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**SELENOPROTEINS IN MAMMALIAN SPERMATOGENESIS:
ROLE OF THE NUCLEAR GP \times 4**

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INTRODUCTION

Spermatogenesis

Spermatogenesis is the process of male germ cell formation and it is divided into three stages, mitotic, meiotic and spermiogenesis phase (fig.1).

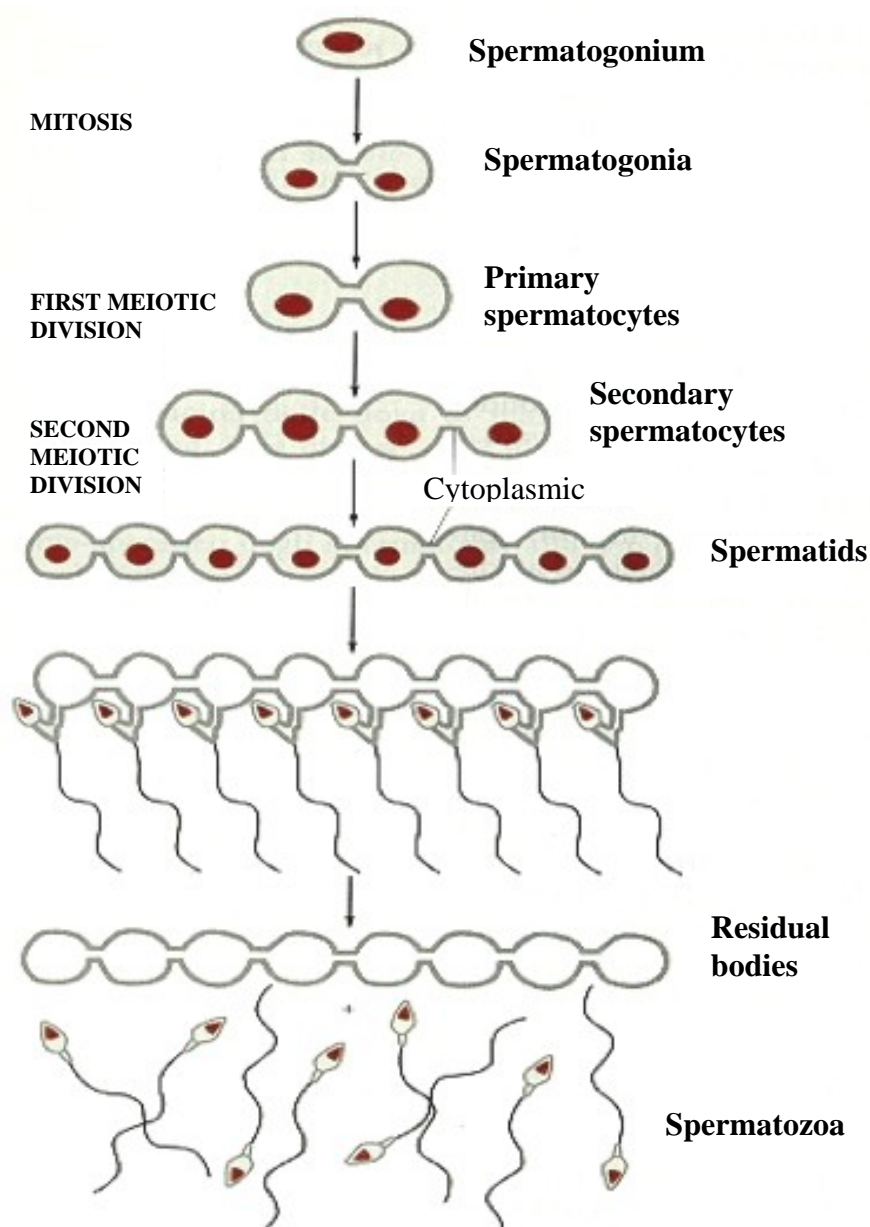


Figure 1-Spermatogenesis scheme

Fetal primordial germ cells give rise to spermatogonia that are present in the testis at birth but do not proliferate until puberty. At this stage of life the complex system of hormonal regulation is activated and promotes the formation of spermatozoa and seminal plasma.

Spermatogonia are divided into two types: differentiating spermatogonia, which will give rise to sperm, and undifferentiated spermatogonia, whose function is to maintain a stem cell population capable of giving life to the next generation of differentiating cells.

In rodents there are three types of spermatogonia: type A spermatogonia, with oval nucleus and finely dispersed chromatin, type B spermatogonia with round nucleus and condensed chromatin and intermediate spermatogonia with fine granules of chromatin thickened to the nuclear membrane. In a cycle of the seminiferous epithelium four consecutive generations of type A spermatogonia (A1,A2,A3,A4) follow one another; each A4 spermatogonium will give rise to the population of intermediate spermatogonia, which in turn produce type B spermatogonia. The latter enter meiosis (prophase I) and give rise to primary spermatocytes. The primary spermatocytes, the largest cells of the spermatogenic line, are characterized by the presence of chromosomes in different stages of condensation.

In prophase I the homologous chromosomes are paired by the synaptonemal complex. The first meiotic division result in a reduction of the genome halving the chromosome set from diploid ($2n$) to haploid (n). The secondary spermatocyte enters the second meiotic division, and gives rise to haploid cells with halved DNA content. In testis sections it is difficult to observe the secondary spermatocytes because they have very short life and remain in interphase for a period of time very small and rapidly enter the second meiotic division.

The spermiogenesis follows meiosis: cells at this stage are called spermatids distinguished by their small size and nuclei with condensed chromatin. Spermatids undergo a complex differentiation process called spermiogenesis, finally becoming spermatozoa. This process includes the acrosome formation, nucleus condensation and elongation, flagellum formation and cytoplasm loss. The final result is the mature sperm that is then released into the lumen of seminiferous tubule.

Throughout spermatogenesis germ cells do not separate physically but remain connected by cytoplasmic bridges. Exchange of soluble factors such as RNA and enzymes occurs through cytoplasmic bridges which ensure synchrony of differentiation leading to

maturation of all the cells of a wave. The seminiferous epithelium in rodents is composed of concentric circles of germ cells found in progressively more advanced stages of development ranging from the periphery to the lumen. The basal layer consist of spermatogonia, follow inward one or two generations of spermatocytes and one or two generations of spermatids. A distinctive feature is that the cells in each layer are exactly in the same stage of development and that the different cellular generations or stages are associated with each other. Each layer is therefore a generation of germ cells developing in synchrony and with a precise relationship with other generations. These clusters of cell types are called *cellular associations*.

Another important feature is that the different cell generations are separated by each other by a constant time interval, that is 8.6 days in mice. The cellular associations vary in different segments of the seminiferous tubule, namely in different cross sections of tubule. In mice, 12 different cellular associations are found. In any particular segment of the tubule, associations follow one after another in time with a precise sequence occupying tubule segments of variable length. Each complete series of adjacent segments containing the typical cellular associations of the cycle is defined as a wave of the seminiferous epithelium. The time interval between two successive appearances of the same association in the same area of the seminiferous tubule is called a *cycle of seminiferous epithelium*.

Chromatin remodeling during spermiogenesis

In advanced stages of spermiogenesis a profound reorganization of the chromatin occurs which undergoes condensation becoming more compact. These changes are accompanied by replacement of histones with protamines. Protamines are sperm specific proteins very rich in arginine and cysteine. However a small percentage of histones (10-15% in humans) remains in the sperm nucleus. The protamines are stored in the nucleus up to fertilization but after this event are completely degraded to be replaced by somatic histones during the formation of male pronucleus (fig.2)

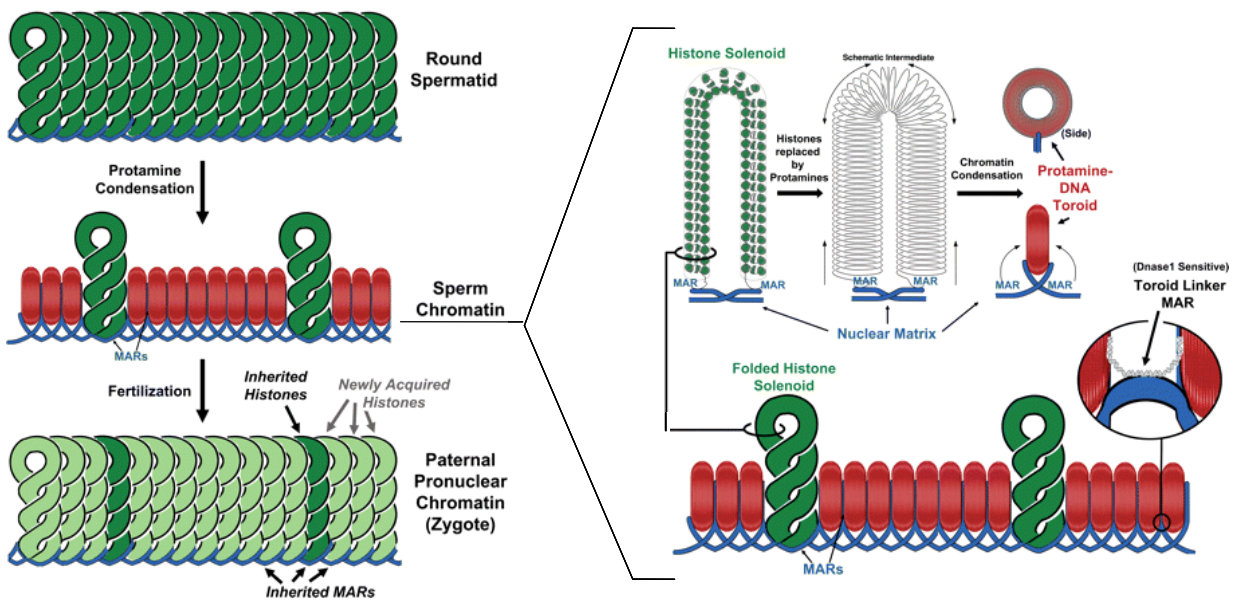


Figure 2-Inheritance of sperm chromatin structural element by the embryo

DNA in round spermatids is packaged by histones (top) but during spermiogenesis, most of these are replaced by protamines (middle, red). After fertilization, the protamines are removed, and histones supplied by the oocyte replace them (bottom, light green). However some histones that were retained in the spermatozoon (middle, dark green) are probably retained in the newly formed paternal pronucleus after fertilization. Sperm nuclear matrix attachment regions (MARs) are probably retained in the paternal pronucleus as well (modified from Ward WS., Mol Hum Reprod, 2010).

Protamines have the function of promoting the condensation of chromosomes in limited space of the male nucleus and protect genome from various harmful agents during the transit of gametes along the male and female genital tracts. At the end of spermiogenesis, most of the cytoplasm is removed from the spermatid forming the residual bodies that is phagocytized by the Sertoli cells. Immediately after the removal of residual bodies, spermatozoa detach from Sertoli cells, which until now had wrapped them, and are released into the tubule lumen. However a small portion of cytoplasm remains attached to the spermatid, which will be eliminated during the maturation of sperm in the epididymis. Mammalian histones are not directly replaced by protamines.

They are first replaced by another group of basic proteins called transition proteins (TP) (Grimes et al.,1977) in elongating and condensing spermatids. TP1 and TP2 are the predominant TPs found in rodent spermatids. TP1 is a 6.2 kDa protein and consists of 20%

arginine, 20% lysine and lacks cysteine, whereas TP2 has a molecular weight of 13 kDa and consists of 10% arginine, lysine 10% and 5% cysteine. TP1 is abundantly expressed and its sequence is highly conserved in many mammals, while the sequence of TP2 is poorly conserved across mammals and its level of expression is different in different species. Both TP1 and TP2 are encoded by single copy genes, *Tnp1* and *Tnp2*, and the genomic locus for TP2 is found in the same gene clusters of the protamines, suggesting that they arise from gene duplication and might therefore share some common function (Zhao et al.,2001). The removal of histones by transition proteins is accompanied by posttranslational changes such as acetylation, ubiquitination and phosphorylation. Furthermore the phosphorylation of transition proteins and protamines is important for chromatin compaction. The free thiols present on the cysteine residues of protamines undergo oxidation by different enzyme systems leading to the formation of disulfide bonds cross-linking responsible for further stabilization and compaction of chromatin (Bedford et al.,1974). Recently it was observed that DNA condensation in male germ cells during the final stages of spermatogenesis is a phenomenon that involves multiple proteins and for some of them the role is known only in somatic cells (Chu et al.,2006).

Sperm nuclear matrix

Inside the nucleus it is possible to distinguish a nonchromatin structure called nuclear matrix. The nuclear matrix is operationally defined as the nuclear structure that remains after the salt extraction of nuclease treated nuclei (Berezney and Coffey., 1974). It consists of a peripheral lamina-pore complex and an internal filamentous ribonucleoprotein network that has not been well characterized. This component of the nuclear architecture provides the internal scaffold of the nucleus. It is a dynamic structure whose morphology and composition vary with the functional state of nuclei from which it is derived. The cell type specificity of nuclear matrix proteins may reflect a nuclear matrix role in the selective activation of those genes which express differentiated function.

The nuclear matrix may be the structural scaffold that determines higher order chromatin organization (Nickerson et al.,1989). Actively transcribed genes are greatly enriched in

nuclear matrix preparations suggesting the association of active chromatin regions with the matrix (Robinson et al.,1982).

In both somatic and sperm nuclei, chromatin is organized into loop domains that are attached every 20-140 kb in length to a proteinaceous nuclear matrix. This organizes the chromatin into functional loops of DNA that help regulate DNA replication (Vogelstein et al.,1980; Kalandadze et al.,1990; Gerdes et al.,1994; Dijkwel and Hamlin 1995) and gene transcription (Chang et al.,1995; Cockerill and Garrard 1986; Nelson et al.,1986). It was demonstrated that each protamine toroid contains a single DNA loop domain (Ward 1993; Sotolongo et al.,2005). Between each protamine toroid , there is a nuclease sensitive segment of chromatin, called toroid linker, which is also the site of attachment of DNA to the *matrix attachment regions* (MARs).The nuclease sensitivity suggests that these protamine linker regions are bound by histones and this is consistent with the wide distribution of histones throughout the genome (Arpanahi et al.,2009). Thus, the organization of sperm DNA into protamine toroids is directly linked to the nuclear matrix. Topoisomerase IIB (TOP2B) is located in the nuclear matrix and it was proposed that it regulates DNA degradation in association with an extracellular nuclease (Shaman et al.,2006) and this degradation initiates at the nuclease sensitive sites of DNA attachment to the nuclear matrix. TOP2B is also required for DNA replication and chromosome segregation, causing transient double-stranded DNA breaks as part of its mechanism. In mammals this process is active in both somatic and germ cells. Furthermore TOP2B is required for the transition from histone-bound to protamine-bound chromatin during spermiogenesis.

Several proteins localize in the nuclear matrix where they play important roles; calcium/calmodulin-dependent protein kinase IV (CaMKIV) and calspermin (CaS) are two proteins expressed in the testis and encoded by a common gene, *Camk4*. CaMKIV is expressed in spermatogonia and spermatids and excluded from pachytene spermatocytes. In germ cells CaMKIV is associated with chromatin but morphological analysis demonstrated that a fraction of CaMKIV in spermatids is localised to the nuclear matrix (Wu and Means,2000), suggesting its involvement in chromatin remodeling.

It is of interest that several evidence support a functional role for the sperm nuclear matrix in the paternal genome during early embryogenesis. Spermatozoa with structurally disrupted sperm nuclear matrices do not support embryonic development after ICSI unlike those with intact matrices (Ward et al.,1999). Furthermore it was demonstrated the importance of an intact nuclear matrix with its associated DNA for the onset of paternal DNA replication after fertilization (Shaman et al.,2007).

Selenium and spermatogenesis

Selenium (Se) is an essential trace element that plays an important role in a number of physiological processes in animals and humans. This element is incorporated into proteins as selenocysteine, thanks to a peculiar translation reprogramming that allows mRNA UGA codon to be specifically recognized by the selenocysteynil-tRNA, instead of canonically functioning as stop signal. The testis represent a specific and privileged target of selenium. The selenium level in the testis is maintained by gonadotropin hormone (Behne et al.,1982) and its concentration increases during puberty with the progress of spermatogenesis (Behne et al.,1986). This element appears to be essential for maintaining a normal spermatogenesis and for male fertility. In case of selenium deficiency, regulatory mechanism strive to maintain an adequate level of this element in the male gonad and, when selenium is administered again, the selenium is supplied to the testis with priority over other tissue (Behne et al.,1982). Dietary selenium is assimilated in the liver and incorporated there as selenocysteine into selenoprotein P (Burk et al.,1991). It then is excreted into the circulation to be taken up by other tissues, probably via receptor-mediated process and degraded. The selenocysteine thus liberated can then be used for local selenoprotein synthesis.

In mammals, normal spermatogenesis is strictly dependent on an adequate intake of selenium (Wu et al.,1979). In Rodents selenium-deficient diet leads to many reproductive disorders including reduced fertility, impaired sperm motility, structural alteration of the midpiece, loss of flagellum and malformation of the sperm head (Wu et al.,1973). Moreover when a low selenium diet was extended for several generations severe testicular

atrophy and a complete disruption of spermatogenesis that could be reversed by a selenium-adequate diet were observed. These alterations, however, were reversible and spermatogenesis was restored by feeding the selenium-adequate diet (Behne et al.,1996). In depleted rats it was observed that sperm chromatin condensation was severely disturbed (Pfeifer et al.,2001).

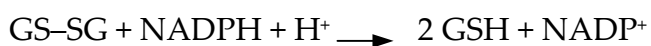
In line with these data showing the importance of selenium for a correct spermatogenesis, in mammalian testis almost the entire selenium content is associated with the GPx4, a member of the large subfamily of the glutathione peroxidases (GPxs).

Glutathione peroxidases (GPxs)

The biological functions of selenium are mediated by a large family of selenoproteins, including the Glutathione Peroxidases (GPxs). Glutathione (GSH) is a tripeptide composed of glutamic acid, cysteine and glycine and it is converted to its oxidized form glutathione disulfide (GSSG).

In the reduced state, the thiol group of cysteine is able to donate a reducing equivalent ($H^+ e^-$) to other unstable molecules, such as reactive oxygen species. In donating an electron, glutathione itself becomes reactive, but readily reacts with another reactive glutathione to form glutathione disulfide.

The biochemical function of glutathione peroxidase is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free hydrogen peroxide to water, according to the following chemical reaction:



In healthy cells and tissue, more than 90% of the total glutathione pool is in the reduced form and less than 10% exists in the disulfide form since the enzyme that reverts it from its oxidized form, glutathione reductase, is constitutively active and inducible upon oxidative stress. In fact an increased GSSG-to-GSH ratio is considered indicative of oxidative stress.

Among the GPxs, some peroxidases are selenium dependent proteins because they contain UGA codon coding for the selenocysteine and include four enzymes, GPx1, GPx2, GPx3,

GPx4. On the contrary GPx5 is a selenium independent protein and is expressed in the cauda epididymis where protect spermatozoa from oxidative injuries that could compromise their integrity and, consequently, embryo viability (Chabory et al.,2009). GPx1 was the first mammalian selenoprotein to be identified in 1957 and has been found to be important in protecting the haemoglobin from the oxidative breakdown brought about by low concentrations of ascorbic acid (Mills et al.,1957). GPx2 is restricted in expression to the gastrointestinal tract and has 65% of the aminoacid sequence and 60% of the nucleotide identical to GPx1 (Avissar et al.,1994). GPx3 is extracellular, especially abundant in plasma (Maddipati et al.,1987) whereas GPx6, in humans, is found in the olfactory epithelium and embryonic tissues (Papp et al.,2007).

Phospholipid hydroperoxide glutathione peroxidase (GPx4)

The selenoprotein phospholipid hydroperoxide glutathione peroxidase (GPx4) was firstly purified from pig liver by Ursini et al. in 1982. Compared with other members of glutathione peroxidases superfamily this monomeric enzyme is highly expressed in the testis and has unique properties, including the ability to reduce the intracellular membrane phospholipids hydroperoxides and to use the thiol groups of proteins, with particular reference to sperm protamines, beside those of glutathione (Godeas et al.,1997; Tramer et al.,2002).

It is remarkable that the testis exhibits the highest specific activity of GPx4 so far measured in mammalian tissues, being almost two orders of magnitude higher than that of brain and liver (Maiorino et al.,2003; Ursini et al.,1995). In addition GPx4 gene expression and enzymatic activity are hormone-dependent. In hypophysectomized rats and in testosterone-deprived rats, testicular GPx4 activity and mRNA content significantly decreased and were partially restored by human chorionic gonadotropin (hCG) or testosterone administration, respectively (Roveri et al.,1992; Maiorino et al.,1998). The *gpx4* gene is composed of 8 exons and encodes for three isoforms having different subcellular localisation, being located in the mitochondria (mGPx4, 23kDa), in the cytosol (cGPx4, 20kDa) and in the nucleus (nGPx4, 34kDa) and differing on their N-terminal aminoacid

sequence. The N-terminuses of the mitochondrial and cytosolic variants derive from the same exon 1A by using different translation sites, whereas the N-terminus of the nuclear isoform is generated by an alternative exon (1B) driven by another promoter located in the first intron of the gene (Maiorino et al.,2003) (fig.3).

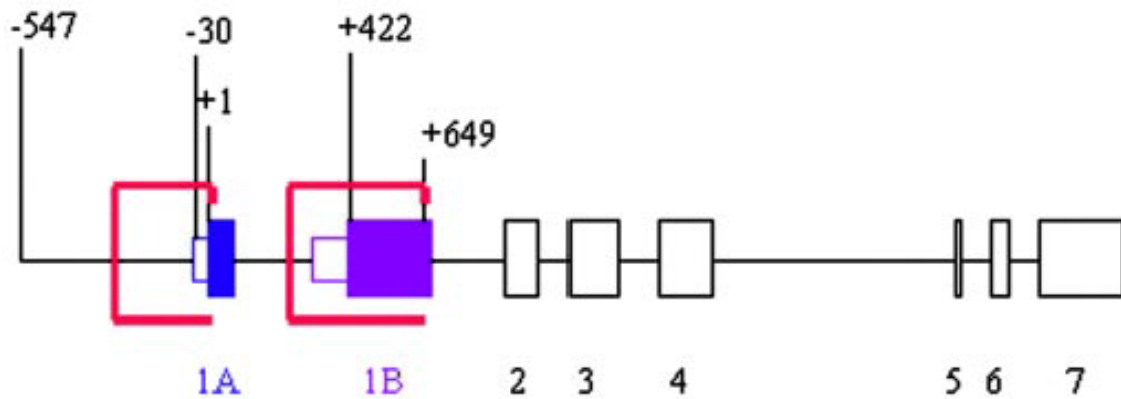


Figure 3-Schematic representation of *gpx4* gene with its promoters (red squares) and two exons 1A and 1B.

The nuclear and mitochondrial proteins have different patterns of expression during male germ cell maturation in rat testis, being nGPx4 expressed only in the haploid phase of spermatogenesis, whereas mGPx4 is also expressed in late primary spermatocytes (Puglisi et al.,2003).

The high expression of GPx4 in the testis underlines the relevance of this gene to spermatogenesis. All three different isoforms efficiently catalyze the reduction of phospholipid hydroperoxides by oxidation of glutathione, clearly indicating that they are involved in the protection of germ cells from oxidative damage.

However, evidence has been accumulating about other roles played by each of the three isoforms. Indeed GPx4 forms behave like moonlighting proteins changing physical characteristics and biological functions depending on the cell type where they are expressed, the intracellular compartment where they are present and the molecules whom they interact with.

mGPx4 exists as a soluble peroxidase in spermatids but persist in mature spermatozoa as an enzymatically inactive, oxidatively cross-linked, insoluble protein. In the midpiece of mature spermatozoa, mGPx4 protein represents more than half of the capsule material

embedding mitochondrial helix and it is cross-linked by disulfide bridges to form protein aggregates (Ursini et al.,1999) together with other capsular proteins (Maiorino et al.,2005). The mechanism of inactivation is thought to be related to glutathione depletion, occurring during germ cell maturation (Shalgi et al.,1989). In fact nGPx4 can use protein thiols as alternate substrates to create protein aggregates that are cross-linked by disulfide bonds and this occurs when the cells are exposed to hydroperoxides at low concentration of glutathione, as is documented for late stages of spermatogenesis (Shibanuma et al.,1994). The role of mGPx4 as a structural protein may explain the impairment of the sperm motility associated with morphological alterations of the midpiece observed in selenium-deficient animals (Wu et al.,1979). In line with this conclusion, a significant reduction of mGPx4 expression has been observed in human spermatozoa of infertile oligoasthenozoospermic patients compared with those of normal fertile males (Imai et al.,2001), emphasizing the clinical relevance of mGPx4 enzyme.

The crucial role of mGPx4 in male fertility has been conclusively demonstrated by a genetic approach. Because deletion of the complete *gp4* gene in mice results in embryonic lethality between 7.5 and 8.5 dpc (Imai et al.,2003; Yant et al.,2003), mice with a specific disruption of mGPx4 were generated. Interestingly mGPx4-KO male mice were infertile with impaired sperm quality and severe structural abnormalities in the midpiece of spermatozoa. Moreover mGPx4-KO spermatozoa display higher protein thiol content and recapitulate features typical of severe selenodeficiency. However, when infertile spermatozoa of mGPx4 KO mice were injected directly into oocytes , they were able to participate in normal development.

Knock-out of *mGPx4* impairs the ability of spermatozoa to participate in fertilization, and in consequence causes infertility of mGPx4-KO males, but has no effect on developmental potential of nuclei of spermatozoa (Schneider et al.,2009). Similar results were obtained by Imai et al (2009), by creating spermatocyte-specific GPx4 knock-out male mice. These mutants are infertile and display a significant decrease in the number of spermatocytes and spermatozoa, which showed significant reductions of forward motility and the mitochondrial membrane potential (Imai et al.,2009).

Liang and his colleagues (2009) generated transgenic mice using mutated *GPx4* genes encoding either the mitochondrial GPx4 or cytosolic GPx4. They demonstrated that transgenic mice with *mGPx4* gene had increased GPx4 protein only in the testes and the *mGPx4* gene failed to rescue the lethal phenotype of the mouse GPx4 null mutation. In contrast, transgenic mice with the *cGPx4* gene had increased GPx4 protein in all tissues and moreover, the *cGPx4* gene was able to rescue the lethal phenotype of the complete *GPx4* gene deletion even if the mice were infertile and exhibited sperm malformations (Liang et al.,2009). They demonstrated that the cGPx4 protein is present in somatic tissue mitochondria and it is essential for survival and protection against apoptosis in mice, whereas the mGPx4 protein is important for male fertility.

As for the nuclear isoform the N-terminal region of nGPx4 contains the nuclear localisation signal and an arginine-rich domain showing more than 50% homology to the protamines sequences. However, no data are available on its subnuclear localisation. The expression of nGPx4 is restricted to the haploid phase of spermatogenesis, appearing firstly at both the mRNA and protein level in round spermatids. nGPx4 expression was maintained in the late stage of spermatogenesis (Puglisi et al.,2003) and was identified in the nuclei of the epididymal spermatozoa of both rats and mice (Pfeifer et al.,2001).

Knock-out mice specifically lacking nGPx4 were fertile but showed impaired chromatin condensation in caput epididymis, but not in cauda epididymis sperm. In addition a significantly higher levels of free thiols in KO spermatozoa isolated from the cauda epididymis was observed (Conrad et al.,2005). These data indicate that nGPx4 acts as a protein thiol peroxidase in vivo and it plays a role in stabilizing nuclear structures in spermatozoa from the caput epididymis before and independently of protein thiol oxidation. The authors suggested that an enzyme other than nGPx4 may be involved in thiol oxidation or, alternatively, that the thiol oxidation process takes place spontaneously. It remains to be investigated whether cauda epididymis sperm from nGPx4-KO mice, reach a correct and stable chromatin condensation status.

AIM OF THE STUDY

In recent years the role of nuclear GPx4 isoform in spermatogenesis has been the interest of several studies. In our laboratory we have previously demonstrated that nGPx4 is expressed in developing germ cells in a stage and cell-specific manner. It was demonstrated that nGPx4 is switched on in round spermatids at both the mRNA level and the protein level (Puglisi et al.,2003) and its expression is maintained in the late stage of spermatogenesis (Pfeifer et al.,2001).

This finding and others such as the demonstration that nGPx4-KO mice are fertile but show impaired nuclear condensation of sperm isolated from the caput epididymis (Conrad et al.,2005), the nGPx4 similar spatio-temporal expression pattern to the protamine, the evidence that its sequence shows more than 50% homology to protamine 1 (Pfeifer et al.,2001) led to propose that nGPx4 is involved in the stabilization of condensed chromatin during sperm maturation.

In this study we first investigated the subnuclear localization of nGPx4 in both somatic cells overexpressing nGPx4 and mouse male germ cells at different steps of maturation (round spermatids and epididymal spermatozoa). By morphological and biochemical analysis we found that nGPx4 was localized at the level of the nuclear matrix.

To gain more insight into the functional role of nGPx4 in chromatin dynamics, spermatozoa were collected from the caput and the cauda epididymides of WT, heterozygous and homozygous nGPx4-KO mice and subjected to an *in vitro* chromatin decondensation assay. Our results show that nGPx4-KO mice sperm decondensed earlier than those from WT at all stages of epididymal maturation. The novel distribution of nGPx4 in the sperm nuclear matrix and the failure of epididymal sperm nuclei from nGPx4-KO mice to appropriately condense prompted us to investigate whether the sperm nuclear structure instability caused by the lack of nGPx4 might impact on the early events occurring after fertilization. In fact it is now well understood that nuclear matrix is crucial for paternal genome function in proper embryonic development (Shaman et al.,2007). Thus we performed *in vitro* fertilization experiments aimed at studying the timing of

sperm nuclear decondensation. In addition, the nuclear matrix attachment regions (MARs) can function as boundaries for genic domains (Bode et al.,2000) and can play key roles in the opening of discrete regions of chromatin in the genome to permit transcription (Krawetz et al.,1999). In particular, MARs bounding the PRM1, PRM2 and TNP2 domain are important to ensure a proper transcription of these genes (Martins et al.,2004). Based on this evidence, we addressed the issue whether nGPx4 might interact with protamines and we thus performed protamine 1 immunoprecipitation experiments in which proteins were prepared by acid extraction of mouse epididymal spermatozoa.

MATERIALS AND METHODS

Animals

Animals used were mice from the CD1 and C57BL/6J strains as well as nGPx4-KO mice. Animals were housed in accordance with the guidelines for animal care of Sapienza University of Rome and were killed by CO₂ asphyxia.

Genotyping

nGPx4 knock out mice were kindly provided by Prof. Marcus Conrad (Munich, Germany). Offspring obtained from heterozygous and homozygous breeding pairs were analysed extracting DNA from mouse tails 2 weeks of age. Each piece of mouse tail was previously digested by incubation in TES solution (50mM Tris pH 8.00, 100mM EDTA, 0.5% SDS) containing proteinase K (10mg/ml) at 55°C overnight. Phenol pH 8.00 was added and centrifuged to separate phases; aqueous phase was recovered and a mix of phenol/chloroform (1:1) was added. After shaking vigorously and centrifuging, the aqueous phase was recovered and DNA was precipitated with 0.3M sodium acetate pH 6.00 and 100% ethanol at RT. DNA was washed with 70% ethanol and finally resuspended in water. Genomic analysis was performed by polymerase chain reaction (PCR) utilizing two primer pair: one specific for the wild type allele (I1f2/Earev1) and the other one specific for the knock out allele (I1f2/eGFPprev) as described by (Conrad et al.,2005).

Plasmid construction

Transfection experiments were performed using the construct generated in our lab by Rossella Puglisi. The construct Flag-nGPx4 contains the mouse nGPx4 full length cDNA with a Flag sequence epitope at 5' end, driven by CMV promoter.

Cell culture

For *in vitro* experiments COS-1 and HeLa cells were used. COS1 and HeLa cells were grown at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM, Sigma) with

high glucose supplemented with 4mM glutamine, antibiotics (penicillin, streptomycin 100µg/ml), hepes 15mM pH 7.7, non essential aminoacids 10mM, gentamicin (40µg/ml) and 10% fetal bovine serum (FBS). Every three days the medium was changed after washing with DMEM medium without serum. After reaching the confluence the medium was removed and cells were detached with a solution of trypsin (2.5mg/ml)/EDTA(0.3mg/ml) for five min at 37°C. Cells were then centrifuged at 1000rpm for 5 min, washed with medium without serum to remove all traces of the enzyme and resuspended in DMEM with serum and plated after appropriate dilution.

Cell transient transfection

The day before transfection, cells were plated at the density corresponding to 95% confluence at the time of transfection.

To obtain the highest transfection efficiency and low cytotoxicity, cell transfection was performed by liposome-mediated method (Lipofectamine™ 2000, Invitrogen) according to manufacturer's instruction. 14µg of plasmid and 35µl of Lipofectamine 2000 were used for 7×10^6 adherent cells on a 100-mm Petri dish. After 6 h of transfection, cells were maintained in growth medium (OptiMEM, GIBCO) without antibiotics supplemented with Na₂SeO₃ at 50nM final concentration, for 48-72 h. After the transfection cells were frozen for a later protein extraction or fixed in 4% paraformaldehyde for immunofluorescence experiments.

Germ cell preparation

Highly purified pachytene spermatocytes and round spermatids (steps 1-8) were obtained from 28-30 days old mouse testes by STAPUT technique (Boitani et al.,1980). Briefly, the cell suspension obtained by enzymatic digestion of testicular tissue was fractionated by velocity sedimentation at unit gravity on 0.5-3% albumin gradient. Purity of cell fraction was verified by flow cytometry and morphology of stained cyospin cell preparations (Puglisi et al.,2003). The cells were washed twice with phosphate-buffered saline (PBS) and then processed as needed. Round spermatids nuclei were prepared following (Platz et

al.,1977) protocol. Very briefly, round spermatids were lysed at concentration of 2×10^7 cells/ml in solution L [5mM MgCl₂, 5mM sodium phosphate, 0.25% Triton X-100, 0.025% STI (soybean Trypsin Inhibitor) and DNase I 1mg/ml (Roche)] containing 0.1mM PMSF. After centrifuging at 600g for 10 min, pellet was suspended in MP solution (5mM MgCl₂, 5mM sodium phosphate, pH 6.5) using Pasteur pipette and then was forced by a syringe through a 25-gauge needle twice. After centrifugation, pellet was washed with MP solution and an aliquot was used for microscopic examination and counting whereas the resulting nuclei suspension was spotted on polylysine coated slides for *in situ* nuclear matrix preparation.

Nuclear matrix preparation from transfected cells, pachytene spermatocytes and round spermatids.

COS-1 cells were transfected with the construct pCMV-Flag-nGPx4 or the control vector and after 48 h of transfection cells were subjected to fractionation. Fractions of cytosol, chromatin and nuclear matrix were prepared following high salt isolation method (Reyes et al.,1997). Cells were homogenized in cytoskeleton buffer (CSK: 10mM Pipes, pH 6.8, 100mM NaCl, 300mM sucrose, 3mM MgCl₂, 1mM EGTA, supplemented with leupeptin, aprotinin and pepstatin (1µg/ml each), 1mM PMSF, 1mM DTT and 0.5% (vol/vol) Triton X-100) for 3 min. The cytoskeletal frameworks were separated from soluble proteins by centrifugation and chromatin fraction was solubilized by DNA digestion with 1mg/ml of RNase-free DNAase I in CSK buffer plus proteinases inhibitors for 15 min at 37 °C. The pellet obtained from ammonium sulfate 0.25M precipitation was subsequently extracted with 2M NaCl in CSK buffer for 5 min at 4°C and then centrifuged. The remaining pellet, considered the nuclear matrix fraction, was solubilized in urea buffer (8M urea, 0.1M NaH₂PO₄, 0.01M Tris, pH 8.00) for SDS-PAGE analysis.

The same procedure was carried out for *in situ* nuclear matrix preparation but in this case cells were extracted directly on permanox slides with the high salt isolation method described above , and then fixed with PFA 4% for 10 min at 4°C for immunofluorescence analysis.

Nuclear matrix preparation of epididymal spermatozoa

Mature spermatozoa were collected from the caudal epididymides and vas deferens of CD1 mice and treated according to Ward et al.,1999 and Shaman et al.,2007. Briefly, after washing at least two times in PBS1X, epididymal spermatozoa were resuspended in a solution containing PBS 1X-0.5mM PMSF, supplemented with the cationic detergent ATAB 0.5% (SIGMA) and 2mM DTT. Spermatozoa suspended in this medium for at least 15' at RT were repeatedly vortexed, then diluted with an half of volume of PBS1X and eventually the entire suspension was layered over a 0.5 ml cushion of ice cold 1M sucrose, 50mM Tris, pH 7.4 and centrifuged at 3000g for 10 min to separate the heads from the tails. The pellet containing the sperm nuclei were resuspended in 2M NaCl, 25mM Tris, pH 7.4, with 2mM DTT and incubated in ice for 25 min for removing protamines. Then, the sperm nuclei were centrifuged at 9000g for 6 min and the pellet was resuspended in TE 1X buffer (10mM Tris, pH 7.5, 1mM EDTA), supplemented with DNase I (100.000 U/ml, Roche) and incubated for 2 h at 37°C. Thereafter, the sperm nuclei were centrifuged at 9000g for 6 min and the pellet was solubilized in sterile water for the following SDS-PAGE analysis. The supernatants resulting from each centrifuging were precipitated with 9 volume of cold absolute ethanol at -20°C.

For *in situ* isolation of nuclear matrix, sperm heads were extracted directly on polylysine coated slides with the method described above, and then fixed with 4% paraformaldehyde (PFA) in PBS for 10 min at 4°C for immunofluorescence analysis.

Immunofluorescence

Immunofluorescence experiments were performed on COS-1 and HeLa cells and epididymal spermatozoa cytocentrifuged on polylysine coated slides subjected to *in situ* nuclear matrix preparation as described previously. The cells were permeabilized for 30 min in PBS containing 5% BSA or donkey serum (Invitrogen) and 0.1% Triton X-100. After washing cells were stained for 1 h at RT with the following primary antibodies: mouse anti-Flag (1:50, SIGMA), rabbit anti-Flag (1:300, SIGMA), rabbit anti-GPx4 (1:20; kindly

provided by Prof. Panfili), rabbit anti-nGPx4 (1:10; PRIMM), rabbit anti-topoisomerase II α (1:250; S.Cruz), rabbit anti-topoisomerase II β (1:100; S.Cruz) or goat anti-lamin B (1:50; S.Cruz) diluted in PBS1X containing 1% BSA and 0,1% Triton X-100 (PBT). Cells were then incubated with the appropriate secondary antibodies: anti-rabbit Alexa fluor 488 (Molecular probes, 1:400), anti-rabbit Alexa fluor 555 (Molecular probes, 1:500), anti-goat Alexa fluor 555 (Molecular probes, 1:1000), anti-mouse Cy3 (Jackson Immunoresearch, 1:1000) diluted in PBT for 1 h at RT in the dark. After washing with PBS 1X cells were incubated with the nuclear staining TOPRO3 (Invitrogen, 1:1000) in PBS for 10 min at RT in the dark. Slides were mounted with Vectashield (Vector Laboratories) and examined under optical fluorescence Zeiss microscope or Leica confocal microscope.

SDS-PAGE and Western blotting

Protein concentration was determined by the bicinchoninic acid method (Pierce) with bovine serum albumin (Pierce) as standard.

Proteins were resolved on 12.5% SDS-polyacrylamide gel. Before separation proteins were denaturated by boiling for 5 min in the loading buffer (5% β -mercaptoethanol, 0.25M Tris pH 6.8, 2% SDS, 10% glycerol, 0.001% bromophenol blue). Separation was performed in a running buffer (25mM TrisHCl pH 11, 0.192M glycine, 0.1% SDS) at 30mA for about 2 hours. The proteins were then transferred onto a nitrocellulose membrane (HybondTM-C extra, Amersham Biosciences) for 2 h at 200mA in a transfer solution composed of 80% running buffer and 20% methanol. For immunoblotting, membranes were blocked with PBS containing 5% nonfat dry milk and 0.1% Tween-20 (Carnation, Nestlè) and probed for 1 h at room temperature with the following primary antibodies: mouse anti-Flag (1:250, SIGMA), rabbit anti-nGPx4 (1:100; PRIMM), goat anti-Lamin B (1:250; S.Cruz) and rabbit anti-Histone H3 (1:5000, SIGMA), diluted in 5% nonfat dry milk, 0.1% Tween-20. Membranes were washed three times for 10 min to remove any non specific binding and then incubated with the appropriate secondary antibodies diluted 5% nonfat dry milk, 0.1% Tween-20 such as anti-mouse (1:1000; DAKO), anti-rabbit HRP (1:2000, Zymed) and anti-goat HRP (1:5000, S.Cruz). After three washes, staining was revealed by enhanced

chemiluminescence system (ECL Plus™, Amersham Biosciences) according to manufacturer's instructions. In particular to detect nGPx4 in epididymal spermatozoa the biotin goat anti-rabbit IgG (1:6000, Zymed) with avidin-biotinylated HRP (Vectastain, VECTOR) and diaminobenzidine (DAB, Roche) colorimetric system was used according to the manufacturer's instructions.

***In vitro* sperm nuclear decondensation assay**

Spermatozoa were collected from adult WT, heterozygous and homozygous nGPx4 KO mice by squeezing separately caput and caudal epididymides in T6 medium (Quinn et al.,1982). Capacitated sperm were decondensed in the presence of 5mmol/L GSH and 10mmol/L heparin (Sigma) in T6 medium plus 1% Triton X-100 at 37°C for 15, 30, 50 and 80 min (Romanato et al.,2001). Controls consisted of parallel incubation with 1% Triton X-100 heparin or GSH alone. After each time period, an aliquot was removed, fixed with 4% PFA in PBS for 10 min at 4°C, cytocentrifuged on polylysine coated slides and analysed by phase-contrast microscopy after DNA staining with Hoechst 33342 (SIGMA). Spermatozoa were classified into three classes of decondensation level (type I-III) besides the normal one, according to the refringency, granular aspect and size of the nucleus. Experiments were run in triplicates and at least 200 cells were evaluated in each sample. Each class of sperm morphology for each time period was expressed as percentage of total cells.

Gamete collection and *in vitro* fertilization

Female CD1 mice (4-8 weeks-old) were superovulated with intraperitoneal injections of 5 IU PMSG, followed 48 h later with 2.5 IU HCG.

For *in vitro* fertilization females metaphase II oocytes were freed from zona pellucida by treatment with Tyrode solution. After washing in HTF medium oocytes were stained with Hoechst for 30 min at 37°C. Sperm were obtained from WT and nGPx4-KO mice of 6-20 weeks. The cauda epididymides were minced in 1ml of HTF medium and were left for 60 min at 37°C to allow swimming-out. Hereafter the sperm suspension of 2 caudae was capacitated and added to a final concentration of 1×10^5 cells /ml. Embryos were fixed with

2% PFA in M2 medium at 4°C after 1h of fertilization and finally whole mounted and analysed under Zeiss microscope to monitor the occurrence of sperm nuclear decondensation.

Preparation and analysis of basic nuclear proteins

We carried out extraction according to Zhao (Zhao et al.,2001). Approximately 35×10^6 mouse epididymal spermatozoa were resuspended in 300 μ l of water containing protease inhibitors and 10mM DTT. Cells were sonicated at 4°C for 5 min (40% amplitude, pulse 30 sec, pause 15 sec) and incubated at 37°C for 30 min. Basic proteins were then extracted in 0.5N hydrochloric acid (HCl) and were forced by a syringe through a 26-gauge needle for six times. After 20 min on ice acid soluble proteins were recovered by centrifugation at 12000g for 10 min at 4°C and precipitated for 2 hours by the addition of 100% trichloroacetic acid (TCA) to a final concentration of 20% TCA. Finally the precipitates were washed with acidified acetone, followed by acetone, dried and resolubilized in water. Protein concentration was determined by the bicinchoninic acid method with BSA as standard. 25 μ g of these proteins were loaded onto a 18% polyacrylamide gel (AU-PAGE) (Meistrich,1989) as a control of immunoprecipitation assay.

For protamine extraction 20×10^6 mouse epididymal spermatozoa were analysed as described by de Yebra and Oliva (1993) suitably modified. Spermatozoa were lysed with 300 μ l of 1mM PMSF in H₂O by hypotonic shock. The sediment was then resuspended in 100 μ l of TEP buffer (20mM EDTA, 1mM PMSF, 100mM Tris, pH 8.00). One volume of 6M guanidine hydrochloride (Sigma), 575mM DTT was added and mixed with a vortex and 1 vol of 552mM sodium iodoacetate (Sigma) and incubated at 37 °C for 30 min. Five volumes of ethanol at -20°C were added and the mixture was incubated at -20°C for 1 min and centrifuged for 15 min. The nucleoproteins of the sediment were then extracted with 500 μ l of 0.5N HCl at 37°C for 5 min and centrifuged 10 min. Proteins were precipitated with 20% TCA for 5 min at 4°C. The proteins were then collected by centrifugation for 10 min, washed twice with 500 μ l of 1% β -mercaptoethanol in acetone, dried and dissolved in 20 μ l of AU-BEM loading buffer[5.5M urea, 20% β -mercaptoethanol and 5% acetic acid

(w/v)]. 4 μ l of this solution were used as a positive control of protamine 1 on the 18% AU-PAGE.

COS-1 cells, transfected with pCMV-Flag-nGPx4 plasmid, were homogenized in RIPA buffer (150mM NaCl, 1% NP40, 0.5% NaDOC, 0.1% SDS, 50mM Tris-HCl pH 8.00) containing protease inhibitors and forced by a syringe through a 26-gauge needle for six times. Cells were sonicated at 4°C for 5 min (40% amplitude, pulse 30 sec, pause 15 sec) and centrifuged at 14000rpm for 40 min at 4°C. With the aim of obtaining proteins resuspended in a buffer compatible with the acid separation as a positive control of nGPx4 in AU-PAGE, 500 μ g of proteins were precipitated with nine volumes of 100% ethanol O/N at -20°C. The sample was centrifuged at 14000 rpm for 30 min and the pellet resuspended in 70% ethanol; after centrifugation for 15 min the pellet was dried and finally resolubized in 40 μ l of AU-BEM loading buffer and half of that loaded onto 18% AU-PAGE.

Protamine immunoprecipitation

Epididymal spermatozoa were extracted as described above and then 500 μ g of proteins were incubated overnight with 2 μ g of goat anti-Protamine 1 antibody (Santa Cruz) adding proteinase inhibitors 100X and PBS 1X until the final volume of 1.5 ml. 25 μ l of Protein G Sepharose 4 Fast Flow (Pharmacia Biotech) were added to the sample and incubation was continued for 3.5 h at 4°C. Finally the bead pellet was resuspended in 20 μ l of 2X sample buffer without bromophenol blue (125mM Tris HCl, pH 6.8, 4% SDS, 20% glycerol) to elute the antigen-antibody complex. The sample was boiled for 5 min, vortex briefly and centrifuged at 8200g for 2 min. The supernatant was precipitated with nine volumes of 100% ethanol O/N at -20°C. The sample was centrifuged, washed in 70% ethanol; the pellet was dried and finally resolubized in 20 μ l of AU-BEM loading buffer. Proteins were separated by electrophoresis in acid-urea 18% polyacrylamide gel (AU-PAGE) (Meistrich,1989) in which, both the molecular size and charge act as bases for protein separation. The separation was performed in running buffer (0.9N acetic acid,) at 150 Volt for 1.5 h. The proteins were then transferred onto a 0.2 μ m microporous polyvinylidene

fluoride (PVDF) membrane (Immobilon P^{SO}, Millipore) for 2 h at 400mA in a transfer solution composed of 0.7N acetic acid.

Immunoblotting was performed as described above. The primary antibodies rabbit anti-nGPx4 (1:100; PRIMM) or goat anti-protamine 1 (1:150, Santa Cruz), and the secondary antibodies anti-rabbit HRP TrueBlot (1:1000, eBioscience) or anti-goat HRP TrueBlot (1:1000, eBioscience) were used.

RESULTS

nGPx4 is associated with the nuclear matrix in haploid male germ cells

In order to analyse the subnuclear distribution of nGPx4, mouse male germ cells at different steps of differentiation were subjected to nuclear fractionation to prepare chromatin and nuclear matrix fractions. Western blotting analysis showed that nGPx4 is present in the nuclear matrix fraction of round spermatids (steps1-8) whereas was absent in pachytene spermatocytes (fig. 4A) in agreement with the observation that nGPx4 begins to be expressed by haploid male germ cells. The purity of chromatin and nuclear matrix fractions were confirmed by using specific markers as histone H3 and Lamin B, respectively. In epididymal spermatozoa a similar localisation was found, when sperm nuclei were analysed by western blotting (fig.4B), using the antibody specific for the nuclear isoform of GPx4 generated against three peptides located in the mouse nGPx4 aminoterminal sequence.

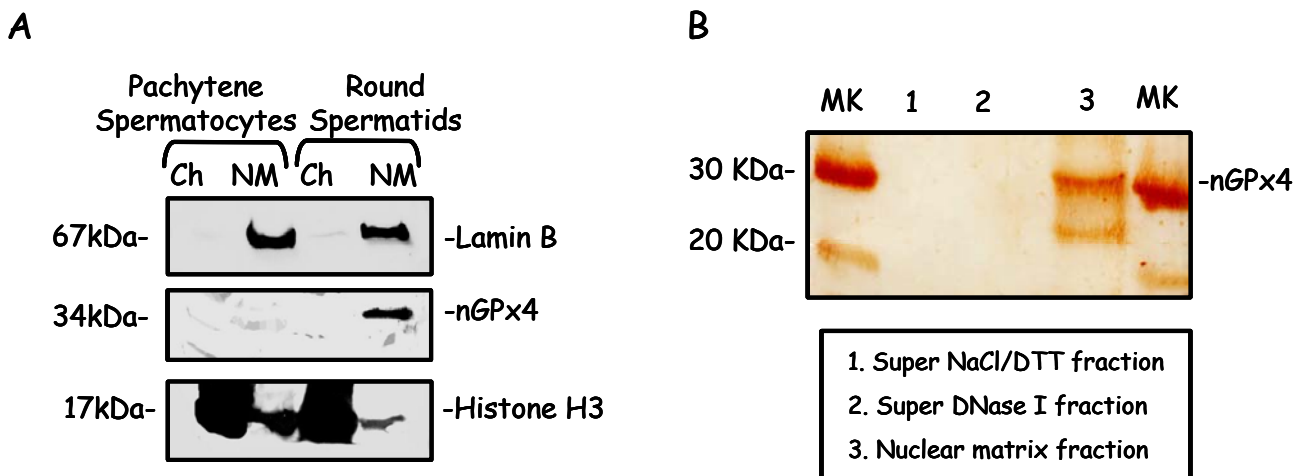


Figure 4. nGPx4 expression in the nuclear matrix of haploid mouse male germ cells. A, western blotting analysis for round spermatids subjected to subnuclear fractionation protocol (Reyes et al.,1997). nGPx4 is present in the nuclear matrix (NM) fraction. To test the purity of each nuclear fraction anti-Lamin B and anti-Histone H3 were used as nuclear matrix and chromatin markers, respectively. Pachytene spermatocytes nuclear fractions were used as negative control, since nGPx4 begins to be expressed in haploid germ cells. B, western blotting analysis for epididymal spermatozoa subjected to subnuclear fractionation in solution following the method described in Shaman et al.,2007.

Biochemical data were confirmed by immunofluorescence analysis. In untreated round spermatids nuclei nGPx4 was undetectable whereas after *in situ* nuclear matrix preparation, when all DNA and protein associated with it were removed, nGPx4 became detectable in the nuclear matrix, in association with lamin B (fig.5).

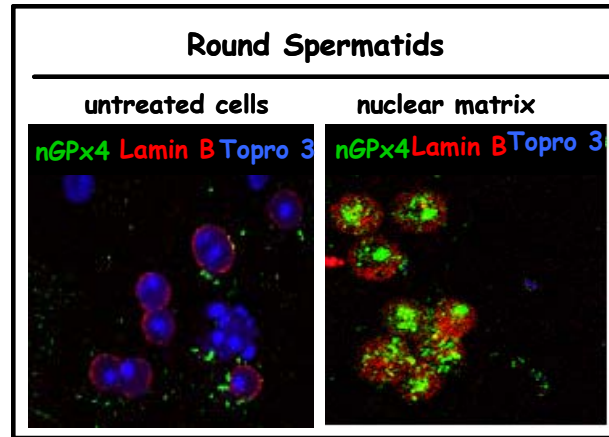


Figure 5. Subnuclear localisation of nGPx4 in round spermatids. Confocal microscopy analysis of round spermatids subjected to *in situ* nuclear matrix preparation. The evidence of successful treatment is TOPRO3 staining showing the absence of DNA and Lamin B signal that recognizes specifically the nuclear matrix. After the treatment the marking with Lamin B increases and nGPx4 signal becomes evident.

Immunofluorescence and confocal microscopy analysis of nGPx4 localisation was performed in epididymal spermatozoa. After removing all cytoplasmic elements with detergents, nuclear morphology was unchanged (fig.6A) and immunostaining with antibody specific for nuclear isoform revealed nGPx4 at a marginal region of sperm heads (fig.6B). No signal was detected with antibody anti-topoisomerase II β (fig.6D) whereas DNA was stained by TOPRO3 staining (fig.6E,F,G). When sperm were stained with the antibody that recognises the aminoacid sequence common to all three GPx4 isoforms, the signal appeared also on the tails (fig.6C). When sperm nuclei were sequentially extracted with high salt and DTT, they appeared swollen (fig.7A) and nGPx4 signal was markedly decreased compared to previous treatment (fig.7B,C), due to chromatin components expanded, and again no signal was evident with anti-topoisomerase II β antibody (fig.7D). When all the DNA was removed, as shown by the absence of TOPRO3 staining (fig.8E,F,G), and only a purified sperm nuclear matrix remained attached to the slide,

sperm nuclei appeared emptied (fig.8A), and showed a clear signal of nGPx4 in the nuclear matrix (fig.8B,C), where also topoisomerase II β was localised (fig.8D).

Overall these results indicate that nGPx4 is a stable member of nuclear matrix proteins throughout the complex series of changes in nuclear protein composition and aggregation that occur during spermiogenesis and sperm passage along epididymis.

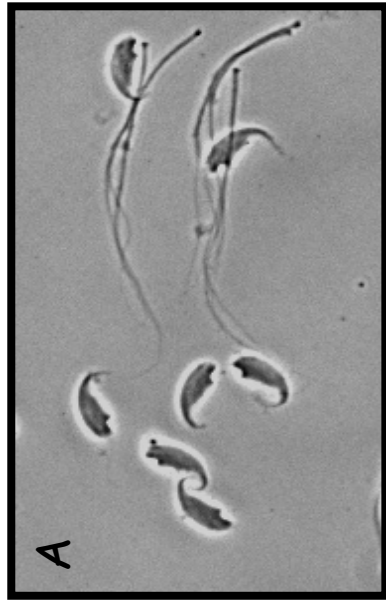
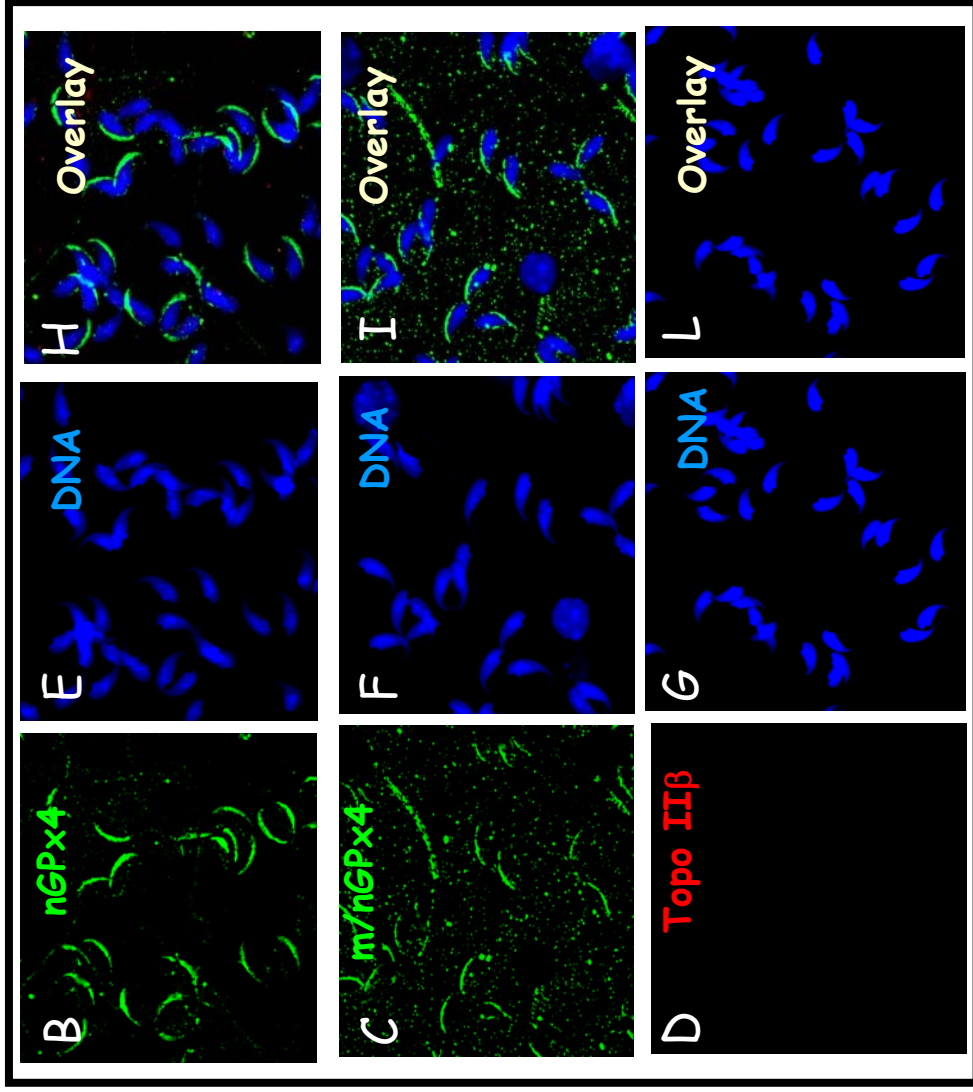


Figure 6. Immunolocalisation of nGPx4 in epididymal spermatozoa ATAB/DTT-treated. Confocal microscopy analysis of mouse epididymal spermatozoa treated with ATAB/DTT and stained with antibody anti-nGPx4 (B) or anti-GPx4 (C), in combination with anti-topoisomerase II β antibody (D) and TOPRO3 (E,F,G). nGPx4 signal is restricted to a marginal region of sperm heads, probably because the nuclei are too compact to allow antibody accessibility. Anti-GPx4 antibody, recognizing both mitochondrial and nuclear isoforms of GPx4, stains also the tails of spermatozoa. No signal was detected with antibody anti-topoisomerase II β (D).

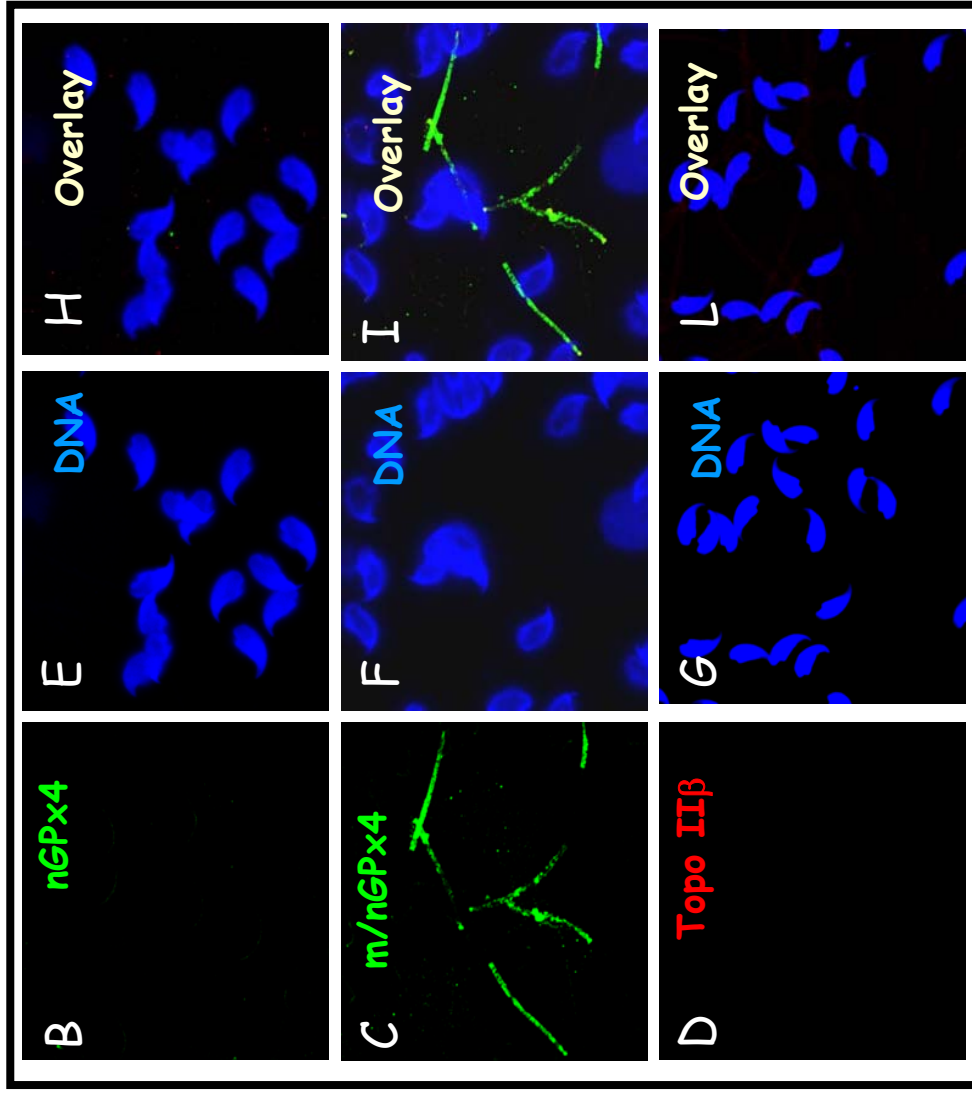


Figure 7. Immunolocalisation of nGPx4 in epididymal spermatozoa NaCl-treated. Confocal microscopy analysis of mouse epididymal spermatozoa treated with NaCl 2M/DTT after ATAB/DTT extraction and stained with antibody anti-nGPx4 or anti-GPx4, always in combination with antibody anti-topoisomerase II β . Sperm nuclei observed under phase contrast are swollen (A) due to the expansion of DNA. For the same reason TOPRO3 signal is increased (E,F,G) whereas nGPx4 signal is decreased compared to previous treatment (B,C). No signal was detected with antibody anti-topoisomerase II β (D).

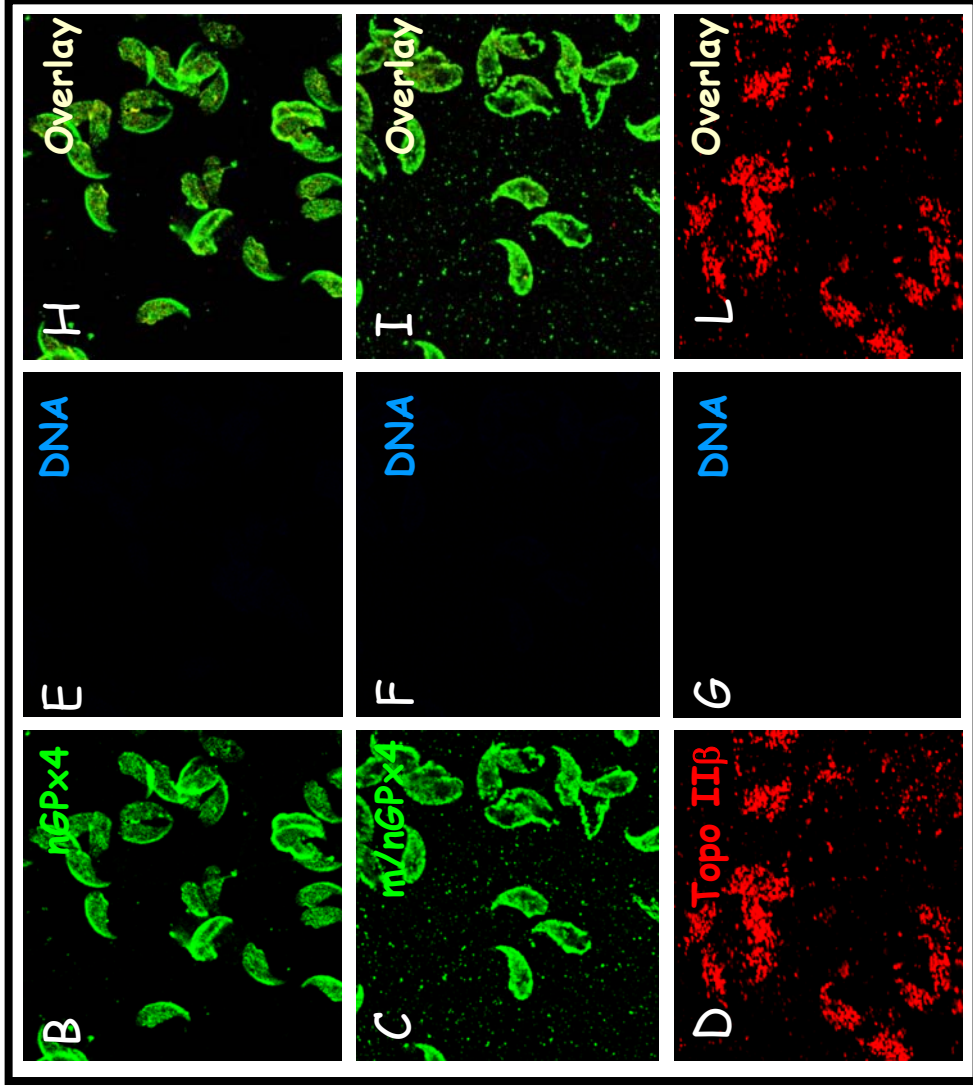


Figure 8. Immunolocalisation of nGPx4 in epididymal spermatozoa DNase I-treated. Confocal microscopy analysis of mouse epididymal spermatozoa treated with DNase I, after the ATAB/DTT and NaCl/DTT steps, and stained with anti-nGPx4 or anti-GPx4 antibodies, in combination with anti-topoisomerase II β antibody. The evidence of successful treatment is TOPRO3 staining showing the absence of DNA (E,F,G). Under phase contrast sperm nuclei are swollen and almost completely emptied (A); anti-nGPx4 (B), anti-GPx4 (C) and anti-topoisomerase II β antibodies (D) showed an higher signal in the nuclear matrix of spermatozoa heads.

nGPx4 is associated with the nuclear matrix in transfected COS-1 cells

The finding that nGPx4 was localised in the nuclear matrix of round spermatids and epididymal spermatozoa led us to verify if this localisation was also observed in somatic cells. Thus the nuclear subfractionation protocol was performed on COS-1 cells transfected with the construct Flag-nGPx4. Biochemical and morphological analysis showed that nGPx4 is present only in the nuclear matrix fraction (fig.9A,B). In conclusion, both overexpression studies *in vitro* and *in vivo* data on germ cells demonstrate that nGpx4 is localised in the nuclear matrix, suggesting novel potential functions of this selenoprotein.

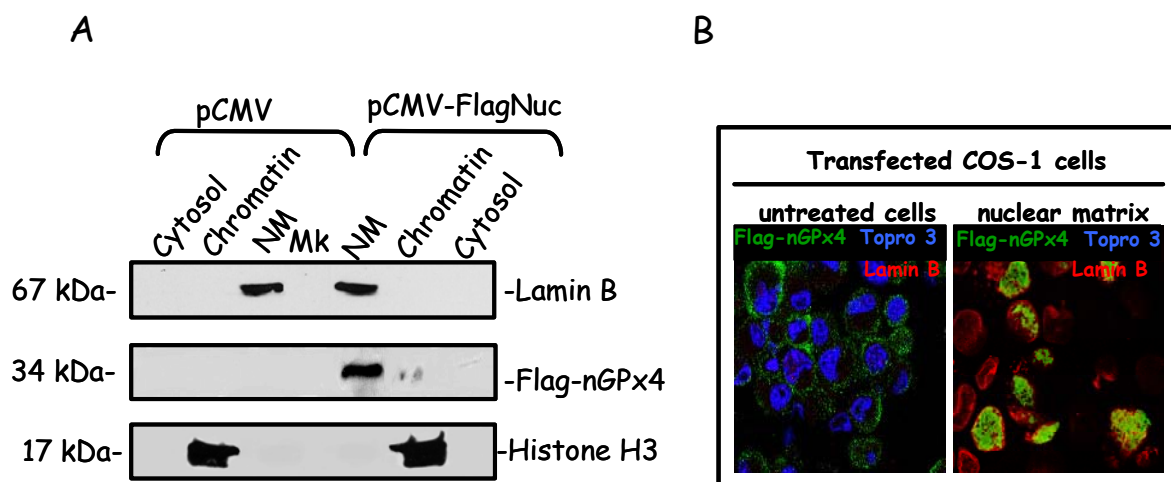


Figure 9. Expression and subnuclear localisation of nGPx4 in transfected COS-1 cells. *A*, western blotting analysis of COS-1 transfected with pCMV-Flag-nGPx4 plasmid subjected to subnuclear fractionation protocol. nGPx4 is present in the nuclear matrix (NM) fraction. To test the purity of each nuclear fraction anti-Lamin B and anti-Histone H3 antibodies were used to detect nuclear matrix and chromatin markers, respectively. *B*, confocal microscopy analysis of COS-1 cells transfected with pCMV-Flag-nGPx4 plasmid subjected to *in situ* nuclear matrix preparation. The evidence of successful treatment is TOPRO3 staining showing the absence of DNA and Lamin B signal that recognizes specifically the nuclear matrix. After the treatment Lamin B and nGPx4 signals colocalised in the nuclear matrix.

nGPx4 is required for DNA condensation in sperm from caput and cauda epididymis

To test the role of nGPx4 in the stability of sperm chromatin, spermatozoa were isolated from the caput and the cauda epididymides of WT, heterozygous and homozygous nGPx4-KO mice and subjected to an *in vitro* chromatin decondensation assay in the presence of glutathione and heparin. Three classes of decondensing sperm nuclear morphology were identified (fig.10A): type I for low decondensation, type II for medium decondensation and type III for high decondensation. In spermatozoa isolated from the

caput epididymis already after 30 min of treatment about 55% of nGPx4-KO sperm had morphology type III, whereas only 15% of heterozygous and none of WT sperm showed this type of morphology (fig.10B). In spermatozoa collected from the cauda epididymis the decondensation kinetics was delayed compared to caput spermatozoa. In fact after 50 min of incubation with decondensing reagents, almost 60% of nGPx4-KO sperm had morphology III, whereas about 70% of heterozygous and only 50% of WT sperm showed an initial decondensation (fig.10C). These data indicate that sperm from both caput and cauda epididymis of nGPx4-KO mice decondensed earlier than those of WT, demonstrating that nGPx4 plays an important role in maintaining sperm chromatin stability.

No nuclear decondensation was observed when sperm were incubated with heparin or GSH alone (data not shown).

The novel distribution of nGPx4 in the sperm nuclear matrix prompted us to investigate whether the sperm nuclear structure instability caused by the lack of nGPx4 might impact on the early events occurring after fertilization. To assess the timing of sperm nuclear decondensation in WT and nGPx4-KO mice we performed IVF experiments with zona pellucida free oocytes and after 1 h of fertilization we obtained a similar results to those obtained in *in vitro* decondensation assays. Fig. 11 shows that nGPx4-KO spermatozoa decondensation was markedly anticipated compared to that of WT. Spermatozoa morphology of nGPx4-KO mice showed in fact 18% of type III and 28.9% of type II respect to 10.5% and 15.6% of WT spermatozoa. Differences were considered statistically significant.

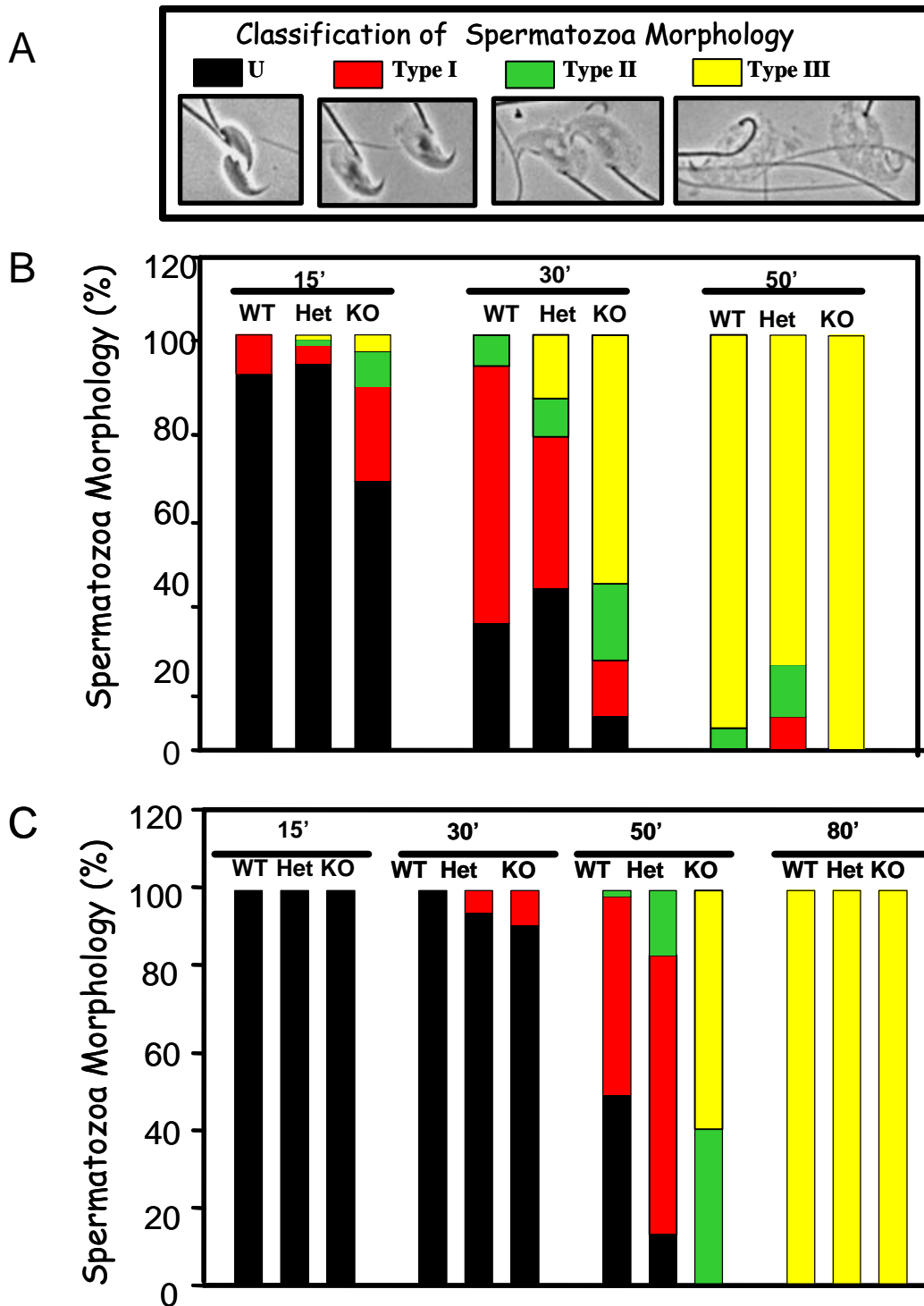


Figure 10. *In vitro* chromatin decondensation assay of caput and cauda epididymides spermatozoa. *A*, nuclear decondensation status of mouse spermatozoa. U = unchanged, type I = low decondensation, type II = medium decondensation, type III = high decondensation. *B* and *C*, decondensation kinetics of capacitated caput (*B*) or cauda (*C*) epididymis spermatozoa of wild type (WT), heterozygous (Het) and homozygous nGPx4 knock out (KO) mice incubated in the presence of GSH and heparin for 15,30,50 and 80 min.

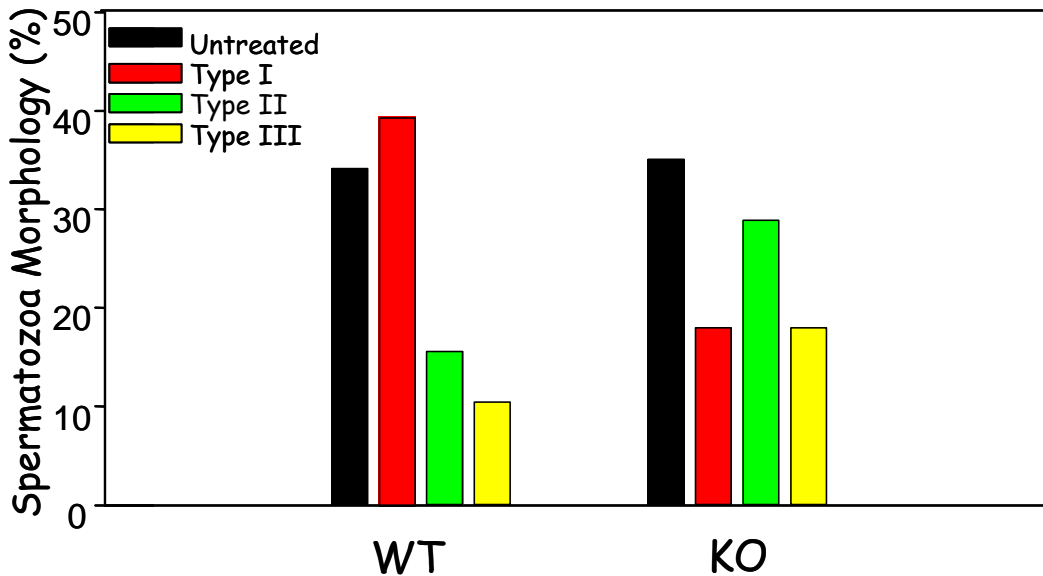


Figure 11. Decondensation of epididymis spermatozoa of wild type (WT) and nGPx4 knock out (KO) mice 1 h after insemination of zona pellucida free oocytes. Sperm nuclear morphology was classified in four classes: U = unchanged, type I = low decondensation, type II = medium decondensation, type III = high decondensation. $P < 0.0001$

nGPx4 co-immunoprecipitates with protamine 1

As the nuclear matrix may play a key role in sperm genome organization and gene potentiation, our intention was to evaluate the possible interaction of nGPx4 with protamines. Protamine 1 immunoprecipitation experiments from mouse epididymal spermatozoa revealed that nGPx4 co-immunoprecipitates with protamine 1 (fig.12), demonstrating the *in vivo* interaction between nGPx4 and protamine 1 and suggesting that nGPx4 can be a key protein in the opening of chromatin regions in the sperm genome to permit transcription.

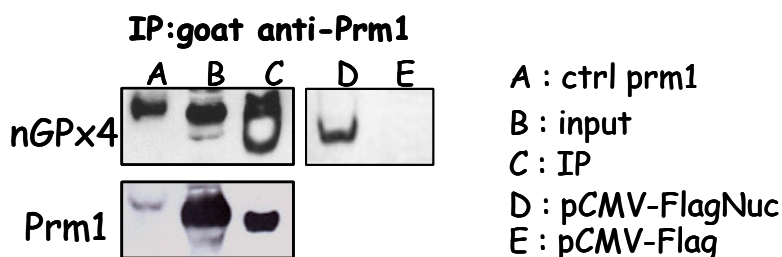


Figure 12. Immunoprecipitation of Protamine1 from mouse epididymal spermatozoa. The goat anti-Prm1 antibody co-immunoprecipitates nGPx4 with protamine 1. The control of protamine1 extraction (A), spermatozoa total lysate (B), anti-Prm1 immunoprecipitate (C) were loaded in duplicate, separated by AU-PAGE and then transferred to PVDF membrane. The blot was probed with rabbit anti-nGPx4 and goat anti-Prm1 antibodies after separating the membrane in the middle. COS-1 total lysates (D,E) were used as control of nGPx4 protein.

DISCUSSION

The interest on the role of the nGPx4 in the male gonad is initially founded on the observation that this selenoprotein is exclusively expressed in the germinal cells during the latest phases of their maturation. This protein has a nuclear location, as shown by both biochemical and morphological analyses, and characteristics very similar to those of protamines. The demonstration of its ability to oxidise *in vitro* the thiol groups of protamines (Pfeifer et al.,2001) has given strength to the hypothesis of its possible role in chromatin condensation during sperm maturation. The analysis of the mouse model in which the *nGPx4* gene has been deleted (Conrad et al.,2005) did not conclusively clarify the role played by the selenoprotein in the mechanism of chromatin condensation. In fact, caput epididymis but not cauda epididymis spermatozoa from nGPx4 mice were shown to be defective in chromatin condensation, making these mutant fertile. Our group has previously demonstrated that the nGPx4 expression is restricted to the haploid phase of spermatogenesis being detected at protein level from round spermatids up to spermatozoa (Puglisi et al.,2003) and its expression is maintained during the maturation up to the epididymal sperm.

With the aim to get further information that allows to clarify the role of the nGPx4 in sperm chromatin remodeling and to reveal yet unknown functions, we have decided to investigate the subnuclear distribution of nGPx4 in round spermatids and epididymal spermatozoa using specific protocols of nuclear fractionation. Our results indicate that nGPx4 is present in the nuclear matrix fraction in both cell types and excluded from the chromatin fraction. Interestingly, we obtained similar results in *in vitro* experiments using somatic cells overexpressing the exogenous protein.

All together, these findings consistently provide new insights into the subnuclear location of nGPx4.

The particular localisation of nGPx4 in the nuclear matrix opens new scenarios on the possible roles of this protein. It is well known that the nuclear matrix is an important subnuclear compartment that regulates the organization and the function of the chromatin

in somatic and germinal cells (He et al.,1990; Bellvè et al.,1992). Particularly, in the sperm nucleus the nuclear matrix organizes the chromatin into functional loops of DNA that help regulate DNA replication and gene transcription (Ward,2010). Recently it has been demonstrated that an intact nuclear matrix is necessary for the progression of the initial events of mouse embryonic development. In fact, such structure is important for sperm nuclear decondensation and subsequent pronucleus formation and for the genome replication (Ward et al.,2007).

In this context it is known that the structural stability of the sperm nucleus depends on the presence of disulfide bridges among nuclear proteins, mostly protamines (Perreault et al.,1987), and that can also be formed by nGPx4, thanks to its ability to oxidise the thiol groups of protamines. Furthermore it was demonstrated that nGPx4, in the absence of glutathione, it is able to form polymers reacting with itself (Mauri et al.,2003) strengthening the concept that this selenoprotein can work as a structural factor. On the basis of these considerations we have hypothesized that nGPx4 localised in the sperm nuclear matrix could be involved in sperm chromatin decondensation after the fertilization. This hypothesis has been tested isolating spermatozoa from the caput and the cauda epididymides of WT, heterozygous and homozygous nGPx4-KO mice and subjected them to *in vitro* chromatin decondensation assay in the presence of glutathione and heparin. We observed that nGPx4-KO sperm nuclei isolated from the caput epididymis were more decondensed than those of WT after 30 min of incubation, confirming Conrad's data (2005). Most surprisingly, we found that also sperm collected from the cauda epididymis of KO mice showed a precocious decondensed morphology respect to WT sperm during incubation with decondensing reagents, although with a delayed time course respect to sperm from the caput epididymis. It is of great interest that we found a similar acceleration of chromatin dispersion when cauda epididymal sperm from nGPx4-KO mice were used to *in vitro* fertilize zona pellucida free oocytes. Thus these findings obtained with two independent experimental approaches clearly indicate that the lack of nGPx4 in the sperm nucleus causes structural chromatin instability making it more susceptible to decondensation. The fact that sperm chromatin decondensation is

anticipated in KO mice could be a consequence of the lower content of disulfide bridges due to the lack of nGPx4.

In line with this, chromatin condensation was found to be altered in sperm from selenium-deficient rats (Watanabe et al.,1991, Pfeifer et al.,2001) and in sperm nGPx4-KO mice (Conrad et al.,2005) and a higher level of free sulphhydryl groups was observed in KO cauda spermatozoa respect to WT (Conrad et al.,2005).

Overall these data favour the idea that nGPx4 might be able to cross-link cysteine residues of protamines as reductants. To this regard, in species such as trout and chicken, that do not contain cysteines in their protamines, nGPx4 and shorter related forms are missing (Bertelsmann et al.,2007).

Another aim of this study was to investigate the possible association of nGPx4 with protamines. The finding that the selenoprotein co-immunoprecipitated with protamine 1 after extraction from mouse epididymal sperm demonstrates the *in vivo* interaction between nGPx4 and protamine1. Although at the first view contradictory, this result is compatible with the fact that the nuclear matrix binds chromatin at sequence-specific regions of attachment, termed matrix attachment regions (MARs). It is of special interest the observation that the human protamine domain is bounded by two sperm specific MARs and maintained it in an open conformation to allow transcription (Schmid et al.,2001; Martins et al.,2004). In line with this, it has been reported that protamine 2 targets to nuclear matrix in elongating spermatids (Wu et Means,2000; Wu et al.,2000). Based on these considerations and our data , we propose that nGPx4 as part of the nuclear matrix may play the role of connecting element to components of chromatin during paternal genome repackaging.

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