HOW IMPORTANT ARE THE HIGHER-ORDER CHROMATIN STRUCTURES FOR THE PROPER GENE EXPRESSION?

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ABSTRACT

In the confined area of the nucleus DNA is organized in chromatin. It maintains the genetic material and by its upper levels of compaction allows proper functioning of the genome. The processes that govern the higher-order chromatin organization are complex and yet not well defined. Importantly, some data show that changes in chromatin organization lead to serious diseases in human which could be a consequence of altered expression of genes localized in chromatin regions with changed structure.

Here, we present the development of a method for higher-order chromatin structure studies in two model systems - Saccharomyces cerevisiae and Drosophila melanogaster.

***Keywords**: DNA, gene expression, higher-order chromatin structures, Saccharomyces cerevisiae*

I. INTRODUCTION

Eukaryotic gene expression is a multi-step process governed by a large set of regulatory factors all playing in a coordinated way, assuring the proper functioning of the genetic material. Its regulation is vital for the cells and the organism because it allows their adaptation to both stress and developmental processes. In the eukaryotic nucleus DNA is organized together with histone proteins in nucleoprotein complex termed chromatin (Jenuwein and Allis, 2001). The main role of chromatin is to preserve DNA and at the same time to allow the correct expression of genes.

Several levels of chromatin condensation are determined in eukaryotes. At the most basic one the molecule of DNA is wound around a histone octamer, called nucleosome. Nucleosome arrays together with DNA define the first level of chromatin condensation, which assures up to 5 times shortage of DNA length (Wollfe, 1995). Further on the molecule of DNA is additionally consolidated by its assembly in thicker structures as the "30 nm" fiber. In fact even this is not enough for DNA incorporation in the nucleus. Certainly upper levels of chromatin compaction are needed for the purpose and these chromatin structures above the "30 nm" fiber are determined as higher-order chromatin organization (Woodcock and Ghosh, 2010). Well known is the fact that linker histones are the fifth class of histone proteins that facilitate, promote and maintain the higher-order chromatin structures (Wolffe, 1995). Little is known about them and research in this field goes on continuously. General experimental approaches for studying higher-order chromatin structures, using light and electron microscopy yield very little information. The reason for this scarce data comes from the fact that nucleosomes and linker DNA cannot be adequately resolved in the compact chromatin.

Here, we present a combination of conventional Comet Assay with *in situ* treatment of cells with nucleases. It led to the development of a novel method for chromatin structure studies, which we named Chromatin Comet Assay (ChCA). Generally, the Comet Assay is an elaborate technique for detection of any kind of DNA damages (Őstling et al., 1984; Olive and Banath, 2006). Nuclease digestion of cells prior to method's implementation allows induction of cuts in chromatin with regard to its compaction status. In this case cells with changed chromatin organization give different

comet images with distinct parameters. Quantitation of results allows assessment of chromatin compaction ratio (Georgieva et al., 2008). In the present study we have applied the method of Chromatin Comet Assay (ChCA) on two model systems: the yeast - *Saccharomyces cerevisiae* and the fruit fly - *Drosophila melanogaster*.

Yeast cells were presented by two strains. Wild type cells with native chromatin organization were compared to mutant cells with knocked-out copy of the gene for the linker histone (*HHO1*). The comparison of these two yeast strains provided a good basis for validation of the method of Chromatin Comet Assay.

The study also involved three *Drosophila* lines: wild type, *delta dfmr1* and flies over-expressing *dfmr1*. FMRP is a highly conserved RNA binding protein. The identification of a *Drosophila* homolog, dFMRP opened the door to detailed genetic investigation of the physiological roles of this protein (Reeve et al., 2005). Its participation in the biogenesis of a large subset of RNA molecules suggests intriguing functions in epigenetic regulation of gene expression. These three lines of *Drosophila melanogaster* gave us a good basis for comparison of their chromatin structures by Chromatin Comet Assay.

The obtained results guarantee the use of the newly developed method in chromatin structure studies and endow its future applications in further chromatin assays.

II. MATERIALS AND METHODS

All reagents and chemicals are purchased from Sigma unless stated otherwise.

Cell cultures and media:

***Saccharomyces cerevisiae* yeast strains:**

The yeast strains used in this research were:

wild type (ABR): *MAT**a** ade2 arg(RV-) leu2-3 112 trp1-289 ura3-52*

HHO1 knock-out: *MAT**a** ade2 arg(RV-) leu2-3 112 trp1-289 ura3-52 ypl127C::K.L.URA3*

HHO1 knock-out cells have been obtained after gene disruption of the gene for the linker histone (*HHO1*) in the wild type cells.

Drosophila melanogaster:

Flies were cultured and tested at 20°C, 68% humidity, on yeast, dark corn syrup and agar food. Three different *Drosophila* lines have been used in this research: wild type, *delta dFMR1* (*ΔdFMR1*) and over-expressing *dFMR1* (*OE dFMR1*). *Delta dFMR1* (*ΔdFMR1*) flies were obtained by deletion of *dFMR1*. *GAL4-dfmr1* overexpression lines were obtained after crosses of *GAL4-elav* and *UAS-dfmr1* on standard fly food and at controlled ambient temperature (22-25°C).

Unless otherwise stated genotypes are as described in Flybase (Drysdale, 2008).

Chromatin studies:

Chromatin Comet Assay (ChCA) on yeast cells:

Yeast cells grown to logarithmic phase ($10x10^6$ cell/ml) were subjected to Comet Assay according to Miloshev et al., 2002.

Comets were observed under Leitz epi-fluorescence microscope (Orthoplan, VARIO ORTHOMAT 2) using 450-490 nm band-pass filter following the staining of DNA with the fluorescent dye SYBR green I (Molecular Probes). Pictures were taken with digital camera, Olympus μ800 at 8 mpx resolution.

Three independent repetitions of the experiments were performed. In each experiment 50 randomly chosen comets per nuclease concentration were taken for quantitation. On the figures representing the yeast ChCA results only one concentration is given for each endonuclease: 100 u/ml for MNase and 50 u/ml for DNase I. These particular endonuclease concentrations were assumed by us as optimal for studying the higher-order chromatin structure of yeast cells.

Chromatin Comet Assay on Drosophila melanogaster cells:

Drosophila melanogaster larvae at eight-day age were subjected to ChCA. Larvae brain cells were isolated under micromanipulator and after washing three times in 1xPBS buffer (2.68 mM KCl, 1.47 mM KH_2PO_4, 1.37 mM NaCl, 8 mM Na_2HPO_4; pH 6.5) were laid over microscopic slides as agarose-cells microgels for Comet Assay analyses as described above with minor modifications required by the type of cells. The modified steps in *Drosophila* Comet Assay included mild lysis conditions and shorter electrophoresis. For the purposes of ChCA the cells in the gels were further digested with deoxyribonuclease I (DNaseI).

Quantitation of Chromatin Comet Assay:

Tail moment:

Tail moment (TM) was calculated by multiplying the percentage of DNA in the tail – IOD_{tail} (integrated optical density of the tail) by the parameter r (Olive et al., 2006). The quantity of DNA in the whole comet was measured by the software Gel Pro Analyzer Version 3.0 – Media Cybernetics, MD, USA. DNA percentage in the tail was calculated as part of the whole DNA content in the comet, which was taken as 100%.

Percentage comets:

The percentage of comets in each probe was calculated as a percentage of the total number of objects observed under the fluorescent microscope.

II. RESULTS AND DISCUSSION

Chromatin Comet Assay on yeast cells:

The Comet assay possesses well-defined advantages in the field of DNA damage detection (Olive et al., 2006). After embedding in agarose cells are lysed and electrophoresed. DNA loops containing breaks escape from the cellular nucleus and form a tail (Shaposhnikov et al., 2008). It is well known that the comet length is not predominantly influenced by the number of DNA breaks, but by the release in supercoiling of chromatin loops (Collins et al., 1997) We have combined the Comet Assay with *in situ* treatment of cells with two traditionally used in chromatin studies nucleases – MNase and DNase I. Both of them have well defined DNA cutting patterns. MNase predominantly makes double-strand cuts in the linker region and is partially inhibited by the presence of linker histones, while DNase I makes single-strand DNA breaks in a 10 bp-step like mode (Wolffe, 1995). The obtained method was entitled as Chromatin Comet Assay (ChCA).

Yeast cells were prepared for Chromatin Comet Assay analyses and were digested with increasing concentrations of MNase and DNase I. Typical yeast chromatin comet assay images are shown on Figure 1A. Images are ordered downward by the increase of the nuclease concentration. It is easily seen that with the increase of the nuclease concentration comets get larger in size with more DNA pulled toward the anode which formed larger comet tails. After quantitation of results we have chosen one concentration per each of the allowing relaxation of chromatin structure and providing typical comet images reflecting the state of chromatin organization in the studied yeast

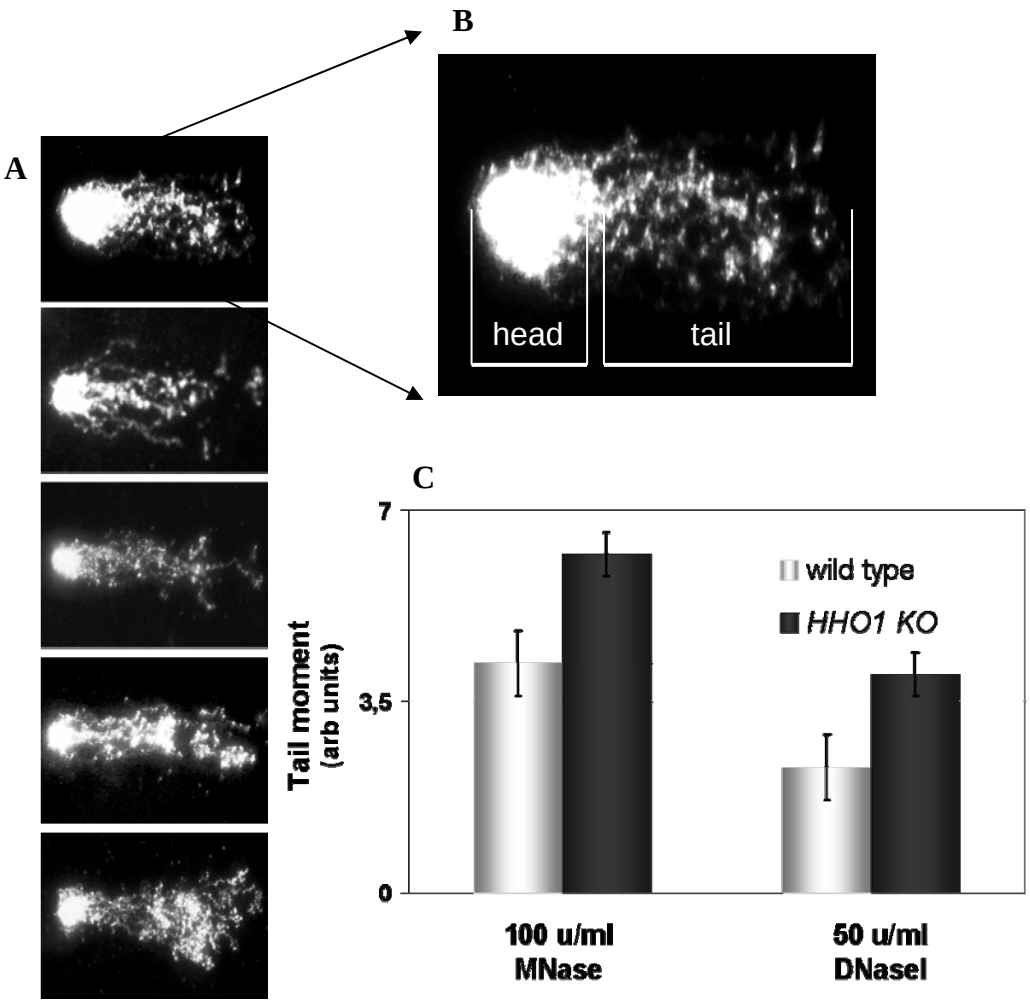

Figure 1. Chromatin Comet Assay on yeast cells.
A: *Typical yeast chromatin comet assay images obtained after digestion of wild type yeast cells with DNase I.*
B: *Characteristic features of yeast comet images: the comet head represents the intact DNA molecule, while the tail stands for DNA loops that have been relaxed by the nuclease and extended toward the anode.*
C: *Quantitation of Tail moment parameter. Comparison between wild type and linker histoneless (HHO1 KO) yeast cells chromatin accessibility.*

strains. All comet images contain a set of characteristic parameters drawn in Figure 1B. For the purposes of this research we have used yeast cells lacking the gene for the linker histone and their progenitor strain. This permitted us to compare their chromatin structures.

Results were quantitated by calculating the parameter Tail moment and are presented on Figure 1C. Yeast cells lacking the gene for the linker histone revealed higher sensitivity to the

action of the two used nucleases. This higher sensitivity is due to more relaxed chromatin structure and is representative of the compaction ratio of their chromatins.

Chromatin Comet Assay on *Drosophila melanogaster* cells:

Three *Drosophila* lines have been used in our research: wild type, *delta dfmr1* and cells over-expressing *dfmr1*. Eight-day cultivation for *Drosophila* larvae proceeded before the ChCA. After these days larvae brains were isolated and brain cells were subjected to ChCA.

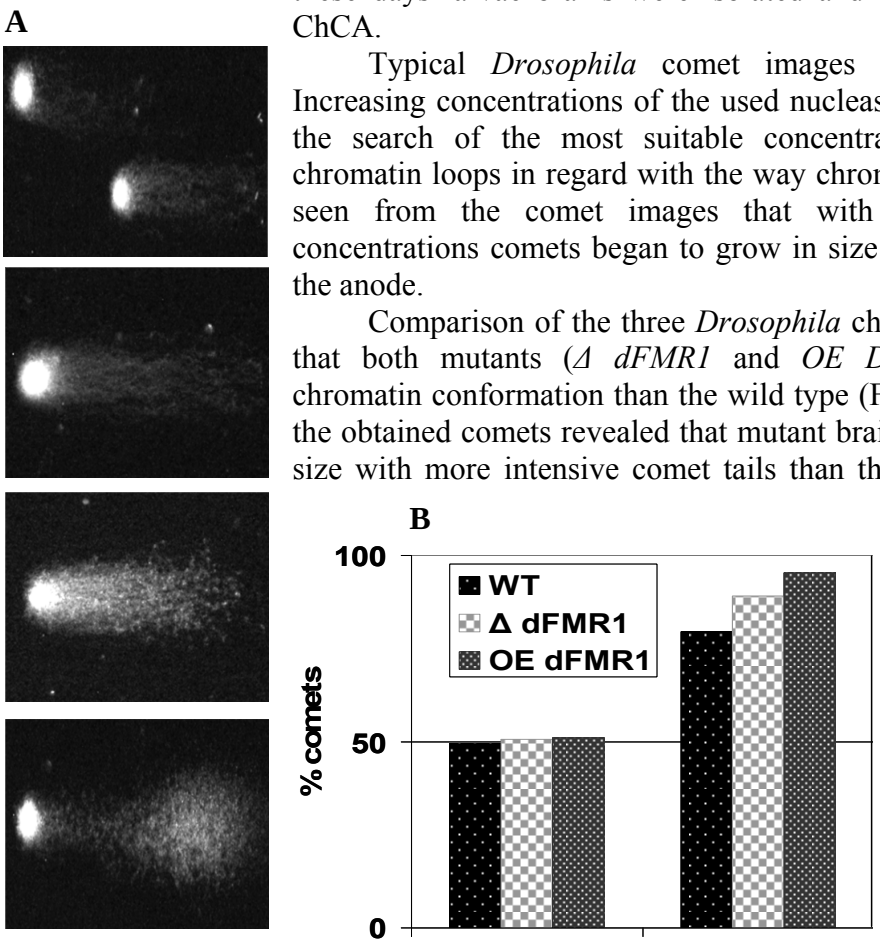

Figure 2. Drosophila Chromatin Comet Assay (dChCA).
A: *Typical comet images obtained after subjecting Drosophila larvae brain cells to ChCA.*
B: *Quantitation of Drosophila ChCA results - assessment of percentage comets in each of the studied Drosophila lines.*

Typical *Drosophila* comet images are shown on Figure 2A. Increasing concentrations of the used nuclease - DNase I were applied for the search of the most suitable concentration allowing relaxation of chromatin loops in regard with the way chromatin is organized. It is easily seen from the comet images that with the increase of DNase I concentrations comets began to grow in size with more DNA extended to the anode.

Comparison of the three *Drosophila* chromatins to DNase I revealed that both mutants (*Δ dFMR1* and *OE DFMR1*) have more relaxed chromatin conformation than the wild type (Figure 2B). Manual scoring of the obtained comets revealed that mutant brain cells gave bigger comets in size with more intensive comet tails than the wild type cells. For ChCA results' quantitation the number of comets has been counted. Percentage of comets has been calculated as described in Materials and methods (Figure 2B). As it is clearly demonstrated on Figure 2B the percentage of comets obtained after DNase I digestion was higher in both mutants. This suggests a very important role of *dFMR1* in structuring of chromatin in brain cells during embryonic development of *Drosophila melanogaster*.

This is the first observation of a role of dFMR1p in chromatin organization of brain cells. These data normally raise intriguing questions about the role of the same protein in human brain development during embryonic stage.

ChCA results with *Drosophila* brain cells proved unambiguously that the method is suitable for chromatin research especially for studying of its higher-order structuring.

IV. CONCLUSIONS

ChCA is a novel method developed by us which allows quick and very sensitive detection of changes in higher-order chromatin organization. The method has been applied on yeast cells: wild type and linker-histoneless mutants and on three *Drosophila melanogaster* lines: wild type and mutants that lack FMR1 or over-express it. The obtained results suggest a great potential of the method for chromatin structure studies. This new method overcomes most of the disadvantages of the other methods for chromatin research because includes single-cell evaluation of bulk chromatin

structure. The advantages of ChCA are that it is easily performed, fast and allows simultaneously manual and automatic scoring. Results are reproducible and helpful in all chromatin structure studies.

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