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GAMETE BIOLOGY

Altered three-dimensional organization of sperm genome in *DPY19L2*-deficient globozoospermic patients

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Abstract

Purpose

To explore the three-dimensional (3D) organization of sperm genome in *DPY19L2*-deficient globozoospermic patients speculating a link between *DPY19L2* and genome organization of sperm nucleus.

Methods

This is a study of chromatin organization in *DPY19L2*-deficient globozoospermic patients and healthy donors using three-dimensional fluorescence in situ hybridization (3D-FISH) combined with confocal laser scanning microscopy followed by 3D image analysis. The 3D structures of sperm nuclei, chromocenter, telomeric regions and chromosome territories (CTs), were reconstructed using IMARIS software, and the relative radial position for each individual signal was calculated. Statistical analysis used a non-parametric Mann-Whitney test was appropriate with significance at $p < 0.05$.

Results

DPY19L2-deficient globozoospermic patients display impaired sperm chromocenter organization resulting in an increased number of chromocenters (5.4 vs 3.5; $p < 0.0001$). Moreover, radial positions of telomeres are modified with a more central position in globozoospermic nuclei. 3D-FISH analysis of five chromosome territories (CTs) (X, Y, 7, 17, 18) showed that *DPY19L2*-deficient globozoospermic sperm nuclei display altered spatial organization of CT X, CT 7 and CT 18.

Conclusions

Our findings strengthen the hypothesis that DPY19L2 might be considered as a LINC-like protein having a crucial role in the organization of nuclear chromatin in sperm nucleus through its interaction with nuclear lamina. Our results might also explain defective embryonic development after intracytoplasmic sperm injection (ICSI) performed with *DPY19L2*-deficient globozoospermic sperm.

Keywords Globozoospermia · *DPY19L2* gene · Chromatin organization · Three-dimensional fluorescence in situ hybridization (3D-FISH) · Nuclear lamina

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Introduction

The organization of nuclear chromatin in mature human spermatozoa is unique and differs dramatically from that of

somatic cells [1–3]. The higher-order of this spatial organization is well defined and characterized by a centrally located chromocenter, which consists of clustered centromeres of non-homologous chromosomes, and peripherally localized telomeric regions [2, 4]. Moreover, chromosomes are arranged non-randomly and occupy defined and preferential spatial localization (chromosome territory (CT)) within the sperm nucleus [3, 4]. Chromatin remodelling occurs during the last phase of spermatogenesis, spermiogenesis, by the replacement of histones by basic sperm-specific protamines [5]. In the resulting mature sperm, DNA becomes supercondensed and genetic activity is completely shut down [6, 7]. This unique genome architecture in sperm is essential for genome integrity, fertility and early embryonic development [8–10]. It was shown that chromatin remodelling occurs concomitantly with the descent of the acrosomal vesicle on the nucleus suggesting that acrosome development and chromatin remodelling are interacting processes [11].

Human globozoospermia is a rare and severe monomorphic teratozoospermia characterized by round-headed spermatozoa lacking an acrosome and causing primary male infertility [12]. Genetic defects of *DPY19L2* represent the main cause of this teratozoospermia by preventing the anchoring of the acrosome to the nucleus [13]. DPY19L2 protein is localized in the inner nuclear membrane and was suggested to have a LINC-like function [14]. LINC (linker of nucleoskeleton and cytoskeleton) complexes or proteins are nuclear envelope-bridging protein structures connecting the nuclear content to the cytoskeleton [15].

If the absence of an acrosome is the main feature of globozoospermic sperm and the major cause of male infertility, previous case reports have suggested that the globozoospermic sperm presents poor chromatin quality and DNA alterations with high percentage of DNA fragmentation [12, 16, 17]. Recently, it was shown that chromatin compaction during spermiogenesis in *DPY19L2* knockout mouse is defective and leads to sperm DNA damage [18]. Several studies have suggested that the order of chromosome deposition into the oocyte and the sperm nucleus could be an epigenetic code that impacts activation of the male genome during the early stages of embryonic development [1, 3, 19]. In fact, chromatin is reprogrammed after fertilization to produce a totipotent zygote with the potential to generate a new organism [20].

It is now recognized that *DPY19L2*-deficient globozoospermic sperm displays chromatin defects that compromise the initiation of embryonic development, but the extent to which the absence of *DPY19L2* modifies the well-orchestrated nuclear organization in humans is still obscure. To answer this question, we studied nuclear chromatin organization in globozoospermic patients and healthy donors in terms of chromocenter organization, telomeric regions and CT localization.

To investigate 3D genome organization, two main strategies are employed, namely, three-dimensional fluorescence in situ hybridization (3D-FISH) and chromosome conformation capture (3C) and its derivatives (4C, 5C, Hi-C) [21]. Although 3C-based approaches have been paramount in our understanding of physical interaction between multiple loci and organization of the genome at the megabase scale, only 3D-FISH can give an overview of the relative positioning of non-interacting territories inside the whole nucleus [21]. Thus, 3D-FISH combined with confocal laser scanning microscopy followed by 3D image analysis is clearly the method of choice to investigate chromatin organization in our study.

Material and methods

Patients and controls

Human sperm cells were prepared from semen obtained from five globozoospermic patients mated with five healthy donors (sperm donors) who gave informed consent to use their sperm for research purposes. Globozoospermic patients included in this study have genetic defects on *DPY19L2*: three patients have a homozygous deletion of the gene; the remaining two patients have composite heterozygous mutations. The five healthy donors with a mean age of 33 years (ranging from 31 to 34 years) were fertile and have had children through spontaneous pregnancies.

Sperm analysis

Routine sperm analyses were carried out in the Laboratory of Reproductive Biology of Cochin Hospital, according to the World Health Organization (WHO) guidelines [22]. Sperm morphology was assessed according to David's modified classification [23]. Selection of control patients required two consecutive normal semen analyses within a period of 3 months, after at least 3 days of abstinence, as recommended by the WHO. The five healthy donors included in our study have normal semen parameters according to the WHO guidelines [22].

3D FISH analysis

Sperm nuclei isolation and decondensation

Since the assessment of nuclear organization in spermatozoa is challenging due to the extreme compactness of chromatin, preparation of sperm cells for hybridization was performed as described previously by Alladin et al. [24], allowing a uniform mild nuclear decondensation required for FISH while preserving the 3D structure of the cells. Briefly, semen samples were washed with RPMI and cells were pelleted by centrifugation then washed

twice with PBS and fixed with 3:1 ethanol/acetic acid. The fixed samples were then loaded on dry microscopic slides and dehydrated in increasing concentrations of ethanol (70%, 90%, 100%). Slides were incubated in 0.5 M NaOH solution at room temperature (RT) for 4 min and dehydrated again.

Separate hybridization with telomere, centromere and whole chromosome paint probes

Hybridization was performed using Human Chromosome Pan-Centromeric probe and Human Chromosome Pan-Telomeric probe (Cambio Ltd., Cambridge, UK). Localization of chromosomes territories (CTs) was analysed using whole chromosome paint (WCP) probes (MetaSystems) (WCPX, WCP7, WCPY, WCP17, WCP18) which were selected knowing preferential localization of chromosomes according to their size and gene density (NCBI data, nlm.nih.gov/genome/guide/human/). Sperm nuclei and the probe were denatured simultaneously at 76 °C for 5 min. Hybridization was carried out in a humid atmosphere at 37 °C for at least 24 h. Posthybridization washes were performed with 0.4xSSC/0.3 Igepal (Sigma-Aldrich Co., LLC, St. Louis, MO) at 72 °C for 2 min 30, then 2xSSC/0.1 Igepal at RT for 1 min. Sperm nuclei were counterstained with the intercalating agent 4′,6′-diamidino-2 phenylindole (DAPI, Abbott Molecular, Abbott Park, IL).

Confocal microscopy

Nuclei were scanned using a three-channel laser scanning confocal microscope (Leica TCS SP2 ABOS; Leica Microsystems, Inc., Buffalo Grove, IL). Images (325 × 325 pixels) were sampled using a ×63 oil immersion objective at a resolution of $xy = 0.2$ μm and $z = 0.3$ μm. For each optical section, images were recorded for one or two fluorochromes (FITC or TRITC). Stacks of optical sections were processed with the dedicated analysis software (LAS-AF Leica Microsystems, Wetzlar, Germany) and collected from nuclei with a regular shape and showing complete hybridization signals in all channels. Overlapping cells and spermatozoa with no fluorescent signals were excluded. Ten to 30 nuclei were scanned per patient and per control for each probe.

3D reconstruction of nuclei, chromocenter, telomeric regions and CTs

The 3D structures of sperm nuclei, chromocenter, telomeric regions and CTs were reconstructed using IMARIS software (Bitplane, Andor Technology Ltd., Belfast, UK). For each nucleus, IMARIS determined volumes of nuclei, chromocenters and CTs and number of individual signals representing pericentromeric heterochromatin (number of chromocenters per nucleus). The choice of a noise/background threshold is known to be crucial in this 3D FISH approach, before we can determine the number and size of the signals. Given that the same operator manually thresholded the images for reconstruction, we considered that the bias introduced during this process was the same for each nucleus and that the datasets were comparable. The degree of nuclear decondensation and variations in nuclear volume were taken into consideration for all measurements; thus, the volume of each CT and chromocenters was normalized over the volume of the corresponding nucleus (relative volume). In order to calculate distances for each nucleus, IMARIS determined the 3D coordinates (x, y, z) from axes A, B and C, the length of axes A, B and C, and the 3D coordinates with respect to the centre of homogeneous mass. For each individual signal (representing CT, pericentromeric heterochromatin, or telomeric region), the 3D coordinates with respect to the centre of homogeneous mass were also recorded. Thus, the relative radial position for each individual signal was calculated (using a Matlab script [The Math Works Inc., Natick, MA]) as the distance between the centre of homogeneous mass of the analysed signal and the centre of homogeneous mass of the nucleus, divided by the length of a line from the centre of the nucleus to the nuclear envelope through the centre of homogeneous mass of the signal.

Statistical analyses

Statistical analysis was performed with GraphPad Prism software version 7 (GraphPad Software Inc., La Jolla, CA) by using a non-parametric Mann-Whitney tests with Bonferroni multiple-comparison adjustments to compare median differences in globozoospermic and control nuclei. The threshold for statistical significance was set to $p < 0.05$.

Ethics statement

This study was approved by the Ethical Review Committee of University School of Medicine (Sfax, Tunisia) and the GERMETHEQUE pilot study committee. GERMETHEQUE Biobank (BB-0033-00081), a partner site of PARIS-COCHIN, provided sperm samples for the purposes of this project, in addition to informed consent from each patient for the use of their materials (CPP 2.15.27). The Biobank registered declaration DC-2014-2202 and authorization AC-2015-2350.

Results

Globozoospermic patients display impaired sperm chromocenter condensation

In sperm nucleus, centromere sequences belonging to non-homologous chromosomes are clustered into a compact chromocenter positioned well inside the nuclear volume. We first tested, using the Human Chromosome Pan-Centromeric

probe, whether globozoospermic patients had altered chromocenter condensation by analysing the relative volume of the chromocenter, the number of signals engaged in the formation of the chromocenters and their radial positions, in each cell. Relative chromocenter volumes in globozoospermic (RV = 0.17 ± 0.06 of the nucleus volume; $n = 81$) and control cells (RV = 0.17 ± 0.05; $n = 97$) are not significantly different between the two types of nuclei ($p = 0.65$). However, the number of chromocenters in globozoospermic cells is significantly higher ($N = 5.4 \pm 1.39$; $n = 81$) than in normal sperm ($N = 3.5 \pm 1.13$; $n = 97$) ($p < 0.0001$) (Fig. 1, Fig. A1, and Fig A2). Chromocenter radial positions showed no significant differences between patients and controls ($r = 0.67 \pm 0.23$ in 97 control nuclei vs $r = 0.68 \pm 0.25$ in 81 globozoospermic nuclei; $p = 0.18$). Our results show that *DPY19L2*-deficient globozoospermic patients display impaired sperm chromocenter organization resulting in an increased number of chromocenters.

Telomeric regions change their radial positioning in globozoospermic sperm nuclei

We then evaluated the radial positions of telomeres in control and globozoospermic cells to assess whether the absence of DPY19L2 in globozoospermic human sperm nuclei alters the interaction of telomeric regions with nuclear lamina. Radial positions of telomeres ($r = 0.79 \pm 0.25$ in 96 control nuclei vs $r = 0.61 \pm 0.22$ in 94 globozoospermic nuclei, $p < 0.0001$) are significantly different between the two types of nuclei with a more central position in globozoospermic nuclei (Fig. 2a). Our data indicate that the absence of DPY19L2 in globozoospermic human sperm nuclei alters the well-organized telomeric regions at the nuclear periphery where they interact in dimers and tetramers [2].

Chromosome territories change their specific spatial positioning in globozoospermic sperm nuclei

In human sperm nuclei, chromosomes are organized non-randomly in distinct CTs that can be delimited as nuclear subvolumes by 3D-FISH. Here, we studied volumes of 5 CTs (X, Y, 7, 17, 18) and their radial positions to test whether the absence of DPY19L2 in globozoospermic human sperm nuclei impairs the spatial organization of CTs. The radial position of CT X ($r = 0.51 \pm 0.11$ in 102 control nuclei vs $r = 0.69 \pm 0.12$ in 90 globozoospermic nuclei, $p = 0.0008$) was modified and adopted a more peripheral position in globozoospermic sperm nuclei (Fig. 2b, Fig. 3, Fig. A3, and Fig. A4), whereas the radial position of CT 7 ($r = 0.82 \pm 0.21$ in 97 control nuclei vs $r = 0.67 \pm 0.12$ in 92 globozoospermic nuclei, $p < 0.0001$) and CT 18 ($r = 0.95 \pm 0.32$ in 107 control nuclei vs $r = 0.79 \pm 0.11$ in 105 globozoospermic nuclei, $p = 0.005$) had a more central position in globozoospermic spermatozoa (Fig. 2c, d and Fig. 4). The radial positions of CT 17

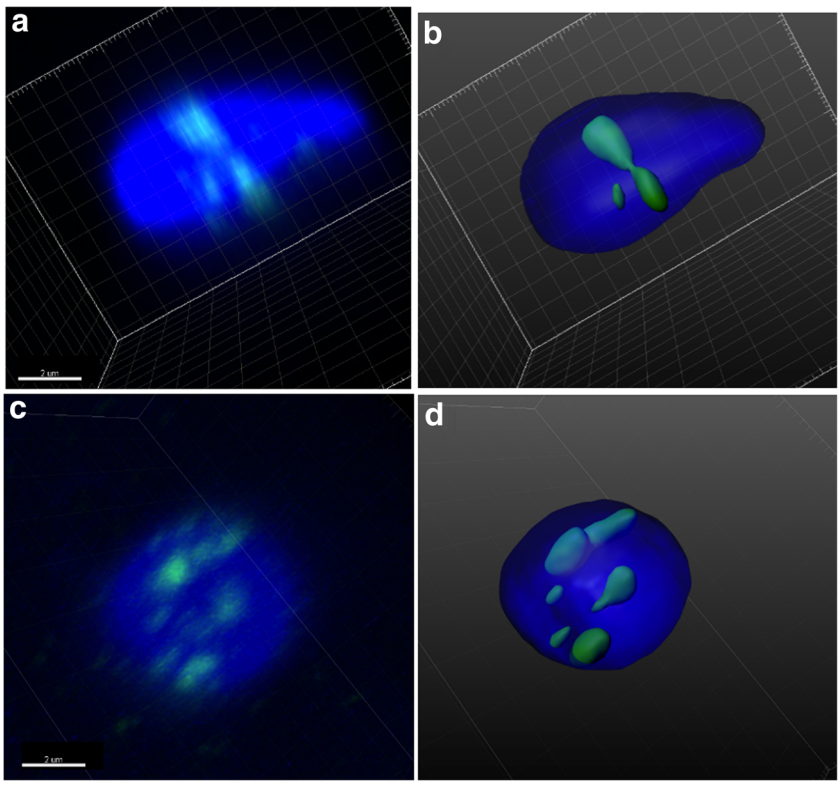

Fig. 1 3D reconstruction of nuclei and chromocenters using IMARIS software (Bitplane). 3D-FISH was performed using Human Chromosome Pan-Centromeric probe (green) on control sperm nuclei ($n = 97$) and globozoospermic sperm nuclei ($n = 81$). DAPI-counterstained nuclei. Scale bar, 2 μm. **a** Raw image of a control sperm nucleus after 3D-FISH. **b** Reconstructed nuclear territory and chromocenters of a control sperm nucleus. **c** Raw image of a globozoospermic sperm nucleus after 3D-FISH. **d** Reconstructed nuclear territory and chromocenters of a globozoospermic sperm nucleus

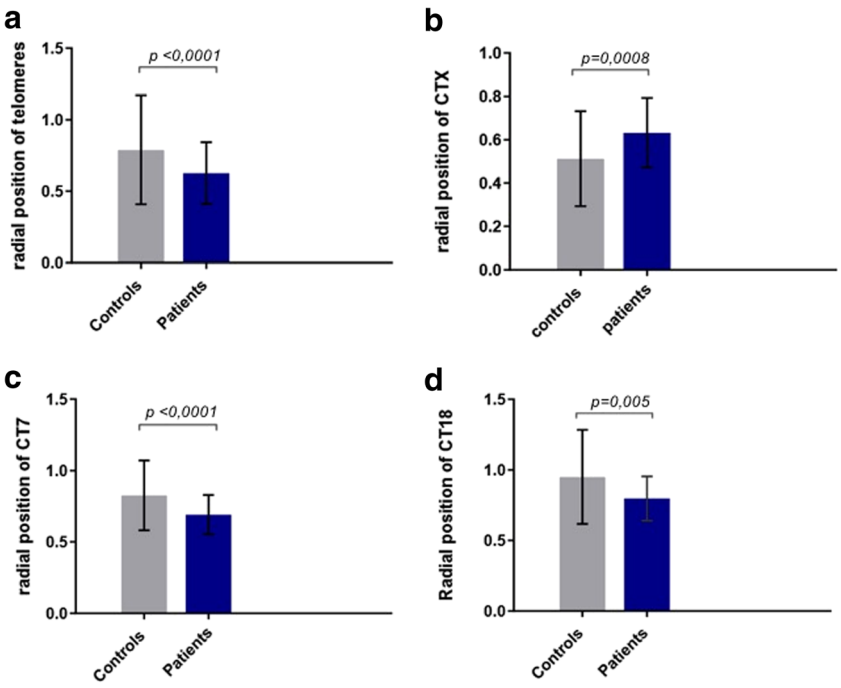

Fig. 2 Comparison of relative radial distribution of telomeric regions (**a**) and chromosome territories (CTs) of chromosome X (**b**), chromosome 7 (**c**) and chromosome 18 (**d**) in control sperm nuclei and globozoospermic sperm nuclei showed a significant difference between the two types of nuclei ($p < 0.05$)

($r = 0.54 \pm 0.25$ in 117 control nuclei vs $r = 0.52 \pm 0.22$ in 107 globozoospermic nuclei, $p = 0.1157$) and CT Y ($r = 0.48 \pm 0.17$ in 95 control nuclei vs $r = 0.49 \pm 0.13$ in 94 globozoospermic nuclei, $p = 0.7$) are not significantly different between the two types of nuclei (Fig. 5). The relative volume of the territory for the five chromosomes is not significantly different between the two types of nuclei (Table 1).

Discussion

It is becoming increasingly clear that perturbation of the well-orchestrated nuclear organization in sperm nucleus may have negative effects on fertility and early embryonic development [10]. While recent studies have been key in opening this relatively new area of study, our current knowledge base is by far

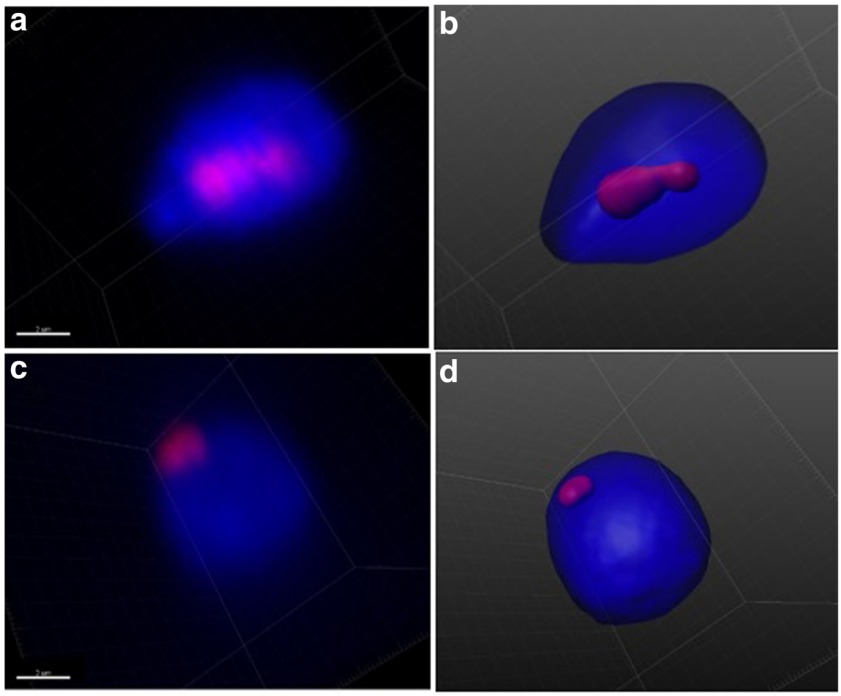

Fig. 3 3D reconstruction of nuclei and chromosome X territory (CTX) using IMARIS software (Bitplane). 3D-FISH was performed using WCPX probe (red) on control sperm nuclei ($n = 102$) and globozoospermic sperm nuclei ($n = 90$). DAPI-counterstained nuclei. Scale bar, 2 μm. **a** Raw image of a control sperm nucleus after 3D-FISH. **b** Reconstructed nuclear territory and CTX of a control sperm nucleus. **c** Raw image of a globozoospermic sperm nucleus after 3D-FISH. **d** Reconstructed nuclear territory and CTX of a globozoospermic sperm nucleus

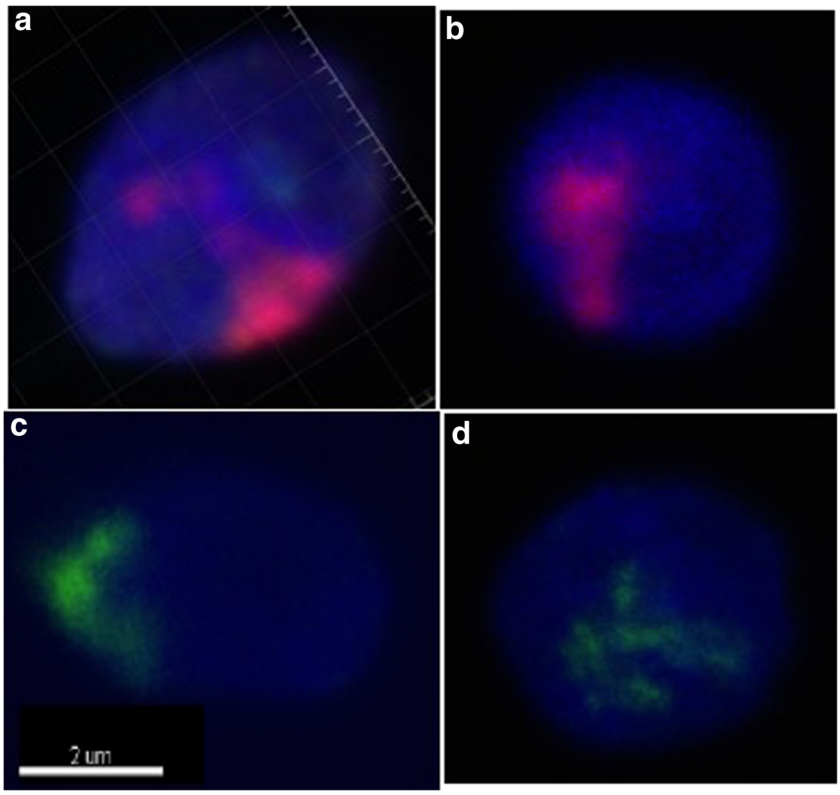

Fig. 4 Raw images of nuclei, chromosome 7 territory (CT 7) and chromosome 18 territory (CT 18). 3D-FISH using WCP 7 probe (red) on control sperm nucleus (**a**) and globozoospermic sperm nucleus (**b**). 3D-FISH using WCP18 probe (green) on control sperm nucleus (**c**) and globozoospermic sperm nucleus (**d**). Relative radial positions of CT 7 and CT 18 are significantly different between control and globozoospermic sperm nuclei with a more central position in patients. Scale bar, 2 μm

still not able to explain, treat or prevent sperm chromatin defects associated with male infertility and defective embryonic development. One key to fully elucidate the sperm epigenome control in the embryo will be to further investigate the epigenetic profiles of various etiologies of infertility. Globozoospermia, a rare cause of male infertility, is known to be associated with poor chromatin quality and DNA alterations with higher percentages of DNA fragmentation [12, 16,

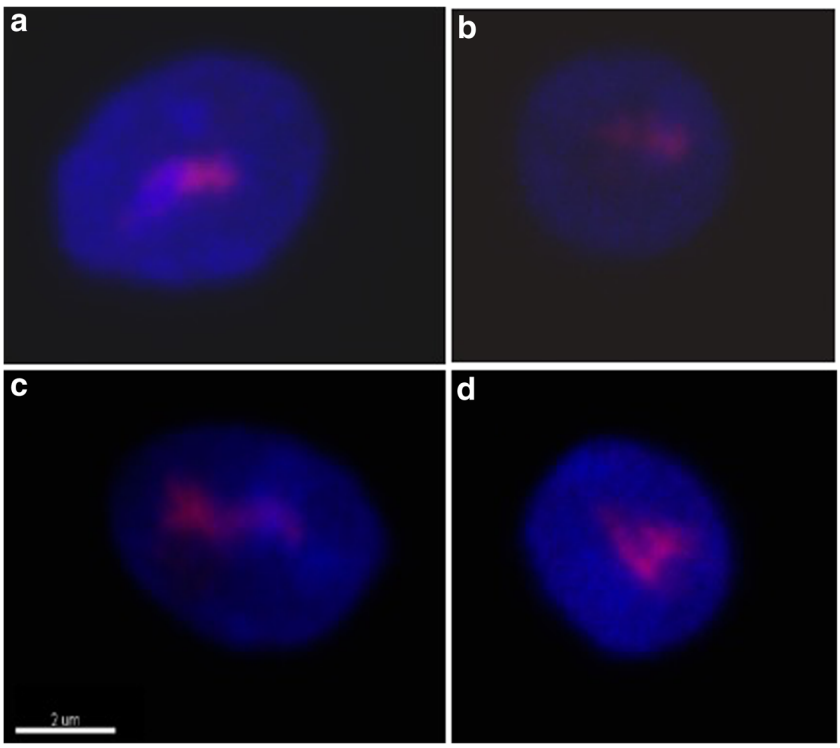

Fig. 5 Raw images of nuclei, chromosome Y territory (CT Y) and chromosome 17 territory (CT 17). 3D-FISH using WCPY probe (red) on control sperm nucleus (**a**) and globozoospermic sperm nucleus (**b**). 3D-FISH using WCP17 probe (red) on control sperm nucleus (**c**) and globozoospermic sperm nucleus (**d**). Relative radial positions of CT Y and CT 17 are not significantly different between control and globozoospermic sperm nuclei. Scale bar, 2 μm

Table 1 Comparison between globozoospermic and control sperm nuclei

	Globozoospermic nuclei		Control sperm nuclei		*p* values
	Mean ± SD	Number of cells analysed	Mean ± SD	Number of cells analysed	
Relative volume of CTX	0.1165 ± 0.0421	90	0.1198 ± 0.0224	102	0.1655
Relative volume of CT 7	0.1232 ± 0.0314	92	0.1246 ± 0.0411	97	0.3144
Relative volume of CT 18	0.0864 ± 0.0463	105	0.0874 ± 0.0367	107	0.1133
Relative volume of CT 17	0.0811 ± 0.0203	107	0.0831 ± 0.0328	117	0.7612
Relative volume of CT Y	0.0509 ± 0.0140	94	0.0513 ± 0.0134	95	0.1723

17]. Genetic defects of *DPY19L2* represent the main cause of this teratozoospermia by blocking sperm head elongation and acrosome formation [25, 26]. It was demonstrated that the absence of this protein in knock-out mouse models and human leads to the destabilization of the nuclear lamina (NL), with persistence of lamin B1 at the whole nuclear periphery in round spermatids, without any polarization [13, 18, 27]. This localization of NL is radically different from that observed in normal spermatozoa, in which lamin B1 changes during spermiogenesis with a progressive polarization to the posterior pole of the elongating spermatids [28]. NL is connected to the cytoskeleton and the centrosome (via SUN-domain proteins which belong to the LINC complex), and to the chromatin (via LEM domain proteins and the lamin B receptor). LINC complexes or proteins are nuclear envelope-bridging protein structures connecting the nuclear content to the cytoskeleton (linker of nucleoskeleton and cytoskeleton) [15]. They are critically involved in a variety of fundamental cellular processes, such as nuclear anchorage, movement and positioning and meiotic chromosome dynamics and maintenance of nuclear shape [29, 30]. Because DPY19L2 protein has been shown to be localized in the inner nuclear membrane and was suggested to have a LINC-like function [14], we hypothesized that a functional defect of this protein may induce an altered chromatin packaging in *DPY19L2*-deficient globozoospermic human sperm nuclei. To the best of our knowledge, the present study is the first to analyse chromosome packaging by 3D-FISH in globozoospermic spermatozoa.

Application of the 3D-FISH technique within mammalian sperm nuclei is a complicated and labour-intensive process because of the extreme compactness of the chromatin; thus, the published literature is limited to a few reports [2, 3, 24, 31]. Different protocols of sperm pretreatment have been used in order to permeabilize spermatozoa and increase the accessibility of the DNA sequences to probes without affecting the 3D structure of the nucleus. It was shown that decondensation with heparin (Zalensky et al. protocol) is associated with low hybridization efficiency [3], while the pretreatment procedure with NaOH [3, 24] leads to a uniform and mild decondensation of sperm nuclei with high hybridization efficiency. For these reasons, we chose to decondense sperm nuclei of patients and controls with NaOH according to a validated protocol by Alladin et al. [24].

In human sperm nucleus, all telomeres are coupled into dimers which are localized at the nuclear periphery where they interact with the nuclear membrane [2]. Telomere dimers in sperm correspond to the interaction between the ends of each chromosome, thus providing the chromosome with a looped hairpin conformation [32]. It was proposed that such a configuration is most likely required for chromosome withdrawal/decondensation during the course of fertilization leading to zygote formation. Our 3D-FISH analysis of telomeres showed that telomeric regions adopted a more central position in globozoospermic sperm nuclei and thus are significantly disturbed in the absence of DPY19L2. The destabilization of the NL in the absence of DPY19L2 might lead to altered anchorage of chromosome ends, resulting in disturbed telomere organization in the sperm nucleus of *DPY19L2*-deficient globozoospermic patients.

Because heterochromatin is tethered to the lamina through lamin A and lamin B receptor mechanisms [33], we then searched for an impact of DPY19L2 deficiency on hetechromatin position. In sperm cells, heterochromatin is organized in chromocenters, composed of several linear arrays of centromeres, with a specific order of non-homologous chromosomes engaged in the formation of these structures [4]. In our study, we showed that the number of observed chromocenters was significantly higher in globozoospermic sperm nuclei than in control cells. No differences in terms of radial positions and relative volume of chromocenters between the two cell types were observed. Our results are in agreement with the recent report by Aladin et al. showing an increased number of chromocenters in immotile human sperm

in comparison with motile spermatozoa, from which Alladin et al. proposed that the optimal nuclear organization be defined as containing one to three chromocenters.

Non-random chromosome positioning with preferred CT localization in the human sperm nucleus is yet another additional element of the well-organized genome architecture of spermatozoa.

Similar to somatic cells, chromosomes are distributed according to their size and gene density: smaller chromosomes are positioned more centrally than larger ones, and most gene-dense chromosomes locate close to the centre of nucleus [4, 31]. It was shown using sperm tail as a reference to identify apical or basal position that chromosome 18 is preferentially located in the basal region of the sperm nucleus, while the X chromosome is preferentially located in the apical region [34]. 3D-FISH analysis of 5 CTs (X, Y, 7, 17, 18) showed that *DPY19L2*-deficient globozoospermic sperm nuclei display altered spatial organization of CT X, CT 7 and CT 18, whereas radial position of CT 17 and CT Y are not significantly different between the globozoospermic sperm and control nuclei. Our findings can be explained by the spatial disorganization of chromocenters and telomeric regions in globozoospermic spermatozoa. Furthermore, the higher-order chromatin organization of the chromocenter and telomeres plays an important role in the sperm nuclear architecture and is implicated in proper intranuclear chromosome positioning with preferred CT localization [4, 32]. It was shown that the X chromosome occupies a preferential position in the anterior half of the volume of the sperm nucleus, a position close to the place of the first contact between sperm and egg [3, 34]. Interestingly, similarities in the location of the chromosome X have been shown in all mammals suggesting a functional significance [35], one possibility being that X chromosome position is critical for establishing X chromosome inactivation. It was also suggested that this position may be related to its high rate of loss from intracytoplasmic sperm injection (ICSI) embryo [34, 36]. The absence of DPY19L2 leads to an abnormal location of the chromosome X in globozoospermic spermatozoa and might explain defective embryonic development after ICSI.

Conclusion

Although studies of sperm nuclear organization are very challenging due to the extreme compactness of chromatin, it is now clear that such studies are not of theoretical importance only. Using 3D-FISH combined with confocal laser scanning microscopy followed by 3D image analysis, we showed that the absence of DPY19L2 leads to a disturbance of the overall sperm nuclear architecture with a lower degree of organization and condensation of the chromocenter resulting in an increased number of chromocenters. We also provide evidences that *DPY19L2*-deficient globozoospermic sperm nuclei display altered spatial organization of telomeric regions and CTs. Our results strengthen the hypothesis that DPY19L2 might be a LINC-like protein having a crucial role in the organization of nuclear chromatin in sperm nuclei through its interaction with NL.

Topographic disorganization of chromosome territories as well as subtle modifications in chromocenters and telomeric regions may also contribute to the defective embryonic development after ICSI performed with *DPY19L2*-deficient globozoospermic sperm.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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