Identification of Changes in Phosphoproteome of Mouse Spermatocytes Upon Inhibition of Sumoylation

Presented to the S. Daniel Abraham Honors Program

in Partial Fulfillment of the

Requirements of Completion of the Program

Stern College for Women

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December 2021

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Abstract

Across the globe, approximately 15% of couples experience infertility issues with about 50% of cases primarily due to a male factor (Agarwal *et al.*, 2021). While some causes of infertility have been attributed to the environment or genetic mutations, there are still many causes that are unknown. To create adequate infertility treatments, a better understanding of spermatogenesis and the proteins involved to promote its progression is necessary. Recent studies have identified SUMO (small ubiquitin-like modifier) proteins and have explored their role in post-translational modifications by binding to other proteins in a process known as sumoylation (Vigodner, 2011). Research suggests that through the inhibition of global sumoylation with the inhibitor Ginkgolic acid (GA), the G2/M transition was arrested in purified mouse spermatocytes *in vitro* (Vigonder *et al*., 2017). These events were determined to be regulated by the crucial kinases, PLK, Aurora kinases, and tyrosine kinases with subsequent research within the lab suggesting that sumoylation regulates several of these kinases. Over the last year and a half our lab has focussed on identifying proteins for which phosphorylation is affected when sumoylation is inhibited in spermatocytes, and regulated by the PLK, Aurora, tyrosine and any other kinases that regulate meiosis. Whether their regulation was directly or indirectly related to sumoylation, and if these targets were known to be involved in any other processes or cell types were also identified with bioinformatics analysis. While target mouse proteins were identified to be involved in both sumoylation and phosphorylation, there is still more to be known about these proteins in germ cells. Future research would focus on each protein's specific role, as well as the specified link between phosphorylation and sumoylation in regulation. Additionally, the ultimate goal would be to study these proteins and connections *in vivo* during mouse and human spermatogenesis.

Introduction

It is estimated that 15% of couples globally are affected by infertility or related problems. While in the past the primary perception was that the woman had to be the one to blame in such a situation (Sennert, 1978), more recent discoveries have estimated that the cause of infertility in approximately 50% of couples is due primarily to a male factor (Agarwal *et al.*, 2021). The causes of male infertility can be due to a wide range of factors, including hormonal disorders, physical problems, lifestyle problems, psychological issues, and genetic abnormalities (Babakhanzadeh *et al.*, 2020). Many of these causes, however, are in fact treatable. Although there has been much effort by researchers to understand and identify the underlying causes of male infertility, as described in **Figure 1** by the Fertility Center of San Antonio, approximately 34% of cases still remain undiagnosed (Male Factor

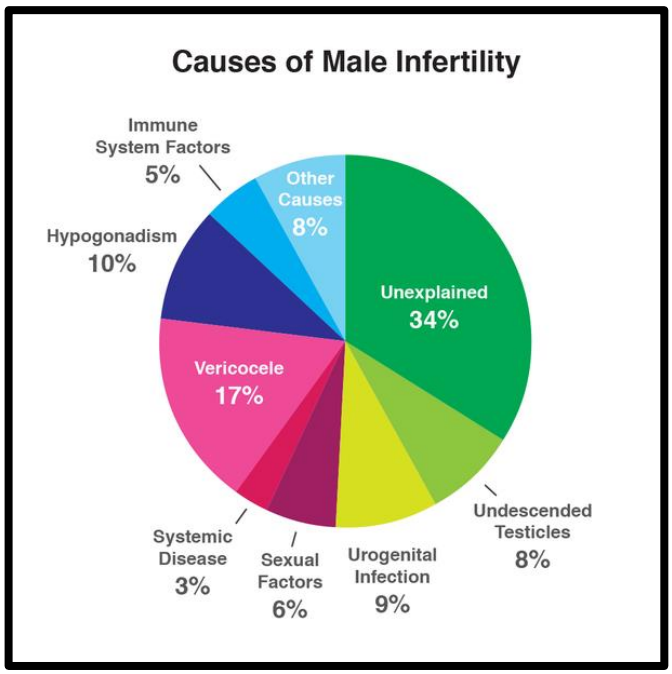

Figure 1: Causes of Male Infertility.
https://www.fertilitysa.com/infertility-treatments/male-factor-infertility

Infertility, n.d.). Thus, there is a need for the precise biological mechanisms, which could be the cause of infertility in men, to be identified and analysed in order to create proper and effective fertility treatments. Many stages and pathways involved with the development of

sperm during spermatogenesis have been identified as possible contributors to infertility (Cheng and Mruck, 2010). Therefore, further research is needed to understand which processes and proteins specifically may be responsible within spermatogenesis. This would then allow us to develop the proper treatments to inhibit these abnormal cellular processes and in turn be an effective way to fight against infertility.

II. Background

A. Known Causes of Male Infertility

Infertility can result from either female or male related issues, with a handful of known causes for male infertility. One of these known reasons is due to a hormonal deficiency or imbalance (Babakhanzadeh *et al.*, 2020). As depicted in **Figure 2,** the hormone axis in the male reproductive system is known as the hypothalamic-pituitary-gonadal axis. As

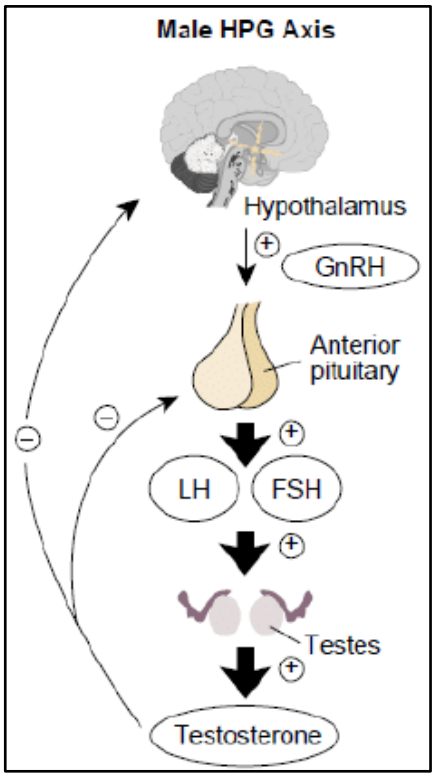

Figure 2: Schematic representation of the hypothalamic–pituitary–gonadal (HPG) axis. (Kong *et al.*, 2004)

the name suggests, the axis consist of the hypothalamus, pituitary, and testicular glands, and is responsible for proper male sexual development and function. Any issue within the axis can lead to infertility. For example, if the brain is not able to produce enough gonadotropic releasing hormone (GnRH), there will be a lack of testosterone, thus resulting in a lack of sperm production (Katz *et al.*, 2017).

According to the National Institute of Child Health and Human Development (NICHD), the most common causes of male infertility are due to genetic or environmental effects that cause the male testes to not function properly. Without proper function of the testes, sperm cannot develop properly either. For instance, epididymitis is a disorder categorized by inflammation of the small, coiled tube at the back of the testicle known as the epididymis. This is usually caused by some type of bacterial infection. Untreated, epididymitis can cause complications, such as abscess formation and testicular infarction. Chronic epididymitis can lead to permanent damage or even destruction of the epididymis and testicle. This could then result in infertility or hypogonadism, diminished functional activity of the gonads, another testicular disorder (Zhao *et al*., 2020).

Furthermore, there are also genetic disorders which contribute to male infertility. The most well-known, for example, is called Klinefelter's syndrome, which accounts for about 14% of male infertility cases. This is when someone is born with an extra X chromosome, resulting in lower production of testosterone and therefore a decreased amount of sperm. This then results in infertility (Babakhanzadeh *et al.*, 2020). Additionally, there are also lifestyle patterns that can result in infertility. For example, the consumption of more than four units of alcohol per day is likely to detrimentally affect sperm quality. This is also true for excessive smoking (Katz *et al.*, 2017). Obesity has also been shown to have a negative effect on fertility (Kahn and Brannigan, 2017).

While these are some of the known causes of male infertility, there are still many cases that cannot be explained. To get to the core of the problem involved with male infertility, a deeper understanding of the underlying mechanisms of spermatogenesis is required.

B. Meiosis and Spermatogenesis

Spermatogenesis is the differentiation and development of haploid spermatozoa from germ cells. In the testes, the male reproductive organ, sperm cells originate and proliferate within the seminiferous tubules, which are coiled tubes. As depicted in **Figure 3** below, there

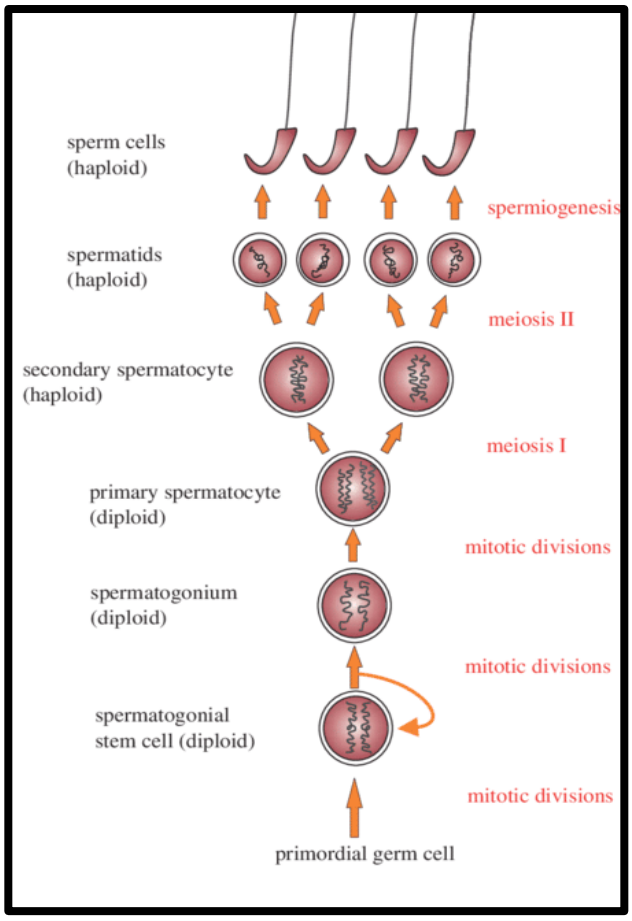

Figure 3: Process of Spermatogenesis
(Cheng and Mruk, 2010)

are three stages of the development of male sperm cells through spermatogenesis. As spermatogonia, at the start of their development, these cells begin to proliferate via mitosis, the replication of stem cells, within the walls of the seminiferous tubules. Following the completion of mitosis, the stem cells have now doubled in size, half continuing as stem cells for future mitotic divisions and the other half developing into germ cells. The second stage of spermatogenesis is meiosis. Meiosis is a cellular process in which a single diploid cell goes through cellular division twice to produce four haploid cells. These cells are the sex, or germ, cells. During the first division, meiosis I, the DNA in the cell is copied, resulting in two identical sets of chromosomes. As the cell moves through the different stages of meiosis, two sister chromatids containing identical genetic information form. As the cell goes through its first division, the sister chromatids stay together resulting in two daughter cells with 23 pairs of chromatids in each cell. These cells then go through meiosis II, another round of cellular division. At the end of meiosis II, four haploid cells are formed. In males, these four cells are known as round spermatids. Round spermatids condense their nucleus, develop tails, loose their cytoplasm, become elongated spermatids, and ultimately become sperm via a process known as spermiogenesis. In the final stage, the spermatids become fully matured and motile spermatozoa in the epididymis. They are then stored there until ejaculation and possible fertilization of the ovum.

C. Sumoylation

Post-translational modifications are modifications on proteins after they were synthesized via addition of different groups or small proteins. Post-translational modifications regulate various aspects of spermatogenesis. One of such modifications is sumoylaion (or covalent attachment of SUM, small ubiquitin-like modifier, proteins). While

the name suggests similarities to ubiquitin, a regulatory protein, SUMO proteins also differ slightly in their amino acid sequence. SUMO proteins have been studied in various somatic cells. Some of their functions include DNA replication, regulation of transcription and translation, and cellular stress response and transport. These proteins also seem to modify proteins without direct attachment (Gill, 2004).

As shown in **Figure 4**, there are many enzymes involved throughout the modification process, known as the sumoylation cycle. The cycle begins when the Ulp1 protease, an enzyme that breaks down proteins, converts the original SUMO protein into SUMO- GG, its mature form. This results in the removal of several c-terminal amino acids. SUMO-activating enzyme (E1), SUMO-conjugating enzyme (E2), and SUMO ligase protein (E3) sequentially bind SUMO proteins to lysine residues, often within the sequence: ψ-K-*X*-D/E, where ψ is a hydrophobic amino acid and *X* can be any amino acid (Wilkinson *et al*., 2008).

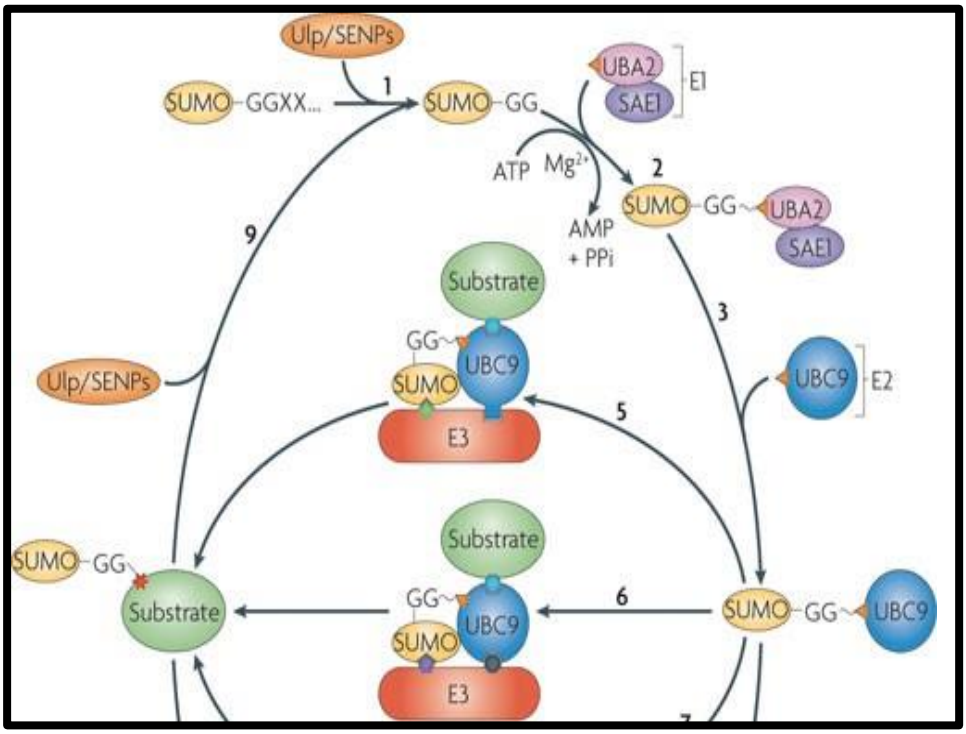

Figure 4: Sumoylation Cycle
(Gareau and Lima, 2010)

Substrates are thus modified by these enzymes, altering protein conformation and stability. This bonding process of SUMO can also be reversed by sentrin-specific proteases (SENPs) which cleave the peptide bonds (Vigodner *et al.,* 2016). With the help of Adenosine Triphosphate (ATP), SUMO peptide forms a thioester bond with the cysteine residue of E1 (a heterodimer of SAE1 and UBA2) and then with E2 (Ubc9). Lastly, SUMO-GG is transferred to the target protein with assistance from E3, a family of SUMO ligase proteins, finally attaching the SUMO protein to the target (Hilgarth *et al.,* 2004).

Like other cellular processes, sumoylation can be affected by other compounds. Ginkgolic acid (GA) was the first inhibitor discovered that specifically inhibited the E1 enzyme activity with SUMO proteins without affecting E1 in ubiquitin (Fukuda *et al*, 2009). GA is a phenolic acid contained in *Ginkgo biloba* L. with neuroprotective, antimicrobial, and antitumoral properties. A structurally simple compound, GA consists of salicylic acid and a long-carbon chain substituent. GA has been identified as an effective inhibitor of sumoylation both *in vitro* and *in vivo.* GA directly binds to enzyme 1 (E1) and prevents the formation of the E1-SUMO complex intermediate during the sumoylation cycle, thereby inhibiting sumoylation (Fukuda *et al*, 2009).

Due to their unique nature and involvement within cellular processes, SUMO proteins have been of interest to scientists for many years (Eifler and Vertegaal, 2016). Four different SUMO paralogs, SUMO 1 to 4, have been identified. SUMOs 1, 2, and 3 have been found on many tissues and seem to resemble each other, while SUMO 4 has been primarily attributed to the kidney, liver, and lymph nodes. SUMO-2/3 is used to describe the isoform of SUMO-2 and SUMO-3 since they are extremely similar proteins. SUMO-1 has been identified as binding to proteins like Ran-GAP1, localizing them in the mitotic spindle, while SUMO-2/3 is mainly found in proteins which migrate to centromeres and kinetochores (Cubaness-Potts *et al* 2015). Over the past few years, SUMO proteins have been identified during

spermatogenesis within cells of the testes. Within Dr. Vigodner's lab, many studies and testing have been performed to identify SUMO targets during various stages of spermatogenesis. Imaging and immunodetection methods have been used to isolate and identify SUMO targets in cells at all stages of spermatogenesis including spermatogonia, spermatocytes, spermatids, and somatic cells within the testes (Vigodner *et al.,* 2016).

Future research on the specific functions of these SUMO proteins within the testes and spermatogenesis in general will help determine the role and importance of sumoylation in the male reproductive cycle when active or inhibited.

D. Phosphorylation

Phosphorylation, as shown below in **Figure 5,** is the addition of a phosphoryl group ($P^{+}O_3^{2-}$) to a molecule and is one of the most important and common modifications for reversible regulation of protein functions. Studies conducted with mammalian cells labelled

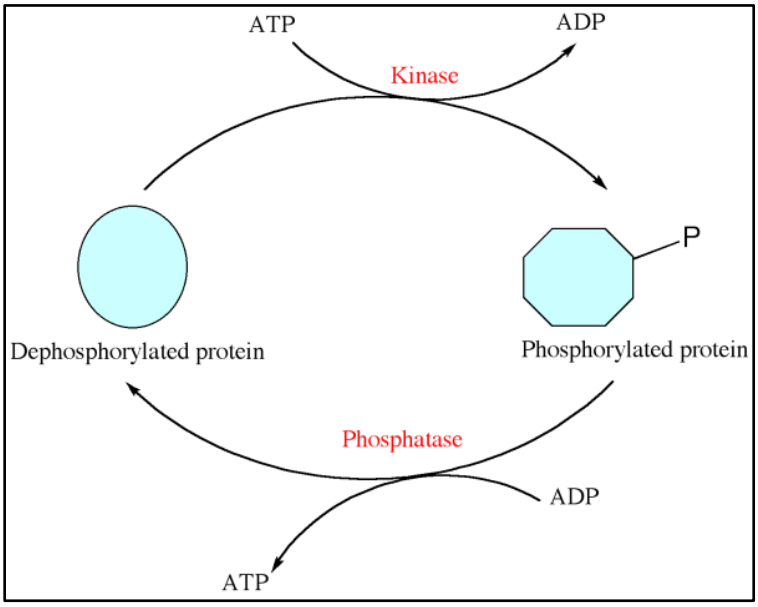

Figure 5: Schematic representation of the addition of a phosphoryl group to a protein.

with [(32)P]orthophosphate indicate that approximately one third of all cellular proteins are

modified by phosphorylation (Sefton, 2001). When induced, phosphorylation is mediated though protein kinases, enzymes that catalyze this transfer of a phosphate group from ATP to the target proteins.

There are two main protein kinases known as serine/threonine kinases and tyrosine kinases. Serine/Threonine kinases phosphorylate the hydroxyl group of serine or threonine amino acids. These events can be regulated by cellular events, as well as chemical signals, such as cAMP/cGMP. Serine/Threonine kinase receptors are essential for the regulation of cell proliferation, apoptosis (programmed cell death), cell differentiation, as well as embryonic development (Capra *et al*., 2006). The other protein kinases, tyrosine kinases, phosphorylate tyrosine amino acid residues and are used in signal transduction. Tyrosine is especially significant since it has a phenol group, an aromatic organic compound with the molecular formula C_6H_5OH, in its side chain. Phosphorylation of the hydroxyl side chain may change the target protein's activity or affect the signaling cascade (Alberts, 2014). Phosphorylation is reversed by protein phosphatases.

Within meiosis, the G2/M1 transition is heavily regulated by post-translational modifications, however the link between these various modifications, such as sumoylation and phosphorylation, is not as well understood. To better understand this connection, Dr. Vigodner's lab previously analysed the role of inhibiting sumoylation in mouse spermatocytes, thereby preventing chromosome condensation and the disassembly of the synaptonemal complex (Vigodner *et al.,* 2017). The results of this study, as shown in **Figure 6**, indicate that inhibition of sumoylation arrests the G2/M1 transition in mouse spermatocytes, as well as affects global phosphorylation. This research also identifies several kinases, such as PLK1 and the Aurora kinases, to be negatively regulated by sumoylation (Vigodner *et al.,* 2017).

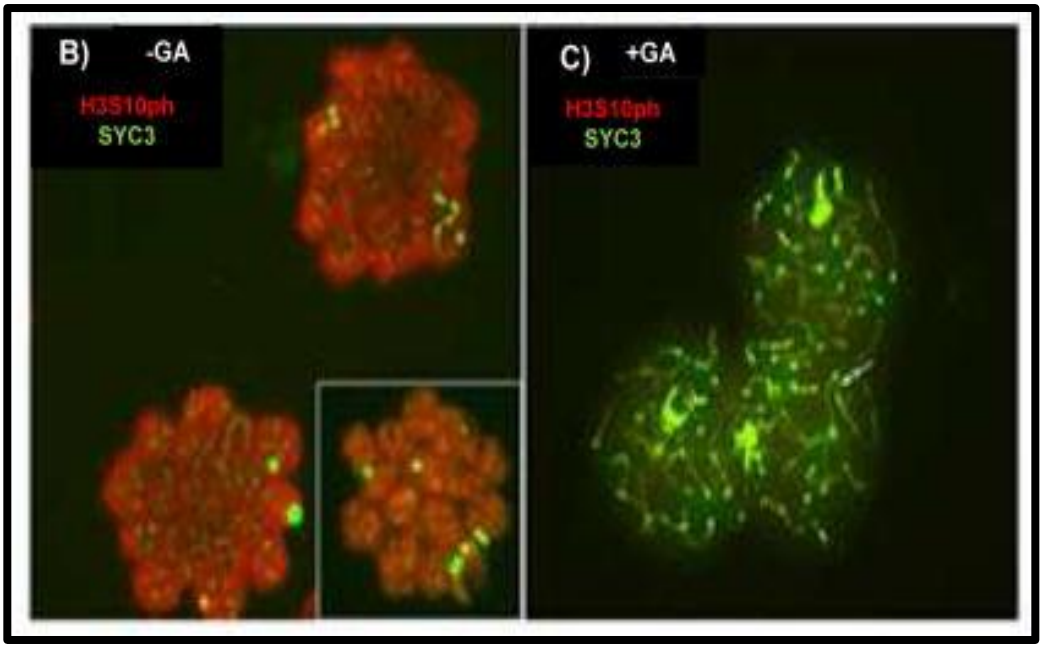

Figure 6: Inhibition of global sumoylation with the inhibitor Ginkgolic acid (GA) arrested the G2/M transition in purified mouse spermatocytes *in vitro.* Chromosomes do not condense (red) and synaptonemal complex does not disassemble (green) (Vigodner *et al*., 2017)

The Polo-like kinase family contains 5 members (PLK1-PLK5) and are involved in various functions in eukaryotic cell division. PLKs regulate events such as, centrosome maturation, checkpoint recovery, spindle assembly, cytokinesis, and apoptosis (Lee *et al*., 2014). Aurora kinases are serine/threonine kinases that are also essential for cellular growth and specifically control chromatid segregation (Tang *et al*., 2017). These findings suggest a possible link between the two post-translational modifications of phosphorylation and sumoylation. Additionally, the question remains which proteins are being phosphorylated specifically in a sumoylation-dependent manner, allowing specialized targeting of problems that could arise within spermatogenesis.

III. Goals

Dr. Vigodner's lab has previously studied and identified certain targets of sumoylation during spermatogenesis. Through the inhibition of global sumoylation with the inhibitor Ginkgolic acid (GA), the G2/M transition was arrested in purified mouse spermatocytes *in vitro*. Those events were determined to be regulated by important kinases, such as PLK, Aurora kinases, and tyrosine kinases. Further research within the lab suggests that sumoylation regulates several of these kinases (Vigodner *et al.,* 2017). During my time in Dr. Vigonder's lab we sought to identify any proteins for which phosphorylation is affected when sumoylation is inhibited in mouse spermatocytes, as well as if any of these proteins could be regulated by the PLK, Aurora, tyrosine or any other kinases that regulate meiosis. Additionally, we sought to determine if their regulation was directly or indirectly related to sumoylation and if these targets were known to be involved in any other processes or cell types. These goals would help for future research involved in male infertility by targeting specific areas where problems could arise or cross over.

IV. Materials & Methods

A. Gel Electrophoresis and Imaging

In order to identify which proteins were being phosphorylated in a sumoylation-dependent manner a two-dimensional gel electrophoresis was performed by Applied Biomics, Inc (Hayward, CA), along with Phospho-profiling and Protein ID. According to the Applied Biomics protocol, samples were prepared with 2D lysis buffer (30 mM Tris-HCl, pH 8.8, containing 7 M urea, 2 M thiourea and 4% CHAPS). The protein concentrations were then measured with Bio-Rad Protein Assay Kit II according to protocol. Each sample was then

mixed with 1.0 uL of diluted CyDye and kept in the dark on ice for 30 minutes. 1.0 ul of 10 mM Lysine was added to each sample to stop the labelling reaction, and then incubated in the dark for another 15 minutes.

The phosphor-profiling gel had an additional 220 g of unlabelled protein added for a total of 250 g. The samples were mixed for the 2D DIGE gel and spun well before loading into the holder. Once the samples were loaded, Isoelectric Focusing (IEF) was run according to the Amersham BioSciences protocol. After the completion of the IEF, samples were incubated and rinsed in the SDS-gel running buffer before transferring into 12% SDS gels.

Following SDS-PAGE, gel images were scanned and then stained using Pro-Q ® Diamond Phosphoprotein Gel Stain. Scanning was performed with Typhoon TRIO and further analysis with DeCyder software.

Spermatocytes were purified from mouse testes and treated with DMSO (control), 30 µm and 50µm of Ginkgolic acid (GA). Using both manual inspection and software, phosphorylated spots in DMSO vs. 30 umGA and DMSO vs. 30 umGA were selected. The spots were then mapped, and their phosphor-ratios were calculated. The proteins were eluted from the matrix and mass spectrometry fragmentation spectra were then acquired for each sample. The peptide mass and fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine to search the database of National Center for Biotechnology Information non- redundant (NCBInr). A list of target proteins was then obtained.

B. Bioinformatics Analysis

After obtaining a comprehensive list of protein targets whose phosphorylation is affected by inhibition of sumoylation from Applied Biomics, Inc (Hayward, CA), we then sought to identify proteins from the comprehensive list that can be regulated by the PLK, Aurora, tyrosine or other kinases that regulate meiosis. This was accomplished using two computer software and databases. The first, The Universal Protein Resource (UniProt), is a comprehensive resource for protein sequence and annotation data. The overall database is composed of three subunits, the UniProt Knowledgebase (UniProtKB), the UniProt Reference Clusters (UniRef), and the UniProt Archive (UniParc), providing researchers with a centralized and inclusive resource for protein sequences and function. The second resource used, PhosphoSitePlus, is a comprehensive and interactive resource for observing experimentally recorded post-translational modifications, primarily of human and mouse proteins.

In order to narrow down the target proteins, each protein name and access number were inserted into the search engine of UniProtKB, encompassing the entire protein knowledgebase. Once the correct protein was found, the mouse cell was made sure to be chosen and analysed. On the UniProt software, the protein's function and interaction with other molecules is presented, as well as any post-translational modifications. From this data, information regarding kinases that phosphorylate the protein was recorded. Any information regarding sumoylation was noted as well.

To cross check the data regarding the phosphorylation of these proteins, PhosphoSitePlus was consulted to determine which kinases were specifically involved for certain targets. Again, target protein names and access numbers were inserted into the search engine, and the corresponding mouse cell was selected. The software then generated a graph with the x-axis representing the protein's residue numbers and the y-axis indicating the

number of references for post-translational modifications. The tabs below the graph allowed us to identify which kinases specifically were interacting upstream or downstream with which proteins. The results of these searches were recorded and analysed.

Following the analysis of the proteins with the two databases, information of the post-translational modification was compiled into an Excel spreadsheet. Working from the chart produced from the comprehensive list of proteins, two more columns were added, one labelled " kinases" and another one labelled "sumoylation." Detailed notes were kept about every protein analysed. After completion of researching each protein on the list, the spreadsheet was further condensed based on importance for the current study.

V. Results

A. Phosphorylated Proteins

After running the two-dimensional gel electrophoresis, staining and a comparative analysis of mouse phosphoproteins was performed by Applied Biomics, Inc (Hayward, CA). Spermatocytes were purified from mouse testes and treated with DMSO (control), 30 μm and 50μm of Ginkgolic acid (GA) and an example of the results of the two-dimensional gel electrophoresis is shown in **Figure 7**.

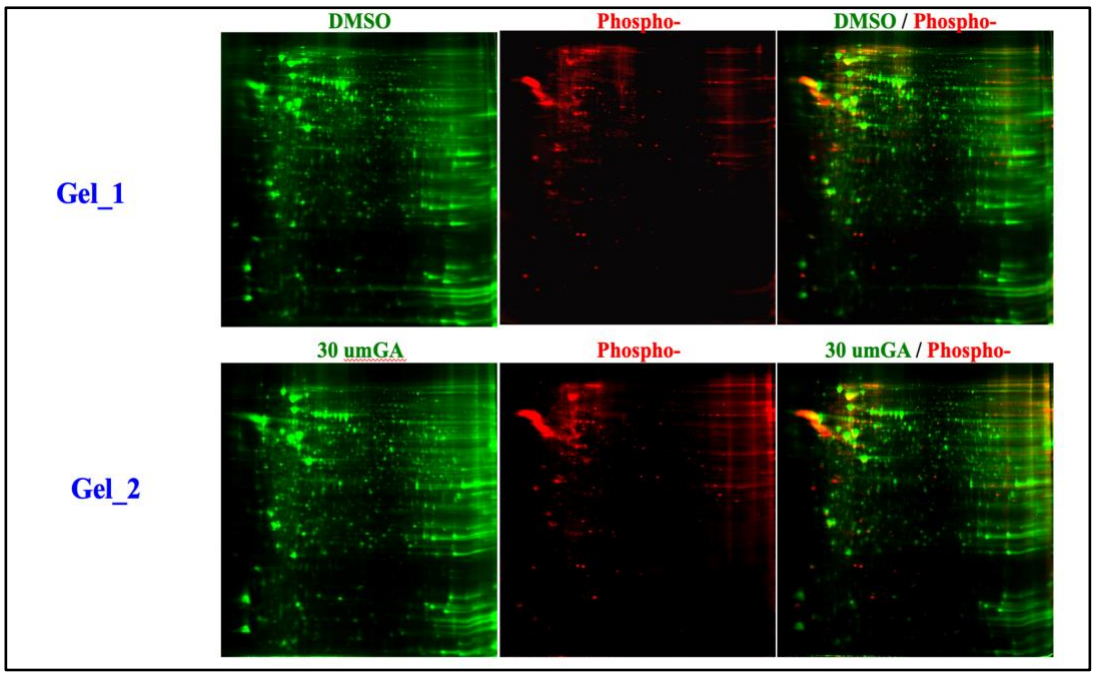

Figure 7: Results of two-dimensional gel electrophoresis of phosphoproteome following inhibition of sumoylation in spermatocytes.
Gel_1: control, protein, and phospho-staining
Gel_2: GA 30, protein, and phospho-staining
(From left to right)

The gels were then analyzed, and proteins were identified through mass spectrometry. Both the protein mass and associated fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine to search the database of National Center for Biotechnology Information non- redundant (NCBInr). Targets with either protein score C.I.% or Ion C.I.% greater than 95 were considered significant. With these results, **Table 1** was created, indicating a comprehensive list of proteins, grouped by general function and categorization, for which phosphorylation is affected in mouse spermatocytes. Analysis of the published and unpublished data from Vigodner's lab was also used to determine whether these targets were previously identified as targets of sumoylation in mouse and human germ cells.

			sumoylated		
RNA/DNA binding,transcription, translation, hisotnes	**accession number**	**MW**	**mouse germ cells**	**human germ cells**	**other cells**
Transcription intermediary factor 1-beta/KAP1	**TIF1B_MOUSE**	**88790.5**	**x**	**X**	**x**
Heterogeneous nuclear ribonucleoprotein H	HNRH1_MOUSE	49,168	x	x	x
Heterogeneous nuclear ribonucleoprotein U	HNRPU_MOUSE	87862.7	x	x	x
Valine--tRNA ligase	SYVC_MOUSE	140127.1			
Splicing factor 1	SF01_MOUSE	70358.1	x		x
Splicing factor U2AF	U2AF1_MOUSE	27,797	x		x
Pre-mRNA-splicing factor	SPF27_MOUSE	26,115	x		
RNA-binding protein EWS	EWS_MOUSE	68,420			
Polyadenylate-binding protein 1	PABP1_MOUSE	70,626		x	x
Nucleosome assembly protein 1-like 1	NP1L1_MOUSE	45,317			
THUMP domain-containing protein 1	THUM1_MOUSE	38,861			
Nucleophosmin	**NPM_MOUSE**	**32,540**			**X**
Eukaryotic translation initiation factor 4E	**IF4E_MOUSE**	**25,038**			**x**
Myelin transcription factor 1-like protein	MYT1L_MOUSE	132,861			
60S acidic ribosomal protein P2	RLA2_MOUSE	11,644	x	x	x
Histone H3.3C	**H3C_MOUSE**	**15,306**	**?**		**x**
Histone H4	**H4_MOUSE**	**11,360**	**?**		**x**
heat shoclk proteins/chaperons/ER proteins					
Transitional endoplasmic reticulum ATPase /Valosine containing pr	TERA_MOUSE	89265.7	x	x	x
Heat shock cognate 71 kDa protein/ Heat shock 70 kDa protein 8	HSP7C_MOUSE	70827.2			x
Hsc70-interacting protein	F10A1_MOUSE	41,630			
T-complex protein 1 subunit epsilon	TCPE_MOUSE	59,586	x	x	x
T-complex protein 1 subunit alpha	TCPA_MOUSE	60,411	x	x	x
Reticulocalbin-2	RCN2_MOUSE	37,248			x
Glutaredoxin-3	GLRX3_MOUSE	37,754			
Endoplasmic reticulum chaperone BiP	BIP_MOUSE	72,378			
ubiquitination					
Ubiquitin-like modifier-activating enzyme 6	UBA6_MOUSE	117,891			
26S proteasome non-ATPase regulatory subunit 13	PSD13_MOUSE	42,782	x		
Proteasome subunit alpha type-5	PSA5_MOUSE	26,394	x	x	x
cytoskeleton/membrane traffic					
Cilia- and flagella-associated protein 36	CFA36_MOUSE	39,575			
Tropomodulin-3	TMOD3_MOUSE	39,478			x
Rho GDP-dissociation inhibitor 1	GDIR1_MOUSE	23,393		x	x
enzymes					
Inositol-3-phosphate synthase 1	INO1_MOUSE	60,893	x		
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10	NDUAA_MOUSE	40,578			
Isocitrate dehydrogenase [NAD] subunit alpha,	IDH3A_MOUSE	39,613			
Pyruvate dehydrogenase E1 component subunit beta	ODPB_MOUSE	38,912			x
Inorganic pyrophosphatase	**IPYR_MOUSE**	**32,646**			
Palmitoyl-protein thioesterase 1	PPT1_MOUSE	34,467			
Leukocyte elastase inhibitor A	ILEUA_MOUSE	42,548			
Phosphoserine phosphatase	**SERB_MOUSE**	**25,080**			

Table 1: List of proteins identified by Applied Biomics, Inc (Hayward, CA), after performing 2D gel electrophoresis, staining and comparative analysis of phosphoproteins following inhibition of global sumoylation with GA inhibitor, arresting the G2/M transition in purified mouse spermatocytes *in vitro.*

B. Specific Targets and Functions

Following the results of the previous experiments and the creation of **Table 1,** a more refined table was created with the UniProt and PhosphoSite databases identifying the target proteins which can be regulated by the PLK, Aurora, tyrosine and other kinases that regulate meiosis. The results of the bioinformatics analysis are expressed in **Table 2**, identifying eight potential protein targets of interests that are regulated by meiotic kinases and interact with SUMO proteins.

Bioinformatics analysis			
	Kinases	**interacts with SUMO?**	**Phosphotase**
Transcription intermediary factor 1-beta/KAP	ATM, MSK1, PKCD	YES	PPP1CA, PP1CB
Heterogeneous nuclear ribonucleoprotein U	**PLK1**	YES	2A (PP2A)
Polyadenylate-binding protein 1	**tyrosine kinase Pyk2**, MAPKAPK2	YES	
Nucleophosmin 1	**PLK1**, **PLK2**, CDK1, NEK2, ROCK2	Yes	
Eukaryotic translation initiation factor 4E	MNL, MNK1, Mtor, **PLK1**, CDK1	YES	
Histone H3.3C	SPIN, **AURKB**, RPS6KA4, RPS6KA5, MAP3K20, PRKCB, DAPK3 , PKN1, JA	Yes	
Histone H4	PAK2	Yes	
Valosine containing protein	14 serines, 14 threonines, 6 tyrosines are phosphorylated	Yes	**PTPL1**

Table 2: Identified protein targets as a result of bioinformatics analysis. These eight protein targets are both regulated by meiotic kinases and interact with SUMO proteins.

The use of PhosphoSitePlus enabled a clear depiction of the protein's regulation by post-translational modifications along the residue positions. As an example, **Figure 8** represents the graph of Polyadenylate-binding protein 1 (PABP1) generated by PhosphoSitePlus.

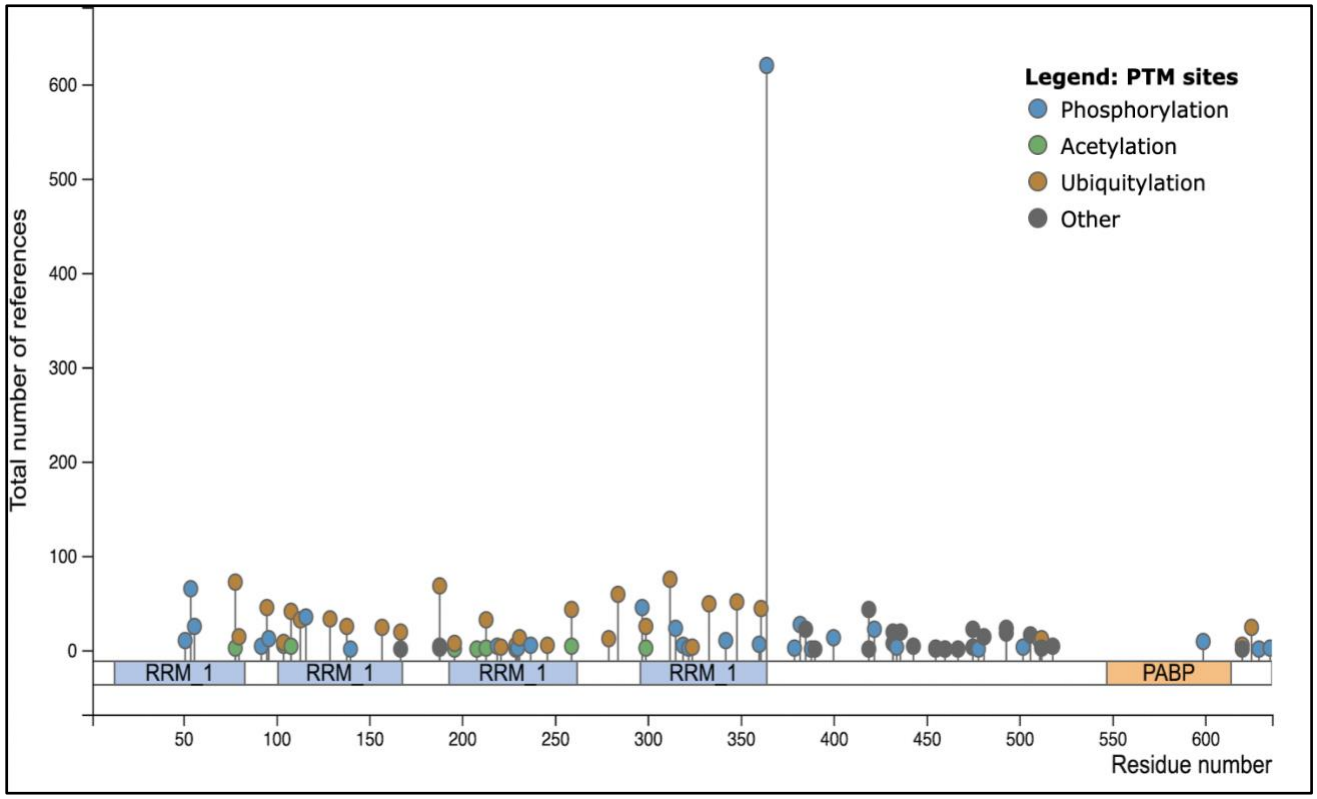

Figure 8: Graphical representation of Polyadenylate-binding protein 1 (PABP1) generated by PhosphoSitePlus.
The colors in the top right corner indicates location of various post-translational modifications
(For example, blue represents areas of phosphorylation.)

The first protein in the table, Transcription intermediary factor 1-beta/KAP, acts as a nuclear corepressor that plays a role in transcription, as well as in the DNA damage response. Once sensing DNA damage, the phosphorylation of KAP-1 on Ser-824 by ATM, ataxia telangiectasia-mutated, kinase enhances cell survival and promotes chromatin relaxation and DNA repair. ATM's most documented function is to mobilize and regulate the cellular response to DNA double-strand breaks (Shiloh and Ziv, 2013). ATM induced phosphorylation on Ser-824 also represses sumoylation, leading to the de-repression of expression of various genes involved in cell cycle control and apoptosis in response to cellular stress (UniProt). Additionally, KAP has E3 SUMO-protein ligase activity and thus sumoylation and desumoylation events directly regulate TRIM28-mediated transcriptional repression.

The next protein, Heterogeneous nuclear ribonucleoprotein U (hnRNP U), is a DNA and RNA binding protein involved in several cellular processes, including nuclear chromatin organization, telomere length regulation, transcription, mRNA alternative splicing and stability, and mitotic cell progression (Chaudhury *et al*., 2010). hnRNP U is extensively phosphorylated on Ser-58 by PLK1. Additionally, there are various amino acid modifications at positions on the peptide that interact directly with SUMO-1 and SUMO-2.

Polyadenylate-binding protein 1 (PABP1) binds the poly(A) tail of mRNA and regulates processes of mRNA metabolism, such as pre-mRNA splicing and mRNA stability. PABP1 is phosphorylated by MAPKAPK2, which is involved in the post-translational regulation of cytokines (Soni *et al*., 2019), as well as Pyk2, a tyrosine kinase that regulates reorganization of the actin cytoskeleton, cell polarization, cell migration, adhesion, spreading and bone remodelling (PhosphoSitePlus).

Nucleophosmin (NPM1) is a molecular chaperone protein with a wide range of functions, including ribosomal protein assembly, prevention of nucleolar protein aggregation, and regulation of the tumor suppressors TP53 and alternative reading frame (ARF) (Brunner and Graubert, 2018). NPM1 is phosphorylated at Ser-4 by PLK1 and PLK2. NPM1 is also phosphorylated by CDK2 at Ser-125 and Thr-198. Additionally, NPM1 is sumoylated by ARF and interacts directly with SUMO-1 and SUMO-2 proteins.

Eukaryotic translation initiation factor 4E (eIF4E) recognizes and binds the mRNA cap during an early protein synthesis and facilitates ribosome binding by promoting the unwinding of mRNA's secondary structures. eIF4E is also phosphorylated by multiple kinases, including PLK1 and regulates spindle association.

Histone H3.3C and H4 are core components of the nucleosome. Nucleosomes wrap around DNA and condense it into chromatin, limiting DNA accessibility. Histones thus play

a critical role in transcription regulation, DNA repair, DNA replication and chromosomal stability. Histone H3.3C is phosphorylated at Ser-11 by AURKB, which is crucial for chromosome condensation and cell-cycle progression during mitosis and meiosis. Histone H4 is phosphorylated by PAK2 at Ser-48. This phosphorylation increases the association of H4 and H3.3C with the histone chaperone HIRA, thus promoting nucleosome assembly of H3.3-H4. Additionally, H4 interacts with SUMO proteins (UniProt).

The last protein in **Table 2**, Valosin containing protein (VCP), is responsible for the fragmentation of Golgi stacks during mitosis, reassembly after mitosis and the formation of the transitional endoplasmic reticulum. VCP is phosphorylated by tyrosine kinases in response to T-cell antigen receptors. The peptide also interacts with SUMO proteins, as indicated by amino acid modifications in various positions on the peptide chain (UniProt).

C. Role of Sumoylation in Regulation of the Identified Targets in Other Cell Types

Following the identification of these targets, we explored any other information known about these proteins in different cell types. For instance, in other cell types, sumoylation and phosphorylation of nucleophosmin/B23 regulate its subcellular localization, cell proliferation and survival. It has been studied that the expression level of B23 is proportional to the cell growth rate, suggesting a positive role in cell growth and proliferation (Okuwaki, 2008).

Further research also suggests that sumoylation of Eukaryotic translation initiation factor 4E (eIF4E) activates mRNA translation. Sumoylation of eIF4E initiates the formation of the active elF4F translation initiation complex. This then promotes the translation of specific proteins that are necessary for cell proliferation and preventing apoptosis. The disruption of the elF4E sumoylation inhibits protein translation and removes the oncogenic functions associated with this protein (Xu *et al*., 2010).

Furthermore, inhibition of sumoylation of VCP leads to an impaired stress response. In normal cells, under oxidative and endoplasmic reticulum stress conditions, sumoylation of VCP initiates the release of VCP to stress granules and promotes the VCP hexamer formation. However, when there are mutations in the VCP N-domain, reducing sumoylation, there is a weakened VCP hexamer assembly, rendering the cells vulnerable to stress (Wang *et al*., 2016).

Additionally, histone sumoylation is associated with transcription repression. Histone H4 is able to bind to E2, the SUMO-conjugating enzyme (UBC9), as well as be sumoylated in an E1, the SUMO-activating enzyme, and E2 dependent manner. Therefore, evidence seems to suggest that histone sumoylation recruits histone deacetylase and heterochromatin protein 1, thereby inducing gene silencing (Shiio and Eisenman, 2003). While these targets may have more known and studied functions within other cell types, not much is known about these proteins in germ cells.

VI. Discussion

In this particular study, we sought to explore a series of questions which could shed light on the role of phosphorylation and sumoylation on mouse spermatocytes. Dr. Vigonder's lab has spent a long time investigating the role of sumoylation in spermatogenesis and its contribution to male infertility. While sumoylation is a post-translational modification that is only more recently studied and less understood, there is accumulating evidence supporting its role during spermatogenesis.

In previous research studies, the inhibition of global sumoylation with the inhibitor Ginkgolic acid (GA), caused purified mouse spermatocytes to be arrested in the G2/M transition *in vitro*. These events were determined to be regulated by the important kinases,

PLK, Aurora, and tyrosine kinases. Further research within the lab suggests that sumoylation is involved in regulating several of these kinases (Vigodner *et al.,* 2017). This information initially led us to further explore the connection between the post-translational modifications of phosphorylation and sumoylation.

The first question we wished to address was which protein's phosphorylation is affected when sumoylation is disrupted. A two-dimensional gel electrophoresis was performed by Applied Biomics, Inc (Hayward, CA), along with Phospho-profiling and Protein ID. By using mass spectrometry, the peptide mass and fragmentation spectra were submitted to GPS Explorer workstation equipped with MASCOT search engine to search the database of National Center for Biotechnology Information non- redundant (NCBInr) and a list of target proteins was then obtained. **Table 1** depicts the results of this experiment with a comprehensive list of phosphorylation affected proteins in mouse spermatocytes.

Since previous research in Dr. Vigodner's lab identified that sumoylation regulates the important kinases of PLK, Aurora, and tyrosine kinases, we looked to identify which proteins from the list in **Table 1** are regulated by these kinases and sumoylation. Starting with the UniProt program, each protein's function and interaction with other molecules was explored. The post-translational modifications regarding phosphorylation and sumoylation were also recorded. Additionally, any specific kinase activity was noted.

Further research into identifying the proteins of interest required the usage of PhosphoSitePlus software and database. Each protein was inserted into the search engine and scanned for the desired post-translational modifications and kinases. With each protein, a graph was generated with the x-axis representing the protein's residue numbers and the y-axis indicating the number of references for post-translational modifications. This allowed us to determine the extent and location of post-translational modifications, including phosphorylation specifically. Additionally, with each protein, a comprehensive list of its

interactions both upstream and downstream was generated and allowed us to analyze protein function and activity. By using the UniProt and PhosphoSite databases, we were able to identify the target proteins which are regulated by meiotic kinases and interact with SUMO proteins, as expressed in **Table 2.** This bioinformatic analysis allowed us to condense the list of proteins from those that experience effects in phosphorylation when sumoylation is inhibited, to a list of more specific proteins that can be involved in the regulation of meiosis.

Additionally, we were able to determine if the target protein's regulation was directly or indirectly related to sumoylation. For instance, Transcription intermediary factor 1-beta/KAP becomes phosphorylated by ATM and has E3 SUMO-protein ligase activity. Therefore, sumoylation and desumoylation events directly interact and regulate TRIM28-mediated transcriptional repression. The next protein identified, hnRNP U, is phosphorylated by PLK1 and has various amino acid modifications at positions on the peptide that interact directly with SUMO-1 and SUMO-2. Next, PABP1 is phosphorylated by Pyk2, a tyrosine kinase, however, there does not seem to be any mention of sumoylation or SUMO proteins within the various software. Therefore, PABP1 may interact indirectly with SUMO proteins, as the tyrosine kinase is known to regulate sumoylation. This is also true for the proteins eIF4E and Histone H3.3C, which are phosphorylated by sumo-regulated kinases PLK1 and AURKB respectively. On the other hand, NPM1 is phosphorylated by PLK1 and PLK2 and sumoylated by ARF, interacting directly with SUMO-1 and SUMO-2 proteins, with the same being true for Histone H4. Lastly, VCP is phosphorylated by tyrosine kinases and interacts with SUMO proteins, indicated by the amino acid SUMO modifications in various positions on the peptide chain.

Finally, a literary search of previously published research was conducted to better understand potential functions of the identified proteins in cell cycle progression. For example, in other cell types, sumoylation of eIF4E activates mRNA translation. Additionally,

sumoylation and phosphorylation of nucleophosmin/B23 regulates its subcellular localization, cell proliferation and survival. Histone sumoylation was found to be associated with transcription repression, while inhibition of the SUMOylation of VCP leads to impaired stress response. These other functions seem to all correlate with essential parts of cell proliferation and survival. This information could be of importance for our study as any effect to these proteins could then influence the progression through meiosis.

VII. Conclusion

Infertility continues to be a problem all over the world, with recent studies shedding more light and understanding on the issue. While male infertility used to be primarily attributed to genetic or environmental conditions, in more recent years, the underlying mechanism of spermatogenesis, and sumoylation specifically, has been explored significantly more in relation to its role in male infertility. In our lab we have studied the importance of various post-translational modifications and the effects on the cells of the male reproductive system. We sought to identify proteins for which phosphorylation is affected when sumoylation is inhibited in spermatocytes. Additionally, from these target proteins we wished to identify proteins regulated by the PLK, Aurora, tyrosine and any other kinases that regulate meiosis.

Based on the experiments and bioinformatics analysis we performed, specific proteins were identified to be regulated by meiotic kinases and interact with SUMO proteins. Upon further research and analysis, it was determined that many of these targets also play a role in cell cycle progression and survival in other cell types. Future studies would focus on each target protein's specific role in germ cells, as well as exploring its regulation by the cross-talk between phosphorylation and sumoylation. The results of this research will be supported *in*

vivo using transgenic mice in which specific protein modifications would be inactivated in a cell-specific manner. Those studies have already been initiated in Vigodner's laboratory. Ultimately, understanding the complex regulation of meiosis and spermatogenesis by various post-translational modifications will allow us to better understand and treat specific cases of male infertility and develop safe contraceptives.

Acknowledgements

Dr. Wachtell

Thank you very much for leading the Honors Program and coordinating all the details that go into such an incredible program. Being a part of the Honors Program has certainly enhanced my experience in Stern College and will have a lasting impact.

Dr. Vigodner

Thank you for your guidance and assistance as my mentor over the last year and a half. You have provided me with the amazing opportunity to be an active member of meaningful science research. I appreciate the time and energy put in to help me learn the necessary techniques and imperative concepts to become a part of the scientific community. Thank you for your advice not only throughout this project, but with my college endeavours and future experiences.

My Parents

Thank you for your unconditional support, love, and encouragement to succeed in all areas of my life. I am lucky to have such incredible role models.

My Husband

Thank you for pushing me to be the very best I can be and to follow my dreams every day. I would not be where I am today without your utmost love and support.

References

Agarwal, A., Baskaran, S., Parekh, N., Cho, C. L., Henkel, R., Vij, S., Arafa, M., Panner Selvam, M. K., & Shah, R. (2021). Male infertility. *Lancet (London, England)*, *397*(10271), 319–333. https://doi.org/10.1016/S0140-6736(20)32667-2

Alberts, Bruce (18 November 2014). *Molecular Biology of the Cell* (Sixth ed.) New York.

Babakhanzadeh, E., Nazari, M., Ghasemifar, S., & Khodadadian, A. (2020). Some of the Factors Involved in Male Infertility: A Prospective Review. *International journal of general medicine*, *13*, 29–41. https://doi.org/10.2147/IJGM.S241099

Brunner, A., Graubert, T., Chapter 58 - Pathobiology of Acute Myeloid Leukemia, Editor(s): Ronald Hoffman, Edward J. Benz, Leslie E. Silberstein, Helen E. Heslop, Jeffrey I. Weitz, John Anastasi, Mohamed E. Salama, Syed Ali Abutalib, Hematology (Seventh Edition), Elsevier, 2018, Pages 913-923.

Capra, Maria; Nuciforo, Paolo Giovanni; Confalonieri, Stefano; Quarto, Micaela; Bianchi, Marco; Nebuloni, Manuela; Boldorini, Renzo; Pallotti, Francesco; Viale, Giuseppe; Gishizky, Mikhail L.; Draetta, Giulio F.; Fiore, Pier Paolo Di (15 August 2006). "Frequent Alterations in the Expression of Serine/Threonine Kinases in Human Cancers". *Cancer Research*. **66** (16): 8147–8154. doi:10.1158/0008-5472.CAN-05-3489

Chaudhury, A., Chander, P., & Howe, P. H. (2010). Heterogeneous nuclear ribonucleoproteins(hnRNPs) in cellular processes: Focus on hnRNP E1's multifunctional regulatory roles. *RNA (New York, N.Y.)*, *16*(8), 1449–1462. https://doi.org/10.1261/rna.2254110

Cheng, C. Y., & Mruk, D. D. (2010). The biology of spermatogenesis: the past, present and future. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *365*(1546), 1459–1463. https://doi.org/10.1098/rstb.2010.0024

Cubeñas-Potts C, Srikumar T, Lee C, et al. Identification of SUMO-2/3-modified proteins associated with mitotic chromosomes. *Proteomics*.15(4):763-772 (2015).

Eifler, K., & Vertegaal, A. (2015). SUMOylation-Mediated Regulation of Cell Cycle Progression and Cancer. *Trends in biochemical sciences*, *40*(12), 779–793. https://doi.org/10.1016/j.tibs.2015.09.006

Fukuda, I., Ito, A., Hirai, G., Nishimura, S., Kawasaki, H., Saitoh, H., Kimura, K., Sodekoa, M., and Yoshida, M. Ginkgolic acid inhibits protein SUMOylation by blocking formation of the E1-SUMO intermediate. *J.chembiol*, 16 (2): 133-140 (2009).

Gareau, J., Lima, C. The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nat Rev Mol Cell Biol* 11, 861–871 (2010). https://doi.org/10.1038/nrm3011

Gill G. (2004). SUMO and ubiquitin in the nucleus: different functions, similar mechanisms?. *Genes & development*, *18*(17), 2046–2059. https://doi.org/10.1101/gad.1214604

Hilgarth, R. et al., 2004, Regulation and Function of SUMO Modification, Journal of Biological Chemistry.

Hornbeck PV, Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. Nucleic Acids Res. 2015 43:D512-20.

Kahn, B. E., & Brannigan, R. E. (2017). Obesity and male infertility. *Current opinion in urology*, *27*(5), 441–445. https://doi.org/10.1097/MOU.0000000000000417

Katz, D. J., Teloken, P., & Shoshany, O. (2017). Male infertility - The other side of the equation. *Australian family physician*, *46*(9), 641–646.

Kong, L., Tang, M., Zhang, T., Wang, D., Hu, K., Lu, W., Wei, C., Liang, G., & Pu, Y. (2014). Nickel nanoparticles exposure and reproductive toxicity in healthy adult rats. *International journal of molecular sciences*, *15*(11), 21253–21269. https://doi.org/10.3390/ijms151121253

Lee, S. Y., Jang, C., & Lee, K. A. (2014). Polo-like kinases (plks), a key regulator of cell cycle and new potential target for cancer therapy. *Development & reproduction*, *18*(1), 65–71. https://doi.org/10.12717/DR.2014.18.1.065

Leslie SW, Siref LE, Soon-Sutton TL, et al. Male Infertility. [Updated 2021 Aug 12]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2021 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK562258/?report=classic

Male Factor Infertility Accounts for Nearly 30 Percent of All Infertility Cases, n.d., Fertility Center of San Antonio, https://www.fertilitysa.com/infe

rtility-treatments/male-factor-infertility

The National Institute of Child Health and Human Development (NICHD), 2017, What are some possible causes of male infertility?, https://www.nichd.nih.gov/health/top ics/infertility/conditioninfo/causes/causes-male (retrieved May 6th, 2020).

Okuwaki M. (2008). The structure and functions of NPM1/Nucleophsmin/B23, a multifunctional nucleolar acidic protein. *Journal of biochemistry*, *143*(4), 441–448. https://doi.org/10.1093/jb/mvm222

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

Sato, T., Katagiri, K., Gohbara, A., Inoue, K., Ogonuki, N., Ogura, A., Kubota, Y., & Ogawa, T. (2011). In vitro production of functional sperm in cultured neonatal mouse testes. *Nature*, *471*(7339), 504–507. https://doi.org/10.1038/nature09850

Sefton B. M. (2001). Overview of protein phosphorylation. *Current protocols in cell biology*, *Chapter 14*,. https://doi.org/10.1002/0471143030.cb1401s00

Sennert, D. and Care, H. (1978). Practical Physick. Printed for William Whitwood, London, England.

Shiio, Y., & Eisenman, R. N. (2003). Histone sumoylation is associated with transcriptional repression. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(23), 13225–13230. https://doi.org/10.1073/pnas.1735528100

Shiloh, Y., Ziv, Y. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nat Rev Mol Cell Biol* 14, 197–210 (2013). https://doi.org/10.1038/nrm3546

Song, H. W., & Wilkinson, M. F. (2012). In vitro spermatogenesis: A long journey to get tails. *Spermatogenesis*, *2*(4), 238–244. https://doi.org/10.4161/spmg.22069

Soni, S., Anand, P. & Padwad, Y.S. MAPKAPK2: the master regulator of RNA-binding proteins modulates transcript stability and tumor progression. *J Exp Clin Cancer Res* 38, 121 (2019). https://doi.org/10.1186/s13046-019-1115-1

Tang, A., Gao, K., Chu, L., Zhang, R., Yang, J., & Zheng, J. (2017). Aurora kinases: novel therapy targets in cancers. *Oncotarget*, *8*(14), 23937–23954. https://doi.org/10.18632/oncotarget.14893

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

UniProt Consortium. UniProt: the universal protein knowledgebase in 2021 Nucleic Acids Res. 49:D1 (2021)

Vigodner, M., 2011, Roles of small ubiquitin-related modifiers in male reproductive function, Int. Rev. Cell Mol. Biol., 288:227-259.

Wang, T., Xu, W., Qin, M., Yang, Y., Bao, P., Shen, F., Zhang, Z., & Xu, J. (2016). Pathogenic Mutations in the Valosin-containing Protein/p97(VCP) N-domain Inhibit the SUMOylation of VCP and Lead to Impaired Stress Response. *The Journal of biological chemistry*, *291*(27), 14373–14384. https://doi.org/10.1074/jbc.M116.729343

Wilkinson, K. A., Nishimune, A., & Henley, J. M. (2008). Analysis of SUMO-1 modification of neuronal proteins containing consensus SUMOylation motifs. *Neuroscience letters*, *436*(2), 239–244. https://doi.org/10.1016/j.neulet.2008.03.029

Xiao, Y., Pollack, D., Andrusier, M., Levy, A., Callaway, M., Nieves, E., Reddi, P., & Vigodner, M. (2016). Identification of cell-specific targets of sumoylation during mouse spermatogenesis. *Reproduction (Cambridge, England)*, *151*(2), 149–166. https://doi.org/10.1530/REP-15-0239

Xu, X., Vatsyayan, J., Gao, C., Bakkenist, C. J., & Hu, J. (2010). Sumoylation of eIF4E activates mRNA translation. *EMBO reports*, *11*(4), 299–304. https://doi.org/10.1038/embor.2010.18

Zhao H, Yu C, He C, Mei C, Liao A and Huang D (2020) The Immune Characteristics of the Epididymis and the Immune Pathway of the Epididymitis Caused by Different Pathogens. *Front. Immunol.* 11:2115. doi: 10.3389/fimmu.2020.02115