamoxifen, the agent used to activate the nuclease involved in inducing the KO state, and observed no change in the stability of the H3K4me3 nucleosomes. When induced, the KO phenotype was verified in Western blots using KDM4-specific antibodies. Based on these data we have concluded that the stability of the +1 nucleosome is regulated by Top2 β in a KDM4-dependent manner. The manuscript summarizing these data has been deposited in biorxiv (KDM4-dependent DNA breaks at active promoters facilitate +1 nucleosome eviction; https://www.biorxiv.org/content/10.1101/2023.07.14.548993v1) and awaits submittal for publication. *I must wait with submittal for the acceptance of our earlier biorxiv manuscript* (EPIGENETIC MODULATION VIA THE C-TERMINAL TAIL OF H2A.Z; https://www.biorxiv.org/content/10.1101/2021.02.22.432230v2) under review at Nature Communications since last November), since the latter clarifies an aspect of the work on the DNA break-destabilized nature of the +1 nucleosomes: why, through the spectacles of their H2A.Z histone variant content the same nucleosomes appear stable? This question was clearly answered in the submitted manuscript by characterizing the heterogeneity of the H2A.Z nucleosomes, i.e. their presence also in heterochromatin. We also hope that the KDM4-related manuscript, considering our aspirations regarding the journal to be targeted, will be better received after being "validated" by an already accepted high-profile paper.

As described in the same biorxiv manuscript, we have also determined how far nucleosomes may be destabilized upon nicking of the genomic DNA. Titrating nucleosome destabilization as a function of the nickase concentration, we could prove that a single nick per loop-size fragments is sufficient to release most of the nucleosomal content of the total chromatin. This observation prompted us to ask how the specific destabilization of the +1 nucleosomes could be interpreted? Having developed a laser scanning cytometric winding assay to study superhelicity in nuclear halo samples, we could prove that the DNA loops protruding from the nuclei under these circumstances are windable, i.e. they are superhelical. Notwithstanding, they stem from regions harboring DNA breaks, as visualized by *in situ* nick translation, at superresolution. Thus, the stem of the loops must be anchored to lamina-associated structures such that the loops themselves remain superhelical.

We have analyzed the effect of superhelical torsion also on the binding of a structural component of chromatin, HMGB1. This protein binds to the internucleosomal linker DNA, and although it has got 2 amino acids that intercalate into the DNA, it proved not to be sensitive to changes of superhelical twist and writhe. On the other hand, its binding was strongly antagonized by the linker histone H1 and binding of the latter was hampered by intercalators. These observations have lead us to conclude that ligand (protein) binding in the internucleosomal chromatin space is controlled primarily by ligand competition (Bosire et al., Sci. Rep., 2022), as opposed to binding of ligands to nucleosomal DNA which is antagonized by the presence of nucleosomes dramatically (Bosire

et al., Plos One, 2019). A methodical aspect of our work on the above proteins has been elaborated in a collaborative work: Zarębski, Bosire et al., Cytometry A, 2021.

Our observation on the strong superhelicity-dependence of nucleosome stability prompted us to investigate if anthracyclins, these medically relevant intercalators, might influence the incidence of interstrand crosslinks generated by cisplatin, also widely used in cancer chemotherapy. This study allowed us to conclude that the two kinds of drugs antagonize each other through a decrease of interstrand crosslinks upon co-treatment, observed at low intercalator concentrations. At high anthracyclin concentrations synergy becomes dominant because of enhanced nucleosome eviction by the anthracycline in the presence of cisplatin. These interactions may have an impact on the efficacy of combination treatment protocols, considering the long retention time of DNA adducts formed by both agents. See Firouzi Niaki et al., Sci. Rep. 2020.

Having realized the strong effect of intercalators on H1 binding (Bosire et al., Sci. Rep., 2022), we have noticed in the Cellminer database that a subpopulation of cell lines, in 10 out of the 60 lines in the database, certain histone genes, but not others, are silenced by DNA methylation. These loci included the genes coding for certain H1 variants. This finding motivated a research program focusing on the role of linker histones in controlling DNA methylation via regulating access to DNA. In contrary with our expectations, we have found no such relationships in a series of experiments involving cell lines with inducible silencing of particular H1 variants or expressing their GFP-tagged forms (obtained in collaboration with Albert Jordan, Barcelona). Then our attention was caught by the fact that among the genes silenced selectively in the 10 cell lines, was ODC1. The idea if polyamines, synthesized primarily through an ODC1 catalyzed pathway, might actually destabilize nucleosomes, came up. Indeed, we could experimentally prove that this is the case. This observation is unexpected in view of the fact that polyamines elicit higher order chromatin compaction, but are in line with their known effect on nucleosome spacing. An unexpected side-product of this work is the observed tight connection between ODC1 expression and one of the genes involved in the regulation of circadian rhythm, PER2. This work was published in Imre et al., Arch Biochem Biophys. 2022.

In summary, as a result of our studies supported by this OTKA grant we have come to the conclusion that the structural constraint imposed on the DNA by the nucleosome from within is a major player determining the binding of ligands from the outside, and a key controller of nucleosome stability is the superhelical state of the internucleosomal DNA, which is regulated by the collaborative actions of topoisomerases and histone demethylases. The connection between the superhelicity of chromatin loops and nucleosome stability integrates regulation at these two distinct levels in the structural hierarchy of chromatin. The effect of superhelicity and changes thereof on the binding of various ligands (HMGB1, H1, DNA-interacting drugs) to chromatin has also been investigated, with ramifications relevant for basic research on the cell nucleus and chromatin structure, as well as on applications of the drugs in medicine.