Phosphorylation and Expression Level of Nucleophosmin is Regulated by SUMOylation in Mouse Spermatogenic Cells

Presented to the S. Daniel Abraham Honors Program

In Partial Fulfillment of the

Requirements for Completion of the Program

Stern College for Women
Yeshiva University
August 16th, 2023

Shaina Matveev

Mentor: Dr. Margarita Vigodner, Cellular Biology

Table of Contents

Abstract

Infertility has long been viewed as a predominantly female condition, even though half of infertility cases are attributed to the male partner. Despite this equal distribution of cases, the current medical understanding of and treatment options for male infertility are deeply lacking compared to that of females. This disparity not only causes there to be fewer options for treating males, but it also often places the burden of treatment for infertile males on their female partners, who may undergo procedures, such as assisted reproductive technology, and face its complications, even when she herself is fertile. This research project, performed in the Vigodner Lab, aims to help bridge this disparity and to contribute to the growing body of much-needed research on male infertility. Because male infertility is a multifactorial condition, continued research surrounding the key pathways involved in male infertility, such as the regulation of spermatogenesis, is imperative in the development of effective treatment options for infertile males. The Vigodner Lab's research focuses on elucidating one means through which spermatogenesis is regulated – through SUMOylation. SUMOylation is a type of post-translational modification (PTM) in which a SUMO protein is bonded to a target protein in order to modify its function. A growing body of research supports the presence of crosstalk between SUMOylation and phosphorylation, a different type of PTM in which a phosphate group is added to a protein in order to modify it. The presence of this crosstalk in spermatocytes was previously studied in a 2-dimensional gel electrophoresis performed in the Vigodner Lab which analyzed the phosphoproteome of spermatocytes before and after inhibition of SUMOylation with GA. It identified several potential downstream targets of SUMOylation-dependent phosphorylation, including nucleophosmin. This research project confirmed that nucleophosmin is a downstream target of SUMOylation-dependent phosphorylation in mouse spermatocytes through the use of western blotting in both cell lines and primary cells.

I. Introduction[1]

Women have been the symbol of fertility since the dawn of civilization. From the large bosomed figurines of antiquity, to the worshiped Aphrodite of the Greeks and Venus of the Romans, to today's curvy models adorning Vogue magazine, femaleness and fertility have been nearly synonymous. Thus, it is no surprise that fertility's antithesis, infertility, came to be viewed as largely a female issue. A problem to be faced by and fixed for women alone. Therefore, with the medical advancements of the modern age, came extensive research into female reproductive biology, yielding great advancements in our understanding of female fertility and fruiting countless advanced treatment options (Turner *et al.,* 2020). However, for every woman struggling with infertility, there is a man struggling too, as in contrast to the long-standing false belief that infertility is a predominantly female issue, half of infertility cases are in fact attributed to the male partner (Leslie *et al.,* 2023).

Despite this equal distribution of cases, the current medical understanding of and treatment options for male infertility are deeply lacking compared to female infertility (Ravitsky & Kimmins, 2019). In fact, approximately 30 – 50% of male infertility cases are diagnosed as idiopathic, meaning that their cause is entirely unknown (Kumar & Singh, 2015). Because of this, the burden of treatment for infertile males often falls on their female partner, who may undergo procedures, such as assisted reproductive technology (ART), and face its associated complications, even when she herself is fertile (Ravitsky & Kimmins, 2019). To correct this imbalance, there is a dire need for increased research to better understand male reproductive biology and infertility in order to improve male treatment options and enable natural conception (Leung *et al.,* 2018) (Turner *et al.,* 2020).

[1] Within this paper, due to the nature of this research, the terms "male / man" and "female / woman" refer to an individual's assigned sex at birth, not their gender identity (Mazure, 2021).

Additionally, such research can help understand and improve men's health overall, as infertility is tied to a myriad of other complications. For example, in the past 40 years, global sperm counts have halved and sperm quality has alarmingly declined, issues not only tied to infertility, but also associated with overall morbidity and mortality (Latif *et al.,* 2017) (Levine *et al.,* 2017). Furthermore, it has been demonstrated that rates of testicular germ cell tumors and cryptorchidism have risen and total testosterone levels have steadily declined since the 1970s - issues both closely tied to infertility (Doria-Rose *et al.,* 2005) (Guardo *et al.,* 2020) (Houman *et al.,* 2020) (Levine *et al.,* 2017). As these male's health issues continue to persist and deepen, the critical need for such research is becoming increasingly apparent.

This research project, performed in the Vigodner Laboratory, aims to contribute to the growing body of much-needed research on male infertility. Because infertility is a multifactorial condition, the identification of the precise mechanisms through which it occurs is difficult. Therefore, continued research on the key pathways involved in spermatogenesis and its regulation is imperative to uncovering the underlying causes of infertility and ultimately finding treatments for them. The Vigodner Laboratory's research focuses on elucidating one means through which spermatogenesis is regulated – through the post-translational modification (PTM) of SUMOylation. A growing body of research supports the presence of crosstalk between this PTM and phosphorylation, a different type of PTM in which a phosphate group is added to a protein. The presence of this crosstalk in spermatocytes was previously studied in a 2-dimensional gel electrophoresis (2-D DIGE) performed in the Vigodner Laboratory which analyzed the phosphoproteome of spermatocytes before and after inhibition of SUMOylation with GA. It identified several potential downstream targets of SUMOylation-dependent phosphorylation, including nucleophosmin. This research project seeks to confirm that

nucleophosmin is a downstream target of SUMOylation-dependent phosphorylation in mouse spermatocytes through the use of western blotting in both cell lines and primary cells.

II. Background

A. Male Infertility

Infertility, defined as the inability to conceive after one year of regular, unprotected intercourse, affects 8 – 12% of reproductive-age people, or over 180 million couples worldwide (Borght & Wyns, 2022) (Keller & Chamber, 2022) (Leslie *et al.,* 2023) (Ombelet, 2001). These cases are equally distributed between being caused solely by a male contributing factor, solely by a female contributing factor, and due to a combination of both male and female contributing factors (Agarwal *et al.,* 2015). Overall, males make up around half of all infertility cases, with cases being categorized as either primary or secondary. Primary infertility describes males who were never fertile, whereas secondary infertility describes men who became infertile later in life (Gowri *et al.,* 2010) (Katib *et al.,* 2014). Male infertility can be due to a wide variety of causes, including physical, hormonal, lifestyle, genetic, and psychological factors, with these factors often being closely associated and intertwined.

Physical abnormalities leading to infertility can hinder the production of sperm or their passage through the testes, epididymis, seminal ducts, or other reproductive structures (Babakhanzadeh *et al.,* 2020) (Singh *et al.,* 2012). The most common physical cause is varicocele, in which the spermatic veins are enlarged, blocking blood drainage from the testes to the abdomen and leading to abnormal sperm morphology and low sperm counts. Other physical abnormalities include congenitally impaired ejaculatory ducts, scarring from infections or surgery, retrograde ejaculation (when semen is ejaculated into the bladder), premature ejaculation (the inability to control ejaculation over 30 seconds following penetration), undescended testicles

(when the testicles fail to descend from the abdominal cavity during fetal development), testicular torsion, and many others (Achermann & Esteves, 2021) (Babakhanzadeh *et al.,* 2020) (Hassanzadeh *et al.,* 2010) (Punab *et al.,* 2016).

Complications causing infertility could also be hormonal. The male reproductive hormones are regulated and provided through the hypothalamic-pituitary-gonadal axis, which involves the hypothalamic, pituitary and gonadal glands, as its name implies. This axis begins as the hypothalamus releases Gonadotropin Releasing Hormone (GnRH), which stimulates the anterior pituitary to release two pituitary gonadotropins - luteinizing hormone (LH) and follicle stimulating hormone (FSH). In the testes, LH then drives the Leydig cells to release testosterone, while FSH stimulates the Sertoli cells to release androgen-binding protein (ABP), which promotes testosterone binding, both needed for sperm production (Corradi *et al.,* 2016) (Hansson *et al.,* 1976). This process is regulated through feedback loops with testosterone inhibiting the hypothalamus and inhibin (produced by the Sertoli cells) inhibiting the anterior pituitary (Figure 1) (Jin & Yang, 2016). Thus, levels of these hormones remain fairly constant throughout an adult male's life (Babakhanzadeh *et al.,* 2020).

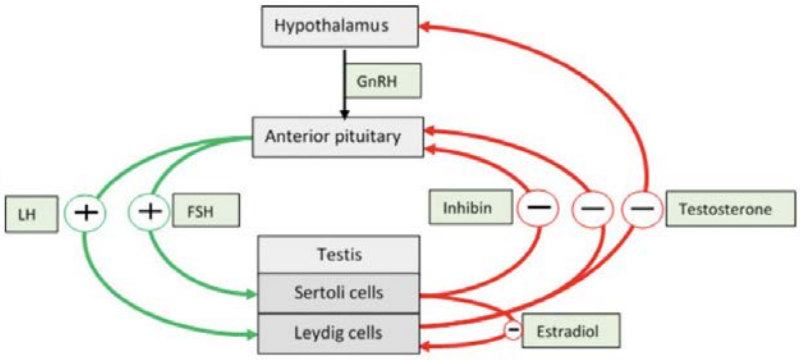

Figure 1.
Simplified diagram depicting the male hypothalamic-pituitary-gonadal axis and its feedback loops (Rosenfield & Pizzuto, 2018).

Any abnormalities in the hypothalamic-pituitary-gonadal axis can lead to infertility. For example, cessation of the hypothalamus to secrete GnRH results in a variety of disorders known as hypogonadotropic hypogonadism (HH), in which testosterone is not produced and infertility results. HH can either be congenital (as in Kallmann syndrome) or acquired, and is treated through the use of sex steroids, gonadotropins, GnRH injections, and testosterone injections (Fraietta *et al.,* 2013). Infertility can also be caused by the inability of the anterior pituitary to produce sufficient amounts of LH or FSH which requires long-term hormonal therapy, tied to a myriad of complications (Babakhanzadeh *et al.,* 2020). Additional hormonal complications that can lead to infertility include hypergonadotropic hypogonadism, elevated levels of LH or FSH, increased concentrations of estrogen, hyperprolactinemia, hypothyroidism, congenital adrenal hyperplasia, insulin disorders, and numerous others (King *et al.,* 2016) (Sengupta *et al.,* 2021).

Several occupational, environmental, and lifestyle factors are additionally associated with male infertility. Studies have demonstrated that alcohol intake, the use of prescription or illicit drugs, smoking, obesity, advanced age, dietary practices, coffee consumption, testicular heat stress, lack of sleep, and radiation are tied to infertility (Balawender & Orkisz, 2020) (Durairajanayagam, 2018) (El-helaly *et al.,* 2010) (Leslie *et al.,* 2023). Additionally, occupational exposure to various chemicals, insecticides, fungicides and pesticides, especially those including benzene and its mixture with toluene and xylene, formaldehyde, chlorpyrifos, carbamates, ethylene glycol-based chemicals, phthalates, flame retardants such as polyaromatic hydrocarbons, and dozens of others place male workers at higher risk of infertility (Marić *et al.,* 2021). Being educated and cognizant of these risks is crucial to males seeking to maintain their fertility.

Additionally, genetic factors are detected in 15% of all male infertility cases. They are grouped into two classifications: chromosomal abnormalities and single-gene mutations. Chromosomal abnormalities, which involve abnormalities of genetic material at the larger chromosomal level, can be either inherited or acquired. They include the aneuploid sex chromosome condition called Klinefelter syndrome, in which males have an extra X-chromosome, as well as Noonan syndrome, which includes an XO-XY mosaic (Babakhanzadeh *et al.,* 2020) (Gun & Gluckman, 1990). Translocations, such as Robersonian and bilateral translocations, inversions, and microdeletions, such as in the AZFa, AZFb, and AZFc segments of the Y-chromosome's long arm, are common causes too. Additional genetic conditions include Young syndrome, Kartagener syndrome, primary ciliary dyskinesia, sertoli cell-only syndrome, mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, and deletions in the FSH, LH, Kal-1, Kal-2, FGFS, GnRH1/GNRHR, PROK@/PROKR2 genes, among dozens of other conditions (Bieniek *et al.,* 2021) (Ceccaldi *et al.,* 2004) (Hawksworth *et al.,* 2018) (Leslie *et al.,* 2023) (Mohammed & Jan, 2023).

The before-mentioned causes are just a sliver of the wide spectrum of male infertility causes. Beyond these are epigenetic factors, infections, additional physical and psychological issues including erectile dysfunction, infrequent coitus and low libido, among numerous others (Babakhanzadeh *et al.,* 2020) (Leslie *et al.,* 2023). These wide-ranging causes are treated through several means. Physical abnormalities are often corrected through surgery, abnormal hormone levels are treated through hormone replacement therapies and medications, underlying conditions such as infections are treated with antibiotics, and psychological matters are managed with medication and counseling, among other treatments. The main option for treating male infertility is the use of assisted reproductive technologies (ARTs), such as *in vitro* fertilization and

intracytoplasmic sperm injections, to impregnate the female partner, both for lack of more male oriented options and due to the high idiopathic diagnostic rates for males. In rare cases, male infertility cannot be overcome and the use of a sperm donor or adoption is recommended (Jain & Sing, 2022) (Luddi *et al*., 2022) (Turner *et al.,* 2020).

B. Spermatogenesis

Spermatogenesis is the process by which spermatozoa are produced (Figure 2) (Table 1). This multi-step process occurs in the germinal epithelium of the seminiferous tubules, located in the testes, where they develop as they progress from the tubular basement membrane to the tubular lumen. Spermatogenesis occurs in three stages: spermatocytogenesis, spermatidogenesis, and spermiogenesis. This process is then followed by spermiation, in which the newly-formed spermatozoa are released into the tubular lumen.

During the first stage, spermatocytogenesis, spermatogonia stem cells proliferate mitotically to produce one type A spermatogonia to maintain the reserve of these stem cells in the basal compartment and one type B spermatogonia. The type B spermatogonia then differentiates into a primary spermatocyte which enters the adluminal compartment to undergo the next stage, spermatidogenesis (Waheeb *et al.,* 2012). During this next stage, the primary spermatocytes undergo meiosis I to produce two secondary spermatocytes each. It is during this step that genetic material is exchanged through crossing-over between the homologous chromosomes in a prolonged prophase. The secondary spermatocytes then undergo meiosis II to produce two haploid spermatids each. An incomplete cytokinesis at the end of this stage ensures that spermatids remain connected by cytoplasmic bridges which allow for further exchange of material and simultaneously maturation (Vantela *et al.,* 2003).

During the final step, spermiogenesis, these immature spermatids differentiate and elongate into spermatozoa. This process involves the formation of the acrosome, which contains hydrolytic enzymes and is later needed for the process of fertilization, and the flagellum, a modified cilia derived from the centrioles which will later give the sperm mobility using the mitochondria concentrated in its mid-piece (Berruti & Paiardi., 2011). Additionally, it involves the loss of the spermatid's excess cytoplasm which is phagocytized by the surrounding Sertoli cells. Once spermatogenesis is complete, these spermatozoa are released into the tubular lumen through spermiation (O'Donnel *et al.,* 2011). They are then stored in the epididymis, where they gain motility and fully mature, only to be released during ejaculation (James *et al.,* 2020).

Additionally, the developing germ cells of Spermatogenesis are supported by a couple non-dividing, somatic cells – the Sertoli cells and the Leydig cells. The Sertoli cells span from the seminiferous tubule's basement membrane to its lumen, where they surround and nurture the developing germ cells, providing them with necessary growth factors and nutrients. Their cells are also connected by specialized adhesion junctions which form the blood testis barrier (BTB) separating the basal and adluminal compartments (Smith & Walker, 2014). The BTB allows for the creation of a unique and protected microenvironment for the regulation of developing germ cells through the secretions of the Sertoli cells, as stimulated by various signal pathways including the cAMP/PKA, ERK1/2, PI3K/Akt, mTORC1/p70SK6, MAPK, AMPK, and TGF-β)/Smad pathways, among others (Jiang *et al.,* 2014) (Meroni *et al.,* 2019) (Ni *et al.,* 2019).

Found outside of the seminiferous tubules are the interstitial Leydig cells of the testes, responsible for the production of the androgen testosterone when stimulated by luteinizing hormone (LH). The binding of LH to Leydig LH receptors stimulates the production of cyclic adenosine monophosphate (cAMP) which raises the levels of cholesterol translocation into the

cell's mitochondria. Once in the inner mitochondrial membrane, the CYP11A1 enzyme metabolizes cholesterol to pregnenolone, which is then converted by various mitochondrial and smooth endoplasmic reticulum enzymes to testosterone (Zirkin & Papadopoulos, 2018). This testosterone is then secreted, where it acts on the Sertoli cells along with FSH to drive spermatogenesis, in addition to other functions (Ge *et al.,* 2008).

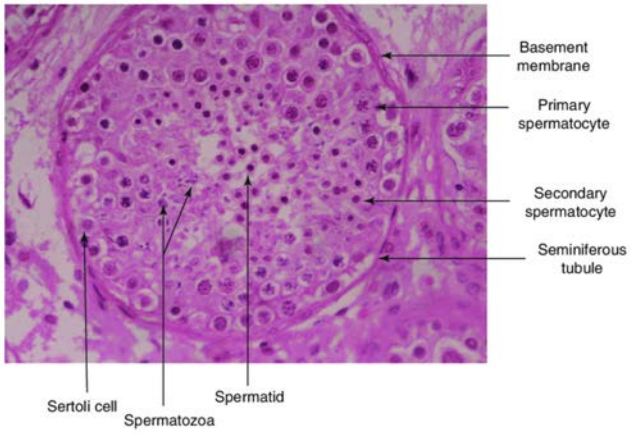

Figure 2.
H&E stained seminiferous tubule cross-section visualizing the various cellular stages of spermatogenesis (Dhole & Kumar, 2017).

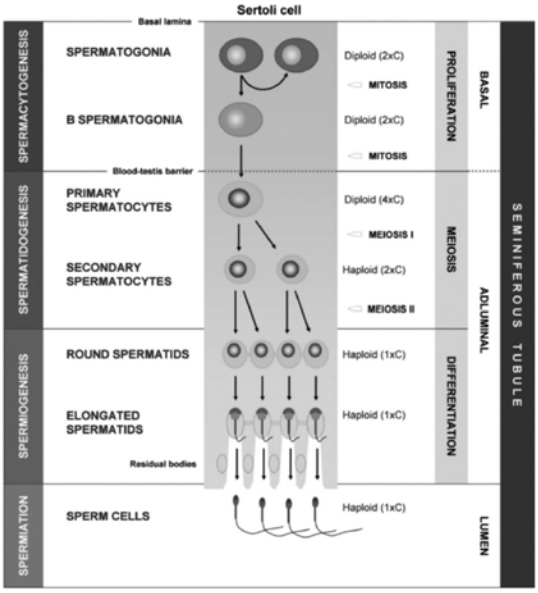

Table 1.
Diagram summarizing the progressing stages of spermatogenesis, with the name and characteristics of each step's germ cells, as well as their location in the seminiferous tubules, being listed (Tapia & Pena, 2009).

C. SUMOylation

Spermatogenesis is regulated by several post-translational modifications (PTMs) – processes through which the function of a target protein is modified after synthesis through the covalent addition of various groups or small peptides (Campbell, 2020). Common PTMs include phosphorylation, glycosylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation and proteolysis (Ramazi *et al.,* 2021). A PTM that has been increasingly studied in recent years is SUMOylation (Yang *et al.,* 2017), which involves the addition of Small Ubiquitin-Like Modifier (SUMO) proteins to a target protein, so named for its similarity in structure and function to the ubiquitin protein of ubiquitination.

Five different SUMO protein paralogues have been identified in humans, labeled SUMO 1-5. While these five paralogues are similar in most regards, they differ slightly in their structure, with each sharing 45-97% sequence similarity, as well as in their function and specific localization within the body (Bouchard *et al.,* 2021). Some functions of SUMO proteins include their role in transcriptional regulation, nuclear-cytosolic transport, stress responses, chromatin inactivation, protein stability, and progression through the cell cycle (Hay, 2005). The SUMO-1 paralogue has been detected in the testis in high concentration where it plays numerous roles, including in spermatogenesis (Vigodner *et al.,* 2006). It is for this reason that the Vigodner Laboratory, in which this research was performed, focuses on the SUMO-1 paralogue.

SUMO-1 has a 97 amino acid (AA) sequence arranged in a ββαββαβ fold similar to ubiquitin (Figure 3) (Tang *et al.,* 2008) (Zhang *et al.,* 2008). The presence of the C-terminal di-glycine motif in ubiquitin is conserved in all SUMO protein's structures, as well, and allows for their conjugation to lysine residues on target proteins. However, SUMO-1 differs in that it has a long and flexible N-terminus extending from its protein core and also the Lys 48 in ubiquitin,

required for forming polymers, is replaced by Gln69 in the same position in SUMO-1, consistent with SUMO-1's inability to form polymers (Bayer *et al.,* 1998). Other SUMO proteins, including SUMO-2 and -3, can form polymers as they have a lysine at position 11 allowing for polySUMOylation, however this too is absent in SUMO-1 (Bouchard *et al.,* 2021). It has been suggested that SUMO-1 can bind to SUMO-2/3 chains as a "chain terminator" (Lee & Dasso, 2013). SUMO-1 and ubiquitin also share a hydrophobic core maintained by internal hydrophobic residues, but the charge topology of each of these proteins differs (Bayer *et al.,* 1998). As for all SUMO proteins, SUMO-1 acts through the SUMOylation / deSUMOylation cycle to affect cellular processes, such as in the cells of spermatogenesis.

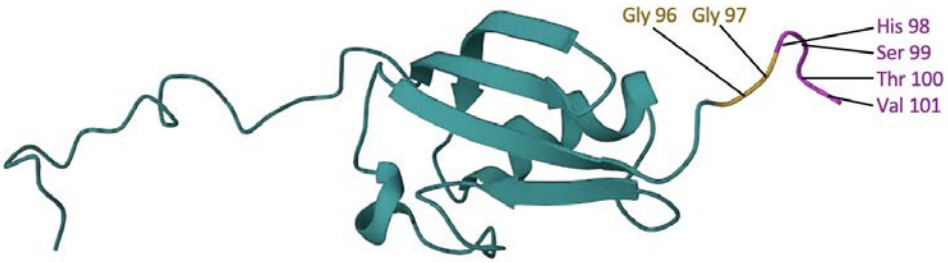

Figure 3.
Homo sapiens SUMO-1 protein ribbon structure, as visualized using Protein Data Bank (PDB). Labeled in yellow is the C-terminal di-glycine motif and labeled in purple is the C-terminal HSTV sequence, cleaved in the mature SUMO-1 protein and present in the SUMO-1 precursor.

The SUMOylation / deSUMOylation cycle is a three step reversible process through which conserved SUMO proteins bond to and modify target proteins (Figure 4). These three steps – initiation, conjugation, and ligation – are each regulated by unique enzymes. Prior to the first step, an AA peptide of the sequence HSTV is cleaved off the C-terminus of the SUMO protein precursor by a SUMO-specific carboxyl-terminal hydrolase (SENP in mammals) to reveal the di-glycine (-GG) motif required for conjugation on the mature SUMO protein (Sajeev *et al* 2021). Once in the SUMO-GG form, the cycle's first step begins with the activation of

SUMO by the activating enzyme (E1), which is a heterodimer consisting of Aos1 and Uba2 subunits. This step results in the ATP-dependent formation of a thioester bond between SUMO and E1's Uba2 subunit. SUMO is then transferred from E1 to a cysteine on Ubc9 (UbcH9 in humans), the conjugating enzyme (E2), in the second step. Finally, the third step involved the ligation of SUMO to its target protein by a member of the SUMO ligating enzyme (E3) family. KAP1 has been identified as being a SUMOligase present in the testes by previous research in the Vigodner Laboratory (Sengupta *et al.,* 2021). It is bonded on the target protein as an isopeptide bond between the C-terminus of SUMO and a target lysine residue commonly present within a ψ-K-X-D/E sequence (in which ψ is a large hydrophobic AA and X is any AA) (Celen & Sahin, 2020) (Hilgarth *et al.,* 2004) (Lee & Dasso, 2013) (Sajeev *et al.,* 2021). This process is reversed through the action of SUMO-specific isopeptidases and proteases, such as the sentrin-specific proteases in mammals (Kunz *et al.,* 2018).

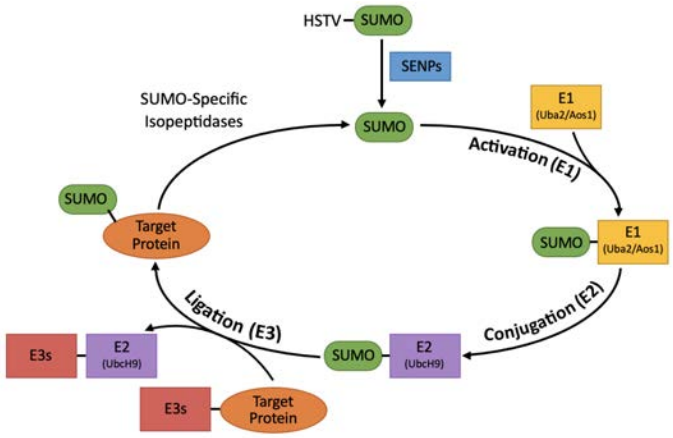

Figure 4.
The SUMOylation / deSUMOylation cycle, depicted with its three steps (activation, conjugation, and ligation) and respective enzymes (E1, E2, E3) shown.

D. Protein Phosphorylation

The most frequently occurring post-translational modification (PTM) in eukaryotes is protein phosphorylation, which is the reversible covalent bonding of a phosphate group (PO_4) to an

amino acid (AA) residue on a protein (Ramazi e*t al.,* 2021) (Ubersax & Ferrell, 2007). In eukaryotes, this occurs mainly on serine, threonine, and tyrosine AA residues through phosphoester bonds (Ardito *et al.,* 2017), though it can happen non-canonically on lysine, arginine, histidine, cysteine, aspartic acid and glutamic acid, as well (Hardman *et al.,* 2019). This addition of a phosphate group to a protein is catalyzed by the ATP-dependant actions of protein kinases, while the reverse action of dephosphorylation is catalyzed by phosphatases, which hydrolyze phosphoric acid monoesters into separated phosphate groups and substrates (Figure 5) (Ardito *et al.,* 2017) (Li *et al.,* 2013).

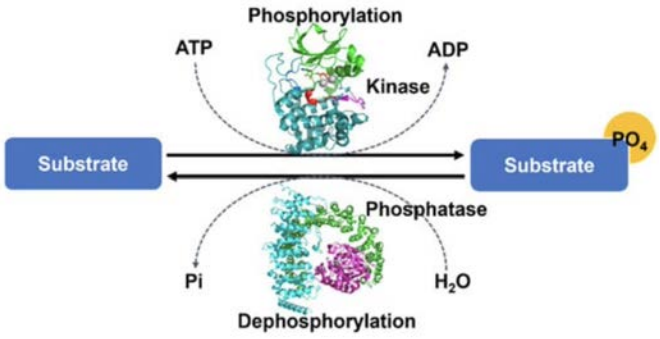

Figure 5.
A simplified mechanism of protein phosphorylation and dephosphorylation, through the action of kinase and phosphatase, respectively (Seok, 2021).

The phosphorylation or dephosphorylation of a protein profoundly affects it, causing it to become activated or deactivated, or modifying its function. It can introduce a conformational change to the structure of the protein, or create new protein-protein interaction surfaces, among other possible changes, affecting its reactivity and ability to bind to other molecules (Cheng *et al.,* 2011) (Kobe *et al.,* 2005). Changing a substrate's phosphorylation state initiates a cascade of cellular and physiological effects. It is a primary mechanism through which a wide range of cellular processes are regulated, such as in metabolism, protein regulation, cell signaling, secretory processes, cellular transport, and numerous others (Pawson & Schott, 2005).

The body contains a diverse range of protein kinases which are commonly divided into a few groups including the Serine / Threonine Protein Kinases (STPKs), the Tyrosine Kinases (TKs), and the Dual Specificity Protein Kinases (DSPKs), among others (Fabro *et al.,* 2005) (Goldsmith *et al.,* 2007) (Thiriet, 2012). As their names' suggest, each class phosphorylates different AA residues. Structurally, nearly all kinases contain an N-terminal lobe, made up of a 5-stranded β-sheet, and a C-terminal lobe made up of mostly α-helices and loops, joined by a hinge region (Figure 6). Between these lobes is a pocket containing a conserved ATP-binding site, as well as an activation segment (Chakraborty *et al.,* 2019) (Kornev & Taylor, 2009) (McClenden *et al.,* 2014). It is through the activation segment that substrate recognition occurs; it helps determine which residues each of the different kinases can interact with and phosphorylate (Izarzugaza *et al.,* 2011). Additionally, kinases themselves are regulated at numerous levels, including through subcellular localization, control of their synthesis, PTMs, and the binding of regulatory proteins (Kostich *et al.,* 2002).

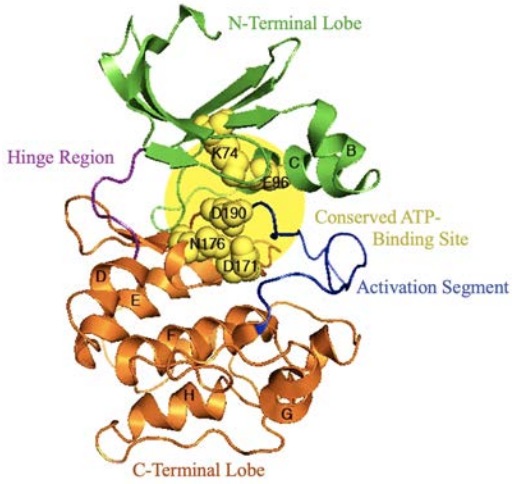

Figure 6.
Human protein kinase model structure showing the N-terminal and C-terminal lobes, as connected by the hinge region, and containing a conserved ATP binding pocket and activation segment for substrate recognition (Izarzugaza *et al.,* 2011).

A growing body of research supports the presence of crosstalk between phosphorylation and SUMOylation. This is suggested in how numerous components of the SUMOylation conjugation system are phosphoproteins and in how some enzymes and regulators involved in protein phosphorylation can be SUMOylated, as well as in the presence of proteins possessing sites for both phosphorylation and SUMOylation (Tomanov *et al.,* 2018) (Uzoma *et al*., 2018). Furthermore, a phosphorylation-dependent SUMOylation motif has been shown to exist in several proteins (Hietakangas *et al.,* 2006) (Mohideen *et al.,* 2009) (Ptak *et al.,* 2021) and it has also been shown that inhibition of SUMOylation, such as through the SUMOylation inhibitor Ginkgolic Acid (GA), affects the phosphorylation of multiple proteins (Yao *et al.,* 2011). Studies have demonstrated that this crosstalk impacts numerous cellular processes, from influencing the transcriptional activity of proteins to affecting protein-protein interactions (Kuo *et al.,* 2012) (Tomasi & Ramani, 2018).

Recent work at the Vigodner Laboratory showed that inhibition of SUMOylation with GA arrested purified mouse spermatocytes at the prophase to metaphase transition *in vitro,* with the cells being unable to disassemble their synaptonemal complexes or condense their chromatin, and the kinases normally activated during this transition, such as Polo-like kinase 1 (PLK1) and Aurora B (AURKB), being significantly inhibited. This additionally affected tyrosine phosphorylation on proteins throughout the cells. However, the specific phosphorylation targets in spermatocytes that are affected by SUMOylation have not yet been identified (Xiao *et al.,* 2017). The Vigodner Laboratory recently, through the use of a 2-D DIGE, analyzed the phosphoproteome of spermatocytes before and after inhibition of SUMOylation, with the SUMOylation inhibitor Ginkgolic Acid (GA), and identified several downstream targets of SUMOylation-dependent phosphorylation, including Nucleophosmin 1 (Figure 7).

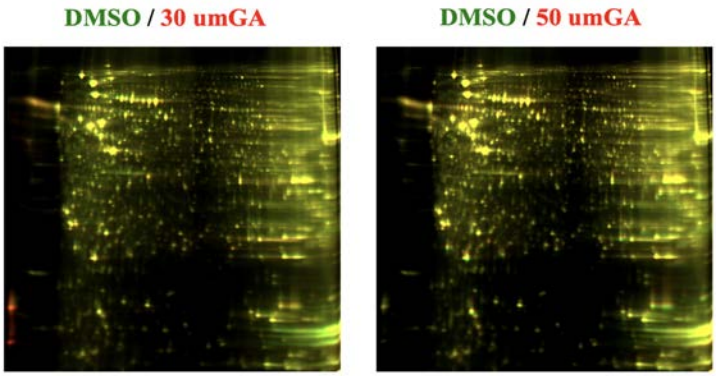

Figure 7.
The 2-dimensional gel electrophoresis of germ cells with two concentrations of GA, revealing changes in the phosphoproteome of these cells associated with SUMOylation inhibition. Nucleophosmin was identified as one of these affected phosphoproteins.

E. Nucleophosmin

The nucleophosmin (NPM) family is a group comprised of three major phosphoprotein members - NPM1, NPM2, and NPM3. All three members share strong sequence and structural homology, including having an N-terminal hydrophobic core domain, required for oligomerization and chaperone activity, followed by a long unstructured segment enriched with acidic stretches, needed for ribonuclease and histone chaperone activity (Figure 8) (Box *et al.,* 2016). These members differ in that only NPM1 and NPM2 contain a basic domain required for nucleic acid binding, and NPM1 alone contains a C-terminal aromatic domain, necessary for nucleolar localization (Okuwaki *et al.,* 2012) (Yip *et al.,* 2011). Of these three members, NPM1 was chosen to be the focus of this research project.

NPM1 is encoded in the 5q35 gene in humans, where the use of alternative codons allows for the production of a couple splice variants from its 12 exons - NPM1.1 and NPM1.3 (a third variant, NPM1.2 has been suggested, however no current biological data supports its existence) (Yip *et al.,* 2011) (Box *et al*., 2016). This nucleolar protein shuffles between the nucleoli, nucleoplasm, and cytoplasm, where it regulates various critical biological functions including

regulating gene transcription, molecular chaperoning, as well as being involved in ribosome biogenesis, apoptosis inhibition, and regulation of the cell cycle (Dermani *et al*., 2021) (Yip *et al.,* 2011). It has further been shown to play a reproductive role as NMP1-knockout mice in the Vigodner Laboratory, who have inactivated NPM1, showed early embryonic fatality.

NMP1 contains several sites for both SUMOylation and phosphorylation. SUMOylation occurs on k230 and k263, with the latter being the main site. Mutation of k263 abolishes its subcellular distribution and makes it more susceptible to caspase-3 cleavage, sensitizing cells to apoptosis, while mutation of k230 (K230R) causes is it to strongly bind to phosphatidylinositol -3,4,5-triphosphate and decreases DNA fragmentation (Liu *et al.,* 2007). For phosphorylation of NMP1, over 40 putative sites exist and several implicated kinases have been identified (Mitrea *et al.,* 2013). In the before-mentioned 2-D DIGE previously performed in the Vigodner Laboratory, NPM1 showed a dose-dependent decrease in phosphorylation as concentration levels of GA was increased, suggesting it is regulated by SUMOylation-dependent phosphorylation in spermatocytes. Additionally, NMP1 is highly expressed in the testes, although its role in meiosis is unknown.

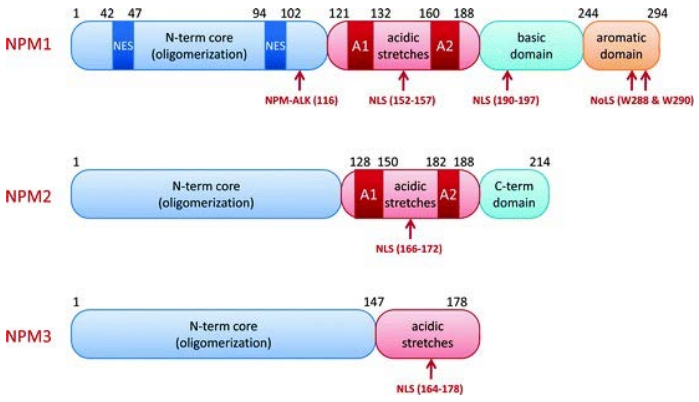

Figure 8.
Diagram depicting the structural domain organization of NPM1, NPM2, and NPM3. Shown, from left to right, are the N-terminal cores, acidic stretches, basic domains, and aromatic domain, differing or conserved in each (Yip *et al.,* 2011).

III. Objective

The purpose of this research project, performed in the Vigodner Laboratory, is to confirm that nucleophosmin is a downstream target of SUMOylation-dependent phosphorylation in mouse spermatocytes through the use of gel electrophoresis and western blotting in both cell lines and primary cells.

IV. Materials and Methods

A. Cell Line

The cell line utilized was the type B spermatogonia-derived GC1 line (ATCC® CRL2053™). These cells were purchased from ATCC (Manassas, VA) and grown at 37°C with 5% CO_2 in DMEM media (11995-065, Life Technologies, Carlsbad, CA) with 5% fetal bovine serum (FBS, 16140-071, Life Technologies), 5% bovine growth serum (SH3054103HI, Fisher Scientific, Carlsbad, CA), 1% penicillin/streptomycin (15140-122, Life Technologies), and 0.5% Fungizone (15290-018, Life Technologies).

B. Germ Cell Purification.

In addition to the use of the GC1 cell line, primary germ cells were purified from euthanized mice and used as well. The C57BL/6NCrl mice were purchased from Charles River (Kingston, NY, USA). The Animal Committee of Albert Einstein College of Medicine (Bronx, NY, USA) approved all animal protocols. Mice at 19–25 dpp (time during the first spermatogenic wave when late spermatocyte complete meiosis and the formation of the round spermatids is ongoing) were sacrificed, and their testes were isolated, decapsulated, and enzymatically digested, first with collagenase (1 mg ml^{-1}) and DNase I (1 μg ml^{-1}) for 4 min to remove Leydig cells, and then with collagenase (1 mg ml^{-1}), trypsin (0.5 mg ml^{-1}), hyaluronidase (1.5 mg ml^{-1}), and DNase I (1

μg ml^{-1}) together for 8 minutes (Figure 9). The enzymes were obtained from Sigma-Aldrich (St. Louis, MO, USA). Both the digestions were performed with constant shaking at 150 rpm in a 34°C water bath (C76 water bath shaker, New Brunswick Scientific, Edison, NJ, USA).

The cells were then filtered through a 70-μm filter, counted, centrifuged at 300g at 4°C for 7 min, and re-suspended in a prewarmed DMEM (Sigma-Aldrich). Approximately 1×10^6 cells were added per one 100mm FBS-coated Petri dish. The dishes were incubated for about 2-3 hours at 34°C. After the incubation, the non-adhesive cells were collected, and the flasks were washed 2 times with gentle agitation. The purity of the isolation was assessed using germ- and Sertoli-specific markers (anti-germ cell-specific antigen antibody [TRA98], and GATA-Binding Factor 4 [GATA4], respectively). Both antibodies were from Abcam (Cambridge, MA, USA), as used in previous studies at the Vigodner Laboratory (Vigodner *et al* 2020).

Figure 9.
The testes of a dissected, euthanized mouse to be isolated, decapsulated, and enzymatically digested.

C. Ginkgolic Acid and Si-RNA Treatments

To assess the effects of SUMOylation on the proteome of cells, Ginkgolic Acid (GA) and siRNA treatments were used to inhibit SUMOylation in experimental cells, to be compared with control cells. For the primary cells, the sumoylation inhibitor Ginkgolic acid (GA) was diluted in DMSO and used at a concentration of 30-100 uM for 2 hours. The concentration was chosen based on

previous Vigodner Laboratory studies (Xiao *et al*., 2016) and at a range that didn't cause massive cell death and detachment. For the cell line, SiRNA treatments were used to inhibit SUMOylation. Eighty pmols of UBC9 (E2), KAP1(E3) or control siRNAs (Santa Cruz Biotechnology; sc-36773, sc-38551 and sc-36869, respectively) were used for the transfection of the GC1 cell line using siRNA transfection reagent (sc-29528) and siRNA transfection medium (sc-36868). The cells were subjected to 6 hours of transfection followed by a 48-hour recovery period prior to analysis, done according to the company instructions. The best dose of siRNAs and the transfection time were determined in pilot experiments, and the downregulation was assessed by western blot analysis.

D. Gel Electrophoresis and Western Blot Analysis

The proteins of SUMOylation-inhibited cells were analyzed through gel electrophoreses and western blots and compared to those of control cells to assess the effects of SUMOylation on the overall proteome of these cells and on NPM1 in particular. Gel electrophoresis was performed under reducing conditions using NuPAGE 4%–12% gradient bis-tris polyacrylamide gels (Thermo Fisher) and MOPS running buffer (Thermo Fisher) as previously described in the Vigodner Laboratory's publications (Sengupta *et al.,* 2021) (Siao *et al.,* 2017). For the primary cells, gels were loaded with triplicate sets of the proteins of control cells, cells treated with 50 μM of GA, and cells treated with 100 μM of GA. For the GC1 line cells, gels were loaded with triplicate sets of the proteins of control cells, cells treated with UBC9 siRNA, and cells treated with KAP1 siRNA.

Rabbit polyclonal anti-SUMO1 (Abcam, ab32058) and anti-SUMO2/3 (Abcam, ab3742) antibodies were used in a 1:500 dilution; a rabbit polyclonal antibody against PCNA (Abcam, ab29) was used at 1:1000 dilution. To visualize nucleophosmin, anti-nucleophosmin (phospho

S125) antibody [EPR1856] (Abcam, ab109546) was used. Equal loading was ensured with monoclonal anti-β-actin (sc-1615, Santa Cruz) or monoclonal anti-β-tubulin (1:2000; Invitrogen) antibody in a 1:1000 dilution. Quantitative densitometry analyses were performed using the Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA), and the density values were normalized to actin or tubulin. In each experiment, controls (untreated samples) were considered as 1, and other samples were normalized to the controls. Each experiment was repeated three times. To calculate the difference between the samples, a paired t-test was used. A value of $p < 0.05$ was considered statistically significant for all experiments.

E. Whole-Cell Protein Lysates and Phosphatase Treatments

Phosphatases were used to inhibit phosphorylation in experimental cells to be compared to control cells. For control cells, whole-cell protein lysates were prepared as previously described, using the whole-cell extraction kit and protease inhibitor from Millipore (2910, Sigma-Aldrich) complemented with phosphatase inhibitors and 2.5 mg ml^{-1} of NEM (a de-sumoylation inhibitor; E3876-100G, Sigma-Aldrich) according to the manufacturer's instructions. For experimental cells, 20 µl of cell lysates, prepared without the phosphatase inhibitor, were incubated with 3µl of rCutSmart™ Buffer buffer (10X) and 3µl of quick calf intestinal alkaline phosphatase (CIP, New England BioLabs,) followed by an incubation for 1 hour at 37°C.

V. Results

A. Western Blot Analysis

Two sets of gels were prepared, one with the proteins of the GC1 cell line (Figure 10a) (containing triplicate sets of the proteins of control cells, cells treated with UBC9 siRNA, and cells treated with KAP1 siRNA) and one with the proteins of the primary cells (Figure 10b)

(containing triplicate sets of the proteins of control cells, cells treated with 50 μM of GA, and cells treated with 100 μM of GA). For both gels, the western blot analyses revealed high molecular weight bands of SUMOylated proteins in the control lane triplicates that were significantly downregulated in the treated, experimental lanes. The treated lanes also all showed increased free SUMO protein concentrations. Additionally, they lacked the upper nucleophosmin band present in the control lanes and showed downregulation in the lower nucleophosmin band. The downregulation in the lower band held true for the cell line triplicates treated with UBC9 siRNA ($p = 0.03$) and KAP1 siRNA ($p = 0.04$) (Figure 10a), as well as the primary cell triplicates treated with 50 μM of GA ($p = 0.007$) and 100 μM of GA ($p = 0.001$) (Figure 10b). The addition of the actin antibody confirmed equal loading amongst all the lanes.

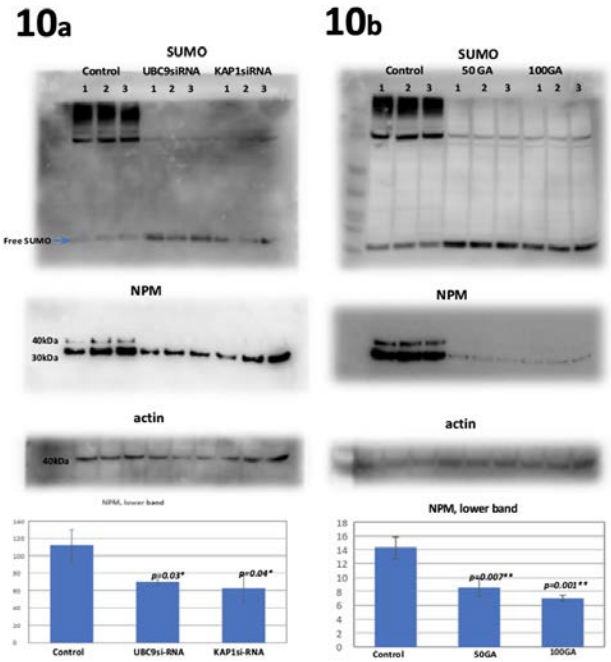

Figure 10.
Results of the western blot. 10a depicts the gel with the GC1 cell line's cells, containing triplicate sets of the proteins of control cells, cells treated with UBC9 siRNA, and cells treated with KAP1 siRNA. 10b depicts the gel with the primary cells containing triplicate sets of the proteins of control cells, cells treated with 50 μM of GA, and cells treated with 100 μM of GA. All treated lanes showed significantly downregulated SUMOylated proteins, increased free SUMO, almost no upper NPM band, and significantly

downregulated lower NPM bands, compared to control cells. Beneath each, the significance of the downregulation of the lower NMP band is confirmed, with p values shown. Actin confirmed equal loading.

B. Phosphatase Treatment

Upon treatment with phosphatase, the upper nucleophosmin band decreased ($p = 0.03$) and the lower nucleophosmin band increased on the western blot ($p = 0.03$), as compared to the control lanes not treated with phosphatase and confirmed with statistical analysis. Actin confirmed equal loading of the sample (Figure 11).

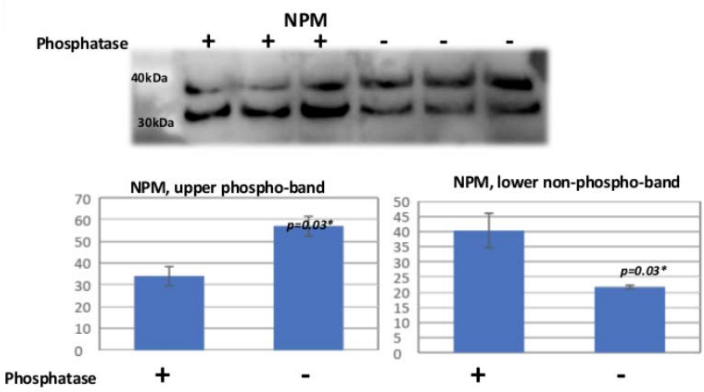

Figure 11.
Results of the phosphatase treatment analysis. Proteins from cells treated with phosphatase were loaded in the first three lanes and control cell proteins were loaded in the last three. The addition of a NMP1 antibody revealed decreased expression of the upper NMP1 band and increased expression of the lower NMP1 band. Actin confirmed equal loading.

VI. Discussion

The purpose of this research project was to confirm that NMP1 is a downstream target of SUMOylation-dependent phosphorylation in mouse spermatocytes through the use of western blotting in both cell lines and primary cells. NMP1 was chosen to be the focus of this project as a 2-dimensional gel electrophoresis previously performed in the Vigodner Laboratory, which analyzed the phosphoproteome of spermatocytes before and after inhibition of SUMOylation

with GA, identified several potential downstream targets of SUMOylation- dependent phosphorylation, including NMP1.

To confirm this finding, two gels were run. One was run with samples of the primary germ cells obtained from the euthanized mice, as treated with SUMOylation-inhibiting GA, and one with samples of the maintained GC1 cell line, as treated with KAP-1 and UBC-9 siRNA, which respectively inhibit the E3 and E2 enzymes implicated in the SUMOylation cycle. The use of western blotting and gel electrophoresis allowed for the proteins of these treated cells to be compared to the proteins present in control cells in which SUMOylation was occurring as per normal. The western blot analyses firstly revealed the profound effects of SUMOylation on the overall proteome of spermatocytes, as the high molecular weight bands of SUMOylated proteins in the control lane triplicates were significantly downregulated in the experimental lane triplicates, confirmed in both gels. Additionally, the experimental lanes showed increased free SUMO protein concentrations, as those SUMO proteins are no longer involved in SUMOylation.

Regarding NMP1, the gel electrophoresis revealed two NMP1 bands in both gels, with the upper band not being present and the lower band showing significant downregulation in the experimental cell lanes. To ascertain whether these NMP1 bands represented the phosphorylated isoform of NMP1 or not, phosphatase treatment tests were performed. This was done as the antibody used in the western blots had only been confirmed to show the phosphorylated NMP1 isoform in humans - not in mice. A western blot was run with samples in which phosphorylation was inhibited with a phosphatase treatment, alongside controls. This test revealed that the upper band is the phosphorylated isoform, as this band decreased when treated with phosphatase, while the lower band is the unphosphorylated isoform, as this band increased when treated with phosphatase. This finding was further confirmed using additional antibodies.

Therefore, the inhibition of SUMOylation caused both a decrease in the phosphorylation of NMP1, as the upper phosphorylated band was not present in the experimental lanes, as well as a decrease in the overall expression level of NMP1, as the lower unphosphorylated band showed significant downregulation in the experimental lanes. NMP1 was thus confirmed to be a downstream target of SUMOylation-dependent phosphorylation in mouse spermatocytes, with both its phosphorylation and expression being affected.

VII. Conclusion

Half of the 180 million cases of infertility worldwide are attributed to the male partner. Despite this, the understanding of and treatment options for male infertility are significantly lacking compared to that of females. Aiming to help bridge this gap, this research project focused on increasing the understanding of the role of one means through which spermatogenesis is regulated - through SUMOylation. A growing body of research suggests the presence of crosstalk between SUMOylation and phosphorylation in regulating processes in various cell types, however its role in the testes is largely unknown. Performed in the Vigodner Laboratory, this project confirmed that nucleophosmin is a downstream target of SUMOylation-dependent phosphorylation in mouse spermatocytes, with both its phosphorylation and expression being affected. This opens up new possibilities for further research, including identifying the role of nucleophosmin in meiosis and elucidating the precise effects of its downregulation. Outside the laboratory, further research could include screening the testicular biopsies of infertile males for mutations in the SUMOylation enzymes, nucleophosmin, and other SUMOylation targets. With further research, the gap in our understanding of male infertility versus female infertility will close and as it does, treatment options will open, bringing hope and solutions to countless individuals seeking to conceive.

Acknowledgements

Dr. Cynthia Wachtell:

Thank you for organizing and running the S. Daniel Abraham Honors Program at Stern College for Women. I feel truly privileged to have had the opportunity to partake in this rigorous program. Its challenging classes, cultural and educational events, and guidance truly enhanced my college experience and will impart me with a lasting impression for years to come.

Dr. Margarita Vigodner:

Thank you for going above and beyond in mentoring and guiding me in completing this project. From providing me with the opportunity to partake in rigorous and meaningful scientific research at your lab, to instructing me in the crucial skills and techniques needed to enter the scientific community, to giving me invaluable advice and support, I truly could not be more grateful.

Dr. Amitabha Sengupta:

Thank you for your infinite patience in teaching me wet lab skills and techniques, as well as for teaching me their backgrounds and applications. I could not be more grateful for your instruction and encouragement.

National Institutes of Health (NIH) and Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD):

Thank you for supporting the work of the Vigodner Lab in which this research project was performed.

Drs. Phyllis and Abraham S. Weissman Summer Student Research Fund in STEM at SCW:

Thank you for supporting my 2022 summer research at the Vigodner lab, your generous scholarship made this project possible.

Amanda Shalumov, Noam Levi, and Sara Chemel:

Thank you for your friendship and support as I worked alongside you in the Vigodner lab, I could not ask for a better group of peers to have spent this time with.

Family, Friends, and Loved Ones:

Thank you for standing by me and supporting me throughout this project, my academic journey, and beyond. This endeavor would not have been possible without your encouragement, your undying belief in my strength and capabilities, and your willingness to help wherever needed, from proofreading a draft to providing a cheery pep-talk when I needed it most. I will always be grateful for the role each of you played in helping me reach this milestone and for all that will follow.

References

Achermann, A. P. P., & Esteves, S. C. (2021). Diagnosis and management of infertility due to ejaculatory duct obstruction: Summary evidence. *International Braz j Urol*, *47*(4), 868–881. https://doi.org/10.1590/s1677-5538.ibju.2020.0536

Agarwal, A., Mulgund, A., Hamada, A., & Chyatte, M. R. (2015). A unique view on male infertility around the Globe. *Reproductive Biology and Endocrinology*, *13*(1). https://doi.org/10.1186/s12958-015-0032-1

Ardito, F., Giuliani, M., Perrone, D., Troiano, G., & Muzio, L. L. (2017b). The crucial role of protein phosphorylation in cell signaling and its use as targeted therapy (review). *International Journal of Molecular Medicine*, *40*(2), 271–280. https://doi.org/10.3892/ijmm.2017.3036

Assidi, M. (2022). Infertility in men: Advances towards a comprehensive and integrative strategy for Precision Theranostics. *Cells*, *11*(10), 1711. https://doi.org/10.3390/cells11101711

Babakhanzadeh, E., Nazari, M., Ghasemifar, S., & Khodadadian, A. (2020). Some of the factors involved in male infertility: a prospective review</p>. *International Journal of General Medicine*, *Volume 13*, 29–41. https://doi.org/10.2147/ijgm.s241099

Balawender, K., & Orkisz, S. (2020). The impact of selected modifiable lifestyle factors on male fertility in the modern world. *Central European Journal of Urology*, *73*(4), 563–568. https://doi.org/10.5173/ceju.2020.1975

Berruti, G., & Paiardi, C. (2011). Acrosome biogenesis. *Spermatogenesis*, *1*(2), 95–98. https://doi.org/10.4161/spmg.1.2.16820

Bieniek, J. M., Lapin, C. D., & Jarvi, K. A. (2021). Genetics of CFTR and male infertility. *Translational Andrology and Urology*, *10*(3), 1391–1400. https://doi.org/10.21037/tau.2020.04.05

Bilbrough, T., Piemontese, E., & Seitz, O. (2022). Dissecting the role of protein phosphorylation: A Chemical Biology Toolbox. *Chemical Society Reviews*, *51*(13), 5691–5730. https://doi.org/10.1039/d1cs00991e

Borght, M. V., & Wyns, C. (2018). Fertility and infertility: Definition and epidemiology. *Clinical Biochemistry*, *62*, 2–10. https://doi.org/10.1016/j.clinbiochem.2018.03.012

Box, J. K., Paquet, N., Adams, M. N., Boucher, D., Bolderson, E., O'Byrne, K. J., & Richard, D. J. (2016). Nucleophosmin: From structure and function to disease development. *BMC Molecular Biology*, *17*(1). https://doi.org/10.1186/s12867-016-0073-9

Campbell, M. (2020, December 4). *An introduction to post-translational modifications*. Proteomics & Metabolomics from Technology Networks. https://www.technologynetworks.com/proteomics/articles/an-introduction-to-post-translational-modifications-343603

Ceccaldi, P.-F., Carré-Pigeon, F., Youinou, Y., Delépine, B., Bryckaert, P.-E., Harika, G., Quéreux, C., & Gaillard, D. (2004). Syndrome de Kartagener et stérilité : Observation, diagnostic et prise en charge. *Journal de Gynécologie Obstétrique et Biologie de La Reproduction*, *33*(3), 192–194. https://doi.org/10.1016/s0368-2315(04)96439-3

Celen, A. B., & Sahin, U. (2020). Sumoylation on its 25th anniversary: Mechanisms, pathology, and emerging concepts. *The FEBS Journal*, *287*(15), 3107–3146. https://doi.org/10.1111/febs.15319

Chakraborty, S., Inukai, T., Fang, L., Golkowski, M., & Maly, D. J. (2019). Targeting dynamic ATP-binding site features allows discrimination between highly homologous protein kinases. *ACS Chemical Biology*, *14*(6), 1249–1259. https://doi.org/10.1021/acschembio.9b00214

Cheng, H.-C., Qi, R. Z., Paudel, H., & Zhu, H.-J. (2011). Regulation and function of protein kinases and phosphatases. *Enzyme Research*, *2011*. https://doi.org/10.4061/2011/794089

Corradi, P. F., Corradi, R. B., & Greene, L. W. (2016). Physiology of the hypothalamic pituitary gonadal axis in the male. *Urologic Clinics of North America*, *43*(2), 151–162. https://doi.org/10.1016/j.ucl.2016.01.001

Dhole, B., & Kumar, A. (2017). Spermatogenesis. In *Basics of Human Andrology* (pp. 135–160). essay, Springer Nature Singapore Pte Ltd.

Di Guardo, F., Vloeberghs, V., Bardhi, E., Blockeel, C., Verheyen, G., Tournaye, H., & Drakopoulos, P. (2020). Low testosterone and semen parameters in male partners of infertile couples undergoing IVF with a total sperm count greater than 5 million. *Journal of Clinical Medicine*, *9*(12), 3824. https://doi.org/10.3390/jcm9123824

Doria-Rose, V. Paul., Biggs, M. L., & Weiss, N. S. (2005). Subfertility and the risk of testicular germ cell tumors (United States). *Cancer Causes & Control*, *16*(6), 651–656. https://doi.org/10.1007/s10552-005-0169-x

Durairajanayagam, D. (2018). Lifestyle causes of male infertility. *Arab Journal of Urology*, *16*(1), 10–20. https://doi.org/10.1016/j.aju.2017.12.004

El-Helaly, M., Awadalla, N., Mansour, M., & El-Biomy, Y. (2010). Workplace exposures and male infertility — a case-control study. *International Journal of Occupational Medicine and Environmental Health*, *23*(4), 331–338. https://doi.org/10.2478/v10001-010-0039-y

Fabbro, D., Cowan-Jacob, S. W., & Moebitz, H. (2015). Ten things you should know about protein kinases: Iuphar Review 14. *British Journal of Pharmacology*, *172*(11), 2675–2700. https://doi.org/10.1111/bph.13096

Fraietta, R., Zylberstejn, D. S., & Esteves, S. C. (2013). Hypogonadotropic Hypogonadism revisited. *Clinics*, *68*, 81–88. https://doi.org/10.6061/clinics/2013(sup01)09

Fukuda, I., Ito, A., Hirai, G., Nishimura, S., Kawasaki, H., Saitoh, H., Kimura, K., Sodeoka, M., & Yoshida, M. (2009). Ginkgolic acid inhibits protein sumoylation by blocking formation of the E1-sumo intermediate. *Chemistry & Biology*, *16*(2), 133–140. https://doi.org/10.1016/j.chembiol.2009.01.009

Ge, R., Chen, G., & Hardy, M. P. (2009). The role of the Leydig cell in spermatogenic function. *Advances in Experimental Medicine and Biology*, *636*, 255–269. https://doi.org/10.1007/978-0-387-09597-4_14

Gerstmeier, J., Seegers, J., Witt, F., Waltenberger, B., Temml, V., Rollinger, J. M., Stuppner, H., Koeberle, A., Schuster, D., & Werz, O. (2019). Ginkgolic acid is a multi-target inhibitor of key enzymes in pro-inflammatory lipid mediator biosynthesis. *Frontiers in Pharmacology*, *10*. https://doi.org/10.3389/fphar.2019.00797

Goldsmith, E. J., Akella, R., Min, X., Zhou, T., & Humphreys, J. M. (2007). Substrate and docking interactions in serine/threonine protein kinases. *Chemical Reviews*, *107*(11), 5065–5081. https://doi.org/10.1021/cr068221w

Gowri, V., Venkiteswaran, K. P., Al-Zakwani, I., Mathew, J., Rahman, K. A., & Al-Marhoon, M. (2010). Comparison of the demographics, semen parameters and hormone profiles in men with primary and secondary infertility. *Sultan Taboos University Medical Journal*, *10*(3), 350–353.

Handelsman, D. J., Conway, A. J., Boylan, L. M., & Turtle, J. R. (1984). Young's syndrome. obstructive azoospermia and chronic sinopulmonary infections. *New England Journal of Medicine*, *310*(1), 3–9. https://doi.org/10.1056/NEJM198401053100102

Hansson, V., Weddington, S. C., French, F. S., McLean, W., Smith, A., Nayfeh, S. N., Ritzen, E. M., & Hagenas, L. (1976). Secretion and role of androgen-binding proteins in the testis and epididymis. *Journey of Reproduction and Fertility Supplement*, *24*, 17–33.

Hardman, G., Perkins, S., Brownridge, P. J., Clarke, C. J., Byrne, D. P., Campbell, A. E., Kalyuzhnyy, A., Myall, A., Eyers, P. A., Jones, A. R., & Eyers, C. E. (2019). Strong anion exchange‐mediated phosphoproteomics reveals extensive human non‐canonical phosphorylation. *The EMBO Journal*, *38*(21). https://doi.org/10.15252/embj.2018100847

Hassanzade, K., Yavari-kia, P., Ahmadi-Asr, Y., Nematzadeh, A., & Alikhah, H. (2010). Features of premature ejaculation in infertile men. *Pakistan Journal of Biological Sciences*, *13*(18), 911–915. https://doi.org/10.3923/pjbs.2010.911.915

Hawksworth, D. J., Szafran, A. A., Jordan, P. W., Does, A. S., & Herati, A. S. (2018). Infertility in Patients With Klinefelter Syndrome: Optimal Timing for Sperm and Testicular Tissue Cryopreservation. *Reviews in Urology*, *20*(2), 56–62. https://doi.org/10.3909/riu0790

Hay, R. T. (2005). Sumo: A History of Modification. *Molecular Cell*, *18*(1), 1–12. https://doi.org/10.1016/j.molcel.2005.03.012

Hietakangas, V., Anckar, J., Blomster, H. A., Fujimoto, M., Palvimo, J. J., Nakai, A., & Sistonen, L. (2005). PDSM, a motif for phosphorylation-dependent sumo modification. *Proceedings of the National Academy of Sciences*, *103*(1), 45–50. https://doi.org/10.1073/pnas.0503698102

Hilgarth, R. S., Murphy, L. A., Skaggs, H. S., Wilkerson, D. C., Xing, H., & Sarge, K. D. (2004). Regulation and function of Sumo Modification. *Journal of Biological Chemistry*, *279*(52), 53899–53902. https://doi.org/10.1074/jbc.r400021200

Houman, J. J., Eleswarapu, S. V., & Mills, J. N. (2020). Current and future trends in men's Health Clinics. *Translational Andrology and Urology*, *9*(S2), S116–S122. https://doi.org/10.21037/tau.2019.08.33

In-text citations: Author/authors. In-Text Citations: Author/Authors - Purdue OWL® - Purdue University. (n.d.).https://owl.purdue.edu/owl/research_and_citation/apa_style/apa_formatting_and_style_guide/in_text_citations_author_authors.html

Izarzugaza, J. M., Hopcroft, L. E., Baresic, A., Orengo, C. A., Martin, A. C., & Valencia, A. (2011). Characterization of pathogenic germline mutations in human protein kinases. *BMC Bioinformatics*, *12*(S4). https://doi.org/10.1186/1471-2105-12-s4-s1

James, E. R., Carrell, D. T., Aston, K. I., Jenkins, T. G., Yeste, M., & Salas-Huetos, A. (2020). The Role of the Epididymis and the Contribution of Epididymosomes to Mammalian Reproduction. *International Journal of Molecular Sciences*, *21*(15), 5377. https://doi.org/10.3390/ijms21155377

Jiang, X.-H., Bukhari, I., Zheng, W., Yin, S., Wang, Z., Cooke, H. J., & Shi, Q.-H. (2014). Blood-testis barrier and spermatogenesis: Lessons from genetically-modified mice. *Asian Journal of Andrology*, *16*(4), 572–580. https://doi.org/10.4103/1008-682x.125401

Jin, J.-M., & Yang, W.-X. (2014). Molecular regulation of hypothalamus–pituitary–gonads axis in males. *Gene*, *551*(1), 15–25. https://doi.org/10.1016/j.gene.2014.08.048

Karimi Dermani, F., Gholamzadeh Khoei, S., Afshar, S., & Amini, R. (2021). The potential role of nucleophosmin (NPM1) in the development of cancer. *Journal of Cellular Physiology*, *236*(11), 7832–7852. https://doi.org/10.1002/jcp.30406

Katib, A. A., Al–Hawsawi, K., Motair, W., & Bawa, A. M. (2014). Secondary infertility and the aging male, overview. *Central European Journal of Urology*, *67*(2), 184–188. https://doi.org/10.5173/ceju.2014.02.art13

Keller, E., & Chambers, G. M. (2022). Valuing infertility treatment: Why Qalys are inadequate, and an alternative approach to cost-effectiveness thresholds. *Frontiers in Medical Technology*, *4*. https://doi.org/10.3389/fmedt.2022.1053719

King, T. F. J., Lee, M. C., Williamson, E. E. J., & Conway, G. S. (2016). Experience in optimizing fertility outcomes in men with congenital adrenal hyperplasia due to 21 hydroxylase deficiency. *Clinical Endocrinology*, *84*(6), 830–836. https://doi.org/10.1111/cen.13001

Kobe, B., Kampmann, T., Forwood, J. K., Listwan, P., & Brinkworth, R. I. (2005). Substrate specificity of protein kinases and computational prediction of substrates. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics*, *1754*(1–2), 200–209. https://doi.org/10.1016/j.bbapap.2005.07.036

Kornev, A. P., & Taylor, S. S. (2010). Defining the conserved internal architecture of a protein kinase. *Biochimica et Biophysica Acta*, *1804*(3), 440–444. https://doi.org/10.1096/fasebj.24.1_supplement.864.3

Kostich, M., English, J., Madison, V., Gheyas, F., Wang, L., Qiu, P., Greene, J., & Laz, T. M. (2002). *Genome Biology*, *3*(9). https://doi.org/10.1186/gb-2002-3-9-research0043

Kumar, N., & Singh, A. (2015). Trends of male factor infertility, an important cause of infertility: A review of literature. *Journal of Human Reproductive Sciences*, *8*(4), 191–196. https://doi.org/10.4103/0974-1208.170370

Kunz, K., Piller, T., & Müller, S. (2018). Sumo-specific proteases and isopeptidases of the SENP family at a glance. *Journal of Cell Science*, *131*(6). https://doi.org/10.1242/jcs.211904

Kuo, C.-Y., Shieh, C., Cai, F., & Ann, D. K. (2012). Coordinate to guard: Crosstalk of phosphorylation, Sumoylation, and ubiquitylation in DNA damage response. *Frontiers in Oncology*, *1*. https://doi.org/10.3389/fonc.2011.00061

Latif, T., Kold Jensen, T., Mehlsen, J., Holmboe, S. A., Brinth, L., Pors, K., Skouby, S. O., Jørgensen, N., & Lindahl-Jacobsen, R. (2017). Semen quality as a predictor of subsequent morbidity: A Danish cohort study of 4,712 men with long-term follow-up. *American Journal of Epidemiology*, *186*(8), 910–917. https://doi.org/10.1093/aje/kwx067

Lee, M.-T., & Dasso, M. (2013). Sumoylation pathway. *AccessScience*. https://doi.org/10.1036/1097-8542.yb133336

Leslie, S. W., Soon-Sutton, T. L., & Khan, M. A. B. (2023). *Male Infertility* . StatPearls Publishing LLC.

Leung, A. K., Henry, M. A., & Mehta, A. (2018). Gaps in male infertility health services research. *Translational Andrology and Urology*, *7*(S3), S303–S309. https://doi.org/10.21037/tau.2018.05.03

Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., Pinotti, R., & Swan, S. H. (2017). Temporal trends in sperm count: A systematic review and meta-regression analysis. *Human Reproduction Update*, *23*(6), 646–659. https://doi.org/10.1093/humupd/dmx022

Li, X., Wilmanns, M., Thornton, J., & Köhn, M. (2013). Elucidating human phosphatase-substrate networks. *Science Signaling*, *6*(275). https://doi.org/10.1126/scisignal.2003203

Liu, X., Liu, Z., Jang, S.-W., Ma, Z., Shinmura, K., Kang, S., Dong, S., Chen, J., Fukasawa, K., & Ye, K. (2007). Sumoylation of nucleophosmin/B23 regulates its subcellular localization, mediating cell proliferation and survival. *Proceedings of the National Academy of Sciences*, *104*(23), 9679–9684. https://doi.org/10.1073/pnas.0701806104

Marić, T., Fučić, A., & Aghayanian, A. (2021). Environmental and occupational exposures associated with male infertility. *Archives of Industrial Hygiene and Toxicology*, *72*(2), 101–113. https://doi.org/10.2478/aiht-2021-72-3510

Mazure, C. M. (2021, September 19). *What do we mean by sex and gender?*. Yale School of Medicine. https://medicine.yale.edu/news-article/what-do-we-mean-by-sex-and-gender/

McClendon, C. L., Kornev, A. P., Gilson, M. K., & Taylor, S. S. (2014). Dynamic architecture of a protein kinase. *Proceedings of the National Academy of Sciences*, *111*(43), 4623–4631. https://doi.org/10.1073/pnas.1418402111

Meroni, S. B., Galardo, M. N., Rindone, G., Gorga, A., Riera, M. F., & Cigorraga, S. B. (2019). Molecular mechanisms and signaling pathways involved in Sertoli cell proliferation. *Frontiers in Endocrinology*, *10*. https://doi.org/10.3389/fendo.2019.00224

Mitrea, D., Royappa, G., Buljan, M., Yun, M., Pytel, N., Satumba, J., Nourse, A., Park, C., Babu, M. M., White, S. W., & Kriwacki, R. W. (2014). Structural polymorphism in the N-terminal oligomerization domain of NPM1. *PNAS*, *111*(12), 4466–4471. https://doi.org/10.2210/pdb4n8m/pdb

Mohammed, S. K., & Jan, A. (2023). *Young Syndrome*. StatPearls Publishing LLC.

Mohideen, F., Capili, A. D., Bilimoria, P. M., Yamada, T., Bonni, A., & Lima, C. D. (2009). A molecular basis for phosphorylation-dependent sumo conjugation by the E2 UBC9. *Nature Structural & Molecular Biology*, *16*(9), 945–952. https://doi.org/10.1038/nsmb.1648

Ndjoko, K., Wolfender, J.-L., & Hostettmann, K. (2000). Determination of trace amounts of ginkgolic acids in ginkgo biloba L. Leaf extracts and phytopharmaceuticals by liquid chromatography–electrospray mass spectrometry. *Journal of Chromatography B:*

Biomedical Sciences and Applications, *744*(2), 249–255. https://doi.org/10.1016/s0378-4347(00)00316-9

Ni, F.-D., Hao, S.-L., & Yang, W.-X. (2019). Multiple signaling pathways in Sertoli cells: Recent findings in spermatogenesis. *Cell Death & Disease*, *10*(8), 541. https://doi.org/10.1038/s41419-019-1782-z

Okuwaki, M., Sumi, A., Hisaoka, M., Saotome-Nakamura, A., Akashi, S., Nishimura, Y., & Nagata, K. (2012). Function of homo- and hetero-oligomers of human nucleoplasmin/nucleophosmin family proteins NPM1, npm2 and NPM3 during sperm chromatin remodeling. *Nucleic Acids Research*, *40*(11), 4861–4878. https://doi.org/10.1093/nar/gks162

Omelet, W. (2011). Global access to infertility care in developing countries: a case of human rights, equity and social justice. *Facts, Views, and Visions in ObGyn*, *3*(4), 257–266.

O'Donnell, L., Nicholls, P. K., O'Bryan, M. K., McLachlan, R. I., & Stanton, P. G. (2011). Spermiation. *Spermatogenesis*, *1*(1), 14–35. https://doi.org/10.4161/spmg.1.1.14525

Pawson, T., & Scott, J. D. (2005). Protein phosphorylation in signaling – 50 years and counting. *Trends in Biochemical Sciences*, *30*(6), 286–290. https://doi.org/10.1016/j.tibs.2005.04.013

Punab, M., Poolamets, O., Paju, P., Vihljajev, V., Pomm, K., Ladva, R., Korrovits, P., & Laan, M. (2016). Causes of male infertility: A 9-year prospective monocentre study on 1737 patients with reduced total sperm counts. *Human Reproduction*, *32*(1), 18–31. https://doi.org/10.1093/humrep/dew284

Ramazi, S., & Zahiri, J. (2021). Post-translational modifications in proteins: Resources, tools and prediction methods. *Database*, *2021*. https://doi.org/10.1093/database/baab012

Ravitsky, V., & Kimmins, S. (2019). The forgotten men: Rising rates of male infertility urgently require new approaches for its prevention, diagnosis and treatment. *Biology of Reproduction*, *101*(5), 872–874. https://doi.org/10.1093/biolre/ioz161

Rosenfield, D. A., & Pizzutto, C. S. (2018). Wildlife Population Control – reproductive physiology under the influence of contraceptive methods in mammalian wildlife, with emphasis on immunocontraception: The best choice? A literature review. *Brazilian Journal of Veterinary Research and Animal Science*, *55*(1), 1–16. https://doi.org/10.11606/issn.1678-4456.bjvras.2018.129431

Sajeev, T. K., Joshi, G., Arya, P., Mahajan, V., Chaturvedi, A., & Mishra, R. K. (2021). Sumo and SUMOylation pathway at the forefront of host immune response. *Frontiers in Cell and Developmental Biology*, *9*. https://doi.org/10.3389/fcell.2021.681057

Sengupta, A., Nanda, M., Tariq, S. B., Kiesel, T., Perlmutter, K., & Vigodner, M. (2021). Sumoylation and its regulation in testicular Sertoli cells. *Biochemical and Biophysical Research Communications*, *580*, 56–62. https://doi.org/10.1016/j.bbrc.2021.09.066

Sengupta, P., Dutta, S., Karkada, I. R., & Chinni, S. V. (2021). Endocrinopathies and Male Infertility. *Life*, *12*(1), 10. https://doi.org/10.3390/life12010010

Seok, S.-H. (2021). Structural insights into protein regulation by phosphorylation and substrate recognition of protein kinases/phosphatases. *Life*, *11*(9), 957. https://doi.org/10.3390/life11090957

Shrivastava, V., Pekar, M., Grosser, E., Im, J., & Vigodner, M. (2010). SUMO proteins are involved in the stress response during spermatogenesis and are localized to DNA double-strand breaks in germ cells. *Reproduction*, *139*(6), 999–1010. https://doi.org/10.1530/rep-09-0492

Singh, R., Hamada, A. J., Bukavina, L., & Agarwal, A. (2012). Physical deformities relevant to male infertility. *Nature Reviews Urology*, *9*(3), 156–174. https://doi.org/10.1038/nrurol.2012.11

Smith, L. B., & Walker, W. H. (2014). The Regulation of Spermatogenesis by Androgens. *Seminars in Cell & Developmental Biology*, *30*, 2–13. https://doi.org/10.1016/j.semcdb.2014.02.012

Tang, Z., Hecker, C. M., Scheschonka, A., & Betz, H. (2008). Protein interactions in the sumoylation cascade - lessons from X-ray structures. *FEBS Journal*, *275*(12), 3003–3015. https://doi.org/10.1111/j.1742-4658.2008.06459.x

Tapia, J. A. (2009). Apoptotic Events in Male Germ Cells and in Mature Mammalian Spermatozoa. In F. J. Peña (Ed.), *Apoptosis: Involvement of Oxidative Stress and Intracellular Ca2+ Homeostasis* (p. 116). essay, Springer, Dordrecht.

Thiriet, M. (2012). Dual-specificity protein kinases. *Intracellular Signaling Mediators in the Circulatory and Ventilatory Systems*, *4*, 379–386. https://doi.org/10.1007/978-1-4614-4370-4_7

Tomanov, K., Nukarinen, E., Vicente, J., Mendiondo, G. M., Winter, N., Nehlin, L., Weckwerth, W., Holdsworth, M. J., Teige, M., & Bachmair, A. (2018). Sumoylation and phosphorylation: Hidden and overt links. *Journal of Experimental Botany*, *69*(19), 4583–4590. https://doi.org/10.1093/jxb/ery167

Tomasi, M. L., & Ramani, K. (2018). Sumoylation and phosphorylation cross-talk in hepatocellular carcinoma. *Translational Gastroenterology and Hepatology*, *3*, 20–20. https://doi.org/10.21037/tgh.2018.04.04

U.S. Department of Health and Human Services. (n.d.). *Sex & gender*. National Institutes of Health. https://orwh.od.nih.gov/sex-gender

Turner, K. A., Rambhatla, A., Schon, S., Agarwal, A., Krawetz, S. A., Dupree, J. M., & Avidor-Reiss, T. (2020). Male infertility is a women's health issue—research and clinical evaluation of male infertility is needed. *Cells*, *9*(4), 990. https://doi.org/10.3390/cells9040990

Uzoma, I., Hu, J., Cox, E., Xia, S., Zhou, J., Rho, H.-S., Guzzo, C., Paul, C., Ajala, O., Goodwin, C. R., Jeong, J., Moore, C., Zhang, H., Meluh, P., Blackshaw, S., Matunis, M., Qian, J., & Zhu, H. (2018). Global identification of small ubiquitin-related modifier (SUMO) substrates reveals crosstalk between SUMOylation and phosphorylation promotes cell

migration. *Mol Cell Proteomics*, *17*(5), 871–888. https://doi.org/10.1074/mcp.ra117.000014

Ventelä, S., Toppari, J., & Parvinen, M. (2003). Intercellular organelle traffic through cytoplasmic bridges in early spermatids of the rat: Mechanisms of haploid gene product sharing. *Molecular Biology of the Cell*, *14*(7), 2768–2780. https://doi.org/10.1091/mbc.e02-10-0647

Vigodner, M., Ishikawa, T., Schlegel, P. N., & Morris, P. L. (2006). SUMO-1, human male germ cell development, and the androgen receptor in the testis of men with normal and abnormal spermatogenesis. *American Journal of Physiology-Endocrinology and Metabolism*, *290*(5), 1022–1033. https://doi.org/10.1152/ajpendo.00527.2005

Vigodner, M., Lucas, B., Kemeny, S., Schwartz, T., & Levy, R. (2020). Identification of sumoylated targets in proliferating mouse spermatogonia and human testicular seminomas. *Asian Journal of Andrology*, *22*(6), 569–577. https://doi.org/10.4103/aja.aja_11_20

Waheeb, R., & Hofmann, M.-C. (2011). Human spermatogonial stem cells: A possible origin for spermatocytic seminoma. *International Journal of Andrology*, *34*(4), 296–305. https://doi.org/10.1111/j.1365-2605.2011.01199.x

Walker, K., Gunn, A. J., & Gluckman, P. D. (1990). XO/XY mosaicism in phenotypic males. *Archives of Disease in Childhood*, *65*(8), 891–892. https://doi.org/10.1136/adc.65.8.891

Xiao, Y., Lucas, B., Molcho, E., & Vigodner, M. (2017). Cross-talk between Sumoylation and phosphorylation in mouse spermatocytes. *Biochemical and Biophysical Research Communications*, *487*(3), 640–645. https://doi.org/10.1016/j.bbrc.2017.04.107

Yang, Y., He, Y., Wang, X., liang, Z., He, G., Zhang, P., Zhu, H., Xu, N., & Liang, S. (2017). Protein sumoylation modification and its associations with disease. *Open Biology*, *7*(10). https://doi.org/10.1098/rsob.170167

Yao, Q., Li, H., Liu, B.-Q., Huang, X.-Y., & Guo, L. (2011). Sumoylation-regulated protein phosphorylation, evidence from quantitative phosphoproteomics analyses. *Journal of Biological Chemistry*, *286*(31), 27342–27349. https://doi.org/10.1074/jbc.m111.220848

Yip, S. P., Siu, P. M., Leung, P. H., Zhao, Y., & Yung, B. Y. (2011). The multifunctional nucleolar protein nucleophosmin/npm/B23 and the nucleoplasmin family of proteins. *The Nucleolus*, *15*, 213–252. https://doi.org/10.1007/978-1-4614-0514-6_10

Zirkin, B. R., & Papadopoulos, V. (2018). Leydig cells: Formation, function, and regulation†. *Biology of Reproduction*, *99*(1), 101–111. https://doi.org/10.1093/biolre/ioy059