IDENTIFICATION OF DNASE II IN MOUSE SPERMATOZOA AND LUMINAL FLUID FROM THE EPIDIDYMIS AND VAS DEFERENS

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

DOCTOR OF PHILOSOPHY

IN

CELL AND MOLECULAR BIOLOGY

DECEMBER 2013

By
Kenneth Dominguez

Dissertation Committee:

W. Steven Ward, Chairperson
Stefan Moisyadi
Helen Turner
Abby Collier
Qingxiao Li

Abstract

The goal of this work was to identify the nuclease associated with sperm DNA degradation in the sperm and surrounding fluid of the vas deferens and cauda epididymis of the mouse. Earlier work in our laboratory demonstrated sperm nucleolytic activity that cleaved chromatin to the size of 50 kbp with Mn^{2+} and Ca^{2+}, which could be reversed using EDTA. We have named this activity Sperm Chromatin Fragmentation (SCF). The reversible nature and activation of SCF using Mn^{2+} and Ca^{2+} are hallmarks of the nuclease topoisomerase IIB. When SCF is active for a period of time a different activity is observed that completely and irreversibly degrades the sperm chromatin. We have named this activity Sperm DNA Degradation (SDD). The complete degradation seen in SDD is much more pronounced in sperm from the vas deferens and the activity is greatly accelerated by the use of EGTA and Ca^{2+}. We found that this combination causes the buffer to become acidic, and it is this acidity that activates the nuclease rather than EGTA and Ca^{2+}. We developed a purification scheme for this nuclease, and then identified it as DNAse II, a known acidic nuclease, by Western blot analysis. The cauda epididymis and vas deferens act as storage areas for sperm, but may also function as a quality control mechanism for defective sperm. This is suggested by the drop in number of defective sperm seen in the cauda epidydimis compared to the caput suggesting they are being marked for degradation. The presence of

DNase II in the vas deferens has yet to be described in the literature. Here we present evidence of its presence and activity in the sperm and luminal fluid of the male reproductive tract.

Table of Contents

List of Figures

List of Abbreviations

BAPTA – 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid. Chelator. More hydrophobic and selective for Ca^{2+} than EDTA or EGTA.

BME – β-Mercapoethanol or 2-mercaptoethanol.

BSA – Bovine serum albumin.

CAD – Caspase activated DNase.

CEAN – Chelated EGTA activated nuclease.

CPAN – Caspase activated nuclease.

DFF40 – DNA fragmentation factor; 40-kDa subunit.

DTT – Dithiothreitol. Reduces disulfide bonds.

EAN – EGTA activated nuclease.

EDTA – Ethylenediaminetetraacetic acid. Chelates free cations.

EGTA – Ethylene glycol tetra acetic acid. Chelates free cations with a higher affinity to calcium than magnesium.

fgl2 –Fibrinogen-like protein 2.

GPI – Glycosylphosphotidyl-inositol.

HMA – Hexamethylene amiloride. A Na-H exchanger inhibitor that causes decreased cellular pH.

HMGB1/2 – High-mobility group protein B1 or B2. Organizes DNA and regulates transcription.

HMW – High molecular weight.

HSP90B1 – Heat shock protein; 90-kDa, beta member 1.

HxK – Histidine, random, and lycine amino acid sequence composing an active site motif. Strongly conserved throughout the PLD superfamily.

ICAD – Inhibitor of caspase activated DNase.

ICSI – Intracytoplasmic sperm injection.

ITMS – Ion trap mass spectrometry.

L30e – The resulting eluent of liver homogenate that has been centrifuged at 60,000 *x g* and the supernatant was filtered through a 30-kDa MWCO filter.

L30r – The resulting retentate of liver homogenate that has been centrifuged at 60,000 *x g* and the supernatant was filtered through a 30-kDa MWCO filter.

MVDP – Mouse vas deferens protein. Secreted apocrinically.

MWCO – Molecular weight cut-off.

NIH – Nation Institutes of Health.

Pel B – Luminal fluid from the vas deferens or epididymis that was centrifuged at 36,000 *x g* giving rise to a pellet that was reconstituted in TKB. The solution was used as is or frozen at -80ºC, then thawed to disrupt vesicle membranes.

Pel B-Pel – Luminal fluid from the vas deferens or epididymis that was centrifuged at 36,000 *x g* giving rise to a pellet that was reconstituted in TKB. The solution was used as is or frozen at -80ºC, then thawed to disrupt vesicle membranes. Pel B was then centrifuged again at 36,000 *x g* giving rise to a pellet that was reconstituted in TKB.

Pel B-Sup – Luminal fluid from the vas deferens or epididymis that was centrifuged at 36,000 *x g* giving rise to a pellet that was reconstituted in TKB. The solution was used as is or frozen at -80ºC, then thawed to disrupt vesicle membranes. Pel B was then centrifuged again at 36,000 *x g* giving rise to a supernatant.

PLD – Phospholipase D. An enzyme superfamily containing a conserved HxK amino acid active site motif.

PUFA – Polyunsaturated fatty acids.

SCF – Sperm chromatin fragmentation.

SDD – Sperm DNA degradation.

SDS – Sodium dodecyl sulfate.

SDS-PAGE – SDS polyacrylamide gel electrophoresis.

SPAM1 – Sperm adhesion molecule 1. Also known as hyaluronidase PH-20. Binds to and degrades the hyaluronic acid rich zona pellucida.

Sup B – Luminal fluid from the vas deferens or epididymis that was centrifuged at 36,000 *x g* giving rise to a supernatant.

TBST – Tris buffered saline and Tween-20.

TGS – Tris-glycine-SDS buffer.

TKB – Tris potassium buffer.

Topo2B – Topoisomerase IIB.

TX-100 – Triton-X 100, a non-ionic detergent.

V100e – The resulting eluent of vas deferens luminal fluid that has been centrifuged at 60,000 *x g* and the supernatant was filtered through a 100-kDa MWCO filter.

Chapter 1. Introduction

1.1. Spermatogenesis

The formation of sperm cells begins with the round diploid spermatogonium, a stem cell, which differentiates into condensed haploid spermatozoa. This process is known as spermatogenesis and is characterized in two stages: spermatocytogenesis and spermiogenesis.

During spermatocytogenesis, precursor spermatogonian stem cells remain undifferentiated by dividing mitotically and remaining diploid [1,2]. Spermatozoan production begins when a round of mitosis produces the primary spermatocyte which then undergoes a round of DNA synthesis and genetic recombination of meiosis I, resulting in two diploid secondary spermatocytes. These cells then divide to form four haploid spermatids. Spermiogenesis, the last stage of spermatogenesis, begins with the differentiation of spermatids, with the aid of Sertoli cells, resulting in the elongation and condensing of the nuclei [3-5]. During spermiogenesis the spermatid ejects the majority of its cytoplasm [6-8] while the histones of the somatic-like nuclear chromatin are replaced with sperm-specific nucleoproteins [4,9-13] which organizes the DNA more tightly and further compresses the chromatin. The increase in chromatin compression has been attributed to the replacement of histones with protamines [14-16] which results in

a distinctive chromatin organization in spermatozoa [17-20]. Protamine organized chromatin forms toroid shaped "doughnuts" which loops in 25 to 50 kb sections [13,21,22].

This compaction results in far less DNA being exposed as it is mostly hidden within toroids [23] and also is resistant to enzymatic digestion by nucleases [24]. The extent of genetic protection provided by the sperm cell has been tested in various ways with viability determined by the ability of the sperm nuclei to decondense and form pronuclei after injection into an oocyte. Hamster, mouse, and human sperm nuclei were shown to be moderately heat stable: spermatozoa that were treated at 90ºC for 30 minutes could still generate viable offspring when injected into oocytes [16,25-27]. Temperatures higher than 90ºC drastically reduced this ability. The viability of mouse and human spermatozoa were also tested against sonication and their ability to participate in embryogenesis was maintained when injected into the oocyte immediately after sonication [16,28]. Lastly, the stability of the chromatin was also successfully confirmed after freeze-drying spermatozoa and injecting into an oocyte.

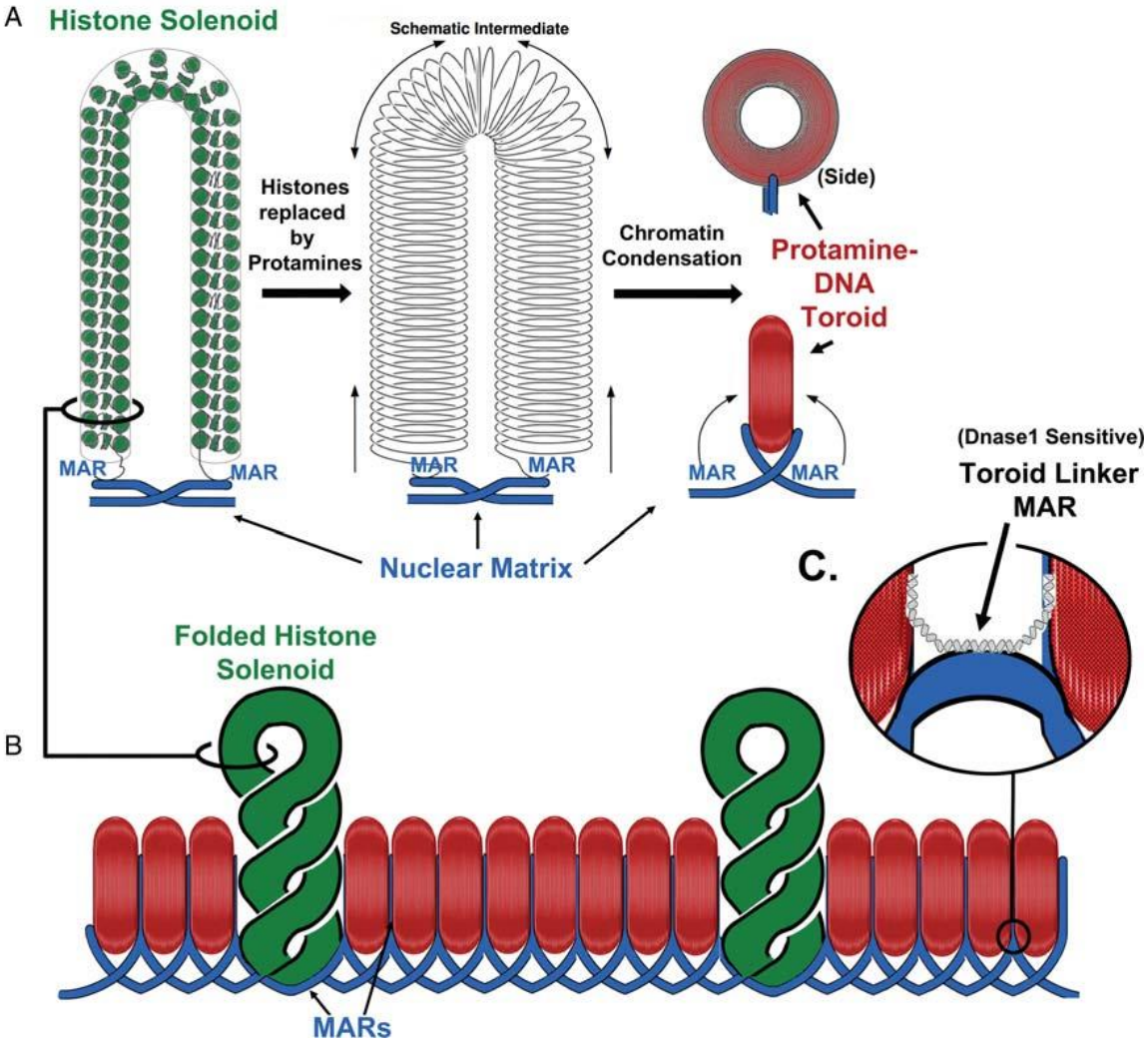
During spermatogenesis, most of the histones are replaced with protamines during the morphological changes that occur during spermiogenesis. The presence of too many histones in mature spermatozoa has been linked to infertility and interpreted as evidence suggesting defective chromatin repackaging during spermiogenesis [24,29-32]. However, recent evidence makes it clear that some

histones are required in mature spermatozoa which has been interpreted as evidence for the possibility of DNA transcription during early embryonic development [33-35]. In either case, however, the points where sperm DNA intersects with the nuclear matrix have less protamine bound DNA and therefore are more sensitive to DNA manipulation by topoisomerase II-beta and nucleases [17,36] or, in the transcriptionally active spermatozoan model, genetic transcription [37-40].

1.2. Chromatin structure in the mature sperm

In order to efficiently transfer the genetic information stored in the sperm cell to the oocyte, and be readily accessible for replication and transcription, the structural organization of the paternal DNA is critically important. Most of the histones in spermatid chromatin are replaced by protamines and the DNA is compacted and organized into toroids that are approximately 50 kbps in length [41-44] (Fig 1.1). The majority of the histones are replaced with protamines, which are arginine rich and present a positive charge that neutralizes the negative phosphodiester backbone of DNA [45,46]. The structure also provides protection to the DNA by positioning it inside of the toroid [23,47]. Interestingly, the requirement needed for DNA to assume the toroid shape appears to be the

Figure 1.1. Histone Solenoid and Protamine Toroid Structures



DNA is interweaved around histones and packaged tightly in the form of solenoids in the nucleus of somatic cells. In sperm cells the majority of histones are replaced with protamines, which allow greater compaction of the DNA and also changes the shape of the chromatin to that of a toroid. The chromatin is attached to specific nuclear matrix attachment regions (MAR) by short strands of linker DNA. This DNA is accessible to nucleases such as DNase I.

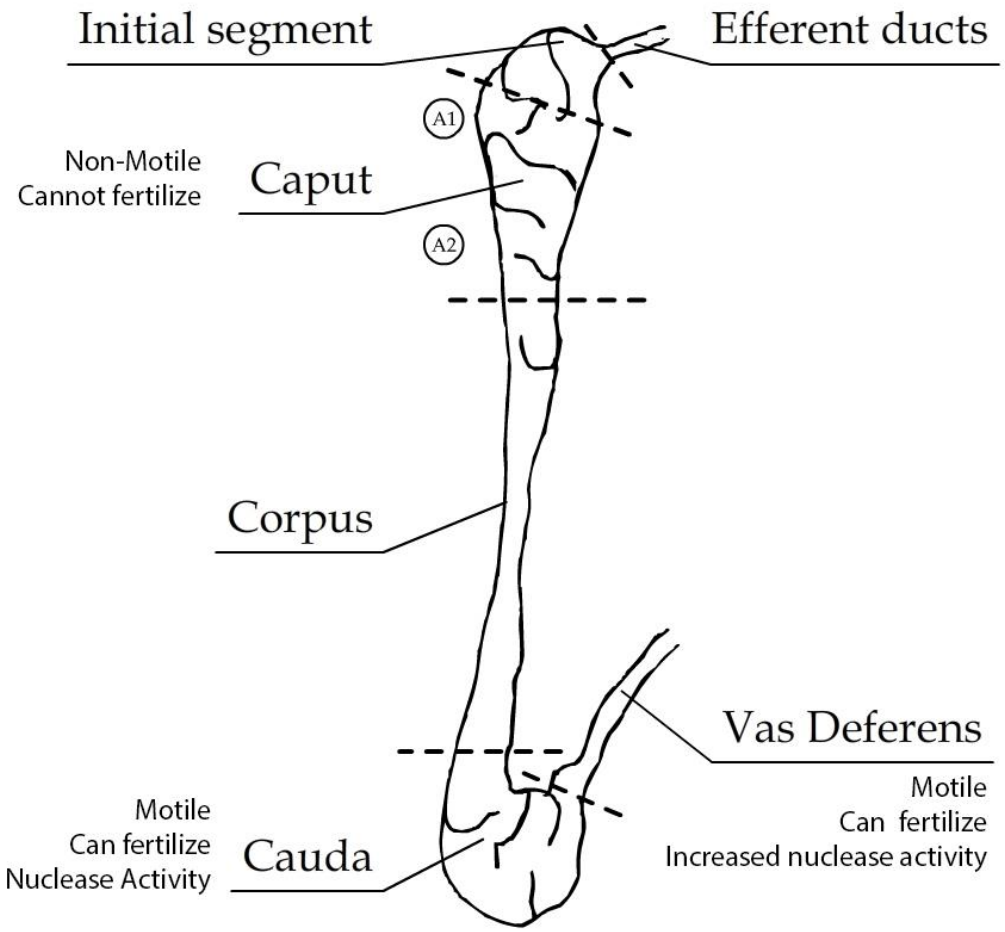
neutralization of the molecules' negative charge as the shape will spontaneously form even in the absence of protamines as long as the charge is neutralized by divalent cations [23,48]. The secondary organization of the toroid loops takes the shape of a package of "lifesavers" [49-51] with each toroid linked and stacked side to side. This provides the maximum protection and also ensures no transcription until after fertilization. Although the majority of sperm DNA is bound with protamine, the linking region between toroids is bound by histones. The current data supports a model where the histone-associated linker region represents functional genes that are used during spermiogenesis [49,51-53] and early fertilization [34,54-58]. This region is also sensitive to nucleases [17,32,57,59-61].

1.3. Spermatozoan Maturation

Spermatozoa exit from the testis fully formed yet immature as they are immotile, cannot locate the oocyte, and cannot undergo an acrosomal reaction in order to penetrate the zona pellucida of the oocyte. It is the migration through the epididymis where the sperm cell undergoes a maturation process, known as capacitation, and exits fully capable of fertilization (Fig. 1.2) [62-69].

The capacitation [56,70-73] of the sperm cell has been the focus of research for many years. Correlations have been made linking sperm cell maturation to interactions of the cells with various elements found in the luminal fluid of the

Figure 1.2. Sperm Matures in the Epididymis



Modified from Arrotéia et al. (2012) The Epididymis: Embryology, structure, function, and its role in fertilization and infertility

Morphologically complete spermatozoa exit the testis via the efferent ducts and enter the initial segment of the epididymis. The sperm travel through the caput, corpus, and accumulate in the cauda of the epidimis. Sperm also accumulate in the vas deferens. Sperm from the caput are not able to move and cannot fertilize the oocyte. Sperm from the cauda are able to move and can fertilize the oocyte. There is also nuclease activity detected in the cauda epididymis and more so in the vas deferens.

reproductive tract. It has long been recognized that epididymal secretions aided sperm cell maturation [65,67,74-76] and aposomes [77-80], apocrine secreted vesicles, are a common method of transferring maturation factors to the sperm cell. Unlike classical merocrine exocytosis, where proteins are processed in the endoplasmic reticulum followed by the Golgi apparatus and finally translocated inside a vesicle to the plasma membrane, during apocrine secretion the protein remains in the cytoplasm and is exported by localizing to apical plasma membrane protrusions, or blebs, which then pinch off into the lumen. Prostasomes, prostate aposomes secreted [81-85] into the luminal fluid after the vas deferens but prior to ejaculation, were identified in semen using fusogenic abilities to transfer proteins to sperm cells in-vitro [86-93] yet few proteins were actually transferred when measured in-vivo [84,94-96]. Prostasomes were also shown to transfer key lipids to the sperm cell [82,83,97,98] with effects on spermatozoan membrane fluidity [84,99] and have been shown to interact with sperm in seminal fluid aiding in promotion of forward motility and prevention of premature acrosome reaction. They also have antibacterial, antioxidant, and immunosuppressive actions [17,78,84,95,96,100-102]. As such, prostasomes have set the precedent for linking sperm maturation with aposomal interactions.

Epididymosomes, another type of aposome, are secreted from the apical epithelial layer of the epididymis and are physically similar to prostasomes but

enter the luminal fluid before ejaculation and differ biochemically from other apopsomes [17,24,103]. Therefore, they are more likely to be related to the normal processing of the sperm cell prior to its journey to fertilize the oocyte. During epididymal transit in mice, epididymosomes have been described as exhibiting an increase in the plasma membrane cholesterol:phospholipid ratio and a decrease in polyunsaturated fatty acids (PUFAs), except sphingomyelin which increases, favoring a loss of fluidity [103-105]. Sperm membranes evolved in the opposite direction during epididymal transit, with an increase in membrane fluidity suggesting the occurrence of a lipid exchange between sperm cells and epididymosomes [103,106]. The concentration of sphingomyelin and cholesterol increases during transit through the epididymis. Lipid rafts [107,108]phospholipid microdomains of the plasma membrane that also exhibit high sphingolipid and cholesterol concentrations, have been found on the surface of epididymosomes [93,109,110] and sperm cells. The rafts, on both epididymosomes and sperm cells, present locations where glycosylphosphotidyl-inositol (GPI)-anchored proteins attach and are suggestive of the possibility that epididymosomes may contribute to the formation of lipid rafts on sperm cells [103,111].

Differences in protein composition contained by epididymosomes from the caput and cauda have been described in the bovine model [112-115]. Evidence for the gain of zona pellucida binding function has been suggested with the presence of caput-derived P25b (zona pellucida binding protein that attaches to the outer

sperm membrane with a glycosylphosphotidyl-inositol anchor) [91,104,113,116] and cauda derived HSP90B1 (chaperone for zona pellucida receptor protein) [113,117]. Protein P25b also has murine and hamster orthologues, SPAM1 (sperm adhesion molecule 1) [116,118,119] and P26h [120-122], respectively. Additionally, epididymosomes from the caput or cauda epididymis have been biotinylated and incubated with sperm from either the caput or cauda epididymis showing that proteins transfer from caudal epididymosomes to both caput and caudal sperm in the same pattern, but biotinylated caput epididymosomes transfer protein differently to caput sperm compared to caudal sperm, further suggesting that protein transfer occurs in a serial manner and has been correlated with sperm maturation during transit through the epididymis [113,123].

After maturation, sperm cells exit the cauda epididymis and enter the vas deferens, which is thought to act as a general reservoir for mature spermatozoa. Extensive research has yet to be done regarding the occurrence of similar aposome/spermatozoan activity in the vas deferens. It has been shown, however, that the vas deferens also has apocrine activity in the production of a protein called MVDP (mouse vas deferens protein), an aldose-reductase present in the cytoplasm of epithelial cells and the luminal fluid [124,125] (however, it was recently shown that when MVDP was knocked out in mice the protein was not necessary for sperm maturation [126,127]). The vas deferens also secretes steroids and removes excess water from the luminal fluid [17,128].

1.4. DNA degradation during Apoptosis

Apoptosis, or the genetically planned death of a cell, has been studied for many years [1,129] and has traditionally been described as morphologically different from necrosis. Apoptosis begins with cell shrinkage due to the fluctuation of osmotically active ions, such as K^+, Na^+, or Cl^-, followed by detachment from the extracellular matrix. With focal adhesion points gone, the cell undergoing apoptosis then assumes a round shape [17,130]. The detachment of the plasma membrane from the cytoskeleton follows next giving rise to membrane protrusions, or blebbing. The apoptotic cell then undergoes condensation in which multiple invaginations of the plasma membrane pinch off into sealed vesicle compartments. The nucleus is also pinched off into its' own compartment where nuclear condensation proceeds with the disassembly of the nuclear matrix, nuclear lamina, and the aggregation of chromatin. The DNA is cleaved into 200 bp segments by caspase activated nucleases (CAD/DFF40/CPAN) [37,39,40] and topoisomerase II. The DNA is also cleaved at internucleosomal regions by topoisomerase II (independent of caspase) [6] as well as nucleases associated with topoisomerase II such as CAD/DFF40 [131,132]. Finally, the compartmentalized vesicles (apoptotic bodies) are phagocytosed by macrophages and non-professional phagocytes. These characteristics separate apoptosis from necrosis, which does not share the same traits and is visibly distinct from apopotosis, as

necrosis involves the swelling of the cell along with the loss of membrane integrity. This ultimately leads to cell lysis and surrounding tissue inflammation.

Even though the events are morphologically distinct, it has been suggested that apoptosis and necrosis are extreme forms of cell death. The signaling involved in apoptosis can be moderated to different strengths giving rise to cells which exhibit both morphologies [133]. As such, it may not be surprising that the machinery used for apoptosis is also used for other purposes as well, such as embryonic development [134], and more specifically, the development of germinal cell lines [135] like spermatozoa [136-140]. As such, it has been observed that nuclease activity is not restricted strictly to the cellular compartment. Nucleases, like DNASE1L3, have been described in extra-cellular seminal fluids [41,141] outside of the sperm cell.

1.5. Role of Topoisomerase II in Apoptosis

Due to the amount of chromosomal DNA that needs to be synthesized and organized during cellular division a detangling tool that prevents entanglements during the manipulation of DNA is required. Topoisomerase II operates in this context by creating a temporary covalent bond with DNA and then a temporary double-strand break allowing for DNA manipulations without entanglements, which is then followed by re-ligation [142]. The size of chromosomal DNA

fragments created after a topoisomerase II excision have been repeatedly measured between 50 and 100 kbp, and the site of the excisions are localized to the internucleosomal linker regions. The size of the DNA fragments and points of excision in cells induced to apoptosis [143,144] also coincides with those of cells undergoing cellular division. Further investigations into the nature of topoisomerase II have led to the conclusion that the enzyme is also active and functioning during apoptosis [1] as well as neuronal development [3,5]. During apoptosis these regions of DNA are bound by topoisomerase II, histone H1, and HMGB1/2 which stimulate caspase activated DNase (CAD) resulting in the production and digestion of high molecular weight (HMW) DNA fragments that correspond in size to chromatin loop domains [6,8].

1.6. Apoptosis in Spermiogenesis

The same protein tools used during normal apoptosis have been identified during spermiogenic development in the testis. During spermiogenesis the Sertoli cells induce normal apoptosis in 50-60% of germ cells during spermatogenesis as the primary spermatocytes enter meiosis I, marking them with Fas leading to apoptosis and phagocytosis [9,11-13]. Many of the enzymes active during apoptosis, including topoisomerase II, have been shown in spermatids undergoing differentiation [14,16] as well as in fully formed sperm cells taken from the epididymis or vas deferens [17]. During spermatocytogenesis caspase activated

proteases, ribonucleases, and nucleases are used to digest unnecessary parts of the developing round spermatid leaving an oblong, fully formed sperm cell.

However, the efficiency is not perfect and some Fas-marked germ cells appear later as mature sperm bearing Fas markers along with abnormal genomic quality [13,21]. Even when leaving the testis, before entering the epididymis, some sperm have been shown to contain double-strand DNA breaks [16,25,27]. It has been suggested that DNA breaks seen in mature sperm chromatin may be the result of incomplete religation of spermatid chromatin during elongation in spermiogenesis [16]. DNA breaks can be artificially induced in mature spermatozoa by activating endogenous nucleases [24,29,31,32]. In species where DNA breaks are found, they have been correlated to infertility in males [33]. It has been hypothesized that in some species spermiogenesis has evolved to increase sperm production rate at the expense of quality, increasing genetic mutations [36] and which may explain the regularity of un-religated DNA breaks.

1.7. Nuclease Activity

Nuclease activity, which occurs during the end stages of apoptosis, can be activated under various conditions. Typical means include caspase (CAD/DFF40/CPAN) [37,39,40] as well as ionic flux activation [41]. Ion induced nuclease activity has been observed in model systems ranging from plants and

bacteria to human cells. Plant systems have been shown to exhibit calcium and magnesium dependent nucleases in apoptotic cells. Extracts from plant cells undergoing apoptosis where able to trigger DNA fragmentation and apoptotic morphology in human cells [45].

As the initiation of apoptosis relies in part on the fluctuating concentrations of ions, ion channels of the mitochondria help control apoptosis by regulating the ion concentration in the cytosol. This, in turn, regulates the activity of proteases and nucleases. These ion channels also regulate the release of apoptogenic factors from the intermembrane space to the cytosol [48].

Rat nuclease DNase gamma, and it's human homologue DNAS1L3 [49,51], are calcium/magnesium dependent nucleases which have been extensively documented in somatic cells, localized in the nucleus, and associated with reproductive cells. Both enzymes cleave DNA in nucleosome segments. Additionally, the sequence of DNase gamma is 45% similar to DNase I and Dnase X [49,51], therefore it has been suggested that DNase gamma and DNAS1L3 are part of the DNase I family as the amino acid residues associated with DNA catalytic hydrolysis are conserved. An important difference between DNase gamma and DNase I lies in the location of activity; DNase gamma is found in the nuclear fraction while DNase I is secreted outside the cell. The precursor region of DNase gamma suppresses activity while two sequences localize the protein to the nucleus. On the other hand, the precursor region of DNase I is hydrophobic which

is suggestive for extracellular secretion [55,58]. DNAS1L3, a type of DNase I, has also been associated with sperm development [17,32,60,61].

1.8. Acidic Nucleases

Most nucleases require divalent metal cations for activation and feature two identical subunits used for double strand cleavage. The proposed mechanism involves the first metal cation coordinating a water molecule that performs a nucleophilic attack on the scissile phosphate to displace the 3'-leaving group, which is stabilized by the second metal cation [63,66] and the same occurs on the other subunit [70,73]. Of the DNA digesting class of enzymes there is a subset that do not require any metal and are known as metal-ion-independent DNases.

These non-metal cation activated nucleases belong to the phospholipase D (PLD) superfamily and use a different mechanism for cleavage involving phosphodiester bond hydrolysis in a two-step mechanism [54,56]. All PLD nucleases operate using two identical HxK motifs in the active site [59] which in some enzymes may be monomeric and located on the same protein, such as human tyrosyl-DNA phosohdiesterase 1 [62,64], or are each located on two homo dimers that are combined at the active site when joined, such as restriction endonuclease Bfil [56,71].

Acidic nucleases catalyze the DNA cleavage reaction in two-steps to generate 5'-phosphate and 3'-OH cleaved DNA. The proposed mechanism involves the histidine in each HxK motif catalyzing the nucleolytic reaction by acting as a nucleophile and attacking the scissile phosphate forming a covalent phosphohistidine intermediate bond while the other histidine acts as proton donor/acceptor stabilizing the reaction [74].

This catalytic mechanism is conserved in the PLD family and is used on phospholipids, DNA, and phosphorylated proteins [77]. DNase II, a nuclease contained in the lysosomes of macrophages, is a member of the PLD family and is one of the early documented nucleases, in the late 1940's, named "acid DNase" [81] with ubiquitous tissue distribution [86]. It is normally activated by low pH in macrophages after engulfing apoptotic cells during apoptosis [94]. This effect can be physiologically induced using HMA (hexamethylene amiloride), which decreases intracellular pH by inhibiting the Na-H exchanger [97]. The loss of DNase II is lethal at the embryonic stage [99].

1.9. Sperm Chromatin Degradation by Topoisomerase II And An As Yet Unidentified Nuclease

The role of topoisomerase II has been well documented in somatic cell division and apoptosis. Its activity has been observed in sperm cells as well. In

spermatozoa, topoisomerase II cleaves DNA in similar fashion as in the somatic cell followed by a full degradation of DNA from other nucleases when incubated with manganese and calcium [17]. The cleavage site taken by topoisomerase II resides on nuclease-sensitive sites where DNA attaches to the nuclear matrix [17,24]. The spatial location of the other nucleases resides on (or is closely associated with) topoisomerase IIB [104,105].

A current method for studying sperm/oocyte interactions during fertilization involves placing sperm nuclei, as is or altered according to experimental design, directly into the oocyte, in a process known as ICSI (intracytoplasmic sperm injection) [106]. What follows next (normally decondensation of chromatin DNA followed by replacement of protamines with histones [107], formation of paternal and maternal pronuclei, and finally zygotic cell division [109,110]) determines whether or not the alterations to the sperm nuclei were critical for fertilization. The minimum requirements for pronuclear formation under experimental conditions require the nuclear matrix with accessible DNA still attached [111]. Interestingly, if topoisomerase IIB is activated to digest DNA in the sperm nuclei prior to ICSI, paternal pronuclei develop but no DNA synthesis occurs. As in somatic cells, other nucleases seem to become activated by topoisomerase IIB after excision if the DNA is not re-ligated within a set period of time, resulting in full DNA digestion. When topoisomerase IIB is activated in sperm taken from the epididymis, more time is needed before full

digestion occurs in contrast with sperm taken from the vas deferens, where less time is needed prior to full DNA digestion [112,114,115]. As sperm taken from the vas deferens initiates full DNA degradation earlier than from the epididymis suggests that more nucleases are present and perhaps are imported into the cell as it waits in the vas deferens prior to its journey to fertilize the zygote.

1.10. Identification of a Nuclease Activity in Epididymal and Vas Defens Luminal Fluid

In this section we will review prior data from our laboratory that provides the foundation for the project to be described. We found a nuclease present in mouse spermatozoa, as well as in the surrounding luminal fluid from the vas deferens, which digests chromatin in conjunction with topoisomerase II that requires Mn^{2+} and Ca^{2+} for full activity. As we isolated an extract containing the activity, we found that the nuclease requires incubation in EGTA, and then in Ca^{2+} to become activated, and so we termed the nuclease EAN (EGTA Activated Nuclease). We hypothesized that EAN could become activated through one of three pathways: (1) EGTA initiates a cell-signaling pathway resulting in the activation of EAN, which then requires Ca^{2+} to digest DNA; (2) EAN is inhibited by a Ca^{2+} binding protein and EGTA releases EAN from inhibition, then additional Ca^{2+} activates EAN to digest DNA. (3) EGTA chelates an unknown divalent cation (not Ca^{2+}) that inhibits EAN, and EAN requires Ca^{2+} to digest DNA.

I tested these possibilities using a plasmid-based assay in which the soluble fraction from the vas deferens luminal fluid (enriched for EAN) was mixed with plasmid DNA and molded into agarose plugs. With EAN and its substrate, DNA, in agarose plugs we could expose them to different conditions to determine the effects of EGTA and Ca^{2+} on EAN. The buffers contained 50 mM Tris, pH 7.5, 100 mM KCl (TKB). When the plugs were first incubated in EGTA (10 to 30 mM), then in $CaCl_2$ (1 to 10 mM), the plasmid DNA was digested. If either EGTA or Ca^{2+} was omitted, the DNA remained intact, verifying that the presence of both EGTA and Ca^{2+} was required for EAN activity.

Understanding the combined action of EGTA and Ca^{2+}, we incubated plugs in EGTA, and then in buffer alone to remove the EGTA, then incubated in Ca^{2+}, we found that there was no DNA digested. When plugs were incubated in EGTA, then buffer, then EGTA again, then in Ca^{2+}, there was EAN activity and the DNA was digested.

All three possibilities described above were inconsistent with these results because the EGTA and Ca^{2+} treatments should not have reversed either a complex cell-signaling pathway or reintroduced an extracted cation. We then looked into a fourth possibility, that EAN was activated by the presence of EGTA and Ca^{2+} in the same buffer. We incubated the plugs in a single buffer containing 2.5 mM EGTA and 2 mM Ca^{2+} that resulted in DNA digestion. Under these conditions most of the Ca^{2+} is chelated by EGTA leading us to the possibility that free Ca^{2+} was not

responsible for nucleolytic activity, instead chelated EGTA was activating EAN. This suggests that a novel nuclease has been found in the mouse epididymis and vas deferens and that we can further characterize it using its unique activation requirement.

Chapter 2. Results I: A novel nuclease activity that is activated by Ca^{2+} chelated to EGTA.

This work has been published in *Systems Biology in Reproductive Medicine* 55:193-199, 2009.

2.1. Introduction

There are several different types of enzymes in the cell that degrade DNA, collectively referred to here as nucleases. They are involved in a host of functions from DNA repair to apoptosis, and are required for proper cell function [104,116]. Most of the nucleases so far identified, with few exceptions, such as prokaryotic exonuclease VII [117], and DNase IIα [116,119], require divalent cations as cofactors for activity, and are inhibited by EDTA and EGTA, which chelate the cation cofactor. The prototypical enzyme of this group is deoxyribonuclease 1 (DNase I), originally isolated from the bovine pancreas [120]. DNase I is a 32 kd protein that requires Ca^{2+} and/or Mg^{2+} for activity, and is completely inhibited by EDTA or EGTA [123]. Both DNase I and DNase IIα are inhibited by Zn^{2+} [124]. It has been proposed that the use of metal cations by metal cation dependent nucleases is required for substrate specificity. The notable substrate specificity of particular base-pair sequences and/or single or double-strand structures are examples of this in nucleases, polymerases, and also in the high copy fidelity of RNA/DNA

polymerases [126]. The current hypothesis for the mechanism of action consists of the first cation functioning in the role of nucleophile formation while the second is needed for transition state stabilization [126].

We have recently found evidence for an apoptotic-like degradation of DNA in mature mouse spermatozoa [17]. Upon incubation of spermatozoa with Mn^{2+}, sperm chromatin is first degraded to 50 kb fragments that can be religated with EDTA, a hallmark of reversible Topo2B cleavage [1]. With further incubation, the sperm DNA is more completely degraded in a typical nuclease digestion pattern that is not reversible [17]. In later studies focusing on the nuclease step of this sperm DNA degradation, we demonstrated that the sperm nuclease could be activated by pretreatment with EGTA followed by incubation with Ca^{2+} [2,60]. In this work, we tested the role of EGTA in activating the sperm nuclease.

2.2. Materials and Methods

2.2.1. Animals

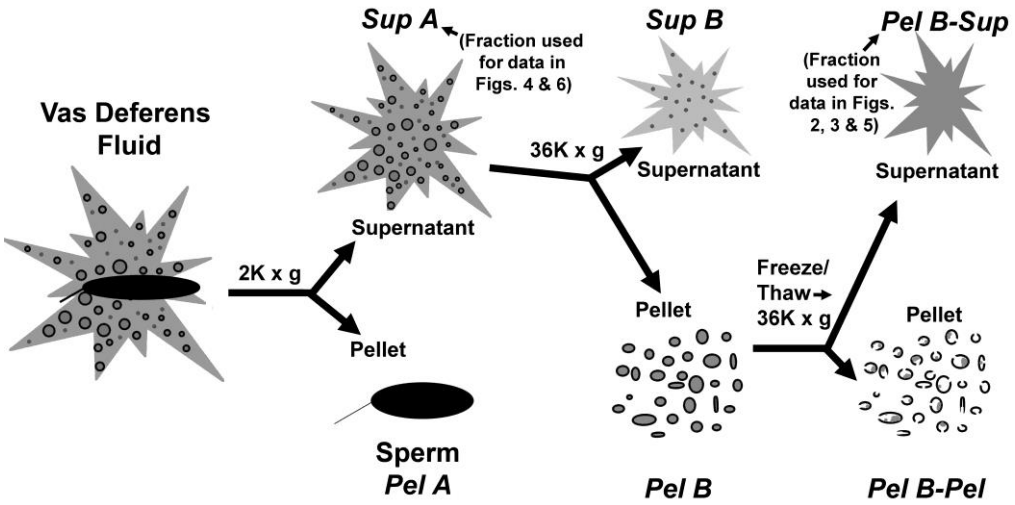
Male B6D2F1 (C57BL/6J X DBA/2) mice were obtained from the National Cancer Institute (Raleigh, NC). Mice were fed *ad libitum* and kept in standard housing in accordance with the guidelines of the Laboratory Animal Services at the University of Hawaii and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research

Council (DHEF publication no. [NIH] 80-23, revised 1985). The protocol for animal handling and the treatment procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Hawaii.

2.2.2. Preparation of Extracts

For extracts from the vas deferens or epididymus, mice were sacrificed using carbon dioxide asphyxiation followed by cervical dislocation. A schematic diagram for the isolation of the vas deferens extract is shown in Figure 2.1. The epididymis and/or vas deferens were excised and fluid from the epididymis and/or vas deferens gently extracted, using tweezers, and diluted to a concentration of approximately 108 spermatozoa/ml in 25 mM Tris, pH 7.4, 150 mM KCl buffer (TKB) and kept on ice. Extracted fluid from the epididymis and/or vas deferens was centrifuged at 2,000 *x g* for 2 minutes at room temperature. This gave rise to a pellet consisting of sperm cells, which was reconstituted in TKB. The resulting supernatant, Sup A, was centrifuged at 36,000 *x g* for 10 minutes at 4º C giving rise to two subfractions, the supernatant, Sup B, and pellet, Pel B. Sup B was used for the size fractionation experiments for which large amounts of enzyme were required (Figs. 4 and 6). The Pel B, thought to contain vesicles, was reconstituted in TKB, frozen at -80º C, then thawed to disrupt vesicle membranes then

Figure 2.1. Schematic diagram of the isolation of the sperm nuclear extract used.



Fluid extracted from the vas deferens was diluted in buffer. The suspension was centrifuged at 2,000 *x g* for 2 minutes at room temperature (RT). The luminal fluid remained in the supernatant, Sup A, while spermatozoa separated to the pellet, Pel A. The SupA was then centrifuged at 36,000 *x g* for 10 minutes at 4ºC. The resulting luminal fluid supernatant, Sup B, was aspirated while the luminal fluid pellet, Pel B, was resuspended in the same volume of buffer and was kept frozen at -80ºC overnight. Pel B was then thawed and centrifuged at 36,000 *x g* for 10 minutes at 4ºC. The resulting supernatant, Pel B-Supernatant, was aspirated while the resulting pellet, Pel B-Pellet, was resuspended in the same volume of buffer.

centrifuged again at 36,000 *x g* for 10 minutes at 4º C, giving rise to the supernatant, designated Pel B-Sup, and pellet, designated Pel B-Pel. Pel B-Sup was the fraction with the highest concentration of CEAN activity, and used for experiments that characterized the activity of CEAN (Figs 2.2, 2.3 and 2.5).

For extracts from the liver and brain, mice were sacrificed as above, and the liver or brain was excised separately. Each were homogenized in 0.5% TX-100 in a homogenizer, centrifuged at 3,000 *x g* for 10 minutes, then centrifuged at 20,000 *x g* for 10 minutes at 4ºC. The supernatants of both were analyzed for activity.

2.2.3. Size fractionation of Extracts

Samples were further fractionated according to size using Vivaspin 500 spin-tubes. Approximately 300 µl of sample was centrifuged through a 100, 50, 30, 10, or 5 kDa filter at 13,000 *x g* for between 10 to 40 minutes at 4ºC. In order to clean the retentates, they were resuspended with approximately 500µl TKB and centrifuged three times until eluent fully removed from retentate. Retentate then resuspended in 300µl TKB.

2.2.4. Nuclease Assay

Plasmids for the nuclease assay were prepared using a CompactPrep Plasmid Midi Kit (Qiagen, Catalog Number 12743). Midi Kit Plasmid procedure was followed resulting in the harvest of purified plasmid DNA (0.51 µg/µl). Nuclease activity was activated by incubating approximately 3 µl of one of the various sperm, liver or brain preparations described above in a total of 20 µl of TKB with 0.624 µg plasmid DNA supplemented with divalent cations (Mn^{2+}, Ca^{2+}, Zn^{2+}, or Cu^{2+}) and/or EGTA. The reaction was incubated at 37ºC for 15 minutes to 2 hours. Cation and EGTA concentrations varied from 2.5 mM to 50 mM in TKB. Evidence of activity was measured by visual detection of digested plasmid DNA separated by electrophoresis on 0.05% ethidium bromide 1% agarose gel.

2.3. Results

2.3.1. Nuclease Activity Requires both EGTA and Ca^{2+}

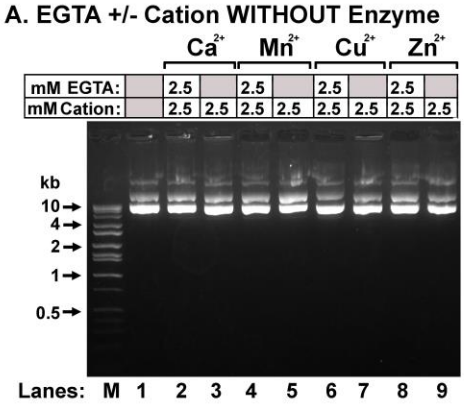
To study the nuclease activity in sperm extracts, we developed a plasmid based assay for DNA degradation [4,60]. A schematic of the isolation of the vas deferens extract used in these studies is shown in Fig 2.1. The enzymatic activity of the nuclease described below was present in all fractions, but highest in Sup A and Pel B-Sup. Pel B-Pel also contained nuclease activity, but required treatment with

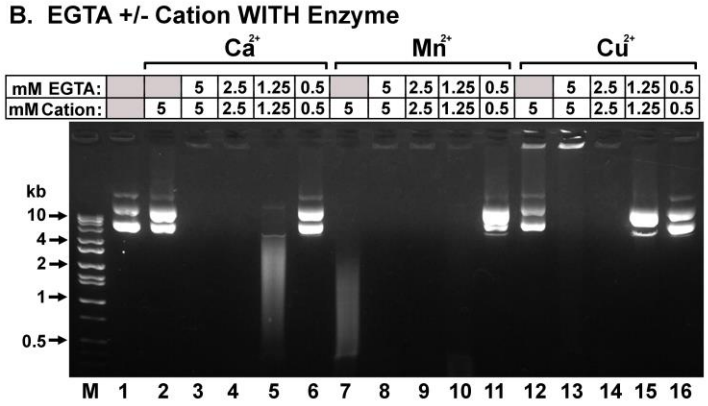
the non-ionic detergent to release it (data not shown). This is consistent with our previous report that the sperm nuclease was sequestered in small vesicles in the vas deferens fluid surrounding the spermatozoa [7,60].

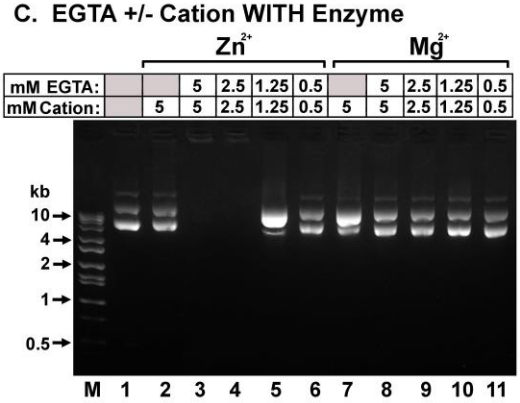
We used a plasmid based assay to determine the role of EGTA in the activation of the nuclease. As a control, we incubated the plasmid with four divalent cations, alone, and with each cation in the presence of EGTA in the absence of tissue extracts. None of these conditions caused DNA degradation (Fig. 2.2A). We next incubated plasmid DNA with the vas deferens sperm extract (Pel B-Sup) in the presence of varying concentrations of EGTA and divalent cations. We found that Ca^{2+}, Cu^{2+}, Zn^{2+} or Mg^{2+}, alone, did not activate nuclease activity in the vas deferens extract (Fig. 2.2B lanes 2 and 12, and Fig. 2.2C, lanes 2 and 7). Mn^{+2} alone did activate a nuclease activity in the extract (Fig. 2.2B, lane 7). We cannot yet establish whether this activity is the same nuclease that requires both EGTA and Ca^{2+} or a different nuclease in the extract. Therefore, for the rest of the work we used Ca^{2+} as the divalent cation, which exhibited no activity alone. The nuclease activity that required EGTA and Ca^{2+} disappeared when the extract was boiled, suggesting the activity depends on a protein.

When the plasmid was incubated in the presence of both EGTA and either Ca^{2+}, Zn^{2+}, or Cu^{2+} the plasmid was digested (Figs. 2.2B and C). For all

Figure 2.2. Nuclease activity requires both EGTA and a divalent cation.







(A) Plasmid DNA was incubated with 2.5 mM Ca^{2+}, Mn^{2+}, Cu^{2+}, or Zn^{2+}, with or without 2.5 mM EGTA, as indicated, without vas deferens extract, for 1 hr at 37ºC. (B and C) Plasmid DNA was incubated with vas deferens extract (Pel B Sup) in the presence of varying concentrations of EGTA and divalent cation for 1 hr at 37ºC, as indicated. In all figures, grey boxes indicate that EGTA or the cation was not added to the sample. Lane M contains molecular weight markers.

combinations, the lowest concentration of chelators and cation was 2.5 mM, although Ca^{2+} exhibited some activity at 1.25 mM. For these cations, the nuclease activity depended on the presence of both the chelators and the cation. Mg^{2+}, one of the most common cofactors for nucleases, did not activate the nuclease either by itself or in the presence of EGTA (Fig. 2.2C, lanes 7 – 11). These data indicated that the nuclease activity required the presence of both EGTA and a divalent cation.

2.3.2. The Nuclease Activity Requires EGTA Chelated to Ca^{2+}

The fact that the nuclease activity required the presence of both EGTA and Ca^{2+} suggested the possibility that it was the chelated complex that activated the enzyme. Furthermore, because EGTA and Ca^{2+} alone did not degrade DNA (Fig. 2.2A, lane 2) but required the presence of the sperm deferens extract (Fig. 2.2B, lanes 3 - 6), it was probable that both chemicals activated the enzyme. We tested this by incubating the vas deferens Pel B-Sup with varying combinations of EGTA and Ca^{2+}. EGTA chelates Ca^{2+} in a 1:1 ratio under normal conditions [4,10,145]. One possible mechanism by which EGTA might activate the nuclease was that EGTA was removing an inhibitory cation, which then allowed Ca^{2+} to bind to the nuclease. To control for this possibility, we tested the nuclease activity in concentrations of Ca^{2+} that were up to 16-fold excess of the EGTA (Fig. 2.3, lanes 6

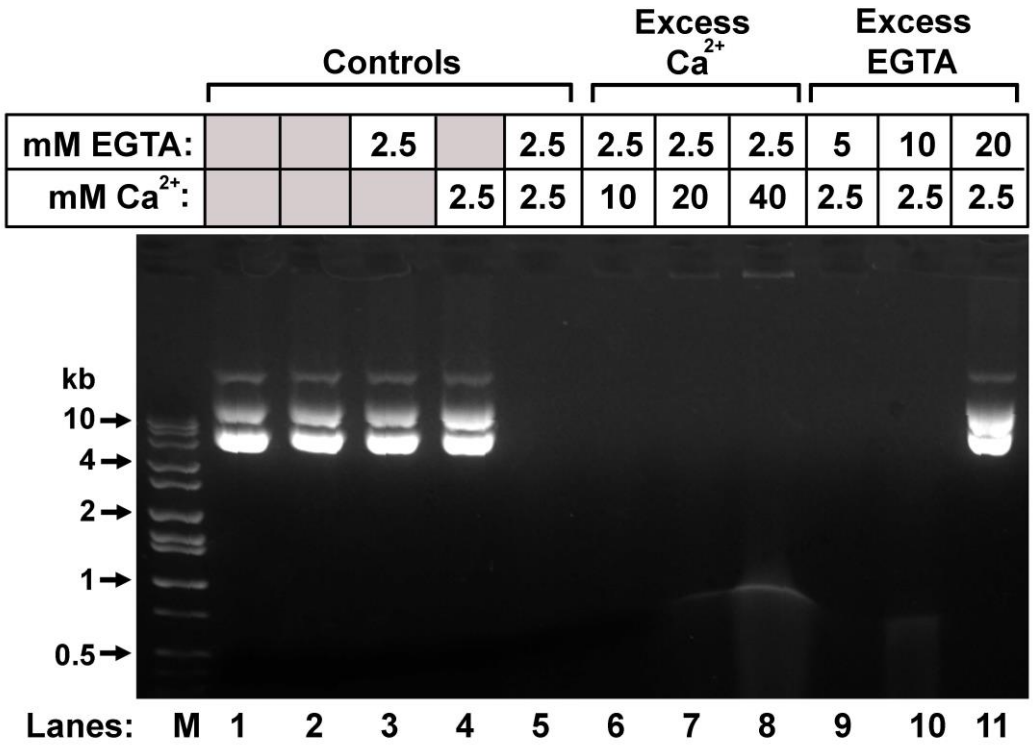
- 8). Under these conditions, all the EGTA would be expected to be saturated in the chelated form. We found that with excess Ca^{2+}, the enzyme was still active, suggesting that EGTA was not removing an inhibitory cation. We also found that the enzyme was active when the extract was incubated in the presence of excess EGTA (Fig. 2.3, lanes 9 and 10). Under these conditions, there is no free Ca^{2+}, suggesting that free Ca^{2+} was not activating the enzyme. The enzyme was inhibited by an 8 fold excess of unchelated EGTA to chelated EGTA (Fig. 2.3, lane 11) suggesting that when free EGTA is present in high enough concentrations it may compete with chelated EGTA to inhibit nuclease activity.

These experiments suggest that the nuclease requires chelated EGTA for activity, and does not require free Ca^{2+}. We therefore term this nuclease CEAN, for Chelated EGTA Activated Nuclease.

2.3.3. EGTA-Ca^{2+} Activation of CEAN is Reversible

To further test the role of EGTA in activating CEAN, we next tested whether EGTA-Ca^{2+} activation of CEAN was reversible. If EGTA-Ca^{2+} activation of CEAN was irreversible, it might suggest that the chelator initiates a signal pathway that results in enzymatic activation that may include phosphorylation or defined proteolytic degradation. However, if the EGTA-Ca^{2+} activation was reversible, it would be more likely that the chelator acted as a direct cofactor. To test this, the vas deferens extract Sup B was incubated with EGTA, Ca^{2+} and plasmid DNA, and

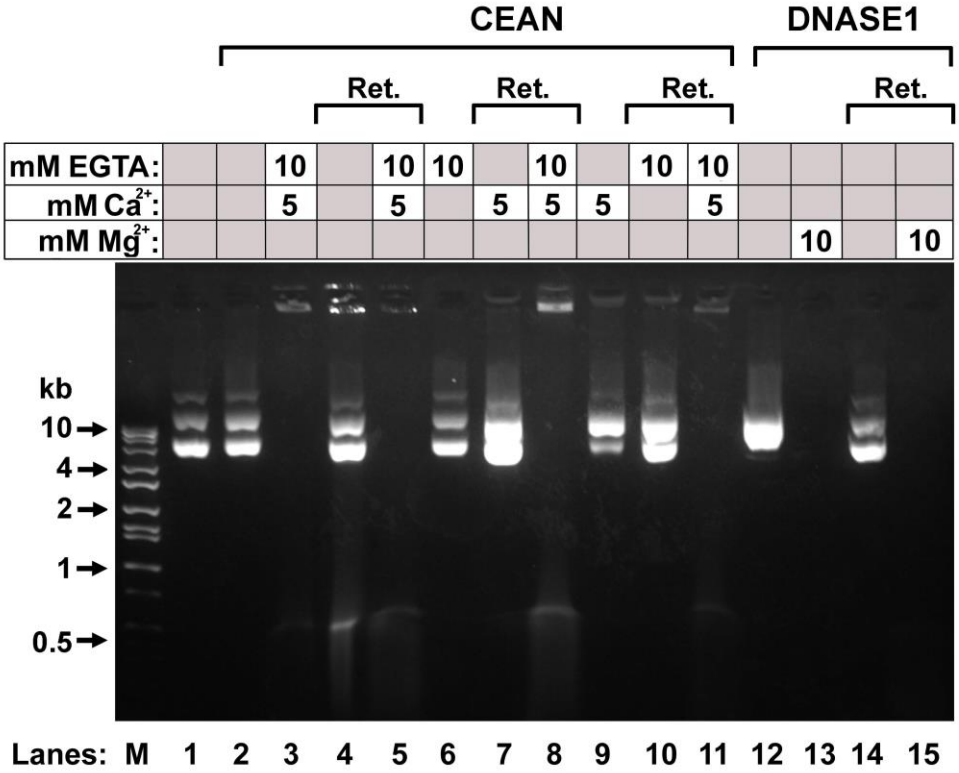
Figure 2.3. Nuclease activity requires Ca^{2+} chelated to EGTA.



Plasmid DNA was incubated with vas deferens extract with varying concentrations of EGTA and Ca^{2+}, as indicated. Lane 1 is a control with no vas deferens extract, and lane 2 is a control with extract (Pel B Sup), but no EGTA or Ca^{2+}. Lanes 3 and 4 contain extract and EGTA or Ca^{2+}, but not both. Lane M contains molecular weight markers. Grey boxes indicate that EGTA or the cation was not added to the sample.

then centrifuged in a microtube containing a 10-kDa filter to remove the EGTA-Ca^{2+} from the enzyme. The less pure Sup B fraction was used because the larger total amount of CEAN activity that was present was needed for spin columns. The retentate was then resuspended in buffer without EGTA or Ca^{2+} and tested for nuclease activity. As shown in Fig. 2.4, lanes 3 and 4, the CEAN digested the DNA before centrifugation but stopped when EGTA-Ca^{2+} was removed. When EGTA-Ca^{2+} was added back to the reaction, the nuclease digestion again proceeded (Fig. 2.4, lane 5). Adding EGTA only or Ca^{2+} only to the vas deferens extract, and then adding Ca^{2+} or EGTA, respectively, after centrifugation also failed to activate the nuclease (Fig. 2.4, lanes 6, 7, 9 and 10), and only when both EGTA and Ca^{2+}, together, were added did the reaction proceed (Fig. 2.4, lanes 8 and 11). As a control, we performed the same experiment with purified DNase I and Mg^{2+}, and demonstrated that removing the Mg^{2+} from the buffer inhibited the activity, but adding Mg^{2+} back reactivated the nuclease (Fig. 2.4, lanes 12-15). These data support the hypothesis that Ca^{2+} chelated to EGTA binds directly to the CEAN as a cofactor.

Figure 2.4. Removal of EGTA-Ca^{2+} from the nuclease reaction by size filtration centriguation.



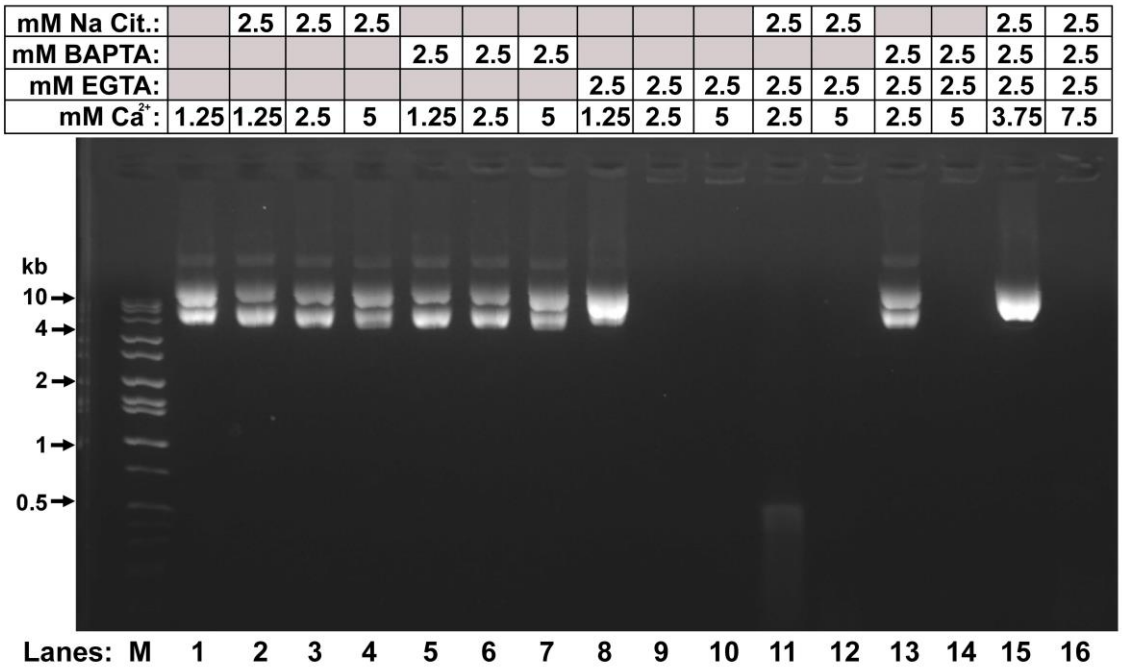
Lane 1, control with plasmid DNA, only. Lane 2, control with vas deferens extract (Sup B) and plasmid DNA with no EGTA or Ca^{2+}. Lane 3, vas deferens extract incubated with EGTA and Ca^{2+} and plasmid DNA for 1 hr at 37ºC, showing DNA digestion. Lane 4, the extract (Sup B) from lane 3 was centrifuged through a 10 kd filter, and the retentate resuspended in buffer containing additional plasmid DNA, and incubated for 1 hr at 37ºC. Lane 5, EGTA and Ca^{2+} was added to the suspension in lane 4 and then incubated for an additional 1 hr at 37ºC. Lane 6, vas deferens extract (Sup B) incubated with EGTA and plasmid DNA for 1 hr at 37ºC, showing DNA digestion. Lane 7, the extract from lane 6 was centrifuged through a 10 kd filter, and the retentate resuspended in buffer containing additional plasmid DNA and Ca^{2+}, and incubated for 1 hr at 37ºC. Lane 8, EGTA was added to the suspension in lane 7 and then incubated for an additional 1 hr at 37ºC. Lanes 9 – 11, as for lanes 6 – 8, but with EGTA and Ca^{2+} reversed. Lane 12, Dnase1 incubated with plasmid DNA for 1 hr at 37ºC. Lane 13, Mg^{2+} added to the suspension in lane 12 and incubated for another hour at 37ºC, now showing DNA digestion. Lane 14, the suspension from lane 13 was centrifuged through a 10 kd filter, and the retentate resuspended in buffer containing additional plasmid DNA, and incubated for 1 hr at 37ºC. Lane 15, Mg^{2+} was added to the suspension in lane 14 and then incubated for an additional 1 hr at 37ºC. Lane M contains molecular weight markers. Grey boxes indicate that EGTA or the cation was not added to the sample.

2.3.4. Enzyme is specific for EGTA/EDTA

We next tested the specificity of CEAN for the chelator. 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*',*N*'-tetraacetic acid (BAPTA) is a stronger Ca^{2+} chelator than EGTA, but has a similar chemical structure to EGTA differing only by the addition of two aromatic rings [15,146]. Citrate is another Ca^{2+} chelator with a very different chemical structure to EGTA. We found that neither BAPTA nor citrate were able activate CEAN in the presence of Ca^{2+} (Fig. 2.5, lanes 2-7). BAPTA did not inhibit plasmid digestion by CEAN in the presence of EGTA and Ca^{2+} (Fig. 2.5, lane 14). However, the data do suggest that BAPTA does chelate Ca^{2+} in this experiment and may even compete with EGTA. When 2.5 mM, each, of EGTA, BAPTA and Ca^{2+} were incubated with vas deferens extract the nuclease was inhibited (Fig. 2.5, lane 13). In this experiment, we would expect BAPTA to compete with EGTA for chelating Ca^{2+}, thereby decreasing the total level of EGTA-Ca^{2+} to less than 2.5 mM, the point where CEAN is not activated (Fig. 2.5, lanes 8 and 9). This conclusion is supported by the restoration of CEAN activity when the Ca^{2+} concentration in this experiment was increased to 5 mM (Fig. 2.5, lane 14). In that experiment, both EGTA and BAPTA would be expected to be saturated with Ca^{2+}, providing the 2.5 mM EGTA-Ca^{2+} required for full activity (Fig. 2.5, lane 9).

These data suggest that it is not simply the chelation of Ca^{2+} that activates CEAN, but that the enzyme has some specificity to the chelator, itself, EGTA complexed to Ca^{2+}.

Figure 2.5. CEAN is specific for EGTA.

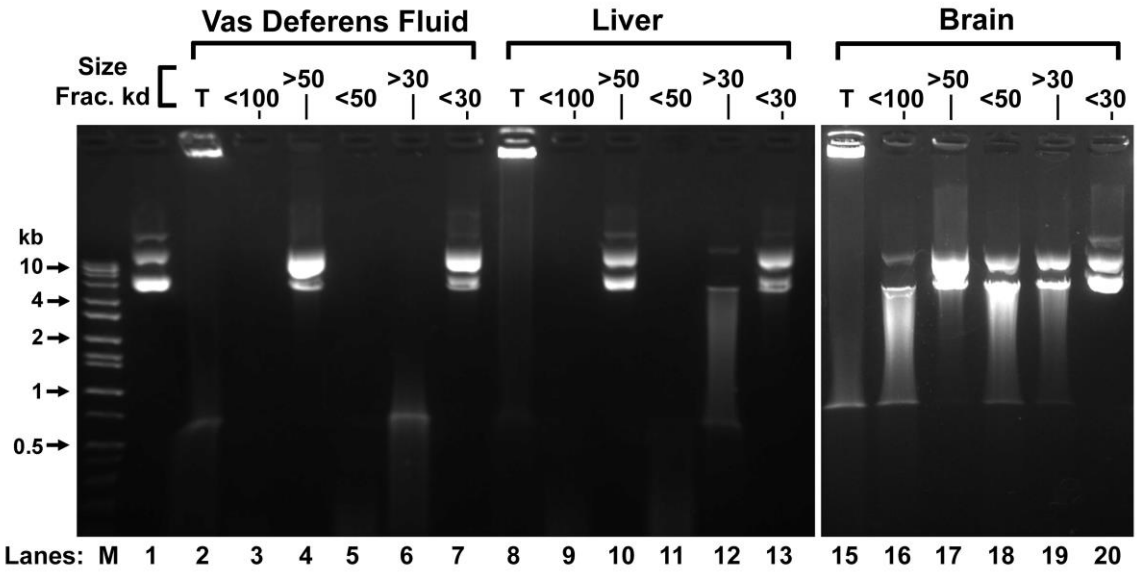


Plasmid DNA was incubated with vas deferens extract (Pel B Sup) in the presence of varying concentrations of EGTA, BAPTA, citrate, and Ca^{2+}, as indicated, for 1 hr at 37ºC. Lane M contains molecular weight markers. Grey boxes indicate that EGTA or the cation was not added to the sample.

2.3.5. CEAN is Present in Several Other Tissues

The novelty of a nuclease that is activated by Ca^{2+} chelated to EGTA in the sperm vas deferens fluid prompted us to examine other tissues for the presence of similar activity. We prepared crude extracts from mouse liver and brain, as described in Methods. We then fractionated the vas deferens, liver and brain extracts using spin columns with different size filters, and tested each for CEAN activity. We found that for both the vas deferens and liver extracts, the CEAN nuclease activity was in the <50 kd and >30 kd fractions (Fig. 2.6, lanes 5, 6, 11 and 12). We found no activity for a CEAN like nuclease in the brain. While this method cannot establish the molecular weight of CEAN, it does suggest that the activities in the two tissues do fractionate the same way in size filtration columns. We also tested several other tissues including heart, intestine, stomach, spleen, and kidney and found that in all these tissues, nuclease activity that required EGTA chelated to Ca^{2+} was present (data not shown). This suggests that CEAN is not restricted to the male reproductive tract, but may be a more ubiquitous enzyme.

Figure 2.6. CEAN activity is present in liver, and is a protein between 50 and 30 kDa.



Vas deferens (lanes 2-7, Sup B), liver (lanes 8-13), and brain (lanes 15-20) extracts were fractionated into different size fractions using size filter centrifugation tubes. Lanes 2, 8 and 15, total extracts. Lanes 3, 9, and 16, 100 kDa eluents. Lanes 4, 10, and 17, 50 kd retentates. Lanes 5, 11, and 18, 50 kDa eluents. Lanes 6, 12, and 19, 30 kDa retentates. Lanes 7, 13, and 20, 30 kDa eluents. All extracts were incubated with plasmid DNA, 5 mM EGTA and 2.5 mM Ca^{2+} for 4 hrs at 37ºC. Lane M contains molecular weight markers. Grey boxes indicate that EGTA or the cation was not added to the sample.

2.4. Discussion

When we first described the existence of a nuclease activity in mouse sperm fluids that could be activated by pretreatment with EGTA and subsequent incubation with Ca^{2+}, we proposed two possible models to explain the role of EGTA in the nuclease activation [18-20,60]. One possibility was that EGTA initiated an activation reaction, perhaps by releasing a calmodulin-like protein from inhibiting the nuclease, which then required Ca^{2+} as a cofactor. The data presented here suggest that this hypothesis is incorrect for two reasons. First, the activation by EGTA-Ca^{2+} is reversible; suggesting that activation by EGTA is not the result of a complicated cell-signaling pathway. Second, the data clearly suggest that it is the chelated form of EGTA-Ca^{2+} that activates the nuclease (Fig. 2.3). This means that it cannot be a sequential reaction in which EGTA has one function, and Ca^{2+} has another.

The second possible model to explain EGTA activation of CEAN was that EGTA chelated an inhibitory cation and Ca^{2+} then served as the cofactor of the nuclease. But our data do not support this model, either. Fig. 2.3, lanes 6 – 8, in particular, indicate that CEAN is activated in a large excess of Ca^{2+} in the presence of EGTA. Under these conditions, EGTA would be expected to be saturated by Ca^{2+} and would not be expected to be available for chelation of another cation. Additionally, Zn^{2+}, which is a typical inhibitory cation for many nucleases activates, rather than inhibits, CEAN (Fig. 2C).

Our data support a model in which the nuclease directly binds to the EGTA-Ca^{2+} complex. We were unable to find another report of a nuclease that is activated by chelated EGTA. The fact that it is also present in the liver, spleen, heart, kidney, stomach and intestine suggests that it is not limited to the male reproductive tract, and may represent a novel class of nuclease not previously described. We propose that the natural cofactor of CEAN is a EGTA like molecule. One candidate is γ-carboxyglutamic acid, a post-translational amino acid modification that can chelate Ca^{2+} [22,147]. The interaction between matrix Gla protein (MGP) and BMP-4 is facilitated by Ca^{2+} chelation of γ-carboxyglutamic acid residues on MGP [26,148]. It is possible that CEAN is activated by a similar protein-protein interaction.

We are currently attempting to isolate CEAN to test the role of EGTA activation of this nuclease more specifically. This is the first report of a nuclease that is activated by chelated EGTA. It suggests that a novel, previously undefined, class of nuclease exists. Based on our previous report [28,60] it is possible that CEAN functions in the apoptotic degradation of cell chromatin. While much more work is remains before these findings are definitive, the data clearly demonstrated the existence of a novel nuclease activity.

Acknowledgements

The authors would like to acknowledge the help of Dr. Joseph Jarrett, of the Department of Chemistry at the University of Hawaii at Manoa for helpful discussion on EGTA chelation chemistry. We would also like to thank Dr. Geoffry De Iuliis of the ARC Centre of Excellence in Biotechnology & Development at University of Newcastle, for helpful discussions on free radical chemistry.

Chapter 3. Results II: Acidic Nuclease Activity

3.1. Introduction

All nucleases need activation to function, usually requiring a co-factor to do so. The majority of nucleases are single or di-cation dependent. Divalent cation dependent nucleases are involved in a wide range of biological functions from digesting DNA and RNA to reversibly cleaving and recombining DNA. There is little correlation between the mechanism of catalysis and function as an individual catalytic mechanism can be adapted in a variety of reactions and biological pathways [30,142].

The biological roles of this class of enzymes vary from DNA repair to apoptosis [34,35,104,116]. There are few exceptions that do not require any cations for activity, such as DNase II [17,116,119]. During apoptosis the enzymatic digestion of DNA is a well-understood event in the somatic cell. Nucleases such as caspase-activated DNase (CAD) play crucial roles during planned cell death. CAD requires cations Zn^{2+} for dimerization [38,149] and Mg^{2+} for activity [42-44,104], and is inhibited by ICAD [37,39,46,104]. Other nucleases such as topoisomerase II are activated during DNA replication, reversibly cleaving DNA during mitosis and meiosis to avoid strand entanglement [23,150,151]. Topoisomerase II is also activated during apoptosis, during which a set of nucleases may interact with the

enzyme to completely degrade the DNA [23,131,132]. Caspase activated DNase (CAD) is such an enzyme. It is bound by topoisomerase II, enhancing CADs cleavage activity [8,50]. There is also evidence of intracellular acidification being involved during the initiation phase of apoptosis [52,53,152,153]. Nucleases such as DNase II represent a different class of enzymes that are cation independent and are activated under acidic conditions. One of the known functions of DNase II is the digestion of leftover apoptotic DNA, downstream of CAD/DFF40, where if left undigested has been linked to autoimmune disease [34,57,154]. Some of the identities of the various nucleases that completely degrade the DNA during apoptosis are known, however, many are not.

We have previously demonstrated that mouse spermatozoa also exhibit an apoptotic-like event we have called Sperm Chromatin Fragmentation (SCF), where chromatin is cleaved by topoisomerase II in the presence of Mn^{2+} and Ca^{2+} and reversed when the cations are chelated with the addition of EDTA [17,57]. We have also identified a separate type of DNA digestion that is irreversible and is activated with EDTA or EGTA and Ca^{2+}. We have named this activity EGTA Activated Nuclease (EAN) and have observed it in the vas deferan luminal fluid as well as other parts of the body [65,67-69,155]. We hypothesize that the EGTA and Ca^{2+} activated nuclease interacts with the protein complex involved in SCF resulting in SDD.

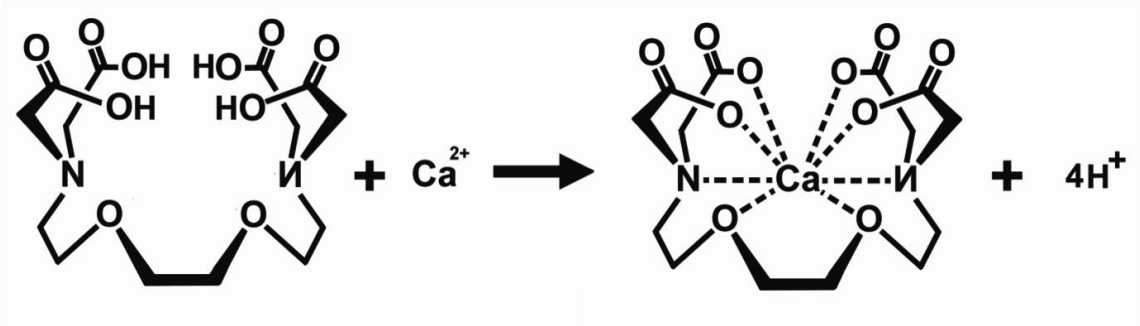
Here, we demonstrate in the liver that the requirement for EAN is in fact low pH rather than EGTA and Ca^{2+}, and that the vas deferens derived nuclease is most likely an acidic nuclease that is metal-ion independent [72,74,142]. We have learned that when EDTA chelates divalent cations the pH is reduced by the release of hydronium ions [65,67,75,76,156] (Fig. 3.1). Furthermore, it is likely that the nuclease is similar to DNase II, a metal-ion independent nuclease that is activated by low pH [78-80,97].

3.2. Materials and Methods

3.2.1. Animals

Male B6D2F1 (C57BL/6J X DBA/2) mice were obtained from the National Cancer Institute (Raleigh, NC). Mice were fed *ad libitum* and kept in standard housing in accordance with the guidelines of the Laboratory Animal Services at the University of Hawaii and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research Council (DHEF publication no. [NIH] 80–23, revised 1985). The protocol for animal handling and the treatment procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Hawaii.

Figure 3.1. Chelation of EDTA Releases Hydronium Ions.



EDTA is in equilibrium with hydrogen as well as with metal ions. Added calcium becomes chelated by free EDTA, which releases hydronium ions and lowers the pH. The opposite is also true; removal of metal from the equilibrium causes EDTA to bind hydronium ions, causing the pH to increase [156].

3.2.2. Preparation of Extracts

For extract from the liver, mice were sacrificed using carbon dioxide asphyxiation followed by cervical dislocation. The liver was excised and homogenized in ice cold 25 mM Tris, pH 7.4, 150 mM KCl buffer (TKB) with 0.25% TX-100 in protease cocktail inhibitor. The homogenate was then centrifuged at 60,000 *x g* for 20 min at 4ºC yielding a supernatant extract which was further processed by size fractionation and either analyzed for activity or used for further preparation.

3.2.3. Size Fractionation of Extracts

Extracts prepared above were further fractionated according to size using Vivaspin 500 or Vivaspin 20 spin-tubes of 5, 10, 30, 50, and 100 kDa Molecular Weight Cut Off (MWCO) (GE Healthcare). Approximately 500 µl or 8 ml of extract was centrifuged through a 100, 50, 30, 10, or 5 kDa filter at 13,000 *x g* for between 10 to 40 min at 4ºC. Eluents were kept on ice. Retentates were resuspended in approximately 500 µl or 8 ml TKB then centrifuged three times until eluent fully removed from retentate. Each retentate was then resuspended in either 500 µl or 8 ml TKB and kept on ice.

3.2.4. Heparin Chromatography

HiTrap Heparin HP column (GE Healthcare, 1 ml bed volume) was equilibrated with 50 mM sodium acetate pH 5 binding buffer at 1 ml/min. Size fractionated extract was injected into the column at a flow rate of 0.5 ml/min using a peristaltic pump. Flow-through fractions were collected and the binding buffer was replaced with elution buffer and the resulting flow through fractions was collected. Elution buffer was then replaced with a salt wash consisting of TKB with 1.5 M $NaCl_2$. The resulting flow through fractions were collected and desalted using a HiTrap Desalting column (GE Healthcare, 5 ml bed volume).

3.2.5. Nuclease Assay

Plasmids for the nuclease assay were prepared using a CompactPrep Plasmid Midi Kit (Qiagen, Catalog Number 12743, Valencia, CA, USA). Midi Kit Plasmid procedure was followed, resulting in the harvest of purified plasmid DNA (0.51 mg/ml). Nuclease activity was activated by incubating approximately 3 µl of one of the various sperm, liver, or brain preparations described above in a total of 20µl of TKB with 200ng plasmid DNA supplemented with divalent cation Ca^{2+} and/or EGTA. The reaction was incubated at 37ºC for 15 min to 2h. Ca^{2+} and EGTA concentrations varied from 2.5mM to 50mM in TKB. Evidence of activity was

measured by visual detection of digested plasmid DNA separated by electrophoresis on 0.05% ethidium bromide 1% agarose gel.

3.2.6. SDS/PAGE

SDS/PAGE, 12% (w/v) acrylamide, was performed according to Laemmli. After electrophoretic separation proteins were silver stained (BioRad Silver Stain Plus).

3.3. Results

In our first attempt to isolate the nuclease we used its activation with EGTA and Ca^{2+} as a unique assay to follow and identify it in different column fractions [60,82-85]. With DNA being the nucleolytic substrate, and since heparin presents itself to the active site similarly as DNA, heparin was chosen as the column type to isolate the nuclease. Immobilized heparin would selectively bind DNA binding proteins as well as nucleases activated by EGTA and Ca^{2+}. The column would then be washed with at least ten column volumes of the same activating buffer to remove any remaining unbound proteins. Finally, an inactive elution buffer missing EGTA and Ca^{2+} would be passed through the column. Without EGTA and Ca^{2+} in the buffer all DNA and heparin binding proteins would remain bound, only

nucleases that require EGTA and Ca^{2+} would be released from the column and eluted.

The activation condition for EAN is 10mM EGTA, 10mM Ca^{2+}, 25mM Tris, and 0.25% TX-100 at pH 7.3. The reaction typically occurs in 20µL in a 1.5 ml eppendorf tube incubated for 1 hour at 37°C. Our initial column activation (nuclease binding) buffer was the EAN reaction solution prepared at liter volumes.

The luminal fluid contained in the vas deferens is of low volume and accumulating enough for chromatography would require a large amount of mice so we chose the liver as the source of starting material to develop our protocol. The liver provides abundant starting mass with identical nucleolytic activity as the luminal fluid from the vas deferens. After the protocol and workflow were optimized using the liver we would return to the vas deferens to fractionate the activity.

Interestingly, when we initially fractionated the liver extract and assayed for activity there was none found in any of the fractions collected. We realized that when the reaction buffer was scaled up from 20 µl to 1 l, although the pH of each individual reagent was neutral, the final pH had shifted to approximately 4.5. The assay was always performed in microliter volumes and so the pH of the final buffer had never been tested. A literature review led us to previously published work warning researchers of acidification following the chelation of EDTA or EGTA [87-

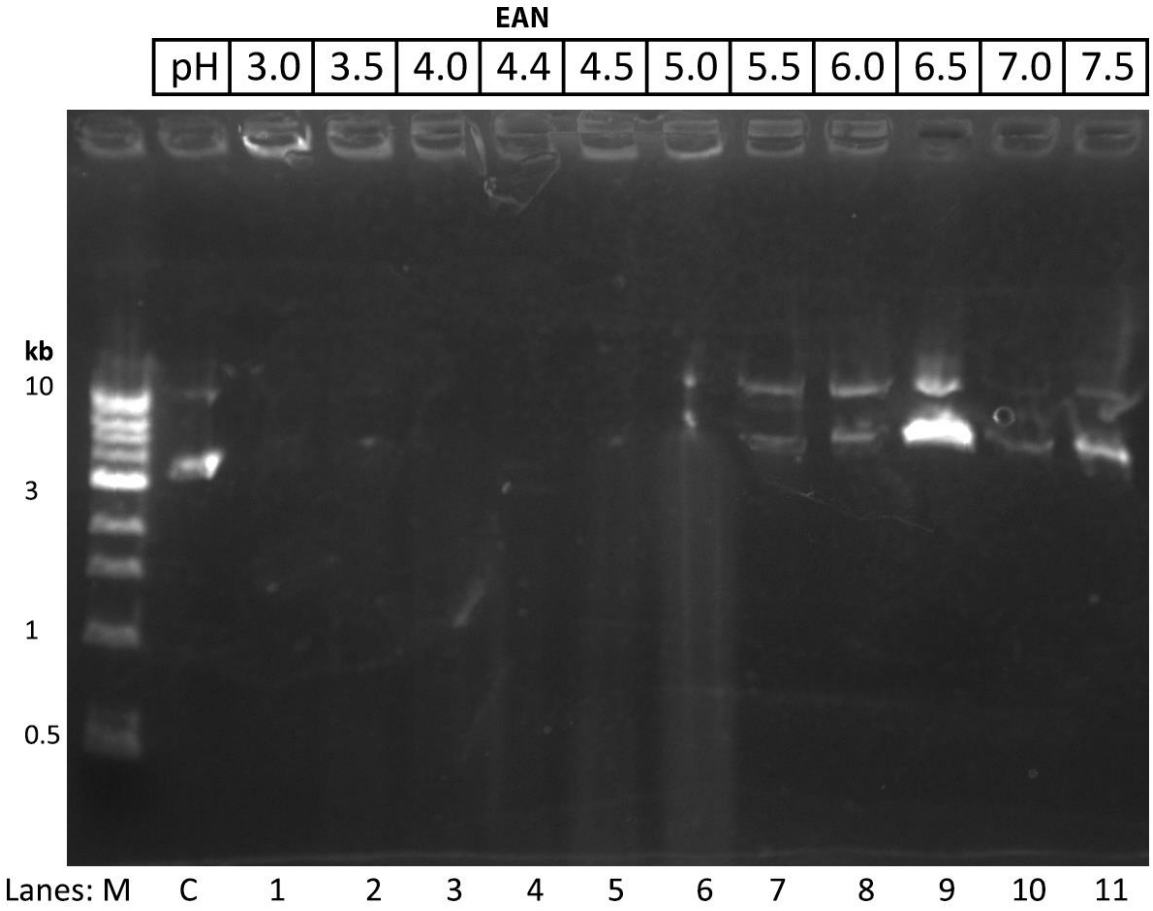
93,156]. The pH shift is caused by the displacement of hydronium ions in the EGTA or EDTA molecule by divalent cations.

Our nuclease may not need EGTA and Ca^{2+} but instead low pH. The protocol for binding the nuclease to the heparin column was adjusted to use a low pH instead of EGTA and Ca^{2+} buffer (binding buffer). Likewise, neutral pH would be used to release the acid dependent nuclease (elution buffer). The immobilized heparin would selectively bind proteins that bind to DNA and nucleases activated by low pH. The column would then be washed with ten column volumes of binding buffer to clear it of remaining unbound proteins while still binding our nuclease. Finally, the inactive (neutral) elution buffer would pass through the column. At neutral pH all DNA and heparin binding proteins would still remain bound, only nucleases that require low pH would be released from the column and eluted.

3.3.1. The nuclease is activated by low pH between pH 3 and 5

We tested whether the nuclease could be activated by low pH in the absence of EGTA or Ca^{2+} (Fig. 3.2). We tested the pH range of the nuclease by incubating the liver extract at 37ºC for one hour with 200 ng of plasmid DNA in 25mM Tris and 0.25% TX-100 with pH ranging from 3 to 7.5 for one hour. The plasmid DNA control is unaffected by low pH (Fig. 3.2, lane C) but when nuclease

Figure 3.2. Acidic Nuclease is Active between pH 3 and 5.



Extract prepared by homogenizing liver in cation-free buffer followed by centrifuging at 60,000 *x g* for 20 min at 4ºC. The resulting supernatant was combined with TKB and 200 ng plasmid DNA between pH 3.0 and 7.5 except for lane 4, which was assayed for EAN activity with unadjusted pH, 10 mM EGTA and 10 mM Ca^{2+}. Acidic nuclease activity is present from pH 3.0 to 5.5 (lanes 1 – 6). EAN activity was tested in lane 4 and has a pH of 4.4. Lanes 7 – 11 did not contain nuclease acitivity. All samples were analyzed by conventional agarose electrophoresis.

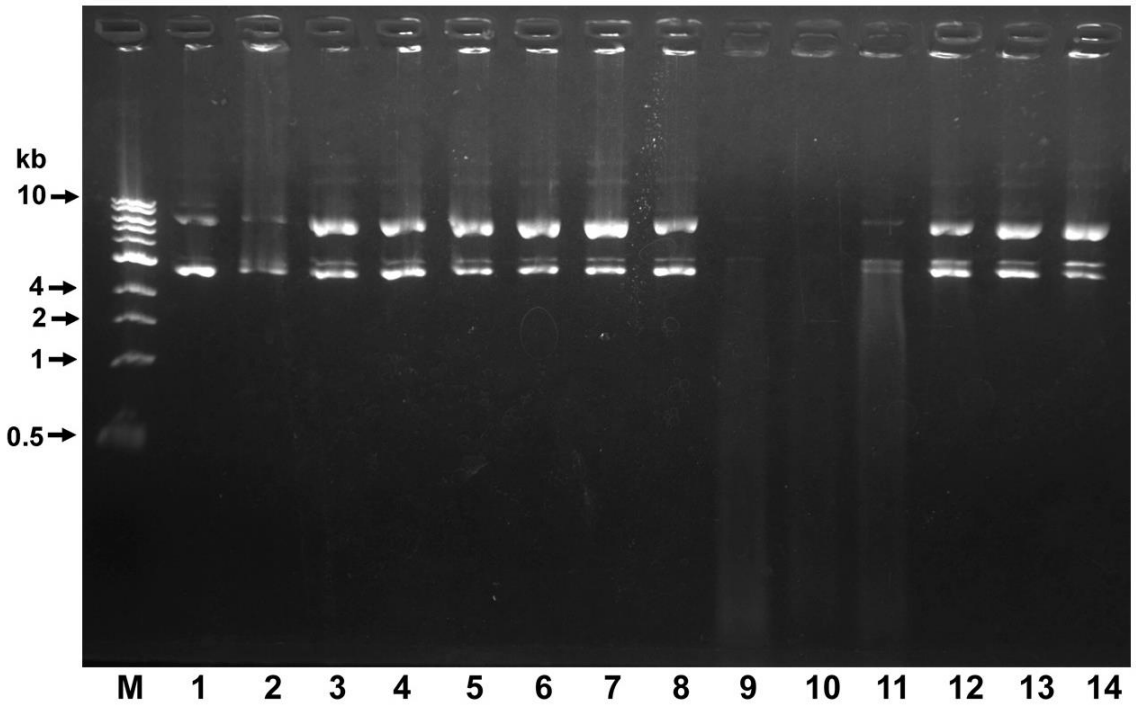
extract is added digestion is detected from pH 3 to 5 (Fig. 3.2, lanes 1 – 3 and 5 – 6). EAN conditions were also used to compare with acid activated nuclease in lane 4 with full plasmid digestion occurring in both. The pH of EAN activity in lane 4 was checked at the beginning and end of the incubation at it remained at 4.4. At pH 5.5 and higher the nuclease is inactive (Fig. 3.2 lanes 7 – 11).

3.3.2. pH Dependent Activity Isolated with Heparin

Understanding the functional pH range of the nuclease, two new column mobile phase buffers were prepared: a binding buffer at pH 5.3 and an elution buffer at pH 7.5, both containing 25mM Tris-HCl, 0.25% TX-100. Prior to fractionation, the pH of the heparin column was adjusted to 5.3 by passing ten column volumes of binding buffer through it. One milliliter of liver extract was then adjusted to pH 5.3 with HCl and injected into the column. Fifteen column volumes of binding buffer were consecutively eluted in three 5 ml aliquots: B1, B2, and B3. Each fraction was concentrated on a 3kDa MWCO filtered spin tube to 1 ml (Fig. 3.3 lanes 2 – 4). Elution buffer was then passed through the column and ten 1 ml fractions were collected (Fig. 3.3 lanes 5 – 14) and tested for pH level. Each fraction was then assayed for acidic nuclease activity by combining 18 µl of each with 2µl of 100 ng/µl plasmid DNA and incubated at 37ºC for one hour. Binding

Figure 3.3. Fractionated pH Dependent Activity

Fraction		B1	B2	B3	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
pH As Is		5.3	5.3	5.3	5.3	5.3	5.2	5.5	7.2	7.5	7.5	7.5	7.5	7.5
pH EQ'd		4	4	4	4	4	4	4	4	4	4	4	4	4



Liver extract acidified to pH 5.3 and fractionated on heparin column using acidic and neutral buffers. "pH As Is" indicates the pH of each fraction upon collection. "pH EQ'd" indicates the adjusted pH used for the assay. The nuclease was retained by the heparin column at pH 5.3 (B1-E4, lanes 2 – 8)) and released at 7.2 (E5-E7). The pH of each fraction was then adjusted to 4 and incubated with plamid DNA for 1h at 37ºC and analyzed by conventional agarose electrophoresis.

buffer fractions B1 – B3 as well as elution fractions E1 – E4 (Fig. 3.3 lanes 2 – 8) tested low pH and did not digest DNA indicating that the nuclease was not eluted and remained active and bound to immobilized heparin in the column. Fractions E5 – E7 tested neutral upon elution and digested DNA when acidified again, indicating that neutral pH deactivated the nuclease allowing it to release the heparin and exit the column. Elution fractions E8-E10 (Fig. 3.3 lanes 12 – 14) tested neutral pH but did not digest DNA suggesting that all the nuclease was eluted in fractions E5-E7.

3.3.3. Protein Complexity of pH Dependent Heparin Fractions

The protein complexity of the eluted fractions compared to the injected liver extract was next examined. Homogenized liver was centrifuged at 60,000 *x g* and the supernatant was filtered through a 30 kDa MWCO spin tube. The eluent was collected (L30e) and resulting retentate was reconstituted in TKB (L30r). The pH of the L30r extract was equilibrated to 4.5 with HCl and injected into a new heparin column equilibrated with acidic binding buffer. Fifteen 1 ml fractions were collected and concentrated on a 3 kDa MWCO filter to 1 ml and labeled fraction B. Neutral elution buffer was then passed through the column and six 1 ml fractions were collected and labeled E1 – E6.

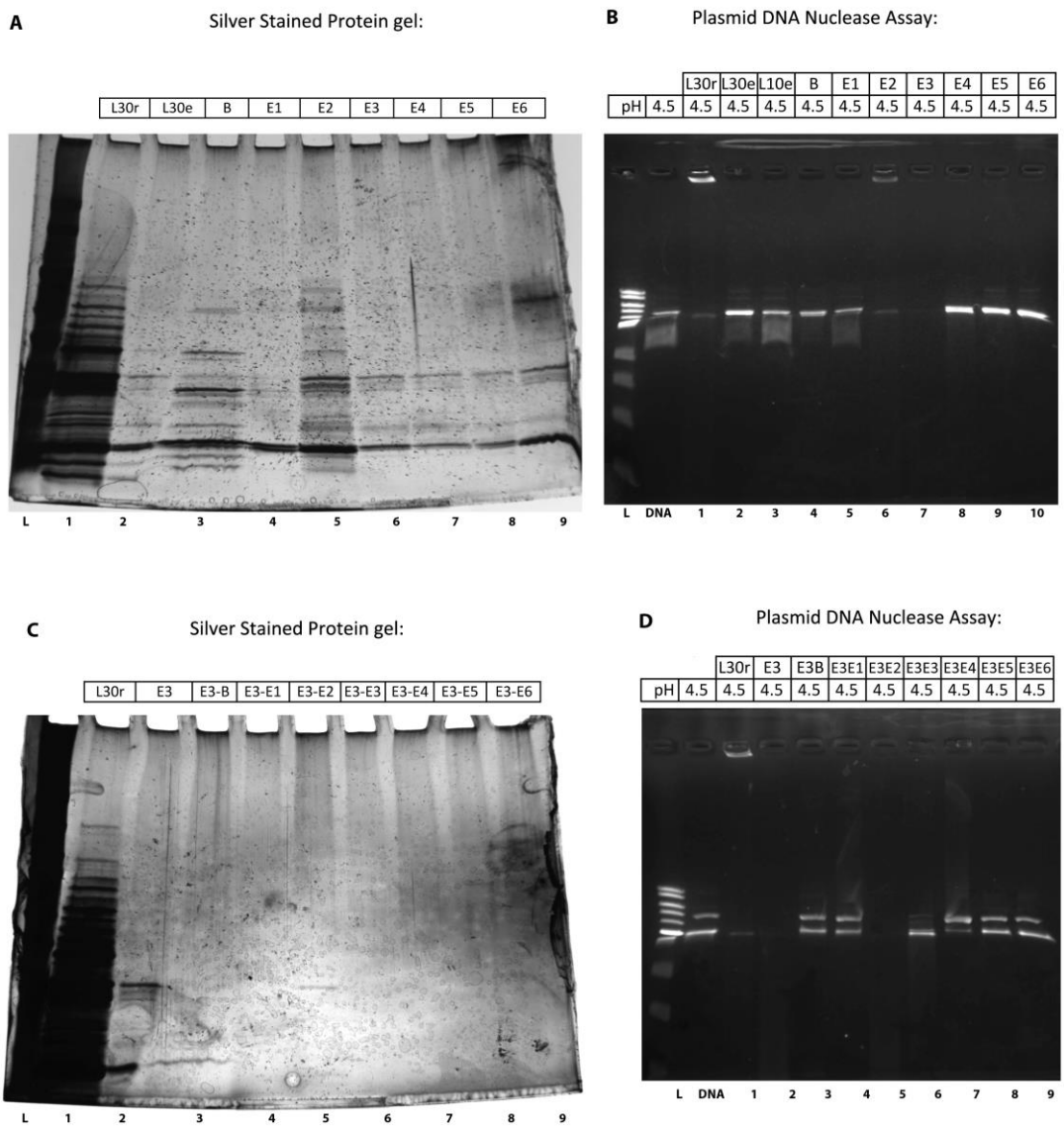
The pH of all fractions were adjusted to 4.5 and assayed for acidic nuclease activity by combining 18 µl of each with 2µl of 100 ng/µl plasmid DNA and

incubated at 37ºC for one hour (Fig 3.4 B). Activity was found in L3or and its elution fractions E2 and E3 (Fig 3.4, B lane 1 and lanes 6 and 7). Extracts L3or, L3oe, and all heparin fractions were also resolved on SDS-PAGE with silver stain (Fig 3.4, A). L3or, B, and E2 (Fig 3.4, B, lane 1 and lane 5) had complex protein profiles compared to elution fraction E3 that had high nuclease activity and a simple protein profile indicating that the protein has been purified by fractionation.

In order to further isolate the nuclease we next repeated the fractionation process on fraction E3. The pH of E3 was equilibrated to 4.5 with HCl and injected into a new heparin column equilibrated with binding buffer. Fifteen 1 ml fractions were collected and concentrated on a 3kDa MWCO filter to 1 ml and labeled fraction E3B. Elution buffer was then passed through the column and six 1 ml fractions were collected and labeled E3E1 – E3E6.

All fractions were assayed for acidic nuclease activity by adjusting the pH of each to 4.5 and combining 18 µl of each with 2µl of 200 ng plasmid DNA and incubated at 37ºC for one hour (Fig 3.4 D). Activity was found in elution fraction E3E2 (Fig 3.4 D, lane 5). L3or and E3 were used as positive controls (Fig 3.4 D, lanes 1 and 2). Extract L3or, original fraction E3, and new fractions were also resolved on SDS-PAGE with silver stain (Fig 3.4, C). Only L3or had a complex

Figure 3.4. Heparin Fraction Protein Complexity



Heparin fractionated liver extract with corresponding activity assay. A. Silver stained fractions from extract. 30kDa retentate (lane 1) and eluent (lane 2). Acidic binding buffer (lane 3) and neutral elution fractions (lanes 4-9). B. Acidic nucelase activity detected in L30r (lane 1) and elution fractions E2 and E3 (lanes 6 and 7). C. Elution fraction E3 (lane 2) was fractionated again on a new heparin column. L30r, E3, and fractions E3-B,and E3-E1 – E3-E6 (lanes 1 – 9) were resolved and stained. D. Nuclease activity detected in L30r (lane 1), E3 from earlier fractionation (lane 2), and E3-E2 (lane 5). All fractions in B and D were adjusted to pH 4.5 and incubated with plasmid DNA for 1h at 37ºC and analyzed by conventional agarose electrophoresis.

protein profile while active fraction E3E2 (Fig 3.4 C, lane 5) had only one protein visibly stained by silver, possibly the nuclease.

3.4. Discussion

These data support the presence of acid activated nucleolytic activity in the liver that is pH dependent, which is indirectly due to the chelation of EGTA. A likely candidate would be a metal-ion independent nuclease such as DNase II that is de-inhibited under acidic apoptotic-like conditions. Such metal-ion independent nucleases are most likely present in their inactive form [84,95,96,157] and are specific in their substrate, preferring to hydrolyze single stranded, unstacked, and base unpaired DNA [82,83,98,142]. There is some literature available describing DNase II activity in the reproductive tract. In avian oocytes DNase II activity has been described digesting all the extra sperm that enters the oocyte, leaving only one sperm cell for fertilization [84,158]. There is also evidence of DNase II present in loach sperm [78,84,95,96,100-102,159]. Here we present a novel and previously undescribed location for a nuclease, the mouse vas deferens.

The pH dependent nuclease may have a role in the male (and perhaps female) reproductive tract involving removal of non-viable sperm. The vas deferens acts as a reservoir for sperm prior to ejaculation. As such, the longer sperm are kept in the vas deferens the more time they are exposed to reactive

oxygen species, many of which are produced by their own mitochondria [103,160]. The pH in the vas deferens is mildly acidic, leaving only the removal of an inhibitor needed for the activation of the nuclease and this may function in the removal of non-viable sperm.

Chapter 4. Results III: Identification of Nuclease

4.1. Identification Schemes

4.1.1. Identification via Mass Spectrometry

Mass spectrometry is an ideal method for identifying protein samples that have been purified to the point were the protein profile complexity has been reduced to a single or handful of bands. The process involves the digestion of protein into polypeptide fragments, which are then ionized and further broken down to individual amino acids that are then measured according to mass. The basic procedure involves resolving the proteins in a sample using SDS-PAGE and then staining with Coomassie Blue followed by excision from the gel and digestion to individual polypeptides using trypsin. The liquid extract is then vaporized, ionized, and then accelerated into an ion beam through an electric and magnetic field to polypeptide fragments then measuring time of flight to calculate the molecular mass of the fragments.

We were not certain which of the stained protein bands was our nuclease. Following the heparin fractionated samples we tested for nuclease activity in each fraction as an indicator for the presence of the nuclease. We also used purified

bovine Dnase II (Sigma) to estimate the size of the enzyme in determining approximately which protein band to excise for analysis.

Three different forms of DNase II have been described in the mouse at different sizes: 45, 30, and 23 kDa [103,161]. The 45-kDa form is synthesized in the ER, processed during transport to the lysosome via the Golgi apparatus for secretion. The 30 and 23-kDa forms are localized to the lysosomes. DNase II has also been observed in the porcine model at sizes 45, 35, and 10 kDa representing various levels of post-translational modification and cleavage, with the 45-kDa pro-protein being the complete and uncleaved form [103,162]. Using this information we cut out stained bands from our heparin fractions that corresponded in size between 30 and 45 kDa for submission to mass spectrometry.

We used the mass spectromety procedure according to the Proteomics Core of the University of Hawaii. Briefly, it involved cutting out protein bands embedded in acrylamide gel, reducing, alkylating, and then digesting with trypsin, followed by extraction of the peptide solution from the gel matrix. The solution was injected into a nanoelectrospray liquid chromatography ion trap mass spectrometry (ITMS) apparatus for analysis.

The results received from screening the mass spectrometry data from the Proteomics Core in the Swiss-Prot database was inconclusive resulting in no identification.

4.1.2. Identification of DNase 2 via Western Blot

Our initial run using mass spectrometry was not conclusive and we chose another route to identify the nuclease. Our data suggested that the protein of interest was DNase II or a DNase II-like nuclease. This is evidenced by the presence of nucleolytic activity during acidic conditions while in the absence of divalent cations. Still, there is no evidence in the literature describing the presence of DNase II in the mammalian male reproductive tract. Normally DNase II is observed in somatic phagocytes engulfing late stage apoptotic cells where it digests the fated cellular nuclei. Here we have evidence suggesting the presence of DNaase II in the male reproductive tract. In order to test for the presence of DNase II or a similar nuclease in the vas deferens we extracted the luminal fluid and prepared the extract using heparin column fractionation as described earlier.

4.2. Methods

4.2.1. Animals

Male B6D2F1 (C57BL/6J X DBA/2) mice were obtained from the National Cancer Institute (Raleigh, NC). Mice were fed ad libitum and kept in standard housing in accordance with the guidelines of the Laboratory Animal Services at the

University of Hawaii and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources National Research Council (DHEF publication no. [NIH] 80–23, revised 1985). The protocol for animal handling and the treatment procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Hawaii.

4.2.2. Preparation of Extracts

For extracts from the vas deferens or epididymis, mice were sacrificed using carbon dioxide asphyxiation followed by cervical dislocation. The epididymis and/or vas deferens were excised and fluid from the epididymis and/or vas deferens gently extracted, using tweezers, and diluted to a concentration of approximately 108 spermatozoa/ml in 25 mM Tris, pH 7.4, 150 mM KCl_2, and 1x protease inhibitor cocktail (TKB) and kept on ice. Extracted fluid from the epididymis and/or vas deferens was centrifuged at 2,000 *x g* for 2 min at room temperature. The sperm pellet was washed with TKB and kept on ice. The resulting supernatant was then centrifuged at 60,000 *x g* for 10 min at 4ºC yielding a second supernatant subfraction containing soluble protein and a second pellet. Both kept on ice.

For extracts from the liver, mice were sacrificed as above, and the liver was excised. The organ was homogenized in TKB with 0.25% TX-100 in a homogenizer

and centrifuged at 60,000 *x g* for 30 min at 4ºC yielding a supernatant containing soluble protein which was kept on ice.

The supernatants of both were further processed by size fractionation and either analyzed for activity or used for further preparation.

4.2.3. Size Fractionation of Extracts

Extracts prepared above were further fractionated according to size using Vivaspin 500 or Vivaspin 20 spin-tubes (GE Healthcare 5, 10, 30, 50, and 100 kDa MWCO). Approximately 500 µl or 8 ml of extract was centrifuged through a 100, 50, 30, 10, or 5 kDa filter at 13,000 *x g* for between 10 to 40 min at 4ºC. Eluents were kept on ice. Retentates were resuspended in approximately 500 µl or 8 ml TKB then centrifuged three times until eluent fully removed from retentate. Each retentate was then resuspended in either 500 µl or 8 ml TKB and kept on ice.

4.2.4. Heparin Chromatography

HiTrap Heparin HP column (GE Healthcare, 1 ml bed volume) was equilibrated with 50 mM sodium acetate pH 5 binding buffer and size fractionated extract was applied to the column at a flow rate of 0.5 ml/min using a peristaltic pump. Minimum buffer pH supported by column is 5. Flow-through fractions were collected. The binding buffer was replaced with elution buffer and the resulting flow through fractions was collected.

4.2.5. Nuclease Assay

Plasmids for the nuclease assay were prepared using a CompactPrep Plasmid Midi Kit (Qiagen, Catalog Number 12743, Valencia, CA, USA). Midi Kit Plasmid procedure was followed, resulting in the harvest of purified plasmid DNA (0.51 mg/ml). Nuclease activity was activated by incubating approximately 3 µl of one of the various sperm or liver preparations described above in a total of 20µl of TKB with 200ng plasmid DNA supplemented with either sodium acetate adjusted to pH 4.5 or with 20 mM $CaCl_2$ and 20 mM EGTA in TKB. The reaction was incubated at 37ºC for 1h. Evidence of activity was measured by visual detection of digested plasmid DNA separated by electrophoresis on 0.05% ethidium bromide 1% agarose gel.

4.2.6. SDS-PAGE

SDS/PAGE, 4-12% (w/v) acrylamide, was performed according to Laemmli in the mini protean gel kit (Bio-Rad). After electrophoretic separation proteins were stained with colloidal Coommassie Blue [108,163].

4.2.7. Western Blot

SDS/PAGE, 12% (w/v) acrylamide, was performed according to Laemmli. After electrophoretic separation proteins were transferred overnight to a nitrocellulose membrane (Amersham, GE HealthCare) using a wet blotter system (BioRad) at 4ºC followed by staining with Ponceau Red. Nitrocellulose membranes were then blocked 1 h at room temperature in either blocking solution PBS (137 mM $NaCl_2$, 3 mM KCl_2, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3) supplemented with 0.1% Tween-20 and 5% non-fat milk or TBS (20 mM Tris, 137 mM $NaCl_2$, pH 7.3) supplemented with 0.1% Tween-20 and 3% (w/v) bovine serum albumin (Sigma) and incubated overnight with primary goat polyclonal antibody raised against a peptide mapping within an internal region of DNase II of human origin (DNase II N-13, sc-19271, dilution 1:100; Santa Cruz). The membrane was then washed three times for ten minutes each with either PBST or TBST at room temperature. The immune complex was revealed with donkey anti-goat secondary antibody coupled with horseradish peroxidase (donkey anti-goat, sc-2020, dilution 1:75,000; Santa Cruz) in either 5% non-fat milk in PBST or 3% BSA in TBST. The membrane was then washed three times for ten minutes each with either PBST or TBST at room temperature followed by treatment with ECL peroxidase substrate (SuperSignal West Pico Chemiluminescent Substrate, 34079, Thermo Scientific) for five minutes prior to imaging for chemiluminescence.

4.2.8. Zymogram

Zymography was conducted according to the method by Boone et al. (1995) with partial modification. Extracts were prepared according to protocol described above, combined with 4x Laemmli sample buffer (Bio-Rad) without DTT or BME, and heated at 65ºC for 10 minutes then loaded on customized Laemmli gels. The gels were made containing 3.5 µg/ml sheared salmon sperm DNA in both 4% stacking and 12% resolving acrylamide gels. The proteins were resolved at 4ºC at 150 V in 1x TGS running buffer (Bio-Rad). Upon completion of electrophoresis the gels were washed three times ten minutes each in DI water to remove SDS, then incubated for one change in 50 mM sodium acetate pH 4.8 (activation buffer) at 37ºC for 30 minutes. The gels were then incubated in new activation buffer overnight at 37ºC. The gels were then soaked in ethidium bromide for 10 minutes, washed with water, and the nuclease activity was detected as a dark area on a fluorescent background when exposed to ultra violet light.

4.3. Results

4.3.1. Detection of DNase II in Liver, Epidydimis, and Vas Deferens

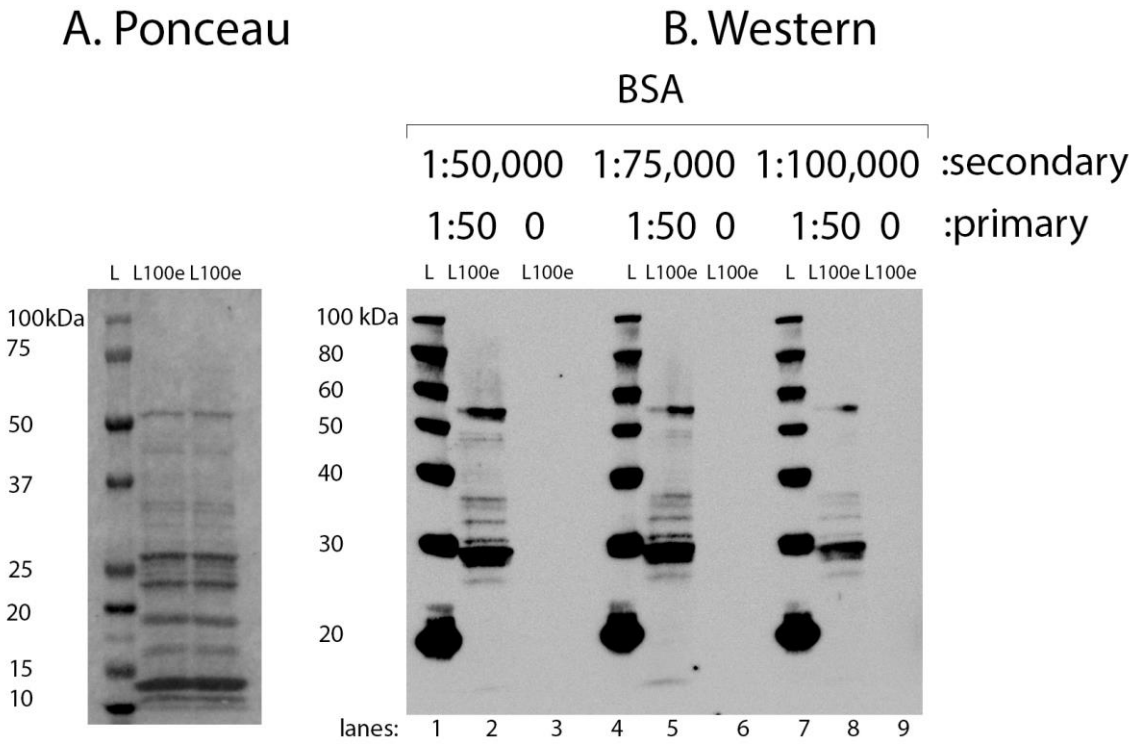
We developed a functional and reproducible protocol for the Western blot of DNase II. Briefly, samples were resolved using SDS-PAGE, transferred to nitrocellulose membranes, visualized with Ponceau Red, and blocked using 3% BSA in TBST. The membranes were incubated overnight at 4ºC with 1:50 dilution

of goat anti-DNase II, followed by 1:75,000 dilution of donkey anti-goat secondary antibody coupled with horse radise peroxidase, and finally the immune complex was revealed by treatment with ECL peroxidase substrate.

Liver extract, a good source of DNase II [93,164], was chosen as a DNase II-positive signal control. Briefly, whole liver was excised and homogenized in TKB. The homogenate was then centrifuged at 60,000 *x g* for 20 minutes at 4ºC and the supernatant was aliqouted to a 100 kDa MWCO spin tube. The resulting eluent (L100e) was collected and kept on ice. The L100e samples were then resolved with SDS-PAGE, transferred to nitrocellulose membrane, and cut into strips containing sets of lanes of combined western and protein ladder (L) and resolved L100e. All membrane strips where blocked with 5% BSA/TBST. Also, each set had a secondary control that is absent anti-DNase II (Fig. 4.1 lanes 3, 6, and 9). DNase II was detected strongly at approximately 55 and 30 kDa in all sets. Minor detection was also noticed at approximately 48, between 38 and 30, 28 kDa, and 12 kDa (Fig. 4.1 lanes 2, 5, 8)

We next prepared extracts from the epididymis and vas deferens by separately centrifuging each at 2,000 *x g* for 2 minutes at RT, aliqouting the supernatants (ES) and (VS) to new tubes, and washing the sperm pellets (EP) and (VP) with TKB. The proteins of L100e, ES, VS, EP, and VP were resolved with SDS-

Figure 4.1. Optimized DNase II Signal in Liver Extract



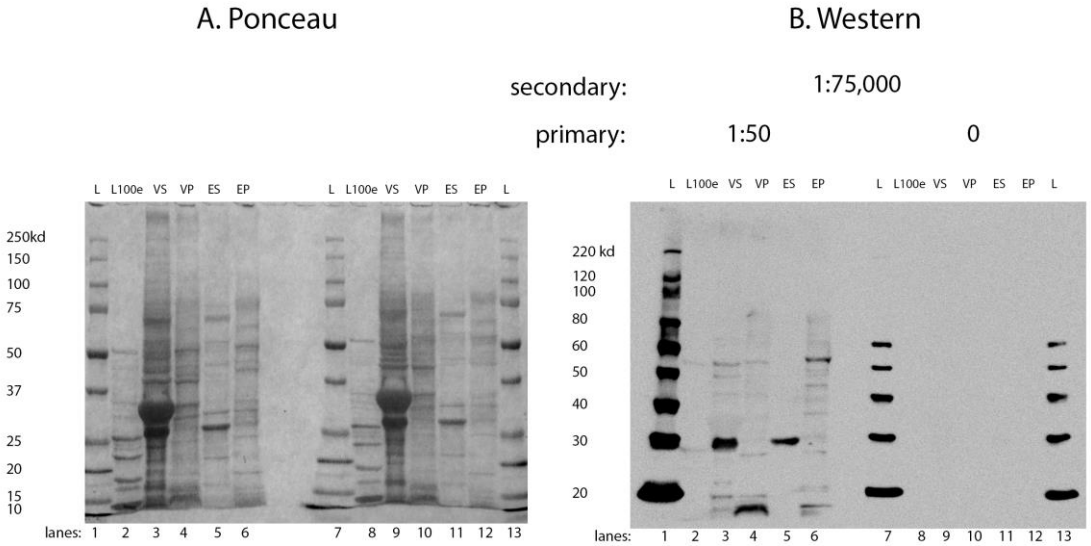
Western analysis of DNase II in mouse liver extract. (A) Proteins resolved with SDS-PAGE and transferred to nitrocellulose and stained with ponceau-S. (B) Following staining, proteins where then probed with or without 1:50 anti-dnase II and then 1:50,000, 1:75,000, and 1:100,000 donkey anti-goat IgG-HRP in 3% BSA/TBST or 5% milk/PBST followed by treatment with ECL peroxidase substrate. Lanes 1 – 3 probed with 1:50,000 secondary, lanes 4 – 6 probeded with 1:75,000, lanes 7 – 9 probed with 1:100,000 secondary, lanes 10 – 12 probed with 1:2,000 secondary in 5% milk/PBST.
L – Combined western and protein ladder, L100e – Liver high-speed supernatant 100 kDa eluent

PAGE, transferred to nitrocellulose membrane, and western blotted. The luminal fluid fractions from both vas deferens and epididymis had a major signal for DNase II at approximately 30-kDa (Fig. 4.2 lanes 3 and 5). A strong band was detected in sperm from the vas deferens at approximately 15 kDa (Fig. 4.2 lane 4). The 15-kDa DNase II was also detected in sperm from the epididymis but much more weakly, however a 50-kDa band grew stronger in the epididymis (Fig 4.2 lane 6). The secondary control indicates no background or non-specific binding (Fig 4.2 lanes 7 – 13).

4.3.2. $Mn^{2+}$and Ca^{2+} Induced Detectable Transfer of DNase II from the Luminal Fluid into the Sperm Cell

Mn^{2+} and Ca^{2+} activated nucleolytic activity has been described in the sperm cell from the epididymis and vas deferens [17,103,115,165]. The cleavage of sperm chromatin DNA into fragments is reversible and has been called Sperm Chromatin Fragmentation (SCF). A secondary activity has been observed occurring soon after SCF that completely digests the sperm chromatin DNA and has been called Sperm DNA Degradation (SDD) [17,113]. The link between SCF and SDD is of interest to our lab and DNase II may be an enzyme responsible for SDD as it has been observed active under conditions that would acidify the environment of the assay, in the presence of EGTA or EDTA and a divalent ion [17,60,91,113,155,156]. We exposed whole extract from the vas deferens and epididymis, consisting of sperm

Figure 4.2. Optimized DNase II Signal in Liver, Vas Deferens, and Epididymis Extracts



Western analysis of DNase II in mouse liver, epididymis, and vas deferens extract. (A) Proteins resolved with SDS-PAGE and transferred to nitrocellulose and stained with ponceau-S. (B) Following staining, proteins where then probed with or without 1:50 anti-dnase II and then with 1:75,000 donkey anti-goat IgG-HRP in 3% BSA/TBST followed by treatment with ECL peroxidase substrate. Lanes 7 – 13 only probed with donkey anti-goat IgG-HRP

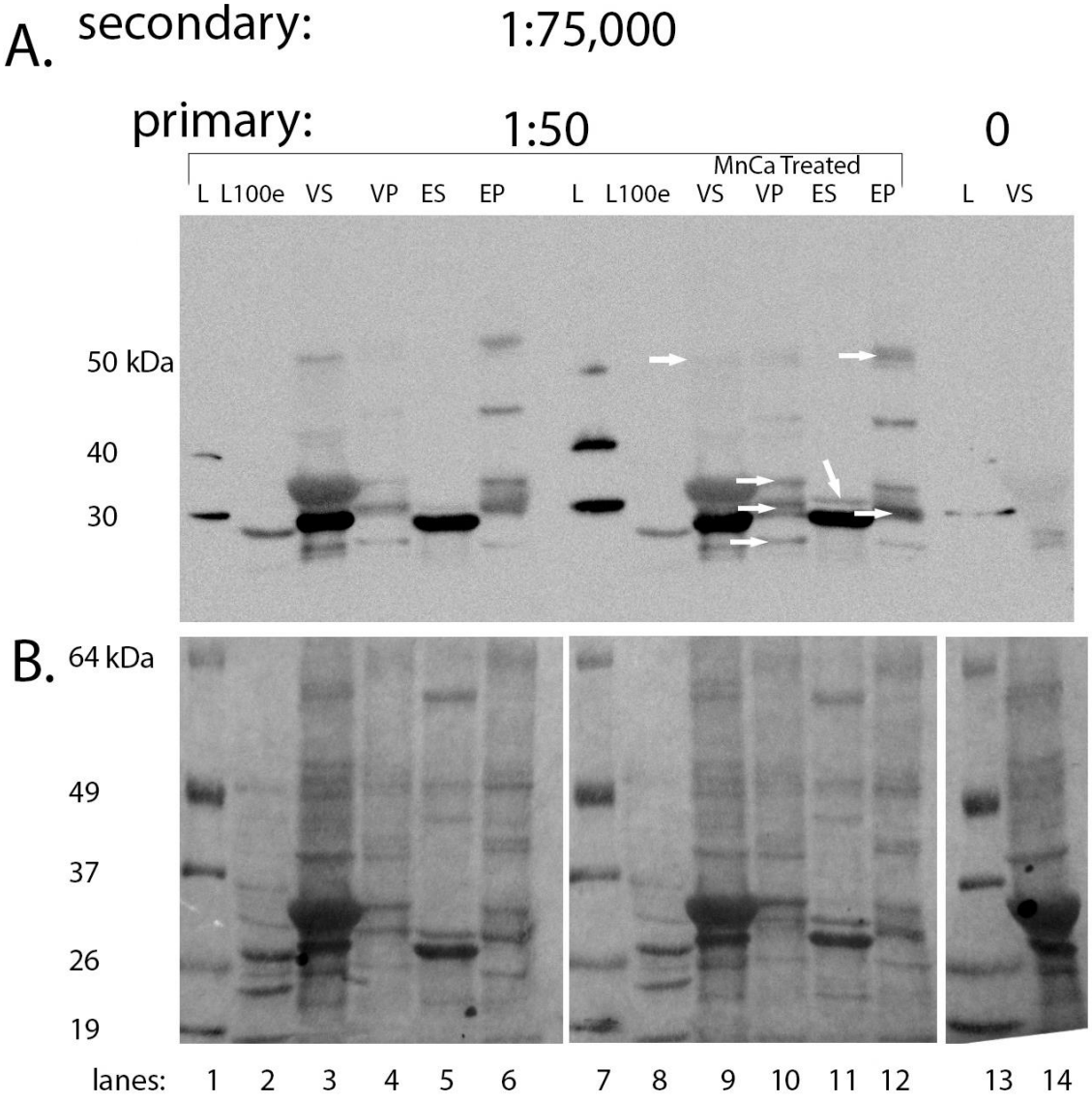
L – Combined western and protein molecular weight ladder, L100e – Liver high-speed supernatant 100 kDa eluent, ES – Epididymis supernatant, EP – Epididymis sperm pellet, VS – Vas deferens supernatant, VP – Vas deferens sperm pellet

cells along with the surrounding fluid, to 10 mM manganese and 10 mM calcium for one hour at 37ºC followed by separation of sperm from fluid via centrifugation at 6,000 *x g* in order to determine if DNase II transferred from the fluid into the sperm cell. This resulted in an observable change in DNase II detection in sperm and luminal fluid from both sources. After the vas deferens extract was treated with Mn^{2+} and Ca^{2+} a decrease of 50-kDa DNase II in the luminal fluid (VS) (Fig. 4.3 lane 9) along with an increase of 35, 30, and 23-kDa DNase II in sperm (VP) (Fig. 4.3 lane 10) was detected. After the epididymal extract was treated with Mn^{2+} and Ca^{2+} an increase in 30-kDa DNase II was detected in the luminal fluid (ES) (Fig. 4.3 lane 11) along with an increase of 50 and 30-kDa DNase II in sperm (EP) (Fig. 4.3 lane 12).

4.3.3. Isolation of DNase II from the Vas Deferens by Heparin Fractionation followed by detection via Western Blot and Zymogram

After heparin isolation of an acidic nuclease was developed using the liver extract we were able to apply the same protocol on extract from the vas deferens. Fluid was extracted from the vas deferens, diluted in TKB, and centrifuged at 60,000 *x g* for 20 minutes at 4ºC. The supernatant was then filtered through a 100-kDa MWCO filter and stored on ice (V100e). Prior to fractionation the heparin

Figure 4.3. MnCa Treated Vas Deferens and Epidydimis



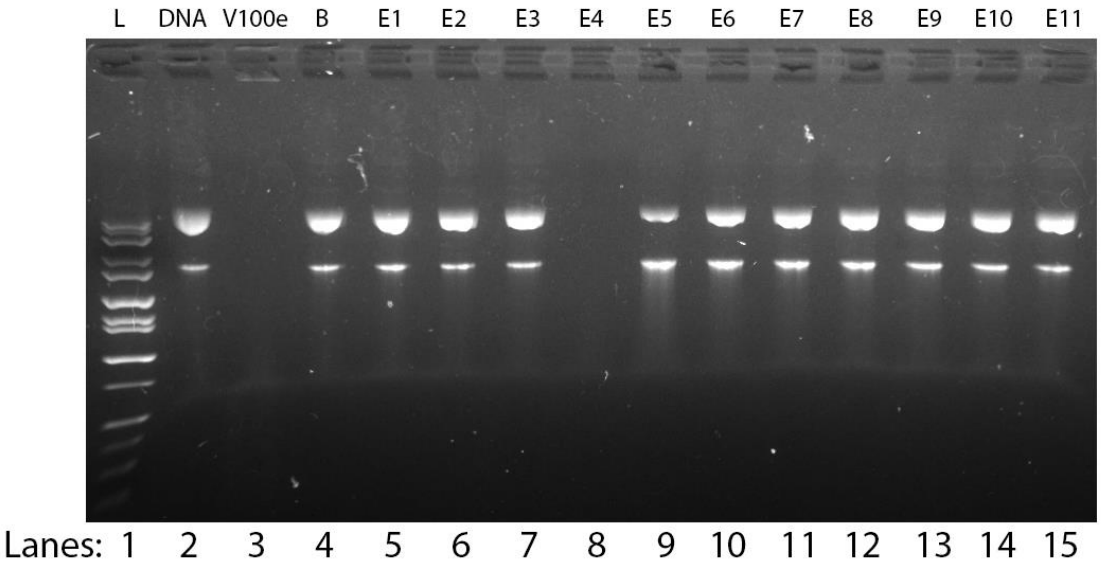
Western analysis of DNase II in mouse liver, epididymis, and vas deferens extract. (A) Following staining, proteins where then probed with or without 1:50 anti-DNase II and then with 1:75,000 donkey anti-goat IgG-HRP in 3% BSA/TBST followed by treatment with ECL peroxidase substrate. (B) Proteins resolved with SDS-PAGE and transferred to nitrocellulose and and stained with Ponceau-S.
Change in DNase II levels detected in sperm from the epididymis and vas deferens. Changes indicated with white arrows.
W – Western blot molecular weight ladder, P – Protein molecular weight ladder, L100e – Liver high-speed supernatant 100 kDa eluent, ES – Epididymis supernatant, EP – Epididymis sperm pellet, VS – Vas deferen supernatant, VP – Vas deferens sperm pellet.

column was adjusted to pH 4.8. The V100e extract was then adjusted to pH 4.8 with HCl and injected into the equilibrated heparin column. Fifteen column volumes of binding buffer was eluted and concentrated to 1 ml with a 3-kDa MWCO spin tube. Elution buffer was then passed through the column and eleven 1 ml fractions were collected. Fifteen ml was collected for the eleventh fraction to ensure that all protein had eluted, and then the fraction was concentrated to 1 ml on a 3-kDa MWCO spin tube. V100e and all its fractions where then assayed for acidic nuclease activity by adjusting the pH of each to 4.8 and combining 18 µl of each with 2 µl of 200 ng plasmid DNA (to assay V100e, 3µl was added to 2µl plasmid and 15µl 50mM sodium acetate pH 4.8) and incubated at 37ºC for 1 hour (Fig 4.4). Activity was only found in V100e and elution fraction E4 indicating that all the nuclease had eluted in one fraction (Fig 4.4 lanes 3 and 8). Elution fractions E5-E11 (Fig. 4.4 lanes 9 – 13) did not digest DNA confirming that all the nuclease was eluted in fraction E4.

We next assayed V100e and fractions B through E7 for activity following protein resolution via zymography. The advantage of the technique is that it allows the correlation of molecular weight with enzyme activity.

The samples were prepared in Laemmli sample buffer without DTT or BME and heated at 65ºC for ten minutes. The sample preparation for zymography is

Figure 4.4. Heparin Fractionated V100e Acidic Nuclease Assay

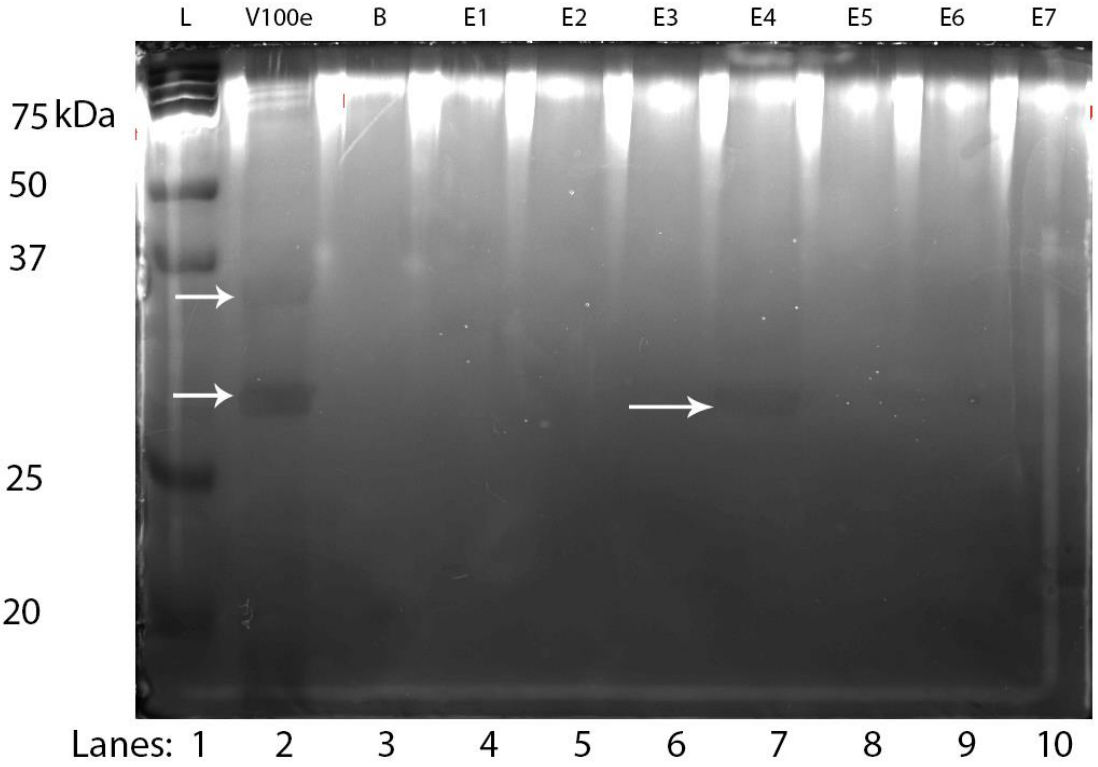


Acidic nuclease activity of V100e (lane 3) and the eluted fractions (lanes 4 – 15). Activity was found in elution fraction E4 (lane 8) indicating that all the nuclease had eluted in one fraction. V100e and all fractions were assayed for acidic nuclease by acidification of all samples to pH 4.8 and combined with 200 ng plasmid DNA at 37ºC for 1 hour and analyzed by conventional agarose electrophoresis.
L – Ladder, V100e – Vas deferens high-speed supernatant 100 kDa eluent

designed to allow some SDS linearization and gel resolution while still being able to renature for activity. The zymogram gel is prepared as per Laemmli with the exception of containing 3.0 µg/ml sheared salmon sperm DNA as substrate. The samples were then resolved at 4ºC at 150 V in 1x TGS running buffer and washed three times ten minutes each in DI water to remove SDS and allow protein folding and renaturing, then incubated for one change in 50 mM sodium acetate pH 4.8 (activation buffer) at 37ºC for 30 minutes. The gels were then incubated in new activation buffer overnight at 37ºC, soaked in ethidium bromide for 10 minutes, washed with water, and the nuclease activity was detected as a dark area on a fluorescent background when exposed to ultra violet light. Two nucleases were detected in V100e and one detected in E4 (Fig. 4.5 lanes 2 and 7). V100e contained acidic nuclease activity at approximately 30 and 35 kDa while E4 contained activity at 30 kDa.

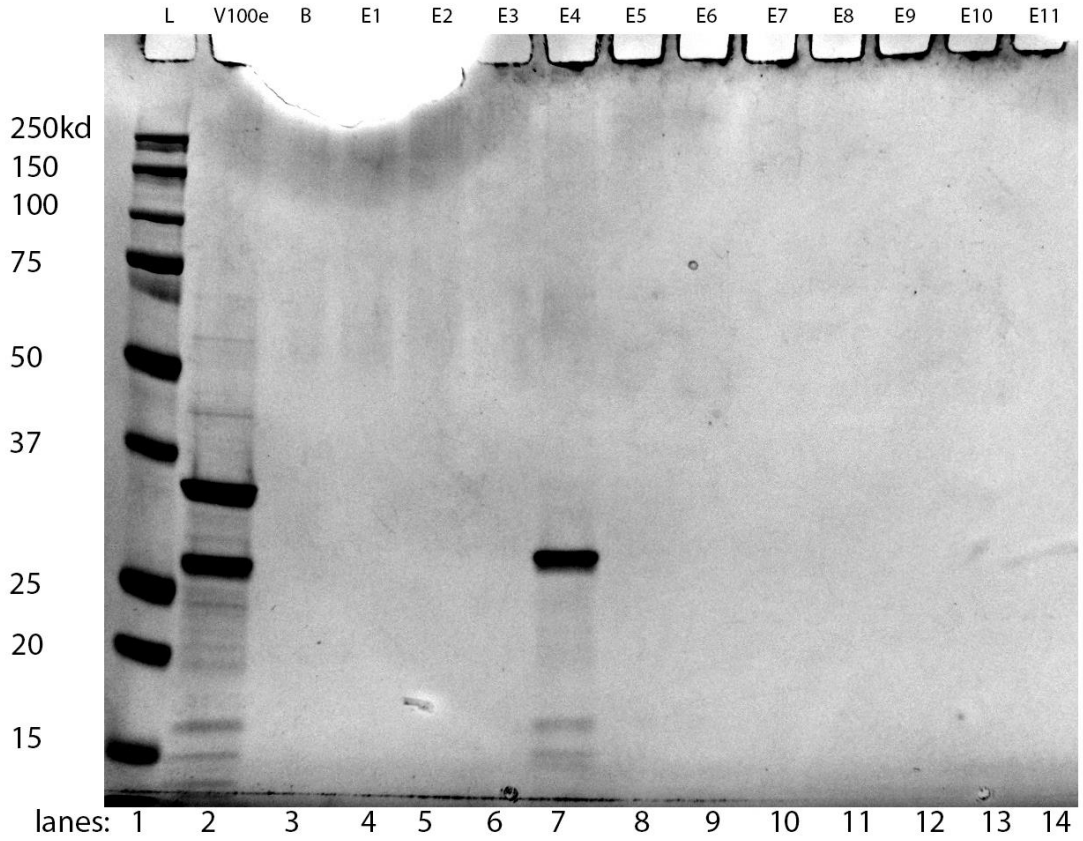
All samples were then prepared in duplicate with Laemmli sample buffer and resolved on SDS-PAGE after which one set was stained with colloidal coommassie blue and the other was transferred to nitrocellulose membrane and stained with ponceau-s in preparation for western blot. After staining with colloidal coomassie blue the protein complexity of the active eluent initial sample E4 was reduced in comparison to the initial sample (Fig 4.6 lanes 7 and 2). V100e contained two major protein bands at 30 and 35 kDa. Most of the protein

Figure 4.5. Heparin Fractionated V100e Zymogram Assay



Zymography of V100e and eluted fractions B – E7. Activity found in V100e and elution fraction E4. Fractions correspond with western blot size location. DNase II activity is indicated by the dark absence of DNA on the gel. V100e has two locations of activity, approximately 30 and 35 kDa, while E4 only has one, 30 kDa. All samples combined with Laemmli buffer and heated at 65ºC for ten minutes and resolved by SDS-PAGE on 12% acrylamide gels embedded with sheared salmon sperm DNA. Photographs taken under UV-light after incubation in activity buffer and ethidium bromide.
L – Protein Ladder, V100e – Vas deferens high-speed supernatant 100 kDa eluent
White arrows – Activity

Figure 4.6. Heparin Fractionated V100e Protein Complexity



Colloidal coomassie stained heparin fractions from V100e. Protein complexity reduced in elution fraction E4 from V100e. Strong protein bands detected at approximately 30 and 35 kDa in V100e (lane 2) and at 30 kDa in elution fraction E4 (lane 7).
L – Protein Ladder, V100e – Vas deferens high-speed supernatant 100 kDa eluent.

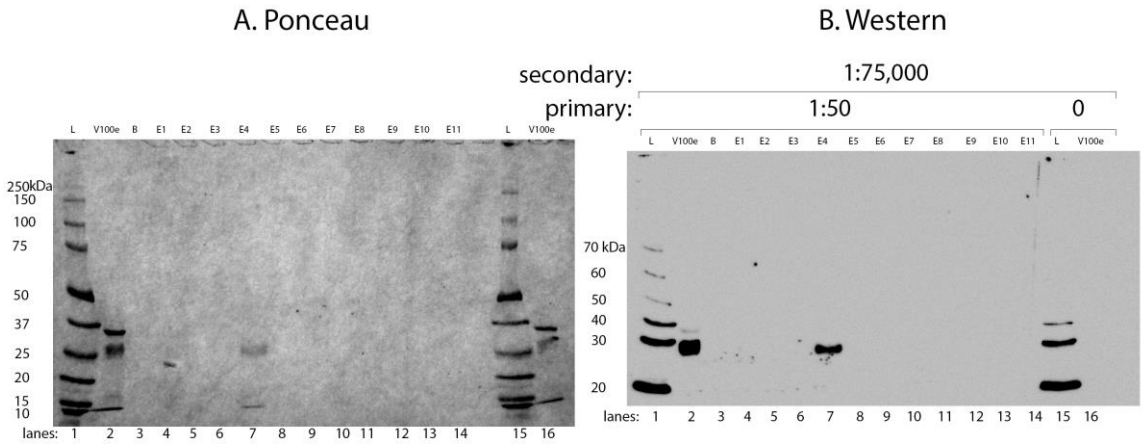
complexity in V100e was removed by fractionation leaving a single band in elution fraction E4 at 30 kDa.

With an optimized western blot protocol we were able to detect for the presence of DNase II in the fraction E4. The nitrocellulose membrane was cut into two sets of strips with one containing lanes of combined Western and protein ladder (L), V100e, and all fraction samples and the other containing combined western and protein ladder and V100e as secondary control. The membrane strips where blocked with 3% BSA/TBST and probed with 1:50 anti-DNase II and 1:75,000 secondary antibody, except for the secondary control which was not probed with primary antibodies. Two bands of DNase II were detected in V100e at 30 and 35 kDa (Fig. 4.7 lane 2). One band was detected in E4 at 30 kDa (Fig. 4.7 lane 7). The secondary control indicates no background or non-specific binding (Fig 4.7 lanes 15 and 16).

4.4. Discussion

In our efforts to identify the nuclease we first attempted mass spectrometry, however our results were inconclusive. In our attempt to isolate a protein sample pure enough for mass spectrometry we attempted column fractionation and in doing so discovered the true means of activating the nuclease. All the previous characterization work led to the initial conclusion that EGTA chelated to Ca^{2+} was

Figure 4.7. DNase II Detected in Heparin Fractionated V100e



Western analysis of DNase II in heparin column elutions of vas deferens extract. (A) Proteins resolved with SDS-PAGE and transferred to nitrocellulose and stained with ponceau-S. (B) Following staining, proteins where then probed with or without 1:50 anti-dnase II and then with 1:75,000 donkey anti-goat IgG-HRP in 3% BSA/TBST followed by treatment with ECL peroxidase substrate. Lanes 15 and 16 only probed with donkey anti-goat IgG-HRP (secondary control). DNase II detected in V100e at approximately 30 and 35 kDa and in E4 at approximately 30 kDa
L – Combined western and protein molecular weight ladder, V100e – Vas deferens high-speed supernatant 100 kDa eluent

activating the nuclease [113,155]. We now know that chelated EGTA lowers the pH of a solution that is not adequately buffered [118,156] and that low pH was actually activating the nuclease. Researching the various types of acid nucleases led to the DNase II family. There are three DNase II-type nucleases: DNase IIα, DNase IIβ, and L-DNase II. DNase IIα, commonly known as DNase II, is the most ubiquitously expressed and secreted enzyme of the family [121,122,161,166,167]. DNase IIβ is expressed in the eye and degrades DNA during lens fiber cell differentiation [113,168]. L-DNase II has also been implicated in apoptosis [125,169] but is not secreted. Our primary choice from the DNase II family was DNase IIα (DNase II) because of its ubiquity and that it is also secreted.

Optimizing a western blotting procedure allowed the detection of DNase II in the epididymis and vas deferens (and also in the liver as a positive control [127,164]). In the epididymis a major DNase II band was detected at 30-kDa in the luminal fluid (Fig. 4.2 lane 5) and at 55-kDa and 15-kDa at lower intensity in the sperm (Fig. 4.2 lane 6). In vas deferens luminal fluid we also detected a major DNase II band at 30-kDa (Fig. 4.2 lane 3). The 15-kDa DNase II was also present in the sperm (Fig. 4.2 lane 4) but at higher intensity. Overall there is more DNase II present in the vas deferens, especially at 30-kDa, which correlates with data indicating that SDD is stronger in sperm extract from the organ.

We also tested for the transfer of DNase II into the sperm cell by incubating whole extract (sperm and its surrounding luminal fluid) from the epididymis or

vas deferens in buffer containing Mn^{2+} and Ca^{2+}, which has been shown to activate SCF as well as SDD. It is also known that EGTA and Ca^{2+} greatly accelerates SDD [17,115,128,165]. Topoisomerase IIB is believed to be responsible for SCF however the enzyme machinery behind SDD is currently under investigation. Here we present preliminary evidence linking DNase II to SDD. After incubating the epididymal and vas deferens extracts in Mn^{2+} and Ca^{2+}, both solutions were separated via centrifugation, immunoblotted, and compared to the same samples not treated with Mn^{2+} and Ca^{2+}. Although most of the DNase II was detected in the luminal fluids, especially at 30-kDa, Mn^{2+} and Ca^{2+} caused a detectable increase in DNase II in the sperm from both the epididymis and vas deferens (Fig. 4.3 B, lanes 10 and 12). This data adds support to the likelihood of DNase II involvement in SDD.

Finally we isolated the enzyme from the vas deferens luminal fluid using heparin column chromatography. Vas deferens luminal fluid was filtered and the 100-kDa eluent was collected and fractionated on a heparin column. The fractions were then assayed for the presence of acidic nuclease activity using a plasmid degradation assay (Fig. 4.4 lanes 3 and 8) and the acidic activity was correlated to molecular weights 30 and 35-kDa using zymography (Fig 4.5 lanes 2 and 7), colloidal coomassie stain (Fig. 4.6 lanes 2 and 7), and western blot (Fig. 4.7 lanes 2 and 7).

According to recent work on DNase II in the mouse [129,161] three forms of DNase II have been elucidated at molecular weights at 45, 30, and 23-kDa. The 45-kDa form is secreted while the 30 and 23-kDa forms are localized to the lysosomes. We found 45-kDa DNase II in the luminal fluid of the vas deferens (Fig. 4.2 and 4.6) and with sperm from the epididymis (Fig 4.3) while 30-kDa DNase II was found in all tested samples (the luminal fluid and sperm of both the epididymis and vas deferens (Figs 4.2, 4.3, and 4.5)). We did not find 23-kDa DNase II in these areas. Finding DNase II with sperm from the cauda epididymis and vas deferens suggests that the nuclease was secreted as soluble protein (45-kDa) or packaged in lysosomes (30-kDa) from the epithelial cells and localized to the sperm cell. The sperm pellet was washed, centrifuged, and the supernatant removed followed by pellet resuspension in buffer three times prior to analysis indicating that the DNase II was bound strongly to the sperm cell, or may have been transferred into the cell. DNase IIs were detected at different weights that have not been described in the literature and can represent different levels of post-translational modification and cleavage unique to the male reproductive tract.

This data confirms the presence of DNase II in the luminal fluid and sperm from the epididymis and vas deferens. Furthermore, we have correlated the activity triggered by EGTA and Ca^{2+} [60,130,155] to the acidic nuclease DNase II. Lastly, we have provided preliminary evidence linking this activity to Sperm DNA Degradation [37,39,40,60].

Chapter 5. Discussion

The nuclease activity we have described here was first seen in the male mouse reproductive tract. The activity was thought to be the product of a nuclease that was first uninhibited by EGTA and then activated by calcium [6,60]. Later it was shown that both EGTA and calcium needed to be present at the same time to chelate the EGTA molecule that was directly activating the nuclease [131,132,155]. Finally we found that chelated EGTA was not necessary and it was in fact the lowering of pH that was solely needed to activate the nuclease.

Our model has continued to evolve from our original hypothesis that the nuclease was a calcium-dependent protein that inhibited the nuclease where EGTA was added to chelate calcium and remove the inhibitory protein leaving the nuclease in an uninhibited state. According to this model, a divalent cation is then introduced to activate the nuclease [60,133]. This model was adjusted to account for our unexpected data that both EGTA and calcium needed to be present at the simultaneously, which in turn suggested that the chelated form of EGTA was responsible for directly activating the nuclease [134,155]. Our current data indicates that the nucleolytic activity was actually due to an acidic nuclease that was activated by the drop in pH from chelating EGTA. This nuclease is

present in the luminal fluid of the vas deferens and to a lesser extent in the epididymis.

The epididymis and vas deferens are known as storage areas for spermatozoa, but also may also function as a quality control mechanism for defective sperm. This is suggested by the drop in number of defective sperm seen in the cauda epidydimis compared to the caput [135,170] and also by the extracellular ubiquitination of sperm seen throughout the epididymis [136-140,171] suggesting they are being marked for degradation.

The presence of an acidic nuclease in the vas deferens has not been directly described, however there is evidence of an abundance of lysosomes in the epithelial cells lining the epididymis and vas deferens [41,141,172] and so it is probable that a DNase II-like protein, which is normally localized within lysosomes, would be present in lysosomal vesicles in the epithelial cells of the epididymis and vas deferens and also excreted into the luminal fluid as soluble protein or contained in microvesicles such as epididymosomes [142,173].

5.1 DNase II relationship to SCF

Topoisomerase IIB can be activated in sperm with Mn^{2+} and Ca^{2+} causing the reversible cleavage of chromatin DNA, and after a window of time full degradation is observed, a process we have named Sperm DNA Degradation.

Interestingly, using EGTA and Ca^{2+} greatly accelerates the SDD reaction [60]. We now know that EGTA and Ca^{2+} lowers the pH of the solution activating an acidic nuclease. Therefore, data presented here support our hypothesis that DNase II is the nuclease activated during SDD.

5.2 Model for DNase II activation

DNase II is typically known for its role in somatic phagocytosis where macrophages engulf apoptotic nuclei. In this context DNase II is found in the acidic lysosomes of the macrophage where it degrades apoptotic chromatin. DNase II is also found on the surface of the skin. Here the nuclease is free from the lysosome and in the active state due to the natural acidity of skin [166]. In this context the nuclease functions to keep the body safe from exogenous DNA and viruses.

Our data indicate that different forms of DNase II are present in sperm and luminal fluid from the epididymis compared to the vas deferens. Interestingly, an approximately 15-kDa DNase II was strongly detected in the sperm of vas deferens (Fig. 4.2 lane 6) but weakly in the epididymis (Fig. 4.2 lane 4). This data parallels the pattern seen in SDD, which is much stronger in the vas deferens than in the epididymis [60]. It is likely that the 15-kDa DNase II is transferred to the sperm cell while in the vas deferens. This transfer most likely occurs in the luminal fluid

when lysosomes containing the enzyme fuse with sperm, since higher molecular weight DNase II is normally secreted in the form of soluble protein and lower molecular weight DNase II is packaged in lysosomes [161].

Although the environment of the vas deferens is acidic, viable sperm are protected because lower molecular weight DNase II is safely contained in lysosomes, while the free floating higher molecular weight form is kept in an inactive state due to inhibition by serum albumin [174], which is found in increasingly higher concentration towards the end of the epididymis as the tube approaches the vas deferens [175]. Activation of the nuclease probably occurs when lower molecular weight DNase II enters the sperm cell after lysosomal fusion or when higher molecular weight Dnase II finds exposed DNA, which it has a higher affinity to than albumin.

The model for SCF parallels apoptosis in somatic cells. Although the conditions for activation of SCF *in vivo* are not yet well understood, in somatic cells DNase II is normally a late stage player during apoptosis. Its role when contained in lysosomes in the macrophage is to remove apoptotic DNA from phagocytosed dying cells. DNase II -/- macrophages cannot degrade engulfed DNA [154]. Additionally, DNase II -/- mice die during later developmental stages due to constitutionally active interferon B (IFN) production because they cannot degrade the apoptoic DNA from enucleated red blood cells [99,161,164,176]. In other words, DNase II is not necessary for apoptosis and is probably not directly

linked to the function of topoisomerase IIB, an integral component of the apoptotic machinery. In its secreted high molecular weight (and free-floating) form, DNase II probably directly attaches to the DNA of damaged sperm that have lost membrane integrity and have exposed chromatin. In lysosomal form, the nuclease probably enters sperm that have been extracellularly primed for DNA degradation. Although phagocytosis of defective sperm has not been detected in the epididymis, an alternate means of dealing with them has been observed. Sperm "death cocoons" have been detected in the epididymis of hamsters. These sperm are non-viable and are coated with fibrinogen-like protein fgl2. The monomeric fgl2 protein of 64 kDa forms 260 and 280 kDa oligomers that coat defective sperm and all sperm fragments [177]. Normally the cell presents phosphatidylserine on the surface of the membrane during apoptosis to signal coagulation activity of macrophage fgl2 [178-180]. Phosphatidylserine has also been detected in damaged sperm [181], which has prompted the authors [177] to speculate that perhaps this acts as a signal for fgl2 binding to damaged sperm, promoting an extracellular protease reaction. However it is not known how the cocoons are eliminated. It is possible that phosphatidylserine and/or fgl2 may signal for DNase II as well.

5.3 Summary

The work presented here indicates the novel involvement of DNase II in a mechanism that degrades DNA in sperm that we have previously named Sperm DNA Degradation (SDD). This activity occurs more strongly in sperm from the vas deferens and is greatly accelerated in the presence of EGTA and Ca^{2+}. The experimental data supporting what we thought was a new class of enzyme that was activated by EGTA chelated to Ca^{2+} remains valid and instead indicates the activity of an acid nuclease that we have identified to be DNase II. Of the three variants of the enzyme, two have been detected and confirmed activatable in the cauda epididymis and vas deferens.

Future work will focus on how DNase II works in conjunction with indicators of damaged sperm, such as phosphatidylserine and protein fgl2, to understand how defective sperm is removed from the male reproductive tract in order to maximize the amount of viable sperm for fertilization. Another direction would be to isolate the DNAse II from vas deferens luminal fluid by immunoprecipitation then attempt to sequence the protein by mass spectrometry to identify the exact form of DNAse II that is present in the vas deferens.

The work in this dissertation identifies DNAse II as the major nuclease in the luminal fluid of the vas deferens, and that it has a role in sperm chromatin

degradation. While we do not yet know the exact mechanism for its action, we now have a target molecule to direct our experimental approaches in defining it.

Bibliography

[1] T.K. Li, A.Y. Chen, C. Yu, Y. Mao, H. Wang, L.F. Liu, Activation of topoisomerase II-mediated excision of chromosomal DNA loops during oxidative stress, Genes Dev. 13 (1999) 1553–1560.

[2] P. Senger, Pathways to pregnancy and parturition, (2004) 368.

[3] X. Yang, W. Li, E.D. Prescott, S.J. Burden, J.C. Wang, DNA topoisomerase IIbeta and neural development, Science. 287 (2000) 131–134.

[4] J.-P. Dadoune, Expression of mammalian spermatozoal nucleoproteins, Microsc Res Tech. 61 (2003) 56–75.

[5] B.-G. Ju, V.V. Lunyak, V. Perissi, I. Garcia-Bassets, D.W. Rose, C.K. Glass, et al., A topoisomerase IIbeta-mediated dsDNA break required for regulated transcription, Science. 312 (2006) 1798–1802.

[6] V.T. Solovyan, Z.A. Bezvenyuk, A. Salminen, C.A. Austin, M.J. Courtney, The role of topoisomerase II in the excision of DNA loop domains during apoptosis, J Biol Chem. 277 (2002) 21458–21467.

[7] D. Sakkas, O. Moffatt, G. Manicardi, E. Mariethoz, N. Tarozzi, D. Bizzaro, Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis, Biol Reprod. 66 (2002) 1061–7.

[8] P. Widlak, W. Garrard, Roles of the Major Apoptotic Nuclease-DNA Fragmentation Factor-in Biology and Disease, Cell Mol Life Sci. (2008).

[9] H. Billig, S.Y. Chun, K. Eisenhauer, A.J. Hsueh, Gonadal cell apoptosis: hormone-regulated cell demise, Hum Reprod Update. 2 (1996) 103–117.

[10] M. Biggiogera, S. Muller, J.L. Courtens, S. Fakan, M.G. Romanini, Immunoelectron microscopical distribution of histones H2B and H3 and protamines in the course of mouse spermiogenesis, Microsc Res Tech. 20 (1992) 259–267.

[11] V. Pentikäinen, K. Erkkilä, L. Dunkel, Fas regulates germ cell apoptosis in the human testis in vitro, Am J Physiol. 276 (1999) E310–6.

[12] D. Sakkas, E. Mariethoz, G. Manicardi, D. Bizzaro, P.G. Bianchi, U. Bianchi, Origin of DNA damage in ejaculated human spermatozoa, Rev. Reprod. 4 (1999) 31–37.

[13] D. Sakkas, J.G. Alvarez, Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis, Fertil Steril. 93 (2010) 1027–1036.

[14] S. McPherson, F. Longo, Chromatin structure-function alterations during mammalian spermatogenesis: DNA nicking and repair in elongating spermatids, Eur J Histochem. 37 (1993) 109–128.

[15] M. Loir, D. Bouvier, M. Fornells, M. Lanneau, J.A. Subirana, Interactions of nuclear proteins with DNA, during sperm differentiation in the ram, Chromosoma. 92 (1985) 304–312.

[16] R.-M. Laberge, G. Boissonneault, On the nature and origin of DNA strand breaks in elongating spermatids, Biol Reprod. 73 (2005) 289–296.
[17] J.A. Shaman, R. Prisztoka, W.S. Ward, Topoisomerase IIB and an extracellular nuclease interact to digest sperm DNA in an apoptotic-like manner, Biol Reprod. 75 (2006) 741–748.
[18] W.S. Ward, Deoxyribonucleic acid loop domain tertiary structure in mammalian spermatozoa, Biol Reprod. 48 (1993) 1193–1201.
[19] T. Haaf, D.C. Ward, Higher order nuclear structure in mammalian sperm revealed by in situ hybridization and extended chromatin fibers, Experimental Cell Research. 219 (1995) 604–611.
[20] D.P. Evenson, K.L. Larson, L.K. Jost, Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques, J Androl. 23 (2002) 25–43.
[21] N. Burrello, G. Arcidiacono, E. Vicari, P. Asero, D. Di Benedetto, A. De Palma, et al., Morphologically normal spermatozoa of patients with secretory oligo-astheno-teratozoospermia have an increased aneuploidy rate, Hum Reprod. 19 (2004) 2298–2302.
[22] W.S. Ward, D.S. Coffey, DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells, Biol Reprod. 44 (1991) 569–574.
[23] I.D. Vilfan, C.C. Conwell, N.V. Hud, Formation of native-like mammalian sperm cell chromatin with folded bull protamine, J Biol Chem. 279 (2004) 20088–20095.
[24] B. Sotolongo, E. Lino, W.S. Ward, Ability of hamster spermatozoa to digest their own DNA, Biol Reprod. 69 (2003) 2029–2035.
[25] E. de Lamirande, C. Gagnon, Paradoxical effect of reagents for sulfhydryl and disulfide groups on human sperm capacitation and superoxide production, Free Radic Biol Med. 25 (1998) 803–817.
[26] K. Yanagida, R. Yanagimachi, S.D. Perreault, R.G. Kleinfeld, Thermostability of sperm nuclei assessed by microinjection into hamster oocytes, Biol Reprod. 44 (1991) 440–447.
[27] M. Aleem, V. Padwal, J. Choudhari, N. Balasinor, M.K. Gill-Sharma, Sperm protamine levels as indicators of fertilising potential in sexually mature male rats, Andrologia. 40 (2008) 29–37.
[28] H. Tateno, Y. Kimura, R. Yanagimachi, Sonication per se is not as deleterious to sperm chromosomes as previously inferred, Biol Reprod. 63 (2000) 341–346.
[29] B. Maione, C. Pittoggi, L. Achene, R. Lorenzini, C. Spadafora, Activation of endogenous nucleases in mature sperm cells upon interaction with exogenous DNA, DNA Cell Biol. 16 (1997) 1087–1097.
[30] L. Silvestroni, G. Frajese, M. Fabrizio, Histones instead of protamines in terminal germ cells of infertile, oligospermic men, Fertil Steril. 27 (1976) 1428–1437.

[31] B. Sotolongo, W.S. Ward, DNA loop domain organization: the three-dimensional genomic code, J Cell Biochem Suppl. Suppl 35 (2000) 23–26.
[32] B. Sotolongo, T.T.F. Huang, E. Isenberger, W.S. Ward, An endogenous nuclease in hamster, mouse, and human spermatozoa cleaves DNA into loop-sized fragments, J Androl. 26 (2005) 272–280.
[33] M.J. D'Occhio, K.J. Hengstberger, S.D. Johnston, Biology of sperm chromatin structure and relationship to male fertility and embryonic survival, Anim Reprod Sci. 101 (2007) 1–17.
[34] S.S. Hammoud, D.A. Nix, H. Zhang, J. Purwar, D.T. Carrell, B.R. Cairns, Distinctive chromatin in human sperm packages genes for embryo development, Nature. 460 (2009) 473–478.
[35] D. Evenson, Alterations and damage of sperm chromatin structure and early embryonic failure, Towards Reproductive Certainty: Fertility & Genetics Beyond 1999: the Plenary Proceedings of the 11th World Congress on in Vitro Fertilization & Human Reproductive Genetics. (1999).
[36] J.P. Blumenstiel, Sperm competition can drive a male-biased mutation rate, Journal of Theoretical Biology. 249 (2007) 624–632.
[37] X. Liu, H. Zou, C. Slaughter, X. Wang, DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis, Cell. 89 (1997) 175–184.
[38] J. Martínez-Heredia, J.M. Estanyol, J.L. Ballescà, R. Oliva, Proteomic identification of human sperm proteins, Proteomics. 6 (2006) 4356–4369.
[39] M. Enari, H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, S. Nagata, A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD, Nature. 391 (1998) 43–50.
[40] R. Halenbeck, H. MacDonald, A. Roulston, T.T. Chen, L. Conroy, L.T. Williams, CPAN, a human nuclease regulated by the caspase-sensitive inhibitor DFF45, Curr Biol. 8 (1998) 537–540.
[41] T. Hashida, Y. Tanaka, N. Matsunami, K. Yoshihara, T. Kamiya, Y. Tanigawa, et al., Purification and properties of bull seminal plasma Ca2+,Mg2+-dependent endonuclease, J Biol Chem. 257 (1982) 13114–13119.
[42] N. Hud, M. Allen, K. Downing, J. Lee, R. Balhorn, Identification of the elemental packing unit of DNA in mammalian sperm cells by atomic force microscopy, Biochem Biophys Res Commun. 193 (1993) 1347–1354.
[43] N. Hud, K. Downing, R. Balhorn, A constant radius of curvature model for the organization of DNA in toroidal condensates, Proc Natl Acad Sci USA. 92 (1995) 3581–3585.
[44] L. Brewer, M. Corzett, E. Lau, R. Balhorn, Dynamics of protamine 1 binding to single DNA molecules, J Biol Chem. 278 (2003) 42403–42408.
[45] F. Domínguez, F.J. Cejudo, Identification of a nuclear-localized nuclease from wheat cells undergoing programmed cell death that is able to trigger DNA fragmentation and apoptotic morphology on nuclei from

human cells, Biochem J. 397 (2006) 529–536.
[46] R. Balhorn, A model for the structure of chromatin in mammalian sperm, J Cell Biol. 93 (1982) 298–305.
[47] J.A. Schmidt, G.F. Browning, P.F. Markham, Mycoplasma hyopneumoniae mhp379 is a Ca2+-dependent, sugar-nonspecific exonuclease exposed on the cell surface, J Bacteriol. 189 (2007) 3414–3424.
[48] Z. Jóźwiak, A. Marczak, [The role of ion channels in apoptosis], Postepy Biochem. 52 (2006) 373–382.
[49] D. Shiokawa, M. Hirai, S. Tanuma, cDNA cloning of human DNase gamma: chromosomal localization of its gene and enzymatic properties of recombinant protein, Apoptosis. 3 (1998) 89–95.
[50] O. Mudrak, R. Chandra, E. Jones, E. Godfrey, A. Zalensky, Reorganisation of human sperm nuclear architecture during formation of pronuclei in a model system, Reprod Fertil Dev. 21 (2009) 665–671.
[51] D. Shiokawa, S. Tanuma, Molecular cloning and expression of a cDNA encoding an apoptotic endonuclease DNase gamma, Biochem J. 332 (Pt 3) (1998) 713–720.
[52] R.P. Martins, S.A. Krawetz, RNA in human sperm, Asian J Androl. 7 (2005) 115–120.
[53] G. Ostermeier, R. Goodrich, M. Diamond, D. Dix, S. Krawetz, Toward using stable spermatozoal RNAs for prognostic assessment of male factor fertility, Fertil Steril. 83 (2005) 1687–1694.
[54] G. Sasnauskas, B.A. Connolly, S.E. Halford, V. Siksnys, Site-specific DNA transesterification catalyzed by a restriction enzyme, Proc Natl Acad Sci USA. 104 (2007) 2115–2120.
[55] B. Polzar, H.G. Mannherz, Nucleotide sequence of a full length cDNA clone encoding the deoxyribonuclease I from the rat parotid gland, Nucleic Acids Res. 18 (1990) 7151.
[56] J.A. Stuckey, J.E. Dixon, Crystal structure of a phospholipase D family member, Nat. Struct. Biol. 6 (1999) 278–284.
[57] A. Arpanahi, M. Brinkworth, D. Iles, S.A. Krawetz, A. Paradowska, A.E. Platts, et al., Endonuclease-sensitive regions of human spermatozoal chromatin are highly enriched in promoter and CTCF binding sequences, Genome Res. 19 (2009) 1338–1349.
[58] S. Shak, D.J. Capon, R. Hellmiss, S.A. Marsters, C.L. Baker, Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum, Proc Natl Acad Sci USA. 87 (1990) 9188–9192.
[59] C.P. Ponting, I.D. Kerr, A novel family of phospholipase D homologues that includes phospholipid synthases and putative endonucleases: identification of duplicated repeats and potential active site residues, Protein Sci. 5 (1996) 914–922.
[60] S.M. Boaz, K. Dominguez, J.A. Shaman, W.S. Ward, Mouse spermatozoa contain a nuclease that is activated by pretreatment with EGTA and

subsequent calcium incubation, J Cell Biochem. 103 (2008) 1636–1645.
[61] V.V. Shastina, N.I. Menzorova, Y.T. Sibirtsev, V.A. Rasskazov, Purification and characteristics of Ca2+,Mg2+- and Ca2+,Mn2+-dependent and acid DNases from spermatozoa of the sea urchin Strongylocentrotus intermedius, Biochemistry Mosc. 68 (2003) 582–592.
[62] D.R. Davies, H. Interthal, J.J. Champoux, W.G.J. Hol, The crystal structure of human tyrosyl-DNA phosphodiesterase, Tdp1, Structure. 10 (2002) 237–248.
[63] L.S. Beese, T.A. Steitz, Structural basis for the 3"-5" exonuclease activity of Escherichia coli DNA polymerase I: a two metal ion mechanism, Embo J. 10 (1991) 25–33.
[64] I. Leiros, S. McSweeney, E. Hough, The reaction mechanism of phospholipase D from Streptomyces sp. strain PMF. Snapshots along the reaction pathway reveal a pentacoordinate reaction intermediate and an unexpected final product, J Mol Biol. 339 (2004) 805–820.
[65] T.G. Cooper, Interactions between epididymal secretions and spermatozoa, J Reprod Fertil Suppl. 53 (1998) 119–136.
[66] V. Derbyshire, N.D. Grindley, C.M. Joyce, The 3"-5" exonuclease of DNA polymerase I of Escherichia coli: contribution of each amino acid at the active site to the reaction, Embo J. 10 (1991) 17–24.
[67] J.-L. Dacheux, J.L. Gatti, F. Dacheux, Contribution of epididymal secretory proteins for spermatozoa maturation, Microsc Res Tech. 61 (2003) 7–17.
[68] J.-L. Dacheux, S. Castella, J.L. Gatti, F. Dacheux, Epididymal cell secretory activities and the role of proteins in boar sperm maturation, Theriogenology. 63 (2005) 319–341.
[69] S. Marengo, Maturing the sperm: Unique mechanisms for modifying integral proteins in the sperm plasma membrane, Anim Reprod Sci. (2007).
[70] A. Pingoud, M. Fuxreiter, V. Pingoud, W. Wende, Type II restriction endonucleases: structure and mechanism, Cell Mol Life Sci. 62 (2005) 685–707.
[71] S. Grazulis, E. Manakova, M. Roessle, M. Bochtler, G. Tamulaitiene, R. Huber, et al., Structure of the metal-independent restriction enzyme BfiI reveals fusion of a specific DNA-binding domain with a nonspecific nuclease, Proc Natl Acad Sci USA. 102 (2005) 15797–15802.
[72] P.E. Visconti, G.D. Moore, J.L. Bailey, P. Leclerc, S.A. Connors, D. Pan, et al., Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway, Development. 121 (1995) 1139–1150.
[73] E.A. Galburt, B.L. Stoddard, Catalytic mechanisms of restriction and homing endonucleases, Biochemistry. 41 (2002) 13851–13860.
[74] G. Sasnauskas, L. Zakrys, M. Zaremba, R. Cosstick, J.W. Gaynor, S.E.

Halford, et al., A novel mechanism for the scission of double-stranded DNA: BfiI cuts both 3'-5' and 5"-3" strands by rotating a single active site, Nucleic Acids Res. 38 (2010) 2399–2410.
[75] P. Hoppe, Fertilizing ability of mouse sperm from different epididymal regions and after washing and centrifugation, J Exp Zool. 192 (1975) 219–222.
[76] R. Yanagimachi, Y. Kamiguchi, K. Mikamo, F. Suzuki, H. Yanagimachi, Maturation of spermatozoa in the epididymis of the Chinese hamster, American Journal of Anatomy. 172 (1985) 317–330.
[77] D.R. Davies, H. Interthal, J.J. Champoux, W.G.J. Hol, Explorations of peptide and oligonucleotide binding sites of tyrosyl-DNA phosphodiesterase using vanadate complexes, J. Med. Chem. 47 (2004) 829–837.
[78] G. Ronquist, I. Brody, The prostasome: its secretion and function in man, Biochim Biophys Acta. 822 (1985) 203–218.
[79] G. Aumüller, H. Renneberg, P.J. Schiemann, B. Wilhelm, J. Seitz, L. Konrad, et al., The role of apocrine released proteins in the post-testicular regulation of human sperm function, Adv Exp Med Biol. 424 (1997) 193–219.
[80] G. Aumüller, B. Wilhelm, J. Seitz, Apocrine secretion--fact or artifact? Ann Anat. 181 (1999) 437–446.
[81] D.G. CATCHESIDE, B. HOLMES, The action of enzymes on chromosomes, Symp. Soc. Exp. Biol. (1947) 225–231.
[82] G. Ronquist, I. Brody, A. Gottfries, B. Stegmayr, An Mg2+ and Ca2+-stimulated adenosine triphosphatase in human prostatic fluid: part I, Andrologia. 10 (1978) 261–272.
[83] G. Ronquist, I. Brody, A. Gottfries, B. Stegmayr, An Mg2+ and Ca2+-stimulated adenosine triphosphatase in human prostatic fluid--part II, Andrologia. 10 (1978) 427–433.
[84] F. Saez, G. Frenette, R. Sullivan, Epididymosomes and prostasomes: their roles in posttesticular maturation of the sperm cells, J Androl. 24 (2003) 149–154.
[85] G. Ronquist, B.O. Nilsson, The Janus-faced nature of prostasomes: their pluripotency favours the normal reproductive process and malignant prostate growth, Prostate Cancer Prostatic Dis. 7 (2004) 21–31.
[86] C. Cordonnier, G. Bernardi, A comparative study of acid deoxyribonucleases extracted from different tissues and species, Can. J. Biochem. 46 (1968) 989–995.
[87] C.H. Yeung, S. Schröter, A. Wagenfeld, C. Kirchhoff, S. Kliesch, D. Poser, et al., Interaction of the human epididymal protein CD52 (HE5) with epididymal spermatozoa from men and cynomolgus monkeys, Mol Reprod Dev. 48 (1997) 267–275.
[88] G. Arienti, E. Carlini, R. Verdacchi, E.V. Cosmi, C.A. Palmerini,

Prostasome to sperm transfer of CD13/aminopeptidase N (EC 3.4.11.2), Biochim Biophys Acta. 1336 (1997) 533–538.
[89] G. Arienti, A. Polci, E. Carlini, C.A. Palmerini, Transfer of CD26/dipeptidyl peptidase IV (E.C. 3.5.4.4) from prostasomes to sperm, FEBS Lett. 410 (1997) 343–346.
[90] A. Minelli, C. Allegrucci, I. Mezzasoma, G. Ronquist, C. Lluis, R. Franco, CD26 and adenosine deaminase interaction: its role in the fusion between horse membrane vesicles and spermatozoa, Biol Reprod. 61 (1999) 802–808.
[91] G. Frenette, R. Sullivan, Prostasome-like particles are involved in the transfer of P25b from the bovine epididymal fluid to the sperm surface, Mol Reprod Dev. 59 (2001) 115–121.
[92] G. Frenette, C. Lessard, R. Sullivan, Selected proteins of "prostasome-like particles" from epididymal cauda fluid are transferred to epididymal caput spermatozoa in bull, Biol Reprod. 67 (2002) 308–313.
[93] R. Sullivan, G. Frenette, J. Girouard, Epididymosomes are involved in the acquisition of new sperm proteins during epididymal transit, Asian J Androl. 9 (2007) 483–491.
[94] S. Nagata, Autoimmune diseases caused by defects in clearing dead cells and nuclei expelled from erythroid precursors, Immunol. Rev. 220 (2007) 237–250.
[95] L. Hermo, R.J. Oko, C.R. Morales, Secretion and endocytosis in the male reproductive tract: a role in sperm maturation, International Review of Cytology. 154 (1994) 106–189.
[96] H. Rejraji, P. Vernet, J. Drevet, GPX5 is present in the mouse caput and cauda epididymidis lumen at three different locations, Mol Reprod Dev. 63 (2002) 96–103.
[97] A. Torriglia, C. Leprêtre, L. Padrón-Barthe, S. Chahory, E. Martin, Molecular mechanism of L-DNase II activation and function as a molecular switch in apoptosis, Biochem Pharmacol. 76 (2008) 1490–1502.
[98] N.L. Cross, Role of cholesterol in sperm capacitation, Biol Reprod. 59 (1998) 7–11.
[99] R.J. Krieser, K.S. MacLea, D.S. Longnecker, J.L. Fields, S. Fiering, A. Eastman, Deoxyribonuclease IIalpha is required during the phagocytic phase of apoptosis and its loss causes perinatal lethality, Cell Death Differ. 9 (2002) 956–962.
[100] R. Fabiani, Functional and biochemical characteristics of human prostasomes. Minireview based on a doctoral thesis, Ups J Med Sci. 99 (1994) 73–111.
[101] C. Kirchhoff, G. Hale, Cell-to-cell transfer of glycosylphosphatidylinositol-anchored membrane proteins during sperm maturation, Mol Hum Reprod. 2 (1996) 177–184.
[102] G. Arienti, E. Carlini, C. Saccardi, C.A. Palmerini, Role of human

prostasomes in the activation of spermatozoa, J Cell Mol Med. 8 (2004) 77–84.
[103] H. Rejraji, B. Sion, G. Prensier, M. Carreras, C. Motta, J.-M. Frenoux, et al., Lipid remodeling of murine epididymosomes and spermatozoa during epididymal maturation, Biol Reprod. 74 (2006) 1104–1113.
[104] P. Widlak, W.T. Garrard, Discovery, regulation, and action of the major apoptotic nucleases DFF40/CAD and endonuclease G, J Cell Biochem. 94 (2005) 1078–1087.
[105] K. Samejima, W. Earnshaw, Trashing the genome: the role of nucleases during apoptosis, Nat Rev Mol Cell Biol. 6 (2005) 677–688.
[106] M.A. Szczygiel, S. Moisyadi, W.S. Ward, Expression of foreign DNA is associated with paternal chromosome degradation in intracytoplasmic sperm injection-mediated transgenesis in the mouse, Biol Reprod. 68 (2003) 1903–1910.
[107] S. Perreault, B. Zirkin, Sperm nuclear decondensation in mammals: role of sperm-associated proteinase in vivo, J Exp Zool. 224 (1982) 253–257.
[108] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature. 387 (1997) 569–572.
[109] P. Adenot, Y. Mercier, J. Renard, E. Thompson, Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos, Development. 124 (1997) 4615–4625.
[110] E. Aoki, R. Schultz, DNA replication in the 1-cell mouse embryo: stimulatory effect of histone acetylation, Zygote. 7 (1999) 165–172.
[111] J.A. Shaman, Y. Yamauchi, W.S. Ward, The sperm nuclear matrix is required for paternal DNA replication, J Cell Biochem. 102 (2007) 680–688.
[112] Y. Yamauchi, J.A. Shaman, W.S. Ward, Topoisomerase II-mediated breaks in spermatozoa cause the specific degradation of paternal DNA in fertilized oocytes, Biol Reprod. 76 (2007) 666–672.
[113] G. Frenette, J. Girouard, R. Sullivan, Comparison between epididymosomes collected in the intraluminal compartment of the bovine caput and cauda epididymidis, Biol Reprod. 75 (2006) 885–890.
[114] Y. Yamauchi, A. Ajduk, J. Riel, M.A. Ward, Ejaculated and epididymal mouse spermatozoa are different in their susceptibility to nuclease-dependent DNA damage and in their nuclease activity, Biol Reprod. 77 (2007) 636–647.
[115] Y. Yamauchi, J.A. Shaman, S.M. Boaz, W.S. Ward, Paternal pronuclear DNA degradation is functionally linked to DNA replication in mouse oocytes, Biol Reprod. 77 (2007) 407–415.
[116] C.J. Evans, R.J. Aguilera, DNase II: genes, enzymes and function, Gene. 322 (2003) 1–15.
[117] A.A. Larrea, I.M. Pedroso, A. Malhotra, R.S. Myers, Identification of two

conserved aspartic acid residues required for DNA digestion by a novel thermophilic Exonuclease VII in Thermotoga maritima, Nucleic Acids Res. 36 (2008) 5992–6003.
[118] P.A. Martin-DeLeon, Epididymal SPAM1 and its impact on sperm function, Mol Cell Endocrinol. 250 (2006) 114–121.
[119] R.G. Oshima, P.A. Price, Alkylation of an essential histidine residue in porcine spleen deoxyribonuclease, J Biol Chem. 248 (1973) 7522–7526.
[120] R.K. Sachs, L.R. Hlatky, B.J. Trask, Radiation-produced chromosome aberrations: colourful clues, Trends Genet. 16 (2000) 143–146.
[121] C. Légaré, B. Bérubé, F. Boué, L. Lefièvre, C.R. Morales, M. El-Alfy, et al., Hamster sperm antigen P26h is a phosphatidylinositol-anchored protein, Mol Reprod Dev. 52 (1999) 225–233.
[122] L. Hermo, D. Jacks, Nature's ingenuity: bypassing the classical secretory route via apocrine secretion, Mol Reprod Dev. 63 (2002) 394–410.
[123] M. Napirei, S. Ludwig, J. Mezrhab, T. Klöckl, H.G. Mannherz, Murine serum nucleases--contrasting effects of plasmin and heparin on the activities of DNase1 and DNase1-like 3 (DNase1l3), Febs J. 276 (2009) 1059–1073.
[124] M. Laskowski, Deoxyribonuclease I, in: P. Boyer, H. Landy, K. Myrback (Eds.), The Enzymes, 3rd ed, Academic Press, New York, 1971: pp. 289–311.
[125] M. Manin, P. Lecher, A. Martinez, S. Tournadre, C. Jean, Exportation of mouse vas deferens protein, a protein without a signal peptide, from mouse vas deferens epithelium: a model of apocrine secretion, Biol Reprod. 52 (1995) 50–62.
[126] W. Yang, J.Y. Lee, M. Nowotny, Making and breaking nucleic acids: two-Mg2+-ion catalysis and substrate specificity, Mol Cell. 22 (2006) 5–13.
[127] C. Baumann, B. Davies, M. Peters, U. Kaufmann-Reiche, M. Lessl, F. Theuring, AKR1B7 (mouse vas deferens protein) is dispensable for mouse development and reproductive success, Reproduction. 134 (2007) 97–109.
[128] S. Andonian, L. Hermo, Principal cells of the vas deferens are involved in water transport and steroid synthesis in the adult rat, J Androl. 20 (1999) 158–176.
[129] J. Kerr, A. Wyllie, A. Currie, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics, Br J Cancer. 26 (1972) 239–257.
[130] C.D. Bortner, J.A. Cidlowski, Cell shrinkage and monovalent cation fluxes: role in apoptosis, Archives of Biochemistry and Biophysics. 462 (2007) 176–188.
[131] F. Durrieu, K. Samejima, J.M. Fortune, S. Kandels-Lewis, N. Osheroff, W.C. Earnshaw, DNA topoisomerase IIalpha interacts with CAD nuclease and is involved in chromatin condensation during apoptotic execution, Curr Biol. 10 (2000) 923–926.
[132] P. Widlak, P. Li, X. Wang, W. Garrard, Cleavage preferences of the

apoptotic endonuclease DFF40 (caspase-activated DNase or nuclease) on naked DNA and chromatin substrates, J Biol Chem. 275 (2000) 8226–8232.
[133] F. Doonan, T.G. Cotter, Morphological assessment of apoptosis, Methods. 44 (2008) 200–204.
[134] J.Z. Parrish, D. Xue, Cuts can kill: the roles of apoptotic nucleases in cell death and animal development, Chromosoma. 115 (2006) 89–97.
[135] I. Rodriguez, C. Ody, K. Araki, I. Garcia, P. Vassalli, An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis, Embo J. 16 (1997) 2262–2270.
[136] E. Arama, J. Agapite, H. Steller, Caspase activity and a specific cytochrome C are required for sperm differentiation in Drosophila, Dev Cell. 4 (2003) 687–697.
[137] J.R. Huh, S.Y. Vernooy, H. Yu, N. Yan, Y. Shi, M. Guo, et al., Multiple apoptotic caspase cascades are required in nonapoptotic roles for Drosophila spermatid individualization, Plos Biol. 2 (2004) E15.
[138] E. Arama, M. Bader, G.E. Rieckhof, H. Steller, A ubiquitin ligase complex regulates caspase activation during sperm differentiation in Drosophila, Plos Biol. 5 (2007) e251.
[139] F. Brugnon, L. Janny, C. Artonne, B. Sion, J. Pouly, G. Grizard, Activated caspases in thawed epididymal and testicular spermatozoa of patients with congenital bilateral absence of the vas deferens and intracytoplasmic sperm injection outcome, Fertil Steril. (2008).
[140] H.-B. Zhang, S.-M. Lu, C.-Y. Ma, L. Wang, X. Li, Z.-J. Chen, Early apoptotic changes in human spermatozoa and their relationships with conventional semen parameters and sperm DNA fragmentation, Asian J Androl. 10 (2008) 227–235.
[141] A.H. Boulares, A.J. Zoltoski, F.J. Contreras, A.G. Yakovlev, K. Yoshihara, M.E. Smulson, Regulation of DNAS1L3 endonuclease activity by poly(ADP-ribosyl)ation during etoposide-induced apoptosis. Role of poly(ADP-ribose) polymerase-1 cleavage in endonuclease activation, J Biol Chem. 277 (2002) 372–378.
[142] W. Yang, Nucleases: diversity of structure, function and mechanism, Q Rev Biophys. 44 (2011) 1–93.
[143] M.A. Lagarkova, O.V. Iarovaia, S.V. Razin, Large-scale fragmentation of mammalian DNA in the course of apoptosis proceeds via excision of chromosomal DNA loops and their oligomers, J Biol Chem. 270 (1995) 20239–20241.
[144] M. Stanulla, J. Wang, D.S. Chervinsky, P.D. Aplan, Topoisomerase II inhibitors induce DNA double-strand breaks at a specific site within the AML1 locus, Leukemia. 11 (1997) 490–496.
[145] D.J. Miller, G.L. Smith, EGTA purity and the buffering of calcium ions in physiological solutions, Am J Physiol. 246 (1984) C160–6.

[146] R.Y. Tsien, New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures, Biochemistry. 19 (1980) 2396–2404.
[147] C. Vermeer, The binding of Gla-containing proteins to phospholipids, FEBS Lett. 173 (1984) 169–172.
[148] Y.Y. Yao, A.A. Shahbazian, K.I.K. Boström, Proline and gamma-carboxylated glutamate residues in matrix Gla protein are critical for binding of bone morphogenetic protein-4, Circulation Research. 102 (2008) 1065–1074.
[149] E.-J. Woo, Y.-G. Kim, M.-S. Kim, W.-D. Han, S. Shin, H. Robinson, et al., Structural mechanism for inactivation and activation of CAD/DFF40 in the apoptotic pathway, Mol Cell. 14 (2004) 531–539.
[150] I.I. Gromova, O.F. Nielsen, S.V. Razin, Long-range fragmentation of the eukaryotic genome by exogenous and endogenous nucleases proceeds in a specific fashion via preferential DNA cleavage at matrix attachment sites, J Biol Chem. 270 (1995) 18685–18690.
[151] T.K. Li, L.F. Liu, Tumor cell death induced by topoisomerase-targeting drugs, Annu. Rev. Pharmacol. Toxicol. 41 (2001) 53–77.
[152] M.-F. Counis, A. Torriglia, Acid DNases and their interest among apoptotic endonucleases, Biochimie. 88 (2006) 1851–1858.
[153] R.A. Gottlieb, J. Nordberg, E. Skowronski, B.M. Babior, Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification, Proc Natl Acad Sci USA. 93 (1996) 654–658.
[154] K. Kawane, H. Fukuyama, H. Yoshida, H. Nagase, Y. Ohsawa, Y. Uchiyama, et al., Impaired thymic development in mouse embryos deficient in apoptotic DNA degradation, Nat. Immunol. 4 (2003) 138–144.
[155] K. Dominguez, W.S. Ward, A novel nuclease activity that is activated by Ca(2+) chelated to EGTA, Syst Biol Reprod Med. 55 (2009) 193–199.
[156] C. Patton, S. Thompson, D. Epel, Some precautions in using chelators to buffer metals in biological solutions, Cell Calcium. 35 (2004) 427–431.
[157] S. Altairac, S. Zeggai, P. Perani, Y. Courtois, A. TORRIGLIA, Apoptosis induced by Na+/H+ antiport inhibition activates the LEI/L-DNase II pathway, Cell Death Differ. 10 (2003) 548–557.
[158] U. Stepińska, B. Olszańska, DNase I and II present in avian oocytes: a possible involvement in sperm degradation at polyspermic fertilisation, Zygote. 11 (2003) 35–42.
[159] Y. Nechaevsky, V. Ivanov, DNase II in spermatozoa of the loach misgurnus fossilis L, Biochemistry Mosc. 64 (1999) 488–493.
[160] R.J. Aitken, A.J. Koppers, Apoptosis and DNA damage in human spermatozoa, Asian J Androl. 13 (2011) 36–42.
[161] S. Ohkouchi, M. Shibata, M. Sasaki, M. Koike, P. Safig, C. Peters, et al., Biogenesis and proteolytic processing of lysosomal DNase II, PLoS ONE. 8 (2013) e59148.

[162] M.-F. Liu, X.-P. Wu, X.-L. Wang, Y.-L. Yu, W.-F. Wang, Q.-J. Chen, et al., The functions of Deoxyribonuclease II in immunity and development, DNA Cell Biol. 27 (2008) 223–228.
[163] D. Nadine, M. Sabine, Fast and Sensitive Colloidal Coomassie G-250 Staining for Proteins in Polyacrylamide Gels, Journal of Visualized Experiments. (2009).
[164] K. Kawane, H. Fukuyama, G. Kondoh, J. Takeda, Y. Ohsawa, Y. Uchiyama, et al., Requirement of DNase II for definitive erythropoiesis in the mouse fetal liver, Science. 292 (2001) 1546–1549.
[165] J.E. Gawecka, J. Marh, M. Ortega, Y. Yamauchi, M.A. Ward, W.S. Ward, Mouse zygotes respond to severe sperm DNA damage by delaying paternal DNA replication and embryonic development, PLoS ONE. 8 (2013) e56385.
[166] H. Fischer, J. Scherz, S. Szabo, M. Mildner, C. Benarafa, A. Torriglia, et al., DNase 2 is the main DNA-degrading enzyme of the stratum corneum, PLoS ONE. 6 (2011) e17581.
[167] K.P. Baker, W.F. Baron, W.J. Henzel, S.A. Spencer, Molecular cloning and characterization of human and murine DNase II, Gene. 215 (1998) 281–289.
[168] S. Nishimoto, K. Kawane, R. Watanabe-Fukunaga, H. Fukuyama, Y. Ohsawa, Y. Uchiyama, et al., Nuclear cataract caused by a lack of DNA degradation in the mouse eye lens, Nature. 424 (2003) 1071–1074.
[169] L. Padrón-Barthe, J. Courta, C. Leprêtre, A. Nagbou, A. Torriglia, Leukocyte Elastase Inhibitor, the precursor of L-DNase II, inhibits apoptosis by interfering with caspase-8 activation, Biochim Biophys Acta. 1783 (2008) 1755–1766.
[170] E. Axnér, Sperm maturation in the domestic cat, Theriogenology. 66 (2006) 14–24.
[171] K.M. Baska, G. Manandhar, D. Feng, Y. Agca, M.W. Tengowski, M. Sutovsky, et al., Mechanism of extracellular ubiquitination in the mammalian epididymis, J Cell Physiol. 215 (2008) 684–696.
[172] S. Andonian, L. Hermo, Cell- and region-specific localization of lysosomal and secretory proteins and endocytic receptors in epithelial cells of the cauda epididymidis and vas deferens of the adult rat, J Androl. 20 (1999) 415–429.
[173] J. Dacheux, C. Belleannée, R. Jones, V. Labas, M. Belghazi, B. Guyonnet, et al., Mammalian epididymal proteome, Mol Cell Endocrinol. (2009).
[174] N. Eshima, A. Muta, M. Anai, Inhibitory effect of bovine serum albumin on acid deoxyribonuclease from rat small intestinal mucosa, J. Biochem. 94 (1983) 345–352.
[175] S. Fouchécourt, S. Métayer, A. Locatelli, F. Dacheux, J.-L. Dacheux, Stallion epididymal fluid proteome: qualitative and quantitative characterization; secretion and dynamic changes of major proteins, Biol

Reprod. 62 (2000) 1790–1803.
[176] Y. Okabe, K. Kawane, S. Akira, T. Taniguchi, S. Nagata, Toll-like receptor-independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation, J. Exp. Med. 202 (2005) 1333–1339.
[177] G.E. Olson, V.P. Winfrey, S.K. NagDas, M.H. Melner, Region-specific expression and secretion of the fibrinogen-related protein, fgl2, by epithelial cells of the hamster epididymis and its role in disposal of defective spermatozoa, J Biol Chem. 279 (2004) 51266–51274.
[178] P.M. Henson, D.L. Bratton, V.A. Fadok, The phosphatidylserine receptor: a crucial molecular switch? Nat Rev Mol Cell Biol. 2 (2001) 627–633.
[179] C.W.Y. Chan, L.S. Kay, R.G. Khadaroo, M.W.C. Chan, S. Lakatoo, K.J. Young, et al., Soluble fibrinogen-like protein 2/fibroleukin exhibits immunosuppressive properties: suppressing T cell proliferation and inhibiting maturation of bone marrow-derived dendritic cells, J Immunol. 170 (2003) 4036–4044.
[180] C.W.Y. Chan, M.W.C. Chan, M. Liu, L. Fung, E.H. Cole, J.L. Leibowitz, et al., Kinetic analysis of a unique direct prothrombinase, fgl2, and identification of a serine residue critical for the prothrombinase activity, J Immunol. 168 (2002) 5170–5177.
[181] G. Barroso, M. Morshedi, S. Oehninger, Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa, Hum Reprod. 15 (2000) 1338–1344.