THE ROLE OF SPERM DNA DAMAGE IN THE ORIGIN OF INFERTILITY ASSOCIATED WITH Y CHROMOSOME LONG ARM DELETIONS IN THE MOUSE MODEL

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAIʻI AT MĀNOA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN DEVELOPMENTAL AND REPRODUCTIVE BIOLOGY

DECEMBER 2013

By

Jonathan M. Riel

Dissertation Committee:

Monika A. Ward, Chairperson
W. Steven Ward
Yusuke Marikawa
Stefan Moisyadi
Rich Allsopp
Thomas Huang
Olivier Le Saux, University Representative

ACKNOWLEDGMENTS

ABSTRACT

In mouse and man Y chromosome deletions are frequently associated with spermatogenic defects. Mice with severe non-pairing Y chromosome long arm (NPYq) deficiencies are infertile *in vivo* and *in vitro*. We have previously shown that sperm from these males, although having grossly malformed heads, were able to fertilize oocytes via intracytoplasmic sperm injections (ICSI) and yield live offspring. However, in continuing ICSI trials we noted a reduced efficiency when cryopreserved sperm were used and with epididymal sperm as compared to testicular sperm. Our initial study tested if NPYq deficiency is associated with sperm DNA damage - a known cause of poor ICSI results. We observed that epididymal sperm from mice with severe NPYq deficiency are impaired in oocyte activation ability, and have an increased incidence of oocyte arrest and paternal chromosome breaks. Comet assays revealed increased DNA damage in both epididymal and testicular sperm, and transmission electron microscopy showed sperm having impaired membrane integrity and abnormal chromatin condensation. We therefore concluded that the increased DNA damage associated with NPYq deficiency might be a consequence of disturbed chromatin remodeling taking place during spermiogenesis.

There are four distinct multi-copy genes found in the long arm of the Y chromosome. One of them, *Sly,* is known to control the expression of sex chromosome genes after meiosis; *Sly* deficiency results in a remarkable upregulation of sex chromosome genes. *Sly* deficiency has also been shown to be the underlying cause of sperm head anomalies and infertility associated with NPYq gene loss. We therefore hypothesized that *Sly* is our target gene. To test this, we examined mice with transgenically (RNAi) silenced *Sly*.

Our analysis of *Sly*-deficient mice demonstrated similar 'sperm DNA damage' phenotype. This confirmed that lack of *Sly* is responsible for the sperm DNA damage/chromatin packaging defects observed in mice with NPYq deletions.

This project provides the first evidence of DNA damage in sperm from mice with NPYq deficiencies and that the multi-copy NPYq-encoded *Sly* gene plays a key role in processes regulating chromatin remodeling and thus maintaining DNA integrity in sperm.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Male factor infertility

Couples that are unable to become pregnant after a year of frequent and unprotected intercourses are considered infertile. Infertility affects up to 15% of all couples worldwide [Rutstein,2004, Spira,1986], and roughly half of these couples experience these difficulties because of the low quality of sperm being produced by the male [Brugh and Lipshultz,2004, Hirsh,2003]

There are several causes for male infertility including environmental, psychological, and genetic factors. Overexposure to certain metals (lead, cadmium, chromium, or copper), pesticides (dibromo-3-chloropropane DBCP, ethylene di-bromade, or carbaryl), solvents (ethylene glycol, hydrocarbon phthalate esters) or estrogens may cause a decrease in sperm quality, count, and function [Sheiner, Sheiner, et al.,2003]. Stress also plays an important role due to its effect on the hypothalamus-pituitary-testicular axis and its role in regulating spermatogenesis [Roberts and McGrady,1996]. There is a list of genetic abnormalities found in men with male infertility that range from gonadotropin releasing hormone deficiency to spermatogenic failure (Mak and Jarvi 1996). The cause of infertility in 10-20% of men with azoospermia (inability to produce any measurable number of sperm) has been traced to deletions in the Y chromosome. Defects in the Y chromosome are considered the most common genetic cause of male infertility [Foresta, Moro, et al.,2001, Krausz, Quintana-Murci, et al.,2000].

1.2 Y chromosome in humans

The Y chromosome in humans spans about 58 million base pairs, and represents approximately 1% of the total DNA. There is a male specific non-recombining region of the human Y chromosome (NRY), which takes up approximately 95% of the chromosome [Tilford, Kuroda-Kawaguchi, et al.,2001]. It can further be broken down into two sections a short arm (Yp) containing 8 Mb, and 14.5 Mb on the long arm (Yq) [Skaletsky, Kuroda-Kawaguchi, et al.,2003]. There are three regions mapped to the Yq, that are defined as azoospermia factors (AZFa, AZFb, and AZFc). These regions are important for male infertility, as they represent areas in the Y chromosome that encode genes playing important roles during spermatogenesis. Deletions of these genes are associated with azoospermia or severe oligozoospermia (low sperm count) [Raicu, Popa, et al.,2003].

Deletions within the male-specific region of the Y chromosome are a common genetic cause of spermatogenic failure in men, making men carrying an affected Y chromosome a target group for assisted reproduction technologies. Many children have already been born after ICSI with sperm from these men. When infertility is caused by a defect in the genes located on the Y chromosome, all male children of an affected father are affected as well, and on reaching sexual maturity, they will require the same assistance that their father needed to achieve conception, leading to a phenomenon called hereditary infertility [Chang, Sauer, et al.,1999, Mau Kai, Juul, et al.,2008, Page, Silber, et al.,1999]. It is therefore of great importance to understand the roles of Y chromosome genes in the etiology of different aspects of male infertility.

The deletions of the Y chromosome are frequently found in both humans and mice. The research aiming to elucidate the functions of the Y chromosome encoded

genes in a mouse model, may ultimately help to understand cases of infertility in men that are linked to Y chromosome deletions.

1.3 Y chromosome in mice

The Y chromosome in mouse is estimated to contain approximately 78 Mb of DNA [Gregory, Sekhon, et al.,2002] and consist of two distinct sections similarly to the Y chromosome in human: the pseudoautosomal region (PAR), and the male specific non-PAR region (NPY). The PAR region is the only part of the Y chromosome that shares homology with the X chromosome, and pairs with it during recombination. The male specific region consists of a short arm (NPYp) and the long arm (NPYq) [Figure 1].

On the NPYp there are seven single-copy genes (*Ube1y1*, *Kdm5d*, *Eif2s3y*, *Uty*, *Ddx3y*, *Usp9y*, and *Sry*), two duplicated genes (*Zfy1 and Zfy2*, and *H2al2y*), and a multicopy gene *Rbmy* [Agulnik, Mitchell, et al.,1994, Bishop and Hatat,1987, Ehrmann, Ellis, et al.,1998, Greenfield, Scott, et al.,1996, Gubbay, Collignon, et al.,1990, Kay, Ashworth, et al.,1991, Mahadevaiah, Odorisio, et al.,1998, Mardon and Page,1989, Mitchell, Woods, et al.,1991] [Figure 1].

The *Sry* gene is also known as the sex-determining gene, and it encodes for the transcription factor that initiates male sex determination [Wallis, Waters, et al.,2008]. Eukaryotic translation initation factor 2, subunit 3, structural gene Y-linked (*Eif2s3y*), is required for spermatogonial proliferation [Mazeyrat, Saut, et al.,2001]. Ubiquitin-activating enzyme E1, Chromosome Y (*Ube1y1*) is known to participate in histone ubiquitination and the exchange of transition proteins and protamines during spermiogenesis [Levy, Navarro, et al.,2000, Odorisio, Mahadevaiah, et al.,1996]. Lysine (K)-specific demethylase 5D (*Kdm5d*) works to demethylate histones and is necessary for

chromatin condensation [Nottke, Colaiacovo, et al.,2009] Ubiquitously transcribed tetratricopeptide repeat gene, Y linked (*Uty*) encodes for peptide repeats that mediate protein-protein interactions [Greenfield, Carrel, et al.,1998]. DEAD (Asp-Glu-Ala-Asp) box polypeptide 3 Y-linked (*Ddx3y*) encodes for proteins that facilitate the DNA packaging in the sperm head [Vong, Li, et al.,2006]. Ubiquitin specific peptidase 9, Y linked (*Usp9y*) is involved in cleaving ubiquitin from a variety of proteins [Hall, Brown, et al.,2003]. Zinc finger protein 1 and 2, Y linked (*Zfy*) are predicted to be transcription factors and participate in the activating the apoptosis pathway for sperm [Vernet, Mahadevaiah, et al.,2011]. *H2al2y* is a novel histone in the H2A superfamily and it is believed that this gene functions in spermatid differentiation [Ferguson, Ellis, et al.,2009]. RNA binding motif, Y linked (*Rbmy*) is the only multi-copy gene found in the short arm, and functions in germline RNA metabolism [Elliott, Ma, et al.,1996, Zeng, Sun, et al.,2008] [Figure 1].

The remaining 90% of the male specific Y, the long arm (NPYq), is made up of repetitive sequences, and is known to encode at least four distinct multi-copy genes: *Ssty*, *Sly, Asty,* and *Orly* [Agulnik, Mitchell, et al.,1994, Conway, Mahadevaiah, et al.,1994, Ellis, Ferguson, et al.,2007, Prado, Lee, et al.,1992, Toure, Clemente, et al.,2005, Toure, Szot, et al.,2004] [Figure 1].

Ssty (spermatid-specific transcripts Y-encoded) gene has more than 100 copies and is specifically transcribed in the testis in round spermatids [Toure, Clemente, et al.,2005]. It has two distinct subfamilies, *Ssty1* and *Ssty2*. *Ssty2* appears to be the most abundant transcript in spermatids but no protein has been identified. *Ssty1* is much less abundant but does produce a protein that has substantial homology to the autosomally
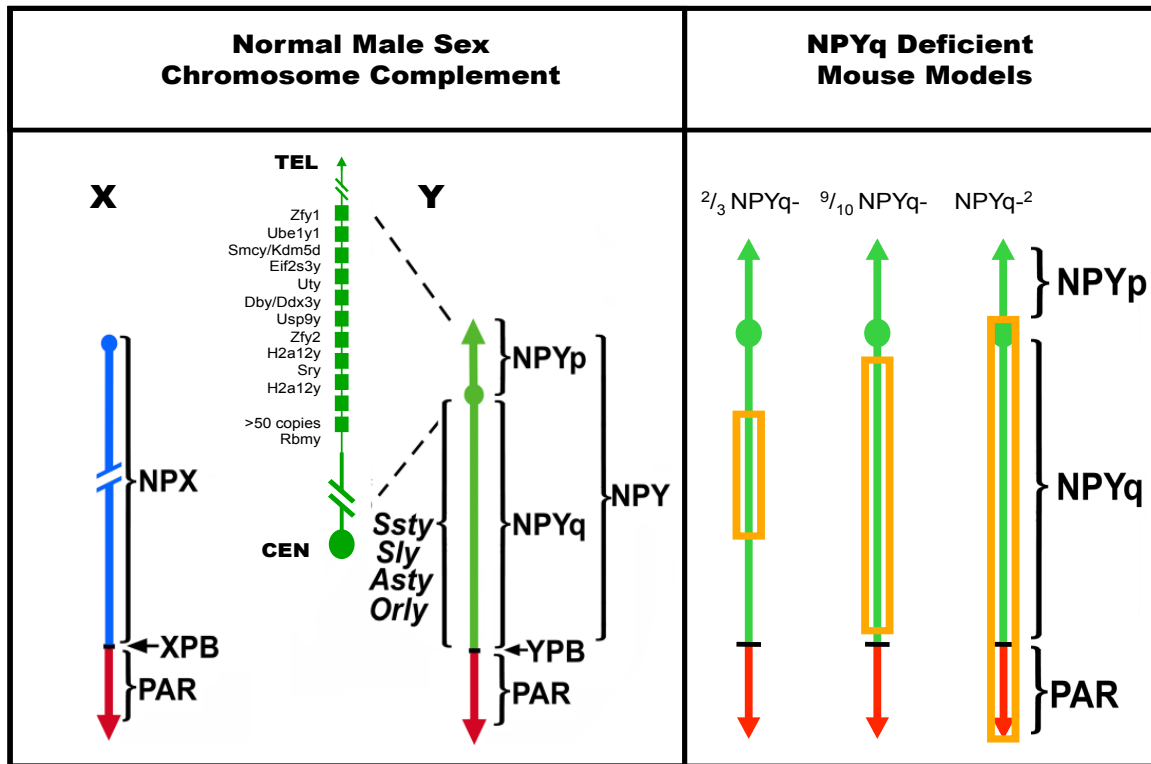
Figure 1. The sex chromosome gene complement of a male mouse and a simplified depiction of the Y chromosomes in NPYq deficient mouse models. The male specific region on the Y chromosome of mice (NPY) can be divided into two parts: the short arm (NPYp) and the long arm (NPYq). Three mouse models with varying deficiencies of the NPYq (2/3NPYq-, 9/10NPYq- and NPYq-2) region are the subject of investigations in this project.

encoded protein SPIN. In mice, SPIN is found in oocytes and early embryos where it localizes to the metaphase spindle [Staub, Mennerich, et al.,2002].

Sly (Sycp3-like Y linked) is testis specific and, like *Ssty1*, is a very abundant transcript in spermatids [Toure, Clemente, et al.,2005]. At least 70 *Sly* copies that have maintained a functional open reading frame are predicted based on the Y chromosome sequence data. *Sly* is a member of the *Xlr* superfamily, and its most closely related member is the multicopy gene *Slx* (formerly called *Xmr*). Both *Sly* and *Slx* encode abundantly expressed spermatid-specific proteins. SLX is cytoplasmic while SLY is found in both cytoplasm and in nuclei; in the spermatid nuclei SLY colocalizes with the sex chromosomes [Cocquet, Ellis, et al.,2012, Reynard, Cocquet, et al.,2009, Reynard, Turner, et al.,2007]. The SLY and SLX are suspected to act antagonistically during spermiogenesis [Cocquet, Ellis, et al.,2012, Ellis, Clemente, et al.,2005].

Asty (Amplified spermatogenic transcripts Y-encoded) is related to the multicopy X-encoded gene *Astx*, with which it shares 92-94% homology. Current evidence suggests that *Astx* is translated but that *Asty* is not. This, however, does not rule out a functional role for *Asty*, especially given the increasing literature on functional noncoding RNAs [Ellis, Ferguson, et al.,2007].

Orly (oppositely transcribed, reassorted locus on the Y) is a chimeric locus on the NPYq that is composed of partial copies of *Ssty1, Sly,* and *Asty* arranged in a sequence. *Orly* is bidirectionally transcribed, giving rise to *Orly* (forward) and *Orlyos* (reverse) transcripts. These transcripts may potentially form double-stranded RNA in partnership with each other or with the progenitor loci *Ssty1*, *Sly*, and *Asty* [Ellis, Ferguson, et al.,2007].

1.4 NPYq deficiency

There are three mouse models with deficiencies in the NPYq that have been studied to date. Male mice carrying a deletion removing approximately two-third of the NPYq (subsequently called 2/3NPYq-) exhibit an increased incidence of sperm headshape defects (mild teratozoospermia), impairment in sperm function, and progeny sex-ratio bias favoring females. As the length of the deletion increases, mice with deletions removing approximately nine-tenth of NPYq (subsequently called 9/10NPY-) or mice with the complete loss of NPYq (subsequently called NPYq-2) become infertile. Their sperm have more severe headshape defects and fail to fertilize eggs in vitro [Conway, Mahadevaiah, et al.,1994, Styrna, Bilinska, et al.,2002, Styrna, Imai, et al.,1991, Styrna, Kilarski, et al.,2003, Styrna, Klag, et al.,1991, Styrna and Krzanowska,1995, Suh, Styrna, et al.,1989, Ward and Burgoyne,2006, Xian, Azuma, et al.,1992].

1.5 Project goals and hypotheses

Our **goal** was to investigate the molecular and genetic origin of infertility in mice with NPYq deficiencies.

In the initial work, we have shown that the infertility of mice with severe NPYq deficiencies can be overcome with intracytoplasmic sperm injection (ICSI) and live offspring from mice lacking the entire NPYq region could be produced [Yamauchi, Riel, et al.,2009]. However, when using preserved epididymal sperm from these males the efficiency of ICSI was poor. We also observed that testicular sperm were more often successful in assisted fertilization than epididymal sperm. There is an evidence in

literature suggesting that impairment of epididymal, but not testicular sperm, coupled with infertility and teratozoospermia may be indicative of DNA damage in sperm and defects in chromatin remodeling [Aoki, Moskovtsev, et al.,2005, Suganuma, Yanagimachi, et al.,2005, Torregrosa, Dominguez-Fandos, et al.,2006].

I therefore **hypothesized** that mice with severe NPYq deletions exhibit sperm DNA damage and abnormal chromatin packaging. In **Chapter 2** of this dissertation I present the results obtained while testing this hypothesis. These data are published:

> Yamauchi, Y.[*], **Riel, J.M**.[*], Stoycheva, Z., Burgoyne, P.S., Ward, M.A. (2010). "Deficiency in mouse Y chromosome long arm gene complement is associated with sperm DNA damage." Genome Biol **11**(6): R66. (* these authors contributed equally)

More than 200 genes are expressed differently in testes from mice with NPYq deficiencies, as compared to wild-type controls. The most striking change being the over-expression of sex chromosome linked genes; among the deregulated genes there are some that are implicated in chromatin remodeling during spermatogenesis. The majority of these genes are also deregulated in mice with a specific small interfering RNA-mediated disruption of the function of the multi-copy NPYq gene *Sly*. *Sly* is one of the four multi-copy genes in the long arm, and it has been shown that its deficiency is associated with similar sperm defects as in NPYq deficient mice [Cocquet, Ellis, et al.,2009]. These *Sly*-deficient mice are near sterile and recapitulate most of the features associated with severe NPYq deficiency, including teratozoospermia, decreased ability to fertilize oocytes in vitro, and altered patterns of chromatin modifications in spermatids.

Thus, I **<u>hypothesized</u>** that *Sly* deficiency is responsible also for sperm DNA chromatin remodeling during spermiogenesis. In **Chapter 3** of this dissertation, I present the results obtained while testing this hypothesis. These data are published:

> **Riel, J. M.**, Yamauchi, Y., Sugawara, A., Li, H.Y., Ruthig, V., Stoycheva, Z., Ellis, P.J., Cocquet, J., Ward, M.A. (2013). "Deficiency of the multi-copy mouse Y gene Sly causes sperm DNA damage and abnormal chromatin packaging." <u>J Cell Sci</u> **126** (Pt 3): 803-813.

The Y-specific (non-PAR) region of the mouse Y chromosome long arm (NPYq) encompasses ~ 90% of the Y-specific DNA content and encodes multiple copies of at least 4 distinct genes expressed in spermatids: *Ssty*, *Sly*, *Asty*, and *Orly*. Although we demonstrated that *Sly* deficiency is associated with a similar sperm DNA damage phenotype as observed in mice with severe NPYq gene loss, the phenotype of *Sly* deficient mice was less severe. If sperm abnormalities in NPYq deficient mice are solely a consequence of *Sly* deficiency, then there should be a correlation between the extent of *Sly* reduction and the severity of sperm defects. *Sly* transcript levels correlated well with the phenotype, but the analysis of SLY protein expression was incomplete.

I **<u>hypothesized</u>** that this milder phenotype of *Sly* deficient mice could be due to either insufficient *Sly*/SLY knockdown, or to participation of another NPYq encoded gene, or both.

In **Chapter 4,** I present the results obtained while testing this hypothesis. These results have not yet been published and the investigations are ongoing. I hope to continue this work during my post-doctoral research.

CHAPTER 2

DEFICIENCY IN MOUSE Y CHROMOSOME LONG ARM GENE COMPLEMENT IS ASSOCIATED WITH SPERM DNA DAMAGE

Abstract

Mice with severe non-PAR Y chromosome long arm (NPYq) deficiencies are infertile *in vivo* and *in vitro*. We have previously shown that sperm from these males, although having grossly malformed heads, were able to fertilize oocytes via intracytoplasmic sperm injection (ICSI) and yield live offspring. However, in continuing ICSI trials we noted a reduced efficiency when cryopreserved sperm were used and with epididymal sperm as compared to testicular sperm. In the present study, we tested if NPYq deficiency is associated with sperm DNA damage – a known cause of poor ICSI results.

We observed that epididymal sperm from mice with severe NPYq deficiency (that is, deletion of nine-tenths or the entire NPYq gene complement) are impaired in oocyte activation ability following ICSI and there is an increased incidence of oocyte arrest and paternal chromosome breaks. Comet assays revealed increased DNA damage in both epididymal and testicular sperm form these mice, with epididymal sperm more affected. In all mice, the level of DNA damage was increased by freezing. Epididymal sperm from mice with severe NPYq deficiencies also suffered from impaired membrane integrity and abnormal chromatin condensation and suboptimal chromatin protamination. It is therefore likely that the increased DNA damage associated with NPYq deficiency is a consequence of disturbed chromatin remodeling.

This study provides the first evidence of DNA damage in sperm from mice with NPYq deficiencies and indicates that NPYq-encoded gene/s may play a role in processes regulating chromatin remodeling and this in maintaining DNA integrity in sperm.

2.1 Background

The DNA of the male specific region of the mouse Y chromosome long arm (NPYq) is highly repetitive and includes multiple copies of four genes: *Ssty, Sly, Asty, Orly* [Ellis, Ferguson, et al.,2007, Toure, Clemente, et al.,2005]. These genes are exclusively expressed in spermatids during the final stages of spermatogenesis [Reynard, Cocquet, et al.,2009]. NPYq deficiency leads to teratozoospermia, subfertility with progeny sex ratio skewed towards females, or to complete infertility [Burgoyne, Mahadevaiah, et al.,1992, Conway, Mahadevaiah, et al.,1994, Styrna, Imai, et al.,1991, Toure, Szot, et al.,2004].

We have previously shown that infertility of mice with severe NPYq deficiencies can be overcome with intracytoplasmic sperm injection; however, the overall efficiency of ICSI [Ward and Burgoyne,2006, Yamauchi, Riel, et al.,2009] was unsatisfactory. This was particularly marked in further ICSI trials with frozen epididymal sperm from males lacking NPYq; despite using artificial oocyte activation, a total of 287 oocytes were injected and 101 embryos transferred into 7 surrogates yielding only 1 pregnancy and 1 viable offspring (Table 1). Poor ICSI success can be due to sperm DNA damage, which is often associated with disturbed chromatin packaging [Aoki, Moskovtsev, et al.,2005, Suganuma, Yanagimachi, et al.,2005, Torregrosa, Dominguez-Fandos, et al.,2006]. Here, we demonstrate that severe NPYq-deficiency results in a high incidence of DNA damage in epididymal sperm, increased sperm damage due to freezing, impaired membrane integrity, poor chromatin condensation and suboptimal sperm chromatin protamination.

2.2 Materials and Methods

2.2.1   Chemicals

Mineral oil was purchased from Squibb and Sons (Princeton, NJ, USA); pregnant mares' serum gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) from Calbiochem (San Diego, CA, USA). All other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise stated.

2.2.2   Animals

Six- to twelve-week-old B6D2F1 (C57BL/6J x DBA/2) females (NCI, Raleigh, NC, USA) were used as oocyte donors for ICSI. The mice of interest in this study were three mutant mice with progressive NPYq deficiency: XY^{RIII}qdel (subsequently called 2/3NPYq-) - these males have a RIII strain-derived Y chromosome with a deletion removing approximately two-thirds of NPYq; XY^{Tdym1}qdelSry (subsequently called 9/10NPYq-) - these males have a 129 strain-derived Y chromosome with an 11-kb deletion removing the testis determinant Sry [Gubbay, Vivian, et al.,1992] that is complemented by an autosomally located Sry transgene [Mahadevaiah, Odorisio, et al.,1998], together with a deletion removing approximately nine-tenths of NPYq [Toure, Szot, et al.,2004]; and $XY*^{X}Sxr^{a}$ (subsequently called NPYq-2) [Yamauchi, Riel, et al.,2009] - in these males the only Y specific material is provided by the Y^{RIII} short arm derived sex reversal factor Sxr^{a} that is attached distal to the PAR of the $Y*^{X}$ chromosome. The $Y*^{X}$ chromosome is an X chromosome with a very large deletion removing most of the X-specific region but leaving an intact PAR and X PAR boundary, together with the X centromere [Burgoyne, Mahadevaiah, et al.,1998]. NPYq-2 males lack the entire Y specific (non-PAR) gene content of Yq in addition to a reduction in copies of $Rbmy$ on

Yp. The control for 2/3NPYq- and NPYq-2 mice is XY^{RIII}, and for 9/10NPYq- is

XY^{Tdym1}*Sry* mice, which carry the same Y chromosome on which the deletion variant

arose. All mice were produced 'in-house' by either breeding or assisted reproduction, and

were on a predominantly C57BL/6 genetic background (more than six generation

backcross from MF1 for all males except for NPYq-2, which were either 62.5% or

81.25% C57BL/6). The mice were maintained in accordance with the NCR 'Guide for

Care and Use of Laboratory Animals' in rooms at 22ºC with 14 h light/10 h dark, and fed

ad libitum.

2.2.3   Gamete collection and embryo culture

Oocyte collection and subsequent oocyte manipulation, including microinjections,

were done in HEPES-buffered CZB medium (HEPES-CZB) [Kimura and

Yanagimachi,1995], with subsequent culture in CZB with an atmosphere of 5% CO_2 in

air [Chatot, Ziomek, et al.,1989]. To obtain testicular sperm a portion of testis was cut off

and minced in ETBS (an EGTA Tris-HCl-buffered solution consisting of 50 mM EGTA,

50 mM NaCl, and 10 mM Tris-HCl buffer, pH 8.2-8.5 [Kusakabe, Szczygiel, et al.,2001]

or HEPES-CZB to release spermatogenic cells. To obtain epididymal sperm the contents

of the caudae epididymides were expressed with needles and placed in HEPES-CZB,

ETBS or phosphate-buffered saline. Spermatozoa were allowed to disperse for 2 to 3

minutes at room temperature. The samples of testicular or epididymal cell suspension

were used for ICSI, comet assay, preparation for transmission electron microscope

(TEM) analysis or sperm nuclear protein isolation immediately after dispersion, or were

subjected to freezing. In some cases epididymal and testicular samples were sonicated

before freezing and/or comet assay (65 output, 10 pulses 1 s each). After sonication the

cell suspension was layered over a two-layer (1.8 to 2.2 M) sucrose gradient and centrifuged (400 g, 20 minutes). The pellet was resuspended in HEPES-CZB or ETBS, and checked under the light microscope to confirm that only sonication-resistant cells (sperm and elongated spermatids) were present.

2.2.4   Epididymal and testicular sperm freezing

Aliquots of 10 µl epididymal sperm or testicular cell suspension in ETBS were loaded in 0.25 ml straws (Edwards Innovations, Spring Valley, VA, USA). Each straw was sealed with Critoseal (Oxford Labware, St Louis, MO, USA) and placed in a plastic holder floating on the surface of the liquid nitrogen for 10 minutes before immersion. For thawing, the straws were removed from the storage container and immersed in a water bath at 37°C for 10 minutes and the contents expressed into a Petri dish. Spermatozoa were used immediately for ICSI or other analyses.

2.2.5   Intracytoplasmic sperm injection

ICSI was carried out as previously described [Szczygiel, Moisyadi, et al.,2003] within 1 to 2 h from oocyte collection and with live sperm randomly chosen for the injections. Sperm-injected oocytes were transferred into CZB medium and cultured at 37°C. The survival of ICSI oocytes was scored 1 to 2 h after the commencement of culture. The activation of ICSI oocytes was scored 6 h after the commencement of culture; the oocytes with two well-developed pronuclei and extruded second polar body were considered activated. The number of two-cell embryos ('fertilized') was recorded after 24 h in culture.

2.2.6 Chromosome analysis

Chromosome preparation and analysis were performed as previously described [Yamauchi, Ajduk, et al.,2007, Yamauchi, Doe, et al.,2007]. The Y chromosome of 9/10NPYq- males and the Y^{*X} chromosome of NPYq-2 males are minute and the latter males also generate some sperm lacking a sex chromosome [Toure, Szot, et al.,2004, Yamauchi, Riel, et al.,2009]. Therefore, for these two genotypes the presence of one small variant and/or lack of one chromosome in the paternal chromosome complement were considered normal. For control males, the chromosomes of a spermatozoon were considered normal when an oocyte contained 40 normal metaphase chromosomes, 20 maternal and 20 paternal. It was not always possible to distinguish between chromosomes of paternal and maternal origin. However, since oocyte chromosomes rarely show structural aberrations at first cleavage metaphase after parthenogenetic activation [Yamauchi, Doe, et al.,2007], any abnormal chromosomes within fertilized oocytes were considered to be of sperm origin. Among the chromosome aberrations, we differentiated between minor (1 to 9 aberrations per karyoplates examined) and multiple (>9, scored always as 10 aberrations per oocyte). In addition to scoring normal versus abnormal karyoplates, we also calculated the incidence of chromosome aberrations, that is, aberration rate, which represents the total number of aberrations divided by the number of oocytes examined.

2.2.7 Comet assay

Sperm DNA fragmentation was assessed using a Trevigen Comet Assay kit (Trevigen, Gaithersburg, MD, USA, catalog no. 4250-050-K) under neutral conditions as previously described [Yamauchi, Ajduk, et al.,2007]. One-hundred DNA tails were

photographed and analyzed per slide and two males were analyzed per genotype. The length of each tail was measured from the center of the comet head to the end of the tail by Image J software and classified into one of the four categories [Yamauchi, Ajduk, et al.,2007].

2.2.8 Transmission electron microscope analysis

Epididymal sperm were fixed in 2.5% glutaraldehyde, 0.1 M sodium cacodylate, 2 mM calcium chloride, pH 7.4, 1 h, then postfixed with osmium tetroxide (1% in 0.1 M cacodylate buffer, 1 h), dehydrated in ethanol, substituted with propylene oxide, and embedded in LX-112 epoxy resin. Ultra-thin sections (60 to 80 nm) were collected on Formvar-coated copper grids, double stained with uranyl acetate and lead citrate, and viewed with a LEO 912 (Zeiss) TEM, and photographed at 8,000× original magnification.

2.2.9 Preparation and analysis of sperm nuclear proteins by immunoblot

All extraction and preparation procedures were performed as described previously [Zhao, Shirley, et al.,2001] except that sperm tails were removed by chemical treatment rather than sucrose centrifugation [Balhorn, Gledhill, et al.,1977]. The proteins were separated by electrophoresis in acid-urea 15% or 20% polyacrylamide gels and either stained with Coomassie blue or used for immunoblotting; an aliquot of sperm proteins corresponding to 4.5×10^6 cells was loaded per each lane. Antibody detection was performed as described previously [Zhao, Shirley, et al.,2001]. Briefly, the nuclear proteins were transferred to a nitrocellulose membrane in 7% acetic acid using Criterion wet blotting unit (BIORAD, Los Angeles, CA, USA). After blocking, the membrane was incubated for 2 h at room temperature with preP2 antibody recognizing the precursor

domain of protamine 2 [Torregrosa, Dominguez-Fandos, et al.,2006] (1:9,000; courtesy

of Marvin Meistrich, University of Texas) and/or antibody Hup2B detecting both

protamine 2 [Stanker, Wyrobek, et al.,1993] (1:500,000; courtesy of Rod Balhorn,

Lawrence Livermore Laboratories). Incubation with the corresponding secondary

antibody conjugated with horseradish peroxidase and detection by chemiluminescence

were carried out as described by the manufacturer (Amersham Pharmacia, Piscataway,

NJ, USA). Band intensities (from Coomassie blue staining or western blot detection)

were quantified using Photoshop software.

2.2.10 Statistics

In the analysis of oocyte activation, oocyte arrest, and incidence of abnormal

karyoplates, Mantel-Haenszel chi square test was used for 'within genotype' comparisons

and Fisher's exact test was used to compare NPYq-deficient genotypes with their

respective controls, and also to analyze the incidence of comet tail types and the TEM

data. ANOVA (Generalized Linear Model as provided by NCSS Statistical Analysis

Software (Kaysville, UT, USA)) with genotype, sperm source (epididymis or testis) and

sperm status (fresh or frozen) as factors was used for the analysis of the oocyte activation

data (after transforming percentage data to angles), comet tail lengths and for

chromosome aberration rates, and for the analysis of western blot band intensities with

gel and genotype as factors.

2.3 Results

2.3.1 Epididymal sperm from males with NPYq deficiencies are less efficient in oocyte activation after ICSI than testicular sperm.

To test for sperm origin or freezing effects on ICSI outcome, injections were carried out with fresh or frozen epididymal sperm, and fresh or frozen testicular sperm. Two males were sampled for each NPY-deficient and the matched control genotypes.

The initial analysis was carried out using the pooled data for the two males of each genotype; the numbers of activated and non-activated oocytes were compared between the NPYq-deficient genotypes and their controls using Fisher's exact test (Figure 2a). For the NPYq-2 versus XY^{RIII} comparison this revealed that fresh epididymal sperm and frozen epididymal sperm from NPYq-2 males were less efficient in oocyte activation than those from XY^{RIII} controls; this also proved to be the case for 9/10NPYq- (P = 0.0001 in all four cases). In 2/3NPYq- neither the frozen nor the fresh epididymal sperm were affected. The degree of impairment of epididymal sperm agrees well with the ranking of the genotypes with respect to the severity of the sperm head abnormalities: NPYq-2 > 9/10NPYq- > 2/3NPYq.

A caveat to this initial analysis is that there were indications of significant inter-male variability, particularly with respect to the two NPYq-2 males. We therefore carried out 'within genotype' comparisons of epididymal and testicular sperm, both fresh and frozen, keeping the individual male data separate, and testing for significant differences using Mantel-Haenszel chi square analysis, which takes account of male to male variation. The control genotypes XY^{RIII} and XY$^{Tdym1}Sry$ did not show any significant differences between epididymal and testicular sperm, whether fresh or frozen, in their ability to activate oocytes. As would be expected from the NPYq deficient versus control comparisons, there was a significant reduction in the oocyte activation efficiency with fresh epididymal sperm as compared to fresh testicular sperm, and with frozen
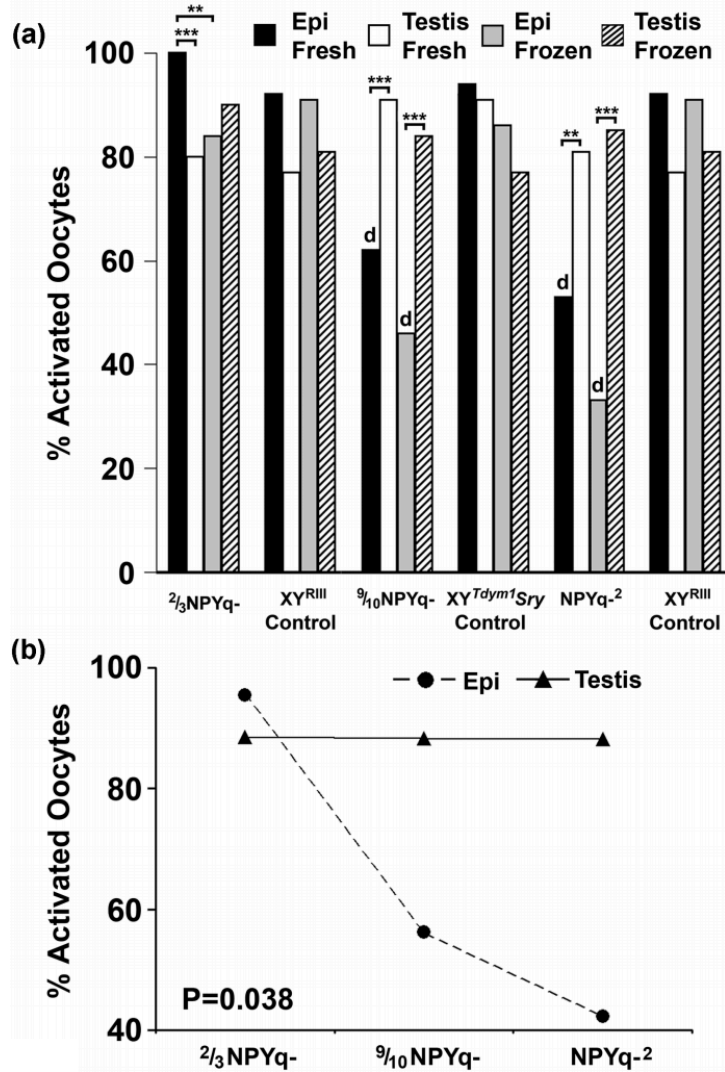
Figure 2. Oocyte activation after ICSI with sperm from mice with NPYq deficiency. (a) Epididymal sperm from males with severe NPYq deficiency (9/10NPYq- and NPYq-2) but not males with moderate NPYq gene loss (2/3NPYq-) were less able to activate oocytes than epididymal sperm from their appropriate controls, as revealed by Fisher's exact test (d, P<0.0001 versus matching sperm type in control). Epididymal sperm from males with severe NPYq deficiency (9/10NPYq- and NPYq-2) but not from males with moderate NPYq gene loss (2/3NPYq-) were less able to activate oocytes than testicular sperm, as revealed by Mantel-Haenszel chi square analysis (**p<0.01, ***p<0.001). (b) Genotype/source interaction revealed by ANOVA, showing that NPYq deficiency preferentially impairs oocyte activation with epididymal sperm (p=0.038). Two males were used per genotype; four sperm groups (epididymal, frozen epididymal, testicular and frozen testicular) were examined per male; approximately 25 (24.88 ± 8.03) oocytes were scored per sperm group per male.

epididymal sperm relative to frozen testicular sperm in NPYq-2 and 9/10NPYq- (Figure 2a). In contrast, the fresh epididymal sperm from 2/3NPYq- gave a significantly higher level of activation than fresh testicular sperm or frozen epididymal sperm.

The above analyses point to NPYq deficiency being a cause of impaired epididymal sperm function, with these effects being proportional to the extent of NPYq gene loss. In the light of this we decided to carry out a single analysis of all the NPYq-deficient male data by ANOVA, with genotype, sperm source (testis or epididymis) and sperm status (fresh or frozen) as factors; an identical analysis was carried out on the two control genotypes for comparison. For these ANOVAs, oocyte activation percentages for individual males were transformed into angles. No significant differences for the three factors were detected among the controls. In contrast, the analysis of the NPYq deficient male data revealed significant effects of genotype (progressively reduced activation with increasing NPYq-deficiency; $p = 0.036$), sperm source (less activation with epididymal sperm than testicular sperm; $p = 0.020$), and a genotype/source interaction (epididymal sperm more affected by NPYq-deficiency than testicular sperm; $p = 0.038$; Figure 2b). Thus, these ANOVA analyses confirm the conclusions from the prior analyses. We conclude that there is a reduction in oocyte activation that increases with the extent of NPYq deficiency, and this effect of NPYq deficiency is largely confined to epididymal sperm.

2.3.2   NPYq deficiency is associated with sperm DNA damage.

Poor activation rates can be circumvented by artificial activation but we have found that even with artificial activation ICSI success rates remained very low with frozen epididymal sperm from NPYq-2 males (Table 1), so we suspected that DNA

Table 1 Intracytoplasmic sperm injection with cryopreserved epididymal sperm from $NPYq^{-2}$ males

Experiment	Number of oocytes injected	Number of oocytes survived (%)[a]	Number of oocytes activated (%)[b, c]	Number of oocytes cleaved (%)[b]	Number of two-cell embryos transferred (number of surrogates)	Number of pregnant surrogates	Number of fetuses
1	64	34 (53)	26 (76)	17 (50)	17 (1)	0	0
2	88	65 (74)	57 (72)	26 (40)	26 (2)	0	0
3	59	44 (75)	41 (93)	25 (57)	25 (2)	1	1
4	76	47 (62)	38 (81)	33 (70)	33 (2)	0	0
1-4	287	190 (66)	162 (85)	101 (53)	101 (7)	1	1

Percentage was calculated from: [a] oocytes injected; [b] oocytes survived. [c]Oocytes were chemically activated with $SrCl_2$; the oocytes were considered activated when they extruded second polar body and had two well-developed pronuclei.

damage may be an important additional factor. We therefore performed comet assays on epididymal and testicular sperm to directly test for DNA damage.

Testicular sperm samples for comet assay were prepared in the same manner as for ICSI so they included other testicular cell types that are not present in the epididymal sperm samples. To test if the presence of these other cell types affects comet assay results, experiments were performed in which a portion of both epididymal and fresh testicular cell suspensions were sonicated under conditions that eliminate these sonication-sensitive cells; comet assays were then performed on non-sonicated and matched sonicated samples, both before and after freezing. Two males of each of the genotypes XY^{RIII}, $XY^{Tdym1}Sry$, and 2/3NPYq- were analyzed; 100 sperm comet tail lengths were measured from each male. The comet tail length data were analyzed by ANOVA with sonication status (sonicated or non-sonicated), genotype, sperm source (testis or epididymis) and sperm status (fresh or frozen) as factors. There were no significant effects of sonication so the fuller analysis including all the NPYq-deficient and control genotypes was therefore carried out without sonication.

We first analyzed the data for the two types of control males and this showed that sperm freezing significantly (p = 0.000001) increased comet tail length (Figure 3a), and that testicular sperm had longer sperm comet tails than epididymal sperm (p = 0.001; Figure 3b). However, there was also a significant (p = 0.013) effect of genotype in that frozen sperm from $XY^{Tdym1}Sry$ males had approximately 25% longer comet tails than those from XY^{RIII}. (A similar increased sensitivity to freezing was also apparent with sperm from 9/10NPYq-, which carry a deleted form of the Y^{Tdym1}.
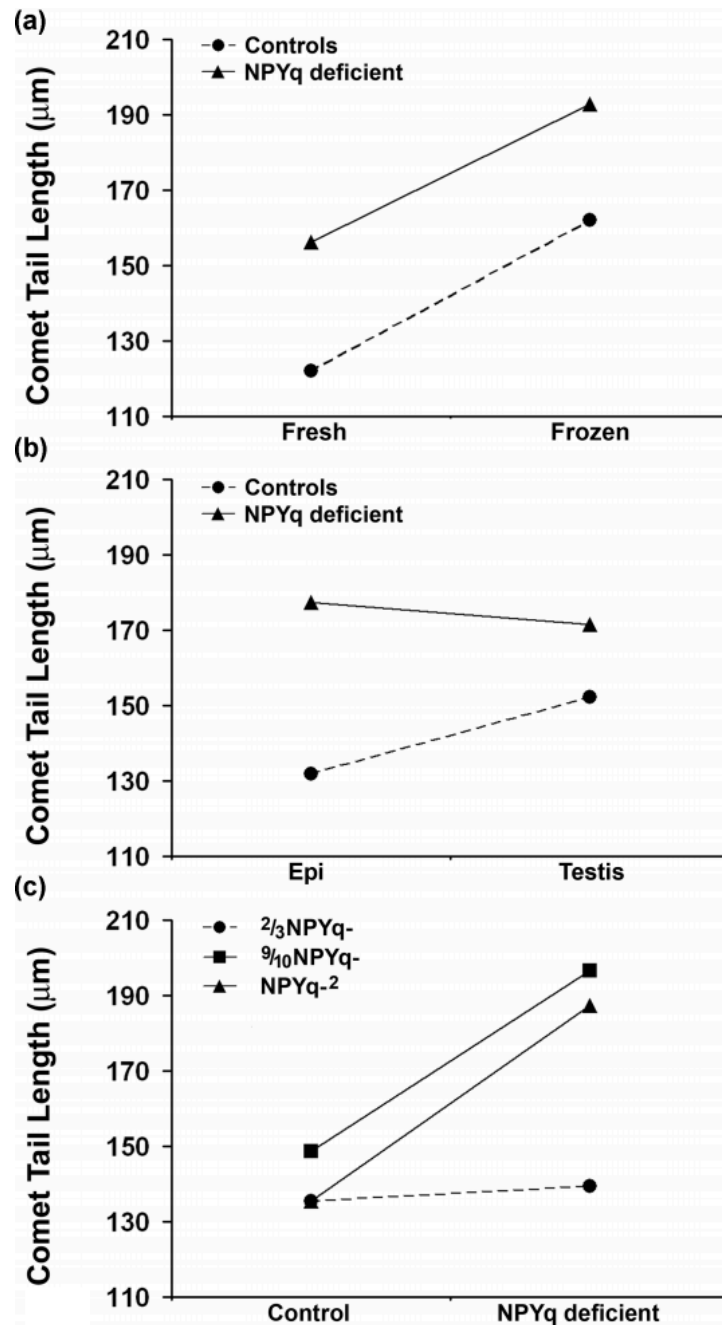
Figure 3. Tail length analysis in comet assay with sperm from mice with NPYq deficiencies – ANOVA analysis. (a) An increase in comet tail length due to sperm freezing in controls (P=0.000001) and NPYq deficient mice (P=0.0084). (b) An increase in comet tail length with testicular as compared to epididymal sperm in controls (P=0.001) but not in NPYq deficient mice. (c) Comparison of NPYq deficient genotypes with their respective controls showing the significant increase in comet tail length in 9/10NPYq- (P=0.0093) and NPYq-2 (P=0.0036). Two males were used per genotype; four sperm groups (epididymal, frozen epididymal, testicular and frozen testicular) were examined per male; 100 sperm were scored per group per male.

We then compared each NPYq-deficient genotype with its matched control. There were no significant differences between 2/3NPYq- and the XY^{RIII} control, but the two remaining NPYq-deficient genotypes had significantly increased sperm comet tail lengths relative to their controls (Figure 3c). Analysis of the three NPYq-deficient genotypes in a single ANOVA showed (as in controls) a significant increase of comet tail length in response to freezing (p = 0.0084; Figure 3a); in contrast to controls there was no significant increase in comet tail length in testicular sperm as compared to epididymal sperm (Figure 3b). Indeed, when the effect of sperm source was compared in analyses within each NPYq-deficient genotype, comet tails were significantly longer in epididymal sperm as compared to testicular sperm from NPYq-2 (p = 0.0034), and this resulted in a highly significant genotype/sperm source interaction (p = 0.0004) when NPYq-2 was compared with its matched XY^{RIII} control.

In addition to comet tail length, classification as to comet tail type can also give an indication of the level of DNA damage. Based on the distribution of comet tail types, differences between epididymal and testicular sperm were observed in mice with severe NPYq deficiencies (Figure 4) but not in 2/3NPYq- mice. Thus, in NPYq-2 and 9/10NPYq-, epididymal sperm yielded significantly fewer comets with tail type 1 (lowest damage) and significantly more comets with tail type 4 (most severe damage). The difference was more pronounced in NPYq-2 than in 9/10NPYq-.

The comet data show that freezing increases DNA damage across all genotypes, that there is an increase in DNA damage relative to controls when the NPYq-deficiency exceeds that of 2/3NPYq-, and that epididymal sperm from mice with severe NPYq deficiency are more susceptible to DNA damage than testicular sperm. We conclude that
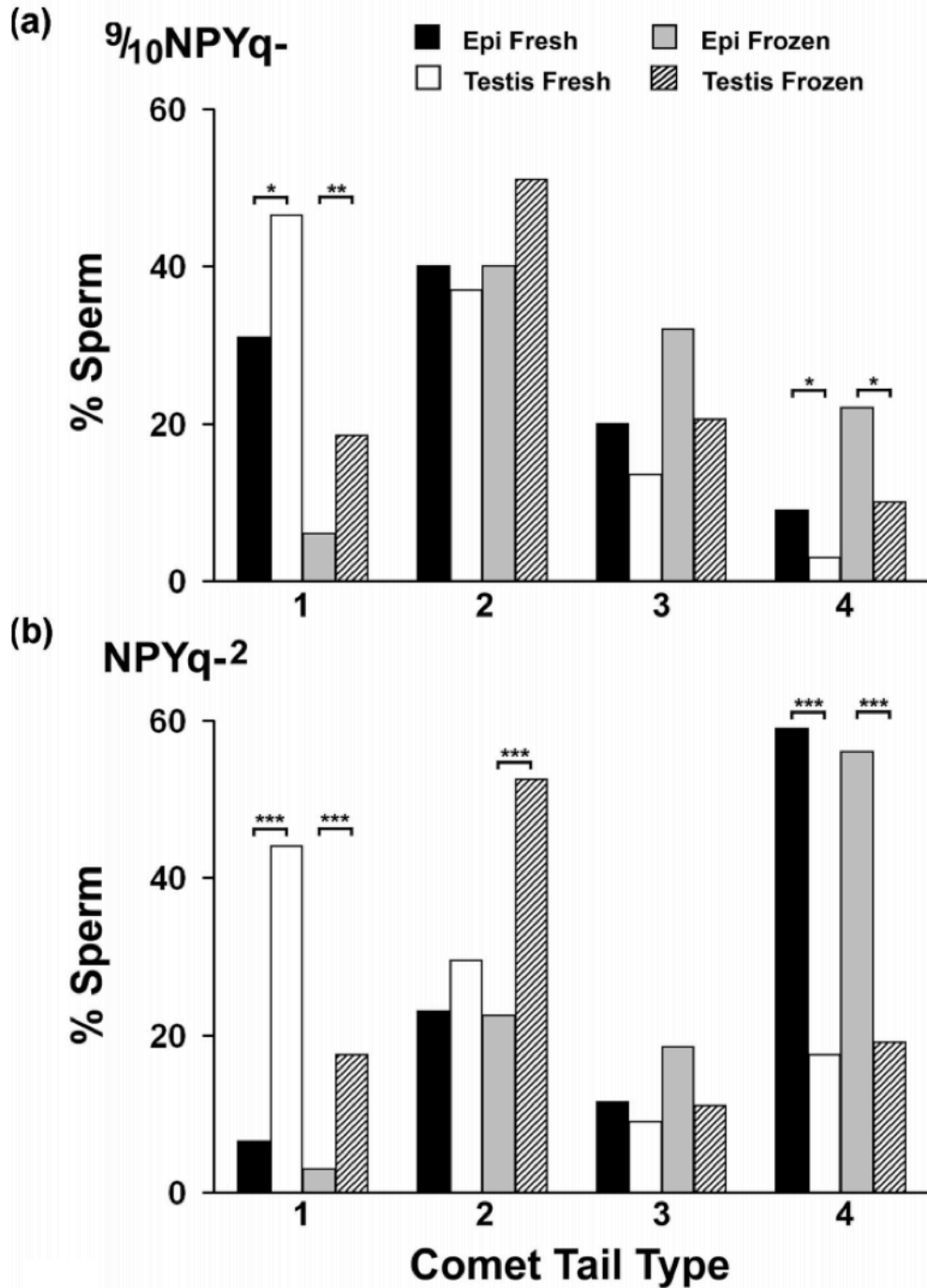
Figure 4. The distribution of comet tail types in mice with severe NPYq deficiencies. Four types of comet tail were differentiated: 1, short tail; 2, long tail, with majority of the DNA still in the head; 3, long tail with DNA evenly distributed through out; 4, long tail with most of the DNA at the distal portion (balloon shapped). The severity of DNA damage increases with tail type, from 1 to 4. Two males were used per genotype; four sperm groups (epididymal, frozen epididymal, testicular and frozen testicular) were examined per male; 100 sperm were scored per group per male. Statistical significance: *$P<0.05$; **$P<0.01$; ***$P<0.001$ (Fisher's two-tailed exact probability test).

severe NPYq deficiency leads to DNA damage that is particularly marked in frozen epididymal sperm, and that this is likely to be the major factor underlying the very poor ICSI outcome using frozen epididymal sperm from NPYq-2 males.

2.3.3 NPYq deficiencies yield a high incidence of oocyte arrest and paternal chromosome breaks after ICSI

When collecting the ICSI activation data, we also collected data on the incidence of early post-fertilization oocyte arrest and of chromosome breakage in the paternal chromosome complements of zygotes, since both are known consequences of sperm DNA damage.

We compared the numbers of arrested and non-arrested oocytes between the NPYq deficient genotypes and their controls using Fisher's exact test (Figure 5). This revealed increased oocyte arrest relative to controls for frozen epididymal sperm from 9/10NPYq- (p = 0.0172) and for fresh and frozen epididymal sperm from NPYq-2 (p = 0.0347 and 0.0005, respectively). We then carried out 'within genotype' comparisons of epididymal and testicular sperm, both fresh and frozen, keeping the individual male data separate, using Mantel-Haenszel chi square analysis (Figure 5). The control genotypes did not show any significant differences in the incidence of oocyte arrest with epididymal as compared to testicular sperm, whether fresh or frozen. However, there was increased oocyte arrest with frozen epididymal sperm as compared to frozen testicular sperm from 9/10NPYq- and NPYq-2 (p < 0.001 and p < 0.0005, respectively). There was also an increase in oocyte arrest with fresh epididymal as compared to fresh testicular sperm in 9/10NPYq- (p < 0.01). Thus, there is increased arrest when there is substantial NPYq deficiency.
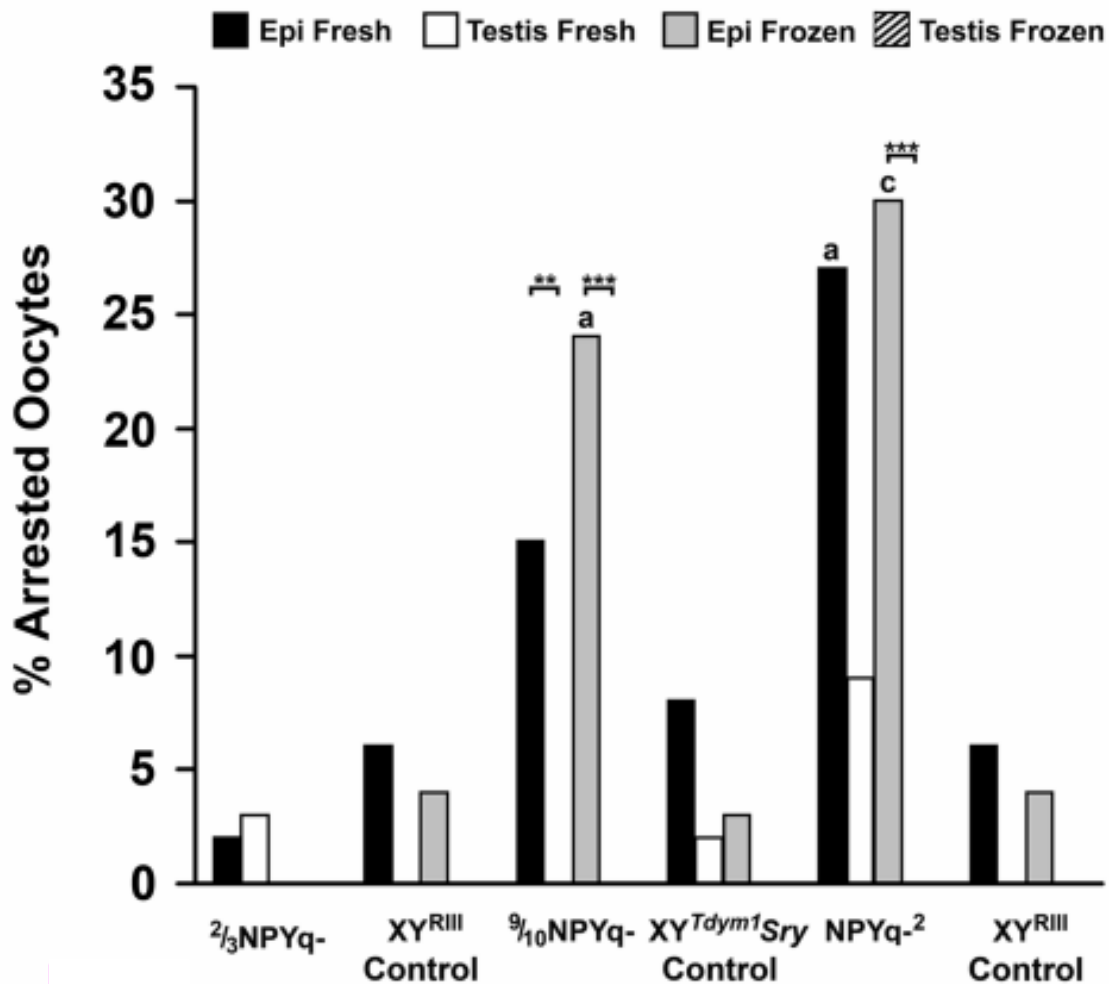
Figure 5. Oocyte arrest after ICSI with sperm from mice with NPYq deficiency.
Epididymal sperm from males with severe NPYq deficiency (9/10NPYq- and NPYq-2)
but not males with moderate NPYq gene loss (2/3NPYq-) led to increased incidence of
oocyte arrest compared to epididymal sperm from their appropriate controls, as revealed
by Fisher's exact probability test. Statistical significance: a=p<0.05; c =p<0.001 versus
matching sperm type in control. Epididymal sperm from males with severe NPYq
deficiency (9/10NPYq- and NPYq-2) but not males with moderate NPYq gene loss
(2/3NPYq-) led to increased incidence of oocyte arrest compared to testicular sperm, as
revealed by Mantel-Haenszel chi square analysis. Statistical significance:**p<0.01;
***p<0.001. Two males were used per genotype; four sperm groups (epididymal, frozen
epididymal, testicular and frozen testicular) were examined per male; 100 sperm were
scored per male; approximately 20 (20.35 +/- 6.94 oocytes were scored per group per
male.

For chromosomal breakage we first compared the number of oocytes with and without breaks in the paternal complement between the NPYq-deficient genotypes and their controls using Fisher's exact probability test (Figure 6). As with the incidence of oocyte arrest, the significant increases in the frequency of oocytes with chromosomal breakage were in 9/10NPYq- and NPYq-. In 9/10NPYq- the effect was restricted to frozen epididymal sperm ($p = 0.0059$), whereas in NPYq-2, epididymal, frozen epididymal and frozen testicular sperm were affected ($p = 0.0009$, $p < 0.0001$ and $p < 0.0001$, respectively).

The paternal chromosome complements originating from NPYq-deficient mice had multiple chromosome and chromatid gaps, breaks and fragments, together with some abnormal chromosome configurations such as rings and exchanges (Figure 7). In order to better reflect the level of chromosome damage, we calculated the incidence of all chromosome aberrations for each sperm category for each male (aberration rate). The resulting data were analyzed by ANOVA.

We first analyzed the data for the control males and this showed that sperm freezing increased the chromosome aberration rate ($p = 0.025$; Figure 8a) but there was a significant sperm source/status interaction ($p = 0.027$), with testicular sperm more sensitive to freezing than epididymal sperm. Comparison of each NPYq-deficient genotype with its matched control revealed that there was no increase in chromosome aberrations in 2/3NPYq-, but there was a 2.7-fold increase in 9/10NPYq- ($p = 0.000145$) and a 7.2-fold increase in NPYq-2 ($p = 0.000019$) (Figure 8b). These markedly different aberration rates resulted in a highly significant effect of genotype ($p = 0.000001$) in the analysis of the three NPY-deficient genotypes in a single ANOVA, with aberration rate
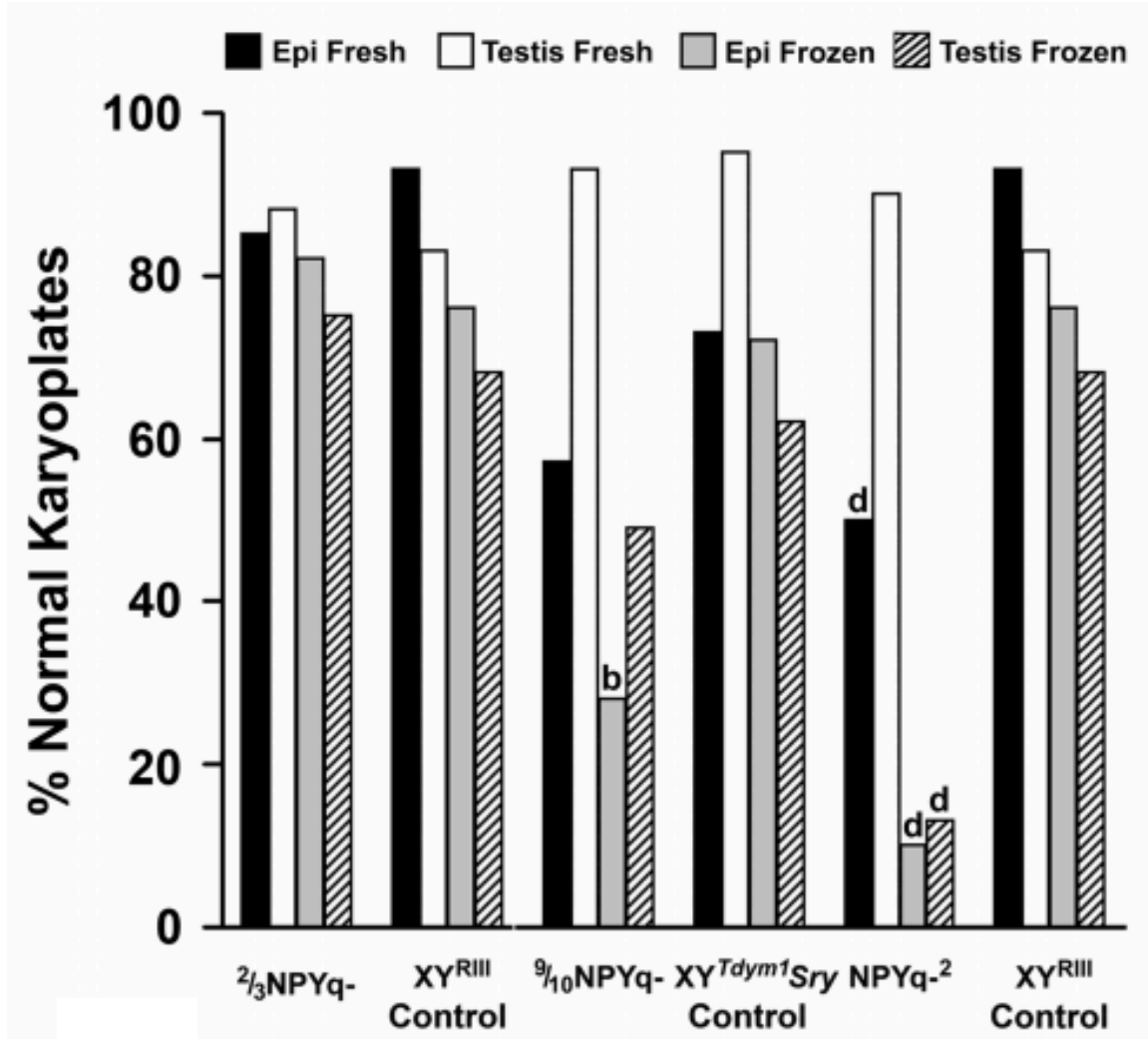
Figure 6. Percentage of normal karyoplates after ICSI with sperm from mice with NPYq deficiency. Sperm from males with NPYq deficiency (9/10NPYq- and NPYq-2) but not males with moderate NPYq gene loss (2/3NPYq-) led to increased incidence of abnormal karyoplates compared to respective sperm types from their appropriate controls, as revealed by Fisher's exact probability test. Statistical significance: b=p<0.01; d=p<0.0001 versus matching sperm type in control. Two males were used per genotype; four sperm groups (epididymal, frozen epididymal, testicular and frozen testicular) were examined per male; approximately 15 (15.88 ± 6.52 oocytes were scored per group per male.
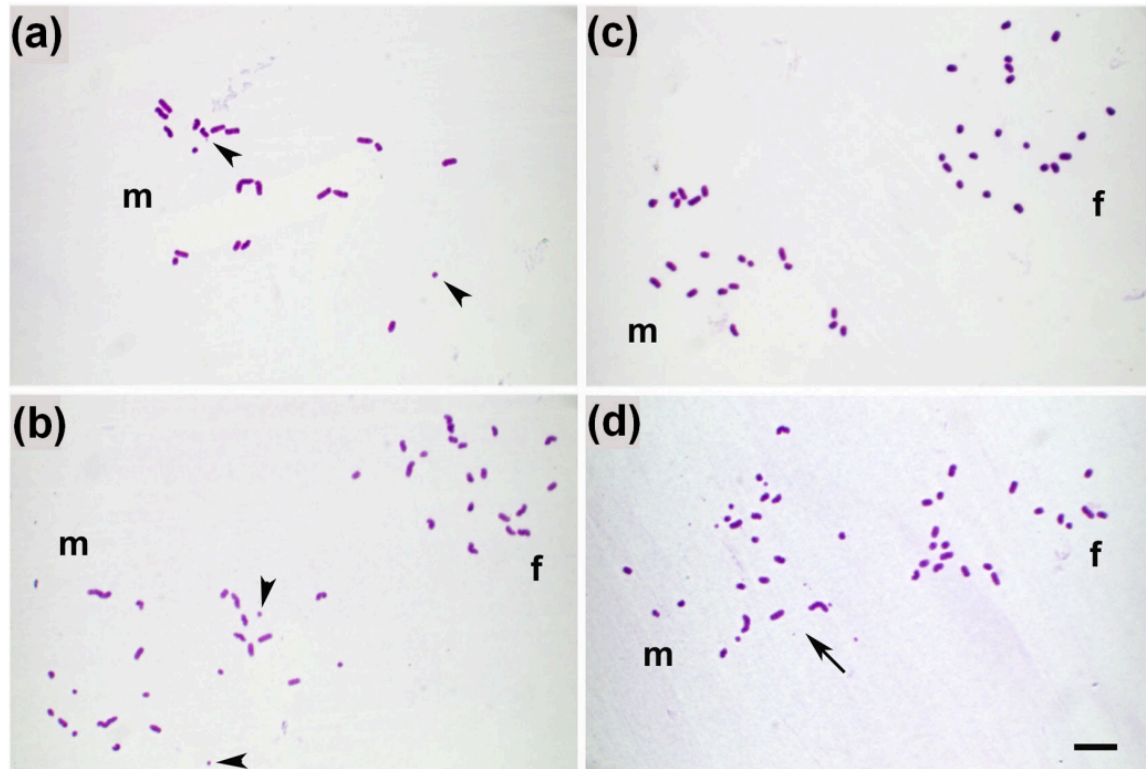
Figure 7. Chromosome analysis after ICSI with sperm from mice with NPYq deficiencies. (a) Fresh epididymal sperm from 9/10NPYq- male. Paternal chromosome complement (m) with 19 normal chromosomes and 3 fragments (examples shown with arrowheads). (b) Fresh testicular sperm from 9/10NPYq- male. Normal paternal (m) and maternal (f) chromosome complements each showing 20 chromosomes. (c) Frozen testicular sperm from 9/10NPYq-male. Normal maternal complement (f, n=20) and paternal karyoplate with 18 normal chromosomes and 6 chromosome fragments (examples shown with arrowheads). (d) Frozen epididymal sperm from NPYq-2 male. Normal maternal chromosomes (f, n=20) and paternal (m) complement with multiple chromosome aberrations (>10 fragments; arrow). Scale bar = 100μm.
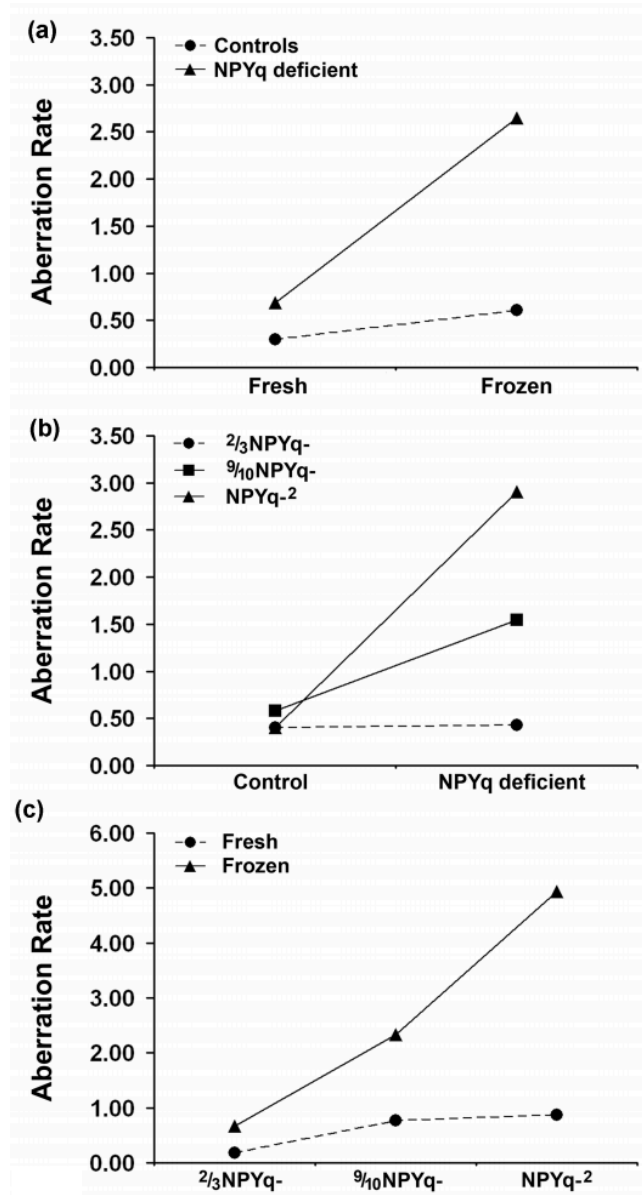
Figure 8. Incidence of paternal chromosome breaks (aberration rate) in zygotes produced by ICSI with sperm from mice with NPYq deficiencies – ANOVA analysis. (a) An increase in chromosome aberration rate due to sperm freezing in controls (p=0.025) and NPYq deficient mice (p<0.000001). (b) Comparison of NPYq-deficient genotypes with their respective controls showing the significant increase in chromosome aberration rate in 9/10NPYq- (p=0.000145) and NPYq-2 (p=0.000019). (c) Genotype/sperm status interaction, showing that with increasing NPYq deficiency the increase in chromosome aberration rates is much more marked with frozen than fresh sperm (p=0.000025). Two males were used per genotype; four sperm groups (epididymal, frozen epididymal, testicular and frozen testicular) were examined per male; approximately 15 (15.88 ± 6.52) oocytes were scored per group per male.

increasing with the extent of NPYq deficiency. However, this increase was predominantly seen with frozen sperm, resulting in a very significant genotype/sperm status interaction (p = 0.000025; Figure 8c) and a very significant affect of freezing overall (p < 0.000001; Figure 8a).

Based on the ANOVA analysis we conclude that in controls the freezing of testicular sperm leads to significant chromosome damage, and that severe NPYq deficiency leads to a marked increase in chromosome damage in response to sperm freezing, with testicular and epididymal sperm now being affected.

2.3.4 Comparison of sperm comet and chromosome aberration data indicates that testicular sperm freezing impairs sperm DNA damage repair in the oocyte

There is substantial evidence showing that oocytes have DNA repair machinery present at fertilization that enables DNA damage in the sperm nucleus to be repaired. The chromosome aberrations present in fertilized oocytes are therefore a manifestation of prior DNA damage that cannot be repaired by the oocyte. For six of the males in the present study the same sperm samples were used for the sperm comet and oocyte paternal chromosome complement analyses, so a direct comparison of these sets of data should highlight those factors that lead to irreparable DNA damage.

The six males for which both sets of data are available are XY^{RIII} (n = 1), 2/3NPYq- (n = 2), 9/10NPYq- (n = 1) and NPYq-2 (n = 2). Because 2/3NPYq- males (with moderate NPYq deficiency) do not manifest any significant differences from XY^{RIII} in either assay, we treated the first three males as one group (group 1, G1). The 9/10NPYq- and NPYq-2 males (severe NPYq deficiency) constituted the second group (group 2, G2), which differed markedly from their controls in both assays. The two

groups were first compared by ANOVA. For sperm comet tail length there was a 38%

increase as a consequence of severe NPYq deficiency (p < 0.000001); epididymal sperm

were preferentially affected in G2 whereas in G1 it was the testicular sperm that had the

longer sperm comet tails (group/sperm source interaction, p = 0.0032). For chromosome

aberration rate there was an almost six-fold increase as a consequence of the NPYq

deficiency (p = 0.000013); sperm from G2 males were much more sensitive to freezing

than those from G1, resulting in a significant group/sperm status interaction (p =

0.00070).

Within group comparisons established that in G1 males there was more DNA

damage (comet assay) in testicular sperm than epididymal sperm (p = 0.0014) and for

both sperm sources the level of damage was markedly increased by freezing (p =

0.00096), but the chromosome aberration rates were only markedly elevated with frozen

testicular sperm (p = 0.031 for source/status interaction), indicating that most of the

damage due to freezing in epididymal sperm was repaired in the oocyte. However, in G2

males, the increase in sperm DNA damage due to freezing was reflected in markedly

increased chromosome aberration rates with both sperm sources (p = 0.001). In addition,

there was a significant increase in chromosome aberrations with fresh epididymal sperm

as compared to fresh testicular sperm (p = 0.000568). These differing effects of sperm

source and sperm freezing between the two groups became apparent when the comet tail

length and chromosome aberration rate data were plotted as a scatter plot (Figure 9). In

summary: frozen testicular sperm from controls and 2/3NPYq- (G1) have DNA damage

that is not resolved in the oocyte, with a consequent increase in chromosome aberrations;

and in males with severe NPYq deficiency (G2), there is a marked increase in sperm
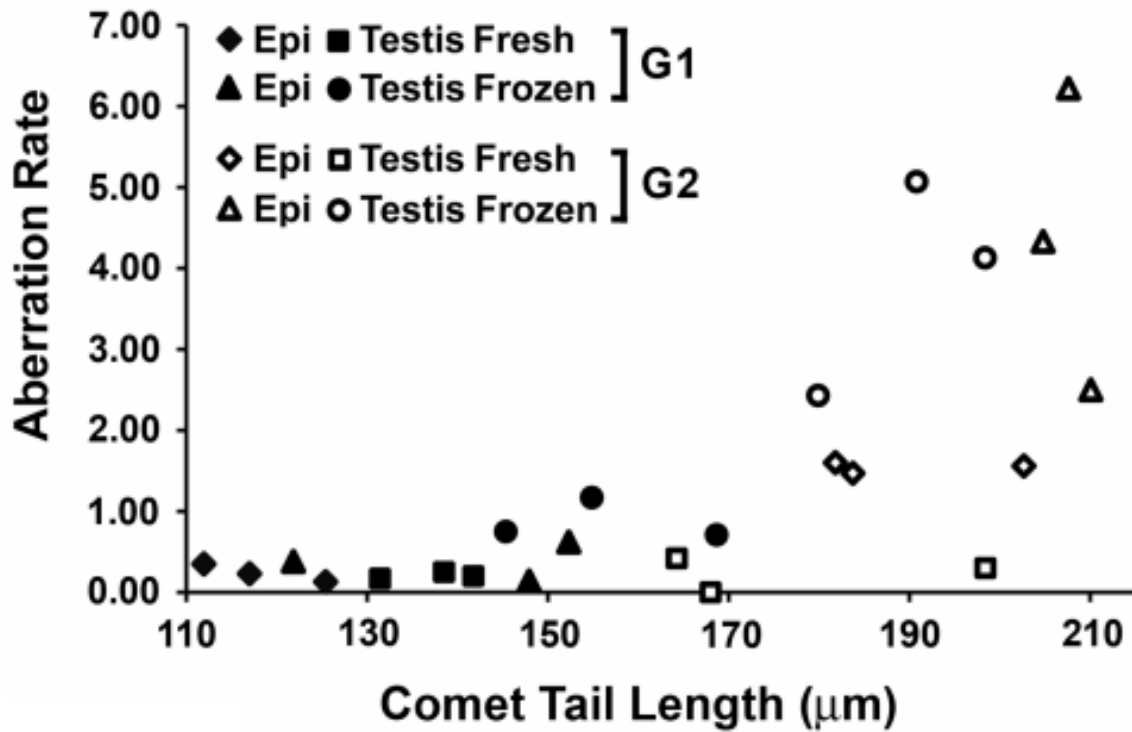
**Figure 9. Comparison of comet assay and chromosome aberration analysis –
ANOVA analysis.** Comet tail length versus aberration rate scatter plot with distinction
between sperm source and sperm status. Group 1 (G1, n=3) = controls + 2/3NPYq-;
group 2 (G2, n=3) = 9/10NPYq- and NPYq-2.

DNA damage in testicular and epididymal sperm. Similarly to G1, frozen testicular sperm have some DNA damage that is not resolved in the oocyte, but in contrast to G1 this is also true of fresh and frozen epididymal sperm.

2.3.5 Sperm from males with NPYq deficiencies have impaired membrane integrity and abnormal chromatin condensation as shown by electron microscopy analysis

Transmission electron microscopy was used to determine membrane integrity and appearance of chromatin in epididymal sperm from mice with NPYq deficiencies. Three males per genotype were examined (except for 9/10NPYq-, for which only two males were tested) and 100 sperm heads were scored per male.

With respect to membrane integrity, we assigned examined sperm heads into three categories reflecting progressive membrane damage: I, intact; B, broken; and D, disintegrating (Figure 10). There were no differences when the incidences of specific categories were compared across control genotypes; almost all sperm had intact membranes (approximately 93%). NPYq-2 mice had predominantly sperm with a disintegrating membrane (approximately 96%) and 9/10NPYq- had the majority of sperm with either broken or disintegrating membrane (>90%). In 2/3NPYq-, most sperm had either intact (approximately 48%) or disintegrating membranes (approximately 44%). All NPYq-deficient genotypes had significantly fewer sperm with intact membrane and significantly more sperm with disintegrating membrane than their respective controls. When NPYq-deficient genotypes were compared against each other, the ranking reflecting the severity of membrane integrity impairment was: NPYq-2 > 9/10NPYq- > 2/3NPYq-.
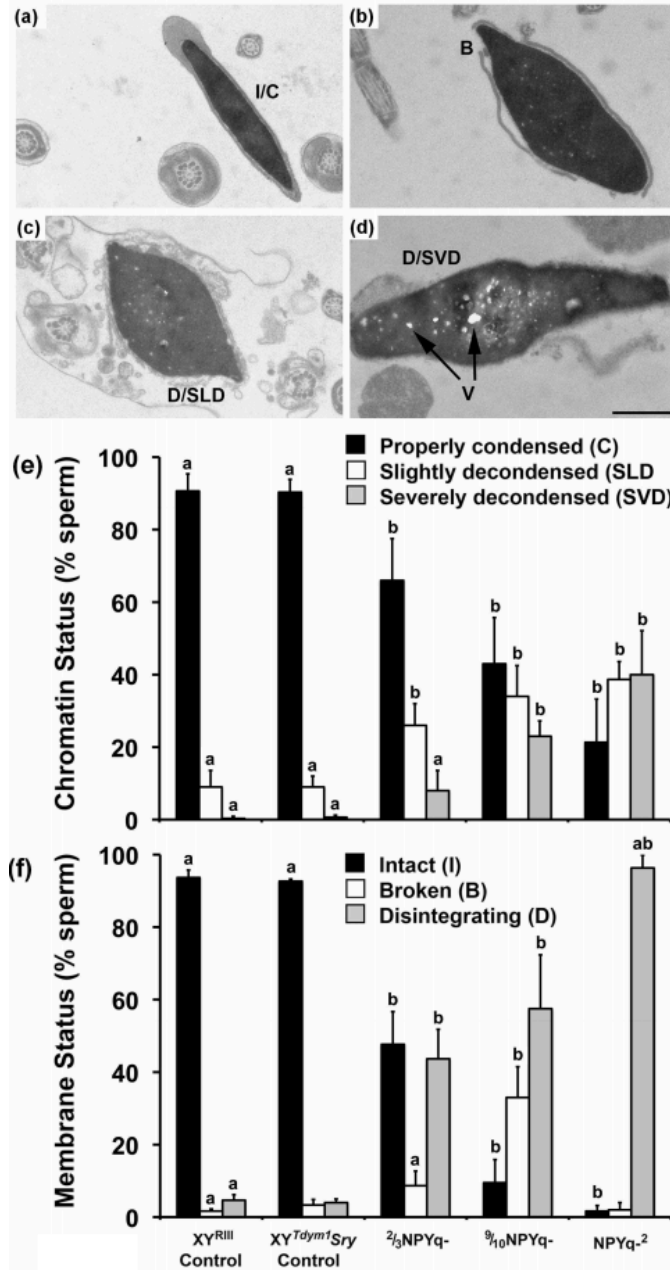
Figure 10. Transmission electron microscopy analysis of sperm from mice with NPYq deficiencies. (a-d) Examples of sperm with various membrane and chromatin condensation deficiencies. (e-f) Analysis of frequency of sperm with various chromatin and membrane integrity deficiencies. When examining chromatin condensation we observed the presence of bright white spots (voids; V), which appeared always in conjunction with severely decondensed chromatin and were present exclusively in sperm from mice with NPYq deficiencies. Scale bar = 1μm. Three males per genotype were examined (except for 9/10NPYq-, for which only two males were tested); 100 sperm heads were scored per male. Each bar represents mean ± standard deviation. Statistical significance: a = different from other categories within genotype; b = different from respective category in control (Fisher's exact probability test).

36

When examining chromatin condensation we categorized sperm into three categories: those with properly condensed (C), slightly decondensed (SLD) and severely decondensed (SVD) chromatin (Figure 10). All controls had the vast majority of sperm with properly condensed chromatin (>90%). In 2/3NPYq- males, sperm with properly condensed chromatin predominated (66%) and less than 10% had severely decondensed chromatin. In 9/10NPYq- and NPYq-2 males more than half of sperm had decondensed chromatin (approximately 57% and approximately 79%, respectively). All NPYq-deficient genotypes had fewer sperm with properly condensed chromatin and more sperm with slightly and severely decondensed sperm than their respective controls.

When membrane integrity and chromatin condensation status were compared, the test for linear trend in proportions [Snedecor and Cochran,1989] confirmed a significant correlation between the maintenance of sperm membrane integrity and proper chromatin condensation (p < 0.001).

Overall, the data show that mice with NPYq deficiencies exhibit membrane damage and abnormal chromatin condensation in sperm, which increases in parallel with the level of NPYq gene loss.

2.3.6 Sperm from males with NPYq deficiencies have impaired protamine processing

To test whether increased sperm DNA damage resulted from abnormal protamination of sperm chromatin, we examined epididymal sperm from mice with NPYq deficiencies for the presence of premature protamine forms, with testis samples providing positive controls. Sperm nuclear protein samples corresponding to the same sperm number were separated on acid-urea polyacrylamide gels. At least two gels were

run and at least three males were tested per genotype (Table 2). No premature protamine P2 bands were detected on Coomassie blue stained gels, in any of the tested genotypes (Figure 11a). However, when the gels were blotted with preP2 antibody, which recognizes premature protamine 2 forms, bands were detected in samples from 9/10NPYq- mice and their controls on two of the four gels (Figure 11c, Table 2). The intensity of preP2 bands was significantly higher for 9/10NPYq- mice than for controls (p = 0.0001). No preP2 was detected in samples from 2/3NPYq and NPYq-2 mice, in the latter genotype perhaps due to the lowest number of mice tested.

Additional evidence of abnormal protamination came from measuring band intensities for mature protamines. On Coomassie blue stained gels, there was no reduction in band intensity relative to controls in 2/3NPYq-, but in 9/10NPYq- the band intensity was reduced by 37% (p = 0.002) and in NPYq-2 males band intensity was reduced by 71%, although this reduction was not statistically significant with the limited number of samples analyzed (Table 2). When the membranes were blotted with Hub2B antibody recognizing mature protamine 2, the same pattern of decreasing levels of the mature form with increasing NPYq deficiency was observed, there being no reduction in 2/3NPYq-, a 12% reduction in 9/10NPYq- (p = 0.00005) and a 44% reduction in NPYq-2 males (not significant) (Figure 11b, Table 2).

In summary, the data indicate that mice with severe NPYq deficiencies have impaired sperm chromatin protamination leading to an increase in premature protamine forms and a decrease in mature protamine forms in epididymal sperm.

Table 2 Western blot analysis of protamines in mice with NPYq deficiencies

Mice examined[a]	Mean band intensity[b] (±SEM) representing mature protamine forms		Mice examined[a]	Mean band intensity[b] (±SEM) representing immature protamine 2 (PreP2)
	Coomassie	Hub2B (protamine 2)		
2/3NPYq- (n = 7)	141.9 ± 4.6	125.3 ± 4.6	-	-
XY[RIII] (n = 6)	138.2 ± 5.0	124.9 ± 5.0		
9/10NPYq- (n = 7)	82.5 ± 4.8*	43.5 ± 0.3***	9/10NPYq- (n = 5)	89.2 ± 4.5**
XY[Tdym1]Sry (n = 6)	131.0 ± 5.2	49.3 ± 0.3	XY[Tdym1]Sry (n = 4)	53.8 ± 5.0
NPYq-2 (n = 3)	22.1 ± 16.2	20.8 ± 6.6	-	-
XY[RIII] (n = 3)	76.0 ± 16.2	36.9 ± 6.6		

[a] At least two separate gels were were run for each NPYq deficient genotype (2/3NPY-, two gels; 9/10NPYq-, four gels; NPYq-^{2}, two gels). For PreP2, data were obtained from only two gels. [b] The data were analyzed by two-way ANOVA with gel and genotype as factors, from which the genotype means and errors are derived. Statistical significance: *P < 0.005; **P < 0.0005; ***P < 0.00005. The lack of statistical significance for the reduction observed in NPYq-^{2} was almost certainly due to the limited number of samples. SEM, standard error of the mean.
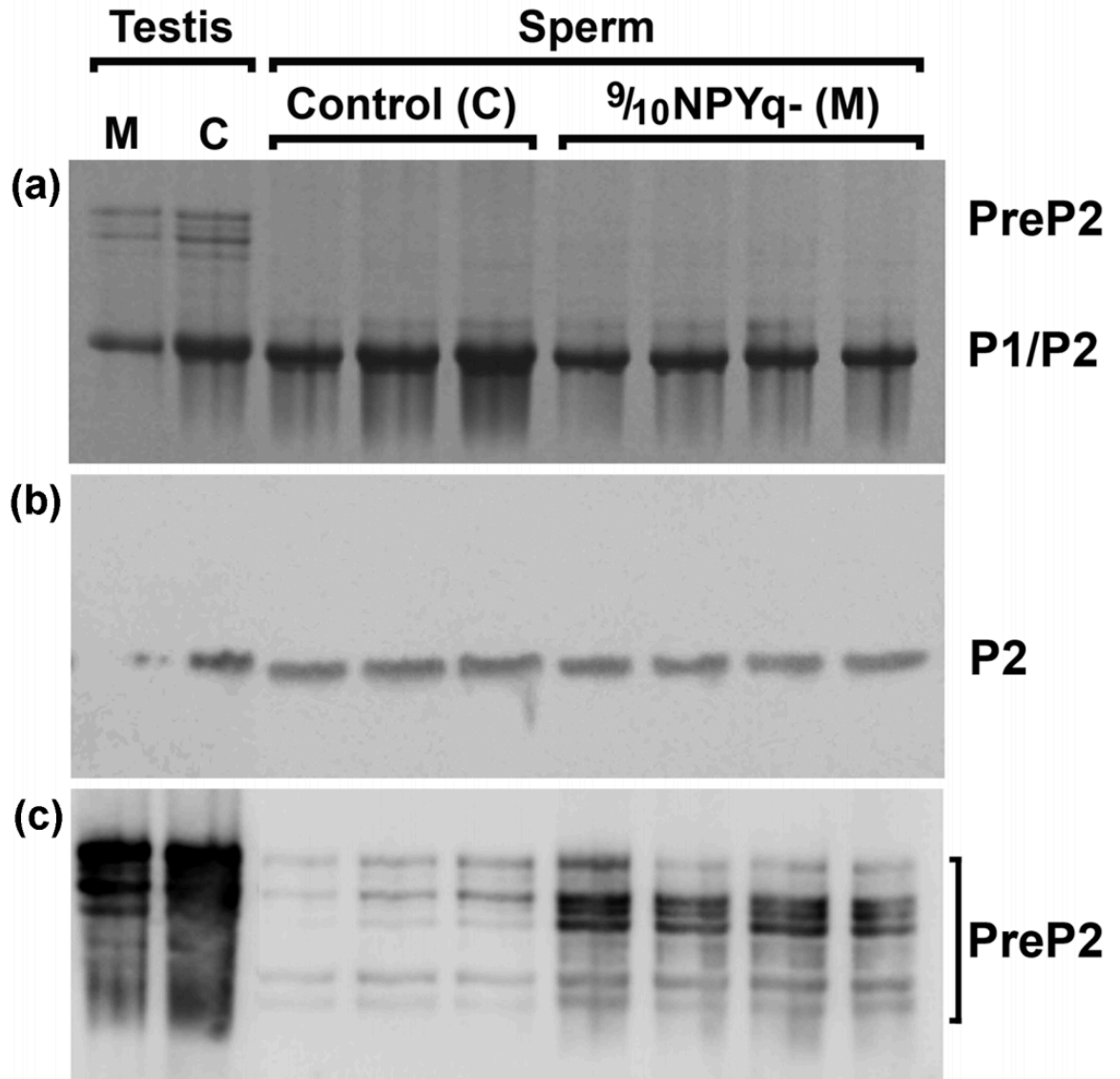
Figure 11. Sperm nuclear protein analysis. A representative acid-urea gel separation of nuclear proteins extracted from epididymal sperm and testes of 9/10NPYq- mutant (M, n=4) and XY^{Tdym1}Sry control (C, n=3) mice. (a) Coomassie blue stained gel. (b) Immunoblot with Hub2B antibody recognizing P2. (c) Immunoblot with preP2 antibody recognizing preP2. P1 and P2, protamine 1 and 2, respectively; PreP2, premature forms of protamine 2.

2.4 Discussion

The aim of the present study was to provide an explanation for the very poor ICSI success when using frozen epididymal sperm from mice with severe NPYq deficiency. The results show that the major underlying cause is an increase in DNA damage that particularly affects epididymal sperm, and that is further increased by sperm freezing. This DNA damage included damage that was not reparable by the DNA repair machinery present in the oocyte, resulting in a marked increase in chromosome aberrations in the paternal chromosome complement following ICSI.

What is the origin of the increased sperm DNA damage in severely NPYq-deficient mice that is already detected in testicular sperm and has increased markedly by the time the sperm have reached the cauda epididymis? The origin of sperm DNA damage is a subject of substantial research and much debate. Aitken et al. [Aitken and De Iuliis,2010, Aitken, De Iuliis, et al.,2009] have proposed a two step model in which sperm exiting the testis with chromatin remodeling defects, such as incomplete protamination, histone retention and poor compaction, subsequently acquire membrane and DNA damage as they transit the epididymis. Several proposed, not mutually exclusive causes of this DNA damage include direct damage by reactive oxygen species [Tremellen,2008] and the action of endonucleases present either within the sperm or in epididymal fluid [Boaz, Dominguez, et al.,2008, Maione, Pittoggi, et al.,1997, Shaman, Prisztoka, et al.,2006, Yamauchi, Ajduk, et al.,2007].

However, there is also evidence that damaged spermatozoa can be selectively removed during epididymal transit [Olson, Winfrey, et al.,2004, Sutovsky, Moreno, et al.,2001], so the level of DNA damage detected in sperm from the cauda epididymis

depends on the balance between the generation of DNA damage and the removal of sperm with DNA damage during epididymal transit. Thiol-cross linking, which takes place during epididymal maturation [Dias, Retamal, et al.,2006, Shalgi, Seligman, et al.,1989, Sivashanmugam and Rajalakshmi,1997], increases overall chromatin compaction. If sperm exiting testes already have some chromatin defect, epididymal maturation may be impaired or may not take place.

In the case of severely NPYq-deficient mice, the primary defect must arise during spermiogenesis since this is when the NPYq-encoded genes are expressed in normal mice [Conway, Mahadevaiah, et al.,1994, Ellis, Clemente, et al.,2005, Reynard, Cocquet, et al.,2009]. In keeping with the two step model for the generation of DNA damage in epididymal sperm [Aitken, De Iuliis, et al.,2009], we have found that epididymal sperm from NPYq deficient mice have defective protamination and chromatin compaction, with associated membrane damage. However, testicular sperm from these mice already have elevated levels of DNA double-strand breaks (DSBs) as detected by the comet assay. Transient DSBs are generated by topoisomerase TOP2B during normal sperm chromatin remodeling and it has been suggested that inefficient repair of these breaks may occur in the context of disturbed protamination [Leduc, Maquennehan, et al.,2008, McPherson and Longo,1992].

The exact mechanistic link between NPYq deficiency and the chromatin changes is difficult to determine because of the major transcriptional changes in spermatids that are associated with NPYq deletions. More than 200 genes were found to be differentially expressed in mice with NPYq deficiencies, as compared to wild-type controls, the most striking change being the over-expression of sex chromosome linked genes; among the

misregulated genes are some that are implicated in chromatin remodeling during spermiogenesis (NPYp-linked *H2al2y*, X-linked *H2al1* and *Cypt*, and autosomal *Hist1h3*, *Hist1h4*, *Chaf1b* and *Speer*) [Cocquet, Ellis, et al.,2009, Ellis, Clemente, et al.,2005]. Given the extent of this transcriptional regulation, it is perhaps unsurprising that there are also changes in the pattern of spermatid chromatin modifications [Reynard and Turner,2009]. The majority of these genes are also misregulated in mice with a specific small interfering RNA-mediated disruption of the function of the multi-copy NPYq gene *Sly*. These mice are near sterile and recapitulate most of the features associated with severe NPYq deficiency, including altered patterns of spermatid chromatin modifications, thus demonstrating that this multi-copy NPYq gene plays a key role in regulating spermiogenic gene expression [Cocquet, Ellis, et al.,2009]. No homologues of *Sly* or the other multi-copy mouse NPYq genes have been identified on the human Y chromosome. Nevertheless, multi-copy testis expressed genes are a feature of the human Y [Kuroda-Kawaguchi, Skaletsky, et al.,2001, Skaletsky, Kuroda-Kawaguchi, et al.,2003] and the multi-copy testis specific gene *RBMY* has a multi-copy homologue (*Rbmy*) on the mouse Y short arm [Mahadevaiah, Odorisio, et al.,1998]. Thus, the two species have in common the fact that genes with a presumed or demonstrated spermatogenic role are often amplified on the Y.

DSBs are extremely hazardous lesions that must be repaired before cell division occurs if cell death is to be avoided. It is well established that oocytes have the DNA damage repair machinery in place to repair DSBs once the sperm chromatin begins to decondense as it is deprotaminated [Barton, Robaire, et al.,2007, Brandriff and Pedersen,1981, Derijck, van der Heijden, et al.,2008, Marchetti, Essers, et al.,2007,

Toyoshima,2009]; this initial repair utilizes non homologous end joining (NHEJ) to re-ligate the broken ends [Derijck, van der Heijden, et al.,2008] and our data suggest this mechanism is sufficient to deal with any DSBs in epididymal sperm from controls, even when the numbers are increased by freezing. However, with fresh epididymal sperm from NPYq-deficient mice there is an elevation in the chromosome aberration rate manifested as broken chromosomes and chromosome rearrangements; with freezing this is further increased, leading to elevated levels of chromosome aberrations also in testicular sperm from control and NPYq-deficient males. These chromosome aberrations are manifestations of unrepaired DSBs. Chromosome rearrangements occur when the 'wrong' DNA ends are ligated, and constitute evidence that the original broken ends have not been maintained in juxtaposition; instead there has been a chance juxtaposition of non-matching ends that are then ligated by NHEJ. The chromosome breaks almost certainly represent the separation of broken DNA ends without the chance finding of an alternative partner. How broken DNA ends might be held in juxtaposition in the protaminated chromatin of the sperm head is not known; nevertheless, the data we have obtained suggest that with incompletely protaminated DNA, as in testicular sperm and in epididymal sperm from NPYq deficient mice, the nuclear matrix/protein scaffold holding the DNA ends together is susceptible to disruption by freezing.

What are the broader implications of the present findings? ICSI is widely used to treat human male infertility due to oligozoospermia or teratozoospermia that is usually associated with sperm defects, such as incomplete protamination and incomplete compaction of the chromatin [Carrell, Emery, et al.,2007, Oliva,2006, Ramos, van der Heijden, et al.,2008]. Thus, the severely NPYq deficient mice studied here are a useful

mouse model for assessing the consequences of these defects for the offspring. It is also important to consider the implications of the additional DNA damage that arises as a consequence of freezing in the NPYq deficient mice as well as in the controls. With freezing and NPYq deficiency, almost none of the embryos transferred reached term; this is unsurprising given the level of chromosome breaks and rearrangements generated, which would be expected to cause early embryonic lethality. This raises a number of issues as to how to maximize ICSI success and how to minimize mutational load in the offspring. In the context of ICSI success it seems clear that in situations where there is increasing DNA damage during epididymal transit, as is the case with NPYq deficiency, it is better to use testicular sperm; however, freezing should be avoided. Improved ICSI success with testicular as compared to epididymal sperm has previously been reported for mice heterozygous for two different but semi-identical translocations [Baart, van der Heijden, et al.,2004] and in mice deficient for transition proteins [Shirley, Hayashi, et al.,2004, Suganuma, Yanagimachi, et al.,2005], as well as in infertile men with sperm DNA damage [Greco, Scarselli, et al.,2005]. A caveat is that in the present study sperm freezing was done without cryoprotectant, but with the method developed to keep DNA damage to a level comparable to freezing with cryprotectant [Kusakabe, Szczygiel, et al.,2001]. Indeed, substantial freezing-induced DNA damage has been reported even with mouse sperm frozen with cryoprotectant [Yildiz, Fleming, et al.,2008] and with cryopreserved human sperm [de Paula, Bertolla, et al.,2006, Donnelly, Steele, et al.,2001, Thomson, Fleming, et al.,2009].

As to mutational load, NHEJ is inevitably mutagenic even when the correct partners are re-ligated [Lieber,2008], so it is expected that the mutational load will

increase as the DNA damage increases; this is supported by a recent study of offspring generated by ICSI using frozen-thawed mouse sperm [Fernandez-Gonzalez, Moreira, et al.,2008]. It has also been argued that disturbances of chromatin remodeling can generate 'epi-mutations' that can contribute to the mutational load across generations [de Boer, Ramos, et al.,2010]. Aside from the implications for treating human infertility by ICSI, our findings raise questions as to the mutational load associated with the use of cryopreserved sperm in general. With the extensive single nucleotide polymorphism mapping panels now available that provide sequence-based, genome-wide markers, it should be possible to screen for DNA sequence changes arising as a consequence of sperm freezing.

2.5 Conclusion

We provide the first evidence on sperm DNA damage in conjunction with deletions of the Y chromosome long arm (NPYq) in mice, with support for the underlying mechanism. NPYq-deficient mice serve as a model for human infertility cases due to Y chromosome deletions and/or cases associated with sperm DNA damage and abnormal sperm chromatin compaction. In addition to demonstrating the involvement of NPYq-encoded genes in regulating chromatin remodeling during spermiogenesis, the study also provides important insights into the regulation of sperm DNA integrity after they are released from the testis, in the epididymis and in the oocyte after fertilization. In the context of other recently published work, our study points to there being an increased mutational load across generations as a consequence of assisted reproduction with sperm resulting from defective chromatin compaction during spermiogenesis or sperm subjected to cryopreservation.

CHAPTER 3

DEFICIENCY OF THE MULTI-COPY MOUSE Y GENE *Sly* CAUSES SPERM DNA
DAMAGE AND ABNORMAL CHROMATIN PACKAGING

Abstract

In mouse and man Y chromosome deletions are frequent and associated with spermatogenic defects. Mice with extensive deletions of non-pairing Y chromosome long arm (NPYq) are infertile and produce sperm with grossly misshapen heads, abnormal chromatin packaging and DNA damage. The NPYq-encoded multi-copy gene *Sly* controls the expression of sex chromosome genes after meiosis and *Sly* deficiency results in a remarkable upregulation of sex chromosome genes. *Sly* deficiency has been shown to be the underlying cause of the sperm head anomalies and infertility asoociated with NPYq gene loss, but it was not known whether it recapitulates sperm DNA damage phenotype.

We produced and examined mice with transgenically (RNAi) silenced *Sly* and demonstrated that these mice have increased incidence of sperm with DNA damage and poorly condensed and insufficiently protaminated chromatin. We also investigated the contribution of each of the two *Sly*-encoded transcript variants and noted that the phenotype was only observed when both variants were knocked down, and that the phenotype was intermediate in severity compared with mice with severe NPYq deficiency.

Our data demonstrates that *Sly* deficiency is responsible for the sperm DNA damage/chromatin packaging defects observed in mice with NPYq deletions and point to SLY proteins involvement in chromatin reprogramming during spermiogenesis, probably through their effect on the post-meiotic expression of spermiogenic genes. Considering

the importance of the sperm epigenome for embryonic and fetal development and the possibility of its inter-generational transmission, our results are important for future investigations of the molecular mechanism of this biologically and clinically important process.

3.1 Background

In the previous chapter we have shown that sperm from mice with severe NPYq deficiencies had DNA damage and abnormal chromatin packaging. Epididymal sperm from mice with severe NPYq deficiency (i.e. 9/10NPYq- or NPYq-2) were impaired in oocyte activation ability following ICSI, and there was an increased incidence of oocyte arrest and paternal chromosome breaks. Comet assay revealed higher DNA damage in both epididymal and testicular sperm from these mice relative to controls, with epididymal sperm the more severely affected. Moreover, epididymal sperm from mutant mice also suffered from impaired membrane integrity, abnormal chromatin condensation and suboptimal protamination, making it likely that the increased DNA damage associated with NPYq deficiency is a consequence of disturbed chromatin remodeling during spermiogenesis.

Sly (*Sycp3*-like Y-linked) is present on NPYq in at least 100 copies, 70 of which retain an open reading frame, and encode a protein that is highly expressed in round spermatids, with nuclear localization at spermiogenesis steps 2/3 to 9 [Cocquet, Ellis, et al.,2009] The SLY protein contains a conserved COR1 domain implicated in chromatin binding [Ellis, Clemente, et al.,2005]. The characterization of 'shSLY mice', in which *Sly* expression has been knocked down by transgenically-delivered short hairpin RNAs, showed that *Sly* deficiency is the major underlying cause of the sperm head

anomalies and infertility associated with NPYq deficiency [Cocquet, Ellis, et al.,2009]. This study preceded our finding of chromatin packaging defects and increased DNA damage in NPYq-deficient mice [Yamauchi, Riel, et al.,2010], and it is therefore not known if shSLY mice recapitulated this aspect of NPYq-deficient phenotype.

In the present study we tested the hypothesis that *Sly* deficiency is responsible for the sperm DNA damage/chromatin packaging defects observed in mice with NPYq deletions, and established *Sly* transcript requirements for this phenotype.

3.2 Materials and Methods

The following techniques were performed similarly as described in Chapter 2: gamete collection and embryo culture, epididymal and testicular sperm freezing, intracytoplasmic sperm injection, sperm chromosome analysis, comet assay, and transmission electron microscope analysis.

3.2.1 Plasmid generation and breeding of transgenic mice

Mice shSLY carry a transgene expressing short hairpin RNAs targeting the specific degradation of *Sly* transcripts. Two shSLY lines were analyzed in the present study: shSLY367 (or sh367), which was previously characterized on an MF1 genetic background [Cocquet, Ellis, et al.,2009] and shSLY344 (or sh344; Figure 12). A U6sh344 construct was produced using a PCR-based approach similar to that described in Harper et al. [Harper, Staber, et al.,2005].

The PCR product was cloned into the pCR2.1 vector and sequenced (TOPO TA Cloning, Invitrogen, USA). The U6sh344 cassette was then subcloned into pEGFP-N1 plasmid (Clontech, Mountain View, CA, USA) using *Afl*II restriction sites. The efficiency and specificity of the construct was checked by co-transfection as previously
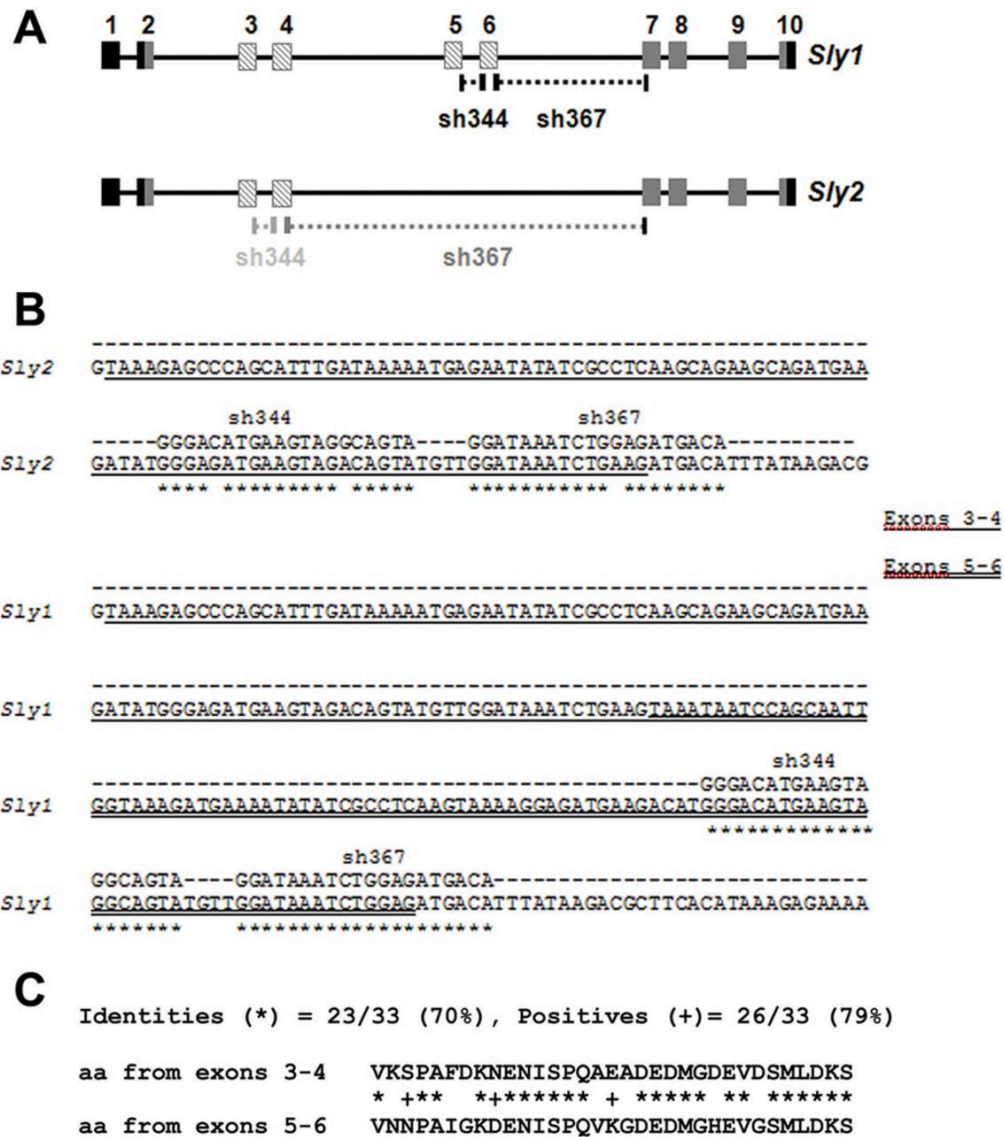
Figure 12. Structure of *Sly* gene. (a) Schematic diagram of the structure of the *Sly* gene (GeneID:100040308) and of its alternative splice variants: *Sly1* and *Sly2*. The boxes numbered 1 to 10 represents exons, with the coding region in gray and alternatively spliced exons in hatched gray. Short hairpin RNAs (sh344 and sh367) were designed to target sequences located in exons 5 and 6 (indicated in black beneath the schematic of the *Sly* gene). Because exons 3-4 share ~86% identity with exons 5-6, sh344 and sh367 can partially recognize sequences in exons 3-4 (indicated in grey beneath the schematic of the *Sly* gene). (b) Alignment of sh344 and sh367 sequences with *Sly1* and *Sly2* cDNA sequences. (c) Alignment of the amino acid sequence derived from exons 3-4 and exons 5-6 of *Sly* gene.

described for other U6shSLY constructs [Cocquet, Ellis, et al.,2009]. The construct was then linearized by *Apa*L1 and *Bsp*H1, on-column purified, and microinjected into fertilized eggs from CBA/Ca6×C57BL/10. Transgenic animals carrying the U6sh344 construct were identified by PCR (Table 3). Both shSLY lines were backcrossed to C57BL/6, to make these mice comparable with NPYq-deficient models used by us before for analysis of sperm DNA damage and chromatin remodeling [Yamauchi, Riel, et al.,2010]. shSLY males on a predominantly C57BL/6 (75–87.5%) genetic background were examined at 2–4 months of age. Non-transgenic siblings served as controls. Female B6D2F1 oocyte donors (National Cancer Institute, Raleigh, NC) for *in vitro* fertilization (IVF) and ICSI were used at 8- to 10-weeks old. The mice were fed *ad libitum* with a standard diet and maintained in a temperature- and light-controlled room (22°C, 14 h light/10 h dark), in accordance with the guidelines of the Laboratory Animal Services at the University of Hawaii and the National Research Council's (NCR) 'Guide for Care and Use of Laboratory Animals'. The protocol for animal handling and treatment procedures was reviewed and approved by the Animal Care and Use Committee at the University of Hawaii.

3.2.2   Western blotting

Western blot analyses were performed as described previously [Reynard, Cocquet, et al.,2009]. Briefly, 10 to 15 µg of testis lysates were run on a 12% SDS/polyacrylamide gel. Following transfer and blocking, membranes were incubated overnight with a primary antibody (anti-SLY1 [Reynard, Cocquet, et al.,2009] at 1:3,000 and anti-β actin at 1:50,000). Incubation with the corresponding secondary antibody

Table 3. Primer sequences

Gene name	Primer name	Primer sequence	Reference
Primers used to produce U6shSLY vector sequences			
U6 Forward primer: 5'-ATCCTCTTAAGTCGACGCCGCCATCTCTAG-3' sh344 Reverse primer: 5'-ATCCTCTTAAGAAAAAAGGACATGAAGTAGGCAGTATCCCATCTGTGGCTTACA GATACTGCCTACTTCATGTCCAAACAAGGCTTTTCTCCAAGG-3' (Riel, Yamauchi et al. 2013) sh367 Reverse primer: 5'-AAAAAAGGATAAATCTGGAGATGACACCCATCTGTGGCTTTACAGTGTCATCTC CAGATTTATCCAAACAAGGCTTTTCTCCAAGGG-3' (Cocquet, Ellis et al. 2009)			
Primers used to identify transgenic animals carrying U6sh344/U6sh367 constructs			
sh344	*sh344-F*	TAGCGCTACCGGACTCAGAT	(Riel, Yamauchi et al. 2013)
	sh344-R	GTCCTCCTTGAAGTCGATGC	(Riel, Yamauchi et al. 2013)
sh367	*sh367-F*	ACGTAAACGGCCACAAGTTC	(Cocquet, Ellis et al. 2009)
	sh367-R	GTCCTCCTTGAAGTCGATGC	
Real-time PCR primers			
Sly Global	*Sly Global-F*	CATTTATAAGACGCTTCACATAAAG	(Cocquet, Ellis et al. 2009)
	Sly Global –R1	TCCTCCATGATGGCTCTTTC	
	Sly Global-R2	ATTCTCCATGATGGCTCTTTC	
Sly Long	*Sly Long F*	GAAGACATGGGACATGAAGTAGG	(Cocquet, Ellis et al. 2009)
	Sly Long R1	TCCTCCATGATGGCTCTTTC	
	Sly Long R2	ATTCTCCATGATGGCTCTTTC	
β-*actin*	*β-actin-F*	GGCACCACACCTTCTACAATG	(Garcia, Collado et al. 2006)
	β-actin-R	GTGGTGGTGAAGCTGTAGCC	
Slx	*Slx-F*	TTCAGATGAAGAAGAAGAGCAGG	(Ellis, Clemente et al. 2005)
	Slx-R	TCCATATCAAACTTCTGCTCACAC	
Slx-like	*SlxL1-F*	TTGGAGGACGCTCATTCTG	(Ellis, Clemente et al. 2005)
	SlxL1-R	ACGACTTGTTGTTGATCATCTCC	
Actrt1	*Actrt1-F*	CTCAAAAATGGTCTGCAACAGC	(Ellis, Clemente et al. 2005)
	Actrt1-R	TCTTGATAGGGGTTCCCTCAAA	
Ssty1	*Ssty1-F*	AGAAGGATCCAGCTCTCTATGCT	(Ellis, Clemente et al. 2005)
	Ssty1-R	CCAGTTACCAATCAACACATCAC	
Ssty2	*Ssty2-F*	CAGGTGCCATTCTTACAGGACTAT	(Ellis, Clemente et al. 2005)
	Ssty2-R	ACCCAGGAACCTATTAAGAAGTCAT	
Asty	*Asty1-F*	GTGGAGTAGAACTCATCATC	(Ellis, Clemente et al. 2005)
	Asty1-R	CAGGAGATGACTAACATAGCA	
Ubb	*Ubb-F*	GAGGGGTGGCTATTAATTATTCG	(Akerfelt, Henriksson et al. 2008)
	Ubb-R	CTAAACTTAAATTGGGGCAAGTG	
Mgclh	*Mgclh-F*	CCTTTACGTGTGACCTTTACCAG	(Ellis, Clemente et al. 2005)
	Mgclh-R	CTGAATATGACATTTCGGATATGGT	
Semi-quantitative PCR primers			
Sly	*Sly-Orf-F*	ATGGCTCTTAAGAAATTGAAGGT	(Riel, Yamauchi et al. 2013)
Sly	*Sly-Orf-R*	TTCTTAGTTCTTGGTCCCCAAGTTC	
β-*actin*	*β-actin-F*	GGCACCACACCTTCTACAATG	(Garcia, Collado et al. 2006)
	β-actin-R	GTGGTGGTGAAGCTGTAGCC	

coupled to peroxidase, and detection by chemiluminescence, were carried out as described by the manufacturer (SuperSignal West Pico, Pierce Biotechnology, Rockford, IL, USA). The analysis of sperm nuclear proteins using acid urea polyacrylamide electrophoresis (AU-PAGE) followed by western blotting were performed exactly as described by us earlier [Yamauchi, Riel, et al.,2010].

3.2.3 Real-time RT-PCR

For real-time reverse transcriptase polymerase chain reaction (RT-PCR), total testis RNA or RNA from round spermatids purified with STAPUT method [Bellve,1993] was extracted using Trizol and DNaseI treatment (Ambion, Austin, TX, USA), and purified using an RNeasy kit (Qiagen, Valencia, CA, USA). Reverse transcription of polyadenylated RNA was performed with Superscript Reverse Transcriptase III, according to the manufacturer's guidelines (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using SYBR Green PCR Master mix on an ABI StepOnePlus machine (Applied Biosystems, Carlsbad, CA, USA). PCR reactions were incubated at 95°C for 10 min followed by 35 PCR cycles (10 s at 95°C and 60 s at 60°C). For analysis of *Sly* expression, two types of PCR reactions were performed: (1) '*Sly1+2*' amplifying both *Sly_v1*and *Sly_v2* transcripts [Reynard, Cocquet, et al.,2009] (primers *Sly* Global) and (2) '*Sly1*' amplifying only *Sly_v1* (primers *Sly* Long). All reactions were carried out in triplicate per assay, and *β-actin* or *Acrv1* (spermatid specific gene not regulated by *Sly*) was included on every plate as a loading control. The difference in PCR cycles with respect to *β-actin* or *Acrv1* (ΔCt) for a given experimental sample was subtracted from the mean ΔCt of the reference samples (non-transgenic siblings) (ΔΔCt). For the quantification of *Sly*knockdown, values were further normalized to ΔΔCt values of the *β-*

53

coupled to peroxidase, and detection by chemiluminescence, were carried out as described by the manufacturer (SuperSignal West Pico, Pierce Biotechnology, Rockford, IL, USA). The analysis of sperm nuclear proteins using acid urea polyacrylamide electrophoresis (AU-PAGE) followed by western blotting were performed exactly as described by us earlier [Yamauchi, Riel, et al.,2010].

3.2.3   Real-time RT-PCR

For real-time reverse transcriptase polymerase chain reaction (RT-PCR), total testis RNA or RNA from round spermatids purified with STAPUT method [Bellve,1993] was extracted using Trizol and DNaseI treatment (Ambion, Austin, TX, USA), and purified using an RNeasy kit (Qiagen, Valencia, CA, USA). Reverse transcription of polyadenylated RNA was performed with Superscript Reverse Transcriptase III, according to the manufacturer's guidelines (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using SYBR Green PCR Master mix on an ABI StepOnePlus machine (Applied Biosystems, Carlsbad, CA, USA). PCR reactions were incubated at 95°C for 10 min followed by 35 PCR cycles (10 s at 95°C and 60 s at 60°C). For analysis of *Sly* expression, two types of PCR reactions were performed: (1) '*Sly1+2*' amplifying both *Sly_v1*and *Sly_v2* transcripts [Reynard, Cocquet, et al.,2009] (primers *Sly* Global) and (2) '*Sly1*' amplifying only *Sly_v1* (primers *Sly* Long). All reactions were carried out in triplicate per assay, and *β-actin* or *Acrv1* (spermatid specific gene not regulated by *Sly*) was included on every plate as a loading control. The difference in PCR cycles with respect to *β-actin* or *Acrv1* (ΔCt) for a given experimental sample was subtracted from the mean ΔCt of the reference samples (non-transgenic siblings) (ΔΔCt). For the quantification of *Sly*knockdown, values were further normalized to ΔΔCt values of the *β-*

53

actin or*Acrv1*. Expression analysis was also done for several X and Y encoded transcripts to test for their upregulation in shSLY mice.

3.2.4 Semi-quantitative RT-PCR

RT-PCR was performed as described for real-time RT-PCR except that *Sly* and *β-actin* were run separately and with cycle number decreased (26 for *Sly* and 24 for *β-actin*) to stop the reaction prior to saturation. The products were electrophoresed, ImageJ software was used to measure band intensity, and *Sly1* and *Sly2* values were normalized to *β-actin*.

3.2.5 Sperm analyses

To analyze sperm number, motility and morphology, cauda epididymal sperm were released into HEPES-CZB, and incubated for at least 10 min at 37°C immediately before analysis. Sperm counts using a hemocytometer were the means of three independent scorings per sample. For analysis of sperm morphology epididymal sperm were stained with silver nitrate as previously described [Mahadevaiah, Odorisio, et al.,1998]. Briefly, the sperm suspension (diluted as necessary with 0.9% NaCl) was smeared on three slides, allowed to dry, fixed in methanol and acetic acid (3∶1), and

stained with silver nitrate. The slides were coded, and 100 sperm heads per slide were viewed at 1000x magnification and scored. Categorization of sperm head morphology was performed as previously described [Yamauchi, Riel, et al.,2009].

3.2.6 CMA3 staining

To indirectly visualize protamine-deficient DNA, CMA3 staining was applied to cryopreserved cauda epididymal sperm as previously described [Simoes, Feitosa, et al.,2009], with modifications. Frozen-thawed sperm were washed with PBS, fixed in

methanol and acetic acid (3 : 1, 5 min, on ice), smeared on microscope slides, and air-dried. The slides were then stained with CMA3 (0.25 mg/ml in McIlvaine buffer, pH 7.0, supplemented with 10 mM $MgCl_2$) for 20 min. The slides were rinsed with buffer and mounted with polyvinyl alcohol mounting medium with DABCO (Sigma, 10981) supplemented with 1.5 µg/ml DAPI. As positive controls we used sperm subjected to deprotamation. To achieve deprotamation sperm were incubated in 5 mM DTT, 0.5% Triton X-100 in PBS for 15 min on ice, then layered on 0.5 ml of 1 M sucrose in 25 mM Tris-HCl, pH 7.4, centrifuged at 3000 *g* for 10 min at 4°C, resuspended with methanol–glacial acetic acid (3 : 1) and fixed for 5 min on ice. Air-dried smears were incubated in 2 M NaCl, 2 mM DTT in H2O for 15 min at room temperature, washed thoroughly with distilled water, air-dried, and stained with CMA3, as described earlier. Microscopy images of the slides were captured using fluorescence Olympus IX81 microscope (Tokyo, Japan) with appropriate filter (460–470 nM), at 400× magnification. For analysis, at least 100 sperm heads of each sample were scored as either positive or negative, followed by measures of fluorescence intensity of each head using MetaMorph Digital Imaging Software (Olympus).

3.2.7   Experimental design and statistical analysis

In the analysis of oocyte activation, oocyte arrest, and incidence of abnormal karyoplates, Mantel-Haenszel χ^2-test, which allows for inter-male variability by keeping the individual male data separate was used for 'within genotype' comparisons and Fisher's exact test was used to assess differences between the genotypes. These two tests were also used to analyze the TEM data. Expression data were analyzed with *t*-test. ANOVA (generalized linear model as provided by NCSS statistical analysis software)

with genotypes, sperm source (epididymis or testis) and sperm status (fresh or frozen) as factors was used for the analysis of comet tail lengths, oocyte activation, arrest and chromosomal integrity, and for chromosome aberration rates. The linear model ANOVA (NCSS) was also used to analyze CMA3 data in epididymal sperm. For comparison of sperm head abnormalities and of sperm motility differences between genotypes were assessed by two-way ANOVA using the GraphPad Prism version 5.0 software. For ANOVA analyses, all percentages were transformed to angles.

The mice of interest in this study were two transgenic *Sly*-deficient genotypes (sh344 and sh367) and their negative siblings as controls. In all experiments except expression analyses the negative siblings of each transgenic line were compared, and if not different were pooled. In order to assess the comet, ICSI and TEM data obtained with *Sly* mice in the context of our earlier data from mice with NPYq deficiencies [Yamauchi, Riel, et al.,2010], we first carried out ANOVA comparisons of the controls from the two studies to see if they were comparable. If this was the case, we then carried out an ANOVA analysis of the NPYq-deficient and *Sly*-deficient genotypes compared with the pooled controls.

3.3 Results

3.3.1 Characterization of a new shSLY line in which *Sly1* but not *Sly2* expression is knockdown

The NPYq specific gene *Sly* encodes two transcript variants, *Sly_v1* and *Sly_v2* [Reynard, Cocquet, et al.,2009], hereafter called *Sly1* and *Sly2* (Figure 12a). Sly1 is a full-length isoform and encodes a ~40 kDa protein (referred to as SLY1), detected by our anti-SLY1 antibody [Reynard, Cocquet, et al.,2009]. Exons 5–6, which arose from a

duplication of exons 3–4 [Ellis, Ferguson, et al.,2007], are spliced out in the *Sly2* isoform.

Our anti-SLY1 antibody does not detect SLY2 protein but *Sly2* can be translated: a

transgene-derived SLY2 protein fused to a FLAG tag was detectable by an anti-FLAG

antibody (Figure 13). We previously reported that, on an MF1 background, transgenic

delivery of *Sly*-specific short hairpin sh367 RNAs led to significant decrease of *Sly* RNA

and protein levels, with both splice variants being affected [Cocquet, Ellis, et al.,2009].

These *Sly*-deficient males presented severe sperm head abnormalities and reduced

fertilizing abilities. Here, we characterized a new line, sh344 (Figure 14a-b), which was

not reported on earlier. As in line sh367, expression of sh344 RNA was associated with a

decrease of global *Sly* transcript level and of SLY1 protein level in the testes: sh367 mice

showed 75% reduction of global *Sly* (*Sly1+2*) and 90% reduction of Sly1 transcripts

while in sh344 the reduction was ~50% of Sly1+2 and ~80% of Sly1 transcripts. No

SLY1 protein was detected in sh367 testes by western blotting, even with prolonged

exposure, whereas in sh344 SLY1 the protein level was decreased by 85% (Figure 14b-

c).

Because exons 3–4 are very similar to exons 5–6 (~86% identity, Figure 12c) we

failed to design real-time PCR primers that were specific to Sly2 transcripts. We

therefore quantified the expression of *Sly1* and *Sly2* transcripts independently with semi-

quantitative RT-PCR, using primers, which span the alternatively spliced exons 5–6.

These experiments demonstrated that, in line sh344, *Sly1* but not *Sly2* transcripts were

knocked down, while both *Sly1* and *Sly2* transcripts were knocked down in line sh367.

Alignment of *Sly2* transcript sequence with sh344 and sh367 RNA sequences shows the
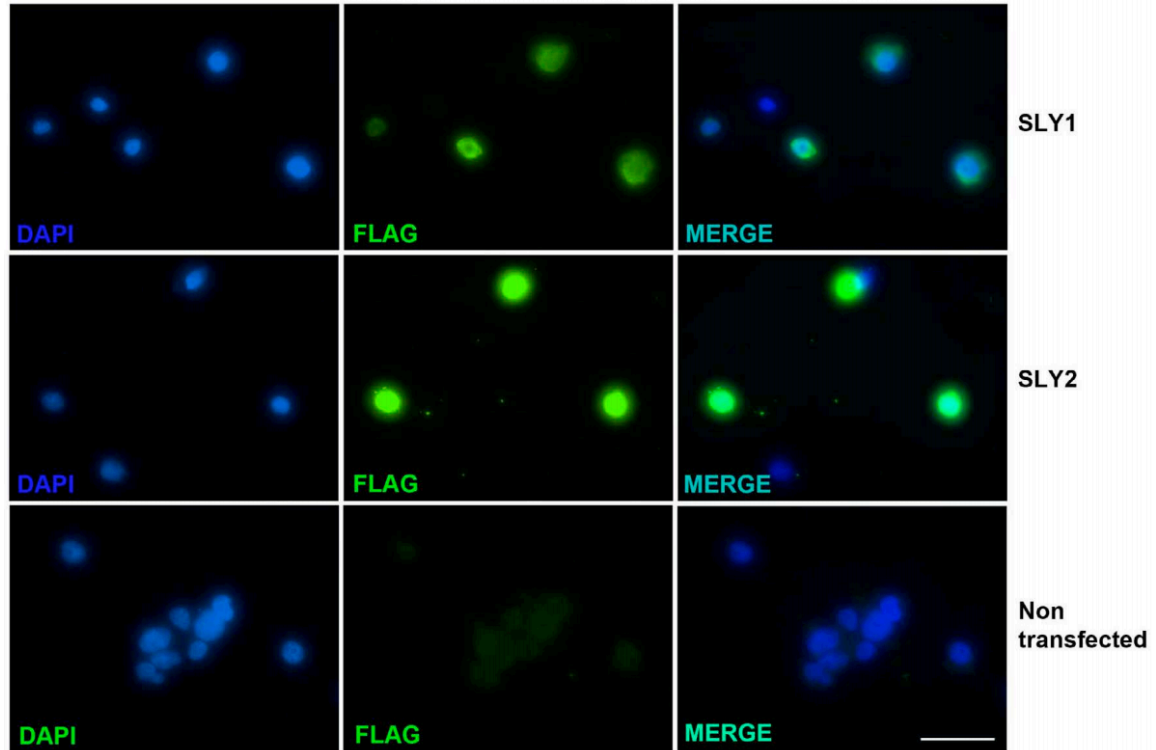
Figure 13. Transgenic SLY expression in transfected cells. HEK293T cells were transfected with a FLAG-*Sly1* and FLAG-*Sly2* constructs and were subjected to immunostaining with an anto-FLAG antibody (green). SLY1 and SLY2 proteins were expressed, evidenced by green fluorescence. The cell nuclei are stained with DAPI (blue). Scale = 50μm.
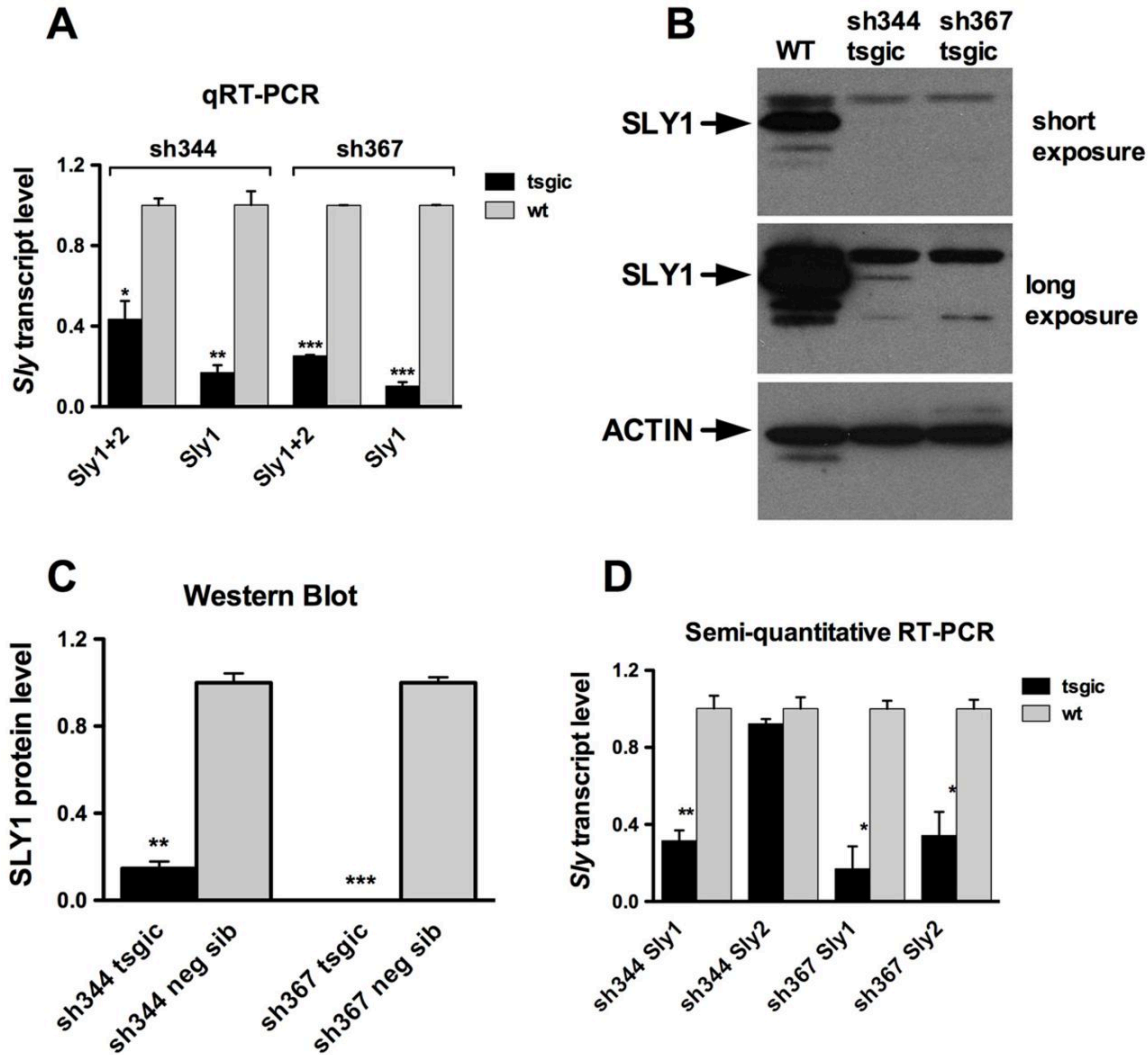
Figure 14. shRNA disrupts *Sly* expression in sh367 and sh344 differently. (a) *Sly* transcripts levels in shSLY transgenic mice (tsgic, *n*=3 for each sh344 and sh367) and controls (neg sib, *n*=2 for each sh344 and sh367) obtained by real-time RT-PCR with actin as a loading control. (b) Western blots of SLY1 protein in testis extract from shSLY transgenic mice and controls with actin as loading control. (c) Levels of SLY1 protein expression in shSLY transgenic mice (tsgic, *n*=4 for sh344 and *n*=3 for sh367) and controls (*n*=2 for sh344 and *n*=3 for sh367) quantified with ImageJ software and normalized to actin signal. (d) *Sly1* and *Sly2* transcript levels in shSLY transgenic mice (*n*=3 for each sh344 and sh367) and controls (*n*=2 for each sh344 and sh367) obtained by semi-quantitative RT-PCR, quantified with ImageJ software and normalized to actin. In A, C and D values are means ± s.e.m. Statistical significance with respect to corresponding control: *P<0.05, **P<0.01, ***P<0.001 (*t*-test). Similar results were obtained for mice on C57BL/6 and MF1 backgrounds. Neg sib, negative siblings.

presence of two mismatches with sh344 sequence while there is only one with sh367

sequence (Figure 12b). Owing to the lack of an SLY2-specific antibody, we could not

measure/observe SLY2 protein level, but one can assume that SLY2 level mimics that

of *Sly2* transcript and, as a consequence, SLY2 protein level is expected to be unchanged

in sh344 testes but decreased in sh367 testes.

These expression analyses demonstrate that, on a predominantly C57BL/6

background, sh367 is deficient for both *Sly1* and *Sly2* (i.e. *Sly*-deficient males) while

sh344 line is only deficient for *Sly1* (i.e. *Sly1*-only-deficient males). To determine the

impact of each *Sly* variant on sperm differentiation, we analyzed and compared the

testicular phenotypes of these two lines.

3.3.2   *Sly*-deficient but not *Sly1*-only-deficient males have increased sperm head shape defects and impaired sperm function *in vitro*

Sly-deficient sh367 mice were previously shown to have impaired sperm function

on MF1 genetic background [Cocquet, Ellis, et al.,2009]. We sought to determine if

C57BL/6 sh367 mice and *Sly1*-only-deficient mice (i.e. sh344) also presented impaired

sperm function. When we assessed fertility *in vitro*, sperm from sh367, but not from

sh344 mice, had significantly impaired ability to fertilize oocytes and those oocytes that

were successfully fertilized exhibited poorer development to blastocyst (Figure 15a). This

sperm dysfunction was not related to decrease in testis size, decrease in cauda epididymal

sperm number or insufficient sperm motility, which were all normal and comparable

between both *Sly*- and *Sly1*-only-deficient lines and controls. Detailed cauda epididymal

sperm head shape analysis differentiating between sperm that were normal, slightly

Sperm number, motility, and ability to fertilize oocytes in vitro	sh367 n=6	sh344 n=3	neg sib n=4
% (No) inseminated oocytes cleaved	11 (31/292)[a]	57 (63/110)[b]	47 (85/180)[b]
% (No) 2-cell developed to blastocyst	68 (21/31)[a]	94 (59/63)[b]	84 (71/85)
Testis weight (mean ± SEM)	95.5 ± 3.8[a]	79.0 ± 2.9[b*]	103.0 ± 8.1[a]
Sperm number / 1 epididymis (x 10^6)	3.4	2.9	2.2
% Motile sperm (total motility)	57.4	54.6	76.8
% Progressively motile sperm	52.8	47.4	69.4
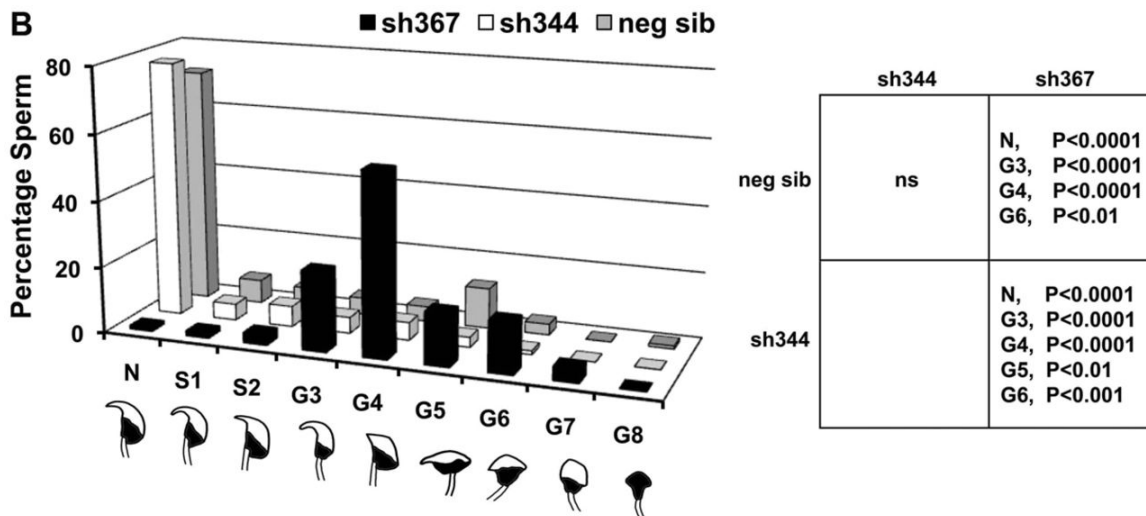
Figure 15. Sperm analyses in *Sly*-deficient mice. The analyses were performed for two transgenic lines with *Sly* deficiency (sh344 and sh367) and non-transgenic siblings controls (neg sib). (a) Analyses of sperm number, motility and ability to fertilize oocytes *in vitro*. Numbers with different superscripts within rows are statistically different (*P*<0.05); *Testes from only two males were weighted. (b) Sperm morphology analysis. Data shown in the *y*-axis are the percentage of sperm analyzed. Three hundred sperm were scored per male, with number of males *n*=3, *n*=3 and *n*=5 for sh344, sh367, and neg sib, respectively. The *x*-axis represents individual categories of sperm head shapes: N, normal; S1–S2, slight defects; G3–G8, gross defects. Examples of each head shape are shown under each sperm morphology designation. The table on the right shows significant differences between the genotypes in the proportion of specific morphology defects (two-way ANOVA). ns, no statistically significant.

deformed, and grossly deformed demonstrated that in *Sly*-deficient sh367 mice virtually all sperm were morphologically abnormal, and had gross head shape defects. However, *Sly1*-only-deficient mice were not significantly different from non-transgenic siblings, both having >85% of morphologically normal sperm (Figure 15b).

To summarize, sperm analyses point to severe sperm head shape defects and impairment in the ability of sperm to fertilize oocytes *in vitro* in *Sly*-deficient, but not in *Sly1*-only-deficient mice.

3.3.3 *Sly*-deficient but not *Sly1*-only-deficient males have increased sperm DNA damage

We have previously shown that testicular sperm from normal wild-type mice have more comet assay detectable sperm DNA fragmentation than cauda epididymal sperm, and that freezing increases DNA damage in sperm from both sources; in mice with severe NPYq deficiencies this sperm DNA fragmentation is markedly increased with epididymal sperm more affected than testicular sperm, and with both types of sperm more susceptible to freezing [Yamauchi, Riel, et al.,2010]. Here, we tested fresh and frozen cauda epididymal and fresh and frozen testicular sperm from *Sly* (sh367)- and *Sly1*-only (sh344)-deficient transgenic lines, with their negative siblings as controls.

As in our previous study, because of the non-normality of the underlying data, we have used male means for the analysis. A preliminary ANOVA analysis revealed no significant differences between sh344 and sh367 negative siblings, so the control data were pooled. We then carried out ANOVA analysis of sh344, sh367, and pooled controls, with genotype, sperm source (testis or epididymis) and sperm status (fresh or frozen) as factors (Figure 16a-c). As in controls described in our previous study, in all genotypes
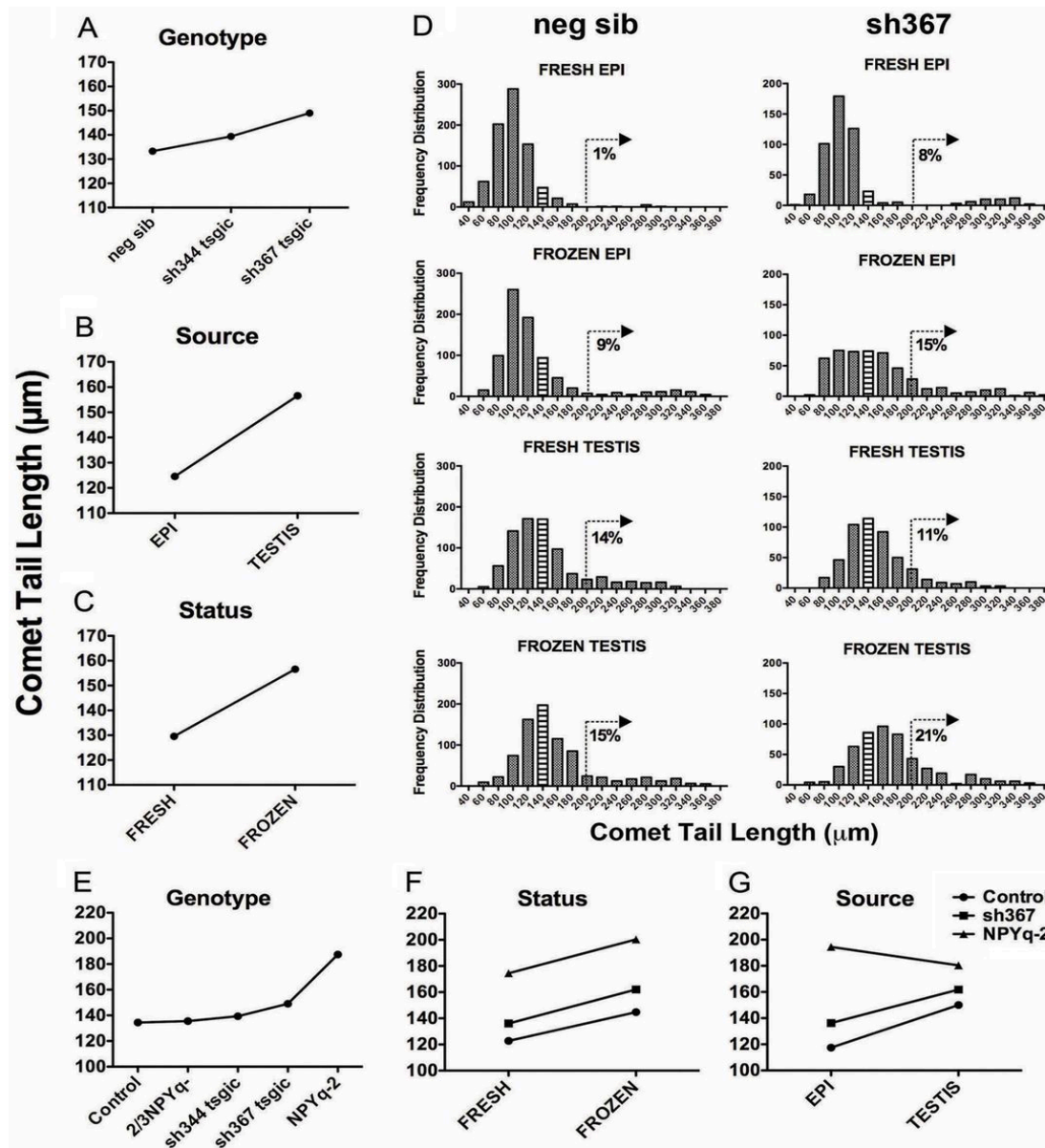
Figure 16. Comet assay analyses of sperm from *Sly*-deficient mice. (a-c) *Sly*-deficient mice, sh344 tsgic and sh367 tsgic, and their non-transgenic siblings (neg sib) were compared for the effects of genotype (a), sperm source (b; epididymal and testicular), and sperm status (c; fresh and frozen). (d) The frequency distribution of all comet tail lengths for each sperm type from neg sib and sh367. (e-g) *Sly*-deficient mouse lines were compared with mice with moderate (2/3NPYq-) and severe (NPYq-2) NPYq deficiency. The controls here were negative siblings of shSLY mice and wild-type controls of mice deficient for NPYq. In f and g, sh344 and 2/3NPYq- data are omitted due to lack of significant difference from controls. The number of males was *n*=8, *n*=10, *n*=3, *n*=5, *n*=2, and *n*=2 for neg, sib, controls, sh344 tsgic, sh367 tsgic, 2/3NPYq- and NPYq-2, respectively; 100 sperm were scored per sperm type (fresh epididymal, frozen epididymal, fresh testicular, frozen testicular) per male. Bin size: 20.

63

there was a significant (p<0.000001) effect of sperm source (testicular sperm more affected than epididymal; Figure 16b) and sperm status (frozen sperm more affected than fresh sperm, Figure 16c). There was also a significant effect of genotype (Figure 16e) *P* p =0.0004) with sh367 mice, but not sh344 mice, having significantly (p =0.00009) longer comet tail lengths than controls. The histograms of the raw data for each sperm type shed light on the nature of sperm DNA damage revealed by the ANOVA analysis (Figure 16d). In the controls the baseline is provided by fresh epididymal sperm, in which 99% (792/800) of sperm fall in a normally distributed population of sperm with comet tails in the range 41–188 μm, and the remaining 1% (8/800) with comet tails ranging from 210 to 308 μm, indicating severe sperm DNA fragmentation. Therefore, as a yardstick for increases in sperm DNA damage for the remaining distributions we have calculated the percentage of sperm with comet tail lengths exceeding 200 μm. Freezing the epididymal sperm shifts the entire distribution a little to the right, and increases the proportion of sperm with severe DNA damage; as a consequence 9% (69/800) of sperm now have comet tail lengths exceeding 200 μm. Comparison of distributions of fresh testicular sperm with fresh epididymal sperm (14%, 113/800 versus 1%, 8/800 >200 μm) indicates a more marked shift of the distribution to the right but a less marked increase in sperm with the highest DNA damage. In agreement with the ANOVA analysis, sperm in sh367 mice are more affected than in controls resulting in further increases in the proportion of sperm with comet tails >200 μm in all categories except fresh testis; nevertheless, for fresh testis it is apparent that the 'normal' part of the distribution has moved to the right which is consistent with the ANOVA result (Figure 16d).

Next, we compared *Sly*-deficient mice with mice with moderate (2/3NPYq-) and severe (NPYq-2) NPYq deficiency (Figure 16e-g). The controls in this comparison were negative siblings of sh344 and sh367 mice and wild-type controls of NPYq-deficient mice; the controls did not differ from each other and were therefore pooled. ANOVA analysis demonstrated a significant (p <0.000001) effect of genotype (Figure 16e) with sh367 and NPYq-2, but not 2/3NPYq- and sh344, mice significantly different from controls. In both *Sly* and NPYq-deficient mice, there was a significant effect of sperm status (Figure 16f; p <0.000001) with frozen sperm having significantly longer comet tail lengths than fresh; the same effect was seen in controls. However, while sh344, sh367 and control mice had overall longer comet tail lengths in testicular sperm than in epididymal sperm, this was not true for NPYq-2 mice, in which epididymal sperm were more affected (Figure 16g; genotype/source interaction *P* p =0.0007).

Overall, comet assay analysis points to *Sly*-deficient but not *Sly1*-only-deficient mice having an increased susceptibility to sperm DNA damage. However, this DNA damage is less severe than in mice with complete NPYq deficiency, and is not preferentially affecting epididymal sperm as was observed in severely NPYq-deficient mice.

3.3.4 *Sly* deficiency is not associated with impairment of early fertilization steps after ICSI and does not lead to increased incidence of paternal chromosome breaks in the zygotes

We have previously shown that when cauda epididymal sperm from mice with severe NPYq deficiencies were injected into the oocytes by ICSI, there was an impairment in oocyte activation, increased incidence of oocyte arrest at the pronuclei

stage, and presence of paternal chromosome breaks in the zygote [Yamauchi, Riel, et al.,2010]. Here, following the design of the previous study, we injected fresh and frozen cauda epididymal and fresh and frozen testicular sperm from *Sly* (sh367)- and *Sly1*-only (sh344)-deficient mice (and transgene-negative siblings serving as controls), into the oocytes and examined early post-fertilization steps.

No significant differences between the same sperm types from *Sly*- and *Sly1*-only-deficient mouse lines and controls, and between epididymal and testicular sperm regardless of whether sperm were fresh or frozen ('within genotype' comparison) were observed in incidence of oocyte activation, oocyte arrest, and oocytes with normal paternal chromosomes. We also carried out a single analysis of *Sly*-deficient mice and controls by ANOVA with genotype, sperm source (testis or epididymis) and sperm status (fresh or frozen) as factors. In all measured end points there was no effect of genotype.

We then placed the data obtained with sh367 and sh344 mice in perspective of data obtained earlier with NPYq-deficient mice [Yamauchi, Riel, et al.,2010] pooling the data obtained after ICSI with four types of sperm, and using as a control pooled controls from both studies. Single ANOVA analysis demonstrated a significant (p <0.001) effect of genotype, with sh367, sh344 and 2/3NPYq mice being no different from controls and significantly less affected than mice with severe NPYq- deficiency (9/10NPYq- and NPYq-2) in incidence of arrested oocytes, oocytes with normal paternal chromosomes and aberration rate.

To assess whether lack of 'ICSI phenotype' in *Sly*-deficient mice was due to milder sperm head shape defects and unintentional preselection during injection, we

compared sh367 frozen cauda epididymal sperm purposely preselected according to their head shape into two groups: sperm with slight and gross (G4–6 shown in Figure 16b) head shape defects. No differences in the incidence of oocytes activated, arrested, and with normal paternal chromosomes were noted after injection of sperm with slight versus gross head shape defects (Fisher's exact test).

Overall, ICSI data imply that contrary to what is seen in mice with severe NPYq deficiency, *Sly* knockdown does not impair sperm function in assisted fertilization. Sperm from *Sly*-deficient mice activate oocytes normally yielding no chromosome damage in the ICSI-generated zygotes.

3.3.5 *Sly* deficient but not *Sly1*-only-deficient mice have poor sperm chromatin condensation

Transmission electron microscopy was used to examine chromatin compaction in cauda epididymal sperm from sh367 *Sly*-deficient mice, sh344 *Sly1*-only-deficient mice, and their negative siblings as controls (Figure 17). Sperm were categorized into three categories: those with properly condensed, slightly decondensed, and severely decondensed chromatin (Figure 17a-c). In controls and sh344 mice the vast majority of sperm had properly condensed chromatin (87% and 77%); less than 1% of sperm had severely decondensed chromatin. In sh367 males, sperm with properly condensed chromatin were significantly less frequent (50%) and the incidence of sperm severely affected increased to 3.4%. Single ANOVA analysis comparing the incidence of sperm with properly condensed chromatin in these three genotypes revealed that sh367 mice were significantly different from control (p =0.00002) and from sh344 (p =0.003; Figure 17d).
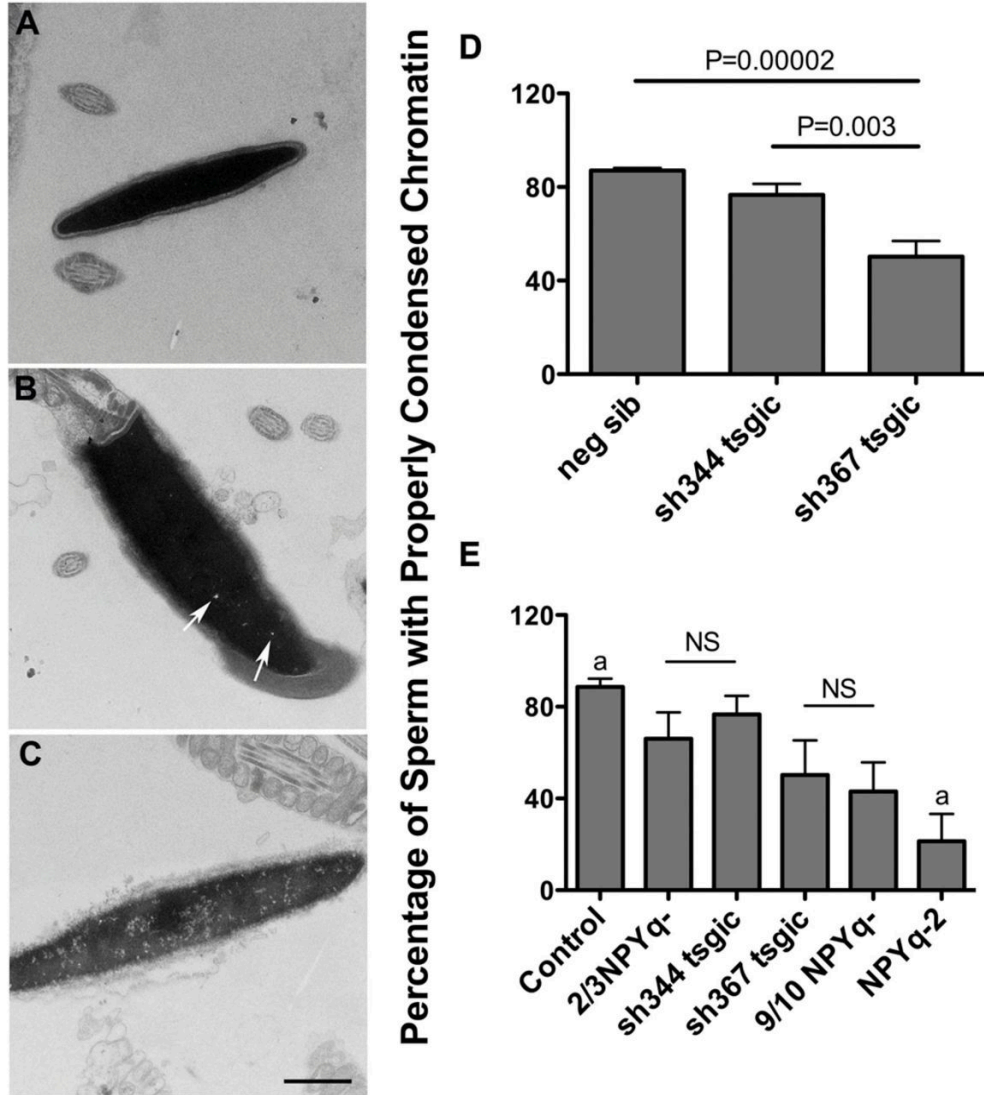
Figure 17. Transmission electron microscopy analysis of chromatin condensation in sperm from *Sly*-deficient mice. (a-c) shows examples of levels of chromatin condensation: (a) properly condensed chromatin; (b,c) slightly (arrows) and severely decondensed chromatin, respectively. (d-e) Two transgenic mouse lines with *Sly* deficiency (sh367 and sh344) and their negative siblings serving as controls were compared in single ANOVA analysis, revealing that sh367 had significantly less sperm with properly condensed chromatin than controls and sh344 (d). When *Sly*-deficient mice were put in the context of mice with moderate (2/3NPYq-) and severe (9/10NPYq- and NPYq-2), single ANOVA analysis showed a significant effect of genotype (p<0.000001), with decrease in proportion of sperm with properly condensed chromatin in order: control>2/3NPYq-=sh344>sh367=9/10NPYq->NPYq-2 (e). Statistical significance: a=different (*P*<0.05) from all other genotypes; NS=non significant. Values are means ± s.e.m. Number of males examined: *n*=7, *n*=3, *n*=5, *n*=7, *n*=13, *n*=3, *n*=2, *n*=3 for neg sib, sh344, sh367, control, 2/3NPYq-, 9/10NPYq-, and NPYq-2, respectively; 100 sperm heads were scored per male. Scale bar: 1 μm.

When the data obtained with *Sly-* and *Sly1-*only-deficient mice were put in the context of data obtained before with sperm from mice with NPYq deficiencies (Yamauchi et al., 2010), single ANOVA analysis showed a significant effect of genotype (p<0.000001; Figure 17e). All tested genotypes were significantly different than controls, which in this analysis were pooled controls from both studies. However, a clear gradation in chromatin condensation deficiency could be observed, with sh344 mice being similar to mice with a moderate NPYq gene loss (2/3NPYq-), sh367 mice being similar to those with extensive NPYq deletion (9/10NPYq-), and mice lacking the entire NPYq gene complement being most affected.

Overall, the data point to *Sly-*deficient but not *Sly1-*only-deficient mice exhibiting impairment in sperm chromatin condensation.

3.3.6   Mice with severe NPYq deficiency and *Sly-*deficient mice have impaired sperm chromatin protamination

Poor chromatin condensation can result from insufficient DNA protamination. Chromomycin A3 (CMA3) is a fluorescent dye that intercalates into DNA but because of its size it can only bind to sperm DNA when it is not completely condensed and supposedly loosely bound to DNA binding proteins. Therefore, CMA3 staining has been used as an indirect measure of the level of protamination in sperm, in humans, mice and other species [Bianchi, Manicardi, et al.,1993, Delbes, Hales, et al.,2007, Noblanc, Peltier, et al.,2012, Simoes, Yamaguti, et al.,2009] Here, we used this approach to examine cauda epididymal sperm from *Sly-* and *Sly1-*only-deficient mice and their negative siblings as controls (Figure 18). Because CMA3 staining was not included in our
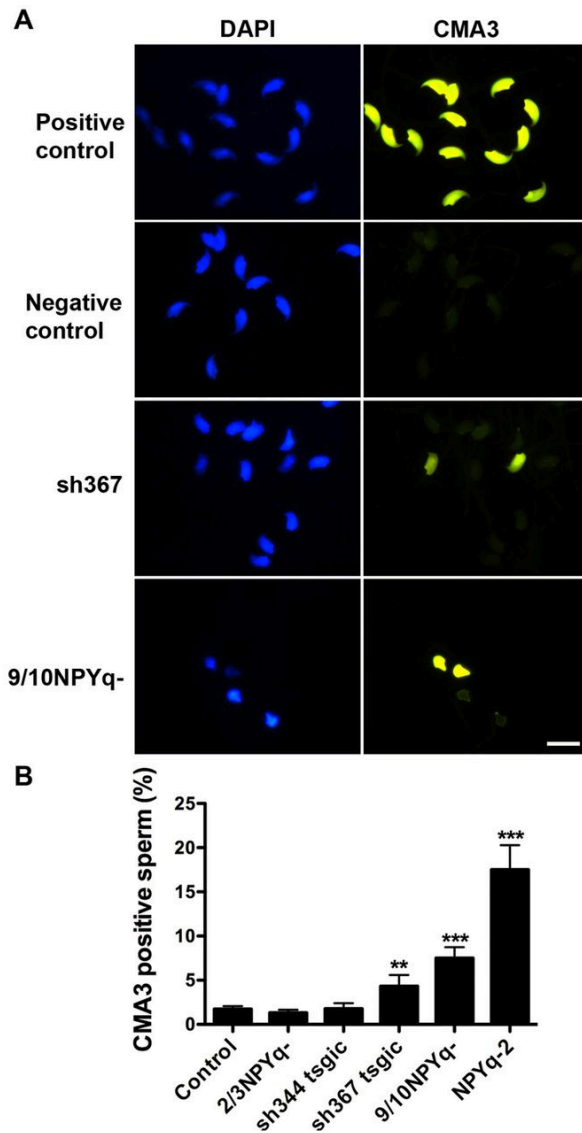
Figure 18. CMA3 staining of sperm from *Sly*-deficient and NPYq-deficient mice. (a) Examples of CMA3 staining (yellow) of epididymal sperm from *Sly*-deficient mice (sh367) and mice with severe NPYq deficiency (9/10NPYq-); the presence of CMA3-positive sperm indicates that these mice have impaired sperm chromatin protamination. Positive controls were sperm treated with 2 mM DTT/0.5% Triton X-100 to reduce disulfide bridges and destabilize sperm chromatin. Negative controls were sperm from negative siblings of sh367 mice. DAPI (blue) was used to stain nuclei. Scale bar: 10 μm. (b) Two *Sly*-deficient lines (sh344 and sh367) and mice with moderate (2/3NPYq-) and severe (9/10NPYq- and NPYq-2) NPYq deficiency were compared to controls. The control represent negative siblings of *Sly*-deficient males and wild-type controls of NPYq-deficient mice, which were not different from each other and were therefore pooled. 100 sperm were scored per male, and the number of males examined per genotype was: $n=27$, $n=9$, $n=6$, $n=3$, $n=6$, $n=6$ for controls, sh344, sh367, 2/3NPYq-, 9/10NPYq-, and NPYq-2, respectively. Statistical significance: **$P<0.01$; ***$P<0.001$ versus control. Values are means ± s.e.m.

previous analysis of NPYq-deficient mice (Yamauchi et al., 2010), we also tested three

NPYq-deficient genotypes and their specific controls. The proportion of CMA3 positive

sperm was similar among the controls justifying pooling for the next comparisons. Single

ANOVA analysis on pooled controls and all *Sly*- and NPYq-deficient genotypes (Figure

18b) revealed a significant effect of genotype (p <0.0001) with sh367 (p =0.0098),

9/10NPYq- (p =0.000013) and NPYq-2 (p <0.000001) having more CMA3 positive

sperm when compared to controls. Mice with moderate NPYq gene loss (2/3NPYq-) and

sh344 mice were not significantly different from controls, and were significantly less

affected than the remaining three genotypes. The results from this multi-genotype

ANOVA were further supported by one-to-one ANOVA comparisons of each genotype

with its specific control. In this analysis sh344 and 2/3NPYq- were not different from

their specific controls, but sh367 (p =0.017), 9/10NPYq- (p =0.0007) and NPYq-2 (p

=0.0001) were.

In support for CMA3 analysis we also examined protamine processing in cauda

epididymal sperm from sh367 mice using acid-urea gel electrophoresis and western

blotting (Figure 19), following the strategy described earlier for NPYq-deficient mice

[Yamauchi, Riel, et al.,2010]. Separated sperm nuclear proteins corresponding to the

same sperm number were assessed on Coomassie-Blue-stained gels and by immunoblot

with Hub2B and PreP2 antibodies recognizing mature and immature protamine P2 forms,

respectively. The analysis was performed with two-way ANOVA, with gel and genotype

as factors. We did not observe significant differences between *Sly*-deficient and control

mice in the intensities of bands representing mature protamines. No preP2 bands

originating from sperm were detected on any of Coomassie-Blue-stained gels. When the
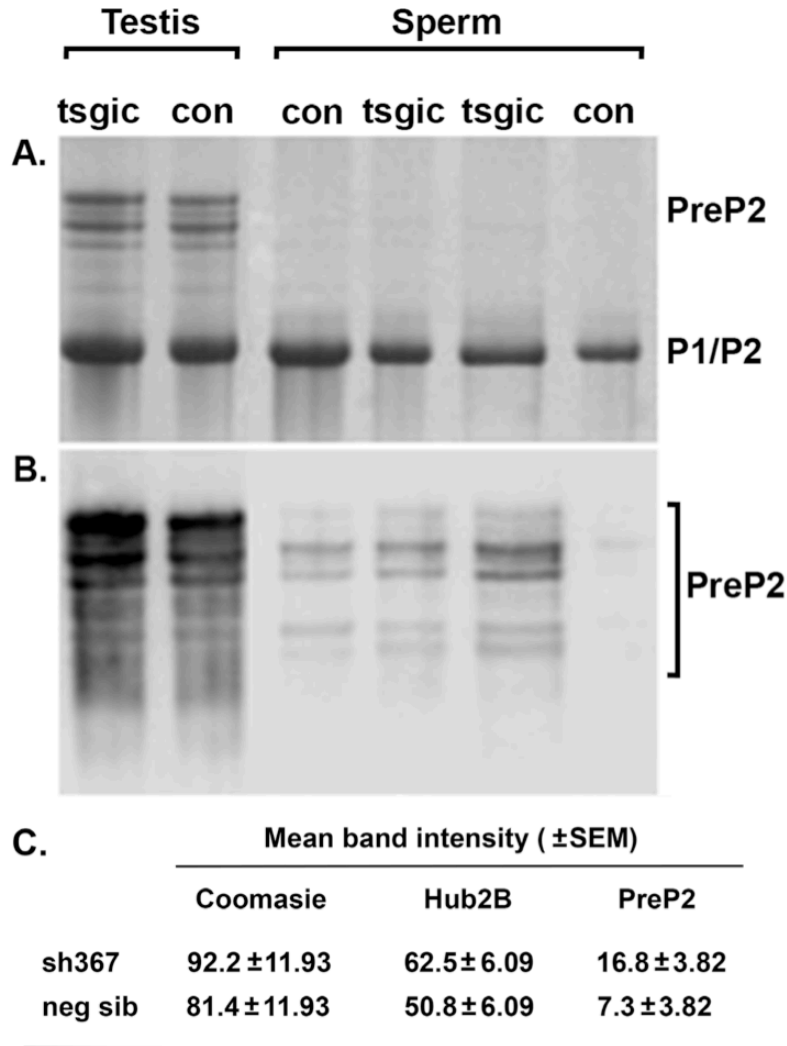
Figure 19. Sperm nuclear protein analysis. Acid-urea gel seperation of nuclear proteins extracted from cauda epididymal sperm corresponding to the same sperm number, and testes from *Sly*-deficient (tsgic) and non-transgenic (con) serving as controls. (a) Coomasie blue stained gel. (b) Immunoblot with preP2 antibody recognizing PreP2. (c) Mean intensity of bands represent mature protamines (Coomasie and Hub2B) and immature protamines (preP2). The analysis was performed with two-way ANOVA, with gel and genotype as factors, from which the genotype means and errors were derived. No statistically significant differences between the genotypes were noted. P1 and P2 is protamines 1 and 2, respectively; PreP2 is premature forms of protamine 2 and the antibody recognizing them, Hub2B is the antibody recognizing mature protamine 2 (gel not shown due to accidental photo loss). Three independent gels were run and 5 males were tested per genotype. No preP2 bands originating from sperm were detected on any of Coomassie blue stained gels. When the gels were blotted with anti-preP2 antibody, bands were detected in sperm from 4 out of 5 *Sly*-deficient males and in 2 out of 5 control males.

gels were blotted with anti-preP2 antibody, bands were detected in four out of five *Sly*-deficient and two out of five control males. Although the difference in average preP2 band intensity did not reach significance, it was more than twice higher in sh367 mice.

Overall, mice with severe NPYq deficiency had significantly increased incidence of CMA3 positive sperm suggesting impaired protamination; *Sly*-deficient mice, but not *Sly1*-only-deficient mice, exhibited the same phenotype but were less severely affected. Impaired protamination in sperm from *Sly*-deficient mice was also shown directly using nuclear protein analysis but, as in CMA3 analysis, it was much less severe than that noted before for mice with severe NPYq deficiency [Yamauchi, Riel, et al.,2010].

3.3.7 *Sly1*-only-deficient males have a minor de-repression of X-and Y-encoded spermiogenic genes compared to global de-repression in *Sly*-deficient males

Sly-deficient males display a remarkable upregulation of sex chromosome genes after meiosis and we have proposed that the multiple defects associated with *Sly* deficiency (head shape malformation, impairment of sperm function, etc.) may likely be a consequence of the massive and global upregulation of spermiogenic genes [Cocquet, Ellis, et al.,2009]. Since *Sly1*-only-deficient males do not display any of the above-mentioned defects we have analyzed the level of expression of several X and Y spermiogenic genes. Results presented in Figure 20 show a de-repression of some of the X and Y encoded genes analyzed in *Sly1*-only-deficient males but this de-repression is significantly less pronounced than in *Sly*-deficient males. Microarray analysis validated the qRT-PCR data, with sh344 males showing the same widespread overexpression of X-

linked spermatid genes previously seen in sh367 [Cocquet, Ellis, et al.,2009] but to a

much lesser extent.
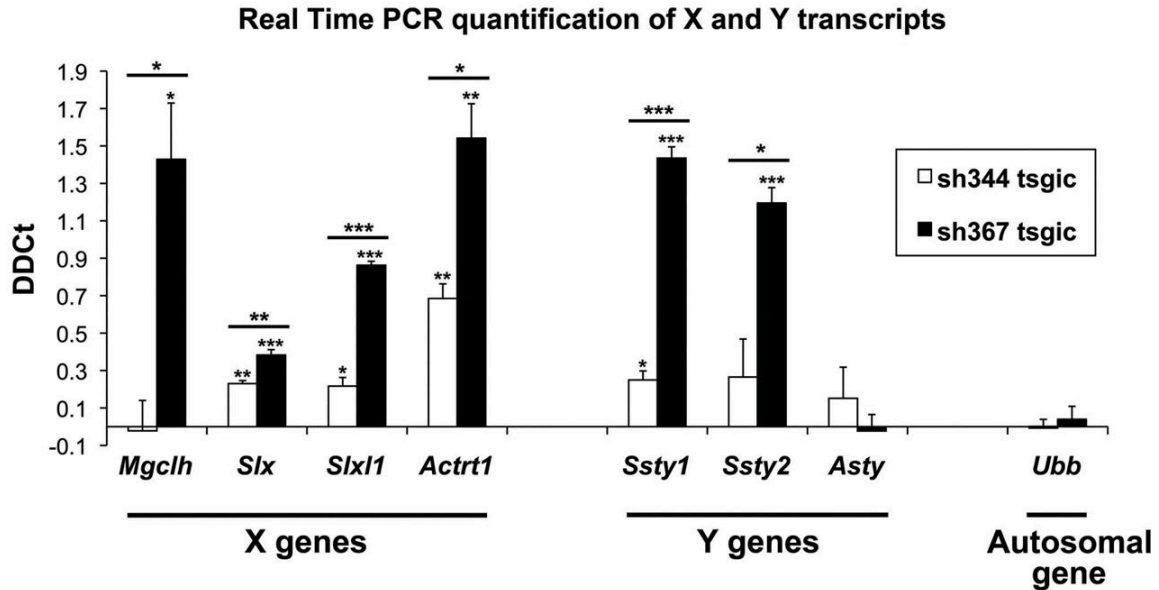
Figure 20. X-Y gene upregulation in *Sly*-deficient mice. Gene expression in testis from two *Sly*-deficient lines (sh344 and sh367) was analyzed by real-time PCR. Values plotted are $\Delta\Delta$Ct ± standard errors. The difference in PCR cycles with respect to the spermatid-specific gene *Acrv1* (ΔCt), the expression of which is not regulated by *Sly*, for a given experimental sample was subtracted from the mean ΔCt of the reference samples (negative siblings of each transgenic line) ($\Delta\Delta$Ct). Three to five animals were analyzed per genotype. Asterisks indicate significant difference between sh344 and sh367 or between *Sly*-deficient mice and control values (*t*-test) with *$P<0.05$, **$P<0.01$ and ***$P<0.001$. Primer sequences are shown in Table 3.

3.4 Discussion

Even relatively minor errors in chromatin remodeling during spermiogenesis are associated with sperm DNA damage and infertility, yet little is known about the etiology. Mice with severe NPYq deletions are infertile due to severe sperm differentiation defects [Ward and Burgoyne,2006, Yamauchi, Riel, et al.,2009]. We have recently observed that sperm from these mice presented abnormal chromatin packaging and DNA damage. Moreover, when these sperm were injected into the oocytes, a significant increase of oocyte arrest at pronuclei stage and of chromosome aberrations in the fertilized eggs were noted [Yamauchi, Riel, et al.,2010]. Here we provide evidence that the deficiency of NPYq encoded gene *Sly* is associated with sperm DNA damage and poor sperm chromatin condensation, and propose that SLY plays a role in spermatid-specific chromatin remodeling.

Sly genes are estimated to be present in between 70 and 100 copies in the mouse genome; the majority of these encode two transcript splice variants, *Sly1* and *Sly2* [Cocquet, Ellis, et al.,2009, Ellis, Bacon, et al.,2011, Scavetta and Tautz,2010]. In the present study, we have produced and characterized a *Sly1*-only-deficient mouse model, and shown that, in contrast to *Sly*-deficient mice, in which both transcript variants are affected, *Sly1*-only-deficient mice do not have sperm differentiation defects. From our analyses, we can draw two hypotheses to explain the phenotypic differences between those two models. *Sly2* may be the important functional variant and its knockdown would be solely responsible for the de-repression of spermiogenic genes and the various subsequent sperm defects observed in *Sly*-deficient mice, or it is the global reduction of both *Sly* variants that matters. Several arguments can be put forward against the former

scenario. First, SLY1 protein co-localizes with the loci that are deregulated in *Sly*-deficient males [Cocquet, Ellis, et al.,2009] and, therefore, is likely to play a role in the regulation of these loci. Second, SLY1 protein structure is very similar to that of SLY2. The COR1 domain, which is presumed to mediate chromatin binding, is identical in SLY1 and SLY2 proteins. SLY1 only differs from SLY2 in the 34 additional amino acids encoded by exons 5–6. Since exons 5–6 arose from a duplication of exons 3–4, the protein region encoded by exons 5–6 shares 70% identity and 79% similarity with the protein region encoded by exon 3–4 (c). All in all, it is more plausible that *Sly1* and *Sly2* genes encode proteins of similar function, and that the phenotype is correlated with the global level of knockdown of *Sly1* and *Sly2*. Our *Sly*/SLY level quantification on whole testes confirms this assumption, with a lower reduction of global *Sly* (*Sly1+2*) transcript level and retention of some of SLY1 protein in phenotypically normal *Sly1*-only-deficient males, and a higher reduction of global *Sly* (*Sly1+2*) and no detectable SLY1 in affected sh367 *Sly*-deficient males.

With respect to sperm chromatin packaging/DNA damage *Sly*-deficient mice recapitulate the NPYq-deficient phenotype only very moderately and much less significantly than other phenotypic features (Table 4). Moreover, *Sly* deficiency is not associated with impairment of early fertilization events after ICSI and does not lead to increased incidence of paternal chromosome breaks in the zygotes that are present in severely NPYq-deficient mice, and this is not due to preselection of sperm with milder head shape defect. The difference in *Sly* transcript levels between mice with severe NPYq gene loss, i.e. deletion removing nine-tenths (9/10NPYq-, 4%) or the entire NPYq (NPYq-2, 0%), and *Sly*-deficient sh367 mice (25%) could explain the differences in the

Table 4. Phenotype characteristics of NPYq- and *Sly*-deficient mice

Phenotypic effects	sh344	sh367	2/3NPYq-	9/10NPYq-	NPYq-2
Sex chromosome gene upregulation	*	***	**	***	****
In vivo fertility	=	***	*	****	****
In vitro fertility	=	***	**	****	****
Sperm number	=	=	=	**	**
Sperm motility	*	*	*	**	**
Sperm head morphology	*	**	*	***	****
Chromatin fragmentation in comet assay	=	*	=	***	****
Oocyte activation in ICSI	=	=	=	***	****
Oocyte arrest in ICSI	=	=	=	***	****
Paternal chromosome breaks in zygotes after ICSI	=	=	=	***	****
Chromatin condensation in TEM	=	**	*	**	***
Chromatin packaging in CMA3 staining	=	**	=	***	****

ICSI, intracytoplasmic sperm injection; TEM, transmission electron microscopy.
Estimated levels of impairment: = not affected; * slightly affected; ** moderately affected; *** severely affected; **** very severely affected. The levels of phenotypic effects of NPYq/*Sly*-deficient mice presented in this table are based on the present study and on our unpublished data, as well as on several previously published manuscripts from our group and few others (Cocquet et al., 2009; Ellis et al., 2005; Grzmil et al., 2007; Reynard et al., 2007; Styma et al., 2002; Styma et al., 2003; Touré et al., 2005; Touré et al., 2004; Ward and Burgoyne, 2006; Xian et al., 1992; Yamauchi et al., 2010; Yamauchi et al., 2009) and therefore should be considered an estimate.

phenotpe in these mice. However, lack of detectable SLY1 protein in all genotypes and similar *Sly1* transcript levels in *Sly*-deficient and 9/10NPYq- mice (10% both) put the dependence of the phenotype exclusively on *Sly*/SLY levels in question. Mice with severe NPYq deficiency lack other genes encoded on the Y long arm, which could contribute to spermiogenic defects in these mice. It, therefore, cannot be excluded that these other NPYq genes, which are not only present but also upregulated in *Sly*-deficient mice [Cocquet, Ellis, et al.,2009, Ellis, Clemente, et al.,2005], mitigate the sperm chromatin packaging/DNA damage phenotype and/or are involved in early embryonic events, either directly or indirectly through the regulation of other loci. The most plausible gene candidate would be *Ssty1/2* (Spermatid-specific transcripts Y-encoded), which is present on the mouse NPYq in more than 100 copies, is specifically transcribed in the testis in round spermatids, and is the only other NPYq gene that is translated. *Ssty1/2* belongs to the *Spindlin* family, one member of which has recently been shown to bind to methylated histone H3 [Wang, Zhou, et al.,2011]; *Ssty1/2* could therefore contribute to chromatin remodeling during spermiogenesis, along with *Sly*. Future work utilizing *Sly* and/or *Ssty1/2* transgene additions to severely NPYq-deficient mice should help clarifying the contribution of *Sly* and *Ssty1/2*.

CHAPTER 4

MILDER PHENOTYPE OF MICE WITH SLY DEFICIENCY CAN BE DUE TO
RETENTION OF RESIDUAL SLY

4.1 Background

To assess which of the NPYq genes is responsible for the infertile phenotype

associated with NPYq deficiency mice were produced, in which the function of NPYq

encoded *Sly* has been disrupted by a transgenically delivered siRNA. The characterization

of these shSLY mice revealed presence of sperm head anomalies, infertility, chromatin

packaging defects and increased sperm DNA damage similar to that noted in NPYq

deletion mutants but less severe. If sperm abnormalities in NPYq deficient mice are

solely a consequence of *Sly* deficiency, then there should be a correlation between the

extent of *Sly* reduction and the severity of sperm defects. *Sly* transcript levels correlated

well with the phenotype. However, the analysis of SLY protein expression was hampered

by lack of a suitable antibody; previously used serum recognized only one of the two

existing SLY isoforms. It was therefore not possible to conclude whether milder

phenotype of shSLY mice was due to insufficient *Sly* knockdown, involvement of

another NPYq gene, or both.

To address this we performed two separate experiments. First, we developed a

new anti-SLY antibody that would recognize both SLY1 and SLY2 protein. This was

geared to help us determine the role of both SLY isoforms and its contributions to

phenotypes examined in our NPYq deficient mice. Secondly, we are carrying out ICSI-

mediated transgene rescue. We created transgenic mice that overexpress SLY proteins in

order to see if transgenic expression of SLY in the context of severely NPYq deficient

genotype will rescue the infertile phenotype of these mice.

4.2 Materials and Methods

  The following techniques were performed similarly as described in Chapter 3: western blotting and real-time RT-PCR.

4.2.1 SLY antibody production and maintenance

  A ClustalW alignment was performed on the amino acid sequences for SLY1, SLY2, and SLX (SLX is a homologous gene encoded on the X chromosome). The amino acid sequence VKSPAFDKNENISPQ (Figure 21), was chosen to act as an antigen that would allow to produce an antibody that specifically targets SLY proteins. Three mice were immunized with the antigen and blood was collected a different time points and tested via ELISA to determine if there was an immune response to the peptide sequence. The spleens from these mice were collected and prepared for fusion with myeloma cells to form hybridomas. The mixture of cells was diluted and clones were grown from single parent cells on microtitre wells. The antibodies that were secreted by the different clones underwent ELISA assays, and lines that gave the strongest signal were selectively grown. Two cell lines producing monoclonal SLY antibodies were established and maintained. Western blots and immunofluorescence analyses of HEK 293T cells transfected with SLY/X constructs were performed to verify that the antibodies recognize specifically SLY1/2 and not SLX.

  Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) was used to maintain and culture hybridomas. Additional medium was added on a daily basis as the hybridomas continued to replicate.

```
SLY1    ----------MALKKLKVIP--------------KEGYLLLLDFDDEDDDIKVSEEA    33
SLY2    ----------MALKKLKVIP--------------KEGYLLLLDFDDEDDDIKVSEEA    33
SLX     ----------MSIKKLWVIP-------------KDGYLLLLDYDSDEE----EEQA    29

SLY1    LSEVKSPAFDKNENISPQAEADEDMGDEVDSMLDKSEVNNPAIGKDENISPQVGDEDMG   93
SLY2    LSEVKSPAFDKNENISPQAEADEDMGDEVDSMLDKSE---------------------   70
SLX     HSEVKPAFGKHENMPPHVEADEDIRDEQDSMLDKSGENVSFSEEWQRFARSVETPMEN-   88

SLY1    HEVGSMLDKSGDDIYKTLHIKRKWMETYVKESFKGSNQKLERFCKTNERERKNINNKFCE   153
SLY2    -----------DDIYKTLHIKRKWMETYVKESFKGSNQKLERFCKTNERERKNINNKFCE   119
SLX     ------WNLLSGEQQVRNASELDLMEVQNPVTHDDGNANPEEVVGDT---RKKINNKLCE   139

SLY1    QYITTFQKSDMDVQKFNEEKEKSVNSCQKEQQALKLSKCSQNQTLEAVKEMHEKSMEVLM   213
SLY2    QYITTFQKSDMDVQKFNEEKEKSVNSCQKEQQALKLSKCSQNQTLEAVKEMHEKSMEVLM   179
SLX     Q------KFDMDIQKFNEEQEKSVNNYQKEQQALKLSECSQSPTMEAIEDMHEKSMEGLM   193

SLY1    NLGTKN-------------    219
SLY2    NLGTKN-------------    185
SLX     NMETNNYDMLFDVDGEETL    212
```

Figure 21. Design of anti-SLY antibody: A ClustalW alignment of the putative SLY1 and SLY2 amino acid sequences with the related SLX protein. The 15 amino acid SLY-specific peptide VKSPAFDKNENISPQ (highlighted in yellow) was used to immunize three mice, producing a SLY antibody that specifically recognizes SLY1 and SLY2 protein. The COR1 domain of SLY1 and SLY2 is in blue font.

4.2.2 Enzyme linked immunosorbent assay (ELISA)

Five antigens were used to coat 96-well plates for ELISA assay: peptide sequence, peptide sequence conjugated to Bovine Serum Albumin (BSA) or Keyhole Limpet Hemocyanin (KLH), and SLY1/2 purified protein. The antigens were coated on to the plate at a concentration of 1.0-0.125 μg per well overnight. Milk in borate buffer (5%) was used to block all unsaturated surface-binding sites. The plates were rinsed with borate buffer and the supernatants of cultured hybridomas or diluted sera (1/400 in 2.5% skim milk/borate buffer) were placed into each well. After an hour incubation, the plates were rinsed with borate buffer. Goat anti-mouse-conjugated to alkaline phosphatase (1/1000 in 2.5% skim milk/borate buffer) was used as a secondary reagent. The substrate used for visualization was para-Nitrophenylphosphate (PNPP), which can be measured with a 405 nm spectrophotometer. Values greater than 1.0 were considered highly reactive, greater than 0.5 somewhat reactive, and below 0.2 non-reactive.

4.2.3 Transfection and culture of HEKcells

HEK 293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) that was supplemented with 10% FBS. At 70-90% confluency, the cells were transfected with 2.0 μg of FLAG-*Sly1*, FLAG-*Sly2*, or FLAG-*Slx* using FuGene (Roche Applied Science). Cells were collected for either western blot analysis or for immunofluorescence analysis after 48-72 hours of transfection.

4.2.4 Immunofluorescence

Transfected HEK 293T cells were fixed in 4% Paraformaldehyde (PFA) and placed on glass slides. After fixation, cells were washed in PBS, and blocked in 0.5% BSA for 1 hour. Primary antibody was applied (anti-SLY that we produced or Sigma

anti-FLAG) at a concentration of 1:500, and slides were incubated at 4°C overnight.

Slides were washed with PBS 3 times, prior to application of secondary antibody at a

concentration of 1:500 (Anti-rabbit/Anti-mouse IgG conjugated to Alexa Fluor 488;

Invitrogen). After overnight incubation at 4°C, the slides were washed and mounted

using Vectashield with DAPI.

4.2.5 Production of NPYq deficient mice transgenic for *Sly*

Transgenic mice that overexpressed *Sly1* and *Sly2*, under the control of the

spermatid-specific promoter (SP10) with and without FLAG tags were created by

pronuclear microinjection. Among several transgenic lines we obtained two lines with

significant *Sly* upregulation (line 30A transgenic for *Sly1* and line 6P transgenic for both

Sly 1 and *Sly2*) and these were propogated. Intracytoplasmic sperm injection (ICSI) was

performed with oocytes from transgenic females from these two lines and sperm from

infertile males with severe NPYq deletions. Among the resulting male progeny there

were NPYq deficient males with and without the *Sly* transgene, and those were used for

phenotype analyses.

4.3 Results

4.3.1 Newly created anti-SLY antibody specifically recognizes SLY1 and SLY2 protein.

In our previous experiments SLY protein expression analysis was hampered by

the lack of a suitable antibody as the only available antibody [Reynard, Cocquet, et

al.,2009] recognized one of the two existing SLY isoforms, SLY1. To address this we

developed a new anti-SLY antibody. After performing a ClustalW alignment of SLY1,

SLY2, and SLX amino acid sequences (Figure 21), the 15 amino acid SLY-specific

peptide VKSPAFDKNENISPQ was used to immunize mice, eventually allowing to obtain a monoclonal antibody that specifically recognized both SLY isoforms.

ELISA was performed on approximately 400 colonies of hybridomas, and roughly 100 colonies produced a polyclonal mixture that positively interacted with SLY protein. Selection continued until there were only 2 lines of monoclonal antibodies that gave us the strongest interaction with SLY proteins.

Four different assays were used to confirm that the antibody specifically targeted SLY and not SLX: ELISA, dot blot, western blot, and immunofluorescence (IF). To test for specificity using dot blot the immunized sera were used against lysates from HEK cells transfected with the following constructs: FLAG-*Sly1*, FLAG-*Sly2*, FLAG-*SlxL1* and FLAG-*Slx* (Figure 22A). To test for specificity using western blot the supernatant from both hybridoma lines that produce anti-SLY, were used against both the lysates from HEK cells transfected with FLAG-*Sly1* and FLAG-*Sly2* (Figure 22B) and the lysates from testes of *Sly*- and NPYq-deficient males (Figure 22C-E). To test for antibody specificity using IF, HEK 293T cells were transfected with FLAG-*Sly1,* FLAG-*Sly2*, and FLAG-*Slx* constructs (Figure 23). Commercially made antibody that recognizes the FLAG-tag recognized all transfected cells, while our anti-SLY antibody gave positive signal with cells that were transfected with *Sly* constructs but not with cells that were transfected with *Slx* constructs.

4.3.2 The milder DNA damage and abnormal chromatin packaging phenotype in *Sly* deficient mice is partially due to the residual retention of SLY protein.

Previously available antibody recognized only SLY1 protein , and was not capable of recognizing the putative SLY2 protein [Reynard, Cocquet, et al.,2009].
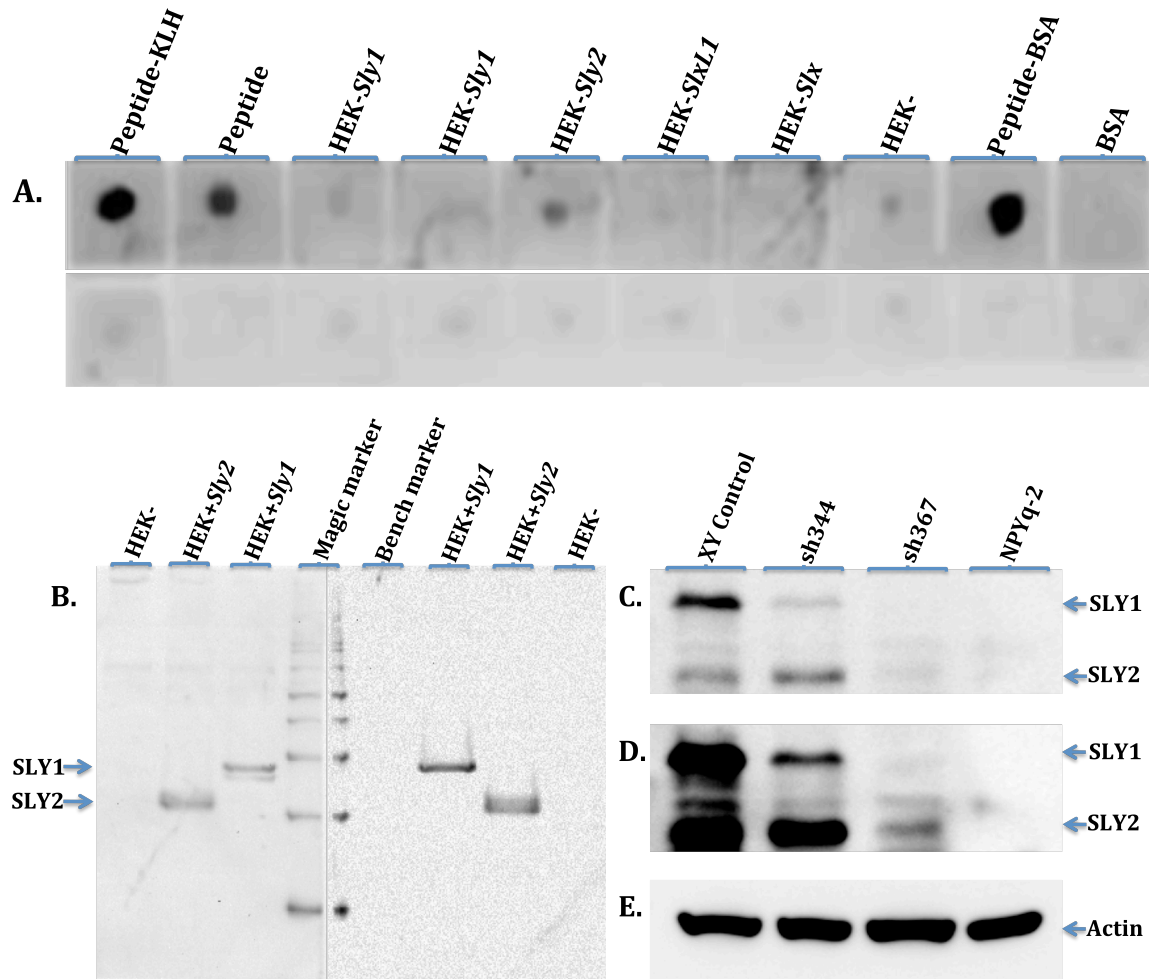
Figure 22. Western and dot blots confirming SLY protein recognition with the new antibody (SLY Ab). (a) Dot blot analysis using serum collected prior (bottom membrane) and after immunization (top membrane). (b) Western blot of SLY1/SLY2 protein from lysates of HEK cells transfected with FLAG-*Sly1* and FLAG-*Sly2* constructs. Both SLY1/SLY2 (arrows) were recognized using FLAG Ab (membrane on the left) and SLY Ab (membrane on the right). Non-transfected cells (HEK-) were used as controls. (c-d) Western blot of SLY1/SLY2 protein from testis extracts of wild-type mice (XY control), sh344 mice, sh367 mice and NPYq deficient males (NPYq-2) at short exposure (c) and long exposure (d), and with actin used as a loading control (e).
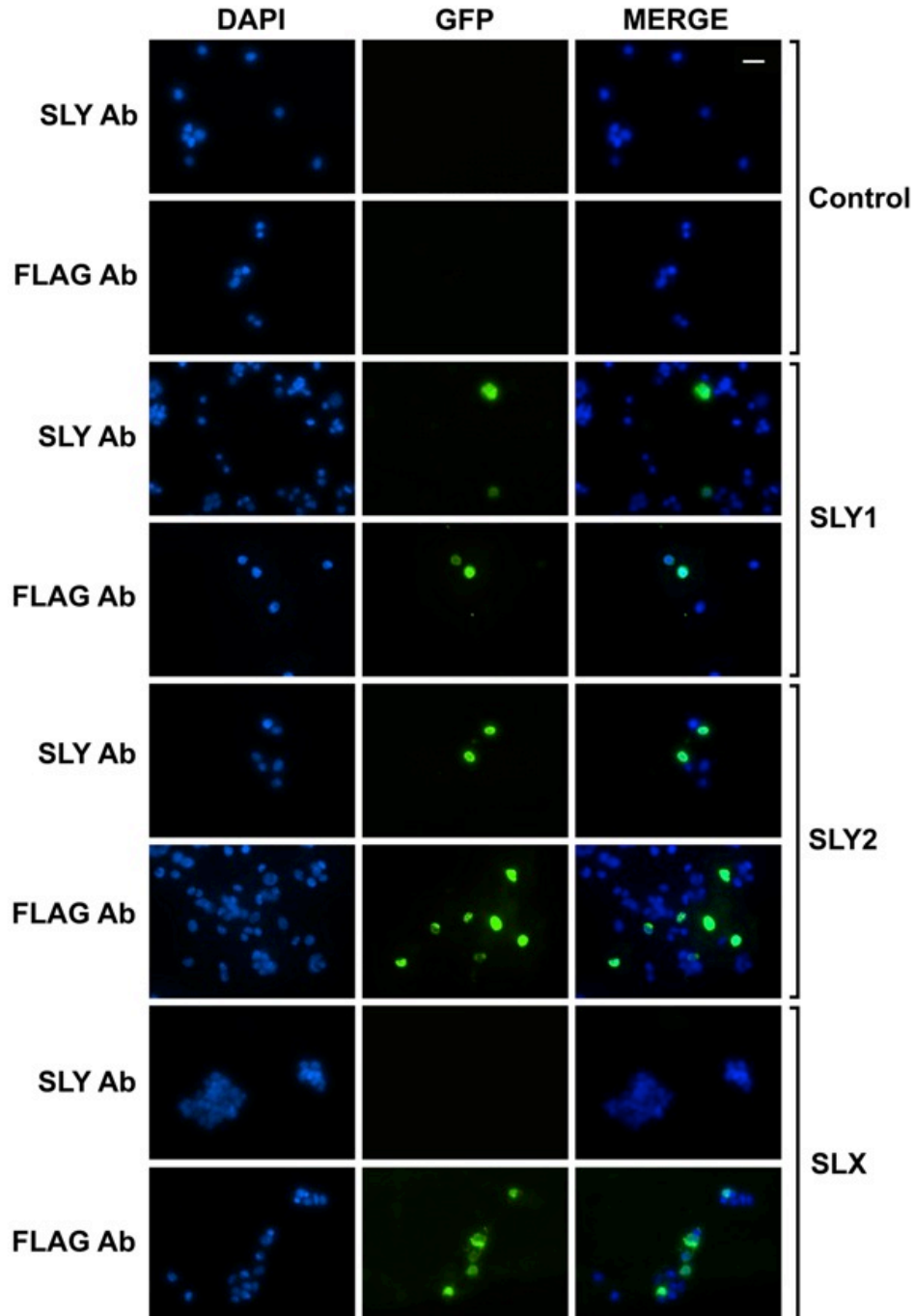
**Figure 23. Transfection of HEK cells with FLAG-*Sly1*, FLAG-*Sly2*, FLAG-*Slx*
constructs follwed by immunostaining using an anti-SLY antibody (SLYAb) and an
anti-FLAG (FLAG Ab).** The cell nuclei are stained with DAPI (blue). Secondary
antibody for both primary antibodies is Alexa Fluor 488 (GFP). Anti-FLAG antibody
detects FLAG-SLY1, FLAG-SLY2, and FLAG-SLX fusion proteins. Anti-SLY antibody
detects SLY1 and SLY2 but no SLX fusion proteins. Control are non transfected HEK
cells. Bar = 100μM.

Therefore, it remained unknown whether there any SLY2 remained in NPYq and *Sly* deficient mice. The SLY specific antibody that we created was therefore used for western blots with protein lysates prepared from testes obtained from mice with NPYq deletions and from shSLY mice (Figure 24). Protein analysis using lysates from shSLY mice confirmed previous qRT-PCR results (Chapter 3, Figure 14d) indicating the differences in SLY knockdown between the lines. Line sh344, *Sly1*-only-deficient mice, displayed a SLY1 protein knockdown only while its SLY2 protein level was not decreased. Line sh367, *Sly*-deficient mice, showed a knockdown in both SLY1 and SLY2 protein levels (Figure 24a-b), as expected.

The levels of SLY1 and SLY2 proteins in *Sly* deficient mice (sh344 and sh367), were compared to those in NPYq deficient mice (2/3NPYq-, 9/10NPYq-, and NPYq-2) and wild-type mice (XY). Mice with a partial NPYq deletion (2/3NPYq-) and a slight phenotype had most of SLY1 (48%) and SLY2 (51%) retained. Mice with extensive NPYq deletions (9/10NPYq-, and NPYq-2) and severe phenotype had no identifiable SLY1 and SLY2 (Figure 24b). Quantification of SLY expression in shSLY mice showed that line sh344, with no phenotype, retained only 6% of SLY1 but SLY2 was overexpressed (156%) while line sh367, with a moderate phenotype, retained no SLY1 and 17% of SLY2 (Figure 24b).

These results indicate that milder phenotype of sh367 mice as compared to mice with severe NPYq deletions may be due, at least partially, to incomplete SLY2 knockdown.

4.3.3 NPYq deficient males transgenic for FLAG-tagged *Sly* transgene driven by SP10 promoter exhibit *Sly* expression rescue but no phenotype rescue.
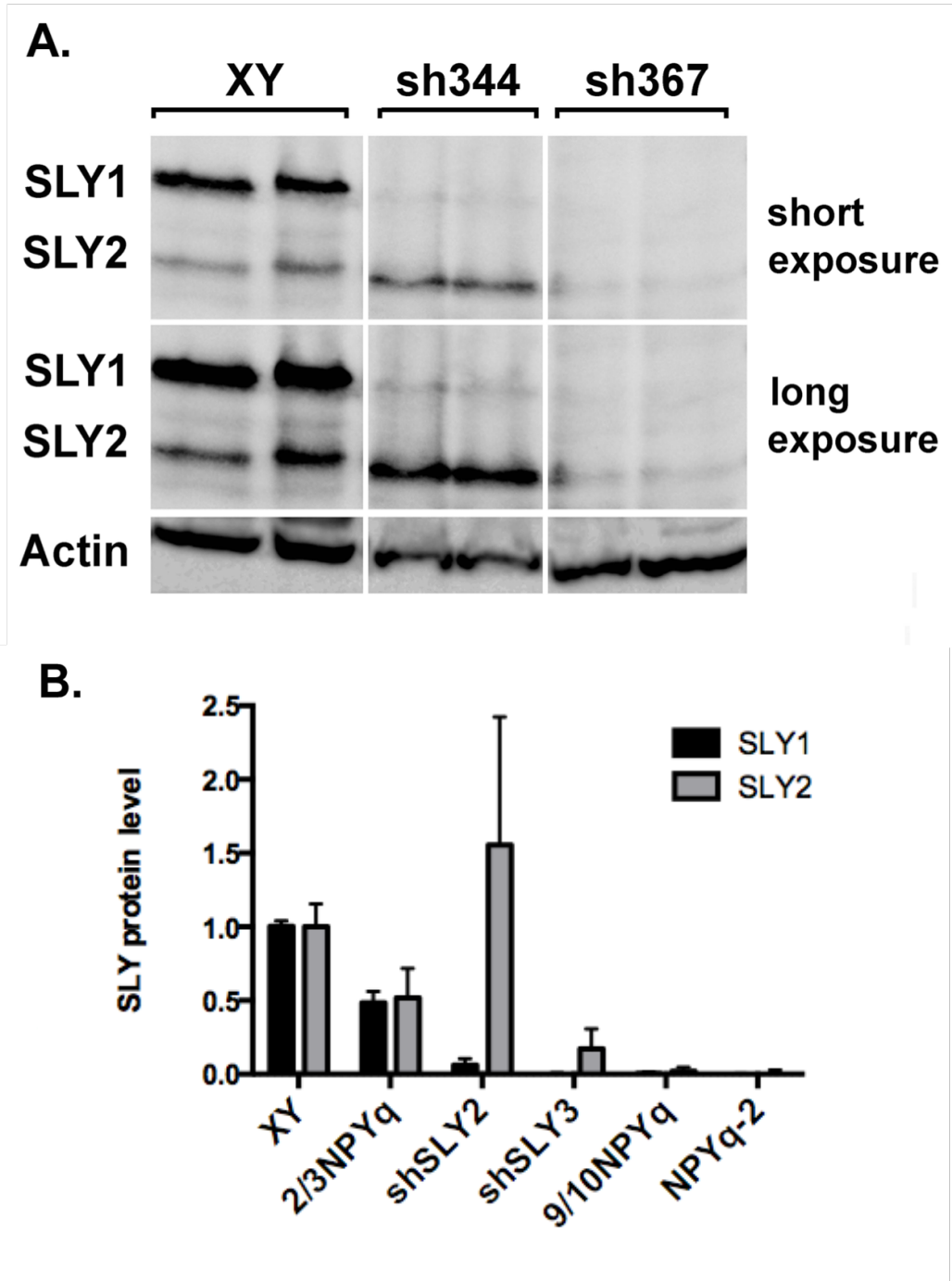
Figure 24. Western blot analysis using newly developed anti-SLY (SLY Ab) antibody. (a) Exemplary western blot of SLY proteins in testis extracts from sh344, sh367 and wild-type (XY) mice. (b) Levels of SLY1 and SLY2 proteins in *Sly* deficient (sh344 and sh367), NPYq deficient (2/3NPYq-, 9/10NPYq and NPYq-2) and control (XY) mice quantified with ImageJ and normalized to actin signal. Values are means of 4-8 replicates +/- SEM. SLY1 and SLY2 levels compared individually across all genotypes were significantly different in all paired comparisons except for (1) sh367 vs. 9/10NPYq- vs. NPYq-2, and (2) sh344 vs. XY (SLY2 only), which did not differ (t-test).

ICSI-generated male progeny with NPY deficiency and the supplementing *Sly* transgene (9/10NPYq- tsgic) and their transgene negative siblings (9/10NPYq-) were examined for rescue of *Sly*/SLY expression and rescue of infertile phenotype via analysis of sperm parameters and their ability to fertilize oocytes in vitro. Expression analysis showed that *Sly1* and *Sly* global *(Sly1+2)* transcripts levels were brought back close to those observed in normal wild-type males (XY). This *Sly* expression rescue was observed using both transgenic lines (30A and 6P) (Figure 25).   Transgenic SLY was translated and similarly as endogenous SLY could be detected in both the cytoplasm and the nuclei of testicular cells. The testes from NPYq deficient transgenic males and transgene negative siblings from both lines (30A and 6P) were processed for comparison of SLY protein levels between cytoplasmic and nuclear fractions (Figure 26). SLY1 and SLY2 were present in both fractions in XY males, but absent in 9/10NPYq negative siblings, as expected. In 9/10NPYq- tsgic 30A (transgenic for *Sly1*), SLY1 was detected in both the cytoplasm and the nucleus, but SLY2 was absent in both fractions. In 9/10NPYq- tsgic 6P (transgenic for *Sly1* and *Sly2)* both SLY1 and SLY2 were present in the cytoplasm and detectable in the nuclei nuclei but at much lower levels.

In spite of successful *Sly*/SLY rescue, the infertile phenotype rescue was not achieved. NPYq deficient males transgenic for *Sly* did not differ from their non-transgenic siblings in testis size, sperm morphology, sperm number and sperm ability to fertilize oocytes in vitro (Table 5 and Figure 27).

Both *Sly*- and NPYq-deficient mice display a remarkable upregulation of sex chromosome genes after meiosis implying that SLY is a key regulator of the sex
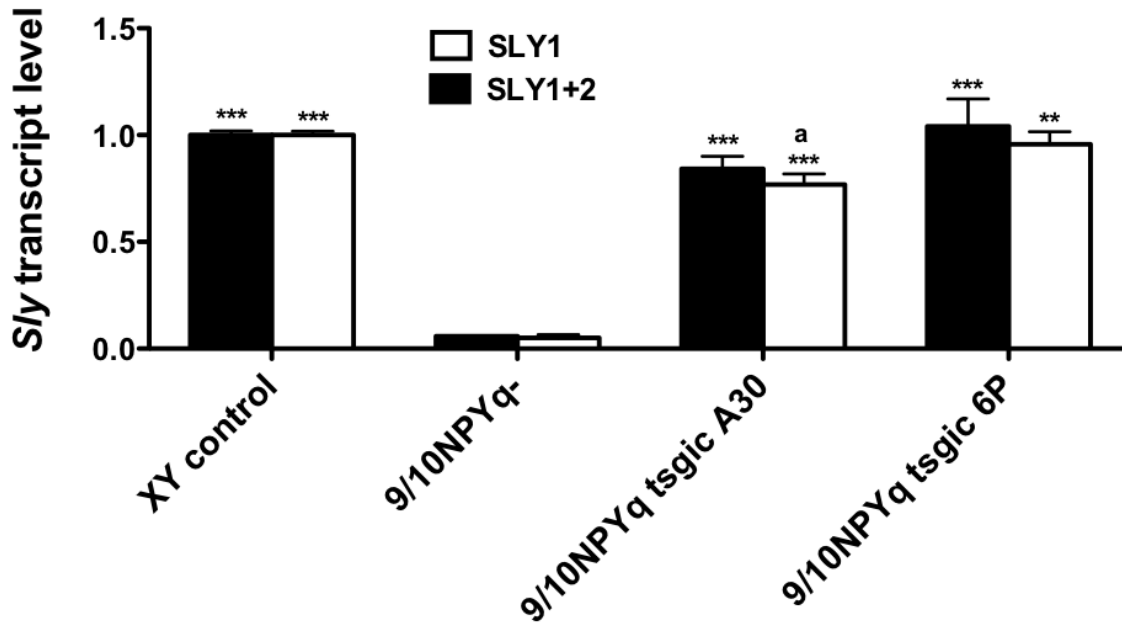
Figure 25. *Sly* gene expression in testis from NPYq deficient mice transgenic for *Sly*.
Sly expression in testes from NPYq deficient males without (9/10NPYq-) and with *Sly*
transgene (9/10NPYq- tsgic 30A or 6P), and from wild-type males (XY control), was
analyzed by real time PCR with actin as a loading control. Three animals were analyzed
per genotype. *** indicate significant difference (p<0.05) vs. 9/10NPYq- and (a)
represents a significant difference (p<0.05) vs. respective group in XY control. Graphs
are means +/- SEM.
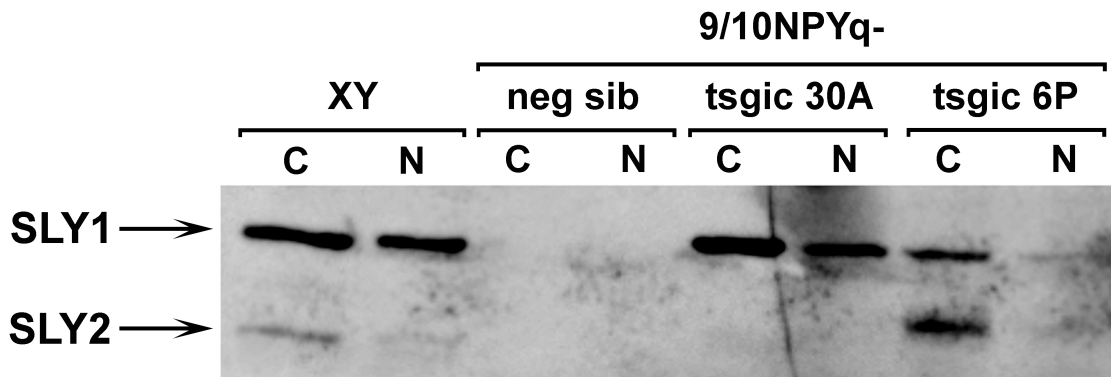
Figure 26. SLY protein expression in testes from NPYq deficient males transgenic for *Sly*. SLY expression in cytoplasmic (C) and nuclear (N) testis protein lysates from NPYq deficient males without (9/10 NPYq-) and with *Sly* transgene (9/10NPYq- tsgic 30A or 6P) and from wild-type males (XY), was analyzed by western blot using a recently developed anti-SLY antibody (SLY Ab). SLY1 and SLY2 are present in both cytoplasmic and nuclei in XY males (with SLY2 much less abundant) but not in 9/10NPYq- negative siblings. In 9/10NPYq- tsgic 30A (transgenic for *Sly1*) SLY1 is clearly observed in both cytoplasmic and nuclear fractions while SLY2 is absent. In 9/10NPYq- tsgic 6P (transgenic for both *Sly1* and *Sly2*) both SLY1 and SLY2 are abundant in the cytoplasm and weakly detectable in the nuclei.
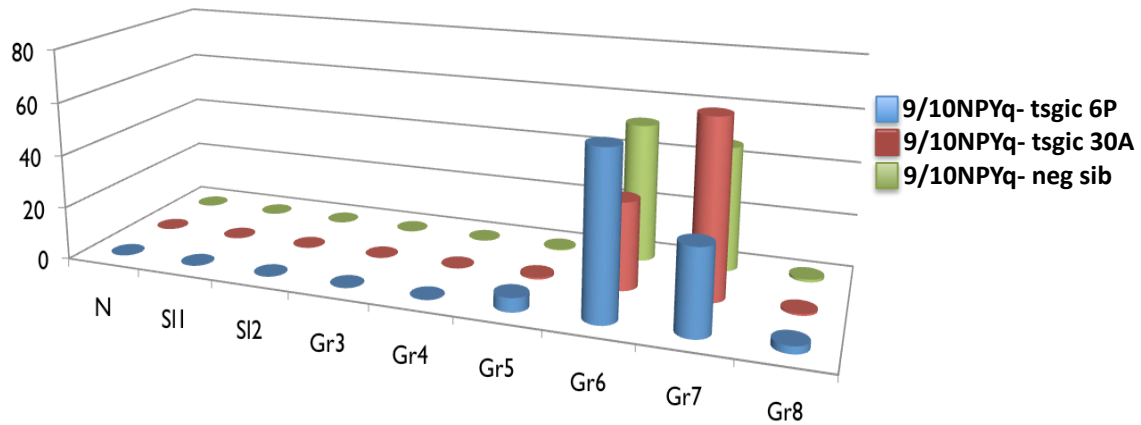
Figure 27. Sperm morphology analysis of transgene rescue mice. Data shown in the y-axis are the percentage of sperm analyzed. One hundred sperm were scored per male, with number of males n=3, n=4 and n=4 for 9/10NPYq- tsgic 6P, 9/10NPYq- tsgic 30A and neg sib, respectively. The x-axis represents individual categories of sperm head shapes; N, normal; Sl1-Sl2, slight defects; Gr3-Gr8, gross defects. Examples of each head shape can be seen in Figure 14.

Table 5. Analysis of sperm number and ability to fertilize oocyte in vitro

Male genotype	Testis Size (mean ± SEM)	Sperm number (x10^6) (mean ± SEM/male)	No. oocytes fertilized	No. (%) oocytes fertilized	No. (%) oocytes cleaved
9/10 NPYq- tsgic 6P (n=4)	58.1 ± 4.39	0.13 ± 0.10	236	0 (0)	0 (0)
9/10 NPYq- tsgic 30A (n=3)	64.0 ± 3.66	0.37 ± 0.04	129	0 (0)	0 (0)
9/10 NPYq- (n=3)	59.5 ± 2.91	1.89 ± 0.04	167	0 (0)	0 (0)
XY control (n=3)	94.5 ± 2.45	NA (1-10x10^6)	170	116 (68)	114 (98)

chromosome gene expression [Ellis, Clemente, et al.,2005, Reynard, Cocquet, et al.,2009]. Indeed, it has been shown that SLY1 overlaps with sex chromatin in wild-type males [Cocquet, Ellis, et al.,2009]. Therefore, the level of expression of a variety of X an Y chromosome genes was examined in testes from NPYq deficient mice transgenic for *Sly*. Both the NPYq deficient mice without (9/10NPYq-) and with *Sly* transgene (9/10NPYq- tsgic 30A) showed upregulation of X chromosome genes (*SlxL1*, *Mgclh*, *Actrt1*, and *170008i05Rik*) as compared to wild-type males (Figure 28). Thus, it is possible transgenic SLY protein in NPYq deficient males transgenic for *Sly* is not functional.

4.4 Discussion/Conclusion

The purpose of these experiments was to help elucidate the role of Y chromosome long arm (NPYq) encoded gene *Sly* in the infertile phenotype of mice with NPYq deletions. Only the *Sly*-deficient (sh367 line) mice displayed the infertile phenotype similar to that of males, however the effects where not as severe, while the *Sly1*-only-deficient males (sh344 line) did not show any phenotype [Riel, Yamauchi, et al.,2013]. Here we provided evidence that a milder phenotype of sh367 is due, at least partially, to retention of residual SLY2. Our results also support the important role of SLY2 isoform, which becomes a target for our future investigations.

Thus far, the transgenic rescue experiments failed to show positive results (i.e. phenotype rescue). We suspect that this may be due to FLAG tag interfering with protein folding and/or function. We have shown that the sex chromosome gene upregulation, which is associated with NPYq gene loss and likely linked to the infertile phenotype, was
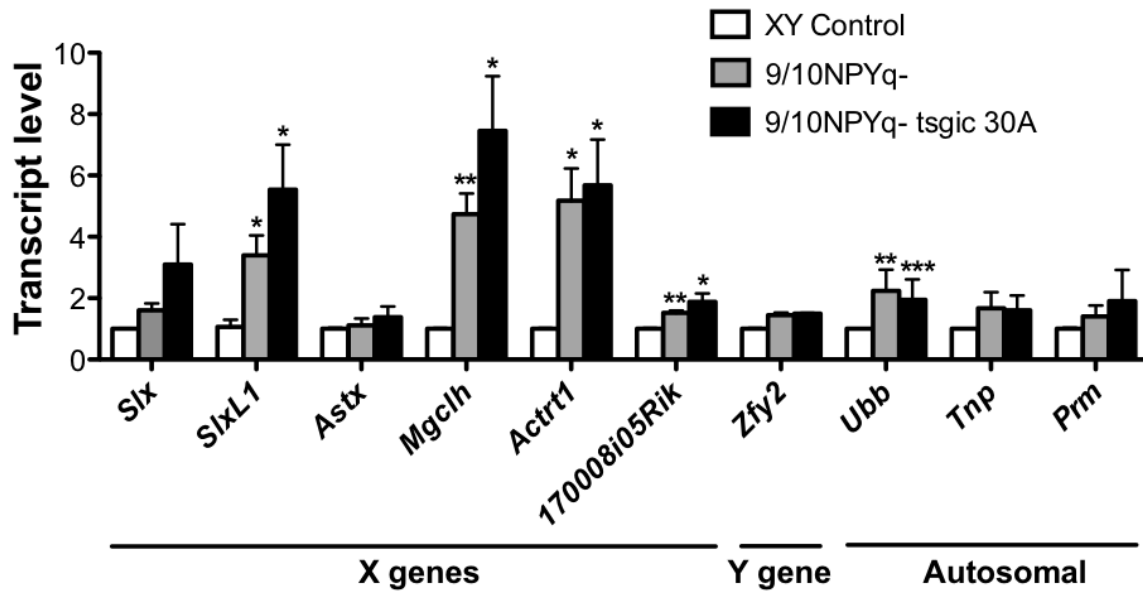
Figure 28. Lack of sex chromosome gene derepression rescue in NPYq deficient males transgenic for *Sly*. The analysis of expression of exemplary sex chromosome genes in testes from NPYq deficient mice without (9/10NPYq-) and with *Sly* transgene (9/10NPYq- tsgic 30A), and from XY control, was analyzed by real time PCR with actin as a loading control. Three animals were analyzed per genotype. Asterisks indicate significant difference between NPYq deficient mice and control values (t-test) with * p<0.05, **P<0.01 and ***P<0.001. There were no differences between 9/10NPYq- and 9/10NPYq- tsgic 30A. Graphs are means +/- SEM. Similar analysis was performed for 9/10NPYq- tsgic 6P and overall no clear sex chromosome genes derepression rescue was observed.

not rescued in transgenic NPYq deficient males, indicating that transgenic SLY, even if capable of entering spermatid nuclei (Figure 26), was unable to be effective in sex chromosome gene regulation as endogenous SLY is.   To alleviate problems with FLAG tag, we are currently generating another set of transgenic mice with SP10-*Sly* transgene not tagged. We have already identified two new *Sly1* and two *Sly2* transgenic lines with a significant *Sly* overexpression, which were propagated to produce transgenic females for ICSI. We anticipate that the phenotype rescue will be achieved, and will coincide with nuclear localization of SLY proteins and rescue of sex chromosome upregulation. These data will clarify the level of *Sly*/SLY involvement in infertile phenotype associated with NPYq deficiency.

CHAPTER 5

CONCLUSION

5.1 Sperm DNA damage and its effects of male fertility.

Sperm DNA is known to contribute half of the genomic content necessary to make up an offspring. Therefore, the integrity of sperm DNA plays an important role in mammalian embryo development and implantation [Ahmadi and Ng,1999]. As part of this dissertation, we provided the first evidence of DNA damage in sperm from mice with NPYq deficiencies, and suggested that NPYq-encoded gene/s may play a role in processes regulating chromatin remodeling and thus in maintaining DNA integrity in sperm [Yamauchi, Riel, et al.,2010]. NPYq deficiency not only leads to DNA damage but also causes teratozoospermia, subfertility with a progeny sex-ratio biases favoring females, and leads to complete infertility [Burgoyne, Mahadevaiah, et al.,1992, Conway, Mahadevaiah, et al.,1994, Styrna, Imai, et al.,1991, Toure, Szot, et al.,2004, Yamauchi, Riel, et al.,2009].

The exact cause of the DNA damage seen in our NPYq deficient mice is yet to be determined; however one of the most interesting observations made during this study is the increase in sperm DNA damage while in transit from the testis to the epididymis. Several theories have been proposed to explain this occurrence. Aitken *et al.* suggested a two-step model, in which testicular sperm with chromatin remodeling defects acquire membrane and DNA damage during this transit [Aitken and De Iuliis,2010]. This DNA damage could be caused by reactive oxygen species [Aitken, De Iuliis, et al.,2009, Tremellen,2008] and/or the action of endonucleases present within the sperm or epididymal fluid [Boaz, Dominguez, et al.,2008, Maione, Pittoggi, et al.,1997, Shaman,

Prisztoka, et al.,2006, Yamauchi, Ajduk, et al.,2007]. It is also possible that thiol-cross linking, which improves chromatin compaction, and normally takes place in the epididymis, does not take place if there is already a chromatin defect in sperm exiting the testis [Dias, Retamal, et al.,2006, Shalgi, Seligman, et al.,1989, Sivashanmugam and Rajalakshmi,1997].

Our discovery that NPYq deficient mice harbor DNA damage directly linked to their infertility make these mice valuable models for investigations of the origin and consequences of human male infertility. The quality of sperm DNA becomes of utmost importance with assisted reproductive techniques (ART), which are commonly used if a couple is infertile. For example, intracytoplasmic sperm injection (ICSI) is a widely used technique to treat human male infertility due to oligozoospermia or teratozoospermia that is usually associated with sperm defects, such as incomplete protamation and incomplete compaction of chromatin [Carrell, Emery, et al.,2007, Oliva,2006, Ramos, van der Heijden, et al.,2008]. Thus, the severely NPYq deficient mice studied here [Yamauchi, Riel, et al.,2010] are a useful mouse model for assessing the consequence of these defects for the offspring. It is also important to consider the implication of the additional DNA damage that arises as a consequence of freezing in the NPYq deficient mice as well as in the controls. With freezing and NPYq deficiency, almost none of the embryos transferred reached term; this is unsurprising given the level of chromosome breaks and rearrangements generated, which would be expected to cause early embryonic lethality. This raises a number of issues as to how to maximize ICSI success and how to minimize mutational load in the offspring.

In the context of ICSI success, it also seems clear that in situations where there is increasing DNA damage during epididymal transit, as is the case with NPYq deficiency, it is better to use testicular sperm; however, freezing should be avoided. Improved ICSI success with testicular as compared to epididymal sperm has previously been reported for mice heterozygous for two different but semi-identical translocations [Baart, van der Heijden, et al.,2004] and in mice deficient for transition proteins [Shirley, Hayashi, et al.,2004, Suganuma, Yanagimachi, et al.,2005], as well as in infertile men with sperm DNA damage [Greco, Scarselli, et al.,2005]. A caveat is that in the present study sperm freezing was done without cryoprotectant, but with the method developed to keep DNA damage to a level comparable to freezing with cryprotectant [Kusakabe, Szczygiel, et al.,2001]. Indeed, substantial freezing-induced DNA damage has been reported even with mouse sperm frozen with cryoprotectant [Yildiz, Fleming, et al.,2008] and with cryopreserved human sperm [de Paula, Bertolla, et al.,2006, Donnelly, Steele, et al.,2001, Thomson, Fleming, et al.,2009].

Although ART has proven useful with couples dealing with infertility, little is known about its effects on the children conceived through these methods. The key problem with ART is that it bypasses the natural selections process that occurs during the sperms transit between the male and female reproductive tract, and up to the point it enters the oocyte [Chandley and Hargreave,1996]. With ART, sperm with damaged DNA can enter the oocyte quite easily, which may result in congenital malformations [Bonduelle, Van Assche, et al.,2002, Hansen, Kurinczuk, et al.,2002, Katalinic, Rosch, et al.,2004, Wennerholm, Bergh, et al.,2000], or chromosomal abnormalities in children produced via ICSI [Bonduelle, Van Assche, et al.,2002, Meschede and Horst,1997]. It is

important that we continue to evaluate the level of DNA damage in sperm, to provide

counsel to infertile couples undergoing ART, and to better inform them of their chances

of success reproduction and health-related risks they may face with their child.

5.2 SLY involvement in sperm DNA damage phenotype of mice with NPYq deficiencies.

As for our NPYq deficient mice, the primary defect must arise during

spermiogenesis, the time period at which the genes encoded in this region are expressed

in normal mice [Conway, Mahadevaiah, et al.,1994, Ellis, Clemente, et al.,2005,

Reynard, Cocquet, et al.,2009]. In this region of the Y chromosome, there are four

distinct multi-copy genes: *Ssty, Sly, Asty* and *Orly* ([Agulnik, Mitchell, et al.,1994,

Conway, Mahadevaiah, et al.,1994, Ellis, Ferguson, et al.,2007, Prado, Lee, et al.,1992,

Toure, Clemente, et al.,2005, Toure, Szot, et al.,2004] [Figure 1]. Of the four multi-copy

genes, the NPYq-encoded *Sly* gene was the target of interest because it was the

deficiency of this gene that caused similar phenotypes as seen in our NPYq deficient

mice. *Sly* controls the expression of sex chromosome genes after meiosis and the

deficiency of this gene resulted in a remarkable upregulation of sex chromosome genes.

Sly deficiency has also been shown to be the underlying cause of sperm head anomalies

and infertility associated with NPYq loss [Cocquet, Ellis, et al.,2009], but it was not

known whether it recapitulates sperm DNA damage.

To determine if *Sly* is responsible for the sperm DNA damage phenotype

observed in mice with severe NPYq deficiency we examined the phenotype of mice with

transgenically knockdown *Sly* [Riel, Yamauchi, et al.,2013]. Our data demonstrate the

Sly deficiency is responsible for the sperm DNA damage/chromatin packaging defects

observed in mice with NPYq deletions and point to SLY proteins involvement in

100

chromatin reprogramming during spermiogenesis, likely through their effect on the postmeiotic expression of spermiogenic genes. Furthermore, the recent development of an antibody that specifically recognizes SLY1 and SLY2 protein has helped clarify why the phenotype in our *Sly* deficient (sh367) transgenic mice was not as severe. Retention of residual SLY1/2 likely lessened the infertile phenotypes associated with severe NPYq deficiency.

No homologues of *Sly* or the other multicopy mouse NPYq genes have been identified on the human Y chromosome. Nevertheless, multi-copy testis expressed genes are a feature of the human Y [Kuroda-Kawaguchi, Skaletsky, et al.,2001, Skaletsky, Kuroda-Kawaguchi, et al.,2003], and the multicopy testis specific gene RBMY has a multi-copy homologue (*Rbmy*) on the mouse Y short arm [Mahadevaiah, Odorisio, et al.,1998]. Thus, the two species have in common the fact that genes with a presumed or demonstrated spermatogenic roles are often amplified on the Y chromosome.

5.3 The mechanism whereby SLY regulates spermatogenesis

How can *Sly*/SLY be involved in sperm DNA damage phenotype? SLY protein has been shown to control the postmeiotic expression of >100 genes, the majority of which are encoded on sex chromosomes. In *Sly*-deficient males, the repressive epigenetic marks normally associated with the sex chromosomes (i.e. CBX1 and H3K9me3) are decreased, which leads to the upregulation of many X- and Y -encoded spermiogenic genes [Cocquet, Ellis, et al.,2009]. The multiple spermiogenesis defects associated with *Sly* deficiency, including the sperm DNA damage and abnormal chromatin packaging described in the present study, are likely a consequence of this massive change in spermiogenic gene expression. In *Sly1*-only deficient males, there are no clear defects of

the spermiogenesis function, and the upregulation of spermiogenic X and Y genes is very limited. In all probability, a certain threshold of upregulation must be reached to significantly alter sperm differentiation and function.

In theory, the sperm differentiation defects observed in *Sly*-deficient and NPYq-deficient mice could result from the deregulation of any of the >100 deregulated genes, or from the collective deregulation of several of them. Nevertheless, several genes appear to be promising individual candidates. For instance, alteration in the expression of *H2al1* and *H2al2y* genes, which encode spermatid specific histone variants [Govin, Escoffier, et al.,2007] could affect sperm chromatin protamination and therefore spermiogenesis. A few autosomal genes were also downregulated in *Sly*-deficient spermatids [Cocquet, Ellis, et al.,2009]. Among them, the gene *Chaf1b* is a very likely candidate to explain sperm DNA damage since it encodes a component of the Chromatin Assembly Factor 1 (CAF-1), which is involved in the DNA damage and repair process [Groth, Rocha, et al.,2007]. RNAi silencing of the CHAF1B in proliferating human cells led to accumulation of double strand DNA breaks, increased level of phosphorylated histone H2AX, and ultimately cell death [Nabatiyan and Krude,2004].

In terms of SLY protein and its role in the sperm DNA damage phenotype, SLY1 protein co-localizes with the loci that are deregulated in *Sly*-deficient males (Cocquet et al., 2009) and, therefore, is likely to play a role in the regulation of these loci. Second, SLY1 protein structure is very similar to that of SLY2. The COR1 domain, which is presumed to mediate chromatin binding, is identical in SLY1 and SLY2 proteins. SLY1 only differs from SLY2 in the 34 additional amino acids encoded by exons 5-6. Since exons 5-6 arose from a duplication of exons 3-4, the protein region encoded by exons 5-6

shares 70% identity and 79% similarity with the protein region encoded by exon 3-4 (Figure 12C). All in all, it is more plausible that *Sly1* and *Sly2* genes encode proteins of similar function, and that the phenotype is correlated with the global level of knockdown of *Sly1* and *Sly2*.

In respect to sperm chromatin packaging/DNA damage *Sly*-deficient mice recapitulate the NPYq-deficient phenotype only very moderately and much less significantly than other phenotypic features (Table 4). The difference in *Sly* transcript levels between mice with severe NPYq gene loss, i.e. deletion removing nine-tenth (9/10NPYq-, 4%) or the entire NPYq (NPYq- 2, 0%), and *Sly*-deficient sh367 mice could explain the differences in the phenotype in these mice. However, lack of detectable SLY1 protein in all genotypes and similar *Sly1* transcript levels in *Sly*-deficient and 9/10NPYq-mice (10% both) put the dependence of the phenotype exclusively on *Sly*/SLY levels in question. Mice with severe NPYq deficiency are lacking other genes encoded on the Y long arm, which could contribute to spermiogenic defects in these mice. It therefore cannot be excluded that these other NPYq genes, which are not only present but also upregulated in shSLY mice [Cocquet, Ellis, et al.,2009, Ellis, Clemente, et al.,2005] mitigate the sperm chromatin packaging/DNA damage phenotype. The most plausible gene candidate would be *Ssty1/2* (Spermatid-specific transcripts Y-encoded), which is present on the mouse NPYq in more than 100 copies, is specifically transcribed in the testis in round spermatids, and is the only other NPYq gene that is translated. *Ssty1/2* belongs to the *Spindlin* family, one member of which has recently been shown to bind to methylated histone H3 [Wang, Zhou, et al.,2011]; *Ssty1/2* could therefore contribute to chromatin remodeling during spermiogenesis, along with *Sly*. Future work utilizing

Ssty1/2 transgene additions to severely NPYq- deficient mice should help clarifying the total contribution of *Sly* and/or *Ssty1/2*.

5.4 Concluding remarks and future plans

Although we were able to conclude that *Sly* deficiency contributes to the sperm DNA damage phenotype seen in our NPYq deficient mice, and that the milder effect was due to the retention SLY proteins, there is still quite a bit of research that should be completed in the future to help clarify the exact role of *Sly*/SLY.

First of all, we were successful in producing transgenic lines that rescued the transcript levels of *Sly1* and/or *Sly2*. However, we failed to show positive phenotype rescue and suspected that this may be due to the FLAG tag interfering with protein folding and/or function. We have shown that the sex chromosome gene upregulation, which is associated with NPYq gene loss, was also not rescued in transgenic NPYq deficient males, indicating that transgenic SLY, even if capable of entering spermatid nuclei, was unable to be effective in sex chromosome gene regulation as endogenous SLY. We are currently in the process of creating new transgenic lines in which transgenic SLY is not FLAG-tagged to determine if this strategy will enable the rescue of the infertile phenotypes.

Secondly, the NPYq region encodes 3 other multi-copy genes that may play roles and contribute to the infertile phenotypes associated with NPYq deficiency. The most likely gene candidate is *Ssty*, as it is the only remaining multi-copy gene encoded in this region that produces a known protein. To determine if *Ssty* contributes to the infertile phenotypes, it would be ideal to create transgenic lines with downregulation of *Ssty*/SSTY expression/translation. If the established lines show a similar phenotype as

our *Sly* deficient mice, it would indicate that Ssty is involved. We would then validate this finding using transgene rescue approach, as we now do with *Sly*.

Lastly, it is important for us to continue investigating the role of *Sly*/SLY, and the mechanism of its contribution to the sperm DNA damage and infertility phenotypes. We are exploring several possible pathways using which *Sly*/SLY may participate in. In the future, we hope to master new techniques that will help to investigate these pathways. With the newly developed SLY antibody in place, we can now perform two important assays. We can look into SLY protein-to-protein interactions through Co-immunoprecipitation, and also look and whether SLY interacts with DNA itself by performing CHIP assay (Chromatin Immunoprecipitation).

All of these suggested future experiments are investigations that are either ongoing or are in the experimental design stage, and I hope to continue this work during my post-doctoral research.

REFERENCES

1. Agulnik AI, Mitchell MJ, Lerner JL, Woods DR, Bishop CE. A mouse Y chromosome gene encoded by a region essential for spermatogenesis and expression of male-specific minor histocompatibility antigens. Hum Mol Genet 1994; 3:873-878.
2. Ahmadi A, Ng SC. Fertilizing ability of DNA-damaged spermatozoa. J Exp Zool 1999; 284:696-704.
3. Aitken RJ, De Iuliis GN. On the possible origins of DNA damage in human spermatozoa. Mol Hum Reprod 2010; 16:3-13.
4. Aitken RJ, De Iuliis GN, McLachlan RI. Biological and clinical significance of DNA damage in the male germ line. Int J Androl 2009; 32:46-56.
5. Aoki VW, Moskovtsev SI, Willis J, Liu L, Mullen JB, Carrell DT. DNA integrity is compromised in protamine-deficient human sperm. J Androl 2005; 26:741-748.
6. Baart EB, van der Heijden GW, van der Hoeven FA, Bakker R, Cooper TG, de Boer P. Reduced oocyte activation and first cleavage rate after ICSI with spermatozoa from a sterile mouse chromosome mutant. Hum Reprod 2004; 19:1140-1147.
7. Balhorn R, Gledhill BL, Wyrobek AJ. Mouse sperm chromatin proteins: quantitative isolation and partial characterization. Biochemistry 1977; 16:4074-4080.
8. Barton TS, Robaire B, Hales BF. DNA damage recognition in the rat zygote following chronic paternal cyclophosphamide exposure. Toxicol Sci 2007; 100:495-503.
9. Bellve AR. Purification, culture, and fractionation of spermatogenic cells. Methods Enzymol 1993; 225:84-113.
10. Bianchi PG, Manicardi GC, Bizzaro D, Bianchi U, Sakkas D. Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human mature spermatozoa. Biol Reprod 1993; 49:1083-1088.
11. Bishop CE, Hatat D. Molecular cloning and sequence analysis of a mouse Y chromosome RNA transcript expressed in the testis. Nucleic Acids Res 1987; 15:2959-2969.
12. Boaz SM, Dominguez K, Shaman JA, Ward WS. Mouse spermatozoa contain a nuclease that is activated by pretreatment with EGTA and subsequent calcium incubation. J Cell Biochem 2008; 103:1636-1645.
13. Bonduelle M, Van Assche E, Joris H, Keymolen K, Devroey P, Van Steirteghem A, Liebaers I. Prenatal testing in ICSI pregnancies: incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. Hum Reprod 2002; 17:2600-2614.
14. Brandriff B, Pedersen RA. Repair of the ultraviolet-irradiated male genome in fertilized mouse eggs. Science 1981; 211:1431-1433.
15. Brugh VM, 3rd, Lipshultz LI. Male factor infertility: evaluation and management. Med Clin North Am 2004; 88:367-385.

16. Burgoyne PS, Mahadevaiah SK, Perry J, Palmer SJ, Ashworth A. The Y* rearrangement in mice: new insights into a perplexing PAR. Cytogenet Cell Genet 1998; 80:37-40.

17. Burgoyne PS, Mahadevaiah SK, Sutcliffe MJ, Palmer SJ. Fertility in mice requires X-Y pairing and a Y-chromosomal "spermiogenesis" gene mapping to the long arm. Cell 1992; 71:391-398.

18. Carrell DT, Emery BR, Hammoud S. Altered protamine expression and diminished spermatogenesis: what is the link? Hum Reprod Update 2007; 13:313-327.

19. Chandley AC, Hargreave TB. Genetic anomaly and ICSI. Human Reproduction 1996; 11:930-932.

20. Chang PL, Sauer MV, Brown S. Y chromosome microdeletion in a father and his four infertile sons. Hum Reprod 1999; 14:2689-2694.

21. Chatot CL, Ziomek CA, Bavister BD, Lewis JL, Torres I. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. J Reprod Fertil 1989; 86:679-688.

22. Cocquet J, Ellis PJ, Mahadevaiah SK, Affara NA, Vaiman D, Burgoyne PS. A genetic basis for a postmeiotic X versus Y chromosome intragenomic conflict in the mouse. PLoS Genet 2012; 8:e1002900.

23. Cocquet J, Ellis PJ, Yamauchi Y, Mahadevaiah SK, Affara NA, Ward MA, Burgoyne PS. The multicopy gene Sly represses the sex chromosomes in the male mouse germline after meiosis. PLoS Biol 2009; 7:e1000244.

24. Conway SJ, Mahadevaiah SK, Darling SM, Capel B, Rattigan AM, Burgoyne PS. Y353/B: a candidate multiple-copy spermiogenesis gene on the mouse Y chromosome. Mamm Genome 1994; 5:203-210.

25. de Boer P, Ramos L, de Vries M, Gochhait S. Memoirs of an insult: sperm as a possible source of transgenerational epimutations and genetic instability. Mol Hum Reprod 2010; 16:48-56.

26. de Paula TS, Bertolla RP, Spaine DM, Cunha MA, Schor N, Cedenho AP. Effect of cryopreservation on sperm apoptotic deoxyribonucleic acid fragmentation in patients with oligozoospermia. Fertil Steril 2006; 86:597-600.

27. Delbes G, Hales BF, Robaire B. Effects of the chemotherapy cocktail used to treat testicular cancer on sperm chromatin integrity. J Androl 2007; 28:241-249; discussion 250-241.

28. Derijck A, van der Heijden G, Giele M, Philippens M, de Boer P. DNA double-strand break repair in parental chromatin of mouse zygotes, the first cell cycle as an origin of de novo mutation. Hum Mol Genet 2008; 17:1922-1937.

29. Dias GM, Retamal CA, Tobella L, Arnholdt AC, Lopez ML. Nuclear status of immature and mature stallion spermatozoa. Theriogenology 2006; 66:354-365.

30. Donnelly ET, Steele EK, McClure N, Lewis SE. Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. Hum Reprod 2001; 16:1191-1199.

31. Ehrmann IE, Ellis PS, Mazeyrat S, Duthie S, Brockdorff N, Mattei MG, Gavin MA, Affara NA, Brown GM, Simpson E, Mitchell MJ, Scott DM. Characterization of genes encoding translation initiation factor eIF-2gamma in mouse and

human: sex chromosome localization, escape from X-inactivation and evolution. Hum Mol Genet 1998; 7:1725-1737.

32. Elliott DJ, Ma K, Kerr SM, Thakrar R, Speed R, Chandley AC, Cooke H. An RBM homologue maps to the mouse Y chromosome and is expressed in germ cells. Hum Mol Genet 1996; 5:869-874.

33. Ellis PJ, Bacon J, Affara NA. Association of Sly with sex-linked gene amplification during mouse evolution: a side effect of genomic conflict in spermatids? Hum Mol Genet 2011; 20:3010-3021.

34. Ellis PJ, Clemente EJ, Ball P, Toure A, Ferguson L, Turner JM, Loveland KL, Affara NA, Burgoyne PS. Deletions on mouse Yq lead to upregulation of multiple X- and Y-linked transcripts in spermatids. Hum Mol Genet 2005; 14:2705-2715.

35. Ellis PJ, Ferguson L, Clemente EJ, Affara NA. Bidirectional transcription of a novel chimeric gene mapping to mouse chromosome Yq. BMC Evol Biol 2007; 7:171.

36. Ferguson L, Ellis PJ, Affara NA. Two novel mouse genes mapped to chromosome Yp are expressed specifically in spermatids. Mamm Genome 2009; 20:193-206.

37. Fernandez-Gonzalez R, Moreira PN, Perez-Crespo M, Sanchez-Martin M, Ramirez MA, Pericuesta E, Bilbao A, Bermejo-Alvarez P, de Dios Hourcade J, de Fonseca FR, Gutierrez-Adan A. Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. Biol Reprod 2008; 78:761-772.

38. Foresta C, Moro E, Ferlin A. Y chromosome microdeletions and alterations of spermatogenesis. Endocr Rev 2001; 22:226-239.

39. Govin J, Escoffier E, Rousseaux S, Kuhn L, Ferro M, Thevenon J, Catena R, Davidson I, Garin J, Khochbin S, Caron C. Pericentric heterochromatin reprogramming by new histone variants during mouse spermiogenesis. J Cell Biol 2007; 176:283-294.

40. Greco E, Scarselli F, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Franco G, Anniballo N, Mendoza C, Tesarik J. Efficient treatment of infertility due to sperm DNA damage by ICSI with testicular spermatozoa. Hum Reprod 2005; 20:226-230.

41. Greenfield A, Carrel L, Pennisi D, Philippe C, Quaderi N, Siggers P, Steiner K, Tam PP, Monaco AP, Willard HF, Koopman P. The UTX gene escapes X inactivation in mice and humans. Hum Mol Genet 1998; 7:737-742.

42. Greenfield A, Scott D, Pennisi D, Ehrmann I, Ellis P, Cooper L, Simpson E, Koopman P. An H-YDb epitope is encoded by a novel mouse Y chromosome gene. Nat Genet 1996; 14:474-478.

43. Gregory SG, Sekhon M, Schein J, Zhao S, Osoegawa K, Scott CE, Evans RS, Burridge PW, Cox TV, Fox CA, Hutton RD, Mullenger IR, Phillips KJ, Smith J, Stalker J, Threadgold GJ, Birney E, Wylie K, Chinwalla A, Wallis J, Hillier L, Carter J, Gaige T, Jaeger S, Kremitzki C, Layman D, Maas J, McGrane R, Mead K, Walker R, Jones S, Smith M, Asano J, Bosdet I, Chan S, Chittaranjan S, Chiu R, Fjell C, Fuhrmann D, Girn N, Gray C, Guin R, Hsiao L, Krzywinski M, Kutsche R, Lee SS, Mathewson C, McLeavy C, Messervier S, Ness S, Pandoh P, Prabhu AL,

Saeedi P, Smailus D, Spence L, Stott J, Taylor S, Terpstra W, Tsai M, Vardy J, Wye N, Yang G, Shatsman S, Ayodeji B, Geer K, Tsegaye G, Shvartsbeyn A, Gebregeorgis E, Krol M, Russell D, Overton L, Malek JA, Holmes M, Heaney M, Shetty J, Feldblyum T, Nierman WC, Catanese JJ, Hubbard T, Waterston RH, Rogers J, de Jong PJ, Fraser CM, Marra M, McPherson JD, Bentley DR. A physical map of the mouse genome. Nature 2002; 418:743-750.

44. Groth A, Rocha W, Verreault A, Almouzni G. Chromatin challenges during DNA replication and repair. Cell 2007; 128:721-733.

45. Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, Vivian N, Goodfellow P, Lovell-Badge R. A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. Nature 1990; 346:245-250.

46. Gubbay J, Vivian N, Economou A, Jackson D, Goodfellow P, Lovell-Badge R. Inverted repeat structure of the Sry locus in mice. Proc Natl Acad Sci U S A 1992; 89:7953-7957.

47. Hall NM, Brown GM, Furlong RA, Sargent CA, Mitchell M, Rocha D, Affara NA. Usp9y (ubiquitin-specific protease 9 gene on the Y) is associated with a functional promoter and encodes an intact open reading frame homologous to Usp9x that is under selective constraint. Mamm Genome 2003; 14:437-447.

48. Hansen M, Kurinczuk JJ, Bower C, Webb S. The risk of major birth defects after intracytoplasmic sperm injection and in vitro fertilization. N Engl J Med 2002; 346:725-730.

49. Harper SQ, Staber PD, He X, Eliason SL, Martins IH, Mao Q, Yang L, Kotin RM, Paulson HL, Davidson BL. RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. Proc Natl Acad Sci U S A 2005; 102:5820-5825.

50. Hirsh A. Male subfertility. BMJ 2003; 327:669-672.

51. Katalinic A, Rosch C, Ludwig M. Pregnancy course and outcome after intracytoplasmic sperm injection: a controlled, prospective cohort study. Fertil Steril 2004; 81:1604-1616.

52. Kay GF, Ashworth A, Penny GD, Dunlop M, Swift S, Brockdorff N, Rastan S. A candidate spermatogenesis gene on the mouse Y chromosome is homologous to ubiquitin-activating enzyme E1. Nature 1991; 354:486-489.

53. Kimura Y, Yanagimachi R. Intracytoplasmic sperm injection in the mouse. Biol Reprod 1995; 52:709-720.

54. Krausz C, Quintana-Murci L, McElreavey K. Prognostic value of Y deletion analysis: what is the clinical prognostic value of Y chromosome microdeletion analysis? Human Reproduction 2000; 15:1431-1434.

55. Kuroda-Kawaguchi T, Skaletsky H, Brown LG, Minx PJ, Cordum HS, Waterston RH, Wilson RK, Silber S, Oates R, Rozen S, Page DC. The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. Nat Genet 2001; 29:279-286.

56. Kusakabe H, Szczygiel MA, Whittingham DG, Yanagimachi R. Maintenance of genetic integrity in frozen and freeze-dried mouse spermatozoa. Proc Natl Acad Sci U S A 2001; 98:13501-13506.

57. Leduc F, Maquennehan V, Nkoma GB, Boissonneault G. DNA damage response during chromatin remodeling in elongating spermatids of mice. Biol Reprod 2008; 78:324-332.

58. Levy N, Navarro A, Bishop CE, Mitchell MJ. The ubiquitin-activating enzyme E1 homologous genes on the mouse Y chromosome (Ube1y) represent one functional gene and six partial pseudogenes. Mamm Genome 2000; 11:164-168.

59. Lieber MR. The mechanism of human nonhomologous DNA end joining. J Biol Chem 2008; 283:1-5.

60. Mahadevaiah SK, Odorisio T, Elliott DJ, Rattigan A, Szot M, Laval SH, Washburn LL, McCarrey JR, Cattanach BM, Lovell-Badge R, Burgoyne PS. Mouse homologues of the human AZF candidate gene RBM are expressed in spermatogonia and spermatids, and map to a Y chromosome deletion interval associated with a high incidence of sperm abnormalities. Hum Mol Genet 1998; 7:715-727.

61. Maione B, Pittoggi C, Achene L, Lorenzini R, Spadafora C. Activation of endogenous nucleases in mature sperm cells upon interaction with exogenous DNA. DNA Cell Biol 1997; 16:1087-1097.

62. Marchetti F, Essers J, Kanaar R, Wyrobek AJ. Disruption of maternal DNA repair increases sperm-derived chromosomal aberrations. Proc Natl Acad Sci U S A 2007; 104:17725-17729.

63. Mardon G, Page DC. The sex-determining region of the mouse Y chromosome encodes a protein with a highly acidic domain and 13 zinc fingers. Cell 1989; 56:765-770.

64. Mau Kai C, Juul A, McElreavey K, Ottesen AM, Garn ID, Main KM, Loft A, Jorgensen N, Skakkebaek NE, Andersen AN, Rajpert-De Meyts E. Sons conceived by assisted reproduction techniques inherit deletions in the azoospermia factor (AZF) region of the Y chromosome and the DAZ gene copy number. Hum Reprod 2008; 23:1669-1678.

65. Mazeyrat S, Saut N, Grigoriev V, Mahadevaiah SK, Ojarikre OA, Rattigan A, Bishop C, Eicher EM, Mitchell MJ, Burgoyne PS. A Y-encoded subunit of the translation initiation factor Eif2 is essential for mouse spermatogenesis. Nat Genet 2001; 29:49-53.

66. McPherson SM, Longo FJ. Localization of DNase I-hypersensitive regions during rat spermatogenesis: stage-dependent patterns and unique sensitivity of elongating spermatids. Mol Reprod Dev 1992; 31:268-279.

67. Meschede D, Horst J. Sex chromosomal anomalies in pregnancies conceived through intracytoplasmic sperm injection: a case for genetic counselling. Hum Reprod 1997; 12:1125-1127.

68. Mitchell MJ, Woods DR, Tucker PK, Opp JS, Bishop CE. Homology of a candidate spermatogenic gene from the mouse Y chromosome to the ubiquitin-activating enzyme E1. Nature 1991; 354:483-486.

69. Nabatiyan A, Krude T. Silencing of chromatin assembly factor 1 in human cells leads to cell death and loss of chromatin assembly during DNA synthesis. Mol Cell Biol 2004; 24:2853-2862.

70. Noblanc A, Peltier M, Damon-Soubeyrand C, Kerchkove N, Chabory E, Vernet P, Saez F, Cadet R, Janny L, Pons-Rejraji H, Conrad M, Drevet JR, Kocer A. Epididymis response partly compensates for spermatozoa oxidative defects in snGPx4 and GPx5 double mutant mice. PLoS One 2012; 7:e38565.

71. Nottke A, Colaiacovo MP, Shi Y. Developmental roles of the histone lysine demethylases. Development 2009; 136:879-889.

72. Odorisio T, Mahadevaiah SK, McCarrey JR, Burgoyne PS. Transcriptional analysis of the candidate spermatogenesis gene Ube1y and of the closely related Ube1x shows that they are coexpressed in spermatogonia and spermatids but are repressed in pachytene spermatocytes. Dev Biol 1996; 180:336-343.

73. Oliva R. Protamines and male infertility. Hum Reprod Update 2006; 12:417-435.

74. Olson GE, Winfrey VP, NagDas SK, Melner MH. Region-specific expression and secretion of the fibrinogen-related protein, fgl2, by epithelial cells of the hamster epididymis and its role in disposal of defective spermatozoa. J Biol Chem 2004; 279:51266-51274.

75. Page DC, Silber S, Brown LG. Men with infertility caused by AZFc deletion can produce sons by intracytoplasmic sperm injection, but are likely to transmit the deletion and infertility. Hum Reprod 1999; 14:1722-1726.

76. Prado VF, Lee CH, Zahed L, Vekemans M, Nishioka Y. Molecular characterization of a mouse Y chromosomal repetitive sequence that detects transcripts in the testis. Cytogenet Cell Genet 1992; 61:87-90.

77. Raicu F, Popa L, Apostol P, Cimponeriu D, Dan L, Ilinca E, Dracea LL, Marinescu B, Gavrila L. Screening for microdeletions in human Y chromosome--AZF candidate genes and male infertility. J Cell Mol Med 2003; 7:43-48.

78. 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; 23:259-270.

79. Reynard LN, Cocquet J, Burgoyne PS. The multi-copy mouse gene Sycp3-like Y-linked (Sly) encodes an abundant spermatid protein that interacts with a histone acetyltransferase and an acrosomal protein. Biol Reprod 2009; 81:250-257.

80. Reynard LN, Turner JM. Increased sex chromosome expression and epigenetic abnormalities in spermatids from male mice with Y chromosome deletions. J Cell Sci 2009; 122:4239-4248.

81. Reynard LN, Turner JM, Cocquet J, Mahadevaiah SK, Toure A, Hoog C, Burgoyne PS. Expression analysis of the mouse multi-copy X-linked gene Xlr-related, meiosis-regulated (Xmr), reveals that Xmr encodes a spermatid-expressed cytoplasmic protein, SLX/XMR. Biol Reprod 2007; 77:329-335.

82. Riel JM, Yamauchi Y, Sugawara A, Li HY, Ruthig V, Stoytcheva Z, Ellis PJ, Cocquet J, Ward MA. Deficiency of the multi-copy mouse Y gene Sly causes

sperm DNA damage and abnormal chromatin packaging. J Cell Sci 2013; 126:803-813.

83.   Roberts G, McGrady A. Racial and gender effects on the relaxation response: implications for the development of hypertension. Biofeedback Self Regul 1996; 21:51-62.

84.   Rutstein SOaIHS. Infecundity, Infertility, and Childlessness in Developing Countries. DHS Comparative Reports No. 9. . 2004.

85.   Scavetta RJ, Tautz D. Copy number changes of CNV regions in intersubspecific crosses of the house mouse. Mol Biol Evol 2010; 27:1845-1856.

86.   Shalgi R, Seligman J, Kosower NS. Dynamics of the thiol status of rat spermatozoa during maturation: analysis with the fluorescent labeling agent monobromobimane. Biol Reprod 1989; 40:1037-1045.

87.   Shaman JA, Prisztoka R, Ward WS. Topoisomerase IIB and an extracellular nuclease interact to digest sperm DNA in an apoptotic-like manner. Biol Reprod 2006; 75:741-748.

88.   Sheiner EK, Sheiner E, Hammel RD, Potashnik G, Carel R. Effect of occupational exposures on male fertility: literature review. Ind Health 2003; 41:55-62.

89.   Shirley CR, Hayashi S, Mounsey S, Yanagimachi R, Meistrich ML. Abnormalities and reduced reproductive potential of sperm from Tnp1- and Tnp2-null double mutant mice. Biol Reprod 2004; 71:1220-1229.

90.   Simoes M, Yamaguti K, Palangana AJ. Rotational dissipation and the Miesowicz coefficients. Phys Rev E Stat Nonlin Soft Matter Phys 2009; 80:061701.

91.   Simoes R, Feitosa WB, Mendes CM, Marques MG, Nicacio AC, de Barros FR, Visintin JA, Assumpcao ME. Use of chromomycin A3 staining in bovine sperm cells for detection of protamine deficiency. Biotech Histochem 2009; 84:79-83.

92.   Sivashanmugam P, Rajalakshmi M. Sperm maturation in rhesus monkey: changes in ultrastructure, chromatin condensation, and organization of lipid bilayer. Anat Rec 1997; 247:25-32.

93.   Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, Repping S, Pyntikova T, Ali J, Bieri T, Chinwalla A, Delehaunty A, Delehaunty K, Du H, Fewell G, Fulton L, Fulton R, Graves T, Hou SF, Latrielle P, Leonard S, Mardis E, Maupin R, McPherson J, Miner T, Nash W, Nguyen C, Ozersky P, Pepin K, Rock S, Rohlfing T, Scott K, Schultz B, Strong C, Tin-Wollam A, Yang SP, Waterston RH, Wilson RK, Rozen S, Page DC. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. Nature 2003; 423:825-837.

94.   Snedecor GW, Cochran WG. Statistical methods. Ames: Iowa State University Press; 1989.

95.   Spira A. Epidemiology of human reproduction. Hum Reprod 1986; 1:111-115.

96.   Stanker LH, Wyrobek A, McKeown C, Balhorn R. Identification of the binding site of two monoclonal antibodies to human protamine. Mol Immunol 1993; 30:1633-1638.

97. Staub E, Mennerich D, Rosenthal A. The Spin/Ssty repeat: a new motif identified in proteins involved in vertebrate development from gamete to embryo. Genome Biol 2002; 3:RESEARCH0003.

98. Styrna J, Bilinska B, Krzanowskaa H. The effect of a partial Y chromosome deletion in B10.BR-Ydel mice on testis morphology, sperm quality and efficiency of fertilization. Reprod Fertil Dev 2002; 14:101-108.

99. Styrna J, Imai HT, Moriwaki K. An increased level of sperm abnormalities in mice with a partial deletion of the Y chromosome. Genet Res 1991; 57:195-199.

100. Styrna J, Kilarski W, Krzanowska H. Influence of the CBA genetic background on sperm morphology and fertilization efficiency in mice with a partial Y chromosome deletion. Reproduction 2003; 126:579-588.

101. Styrna J, Klag J, Moriwaki K. Influence of partial deletion of the Y chromosome on mouse sperm phenotype. J Reprod Fertil 1991; 92:187-195.

102. Styrna J, Krzanowska H. Sperm select penetration test reveals differences in sperm quality in strains with different Y chromosome genotype in mice. Arch Androl 1995; 35:111-118.

103. Suganuma R, Yanagimachi R, Meistrich ML. Decline in fertility of mouse sperm with abnormal chromatin during epididymal passage as revealed by ICSI. Hum Reprod 2005; 20:3101-3108.

104. Suh DS, Styrna J, Moriwaki K. Effect of Y chromosome and H-2 complex derived from Japanese wild mouse on sperm morphology. Genet Res 1989; 53:17-19.

105. Sutovsky P, Moreno R, Ramalho-Santos J, Dominko T, Thompson WE, Schatten G. A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. J Cell Sci 2001; 114:1665-1675.

106. Szczygiel MA, Moisyadi S, Ward WS. Expression of foreign DNA is associated with paternal chromosome degradation in intracytoplasmic sperm injection-mediated transgenesis in the mouse. Biol Reprod 2003; 68:1903-1910.

107. Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA, Clark AM. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod 2009; 24:2061-2070.

108. Tilford CA, Kuroda-Kawaguchi T, Skaletsky H, Rozen S, Brown LG, Rosenberg M, McPherson JD, Wylie K, Sekhon M, Kucaba TA, Waterston RH, Page DC. A physical map of the human Y chromosome. Nature 2001; 409:943-945.

109. Torregrosa N, Dominguez-Fandos D, Camejo MI, Shirley CR, Meistrich ML, Ballesca JL, Oliva R. Protamine 2 precursors, protamine 1/protamine 2 ratio, DNA integrity and other sperm parameters in infertile patients. Hum Reprod 2006; 21:2084-2089.

110. Toure A, Clemente EJ, Ellis P, Mahadevaiah SK, Ojarikre OA, Ball PA, Reynard L, Loveland KL, Burgoyne PS, Affara NA. Identification of novel Y chromosome encoded transcripts by testis transcriptome analysis of mice with deletions of the Y chromosome long arm. Genome Biol 2005; 6:R102.

111. Toure A, Szot M, Mahadevaiah SK, Rattigan A, Ojarikre OA, Burgoyne PS. A new deletion of the mouse Y chromosome long arm associated with the loss of Ssty expression, abnormal sperm development and sterility. Genetics 2004; 166:901-912.

112. Toyoshima M. Analysis of p53 dependent damage response in sperm-irradiated mouse embryos. J Radiat Res 2009; 50:11-17.

113. Tremellen K. Oxidative stress and male infertility--a clinical perspective. Hum Reprod Update 2008; 14:243-258.

114. Vernet N, Mahadevaiah SK, Ojarikre OA, Longepied G, Prosser HM, Bradley A, Mitchell MJ, Burgoyne PS. The Y-encoded gene zfy2 acts to remove cells with unpaired chromosomes at the first meiotic metaphase in male mice. Curr Biol 2011; 21:787-793.

115. Vong QP, Li Y, Lau YF, Dym M, Rennert OM, Chan WY. Structural characterization and expression studies of Dby and its homologs in the mouse. J Androl 2006; 27:653-661.

116. Wallis MC, Waters PD, Graves JA. Sex determination in mammals--before and after the evolution of SRY. Cell Mol Life Sci 2008; 65:3182-3195.

117. Wang SS, Zhou BO, Zhou JQ. Histone H3 lysine 4 hypermethylation prevents aberrant nucleosome remodeling at the PHO5 promoter. Mol Cell Biol 2011; 31:3171-3181.

118. Ward MA, Burgoyne PS. The effects of deletions of the mouse Y chromosome long arm on sperm function--intracytoplasmic sperm injection (ICSI)-based analysis. Biol Reprod 2006; 74:652-658.

119. Wennerholm UB, Bergh C, Hamberger L, Lundin K, Nilsson L, Wikland M, Kallen B. Incidence of congenital malformations in children born after ICSI. Hum Reprod 2000; 15:944-948.

120. Xian M, Azuma S, Naito K, Kunieda T, Moriwaki K, Toyoda Y. Effect of a partial deletion of Y chromosome on in vitro fertilizing ability of mouse spermatozoa. Biol Reprod 1992; 47:549-553.

121. Yamauchi Y, Ajduk A, Riel JM, Ward MA. Ejaculated and epididymal mouse spermatozoa are different in their susceptibility to nuclease-dependent DNA damage and in their nuclease activity. Biol Reprod 2007; 77:636-647.

122. Yamauchi Y, Doe B, Ajduk A, Ward MA. Genomic DNA damage in mouse transgenesis. Biol Reprod 2007; 77:803-812.

123. Yamauchi Y, Riel JM, Stoytcheva Z, Burgoyne PS, Ward MA. Deficiency in mouse Y chromosome long arm gene complement is associated with sperm DNA damage. Genome Biol 2010; 11:R66.

124. Yamauchi Y, Riel JM, Wong SJ, Ojarikre OA, Burgoyne PS, Ward MA. Live offspring from mice lacking the Y chromosome long arm gene complement. Biol Reprod 2009; 81:353-361.

125. Yildiz C, Fleming C, Ottaviani P, McKerlie C. Fresh and frozen-thawed sperm quality, nuclear DNA integrity, invitro fertility, embryo development, and live-born offspring of N-ethyl-N-nitrosourea (ENU) mice. Cryobiology 2008; 57:156-162.

126.   Zeng M, Sun H, Chen S, Wang X, Yang Y, Liu Y, Tao D, Yang Z, Zhang S, Ma Y. Identification of target messenger RNA substrates for mouse RBMY. Mol Hum Reprod 2008; 14:331-336.

127.   Zhao M, Shirley CR, Yu YE, Mohapatra B, Zhang Y, Unni E, Deng JM, Arango NA, Terry NH, Weil MM, Russell LD, Behringer RR, Meistrich ML. Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice. Mol Cell Biol 2001; 21:7243-7255.