Spermatogenesis 4, e28460; March 2014; © 2014 Landes Bioscience

The nuclear form of glutathione peroxidase 4 colocalizes and directly interacts with protamines in the nuclear matrix during mouse sperm chromatin assembly

Rossella Puglisi[1], Irene Maccari[1], Simona Pipolo[1], Franco Mangia[2], and Carla Boitani[1,*]

[1]DAHFMO; Section of Histology & Medical Embryology; University of Rome "La Sapienza"; Rome, Italy; [2]Dept Psychology; Section of Neuroscience; University of Rome"La Sapienza"; Rome, Italy

Keywords: protamines, nGPx4, heterologous expression, nuclear matrix, sperm chromatin assembly

The testis-specific nuclear form of Phospholipid Hydroperoxide Glutathione Peroxidase (nGPx4) is associated with the nuclear matrix during spermiogenesis and is implicated in sperm chromatin condensation. In this study, we have addressed the question whether nGPx4 directly interacts with protamines by transiently sharing a nuclear matrix localization. We first expressed tagged protamine 1-myc and protamine 2-V5 in HeLa and COS-1 cells and showed by both confocal microscopy and immunoblotting analyses that protamines were produced in vitro and colocalized correctly to the nucleus. Co-transfection experiments demonstrated that protamine 1 was physically associated with flag-nGPx4 specifically at the level of nuclear matrix. The peculiar presence of protamines together with nGPx4 in this subnuclear compartment was also confirmed in mouse elongated spermatids by immunofluorescence, suggesting that nGPx4 is a physiological component of a novel protein complex relevant to chromatin assembly in condensing haploid cells. Also, in epididymal sperm, nGPx4 and protamine 1 co-immunoprecipitated, indicating that nGPx4, although localized to a subnuclear compartment different from that of protamines, represents a constant link between nuclear matrix and chromatin in mammalian male gamete.

Introduction

A key feature of mammalian sperm is its unique chromatin structure, having the vast majority of DNA remarkably and efficiently packaged into toroids by highly basic proteins called protamines, whereas only a small percentage of DNA is bound by histones in nucleosomal configuration. Sperm chromatin is organized into loop domains attached to a proteinaceous scaffold, the nuclear matrix, which structurally orders and functionally affects the paternal genome.[1,2] There is increasing interest in studying the regulation of sperm chromatin organization, because any alterations in protein composition or the structural integrity of sperm chromatin may contribute to male subfertility/infertility. In fact, faulty nuclear remodeling, abnormal chromatin condensation pattern, and incomplete protamination are commonly observed in sperm from infertile mice and humans.[3-7] More recently, a novel role of sperm chromatin in carrying epigenetic information for the zygote has emerged. Distinct DNA methylation and histone modifications were revealed in human and mouse sperm genome, specifically at regulatory sequences of genes important for embryo development.[8-10] It is also of interest that defects of sperm DNA methylation at imprinted loci have recently been shown to be associated with abnormal chromatin packaging in infertile patients.[11]

Studying protamine partners in sperm chromatin functional dynamics, we recently reported that the nuclear form of glutathione peroxidase 4 (nGPx4) plays a relevant role in chromatin compaction during spermiogenesis. Sperm from cauda epididymis of nGPx4-knockout mice appeared to be prone to decondense faster than those from wild-type mice following gamete fusion at fertilization, as well as in an in vitro chromatin decondensation assay.[12] This finding, and a number of other evidence, support the concept that nGPx4 participates, with protamines, in the process of disulfide bond formation resulting in the proper stability of sperm chromatin.[12,13] Of note, we also observed that nGPx4 has a specific subnuclear distribution in the nuclear matrix of haploid male germ cells, from round spermatids to cauda epididymal sperm.[12] Although the reason for this localization remains unclear, it is indeed intriguing, because nuclear matrix binds chromatin at sequence-specific regions of attachment, the so-called matrix attachment regions (MARs), which exhibit a variety of functions, including

*Correspondence to: Carla Boitani; Email: carla.boitani@uniroma1.it
http://dx.doi.org/10.4161/spmg.28460
Submitted: 01/16/2014; Revised: 03/04/2014; Accepted: 03/06/2014

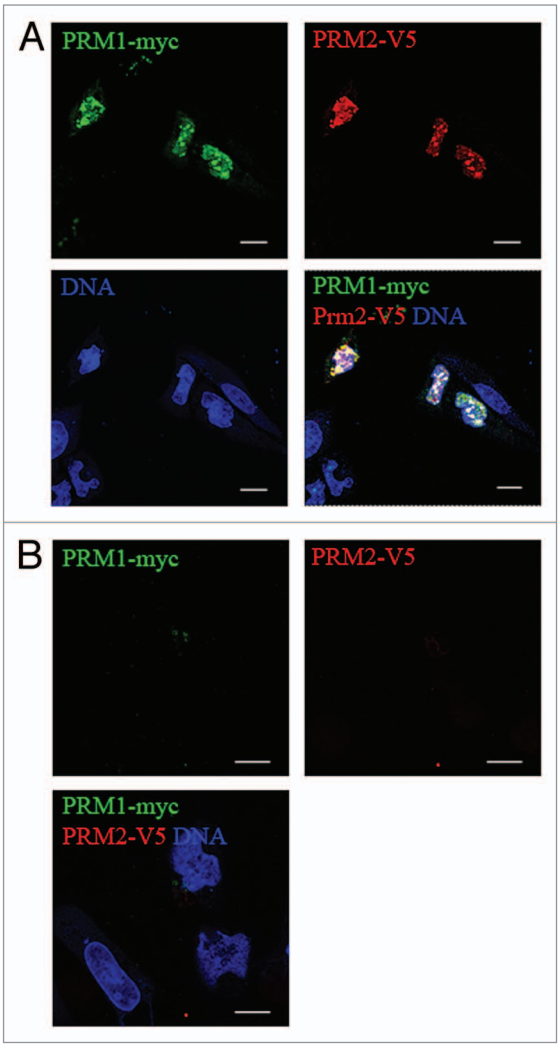

Figure 1. Protamine 1 and protamine 2 are expressed in vitro in a heterologous cell system. Confocal microscopy analysis of HeLa cells transfected for 48 h with (**A**) the vector pBUDCE4.1-PRM1myc-PRM2V5 and (**B**) the control empty vector. Cells were immunostained with anti-c-myc (green) and anti-V5 (red) antibodies and counterstained with TOTO-3 (blue). Note that the two protamines colocalized specifically to the nucleus showing an overlapping diffuse nucleoplasmic signal and a variable number of bright dots. (Scale bars: 20 μm.)

chromosome organization into discrete domains and chromatin potentiation.[14,15] As an example, the sperm genome displays two haploid-specific MARs flanking the protamine domain, a DNA region containing a cluster of three genes encoding the proteins responsible for DNA packaging protamine 1, protamine 2, and transition protein 2.[16,17] Interestingly, a modification of the configuration of these MARs causes a change in the expression of these genes,[16] indicating that genome–nuclear matrix interactions play a fine regulatory role in sperm chromatin condensation. During spermatid differentiation, chromatin remodeling requires a coordinated spatial and temporal interplay between and within different cellular and subnuclear compartments. In fact, mice lacking the mouse germ cell less-1 (mGCL-1) protein, a nuclear matrix component that binds the nuclear membrane, show aberrant chromatin reorganization during spermatogenesis, accompanied by abnormal expression of protamines and retention of transition protein 2 in late spermatids.[18] In addition, protamine 1 itself transiently associates with lamin B receptor on the inner nuclear membrane in condensing spermatids before being deposited on chromatin.[19] Premature expression of protamine 1 also causes precocious DNA condensation, leading to fertility defects.[20] In contrast to protamine 1, protamine 2 is synthesized in spermatids as a precursor molecule substantially larger, enters the nucleus, binds to the condensing DNA, and then undergoes proteolytic cleavage and phosphorylation.[21,22] Interestingly, an increased amount of pre-protamine 2 forms caused by an abnormal processing has been described in sperm nuclei from infertile patients.[23]

Based on the above findings, we have hypothesized a functional/physical relationship between protamines and nGPx4 at the level of nuclear matrix in the process of chromatin assembly during spermiogenesis. In this paper, we have experimentally tested this hypothesis by in vitro expressing/co-expressing protamine 1, protamine 2, and nGPx4 in HeLa and COS-1 cell lines, and then analyzing the subnuclear distribution and relationship of these proteins. In addition, we have also analyzed the association of nGPx4 with protamine 1 by immunoprecipitation in mouse epididymal sperm.

Results

In vitro expression of protamine1(PRM1) and protamine 2 (PRM2)

The study of protamine–DNA assembly during spermiogenesis is hampered by the fact that these proteins are expressed only in spermatids, namely cells that cannot survive in long-term in vitro cultures and are thus unaccessible to most experimental in vitro manipulations. We thus reasoned that expressing protamines in heterologous cell lines might be of help to identify molecular factors putatively involved in the process of sperm chromatin compaction. As to our knowledge there was no previous report of protamine expression in heterologous cell systems, likely because of the very peculiar structure and amino acid composition of these proteins, we generated several DNA constructs and performed two independent sets of experiments based on the transfection of HeLa and COS-1 cell lines, which do not express protamines. HeLa cells were transfected with a dual promoter vector encoding both protamine 1-myc and protamine 2-V5 for 48 h and then immunostained with anti-myc and anti-V5 antibodies. A robust expression of both protamines was detected, having a strictly nuclear colocalization (**Fig. 1A**). No signal was observed in cells transfected with the empty vector (**Fig. 1B**). Similar features were also observed when cells were transfected with vectors coding for either single protamine (data not shown). We observed that higher and more consistent levels of recombinant protamines were produced after transfection with either dual or single promoter constructs by COS-1 cells compared with HeLa cells. Both cell lines were viable throughout the entire transfection time up to 72 h, remaining attached to the culture dishes, morphologically

undamaged, and showing no apparent signs of cell degeneration (**Fig. S1**). We therefore conclude that protamines can actually be produced in sufficient quantity and correctly directed to the nucleus in heterologous cell systems.

To further prove that protamines are expressed by heterologous cells, basic nuclear proteins were extracted from COS-1 cells transfected for 72 h with a single promoter vector encoding protamine 1-myc and analyzed by western blotting (**Fig. 2**). The anti-protamine 1 antibody recognized a band having a gel migration slower than protamine 1 extracted from mouse epididymal sperm used as positive control, likely due to the presence of the myc-tag and/or an incorrect process of post-translation modifications by COS-1 cells. The anti-myc antibody detected a band only in COS-1 cells transfected with the vector for protamine 1.

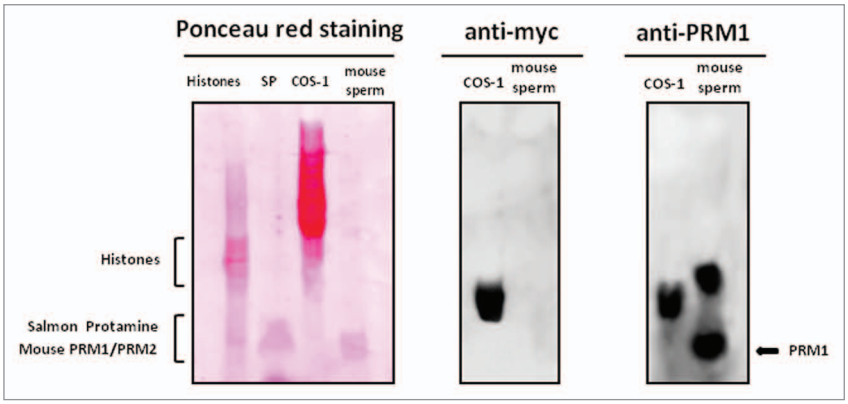

Figure 2. Western blot analysis of protamine 1 expressed in vitro by COS-1 cells transfected with pBUDCE4.1-PRM1myc vector for 72 h. Ponceau red staining of the immobilon membrane is shown to visualize the migration markers: histones, salmon sperm protamine (SP), and mouse sperm protamines. Filters were immunoblotted with either the anti-c-myc antibody, which recognized only the protein synthesized by COS-1 cells, or the anti-protamine 1 antibody, which recognized protamine 1 of both COS-1 cells and mouse sperm. Protamine 1 extracted from mouse epididymal sperm as described in Materials and Methods, was used as immunoblot positive control. The additional band having slower migration than PRM1 likely reflects a different degree of protamine denaturation on the AU-PAGE. The protein synthesized by COS-1 cells showed a different electrophoretic mobility compared with PRM1 extracted from mouse spermatozoa, likely due to the presence of the myc-tag and/or the inability of COS-1 cells to post-translationally modify protamines.

Nuclear colocalization of tagged nGPx4 and protamines

Given that tagged protamines 1 and 2 can be expressed in two different mammalian cell lines, we addressed the question whether these proteins interacted with the nuclear isoform of GPx4, looking for nuclear colocalization. To this aim, we first analyzed the subcellular localization of nGPx4 expressed in vitro by HeLa cells transfected with a vector encoding Flag-nGPx4. Double immunostaining using anti-flag antibody and anti-lamin B to label the nuclear periphery revealed a clear distribution of nGPx4 within the nucleus (**Fig. 3**). Next, we performed a confocal microscopy analysis of HeLa cells co-transfected with Flag-nGPx4 and the dual promoter construct encoding protamine 1-myc and protamine 2-V5 (**Fig. 4**). A strong immunostaining of both nGPx4 and protamine 1 was visualized in the nucleus, having a high degree of colocalization (**Fig. 4A**). A similar co-immunoreactivity was also observed when cells were stained with anti-flag and anti-V5 antibodies (**Fig. 4B**). Thus, when overexpressed in heterologous mammalian cells, protamine 1, protamine 2, and nGPx4 precisely and exclusively colocalized to the nucleus.

We previously reported that nGPx4 is specifically associated with nuclear matrix in testicular haploid germ cells and epididymal sperm.[12] Therefore, following the observation that a similar subnuclear localization was also observed in COS-1 cells transfected with Flag-nGPx4,[12] we asked whether protamines exogenously expressed in COS-1 cells were associated with chromatin, as they do in mature sperm, or colocalized with nGPx4 at the level of the nuclear matrix. This question was addressed by in situ preparation of the nuclear matrix fraction from COS-1 transfected with the construct encoding protamine 1-myc. Histones were extracted and DNA was removed in order to unmask lamin B in the internal nuclear matrix. Immunofluorescence analysis showed that protamine 1 was actually associated with the nuclear matrix (**Fig. 5**). In addition, when COS-1 cells were co-transfected with Flag-nGPx4 and protamine 1-myc, and then subjected to in situ sequential extraction to remove all DNA and histones to leave the nuclear matrix, immunodetection of the two proteins showed that nGPx4 and protamine 1 colocalized to this specific nuclear compartment (**Fig. 6**).

Interaction of nGPx4 with protamines in mouse elongated spermatids and epididymal spermatozoa

The unexpected finding that nGPx4 and protamines 1 and 2 colocalized to the nuclear matrix of COS-1 cells; prompted us to analyze whether such localization actually represented an early and transient step of chromatin assembly during spermiogenesis in vivo, when protamines are first synthesized. To this aim, we performed a double immunofluorescence staining of in situ nuclear matrix preparations from sonication-resistant spermatids (SRS) (**Fig. 7**). It appeared that protamine 2 and nGPx4 actually colocalized to the nuclear matrix at this stage, in agreement with results obtained with COS-1 cells, opening the possibility that protamines directly interact with nGPx4 prior the final deposition into sperm chromatin. Even though this question was not directly addressed in SRS because of the paucity of these cells, we investigated whether this interaction was also maintained at later stages of germ cell maturation. Basic nuclear proteins from whole epididymal sperm were extracted and subjected to immunoprecipitation using the anti-protamine 1 antibody. Western blot analysis of immunoprecipitates with either anti-nGPx4 or anti-protamine 1 revealed that protamine 1 actually co-immunoprecipitated with n-GPx4 (**Fig. 8**).

Discussion

The nuclear matrix represents a highly dynamic network of proteins, which, among many functions, organizes and recruits

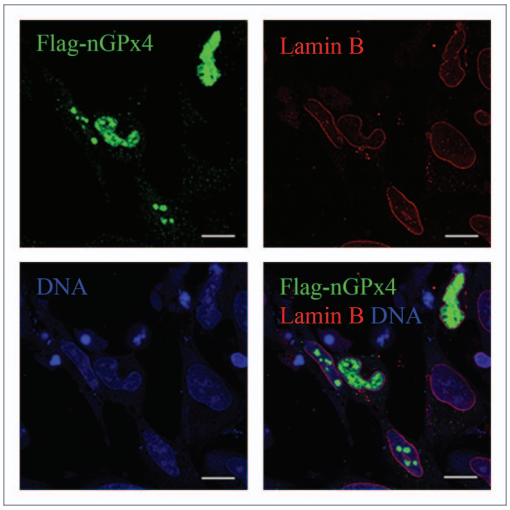

Figure 3. nGP4 localizes to the nucleus when expressed in vitro after transfection of HeLa cells with the vector pCMV-Flag-nGPx4. Cells were stained with anti-flag (green) and anti-lamin B (red) antibodies and analyzed at the confocal microscopy. Lamin B staining was limited to the nuclear periphery, whereas nGPx4 staining was within the nucleus. (Scale bars: 20 μm.)

other protein complexes that modify chromatin packaging. It is thus reasonable to hypothesize that the nuclear matrix is also involved in the process of histone substitution by protamines during spermiogenesis. In agreement with this idea, we previously found that nGPx4, an enzyme necessary for proper DNA condensation in sperm, is constantly located to the nuclear matrix during spermiogenesis and in mouse epididymal sperm,[12] opening the question whether protamines directly interact with nGPx4 by transiently sharing a nuclear matrix localization. In this report, we demonstrate the ability of nGPx4 to interact with protamines when the proteins are expressed in a heterologous in vitro cell system and in mouse late-step spermatids and epididymal sperm. Protamine 1 and protamine 2 are synthesized during mammalian spermiogenesis in elongating spermatids, and are then rapidly transferred to the nucleus, where they undergo a variety of post-translational modifications, including phosphorylation/dephosphorylation, and, as for protamine 2, a proteolytic processing. These post-synthetic events are important for nucleoprotamine assembly,[21] and for the process of sperm nuclei condensation, in which additional molecular partners are likely to be also involved. Due to the complexity of this process, we reasoned that protamine and nGPx4 expression in a heterologous mammalian system might be of help to identify intermediate steps of sperm chromatin assembly that are difficult to be revealed in late spermatids. Indeed, data obtained in this study indicate for the first time that protamines can be expressed successfully in mammalian cell lines without apparent cell toxicity, making transfected HeLa and COS-1 cells a good experimental paradigm for studying protamine processing, including structural modification, phosphorylation, disulfide bond formation, and physical/functional interaction with DNA and other proteins.

We have found that when expressed in COS-1 cells or HeLa cells, protamine 1 and protamine 2 colocalize together with nGPx4 to the nucleus at the specific level of nuclear matrix. The

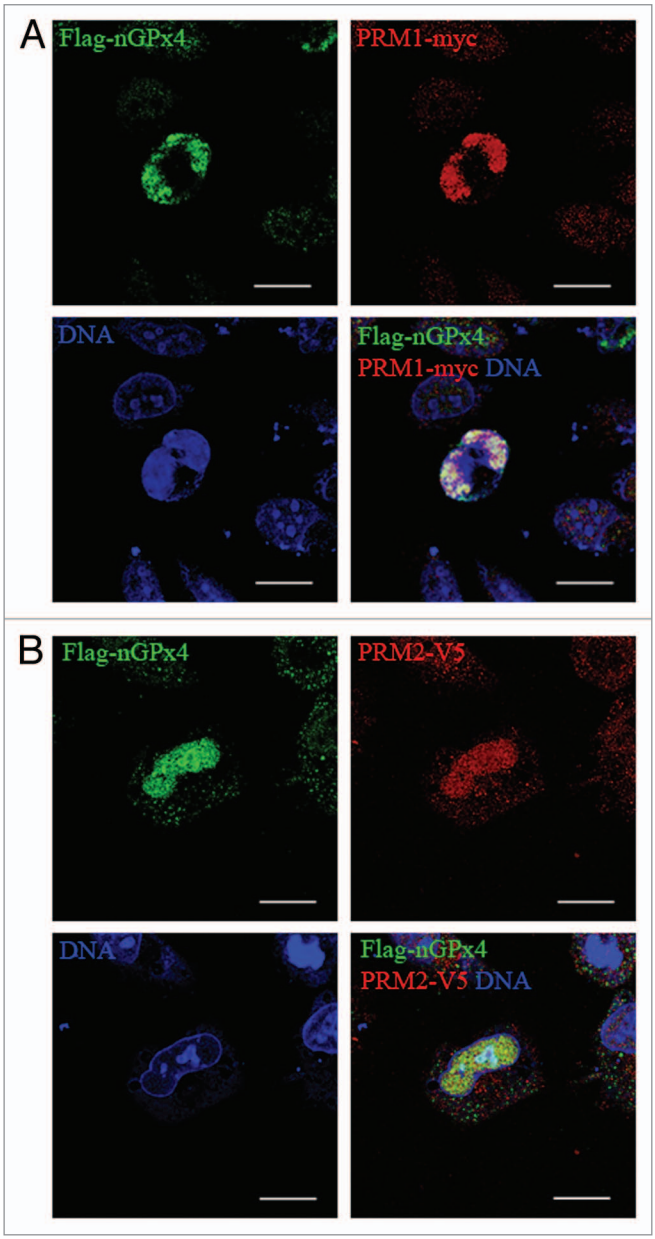

Figure 4. nGPx4 colocalizes esclusively to the nucleus together with both protamine 1 and protamine 2 in a heterologous cell system. HeLa cells were double transfected with the vectors pBUDCE4.1-PRM1myc-PRM2V5 and pCMV-flag-nGPx4 for 48 h and double stained in (**A**) with anti-flag (green) and anti-c-myc (red) antibodies; in (**B**) with anti-flag (green) and anti-V5 (red) antibodies. DNA was stained with TOTO-3 (blue). (Scale bars: 20 μm.)

localization of protamines to this subnuclear compartment was surprising, because we had previously observed that these proteins are associated with chromatin fraction, but not nuclear matrix, in sperm.[12] A likely explanation of present findings is that expressing protamines in a heterologous system somehow blocks the process of protamine assembly at an intermediate stage(s) because of the lack of other factor(s) required for the completion of chromatin compaction in somatic cells. Consistent with our observations, it was previously described that protamine 1 is transiently

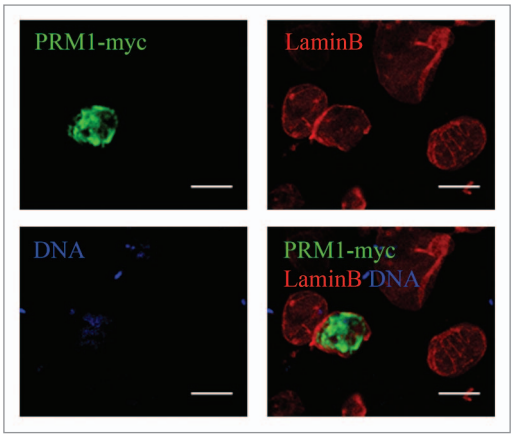

Figure 5. Protamine 1 is located to the nuclear matrix in a heterologous cell system. COS-1 cells were transfected with pBUDCE4.1-PRM1myc, spotted on a slide, and subjected to in situ extraction and DNA digestion as described in Materials and Methods, and finally double stained with anti-myc antibody (green), anti lamin B antibody (red), and Toto-3 (blue). Lamin B was used as marker of the nuclear matrix. (Scale bars: 10 μm.)

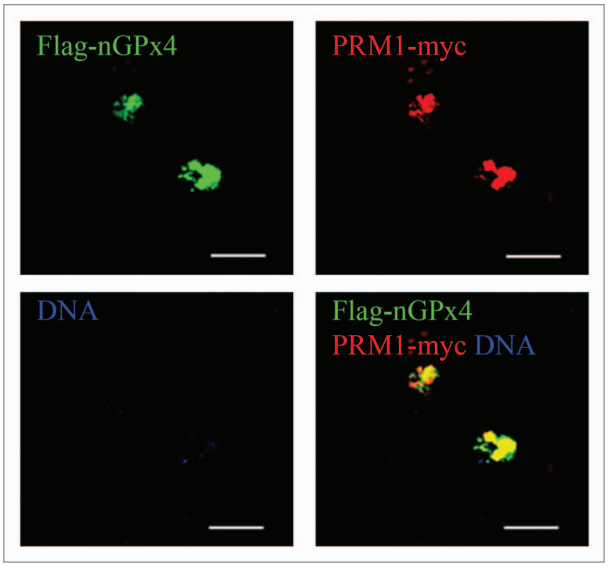

Figure 6. nGPx4 associates with protamine 1 at the level of the nuclear matrix in a heterologous cell system. COS-1 cells were co-transfected with pCMV-Flag-nGPx4 and pBUDCE4.1-PRM1myc, subjected to in situ nuclear matrix preparation as described in Materials and Methods, immunostained with anti-flag (green) and anti-myc (red antibodies), and analyzed by confocal microscopy. The removal of DNA was assessed by TOTO-3 staining (blue). (Scale bars: 20 μm.)

associated with lamin B receptor, a nuclear membrane protein located to the internal nuclear matrix, in elongating spermatids.[19] We thus propose that nuclear matrix represents an intermediate station where factors required for sperm chromatin condensation, including protamines and nGPx4, interact with each other before protamines are finally deposited on chromatin. This idea is also strengthened by the fact that, besides HeLa and COS-1 cells, protamine association with the nuclear matrix is also a feature of elongating spermatids, likely representing a physiological and transient step of protamine processing during spermiogenesis. It is also worth to note that, once initially established at the level of nuclear matrix, nGPx4 and protamine interaction is thereafter maintained in mature sperm, in spite of the different subnuclear compartments these two proteins belong to, namely the internal nuclear matrix and chromatin, respectively,[12] reinforcing the possibility that nGPx4 plays a structural role in sperm. Nuclear matrix is involved in the topological organization and function of DNA.[24] Of particular interest, in sperm genome, the protamine1-protamine 2-transition protein 2 cluster is flanked by two haploid-specific nuclear matrix attachment regions (MARs) that have been shown to be key in regulating the expression of this suite of genes.[16] Although it is not known if nGPx4 is a MAR binding protein, we propose that nGPx4 directly participates in the process of histone-to-protamine substitution by physically interacting with protamines and then targeting them to chromatin during spermiogenesis. In line with this idea, nGPx4 is expressed concomitantly with protamines in late step spermatids.[25]

It is well known that protamines are able to bind DNA providing proper chromatin condensation in spermatids and sperm. Also, nGPx4 appears to ensure the correct DNA compaction in sperm, as demonstrated by our previous findings,[12] although the precise molecular mechanisms underlying such events have not yet been fully clarified. In contrast, somatic cells transfected with either protamines or nGPx4 alone or in combination were unable to undergo apparent morphological changes of chromatin status. It is likely that histones bound to DNA hinder the protamine binding to DNA. Another possibility is that other molecular partners are missing in the in vitro system, thus making it difficult a process of chromatin remodeling.

Materials and Methods

DNA constructs

The Flag-tagged nGPx4 expression vector was prepared as previously described.[12] The *myc*-tagged protamine 1 (PRM1) expression construct driven by CMV promoter was prepared by cloning the full-length *prm1* cDNA sequence, previously generated by RT-PCR from mouse total germ cell, by using primers pair containing *Hind III* and *Xba I* restriction sites, respectively, into pBUDCE4.1 vector (Invitrogen, #V532–20), in frame with 3′ end *myc* epitope coding sequence (**Fig. S2**).

The *V5*-tagged protamine 2(PRM2) expression construct driven by human EF-1a promoter was prepared by cloning the full-length *prm2* cDNA sequence, generated by PCR from PCR4-PRM2-topo vector (kindly provided by Prof Norman Hecht) using primers pair containing *Not I* and *Xho I* restriction sites, respectively, into pBUDCE4.1 vector in frame with 3′ end *V5* epitope coding sequence (**Fig. S2**). Nucleotide sequencing of both protamine 1 and 2 cDNA constructs demonstrated 100% identity to that described by Yelick PC et al.[22] and verified correct in frame insertion into the restriction sites.

Cell preparation, culture, and transfection

Haploid male germ cells were obtained from CD1 mice (Charles River Laboratories). Animals were housed in accordance

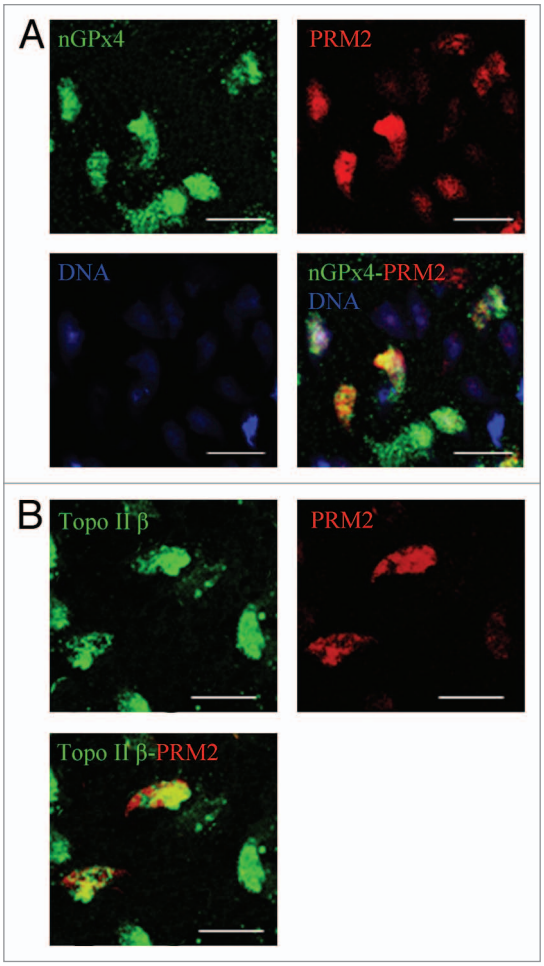

Figure 7. nGPx4 associates with protamine 2 at the level of nuclear matrix in sonication resistant spermatids (steps 12–16, SRS) isolated from adult mouse testis. Nuclear matrix was prepared from SRS as described in Materials and Methods and double immunofluorescence was performed to detect nGPx4 (green) and protamine 2 (red) (**A**). We used topoisomerase IIβ (topo IIβ) (green) to assess the accuracy of nuclear matrix preparation (**B**). The double staining with anti-nGPx4 and anti-TopoIIβ was not possible, because both antibodies had been generated in rabbit. (Scale bars: 10 μm.)

with the Sapienza University guidelines for animal care and were sacrificed by asphyxia with CO_2. Sonication-resistant spermatids (SRS) were prepared from adult mouse testes as previously described.[12] Spermatozoa were collected from cauda epididymis and vas deferens of adult mice by squeezing and mincing tissues in PBS. Released sperm were then washed twice in PBS by centrifugation at 1000 g for 15 min.

COS-1 cells and HeLa cells were grown at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium with high glucose, supplemented with 10% fetal bovine serum. For immunofluorescence analysis, 1 x 10^5 cells were cultured in 8 wells permanox chamber slide systems (Nunc Lab-Tek, Sigma) and transfected with 0.4 μg of the appropriate plasmid by liposome-mediated method (Lipofectamine™ 2000, Invitrogen, #11668-019) as previously described.[12] Transfection efficiency was 24 +/− 4.06% of cells expressing protamines (calculated in 5 independent experiments), as determined by flow cytometry analysis using anti-V5 antibody.

Flow cytometry analysis

HeLa and COS-1 cells were transfected with a dual promoter vector encoding both protamine 1-myc and protamine 2-V5 for 48 h. After fixation in 4% paraformaldehyde (PFA), cells were incubated with monoclonal anti-V5 antibody (1:50, Sigma; #V8012) for 30 min at 37 °C, washed, incubated with a biotinylated anti-mouse antibody (1:50, Jackson ImmunoResearch, #715-065-150), washed again, incubated with streptavidin-Phycoerythrin-Cy5 (1:100, Becton Dickinson #554062). Mouse IgG1 was used as isotype control. Fluorescence was analyzed by a Becton Dickinson FACS Vantage SE flow cytometer.

Immunofluorescence analysis

After transfection cells cultured on permanox chamber slides were fixed with 4% paraformaldehyde (PFA) for 10 min at 4 °C, then washed with PBS, and directly processed for immunostaining. Cells were incubated for 30 min in PBS containing 5% BSA and 0.1% Triton X-100 and then incubated for 1 h at room temperature (RT) with the following primary antibodies: rabbit anti-Flag (1:300; Sigma, #F7425); mouse anti-c-myc (1:30; Santa Cruz, #sc-40); goat anti-V5 (1:300; Bethyl, # A190-119A); goat anti-lamin B (1:50; Santa Cruz, #sc-6217); goat anti-protamine 2 (1:200; Santa Cruz, #sc-23104), rabbit anti-nGPx4 (1:100; Primm); rabbit anti-topoisomerase IIβ (1:100; Santa Cruz, #sc-13059). Cells were washed with PBS and then incubated for 1 h at RT with the appropriate secondary antibody diluted in PBS containing 1% BSA and 0.1% Triton X-100: anti-rabbit Alexa Fluor 488-conjugated antibody (1:500; Molecular Probes, #A21206); anti-goat Alexa Fluor 555-conjugated antibody (1:1000; Molecular Probes, #A21432); anti-mouse Alexa Fluor 488-conjugated antibody (1:500, Molecular Probes, #A21202) and anti-mouse Cy3-conjugated antibody (1:1000; Jackson Immunoresearch, #115-165-146). TOTO-3 (1:1000; Molecular Probes, #/3604) was used to stain DNA. Slides were mounted with Vectashield (Vector Laboratories, #H-1000) and analyzed under a Zeiss optical fluorescence microscope and a Leica confocal-microscope.

Protein extraction, western blotting, and immunoprecipitation assay

Basic nuclear proteins were extracted from transfected COS-1 cells and from epididymal spermatozoa according to Yu YE et al.[26] Cells were sonicated and treated with 0.5N hydrochloric acid (HCl). Proteins extracted were precipitated with 20% trichloroacetic acid (TCA) and washed with acidified acetone followed by acetone. For protamine 1 detection, epididymal sperm were processed as described by de Yebra and Oliva[27] with minor modifications. Cells were treated with 6M guanidine-HCl followed by 0.5N HCl extraction. Proteins were precipitated with 20% trichloroacetic acid and then collected by centrifugation.

Proteins were fractionated by 18% acetic acid-urea PAGE (AU-PAGE)[28] and finally transferred onto polyvinylidene fluoride Immobilon-P membranes (Millipore, #ISEQ10100). Blotted membranes were incubated for 1 h at RT in 5% non-fat dry milk and 0.1% Tween-20 with the following primary antibodies: mouse anti-c-myc (1:200, Santa Cruz); goat anti-protamine 1 (1:150; Santa Cruz, #sc-23107). After several washes filters were incubated

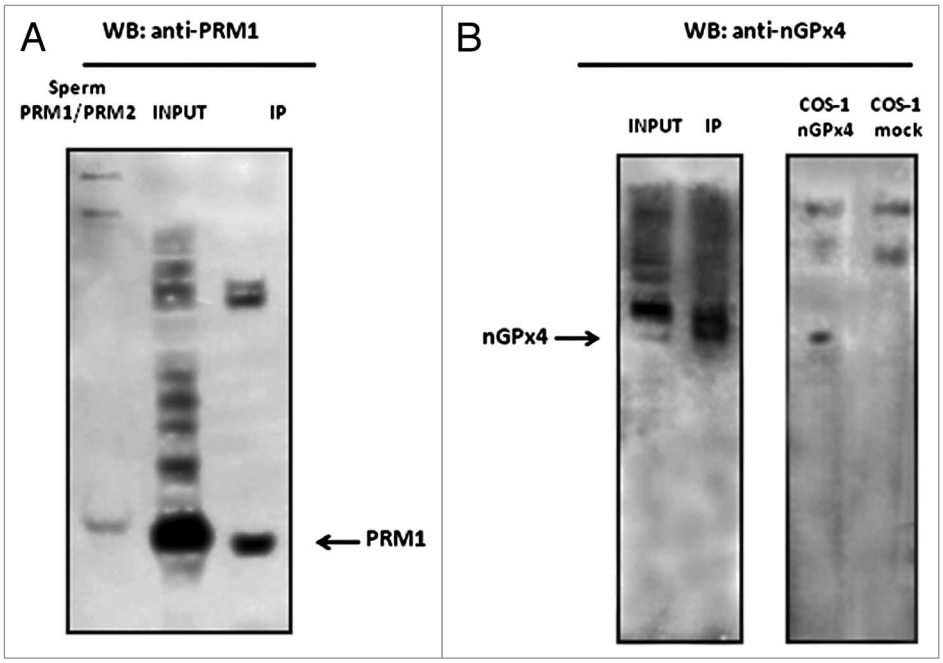

Figure 8. nGPx4 co-immunoprecipitates with protamine 1 in mouse epididymal spermatozoa. Whole sperm lysate (input) was subjected to immunoprecipitation (IP) with anti-protamine 1 antibody, as described in Materials and Methods, and then analyzed by AU-PAGE and western blotting using anti-protamine 1 (**A**) or anti-nGPx4 (**B**) antibodies. Protamines extracted from mouse sperm (Sperm PRM1/PRM2) and basic nuclear proteins extracted from COS-1 cells transfected with either pCMV-Flag-nGPx4 (COS-1 nGPx4) or empty vector (COS-1 mock) as described in Materials and Methods, were used as migration markers of protamine 1 and nGPx4, respectively.

with the appropriate secondary antibodies: anti-mouse and anti-goat horseradish peroxidase-conjugated (1:1000, DAKO, #P0161, and 1:3000, Santa Cruz, #sc-2020, respectively). Protein bands were detected by chemiluminescent method (GE Healthcare, #RPN2232) according to the manufacturer's recommendations.

For protamine 1 immunoprecipitation, 50 µg of sperm basic protein extracts (see above) were incubated with 2 µg of goat anti-protamine 1 overnight at 4 °C under constant rotation. Protein G-Sepharose 4 Fast Flow (GE Healthcare, #17-0618-01) was added to the extracts together with the antibody and incubated for further 3.5 h. Beads were washed five times with PBS and proteins were eluted by boiling in SDS sample buffer for 5 min. Extracts were then centrifuged and supernatants were precipitated in 100% ethanol overnight at -20 °C, centrifuged, and the pellets were dried. Proteins were dissolved in AU-BEM buffer (5% acetic acid, 5.5 M urea, 20% β-mercaptoethanol), fractionated on AU-PAGE, and processed for western blot analysis as described above. The primary antibodies used were rabbit anti-nGPx4 (1:100, Primm) and goat anti-protamine 1 (1:150, Santa Cruz). Secondary anti-rabbit or anti-goat True Blot IgGs conjugated to horseradish peroxidase (eBioscience, #18-8816, #18-8814, respectively) were used at the dilution of 1:1000.

In situ nuclear matrix preparation

Two different protocols were used to prepare in situ nuclear matrix fraction from COS-1 cells and mouse spermatids, respectively.

Protocol I

COS-1 cells were cytocentrifuged on polylysine-coated slides and subjected to in situ sequential extraction of DNA and proteins. After 3 min incubation in CSK buffer at 4 °C,[12] cells were treated with 1 mg/ml RNase-free DNase (Roche, #104159) for 15 min at RT, adding 0.25M ammonium sulfate for further 5 min. After 2M NaCl extraction for 5 min at 4 °C, the remaining material is considered the nuclear matrix fraction.

Protocol II

Sonication resistant spermatids (SRS) were spotted on polylysine-coated slides and incubated in 0.5% ATAB, 0.5 mM PMSF, and 2 mM DTT. Cells were then treated with 2M NaCl, 25 mM TRIS-HCl pH 7.4, and 2 mM DTT for 30 min at RT, followed by 1 h incubation with DNase at 37 °C. Nuclear matrices were fixed with 4% PFA for 10 min at 4 °C for immunofluorescence analysis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We are grateful to Prof Mario Stefanini and Prof Maria Teresa Fiorenza for helpful discussion. We thank Prof Norman Hecht for the generous gift of the PCR4-PRM2-topo vector. We also thank Ms Tiziana Menna for her technical assistance and Ms Stefania De Grossi for her valuable support of the confocal microscopy analysis. This work was supported by grants from Prin 2010–2011 to Boitani C.

Supplemental Material

Supplemental material may be found here: http://www.landesbioscience.com/journals/spermatogenesis/article/28460/

References

1. Johnson GD, Lalancette C, Linnemann AK, Leduc F, Boissonneault G, Krawetz SA. The sperm nucleus: chromatin, RNA, and the nuclear matrix. Reproduction 2011; 141:21-36; http://dx.doi.org/10.1530/REP-10-0322
2. Ward WS. Function of sperm chromatin structural elements in fertilization and development. Mol Hum Reprod 2010; 16:30-6; http://dx.doi.org/10.1093/molehr/gap080
3. Carrell DT, Liu L. Altered protamine 2 expression is uncommon in donors of known fertility, but common among men with poor fertilizing capacity, and may reflect other abnormalities of spermiogenesis. J Androl 2001; 22:604-10
4. Ramos L, van der Heijden GW, Derijck A, Berden JH, Kremer JA, van der Vlag J, de Boer P. Incomplete nuclear transformation of human spermatozoa in oligo-astheno-teratospermia: characterization by indirect immunofluorescence of chromatin and thiol status. Hum Reprod 2008; 23:259-70; http://dx.doi.org/10.1093/humrep/dem365
5. Oliva R. Protamines and male infertility. Hum Reprod Update 2006; 12:417-35; http://dx.doi.org/10.1093/humupd/dml009
6. Aoki VW, Liu L, Carrell DT. Identification and evaluation of a novel sperm protamine abnormality in a population of infertile males. Hum Reprod 2005; 20:1298-306; http://dx.doi.org/10.1093/humrep/deh798
7. Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N, Bizzaro D. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. Biol Reprod 2002l; 66:1061-7; http://dx.doi.org/10.1095/biolreprod66.4.1061
8. Arpanahi A, Brinkworth M, Iles D, Krawetz SA, Paradowska A, Platts AE, Saida M, Steger K, Tedder P, Miller D. Endonuclease-sensitive regions of human spermatozoal chromatin are highly enriched in promoter and CTCF binding sequences. Genome Res 2009; 19:1338-49; http://dx.doi.org/10.1101/gr.094953.109
9. Brykczynska U, Hisano M, Erkek S, Ramos L, Oakeley EJ, Roloff TC, Beisel C, Schübeler D, Stadler MB, Peters AH. Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. Nat Struct Mol Biol 2010; 17:679-87; http://dx.doi.org/10.1038/nsmb.1821
10. Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, Cairns BR. Distinctive chromatin in human sperm packages genes for embryo development. Nature 2009; 460:473-8
11. Hammoud SS, Purwar J, Pflueger C, Cairns BR, Carrell DT. Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. Fertil Steril 2010; 94:1728-33; http://dx.doi.org/10.1016/j.fertnstert.2009.09.010
12. Puglisi R, Maccari I, Pipolo S, Conrad M, Mangia F, Boitani C. The nuclear form of glutathione peroxidase 4 is associated with sperm nuclear matrix and is required for proper paternal chromatin decondensation at fertilization. J Cell Physiol 2012; 227:1420-7; http://dx.doi.org/10.1002/jcp.22857
13. Conrad M, Moreno SG, Sinowatz F, Ursini F, Kölle S, Roveri A, Brielmeier M, Wurst W, Maiorino M, Bornkamm GW. The nuclear form of phospholipid hydroperoxide glutathione peroxidase is a protein thiol peroxidase contributing to sperm chromatin stability. Mol Cell Biol 2005; 25:7637-44; http://dx.doi.org/10.1128/MCB.25.17.7637-7644.2005
14. Drennan KJ, Linnemann AK, Platts AE, Heng HH, Armant DR, Krawetz SA. Nuclear matrix association: switching to the invasive cytotrophoblast. Placenta 2010; 31:365-72; http://dx.doi.org/10.1016/j.placenta.2010.02.012
15. Linnemann AK, Krawetz SA. Maintenance of a functional higher order chromatin structure: The role of the nuclear matrix in normal and disease states. Gene Ther Mol Biol 2009; 13:231-43
16. Martins RP, Ostermeier GC, Krawetz SA. Nuclear matrix interactions at the human protamine domain: a working model of potentiation. J Biol Chem 2004; 279:51862-8; http://dx.doi.org/10.1074/jbc.M409415200
17. Martins RP, Krawetz SA. Decondensing the protamine domain for transcription. Proc Natl Acad Sci USA 2007; 104:8340-5; http://dx.doi.org/10.1073/pnas.0700076104
18. Kimura T, Ito C, Watanabe S, Takahashi T, Ikawa M, Yomogida K, Fujita Y, Ikeuchi M, Asada N, Matsumiya K, et al. Mouse germ cell-less as an essential component for nuclear integrity. Mol Cell Biol 2003; 23:1304-15; http://dx.doi.org/10.1128/MCB.23.4.1304-1315.2003
19. Mylonis I, Drosou V, Brancorsini S, Nikolakaki E, Sassone-Corsi P, Giannakouros T. Temporal association of protamine 1 with the inner nuclear membrane protein lamin B receptor during spermiogenesis. J Biol Chem 2004; 279:11626-31; http://dx.doi.org/10.1074/jbc.M311949200
20. Lee K, Haugen HS, Clegg CH, Braun RE. Premature translation of protamine 1 mRNA causes precocious nuclear condensation and arrests spermatid differentiation in mice. Proc Natl Acad Sci USA 1995; 92:12451-5; http://dx.doi.org/10.1073/pnas.92.26.12451
21. Green GR, Balhorn R, Poccia DL, Hecht NB. Synthesis and processing of mammalian protamines and transition proteins. Mol Reprod Dev 1994; 37:255-63; http://dx.doi.org/10.1002/mrd.1080370303
22. Yelick PC, Balhorn R, Johnson PA, Corzett M, Mazrimas JA, Kleene KC, Hecht NB. Mouse protamine 2 is synthesized as a precursor whereas mouse protamine 1 is not. Mol Cell Biol 1987; 7:2173-9
23. de Mateo S, Ramos L, de Boer P, Meistrich M, Oliva R. Protamine 2 precursors and processing. Protein Pept Lett 2011; 18:778-85; http://dx.doi.org/10.2174/092986611795713998
24. Anachkova B, Djeliova V, Russev G. Nuclear matrix support of DNA replication. J Cell Biochem 2005; 96:951-61; http://dx.doi.org/10.1002/jcb.20610
25. Puglisi R, Tramer F, Panfili E, Micali F, Sandri G, Boitani C. Differential splicing of the phospholipid hydroperoxide glutathione peroxidase gene in diploid and haploid male germ cells in the rat. Biol Reprod 2003; 68:405-11; http://dx.doi.org/10.1095/biolreprod.102.006544
26. Yu YE, Zhang Y, Unni E, Shirley CR, Deng JM, Russell LD, Weil MM, Behringer RR, Meistrich ML. Abnormal spermatogenesis and reduced fertility in transition nuclear protein 1-deficient mice. Proc Natl Acad Sci USA 2000; 97:4683-8; http://dx.doi.org/10.1073/pnas.97.9.4683
27. De Yebra L, Oliva R. Rapid analysis of mammalian sperm nuclear proteins. Anal Biochem 1993; 209:201-3; http://dx.doi.org/10.1006/abio.1993.1104
28. Meistrich ML. Histone and basic nuclear protein transitions in mammalian spermatogenesis. In: S.Hnilica GSSJLS, editor. Histones and other basic nuclear proteins. Orlando, Flo: CRC Press; 1989. p. 165-82.