Increased Age Is Associated With Epigenetic and Structural Changes in Chromatin From Neuronal Nuclei

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ABSTRACT

Chromatin organization has been considered to play a major role on aging, by regulating DNA accessibility to transcription and repair machinery. Such organization can be modulated by epigenetic events, such as DNA methylation and histone post-translational modifications. Since changes on gene expression profiles have been described in aged neurons, our aim was to study the age-dependent relationship between structural and epigenetic alterations on chromatin of cortical neurons from mice. For this purpose, isolated neuronal nuclei from mice of two ages were studied by image analysis after cytochemistry, or assessed for chromatin accessibility by enzymatic digestion. Additionally, two epigenetic marks, for open and for densely packed chromatin fibers were quantified. Results indicate epigenetically driven alterations on chromatin organization of cortical neurons with advancing age, whose fibers seem to undergo redistribution and unpackaging. Since increased transcriptional activity is not characteristic of aged neurons, these loosened chromatin fibers may be associated with impaired genome stability, as well as with increased accessibility of repair machinery to a life span damaged DNA. J. Cell. Biochem. 115: 659–665, 2014.
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Aging is a phenomenon inherent to all living beings and represents a major risk factor for several chronic diseases, such as type-2 diabetes, cardiovascular and neurodegenerative diseases, and many types of cancer [Feser et al., 2010; Harries et al., 2011; Sikora et al., 2011]. It is characterized by a series of biochemical and structural alterations on several organelles, being the nucleus one of the most affected. Such alterations induce a cell cycle arrest and the cell reaches the replicative senescence [Dimauro and David, 2009], leading to a gradual loss of function of the tissue, due to decreased regenerative and proliferative capacities.

It is believed that the aging process is strongly associated with modifications on the chromatin structure [Dimauro and David, 2009; Shin et al., 2011], which can be a result of accumulating errors and epigenetic changes, leading to altered gene expression profile and onset of an aged phenotype [Shin et al., 2011]. Genes responsible for cell repair, stress response, as well as nutrient recognition and uptake have been among the main genes involved with aging and age-associated diseases, thus highlighting the importance of cellular metabolism on the aging process [Kenyon, 2010].

Gene expression is closely regulated by structural organization of chromatin, which can control the accessibility of transcription factors to genes [Di Bernardo et al., 2012]. More condensed chromatin states tend to reduce transcription rate, and increase its stability, whereas a less condensed chromatin tends to be transcription permissive and less stable. Chromatin packing states depend on the interaction of DNA with histones and other nuclear proteins, and such interaction

can be regulated epigenetically [Penner et al., 2010]. Epigenetic control of chromatin structure and gene expression can result from DNA methylation of cytosine residues, histone post-translational modifications (PTMs) and non-coding small interference RNAs [Di Bernardo et al., 2012; Huidobro et al., 2012].

Histone PTMs occur mainly on amino acid residues within histone tails. The main modifications include methylations, acetylations, and phosphorylations, but others may be present [Di Bernardo et al., 2012]. Each of these modifications can induce several effects, depending on the combination of modified residues [Berger, 2007; Kouzarides, 2007; Gibney and Nolan, 2010]. The pattern of distribution and types of epigenetic marks determines the cell phenotype, which can be changed in order for the cell to adapt to different physiological or pathological conditions or under different stimuli [Jenuwein and Allis, 2001; Evertts et al., 2013].

Although the aging process has previously been associated with alterations on chromatin structure, data from the literature are still conflicting. While some authors showed evidence of increased genome instability and chromatin unpackaging in aging neurons [Miyoshi and Fukuda, 1986], other studies described increased chromatin condensation on these cells [Thakur, 1984; Thakur et al., 1999]. While mouse hepatocytes seem to undergo chromatin decondensation with aging [Moraes et al., 2007; Ghiraldini et al., 2012], some senescent human, rat, and primate cells have been reported to show more condensed chromatin [Narita et al., 2003; Adams, 2007].

Chromatin structural alterations have been observed on important progeroid syndromes, such as Hutchinson-Gilford's, Werner syndrome and ataxia telangiectasia. During the aging process the alterations found on these syndromes partially mimic that of aged cells from healthy individuals, suggesting a strong relationship between aging and chromatin structure [Sedivy et al., 2008; Shin et al., 2011].

In addition, previous works describe that memory and synaptic plasticity processes require transcription of specific genes, whose activity is reduced on aging and models of memory loss [Penner et al., 2010]. Given that chromatin structure regulates gene transcription, it is hypothesized that epigenetic modifications on the central nervous system may have key roles on the development of neurodegenerative disorders, such as Alzheimer's disease and schizophrenia [Cheung et al., 2010; Penner et al., 2010; Kosik et al., 2012].

Age-associated cognitive decline is becoming a major problem due to the increased population longevity. Thus understanding the mechanisms involved on the aging process becomes increasingly important, since it could lead to the development of therapeutic measures that prevents or delays the onset of age-associated diseases and increase life quality on the elderly population. Therefore, on the present work, we sought to evaluate structural and epigenetic changes on neuronal chromatin from mice of two different ages. Our present results point to chromatin reorganization with increased age, which is associated with decreased packaging, and changes on epigenetic marks. Such changes could contribute to an altered gene expression profile, which could drive the aging process in neural cells.

MATERIALS AND METHODS

ANIMALS

We used young (4–8 weeks old) and old (55–65 weeks old) Balb/c female mice, obtained from the Multidisciplinary Center of Biological Investigation (CEMIB) of the University of Campinas (Brazil). The animals were reared under standard controlled conditions, fed extruded chow (Nuvital1, Colombo, Brazil) and given water ad libitum. Nearly 20 animals for each group were used in order to guarantee enough brain tissue for nuclei extraction protocols. Animals were killed by decapitation. The protocols involving animal care and use in the present study were approved by the Committee for Ethics in Animal Use of the University of Campinas (registration no. 1608-1) and met the guidelines of the Canadian Council on Animal Care.

NUCLEI ISOLATION

Cortexes from young and old mice were dissected from fresh brain tissue and freed from white matter and meninges. The nuclei isolation protocol was adapted from Thompson [1973] and Pearson et al. [1983]. Briefly, the cortexes were suspended in sucrose solution (2 M sucrose, 1 mM $MgCl_2$, 10% Triton X-100, 0.25 mM PMSF) and homogenized using a Potter type homogenizer. The homogenate was then filtered through a layer of mousseline and ultracentrifuged for 30 min at 64,000g, 4°C. The supernatant was discarded and the pellet was resuspended in sucrose solution (2.4 M sucrose, 1 mM $MgCl_2$, 0.25 mM PMSF). This crude nuclear suspension was applied to a centrifuge tube and another sucrose solution (1.8 M sucrose) was gently poured on top of the first one in order to form a sucrose gradient. The gradient was then ultracentrifuged for 30 min at 85,000g, 4°C.

Two nuclei populations resulted from the second centrifugation: a population in the interface between the two sucrose cushions containing neuronal nuclei and a pelleted population enriched in glial nuclei. The 1.8 M sucrose solution on top of the neuronal nuclei population was discarded and the 2.4 M sucrose solution was transferred to a new tube and diluted in two volumes of 0.32 M sucrose, 1 mM $MgCl_2$, 0.25 mM PMSF. The suspension was then centrifuged for 3 min at 2,000g. The pellet containing the neuronal nuclei was then stored at −20°C in 0.32 M sucrose, 1 mM $MgCl_2$, 0.25 mM PMSF, and 30% glycerol, until use.

CYTOCHEMISTRY AND IMAGE ANALYSIS

Isolated neuronal nuclei were spread onto histological slides, fixed in absolute ethanol/glacial acetic acid (3:1, v/v) for 1 min, and washed in 70% ethanol for 5 min. Fixed and air dried nuclear smears were then subjected to the Feulgen reaction, with hydrolysis done in 4 M HCl at 25°C for 55 min to detect DNA.

For video image capture and analysis, Leica equipment and Leica and ImageJ software were used, respectively. Images were obtained with a Leica DM500 microscope equipped with a 100/1.25 Plan objective, pre-focused and pre-centered condenser, LED illumination (3 W, 6,000 K temp), an illumination control knob for light intensity held constantly at maximum level, and an iris diaphragm of condenser rotated into position 100×. Illumination conditions, including those of the environment, were kept constant for all nuclei

analyzed. Images to be processed were fed from the microscope into an Intel Core i7 Desktop through the Leica ICC50 HD High Definition Digital Microscope Camera and Leica LAS EZ capture software (v 1.8.1, Leica Microsystems Limited, Switzerland). Capture conditions were as follows: exposure 6.8 ms, gain 1.5×, gamma 2.00, and saturation 100.00. Under the optical conditions used, 1 μm corresponded to 15.9 pixels. ImageJ software provided quantitative information on nuclear area (in square micrometers) and mean gray value per nucleus that was subsequently converted into optical density (OD = absorbance). Conversion of mean gray values into absorbances was made by using the software's "Optical Density Calibration" tool, based on a Kodak No. 3 Calibrated Step Tablet, which has 21 steps with an OD range from 0.05 to 3.05 OD. Integrated OD (IOD; in this case, Feulgen-DNA values) was obtained as the product of the absorbances by the nuclear areas. Contrast and entropy, textural features based on Gray Level Correlation Matrices (GLCM), were also estimated. Contrast is a measure of the contrast or the amount of local variation present in the image [Haralick et al., 1973], and reflects the variability in the degree of chromatin packing per nucleus. Entropy measures the level of spatial disorder of gray levels in the GLCM, and reflects how much heterogeneous a Feulgen-stained nucleus can be. Nearly 1000 nuclei per age were selected at random for image analysis such that selection was not limited to a few areas of the smears.

MICROCOCCAL NUCLEASE ASSAY

Digestion of chromatin with microccocal nuclease (MNase) was done according to Thakur et al. [1999] with modifications. Isolated nuclei were incubated with 0.5 U of MNase per mg of DNA for 2, 7, and 10 min and the reaction was stopped with 20 mM EDTA. DNA was extracted with chloroform and precipitated with 0.3 M sodium acetate and ethanol. DNA fragments were resolved on 1.8% agarose gels and band quantification was done using ImageJ software. Genomic DNA (not mixed with MNase) was used as a control. The chromatin accessibility index (CAI) of the two age groups was calculated dividing the sum of DNA digested areas by the uncut areas.

WESTERN BLOT ANALYSIS

Isolated nuclei were denatured by boiling for 5 min at 95°C in 0.06 M Tris–HCl buffer (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.025% Bromophenol blue, and 5% 2-mercaptoethanol [Laemmli, 1970], separated by electrophoresis on a 17% SDS–polyacrylamide gel (SDS–PAGE), and transferred onto a nitrocellulose membrane in 25 mM Tris buffer that contained 15% methanol, 1% SDS, and 192 mM glycine. Membranes were stained in Ponceau S for evaluation of transfer quality. After blocking for 1 h with 4% skim milk in Tris-buffered saline-Triton X-100 (TBST; 50 mM Tris pH 8.0, 150 mM NaCl, and 0.1% Triton X-100), the membrane was incubated overnight in a cold chamber with rabbit anti-H3K9me3 or anti-H3K9Ac IgG (Millipore™) diluted in the blocking solution (1:1,000), then washed three times (5 min each) on TBST (no milk) and incubated with goat secondary anti-rabbit IgG conjugated with horseradish peroxidase (Millipore™) diluted on TBST (1:7,500). All membranes were incubated with rabbit anti-H4 (Millipore™) for input control (1:5,000).

After incubation with the secondary antibody, membranes were washed three times (5 min each) on TBS and bands were revealed with luminol/hydrogen peroxide (SuperSignal® West Pico Trial Kit, Thermo Scientific), imprinted on high performance chemiluminescence film (GE Healthcare®), digitalized and quantified using ImageJ software.

STATISTICAL ANALYSIS

Data from image analysis are presented as mean ± standard error of the mean. The statistical significance between group comparisons was determined by one-way analysis of variance (ANOVA), followed by post hoc Tukey's multiple comparison test. MNase assay and Western blot data were analyzed by means of a Mood's non-parametric test. A bootstrap method of resampling was applied to calculate standard error of the median for these samples. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

CHROMATIN FROM NEURONAL NUCLEI UNDERGOES DECONDENSATION AND REORGANIZATION WITH INCREASING AGE

Images from Feulgen-stained neuronal nuclei from young and old mice (Fig. 1) were analyzed by means of the nuclear areas, absorbances (mean and total per nucleus), and textural parameters (contrast and entropy). As summarized in Table I, nuclear areas are significantly higher in older animals. As expected, this trend is followed also by an increase in integrated optical density (IOD), which reflects DNA content per nucleus. The slight increase on the nuclear content in aged mice may give evidence for an age-dependent aneuploidy, which has been already described in the literature [Rehen et al., 2001; Faggioli et al., 2011].

The ratio nuclear area/IOD was higher in aged animals, thus implying that neuronal nuclei from old mice increase in size without a proportional increase in nuclear DNA content. That means a more spread and possibly decondensed chromatin, which is supported by the finding of the lowest mean OD values in old mice, when compared with younger animals. This unpackaging could be either, decondensation of all nuclear areas, irrespective of their previous packaging level, an even higher decondensation of previously decondensed areas, or decondensation of naturally condensed areas (e.g., heterochromatin). Decreased contrast values with aging, along with decreased mean absorbances (OD), point out to decondensation of previously condensed areas, as these parameters reflect, respectively, the difference between condensed and non-condensed regions with respect to their packing level, along with chromatin condensation level in the nucleus as a whole. Additionally, decrease of entropy in the old animals indicates a more homogeneous chromatin distribution.

In order to confirm this chromatin unpackaging, isolated neuronal nuclei subjected to MNase treatment were also analyzed, since the regions of chromatin that contain actively transcribing genes have been shown to be more sensitive to MNase, which cleaves the DNA at internucleosomal regions [McGhee and Felsenfeld, 1980].

The results shown in Figure 2, clearly indicate that chromatin from aged neurons is more accessible to MNase digestion than
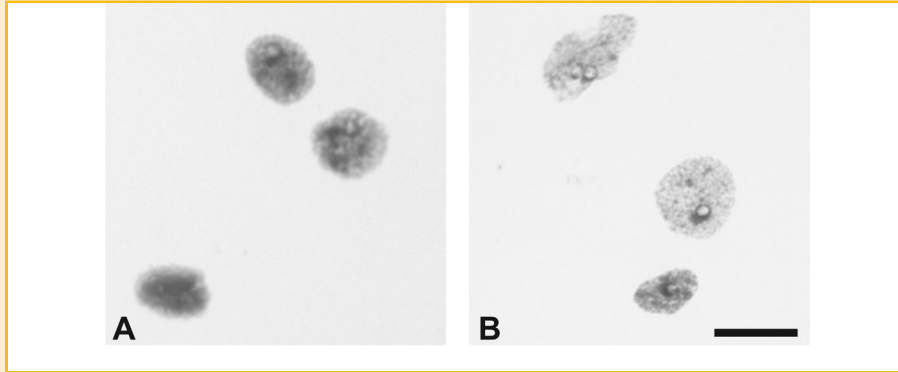
Fig. 1.   Images of Feulgen-stained nuclei from young (A) and old (B) mice. Some neuronal nuclei from old mice are bigger and their chromatin is less packed. Scale bar indicates 10 µm.

that from young neurons (Fig. 2C). Such difference that can be perceived under higher incubation times (Fig. 2B), is due to a more open and loosely packed chromatin in neurons from aged mice, thus being in agreement with the results obtained with image analysis.

AGE-RELATED CHANGES IN CHROMATIN STRUCTURE ARE ASSOCIATED WITH EPIGENETIC CHANGES ON HISTONE TAILS

Considering the role of epigenetic marks on chromatin organization, as well as on gene expression, two marks, one for open chromatin/gene transcription, and other for densely packed chromatin/gene silencing, respectively, acetylation and trimethylation of histone H3 on lysine 9 (H3K9Ac and H3K9me3) [Richards and Elgin, 2002; Berger, 2007; Huidobro et al., 2012] were measured on isolated neuronal nuclei from young and old mice.

It was found that while trimethylation of H3K9 decreases with increasing age, there is a high age-related increase in acetylation levels of H3K9 (Fig. 3). These results are in agreement with the data indicating more open chromatin organization in aged neurons, since acetylation marks on H3K9 are associated with open chromatin fibers. At the same time, since the modifications coincide, it would be necessary that a histone demethylation occurred before the histone acetylation could take place.

TABLE I. Image Analysis of Feulgen-Stained Nuclei

Parameter	Young (n = 893)	Old (n = 864)
Nuclear area (µm²)	44.84 ± 0.534	61.94 ± 0.687
Absorbances (OD)	0.36 ± 0.003	0.27 ± 0.002
Feulgen-DNA values (IOD)	14.71 ± 0.039	15.78 ± 0.045
Contrast	21.56 ± 0.669	16.18 ± 0.522
Entropy	7.22 ± 0.007	7.04 ± 0.009

Values represent arithmetic mean ± standard error of the mean. P is less than 0.001 for all comparisons.
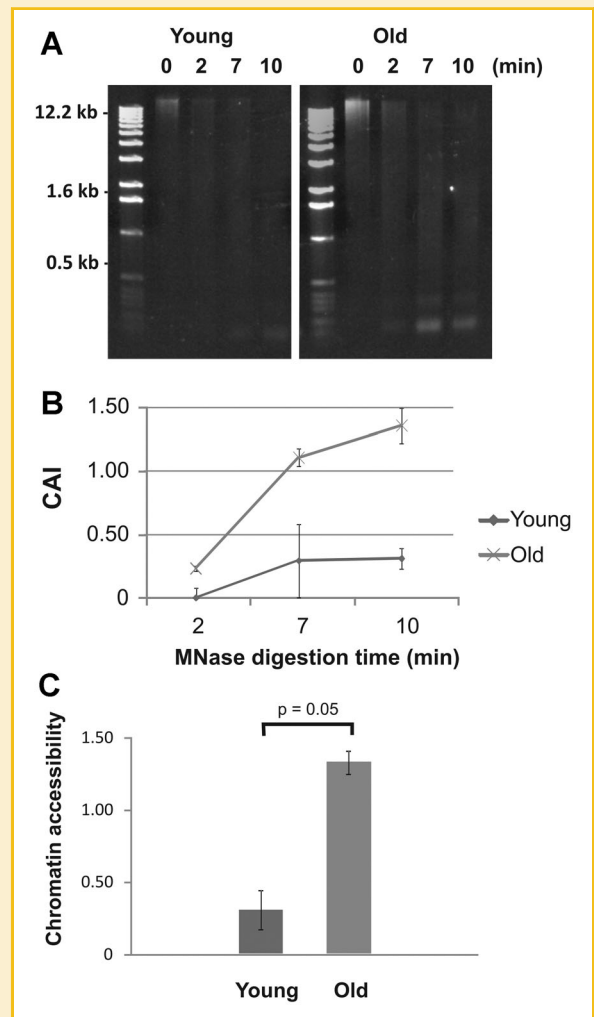IOD, integrated optical densities; n, number of nuclei analyzed; OD, optical densities.

Fig. 2.   Chromatin accessibility assay. (A) Separation of DNA fragments on agarose gels after digestion of isolated nuclei for 0, 2, 7, and 10 min with MNase. (B) Median chromatin accessibility index (CAI) for each age group on 2, 7, and 10 min digestion. (C) Median CAI for each group after a 10-min digestion, when the differences are higher. Three sets of experiments were done for each group. Error bars indicate standard error of the median.
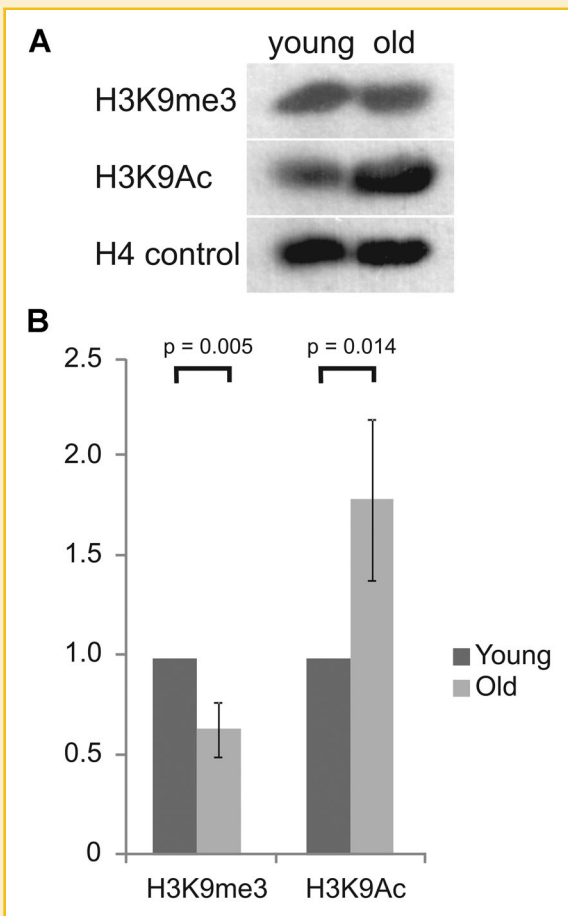
Fig. 3. Quantification of epigenetic marks on neuronal nuclei from young and old mice. (A) Immunoblotting of SDS–PAGE fragmented nuclei using antibodies anti–trimethyl–H3K9 (H3K9me3) and acetylated H3K9 (H3K9Ac). An antibody that recognizes H4 was used as the loading control. (B) Densitometry of bands detected after chemoluminescence. Values above bars are medians. Band intensities for young animals were normalized to a value of 1. Four sets of experiments were done for each group. Error bars indicate standard error of the median.

DISCUSSION

The results indicate that the chromatin of neuronal nuclei from mice undergoes unpackaging as the animal ages, mainly on previously condensed regions. These results support previous data from the literature, which describe that aging is accompanied by loss of heterochromatic areas in other experimental models, although these results were found only for non-neuronal cells [Villeponteau, 1997; Burgess et al., 2012; Tsurumi and Li, 2012]. In *Drosophila* cells, for instance, it has been shown that heterochromatin formation and maintenance promote longevity [Larson et al., 2012].

The quantification of epigenetic marks demonstrated that H3K9Ac, a modification related to chromatin opening and gene activation [Berger, 2007], is increased in aged neuronal nuclei, which supports the present finding of chromatin unpackaging observed in old mice.

While some authors have reported a decrease in H3K9Ac levels in nuclei from aged animals [Kawakami et al., 2009], others have found increased levels of this epigenetic mark with aging [Ghiraldini et al., 2012, 2013], which is in agreement with our present observations, although the results have been obtained from different cell types. It has been shown that the increase in H3K9Ac abundance in aged animals is related to a decreased activity of the histone deacetylase Sirt1 along aging [Corbi et al., 2012; Ghiraldini et al., 2012, 2013; O'Sullivan and Karlseder, 2012], albeit deacetylating activity of sirtuins have been associated more with H4K16 rather than with H3K9 [Vaquero et al., 2007].

Additionally, we have found that H3K9me3, a modification necessary for heterochromatin formation [Kreiling et al., 2011; Huidobro et al., 2012], was decreased on aged neuronal nuclei, supporting the idea of an age-related heterochromatin loss and corroborating the global chromatin unpackaging state observed in this study. Decreased levels of H3K9me3, along with increased levels of histone acetylation, are characteristic of some important progeroid syndromes, such as Hutchinson-Gilford's [Han and Brunet, 2012], and have been observed in aged oligodendrocytes [Shen et al., 2008], supporting a strong relationship between H3K9 demethylation and aging. However, data from the literature are contradictory, since there is description of both increase and decrease of H3K9me3 marks along aging [O'Sullivan and Karlseder, 2012].

Such a chromatin decondensation with aging is intriguing for two reasons: first, open chromatin is usually associated with gene transcriptional activation [Li et al., 2007], which maybe not the case of aged cells and, second, senescence-associated heterochromatin foci (SAHF) are characteristic of some aged cells [Sedivy et al., 2008]. On the first case, we can argue that histone acetylation is not necessarily associated with gene transcription. In fact, for some genes, the effect can be the opposite [Ellis et al., 2008], which means that the increase in H3K9Ac may not necessarily be associated with increased gene expression. Second, we can argue that SAHF formation do not necessarily eliminate the possibility of a general chromatin unpackaging. As proposed by Sedivy et al. [2008], cellular senescence leads to a redistribution of heterochromatic areas, which is supported by present findings on changes in the nuclear entropy with age. In addition, it is possible that the decrease on chromatin packing state is associated with decreased chromatin stability, which is characteristic of aged cells [Miyoshi and Fukuda, 1986; Oberdoerffer et al., 2008].

Another possible explanation is that the more open chromatin state could be a cell attempt to render it more accessible to repair machinery. Such conclusion is plausible considering the great load of damage that chromatin accumulates along life span [Ghiraldini et al., 2013]. In fact, it has been described that an increase in histone acetylation in aged cells is associated with increased repair [Di Bernardo et al., 2012], although it has been published elsewhere that all pathways of DNA repair become less efficient with age, leading to accumulation of mutations and chromosomal alterations (see Gourbunova et al. [2007] for a revision). Thus, it is still hard to establish the cause–effect relationship concerning chromatin structure and aging. We do not exclude the possibility that, along aging, there is a loss of proteins responsible for the maintenance of heterochromatin, leading to epigenetic imbalance, and impacting

negatively several nuclear functions, thus contributing with the aged phenotype.

CONCLUSIONS

Present results suggest a decrease in heterochromatin areas in neuronal cells of mice with aging. This remodeling could allow chromatin more prone to damage and accumulation of errors, leading to increased genomic instability. At the same time, chromatin may become more accessible to repair mechanisms, although this phenomenon may reveal less efficiency with advancing time. However, it is not currently possible to conclude if such alterations are a mere consequence of the aging process or if they are inducing the aged phenotype in cortical neurons from mice. Additional studies are, therefore, necessary to bring light on this matter.

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