The Role of DNase X in Skeletal Muscle Addressed by Targeted Disruption of the Gene in a Mouse Model

By

Iran Rashedi

A thesis submitted to

The Faculty of Graduate Studies of the University of Manitoba

In partial fulfillment of the requirements for the degree of

Master's of Science

Department of Biochemistry and Medical Genetics

Faculty of Medicine

University of Manitoba

Winnipeg

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION

The Role of DNase X in Skeletal Muscle Addressed by Targeted Disruption of the Gene in a Mouse Model

BY

Iran Rashedi

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

Of

MASTER OF SCIENCE

This work is dedicated to my dearly loved family whose ever-lasting support gives me the chance to follow my dreams, and to all my teachers who have taught me great lessons both in my life and my academic career.

Acknowledgements

I would like to express my gratitude to all those who gave me the possibility to complete this work. I am deeply indebted to my former supervisor, Dr. Marek Los, whose guidance, encouragement and endless support provided me the opportunity to do this research in the first place, and helped me through all the time of this work. I am enormously grateful to my supervisor, Dr. Phillip Gardiner, whose support in the difficult time of this work helped me to complete this research and write my thesis. I am obliged to my advisory committee members, Dr. Klaus Wrogemann, Dr. Jimming Kong and Dr. Jeffrey Wigle for all their valuable advice as well as many other aspects of their care and consideration without which this work would likely not have matured. I would also like to express my gratitude to Dr. Judy Anderson for her valuable inputs and generous sharing of her knowledge with me especially at the very first steps of this project.

I wish to thank the Department of Biochemistry and Medical Genetics for all the encouraging support they provided me to get this job done. I specifically want to thank Tuntun and Jan who were always there to offer their help, share their supporting thoughts and give me the motivation for further challenges. I have furthermore to thank Mary in Manitoba Institute of Cell Biology for all her assistance in paperwork and expediting the ordering process during the last couple of months of this work.

I owe a lot to my lab fellows with whom I've spent numerous good moments during both working and fun hours. Especially, I would like to thank Reddy, Evan, Saeid and Soumya for the valuable discussions we had. We shared our thoughts and feelings in all ups and downs, and learned a lot going through an exceptional experience.

I would like to acknowledge all the financial support I was provided over the period of my work here including Manitoba Health Research Council Studentship and the Government of Manitoba Graduate Scholarship.

And finally, my special thanks are due to my family and friends for the love and care they shared with me and for their patience and encouragements which inspired me to go ahead and accomplish this work.

Table of Contents

Abstract

DNase X is a DNase I-like endonuclease which is highly expressed in cardiac and skeletal muscles. The protein is highly similar to DNase I and exhibits the same enzymatic properties known for DNase I *in vitro*. A handful of previous studies has provided information on the cellular localization of the protein, and its biochemical and functional features as a DNA hydrolyzing enzyme; however, the biological role of DNase X is not yet known. To address the function of DNase X *in vivo*, we have generated a DNase X mutant mouse by targeted disruption of the gene. DNase $X^{(-/-)}$ mice are viable, fertile, and develop normally after birth, though are lighter in body weight than their wild-type littermates. We have shown that DNase X is dispensable for skeletal muscle development, and macroscopic structure and morphology of muscles of the hindlimb in DNase $X^{(-/-)}$ mice are comparable with wild-type muscles. DNase $X^{(-/-)}$ mice displayed reduced fatigue tolerance when tested for their running performance. Histology revealed that a notable proportion of fibers in soleus of older DNase $X^{(-/-)}$ mice have evidence of damage/regeneration, which affected both slow and fast fibers equally. This phenotype was most noticeable in soleus but also appeared in some other leg muscles in DNase $X^{(-/-)}$ mice after treadmill running whereas no similar changes were observed in any wild-type muscles studied. We carried out quantitative real time RT-PCR, and found no evidence of sex- and age-association in the expression of DNase X in skeletal muscle. We examined the possibility of functional redundancy by evaluating the mRNA expression profile of other members of DNase I family in DNase $X^{(-/-)}$ mice, and found no significant variation between the wild-type and mutant muscles. We also found that nuclease activity was diminished in tissue extracts of DNase X deficient muscles, whereas no similar difference

was observed in other tissues. These findings suggest that DNase X is involved in the normal physiology of skeletal muscle by preventing age- and stress-related fiber damage in certain muscle types. This is the first report on DNase X null mice; however, additional investigations will need to be performed to further characterize the muscle-specific phenotype in these mice, and to unravel the mechanisms contributing to this unique phenotype.

List of Figures

List of Tables

Abbreviations

aa	amino acid
AIF	apoptosis-inducing factor
AKAP	A-kinase anchor protein
AMID	AIF-homologous mitochondrion-associated inducer of death
Apaf-1	apoptosis protease activating factor-1
bHLH	basic helix-loop-helix
bp	base pair
BSA	bovine serum albumin
CAD	caspase-activated DNase
CaMK	calcium/calmodulin-dependent protein kinase
CIDE	cell death-inducing DFF45-like effector
CSA	cross sectional area
Ct	cycle threshold
dATP	deoxyadenosine triphosphate
DFF	DNA fragmentation factor
DLAD	DNase2-like acid DNase
DNase	deoxyribonuclease
DNaseIl2	DNase I-like 2
dsDNA	double strand DNA
EDL	extensor digitorum longus
Endo G	endonuclease G
ES	embryonic stem cell

FGF	fibroblast growth factor
GAAD	granzyme A-activated DNase
GPI	glucosylphosphatidylinositol
HAT	histone acetyltransferase
HDAC	histone deacetylase
ICAD	inhibitor of CAD
IGAAD	inhibitor of Granzyme A-Activated DNase
IGF	insulin-like growth factor
kb	kilobase
KO	knockout
L-DNase II	LEI-derived DNase II
LEI	leukocyte elastase inhibitor
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MEF	myocyte enhancer factor
MHC	myosin heavy chain
MRF	muscle regulatory factor
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
MyoD	myoblast determination protein
Myf	myogenic factor
neo	neomycin resistant gene
NFAT	nuclear factor of activated T cells

NM23-H1	H1 member of nucleoside diphosphate kinase gene-protein family
NOS; e/nNOS	nitric oxide synthase; endothelial/neuronal NOS
Pax	paired-box homeodomain
PBS	phosphate buffered saline
PDGF-A	platelet-derived growth factor-A
QC	quadriceps
qRT-PCR	quantitative real-time PCR
rhDNase I	recombinant-human DNase I
ROS	reactive oxygen species
RT	room temperature
SLE	systemic lupus erythematosus
SR	sarcoplasmic reticulum
ssDNA	single strand DNA
TA	tibialis anterior
TNF-α	tumor necrosis factor alpha
UTR	untranslated region
WT	wild-type
Y2H	yeast two-hybrid

Literature Review

1) Deoxyribonucleases

Deoxyribonuclases (DNases) are enzymes that cleave the phosphodiester bond in the DNA backbone and promote the hydrolytic cleavage of the DNA molecule, and play important roles in many biological processes during cell growth and development (Buttin and Kornberg, 1966; El-Shemerly et al., 2005; Lehman, 1967; Neale et al., 2005; Yan et al., 2006). These include DNA synthesis and repair, maintaining chromosome stability and suppressing tumor development, and regulating intracellular metabolism of nucleic acids. The involvement of nucleases in programmed cell death indicates the crucial role of these proteins in tissue remodeling and homeostasis (Enari et al., 1998; Jacobson et al., 1997; Liu et al., 1998a; Peitsch et al., 1993). The importance of the DNA-degrading function of DNases in normal physiology and in the prevention of pathologic conditions such as progression of autoimmune responses have been addressed by studying mutations which disrupt DNases function (Kawane et al., 2001; Napirei et al., 2000; Nishimoto et al., 2003; Shin et al., 2004; Yasutomo et al., 2001). A wide variety of nucleases have been identified thus far. Effective control of these nucleases in cellular processes *in vivo* is ensured by several mechanisms evolved to regulate the expression as well as the activity of these enzymes. These include but are not limited to the control at the gene expression level and the existence of alternative transcripts, and regulation of translation as seen in DNase I (Kominato et al., 2006) and DNase I-like1 (Coy et al., 1996), the specific activation requirement such as pH as seen in DNase II enzymes (Evans and Aguilera, 2003), the moderating effect of chaperone and/or inhibitor molecules as seen in the interaction of CAD/ICAD (Enari et al., 1998; Liu et al., 1997), and finally the

sequestration in subcellular organelles such as compartmentalization of EndoG in the mitochondrial intermembrane space (Ohsato et al., 2002). DNases differ in their biochemical properties such as substrate specificity, mechanism of action, biological functions and the expression pattern in different tissues. Based on their similarities either at the sequence and structure levels or their functional characteristics, these proteins can be classified into several groups. In one classification, DNases are categorized by their dependency on metal ions as cofactors for their enzymatic activity. Endonucleases in these groups may act independently of bivalent cations, or may require the presence of Mg^{2+}, or both Ca^{2+} and Mg^{2+} ions for their function (Counis and Torriglia, 2006). Brief descriptions of the best known DNases in each group and their features will follow.

1-1) Cation-independent DNases

DNase II or the "acid DNase" family is a well known example in this group. DNase II family consists of three enzymes that promote DNA hydrolysis under acidic conditions. These enzymes reside within the lysosomes and are most active at low pH range (4.8-5.6), and do not require bivalent cations for their activity (Liao et al., 1989). Enzymes in this group include DNase II (also called DNase II-α) (Liao, 1985; Yasuda et al., 1992; Yasuda et al., 1998), DNase II-β (DLAD/DNase II-like acid DNase) (Krieser et al., 2001; Tanuma and Shiokawa, 1999), and L-DNase II (LEI-derived DNase II) (Torriglia et al., 1998). L-DNase II is derived from a precursor molecule, Leukocyte Elastase Inhibitor, which initially has an anti-protease activity. Proteolytic cleavage of LEI and a subsequent conformational modification abolishes the anti-protease activity of the protein and produces L-DNase II which has endonuclease properties. This conversion

can also be induced *in vitro* in acidic pH (Padron-Barthe et al., 2007; Torriglia et al., 1998). DNase II nucleases are encoded by distinct genes and cleave dsDNA nonspecifically to produce short oligonucleotides with 3'-P/5'-OH termini (Barry and Eastman, 1993; Harosh et al., 1991). DNase II and LEI/L-DNase II are ubiquitously expressed (Torriglia et al., 1998; Yasuda et al., 1998) while DLAD shows a restricted tissue distribution being mostly found in lens, salivary gland followed by lung, prostate, and lymph node (Counis and Torriglia, 2006; De Maria and Bassnett, 2007; Krieser et al., 2001; Reynolds et al., 1996). L-DNase II degrades DNA in apoptotic cells while DNase II functions in phagocytes to clear the engulfed DNA of apoptotic cells (McIlroy et al., 2000; Odaka and Mizuochi, 1999).

1-2) Mg^{2+}-dependent DNases

One of the best described nucleases in this group is CAD (murine), which is also known as DNA fragmentation factor 40 (DFF40, human) (Mukae et al., 1998; Scholz et al., 2002). CAD is the main endonuclease that promotes cell-autonomous DNA fragmentation and plays a major role during apoptosis (Earnshaw, 1995; Enari et al., 1998). It generates double-stranded breaks in the internucleosomal linker regions across chromatin to produce blunt ends with 5′-P and 3′-OH free ends. It first creates 50- to 300-kb products which are then further cleaved to oligonucleosomal fragments (Widlak et al., 2000). CAD has been shown to have a limited tissue distribution; it was mainly found in heart, placenta, kidney, pancreas, spleen, prostate, ovary, colon, and peripheral blood leukocytes while no expression was detected in several tissues including lung, liver, skeletal muscle and thymus (Mukae et al., 1998). In living cells, CAD resides in the nucleus (Liu et al., 1998b; Samejima and Earnshaw, 1998) and its nuclease activity is

suppressed by the formation of a complex with the ICAD/DFF45 (Enari et al., 1998; Liu et al., 1997) through a shared homologous domain at their N-termini (CAD or CIDE domain) (Inohara et al., 1998; Mukae et al., 1998). ICAD inhibits DNase activity of CAD not by blocking its active site but by impairing its binding to substrate DNA (Enari et al., 1998; Sakahira et al., 2001). In the presence of apoptotic stimuli; however, activated caspases promote the proteolytic cleavage of ICAD that reduces its binding affinity for CAD (Liu et al., 1997; Sakahira et al., 1998). Two forms of ICAD (ICAD-L/DFF45, ICAD-S/DFF35) have been identified both of which are cleaved by caspases. This leads to homo-oligomerization and activation of CAD and the subsequent initiation of DNA degradation (Enari et al., 1998; Liu et al., 1997; McCarty et al., 1999; Sakahira et al., 1998). GAAD (granzyme A-activated DNase) is another DNase in this group which is activated by a similar mechanism through granzyme A-mediated cleavage of its inhibitor, IGAAD (Fan et al., 2003). GAAD/NM23-H1 was initially identified as a tumor metastasis suppressor protein (Steeg et al., 1988) which probably mediates its function by repressing transcriptional activity of PDGF-A (Ma et al., 2002). Upon activation, GAAD is able to induce single-stranded nicks in DNA molecules and produces multi-kb fragments which may not be detected by conventional assays to identify apoptotic DNA ladders (Lieberman and Fan, 2003).

Endonuclease G (EndoG) is another well known example of Mg^{2+}-dependent nucleases which is highly conserved in mammals (Huang et al., 2002; Low et al., 1988). The protein is located in the mitochondrial intermembrane space (Ohsato et al., 2002) but upon activation of apoptosis it is released into the cytosol from where it translocates into the nucleus to induce DNA degradation in a caspase-independent manner (Li et al., 2001;

van Loo et al., 2001). EndoG nicks ss-DNA and shows a preference to cleave guanine-rich sequences and produces 5'-P/3´-OH ends (Ruiz-Carrillo and Renaud, 1987). EndoG is ubiquitously expressed and is most abundant in the adult liver, heart and skeletal muscle (Apostolov et al., 2007; Prats et al., 1997). The normal biological function of EndoG is not clearly understood; whereas studies with endonuclease $G^{-/-}$ mice suggest the function of the enzyme to be unrelated to mtDNA replication (Irvine et al., 2005), other studies indicate that it plays a role in cell proliferation (Cote and Ruiz-Carrillo, 1993; Huang et al., 2006).

Apoptosis-inducing factor (AIF) is another mitochondrial-confined endonuclease in this group. In the presence of Mg^{2+}, AIF can bind to DNA in a non sequence-specific manner and induce high-molecular weight (~ 50kb) DNA fragmentation independent of caspases (Susin et al., 1999). When cell death is induced, AIF is released and translocates into the nucleus where it is believed to recruit and activate other endonucleases to promote DNA fragmentation (Vahsen et al., 2006; Ye et al., 2002). This nuclear multiprotein complex, called the degradasome, is a repertoire of interacting nucleases and other proteins such as cyclophilin A which has been shown to be essential for proapoptotic activity of AIF (Cande et al., 2004; Parrish et al., 2003). AIF is ubiquitously expressed and has a dual function in cellular processes. In addition to its proapoptotic role when translocated into the nucleus (Susin et al., 2000; Yu et al., 2002), AIF is involved in oxidoreductase activity of the cell through its FAD-binding domain when located in the mitochondrial intermembrane space (Miramar et al., 2001; Susin et al., 1999). The importance of AIF function in physiology of mitochondrial respiration has

been shown in animal studies in which the AIF-mutant mice show a reduced tolerance to oxidative stress (Klein et al., 2002).

Other nucleases in this group may include AMID, a homolog of AIF, which is believed to reside primarily in the cytoplasm and triggers apoptosis upon overexpression (Ohiro et al., 2002; Wu et al., 2002). Ape1 (Apurinic/Apyrimidinic endonuclease) is another Mg^{2+}-sensitive DNase, best known for its DNA repair function, which has been shown to promote apoptotic DNA fragmentation (Demple et al., 1991; Robson and Hickson, 1991; Yoshida et al., 2003). Some of these nucleases have been shown to be indispensable for normal development, yet the biological significance of others remain to be understood.

1-3) Mg^{2+}/Ca^{2+}-dependent DNases

This group comprises diverse nucleases including DNase I-like enzymes, and many other endonucleases which have been partially identified and are known by their molecular weights. This latter group includes but is not limited to NUC 18 (an 18 kDa endonuclease) (Gaido and Cidlowski, 1991; Montague et al., 1997), NUC 58 (Deng and Podack, 1995), NUC 70 (Urbano et al., 1998), 27 kDa (Ribeiro and Carson, 1993) and 97 kDa (Pandey et al., 1997) endonucleases. These DNases have been isolated and purified from different tissues and promote different biological functions. For instance, NUC18 activation has been observed in the course of glucocorticoid-induced apoptosis in thymocytes (Compton and Cidlowski, 1987), while NUC70 is exclusively expressed in hematopoetic cells and although has not proven to play a key role in apoptosis, this protein translocates into the nucleus when cell death is induced (Urbano et al., 1998).

1-3-1) DNase I family of endonucleases

DNase I family consists of four distinct enzymes which share extensive similarities with each other. These include DNase I (Price, 1975), DNase I-like1/DNase X (Parrish et al., 1995), DNase I-like2 (Rodriguez et al., 1997) and DNase I-like 3/DNase γ (Baron et al., 1998; Rodriguez et al., 1997) which share 38-56% identity at the amino acid level [Fig. 1]. All the proteins in this family have N-terminal signal peptides, and the residues at their likely active sites are well-conserved (Liu et al., 1999; Shiokawa and Tanuma, 2001). Furthermore, there are conserved regions with no currently assigned function among these nucleases. These nucleases also exhibit identical enzymatic properties. They are active in the presence of Ca^{2+} and Mg^{2+}/Mn^{2+} ions and are inhibited by Zn^{2+} ions. They have a preference to cut dsDNA and to cleave it to produce 5'-P and 3'-OH termini (Kishi et al., 2001; Price, 1975; Shiokawa and Tanuma, 2001). Endonucleases in this group exhibit optimum activity at neutral pH with an exception being DNase I-like 2 which is most active at acidic pH range. DNase I nucleases are encoded by distinct genes which are expressed at various levels in a wide variety of tissues; however, high levels of expression of these enzymes can be exclusively found in certain tissues/organs (Parrish et al., 1995; Rodriguez et al., 1997; Shiokawa and Tanuma, 2001; Zeng et al., 1997). Given the similarities between the members of this family, and since DNase I is the only nuclease in this group whose structure is resolved and whose function has been extensively studied, the key properties of this DNase will be discussed in more detail.

Figure 1. Comparison of primary structures of nucleases in human DNase I family. Multiple alignment was performed using Clustal W program. These proteins share 37-56% identity at the amino acids level. Position of structurally and functionally important residues are indicated by arrowheads and boxes, and are based on the known structure of DNase I. These residues are well conserved in sequences of other DNases in this family. Blue box, putative signal sequences predicted by PSORT II program (http://psort.nibb.ac.jp/). Intracellular; red box, residues in two active sites (boxes with asterisks indicate residues in main active site); green box, residues forming disulfide bridge; arrowheads, positions of residues forming two structural Ca^{2+}-binding sites. The RefSeq accession numbers for sequences are: DNase I, NP_005214; DNase X, NP_001009932; DNaseII2, NP_001365; DNaseII3, NP_004935.

1-3-1-1) DNase I

DNase I is a secretory glycoprotein and is the major nuclease present in serum, urine and body secreta (Kishi et al., 1990a; Kishi et al., 1990b). It is one of the best characterized enzymes with known structural, functional and biochemical properties. *DNASEI* is a polymorphic gene that code for a 282-amino acid protein in human with six known codominant allelic variants (Iida et al., 1997; Yasuda et al., 1995a; Yasuda et al., 1995c). It was the first DNase whose 3D structure was crystallographically resolved (Oefner and Suck, 1986; Suck et al., 1984). Several DNase I isozymes are known that differ in their primary structures and/or carbohydrate chain contents (Abe and Liao, 1983; Kishi et al., 1995; Yasuda et al., 1995b); nevertheless, all different isotypes display similar physiological and catalytic properties in biological conditions (Price et al., 1969a; Salnikow et al., 1970).

DNase I cleaves DNA in a non base/sequence-specific manner; however, it shows a preference to cleave dsDNA at the 5' side of pyrimidines, and seems to recognize sequence-specific structural features such as local variations in the twist angle between adjacent base pairs in dsDNA (Lomonossoff et al., 1981; Suck et al., 1984). The hydrolytic reaction of DNA cleavage is performed via a chain of proton acceptation/donation involving Glu, His, and water molecules. DNase I interacts with the substrate dsDNA through a shallow groove between two β-sheets in its structure, with the interacting surface covering about six base pairs at one side of the DNA double helix. This binding results in the widening of the minor groove and bending of the DNA double helix far from the binding surface (Suck and Oefner, 1986; Weston et al., 1992). The tight fit of the interacting surfaces is an important factor to determine the feature and

frequency of cutting by DNase I. It has been shown that the variations in the minor groove width as well as the flexibility of dsDNA are recognized by the enzyme and are main elements determining the DNase I hypersensitive regions (Lomonossoff et al., 1981; Nelson et al., 1987). Residues directly involved in catalytic reaction and those assisting the binding of the enzyme to its substrate are highly conserved among different species and have been revealed through different experiments including mutation analysis (Suck and Oefner, 1986).

DNase I has two strong Ca^{2+}-binding sites (K_D= 1.4×10^{-5}) which are essential for both enzymatic activity and structural integrity of the protein. In addition to stabilizing the loop regions in the molecule, Ca^{2+} protects DNase I from proteolytic digestion, and also prevents the reduction of essential disulfide bond in the molecule and assists the refolding of the semi-reduced enzyme (Oefner and Suck, 1986; Price et al., 1969b). These structural Ca^{2+} ions, however, are different from the calcium found at the catalytic center which is essential for enzymatic activity. The Ca^{2+} at the active site seems to be involved in correct positioning of the phosphodiester bond with respect to the enzyme, and facilitating the nucleophilic attack by its positive charge (Suck and Oefner, 1986). In addition to binding to Ca^{2+} and DNA, DNase I binds to both monomeric (G-actin) and polymeric (F-actin) forms of actin with high affinity (K_D about 10 pM). The 1:1 complex between G-actin and DNase I is able to inhibit both polymerization of actin monomers and nuclease activity of DNase I, and binding of the enzyme to F-actin is strong enough to depolymerize F-actin polymers (Lazarides and Lindberg, 1974; Mannherz et al., 1975). The biological significance of this interaction is not known yet. It might function either as an inhibitory mechanism to control the nucleolytic activity of DNase I during cell cycle

or it may imply a regulatory role for formation and function of actin filaments (Lazarides and Lindberg, 1974).

1-3-1-1-1) Clinical importance of DNase I

DNase I is generally regarded to have major role in the digestion of dietary DNA; however, the enzyme has been purified from different mammalian tissues suggesting other functions for the protein in biological processes (Nadano et al., 1993; Polzar et al., 1994). In humans, more than 60 SNPs are known for DNase I gene, which are found at 5'-UTR, introns and the coding sequences. These polymorphisms along with mutations in gene sequence have been shown to be associated with susceptibility to specific diseases including systemic lupus erythematosus (SLE) (Yasutomo et al., 2001), gastric and colorectal carcinoma (Tsutsumi et al., 1998; Tsutsumi et al., 2000), and myocardial infarction (MI) (Kumamoto et al., 2006) in certain populations. DNase I has been considered as a novel diagnostic tool and therapeutic agent in certain clinical conditions. These applications are mainly based on the analysis of DNase I activity in body fluids and/or phenotype identification by isoelecteric focusing separation of different isozymes (Basso et al., 1985; Funakoshi et al., 1979a; Tsutsumi et al., 2000). The abrupt increase of DNase I in the serum of patients with acute MI makes it a potential marker for early-phase of myocardial ischemia/infarction (Kawai et al., 2004). Additionally, significant increase in serum DNase I activity was detected during the development of breast cancer (Ramandanis et al., 1982) whereas cases of pancreatitis and glomerulonephritis have been correlated with reduced levels of DNase I activity in blood (Chitrabamrung et al., 1981; Funakoshi et al., 1979b). As a therapeutic agent, aerosolized recombinant-human DNase

I (rhDNase I) has been widely used to improve symptoms in cystic fibrosis by decreasing the viscosity of mucosal secretion (Laube et al., 1996; Wilmott et al., 1996). Moreover, rhDNase I has shown dramatic effects in the management of both the acute and refractory forms of asthma (Patel et al., 2000).

1-3-1-2) DNase X

DNase X is the first human DNase protein identified to be homologous to DNase I (thus called DNase I-like1) (Parrish et al., 1995). The protein is 38% identical and 58% similar in primary sequence to that of DNase I, and exhibits the same bivalent cation dependency as DNase I (activated by Ca^{2+} and Mg^{2+} in a synergistic manner and strongly inhibited by Zn^{2+}) (Coy et al., 1996; Parrish et al., 1995; Shiokawa and Tanuma, 2001). Similar to DNase I, the nuclease activity of DNase X is inhibited by G-actin, though the actin-binding residues in DNase I molecule are not well conserved in the DNase X sequence. DNase X gene is located at q28 region of the human X chromosome and is highly expressed in both cardiac and skeletal muscles, where the other DNase I family members are barely expressed (Los et al., 2000; Parrish et al., 1995; Pergolizzi et al., 1996; Shiokawa and Tanuma, 2001). This muscle-specific DNase is also found at low to moderate levels in most tissues, with the lowest expression levels in brain and thymus (Los et al., 2000; Shiokawa et al., 2005). Several alternative transcripts of DNase X have been identified in different tissues which differ in the 5′-UTR and all code for an identical protein (Coy et al., 1996; Los et al., 2000; Pergolizzi et al., 1996). Many of the features of DNase X gene/protein are conserved among mammals (Shiokawa et al., 2005). The murine DNase X is > 66% identical to human DNase X at the amino acid

sequence level and exhibits the same distribution being highly expressed in cardiac and skeletal muscles (Los et al., 2000; Shiokawa et al., 2005).

Figure 2. Schematic representation of DNase X mature protein. DNase X contains a predicted N-terminus signal peptide, which is removed from the mature protein. A hydrophobic sequence at the C-terminus of the protein may serve as a transmembrane (TM) segment, or GPI-modification site (GMS) to anchor the protein in the membrane. The presence of a helix-loop-helix (HLH) motif in DNase X structure suggests that the protein may function in a dimer complex. The hatched box represents region containing active sites, S-S bridge, and Ca2+-binding sites.

Human DNase X is a 302-aa protein with a predicted N-terminus signal peptide, a C-terminus hydrophobic stretch which may serve as a membrane-associated segment, and a helix-loop-helix domain (Parrish et al., 1995) [Fig. 2]. All mammalian DNase X proteins have potential sites for N-glycosylation, and the leader peptide and the C-terminal hydrophobic region seem to be conserved in their sequences (Shiokawa et al., 2005). DNase X has been shown to be glycosylated, although it is not proven to be secreted form the cell (Los et al., 2000; Shiokawa and Tanuma, 2001). It has been reported that DNase X is a GPI-anchored membrane protein whose function is to provide a barrier to inhibit endocytosis-mediated transfer of foreign DNAs by hydrolyzing them

in endocytic vesicles (Shiokawa et al., 2007). DNase X has been reported to be able to induce apoptosis upon overexpression and cause internucleosomal DNA-fragmentation in isolated nuclei (Los et al., 2000); however, involvement of DNase X in apoptosis was not detected in a separate study using a different cell line (Shiokawa and Tanuma, 2001).

1-3-1-3) DNase I-like 2

DNase I-like 2 (DNaseII2) is the only member in the DNase I family and the only enzyme in divalent cation-dependent nucleases group that has acidic pH optimum (pH 5-6) (Shiokawa et al., 2004). *DNASEII2* codes for a protein which is 56% identical to DNase I in amino acid sequence (Rodriguez et al., 1997). Several splice variants of *DNASEII2* have been identified that code for at least two different proteins (299 and 278 aa) that exhibit identical enzymatic properties (Shiokawa et al., 2004). The proline-rich domain which is a unique feature of DNaseII2 is absent in the short form of the protein (DNaseII2S). DNaseII2 was primarily found to be expressed in fetal and adult brain; however, later studies demonstrated a low level of expression in almost all tissues. DNaseII2S seems to be exclusively expressed in myeloid cells (Rodriguez et al., 1997; Shiokawa et al., 2004). DNaseII2 is located in the cytoplasm, and similar to DNase I is secreted *in vitro* (Shiokawa and Tanuma, 2001). In cultured cells, DNaseII2 did not translocate into the nucleus during apoptosis (Shiokawa and Tanuma, 2001); yet it is likely that this nuclease becomes activated and plays a role in apoptosis *in vivo* following the intracellular pH changes during the cell death process. Shiokawa *et al.* showed that the expression of DNaseII2 is induced by TNF-α, most likely through the interaction with a potential NFκB binding site in the 5′ flanking sequence of the gene (Shiokawa et al.,

2004). Subsequently, the authors have proposed that DNaseII2 becomes activated via the NFκB pathway and secreted into the extracellular space to play a role in the clearance of harmful DNA of damaged cells and pathogens.

1-3-1-4) DNase I-like 3

DNase I-like 3 (DNaseII3/DNase γ) is a 305-aa protein which is 46% identical to DNase I at the amino acid level (Rodriguez et al., 1997). The protein contains two functional nuclear localization signals which are not found in other members of the DNase I family (Rodriguez et al., 1997; Shiokawa et al., 2003). DNaseII3 is predominantly expressed in lymphoid organs such as the spleen, lymph node, thymus and bone marrow (Shiokawa and Tanuma, 2001; Zeng et al., 1997). It is primarily localized in the nucleus and has been shown to become activated and promote DNA fragmentation during apoptosis (Shiokawa et al., 2003; Yakovlev et al., 1999). The biological role of DNaseII3 is not known; however, it has been reported to be involved in the generation of double strand breaks in variable region of immunoglobulin genes contributing to the rearrangement of immunoglobulin genes through somatic hypermutation (Okamoto et al., 2005). DNaseII3 is scarcely expressed in adult muscle cells where DNase X is highly expressed. Nevertheless, it has been shown that DNaseII3 is upregulated in C2C12 cultured myoblasts during myogenic differentiation (Shiokawa et al., 2002). DNaseII3 is suggested to be involved in apoptosis associated with myogenesis, and induce DNA degradation in myoblasts that fail to commit to differentiation.

1-4) Animal models to study DNases function

1-4-1) DNase II

DNase II has been shown indispensable for erythropoiesis in mouse fetal liver. DNase II-null mice showed multiple organ defects including the kidney, diaphragm, lungs and severe anemia (Kawane et al., 2001; Krieser et al., 2002). While heterozygous mutant mice were healthy, homozygous DNase II deficient mice died either *in utero* or just after birth owing to severe anemia and/or asphyxia. Histologic examination of different tissues in these mice revealed the presence of many foci with central macrophages containing inclusions that seemed to be engulfed but undigested pycnotic nuclei. The defect seemed to be in phagocyte-mediated degradation of DNA expelled from erythroid precursor cells by macrophages lacking DNase II, and not an autonomous DNA fragmentation defect in DNase II deficient erythrocyte lineage. This accumulation of undigested DNA seems to trigger an acute cellular response by upregulating interferon-inducible genes leading to animal death. This was identified by generating mouse deficient in both DNase II and interferon type 1 receptor, which was viable despite the presence of undigested DNA in its tissues (Kawane et al., 2001; Yoshida et al., 2005).

1-4-2) CAD

The physiological importance of engulfed-mediated DNA degradation and the involvement of DNase II which acts as the main enzyme in this process was further showed by generating $CAD^{(-/-)}$ mice as well as DNase $II^{(-/-)}/CAD^{(-/-)}$ double-knockout mice. CAD deficient mice were viable and developed normally whereas the phenotype of DNase $II^{(-/-)}/CAD^{(-/-)}$ embryos was similar to that of DNase $II^{(-/-)}$ mice (Kawane et al.,

2003). Kawane et al. showed that CAD deficient thymocytes did not undergo apoptotic DNA fragmentation; however, apoptotic cells were phagocytosed by macrophages and their DNA was successfully degraded. Further analysis showed that the development of thymus and T cells were significantly impaired in embryos lacking both DNase II and CAD. It was suggested that CAD in apoptotic thymocytes and DNase II in macrophage lysosomes act cooperatively to degrade the DNA of apoptotic cells during T cell development (Kawane et al., 2003). Recently, it was shown that inducible deletion of DNaseII gene in mice triggered the development of chronic polyarthritis similar to human rheumatoid arthritis (Kawane et al., 2006). It has been proposed that the initial event in the pathogenesis of polyartheritis phenotype is the failure of macrophages to degrade DNA during erythropoiesis. This results in upregulation of TNFα which in turn induces the generation of various proinflammatory cytokines by synovial cells.

1-4-3) DLAD

$DLAD^{(-/-)}$ mice showed no gross developmental abnormality but developed a progressive cataract (Nishimoto et al., 2003). The presence of undigested DNA in the epithelial layer of lens suggested that DLAD is involved in the degradation of DNA during differentiation of lens cells. In the absence of DLAD, undigested DNA accumulates in the fiber cells, which subsequently blocks the light path.

1-4-4) EndoG

EndoG mutant mice have been developed separately by three different groups (David et al., 2006; Irvine et al., 2005; Zhang et al., 2003). Studies by David *et al.* and

Irvine *et al.* demonstrated similar results which differed from those of study by Zhang *et al.* The original description of EndoG$^{(-/-)}$ mice by Zhang *et al.* delineated that these mice were not viable and died at an early stage (E2.5-E3.5) of embryonic life. They showed that the sensitivity to cell death stimuli was reduced in different cells in EndoG heterozygote mice, and suggested that EndoG is required for embryogenesis, and is involved in normal development by regulating the apoptosis at early developmental stages (Zhang et al., 2003). On the contrary, the results of the two other studies (David et al., 2006; Irvine et al., 2005) revealed that EndoG mice were viable and developed normally after birth. They showed that EndoG$^{(-/-)}$ cells exhibited a similar susceptibility to pro-death insults as compared to wild-type cells, and concluded that EndoG is dispensible for early embryogenesis, and that loss of protein function does not affect apoptosis in EndoG deficient cells. The discrepancy seemed to be a result of different targeting strategies used by these groups which resulted in the disruption of a neighboring gene (*D2Wsu81e*) with a partial overlap with *EndoG*, which was not compatible with life in mice generated by Zhang *et al.*

1-4-5) DNase I

DNase I-deficient mice showed an SLE-like phenotype with antibodies against nuclear antigens, and immune-complex glomerulonephritis (Napirei et al., 2000). The expression of DNase I was reduced to half in DNase I heterozygous mice compared with the wild-type littermates, and the mutant mice developed the phenotype in a DNase I dose dependent manner with the incidence of the disease being lower in heterozygous than in homozygous animals. Napirei *et al.* suggested a protective role for DNase I to

prevent the stimulation of an autoimmune response by removing the DNA from soluble or deposited nucleoprotein complexes. This may be of a particular importance at sites with high cellular turnover such as gasterointestinal and genitourinary tracts, where DNase I is expressed at high levels (Polzar et al., 1994; Takeshita et al., 1997). The involvement of DNase I in the pathogenesis of an SLE-type autoimmune phenotype was frequently evidenced by identifying DNase I gene mutations in SLE patients (Bodano et al., 2006; Shin et al., 2004; Yasutomo et al., 2001).

2) Skeletal muscle

Skeletal muscle is a syncytium of highly-arranged myofibers with postmitotic nuclei. It is rich in connective tissue, and is highly vascularized which provides it efficient access to oxygen and other nutrients. Skeletal muscle is a heterogeneous tissue composed of a variety of muscle fibers that differ in their metabolic and contractile properties (Brooke and Kaiser, 1970). As they mature, myofibers become innervated by single motor neuron whose activity is a factor to determine the characteristics of myofibers to form motor units. Myofibers are classically defined on the basis of the expression of specific myosin heavy chain (MHC) isoforms, and are divided into two major groups of slow-twitch (type I) and fast-twitch (type II) fibers. Type II fibers are further divided into several subtypes including type IIA, type IIX/IID and type IIB fibers (Bar and Pette, 1988; Gardiner, 2001; Schiaffino et al., 1989). Alongside the variability in the expression of MHC genes, muscle fibers differ in their content of other muscle-specific proteins as well. The slow- and fast-twitch fibers express different isoforms, or various concentrations of most of the myofiber proteins including tropomyosin, myosin light chain, membrane proteins

mediating calcium trafficking, and many metabolic enzymes (He et al., 2000; Okumura et al., 2005; Pette and Staron, 1997). This diversity enables different muscle groups, which contain various proportions of these fibers, to exhibit a variety of functional properties. Slow-twitch fibers have a low velocity of shortening and develop modest power. These fibers are rich in mitochondria and enzymes for oxidative metabolism, which make them resistant to fatigue and therefore suitable for long-term activities that need endurance. Fast-twitch fibers provide powerful, rapid and short-lasting contractions and sustain muscle performance in anaerobic activities. Type II fibers differ in their content of oxidative and glycolytic enzymes and subsequently exhibit various degrees of resistance to fatigue. Type IIB fibers have a pure glycolytic metabolism and high velocity of contraction whereas type IIA fibers benefit both oxidative and glycolytic pathways and possess the slowest speed of contraction among fast fibers (He et al., 2000; Takekura and Yoshioka, 1987). The main characteristics of these fiber types are summarized in Table 1.

Alongside the fibers that express a single MHC isoform, there is a wide spectrum of hybrid myofibers in a muscle that coexpress two different MHC isoforms (Billeter et al., 1980; Bottinelli et al., 1994). During embryonic muscle development, many of these subtypes can be identified in muscle fibers; however, shortly after birth the majority of myofibers exhibit an established phenotype (Biressi et al., 2007b; Garry et al., 1996; Sacks et al., 2003; Wigston and English, 1992). In adult muscle, myofibers can remodel their phenotype and switch from one type to another in response to physiological demands such as activity or hormonal alterations. These changes occur as a result of gene expression reprogramming which alters metabolic and contractile properties of muscle

fibers and lead to the appearance of innumerable fiber type transients (Mu et al., 2007; Pette and Staron, 1997; Shi et al., 2008).

2-1) Skeletal muscle remodeling

2-1-1) Myogenesis

During embryogenesis, skeletal muscle passes through several defined steps that are tightly regulated. Skeletal muscle development starts with mesoderm-derived progenitor cells that become committed to the myogenic lineage and form myoblasts. Subsequently, myoblasts cease proliferation, and differentiate and fuse to form myotubes, which further develop into highly specialized, multinucleated myofibers (Biressi et al., 2007a). The myogenic program is initiated by a complex series of spatiotemporal signaling events in the paraxial mesoderm and the somites, which provides a permissive and inductive environment to initiate muscle specification and morphogenesis (Linker et al., 2003). The interplay between promoting and suppressing signals is mainly derived from neighboring structures such as neural tube, floor plate, surface ectoderm and notochord coordinates myotome formation (Brent and Tabin, 2002; Munsterberg and Lassar, 1995). The signaling events involve several pathways including Sonic Hedgehog (shh) and Wnts that activate downstream molecules to switch on the muscle-specific gene program (Cossu and Borello, 1999; Fan et al., 1995; Munsterberg et al., 1995; Reshef et al., 1998). In vertebrates, a number of muscle regulatory factors (MRFs), which belong to the basic helix-loop-helix (bHLH) family of transcription factors, play a central role in promoting myogenesis (Braun et al., 1989; Davis et al., 1987; Edmondson and Olson, 1989; Rhodes and Konieczny, 1989; Tajbakhsh et al., 1998; Wright et al., 1989). MRFs

consist of 4 transcription factors, MyoD (Myf1), Myf5, Myogenin and MRF4 (Myf6), which are exclusively expressed in the skeletal muscle lineage and are capable of inducing myogenesis in non-myogenic cell lines (Russo et al., 1998; Young et al., 1998). These myogenic transcription factors activate the muscle-differentiation gene program via association with ubiquitous E-proteins and binding E-boxes in the regulatory sequences of target genes (Lassar et al., 1991). Studies of animal models with mutations in MRF genes have proven the critical role of MRFs during skeletal muscle development, and revealed a functional redundancy as well as a hierarchy among MRF family members (Hasty et al., 1993; Nabeshima et al., 1993; Olson et al., 1996; Rawls et al., 1995; Rudnicki et al., 1993; Venuti et al., 1995). In addition to and in association with bHLH MRFs, there are many other transcription factors such as MEF (myocytes enhancer factor) family and Pax proteins which are expressed at different stages of muscle development during embryonic and postnatal life (Black and Olson, 1998; Jostes et al., 1990; Naidu et al., 1995; Relaix et al., 2006). It has been shown that the consensus binding sequences for these transcription factors are present within the regulatory regions of many muscle specific genes.

2-1-2) Skeletal muscle adaptability

Skeletal muscles show a major capacity to modify their molecular, metabolic and functional phenotype, and undergo remodeling in response to environmental changes. It has been known for many years that alteration in innervation pattern of muscles results in a gradual transformation of the fiber type (Pette and Vrbova, 1985). A similar phenotype change occurs when muscles are exposed to chronic electrical stimulation or exercise

training (Pette et al., 1972; Pette and Vrbova, 1985; Windisch et al., 1998). This remodeling is mediated by contraction-induced alterations in intracellular calcium or reactive oxygen species which serve as signals to activate various pathways that induce carefully orchestrated changes in protein composition of myofibers. These changes are rapid at the gene expression level, but it takes longer (weeks to months) for muscles to change the intracellular level of protein isoforms and adapt a new phenotype (Pette and Staron, 1997). A variety of signaling pathways including Ras/MAPK, IGF/AKT/mTOR, calcineurin/HDAC and CaMK/NFAT, and multiple transcription factors, coactivators and corepressors are involved in muscle fiber specification and remodeling. MEF2 is a well recognized component in this fiber transition process whose function in activating the muscle gene program is regulated by activation and suppression pathways in response to intracellular Ca^{2+} fluctuations incurred by physiological signals. The activity of nuclear MEF2 is inhibited by its binding to class II HDAC proteins. The activity of HDACs is regulated by their phosphorylation status which in turn can be affected by changes in cytosolic [Ca^{2+}] (Lu et al., 2000; McKinsey et al., 2000a). Elevated Ca^{2+} level activates CaMK-IV leading to the phosphorylation of HDACs, which subsequently results in dissociation of these proteins from MEF2 allowing it to initiate fiber-specific gene program through its associating with other proteins. The inhibitory effect of HDACs can also be reversed by activation of the calcineurin/NFAT pathway (Chin et al., 1998). Calcium binding to calmodulin activates Ca^{2+}/calmodulin-activated phosphatase calcineurin which dephosphorylates NFAT. This results in the translocation of NFAT from the cytoplasm to the nucleus where it interacts with and recruits coactivators possessing HAT activity to MEF2 triggering its dimerization and association with other

muscle-specific transcription factors (Dolmetsch et al., 1997; McKinsey et al., 2000b). The activity of MEF2 is also regulated by p38 MAPK which phosphorylates MEF2 on various threonine residues in its transcriptional activation domain and enhances its transcriptional activity (Zhao et al., 1999).

2-2) Muscle damage

The mechanisms underlying muscle damage are poorly characterized. It has been known for many years that damaged muscles have elevated levels of intracellular calcium, which has a central role in the development of myofiber damage (Duncan, 1978). The role of calcium and pathways involved in promoting muscle damage, and the mechanisms contributing to influx of calcium form extracellular matrix have been widely investigated. Several ion channels including stretch-activated channels as well as induction of microlesions in myofiber membrane have been speculated to be ports of calcium entry into the cytosol (Duncan and Jackson, 1987; McNeil and Khakee, 1992; Yeung et al., 2005). Membrane defects appear as a natural consequence of muscle activity, which is particularly pronounced in stretched/eccentric muscle contractions. It has been reported that in normal fibers, these breaches are repaired in less than 1 minute, and the slower rate of repair is associated with fiber damage and dystrophic phenotype in muscle (Bansal et al., 2003). In addition to mechanical shear, development of membrane defects may be induced by increased production of free radicals such as nitric oxide and oxygen species in muscle cells. Reactive oxygen species (ROS) are generated within the cell as byproducts of several metabolic and enzymatic pathways; however, the majority of cellular ROS are generated within the mitochondrial electron transfer chain. ROS

promotes lipid peroxidation and oxidation of structurally important proteins in cellular membranes. This results in physicochemical changes in lipid bilayer and leads to increased permeability of these membranes (Mason et al., 1997). Moreover, nitric oxide is produced by two NOS isoforms (eNOS and nNOS) which are constitutively expressed in myofibers. Elevated [Ca^{2+}] induces nitric oxide production through calmodulin binding-dependent activation of both NOS isoforms. Increased levels of ROS and nitric oxide in skeletal muscle, as seen in physical exercise, affect muscle physiology at both tissue and cellular levels and mediate oxidative injury in muscle (Davies et al., 1982; Jenkins and Goldfarb, 1993; Reid and Durham, 2002). ROS and nitric oxide both induce calcium channel opening at high concentrations, and interact with each other to form highly-reactive free radical derivatives which further disturb ion homeostasis and damage membrane integrity. This leads to the sustained influx of extracellular calcium which overrides the buffering capacity of the cell to maintain physiologic levels of intracellular calcium. Elevated [Ca^{2+}] may act as a positive feedback and further exacerbate ROS generation by dysregulating mitochondrial function (Brookes et al., 2004). This increase in cytosolic amounts of calcium activates calpains which can degrade cytoskeletal proteins and drive tissue degeneration (Belcastro, 1993; Carafoli and Molinari, 1998). Additionally, elevated [Ca^{2+}] can activate phospholipase A, leading to additional disruption of membrane structure (Duncan and Jackson, 1987; Howl and Publicover, 1990).

Muscle injury is characterized by loss of force, release of soluble intracellular enzymes, calcium influx as well as ultrastructural, morphologic and histologic changes (Friden et al., 1983; Jones et al., 1983; Wood et al., 1993). The major histopathologic

event in muscle degeneration is necrosis of muscle fibers accompanied by the recruitment of mononucleated cells (Tidball, 1995). Muscle degeneration is followed by the activation of repair processes which are characterized by molecular and morphological changes such as appearance of fibers with small diameter and centrally located nuclei. Skeletal muscle shows increased susceptibility to injury, and reduced capacity for regeneration with age. Age-related decline in physiological function is postulated to be a result of impaired mitochondrial function due to accumulation of mtDNA mutations, and increased oxidative stress and free radical production (Esposito et al., 1999; Harman, 1956; Jenkins and Goldfarb, 1993; Marzani et al., 2005; Miquel et al., 1980; Tanaka et al., 1996). Mitochondria, as the major source of oxidants, are targets for the toxic effects of free radicals themselves. The increased level of free radicals further compromises mitochondrial function by depressing its enzyme activity, damaging mtDNA and mitochondrial membranes. Active metabolic state and high oxygen utilization rate in skeletal muscle provide the potential for generation of free oxidative radicals in this tissue. Free radical production varies in fast and slow muscles which may confer fiber type-specific susceptibility/resistance to oxidative damage (Perez et al., 2002). The vulnerability to oxidative damage also depends on the muscle antioxidant properties which differ between various muscles (Ji et al., 1992).

2-3) Skeletal muscle regeneration

Skeletal muscle has a remarkable capacity for regeneration and maintaining muscle performance after injury. Upon muscle injury and degeneration, a finely-orchestrated series of cellular response becomes activated that leads to the repair of

damaged fibers. In adult skeletal muscle, a class of cells with stem cell characteristics, termed satellite cells are associated with muscle fibers and are responsible for regeneration of mature fibers (Mauro, 1961; Seale and Rudnicki, 2000). Muscle injuries have been shown to trigger the release of soluble factors that provide signals to activate satellite cells. Activated satellite cells proliferate and give rise to new myonuclei which subsequently differentiate into myoblastic cells. These new myoblastic cells either fuse with each other to form new myofibers or fuse with an existing damaged fiber to repair and replace it (Hawke and Garry, 2001). Activation of satellite cells initiates a myogenic program which is associated with upregulation of muscle transcription factors including MRFs, and the subsequent expression of muscle-specific genes. Satellite cells which become activated and form the adult myoblasts can be identified by high expression of two of MRFs, MyoD and Myf5 (Cornelison and Wold, 1997; Fuchtbauer and Westphal, 1992; Grounds et al., 1992). Terminal differentiation is completed with the activation of cell cycle-dependent kinase inhibitors that induce myofibers to permanently withdraw from the cell cycle and enter postmitotic state (Fuchtbauer and Westphal, 1992; Grounds et al., 1992; Lassar et al., 1994; Yablonka-Reuveni and Rivera, 1994; Zhang et al., 1999).

Regeneration in skeletal muscle is associated with histologic changes which are used to identify the repair process. The regeneration phase is similar in different muscles; however, the kinetics of the process vary depending on the cause and extent of the damage as well as the strain in animal models (Irintchev and Wernig, 1987; Lefaucheur and Sebille, 1995; Roberts et al., 1997). Degenerated fibers can be found within few days after injury followed by the appearance of regenerating fibers which leads to a normal muscle by few weeks to months (Carlson and Gutmann, 1975; Harris and Johnson, 1978).

The main morphologic features include myofibers which are small in size and express embryonic forms of MHC and the appearance of centrally placed myonuclei that can be identified on muscle cross-sections (Maier et al., 1986; Winchester and Gonyea, 1992). Fiber splitting/branching is also seen in regenerative fibers, a phenomenon which occurs as a result of incomplete fusion of fibers surrounded by the same basal lamina (Blaivas and Carlson, 1991; Blaveri et al., 1999). After the fusion of myogenic cells is completed, myofibers grow and increase in size, and their myonuclei move to the periphery of the fiber where they are located in normal fibers. These mature regenerated myofibers are indistinguishable from undamaged fibers both morphologically and functionally (Charge and Rudnicki, 2004; Lancut et al., 2004). The normal muscle architecture is restored by a variety of trophic factors such as IGF, neural-derived factors and FGF, which are involved in maintaining the balance between growth and differentiation of satellite cells (Hawke and Garry, 2001).

3) Apoptosis in skeletal muscle and the involved nucleases

In multicellular organisms, apoptosis plays a crucial role in developmental processes and tissue homeostasis. In skeletal muscle, however, the role of programmed cell death is less understood. Skeletal muscle is a unique tissue in that muscle fibers are multinucleated cells in which the myofiber cytoplasm is virtually segregated into many domains each supported by a single nucleus (myonuclear domain). The presence of hundreds of myonuclei in each muscle fiber, some of which may undergo apoptosis while the others and the whole fiber are unaffected, displays a unique feature of apoptotic process in muscle. Apoptosis is induced in skeletal muscle in a variety of physiological

and pathological conditions including exercise, atrophy, denervation, and dystrophinopathies (Borisov and Carlson, 2000; Podhorska-Okolow et al., 1998; Smith et al., 2000; Tews and Goebel, 1997; Tews et al., 1997). In adult skeletal muscle, apoptosis has been shown to be increased with senescence, and proposed to be associated with age-related muscle wasting through progressive loss of myocytes and myonuclei (Dirks and Leeuwenburgh, 2002; Dupont-Versteegden, 2005; Pollack et al., 2002).

Despite the abundance of investigations over the past years to reveal the mechanism of apoptosis in muscle cell, the intracellular death effectors and the pathways involved are poorly characterized. Several lines of evidence have indicated that both caspase-dependent and -independent pathways might be involved in muscle fiber apoptosis (Chung and Ng, 2006; Dirks and Leeuwenburgh, 2002). Mitochondria, whose dysfunction has also been reported to be associated with aging, may play a central role in the progression of apoptosis in muscle cells, and may proceed cell death via activation of both caspase-dependent and -independent pathways. Mitochondria activate the caspase-dependent pathway through releasing cytochrome c which forms a complex with Apaf-1 and dATP, and binds to and activates procaspase-9. Activated caspase-9 subsequently activates effector caspase-3 to execute the cell death process (Li et al., 1997). The involvement of mitochondria in caspase-independent cell death is mediated through EndoG and AIF, which can be released from mitochondria, and induce DNA fragmentation upon their translocation into the nucleus (Li et al., 2001; Susin et al., 1999; van Loo et al., 2001). The latter pathway might be of particular relevance in myonuclei apoptosis since it allows the elimination of nuclei without devastating the cytoplasmic proteins and the entire muscle fiber by caspases. Several reports have described the

increased levels of the mediators of both cell death pathways and the concomitant DNA fragmentation in muscles of aged animals (Chung and Ng, 2006; Dirks and Leeuwenburgh, 2002; Leeuwenburgh et al., 2005; Siu et al., 2005). Furthermore, it has been shown that the involvement of apoptosis executers and activated pathways vary during the course of muscle development, and between young and old muscles (Fimia et al., 1996; Leeuwenburgh et al., 2005; Shiokawa et al., 2002; Siu et al., 2005). Upon induction of apoptosis, myoblasts exhibit different patterns of DNA fragmentation that is correlated with the stage of myogenic differentiation, which reflects the involvement of more than one nuclease in the apoptotic process in muscle cells (Fimia et al., 1996; McArdle et al., 1999). Fimia *et al.*, for instance, reported a high level of nuclease activity in nuclear extracts from both differentiated and undifferentiated myoblasts attributable to the endonucleolytic activity of NUC 18 (Fimia et al., 1996). Shiokawa *et al.* showed an induction in DNaseII3 during myogenic differentiation and suggested that DNaseII3 is responsible for catalyzing apoptotic DNA fragmentation in differentiating myoblasts (Shiokawa et al., 2002). They also showed that overexpression of DNaseII3, but not DNase X (which is highly expressed in adult myocytes), confers DNA-ladder forming ability to proliferating myoblasts. These observations could be attributed to many possible modifications including the alteration in the level or activity of nucleases, the availability of their substrate, and adaptive regulatory responses during muscle development. A more detailed mechanism of apoptosis in skeletal muscle as well as the pre-proapoptotic signaling and the exact role of this process in muscle cells remain to be addressed in future studies.

Table 1. ***Main characteristics of skeletal muscle fiber types.***

Fiber type	MHC isoform	Contraction	Metabolism	Function	Reference
I	MHC*7/I*	Slow-twitch	Oxidative; Rich in mitochondria, myoglobin, & blood supply, SO fibers	Highly resistant to fatigue, suitable for aerobic activities, maintaining body posture	(Nemeth et al., 1979; Pette and Staron, 2000; Schiaffino and Reggiani, 1996)
IIA	MHC*2/IIa*	Fast-twitch; faster than type I, slower than type IId & IIb	Oxidative/glycolytic; Rich in mitochondria & blood supply, FOG fibers	Resistant to fatigue, sustaining aerobic activities	(Bar and Pette, 1988; Nemeth et al., 1979; Schiaffino et al., 1989)
IIX/IID	MHC*1/IIx/IId*	Fast-twitch; slower than type IIb	Glycolytic; Less dense in mitochondria & myoglobin, rich in glycogen & glycolytic enzymes, FG fibers	Fatigues rapidly, suitable for anaerobic activities	(Bar and Pette, 1988; Nemeth et al., 1979; Schiaffino et al., 1989)
IIB	MHC*4/IIb*	Fast-twitch; fastest type II fiber	Glycolytic; Rich in glycogen & glycolytic enzymes, FG fibers	Fatigues rapidly, suitable for anaerobic activities, short, strong & rapid contractions	(Bar and Pette, 1988; Schiaffino et al., 1989)

MHC, myosin heavy chain; SO, slow oxidative; FOG, fast oxidative glycolytic; FG, fast glycolytic.

Study design

Rationale

DNase X is present at a basal levels in many tissues (Los et al., 2000; Shiokawa and Tanuma, 2001) which may indicate its involvement in general cellular activities. However, the protein is highly expressed in cardiac and skeletal muscles (Los et al., 2000; Parrish et al., 1995; Pergolizzi et al., 1996; Shiokawa and Tanuma, 2001), a feature that is known to be conserved among mammals (Shiokawa et al., 2005). This conserved tissue specificity implies an exclusive role for DNase X in these tissues. Human DNase X shares a good homology with DNase I whose involvement in the development of autoimmune disorder, SLE, has been previously reported. Considering the structural and functional similarities between these two proteins, DNase X may also be involved in a comparable process, specifically in skeletal muscle. Additionally, murine DNase X is more than 66% identical to the human homologue and exhibits the same expression pattern as human protein. This latter fact allows us to generate and use an animal model to study the possible function of DNase X in human tissues. Based on this rationale, a DNase X mutant mouse was generated to address the physiological role of this protein *in vivo*.

Hypothesis

We hypothesized that DNase X is important for skeletal muscle physiology, and lack of it function may contribute to the development of muscle-related pathologies.

Objectives

The objectives of this study include:

1) To perform a morphological analysis of DNase X deficient mice with emphasis on skeletal muscle.

2) To perform a systemic histological examination of major organs as well as different fast- and slow-twitch muscles of the hindlimb.

3) To test the physical activity performance of DNase $X^{(-/-)}$ mice.

4) To determine the expression profile of the *DNASEX* gene in normal tissues, as well as the expression of its DNase I-like homologues in DNase X mutant muscles.

5) To measure the nuclease activity in DNase X deficient muscles.

Materials and Methods

1) *Animals*

Inbred C57BL/6J mice were used for this study. All mice were bred, fed and housed according to the guidelines of the Canadian Council on Animal Care. The experimental protocols were approved by the Animal Care Committee of the University of Manitoba.

1-1) *Generation of DNase X mutant mouse*

Transgenic animals were generated by standard techniques using a commercial provider (mice and more/ Hamburg, Germany). The targeting vector was constructed to contain the neomycin resistant gene (*neo*) and 4 of the downstream coding exons (exons 5-8). A *HSV-TK* cassette was inserted downstream of the modified gene for negative selection [Fig. 3a]. Targeting construct was eletroporated into E14 embryonic stem (ES) cells, and cells were cultured to obtain G418-positive clones. The homologous recombination event was verified by PCR (data not shown) and the recombinant clone was injected into C57BL/6 recipient blastocysts which were transferred into pseudopregnant females. Genotyping of offspring was carried out by PCR amplification of genomic DNA using primers that flanked the *neo* insertion site in intron 1 and 4 as well as primers in deleted exons and the neo sequence [Fig. 3b,c]. The absence of DNase X mRNA in mutant mice was verified by a RT-PCR assay [Fig. 3d].

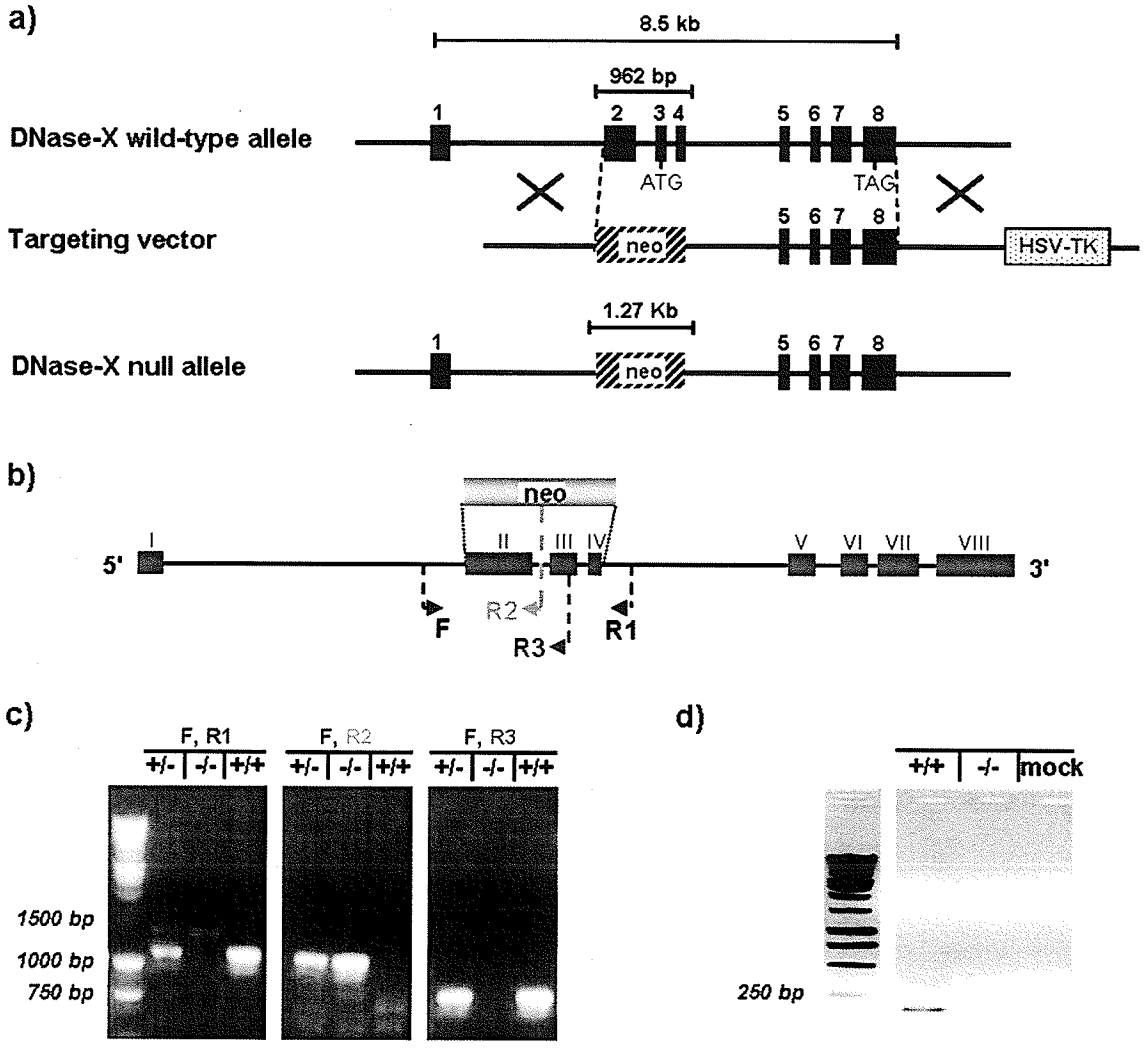


Figure 3. Deletion of DNase X gene (Parrish et al, 1995; Coy et al., 1996) by gene targeting. a) Schematic presentation of *DNASEX* wild-type allele, targeting vector, and the structure of the targeted gene. Exons are shown by numbers. The transcription initiation and stop codons are indicated. b) Positions of the primers used for genotyping DNase X wild-type (+/+), heterozygous (+/-), and knockout (-/-) mice. F, forward primer; R, reverse primer. Blue and red boxes represent the untranslated and translated regions, respectively. c) The results of PCR assay of genomic DNA. c, left panel) Primer set F & R1 amplified a 1400- and 1090-bp segment in mutant and wild-type alleles, respectively. The heterozygous mice were identified by the detection of two bands related to the presence of two alleles, whereas only one band corresponding to each allele was seen in either wild-type or knockout mice. c, middle panel) Primer pairs F & R2 were able to amplify a 1050-bp sequence in knockout allele, and hence the band is absent in wild-type samples. c, right panel) Primers F & R3 amplified a 870-bp sequence on wild-type allele resulting in the detection of a single band in both wild-type and heterozygote mice. d) Lack of DNase X mRNA was confirmed by RT-PCR. The band in wild-type sample corresponds to a 191-bp product amplified by DNase X specific primers, which is absent in mutant as well as mock (no template) samples.

1-2) Verification of the transgene at the genomic and mRNA level

DNA was extracted form the tail samples of 20 day-old pups using a DNA extraction kit (DNeasy Tissue Kit, Qiagen, Mississagua, ON). Genotyping was performed using a forward (F) and several reverse (R1-R3) PCR primers from different regions on normal and mutant alleles. Each primer set amplified a desired segment on wild-type and transgene alleles. The PCR amplification products were detected as variably sized bands by electrophoresis on 1.8% agarose gel containing Ethidium Bromide [Fig. 3c]. Primer sequences used were as follows: forward (F) in intron 1 sequence: 5′-CACCTGGGGAAGATGAAAAA -3′, reverse-1 (R1) in intron 4 sequence: 5′-GGGCTCCAGCAATTCATCTA -3′, reverse-2 (R2) in *neo* sequence: 5′-TATCACGGGTAGCCAACG -3′, reverse-3 (R3) in coding sequence of exon 3: 5′-GCACCATGATATCACATCGG -3′. Amplification condition was as follows: 36 cycles of: 30 sec 94 °C, 1 min 58 °C, 2 min 72 °C; and 3 min 72 °C.

The absence of DNase X mRNA in DNase $X^{(-/-)}$ mice was demonstrated by RT-PCR approach, using primers that amplify a 191-bp region expanding from exon 4 to the junction of exon 5 and exon 6 in DNase X cDNA. Total RNA was prepared after homogenization of the tissue using the RNeasy Protect Mini Kit (Qiagen, Mississagua, ON). To eliminate genomic DNA contamination, samples were treated with RNase-Free DNase (Qiagen, Mississagua, ON). The experiment was performed using a one-step RT-PCR kit (BioRad), following the recommendations of the supplier. Magnesium ion was added to the reaction mixture by a final concentration of 1.5mM. The primer sequences were as follow: 5′-CCAGGAGTTACAGCTTCCTAAACAGCTC-3′ on sense, and 5′-CACACTTGGAAGAGTTTTGCTAGGGAGAG-3′ on antisense. Amplification

condition was: 50 °C for 10 min; 95 °C for 5 min; 40 cycles of: 95 °C for 10 sec, 59 °C for 15 sec, 72 °C for15 sec; and 95 °C for; 59 °C for 1 min. The PCR products were detected on 1.8% Ethidium Bromide-containing agarose gel [Fig. 3d]. The PCR product was sequenced by standard procedures at DNA Sequencing Facility of Manitoba Institute of Cell Biology, University of Manitoba.

2) Body and organ wet weight

All animals were housed in a 12-hour light/dark cycle with ambient temperature and given free access to food and water. For the study of body weight alterations, age-matched wild-type and DNase $X^{(-/-)}$ mice were used at 6, 13 and 27 weeks of age. For the study of organ weights, adult mice of 9 month-old were weighed and euthanized, and all major organs as well as hindlimb muscles of gastrocnemius (GN), quadriceps femoris (QC), tibialis anterior (TA), extensor digitorum longus (EDL) and soleus were isolated, cleaned and weighed. The obtained values of organ weights were corrected for the total body weight and the normalized values were used for the analysis.

3) Fiber enumeration

Male mice at the age of 12-13 weeks and 1 year were used for this experiment. After euthanasia, TA, EDL and soleus of both hindlimbs were isolated, and cleaned from the surrounding fascia and connective tissue. Fresh muscles were macerated by a modified method of acid digestion (Timson, 1982) originally described by Gollnick et al. (Gollnick et al., 1981). Briefly, the whole muscles were placed in 15% nitric acid for 4 hours at room temperature (RT), rinsed thoroughly with and placed in double distilled

water until being analyzed. Before the analysis, dishes containing tissues were coded to ensure minimizing the examiner bias. Under a dissecting microscope, muscles were cleaned from undigested connective tissues, and muscle bundles were carefully dissected and placed in groups of similar length. A minimum of total 170 full-length myofibers from different groups were counted in each muscle. The counted fibers and the remaining part of the muscle were air-dried overnight at RT and weighed. The total number of myofibers composing the muscle was estimated by a simple math with the weight of counted fibers and that of all fibers together and the number of the fibers counted. A minimum of 3 animals were studied in each group and the average values of each muscle pair (from both legs) were used for comparison between wild-type and DNase $X^{(-/-)}$ mice.

4) Preparation of histology sections

Animals were euthanized by CO_2 asphyxiation and major organs as well as muscles of the hindlimb were collected. For histology examination, tissue samples were dehydrated, fixed in 10% neutral phosphate-buffered formalin and embedded in paraffin. Sections were cut at a thickness of 5 μm and stained with standard hematoxylin and eosin procedure. Sections were visualized using a bright-field microscope (Zeiss Axioskope 2) attached to a Polaroid DMC2 digital color camera.

5) Immunohistochemistry

Isolated muscles were embedded in OCT and snap frozen in isopentane precooled in liquid nitrogen. 10 μm-thick sections were prepared from muscle midpoint at –20°C using a cryostat, mounted on SuperPlus glass slides (Fisher Scientific, Pittsburgh, PA)

and allowed to air-dry overnight at RT. Samples were stored in -80°C until further processed. For immunofluorescence staining, sections were fixed in ice-cold acetone and washed three times with cold PBS followed by blocking in PBS containing 0.1% tween20 and 1% BSA for one hour. Antibodies were diluted in the blocking solution and incubation was carried out in a humidified chamber either for one hour at RT or at 4°C overnight. The primary antibodies and the concentrations used were as follow: rabbit polyclonal anti-dystrophin antibody (Abcam, 1:250), mouse anti-skeletal muscle slow myosin monoclonal antibody (Sigma, 1:250). Each time before proceeding to the next immunolabeling, slides were washed 3 times, 3 minutes each time. The secondary antibodies and the concentrations used were as follow: sheep anti-rabbit IgG (Cy3-conjugated, Sigma, 1:250), goat anti-mouse IgG (Cy5-conjugated, Sigma, 1:200). Sections were washed and dehydrated by serial changes of 70%, 95%, and 100% ethanol and cover-slipped with Vectashield mounting medium containing DAPI. Immunohistochemistry analyses were conducted using a fluorescent microscope (Zeiss Axioplan II) attached to a Zeiss AxioCam HRm camera, and AxioVision 4.5 software for image capturing on a Pentium IV computer.

6) Fiber type composition and area, cytoplasm to nucleus ratio

Male mice, aged 6 weeks and 1 year, were used to study the morphological changes in skeletal muscle. Cross sections of EDL, plantaris and soleus were stained with anti-MHC*I* antibody, as described under immunohistochemisty section. Fibers were classified based on the reaction with MHC*I* antibody, and the number of type II fibers was calculated as the difference between the total number of fibers and the number of

stained/type I fibers. For each section, images were taken from 4-6 randomly-selected areas at 20×. Fiber type composition was determined by counting all fibers that appeared in each field of the muscle cross section, and the data were presented as the ratio of each fiber type to total fibers counted. The area of single fibers was marked and determined using NIH ImageJ software, and was determined for all the fibers observed in each separate image field. Data for fiber cross-sectional area (CSA) were plotted as the mean CSA of each fiber type. Nuclear domain, expressed as the area of CSA, was determined as the total CSA of fibers divided by the number of nuclei (nuclei confined to sarcolemma and not those in interstitial area). The proportion of fibers with internal nuclei was quantified and presented as the percentage of total myofibers with centrally located nuclei. The proportion of each fiber type with central nuclei was plotted as the percentage of each fiber type that carried internal nuclei. During the study, slides were coded to avoid any examiner bias.

7) Voluntary wheel training

A total number of 121 wild-type and 220 DNase $X^{(-/-)}$ mice (17-22 wild-type and 30-54 knockout mice in each group) at 6, 13 and 27 weeks were used for this experiment. Mice were placed in individual cages (45 × 23.5 × 20.5 cm) equipped with running wheels (12.7 cm diameter) as described previously (Archer et al., 2006), and were allowed voluntary access to the wheel for 24 hours. Two small magnets, attached on the opposite sides of the wheel circumference, triggered (twice per rotation) a magnetic switch (Guard™) connected to a counter-recorder (Omron™ Type H7EC-BLM). Total distance run in 24 hour was calculated using simple mathematics with obtained values of

the number of rotations and wheel diameter. Distance run was normalized to body weight to account for variability in wheel loading that could affect running performance among different-sized mice either within or between groups.

8) Treadmill running

A total of 64 wild-type and DNase X mutant mice (8 mice in each group), at age of 6 and 36 weeks were used for this experiment. The whole body weight in wild-type mice ranged from 21.8-24.5g in 6 week-old males, 18.4-20.3g in 6 week-old females, 42.3-45.1 in 36 week-old males and 28.8-32.5 in 36 week-old females. The ranges of body weight in knockout mice were 19.9-22.9 in 6 week-old males, 17.6-19.3 in 6 week-old females, and 38.6-42.6 and 26.6-30.2 in 36 week-old males and females, respectively. Mice were exercised on a motor-driven rodent treadmill for three bouts of 10 min training with one day interval in between (1 bout per day) to acclimate to the test condition, and were allowed 2 days of recovery before the running test was initiated. In the test protocol, the running speed started at 10 m/min (10° uphill grade), and increased by 2 m/min to 17 m/min, and maintained at this speed. Mice were elicited to run in the dark and by touching their back with a pencil, and ran until they could no longer maintain the speed and were unable to run further. Each mouse was tested twice, with one day interval between the tests, and the average distance run in two experiments was used for analysis. Mice that could not keep pace with the increasing speed or did not maintain running at the final speed for 2 min were eliminated from the study. Distance was calculated by multiplying the running speed by the time mice ran on that speed.

9) Induction of fiber damage by running exercise

Three bouts of treadmill running exercise were used to induce muscle damage in both wild-type and DNase $X^{(-/-)}$ mice. 11-13 month-old mice were allowed to run up to 15 min in each trial under the condition described earlier, and muscle tissues were isolated and prepared for analysis.

10) RNA isolation, and quantitative real-time RT-PCR of DNase I family

Total RNA was isolated from the fresh tissues using RNA isolation kit (Qiagen, Mississagua, ON) according to manufacturer's recommendations. Samples were treated with RNase-Free DNase (Qiagen) to eliminate genomic DNA contamination. The purity and concentration of RNA was verified by the ratio of optical densities at 260 and 280 nm (OD260/OD280) using GeneQuant Pro spectrophotometer (Biochrome) and equal amounts of template were used for the experiments. Primer sequences for PCR are shown in Table 2. Primers were selected from the coding sequences of the corresponding gene with one of the primers in each pair spanning over the region of exon/exon junction. Real time quantitative RT-PCR was performed using an iCycler Real-Time PCR instrument (BioRad, CA) and a one-step RT-PCR kit (BioRad), according to manufacturer's instructions. A final 25µl of RT-PCR reactions were prepared on ice and a final 1.5mM magnesium ion concentration was implemented to the reaction mixture. Amplification condition was as follows: 10 min 50 °C; 5 min 95 °C (to activate the hot-start iTaq™ DNA polymerase); 40 cycles of: 10 sec 95 °C, 15 sec 59 °C, 15 sec 72 °C; 1 min 95 °C; 1 min 59 °C. Melting curve data was collected at 59°C. The threshold was set automatically within the logarithmic phase. PCR products were detected on 1.8% agarose gel

containing ethidium bromide and signals were visualized by UV irradiation on a GelDoc 2000/ChemiDoc System (Bio-Rad, Mississauga, ON). The authenticity of amplified products was verified by electrophoresis and detection of a single band of expected size on agarose gel as well as direct sequencing of the amplicons following the purification of DNA in the corresponding bands using DNA Gel Extraction Kit (Qiagen).

Table 2. ***Primer information for real-time PCR.***

Gene	Amplicon length (bp)	Strand	Sequence (5' - 3')	RefSeq accession number
DNase I	148	sense anti-sense	TCAGATTGGCTTTCAGGATGCGGTAC GAGAGGGTAGCATTGGACATCTTAGTCTCC	NM_010061
DNase X	191	sense anti-sense	CCAGGAGTTACAGCTTCCTAAACAGCTC CACACTTGGAAGAGTTTTGCTAGGGAGAG	NM_027109
DNaseIl2	181	sense anti-sense	CTAGCTGTGCCACCAAGGAGC CAGTCATGCGCCTTCACGTACTTG	NM_025718
DNaseIl3	162	sense anti-sense	GGAAATCAAGGACAGCAGCAACAACATC GACACCAGCTTCTCCTTGTAGACGAAG	NM_007870
Cyc C	176	sense anti-sense	CAACAGGAGAGAAAGGCTACGGGTAC GACCCAGCCAATGCCATAGTGC	NM_008908

DNaseIl2, DNase I-like2; DNaseIl3, DNase I-like3; Cyc C, cyclophilin C.

Data analysis and relative quantification was performed using the comparative *Ct* method (also known as $\Delta\Delta Ct$ method) (Livak and Schmittgen, 2001). The fold changes of each DNase gene mRNA in wild-type and DNase X knockout samples were normalized using the *Ct* values of the cyclophilin C transcripts as internal control, and were calculated by the formula: fold change = $2^{-[\Delta\Delta Ct]}$ where $\Delta\Delta Ct$ = [*Ct* gene of interest (in sample of interest) - *Ct* reference gene (in sample of interest)] - [*Ct* gene of interest (in reference sample) - *Ct* reference gene (in reference sample)]. In this study, the gene of

interest relates to each DNase, the reference gene is cyclophilin C, the sample of interest is DNase X deficient tissue and the reference sample is wild-type tissue. Samples were run in duplicate and the mean values were used for the analysis. All experiments included negative controls with no template to exclude false-positive results. Experiment was performed in 6 week-old and 1 year-old mice and data was obtained from 3-4 mice in each group.

11) Nuclease activity assay

A number of 3 or 4 adult male mice aged 8 months were used for the assay. Fresh tissues were homogenized in a manual ground glass tissue homogenizer, and incubated with 3ml/g RIPA buffer on ice for 20 min. The composition of RIPA buffer was: 0.5% Sodium Deoxycholate, 0.1% SDS, 1% Triton X-100, 1% NP-40, 1 mM EDTA, 1 mM EGTA, 250 mM NaCl, 20 mM Tris HCl (pH 7.5), 30 mM Sodium Fluoride (NaF), 1 mM Sodium Orthovanadate (Na_3VO_4), 10 mM Tetrasodium Pyrophosphate, protease inhibitor cocktail (Sigma Co.). The suspensions were centrifuged for 15 min at 13000 rpm and the total protein extract in the supernatant was used to evaluate the nuclease activity of the tissue. Nuclease activity was assayed by measuring the degradation of BamH1-linearized pTRE2pur plasmid DNA (Clontech) which was determined by agarose gel electrophoresis. A total volume of 20 µl reaction mixture containing separated buffers for each DNase, as described previously (Shiokawa and Tanuma, 2001), was prepared on ice. 1 µg of plasmid DNA was incubated with 10 µg of tissue extract for 15 min at 37°C, and the enzymatic reaction was terminated by addition of 5 µl EDTA 250mM and chilling on ice. Two reactions containing plasmid

DNA and either 0.5 unit of DNase I enzyme (Sigma, AMP-D1) or the lysis buffer were used as positive and negative controls, respectively. The reaction mixtures were loaded in ethidium bromide-containing 1% agarose gel, and signals were visualized by u.v. irradiation on a GelDoc 2000/ChemiDoc System (Bio-Rad, Mississauga, ON). Semi-quantification of DNA degradation was performed by densitometry using the Quantity One software (version 4.6; Bio-Rad). The difference in band intensity between negative and positive controls in each gel was assumed as equivalent to the activity of 0.5 unit of enzyme in the positive control reaction, and was used as a "base value" to quantify the DNase activity in other reactions. The nuclease activity of each reaction was calculated with a simple math using the difference in band densities between the corresponding lane and non-degraded DNA in negative control, and the "base value" in each gel. Values obtained from three independent experiments were used for the analysis. The sum of nuclease activities calculated for all four DNases in a tissue sample was used as the total nuclease activity of the corresponding tissue for comparison.

12) Statistical analysis

Statistical analysis was performed using the GraphPad Prism statistical program (version 4.00 for Windows, San Diego CA). The variation of fiber number between a pair of muscles within an animal was assessed by paired *t*-test. Muscle fiber number between wild-type and DNase $X^{(-/-)}$ mice was analyzed using an unpaired *t*-test. Treadmill running data was analyzed using an unpaired *t*-test and one-way ANOVA and Tukey-Kramer post hoc test. Data are expressed as mean ± SEM. Statistical significance was set at $P \leq 0.05$ and is expressed either as *P* value or stars (*: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

Results

Targeted disruption of DNASEX in mouse

DNase $X^{(-/-)}$ mice were generated by homologous DNA recombination in embryonic stem cells. The targeting construct was designed to delete the first 2 of the 6 coding exons of *DNASEX* gene [Fig. 3a]. An ES clone carrying the desired homologous recombination was used to generate a chimera which gave rise to DNase $X^{(+/-)}$ pups. The gene targeting in the offspring was verified by genomic DNA PCR [Fig. 3c] and the absence of DNase X mRNA was identified by RT-PCR [Fig. 3d]. DNase X null mice were born at the expected Mendelian frequency, were viable and fertile, and did not exhibit any gross postnatal developmental abnormalities.

Whole body and organ weight

DNase $X^{(-/-)}$ mice were lighter than their age-matched wild-type counterparts [Fig. 4]. The results of whole body weight analysis are summarized in Table 3. The differences in mean body weight were significant in younger animals (6 and 13 week-old) in both male and female mice. The differences were more dramatic in male mice as compared to female mice and represented 9.5% (P <0.001) and 7.2% (P <0.01) reduction in mean body weight in 6 and 13 week-old animals, respectively. In the female group, differences represented 4.1% (P <0.01) and 5.1% (P <0.05) reduction in mean body weight of knockout mice compared to that of wild-type mice at age of 6 and 13 weeks, respectively. The analysis of growth curve showed no significant difference in the weight gain by age in wild-type and DNase $X^{(-/-)}$ mice (data not shown). The results showed that mean body weight increased by 74.1% in knockout and 64.1% in wild-type male mice between 6 and

27 weeks of age. In female mice, mean body weight increased by 46.7% in knockouts vs. 50.3% in wild-types between these two age points.

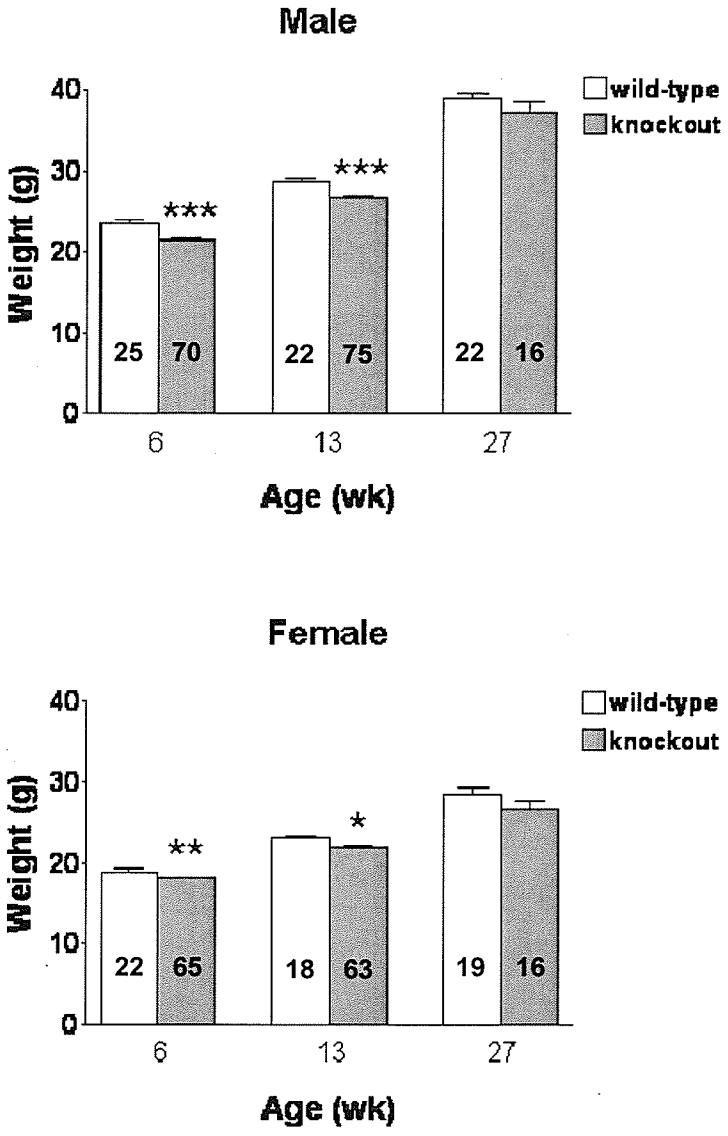


Figure 4. Comparison of whole body weight. DNase $X^{(-/-)}$ mice (solid bars) weighed less than wild-type (open bars) counterparts of the same age. The differences were notable in younger mice, and in male mice. The numbers inside the bars represent the number of animals examined. Error bars represent SEM. *The difference is significant between two groups (*P <0.05; **P <0.01; ***P <0.001).

Table 3. ***Mean body weight (g).***

	6 week-old	13 week-old	27 week-old
Male			
WT	23.72 ± 0.3 (n=25)	28.78 ± 0.3 (n=22)	38.94 ± 0.6 (n=22)
KO	21.46 ± 0.2*** (n=70)	26.7 ± 0.2*** (n=75)	37.36 ± 1.1 (n=16)
Female			
WT	18.97 ± 0.2 (n=22)	23.11 ± 0.2 (n=18)	28.53 ± 0.7 (n=19)
KO	18.18 ± 0.1** (n=65)	21.92 ± 0.2* (n=63)	26.67 ± 0.9 (n=16)

Values are means ± SEM; WT, wild-type; KO, DNase X knockout; * The value is significantly different from the value in wild-type mice (* P <0.05; ** P <0.01; *** P <0.001).

Organ wet weights are plotted as the ratio of organ wet weight to total body weight. The results showed variability in organ weights in DNase X knockout as compared to wild-type mice [Fig. 5]. However, none of the differences, were statistically significant (P >0.05).

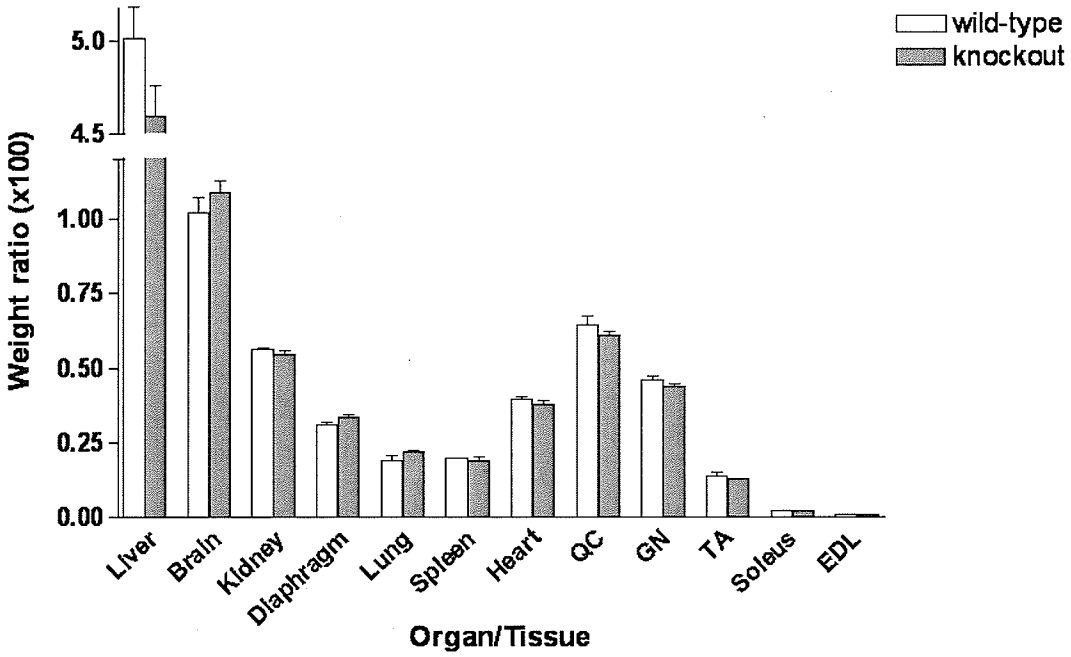


Figure 5. Organ wet weights. The variations in organ weights normalized to the whole body weight were not statistically significant between DNase X wild-type (open bars) and knockout (solid bars) mice. The muscles of the leg weighed uniformly less in DNase $X^{(-/-)}$ mice. Data are obtained from a minimum of three mice at 9 months of age. Error bars represent SEM. QC, quadriceps femoris; GN, gastrocnemius; TA, tibialis anterior; EDL, extensor digitorum longus.

Fiber enumeration in muscles of the leg

DNase X is abundantly expressed in skeletal muscle. We examined the muscles of the hindlimb for any morphological changes in the absence of DNase X. Our data showed no significant variation in fiber number between wild-type and knockout mice. Also, the mean fiber number did not change with age and was only slightly higher in older muscles (data not shown). In comparison between wild-type and DNase X deficient muscles, the mean fiber numbers of examined muscles were not significantly different. The results of fiber number determination from soleus, EDL and TA muscles are presented in Table 4.

Table 4. ***Fiber enumeration.***

	13 week-old	1 year-old
Soleus		
WT	893.6 ± 23.1 (n=4)	910.1 ± 29.7 (n=4)
KO	839 ± 16.5 (n=4)	843.5 ± 22.4 (n=4)
EDL		
WT	838.2 ± 23.5 (n=3)	853.3 ± 22.4 (n=4)
KO	804 ± 16.6 (n=3)	816.8 ± 27.7 (n=3)
TA		
WT	2635 ± 76.7 (n=4)	2653 ± 30.9 (n=4)
KO	2515 ± 36.5 (n=4)	2539 ± 65.2 (n=3)

Values are means ± SEM; EDL, extensor digitorum longus; TA, tibialis anterior; WT, wild-type; KO, DNase X knockout.

Fiber type composition and CSA

We evaluated the effect of DNase X on fiber type composition and single fiber CSA in normal and mutant muscles. Fast and slow fibers were identified based on the reaction of MHC*I* in type I/slow fibers with its specific antibody [Fig. 6]. The EDL, plantaris and soleus muscle in wild-type and DNase $X^{(-/-)}$ mice showed no significant variation in the proportion of fibers expressing fast and slow MHCs. In plantaris of both

wild-type and knockout mice, less than 2% of fibers showed a slow phenotype whereas in EDL, no type I fiber was found (data not shown). In soleus, type I fiber composition showed a similar increase with age in wild-type and knockout mice (24% in wild-type and 18% in knockout). These data are presented in Table 5.

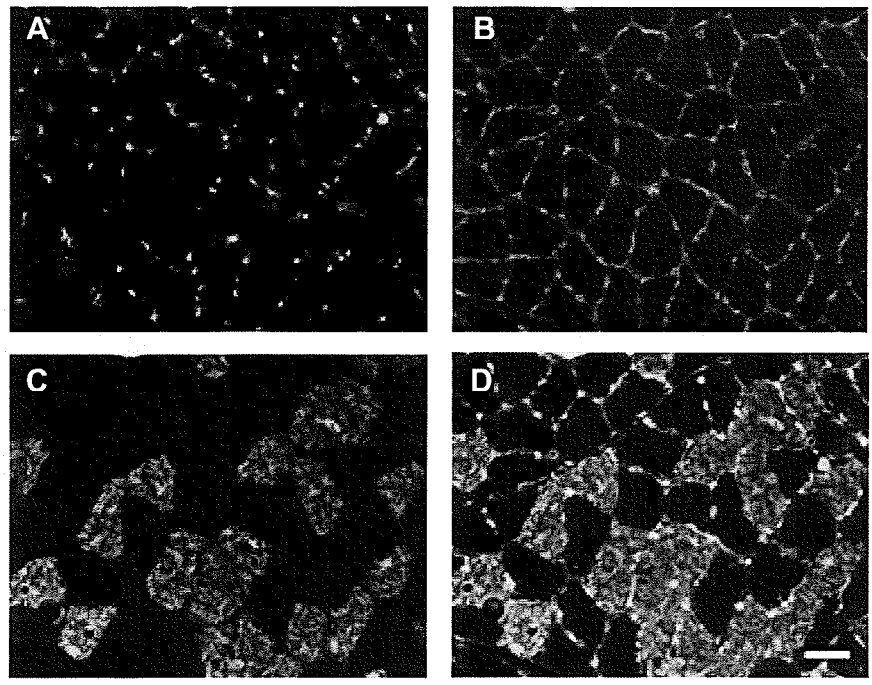


Figure 6. Immunostaining of skeletal muscle fiber types. Muscle cross sections were stained with anti MHC type I (slow) mAb. A-D: transverse section of soleus in 1 year-old mouse. A: nuclei stained with DAPI. B: sarcolemma stained with Cy3-conjugated antibody against dystrophin. C: type I fibers reacted to Cy5-conjugated MHC*I* antibody; type I fibers stained green and type II fibers remained unstained. D: merged image. Scale bar = 30 μm.

Table 5. ***Fiber type composition in soleus.***

	6 week-old	1 year-old
Type I		
WT	39.74 ± 1.6% (n=3)	49.32 ± 2.7% (n=4)
KO	42.40 ± 4.8% (n=4)	50.22 ± 2.2% (n=5)
Type II		
WT	60.26 ± 1.6% (n=3)	50.68 ± 2.7% (n=4)
KO	57.60 ± 4.8% (n=4)	49.78 ± 2.2% (n=5)

Values are means ± SEM (expressed as a percentage of the total number of fibers); WT, wild-type; KO, DNase X knockout.

Single fiber CSA was examined in EDL, plantaris, TA and soleus muscles of young (6 week-old) and older (1 year-old mice). In young mice, no significant variation was found in CSA of fibers between wild-type and mutant muscles. In 1 year-old mice, mean fiber CSA was comparable in EDL, plantaris and TA of wild-type and DNase $X^{(-/-)}$ mice [Table 6]. In these muscles, the cytoplasm area per nucleus ratio was also similar in knockout and wild-type muscles (data not shown). In soleus of older mice; however, the mean CSA was significantly lower in knockout mice [Fig. 7]. The difference was equally seen in type I and type II fibers. Frequency distribution analysis revealed a notable proportion of fibers with CSA smaller than 500 μm^2 in soleus of knockout mice [Fig. 8a]. This marked increase in the number of fibers with small CSA (< 500 μm^2) in knockout soleus (18.38 ± 3.6% in knockouts vs. 1.680 ± 0.5% in wild-types; P <0.01) led to the appearance of a bimodal frequency distribution curve in DNase X deficient soleus and a unimodal pattern in wild-type muscle [Fig. 8b]. To test whether the incidence of small fibers accounted for the difference observed in mean fiber CSA between wild-type and knockout soleus, we reevaluated fiber CSA in soleus after excluding the small-diameter fibers from both samples, and found no difference between CSAs of myofibers in wild-

type and DNase $X^{(-/-)}$ soleus. This indicated that the difference in fiber CSA in soleus of older animals in wild-type and DNase $X^{(-/-)}$ mice was mainly due to the increase in the number of small fibers in knockout muscle. The ratio of mean CSA of type I fibers to that of type II fibers remained unchanged with age in both wild-type and knockout soleus.

Table 6. ***Mean CSA of single fibers (μm^2) in muscles of 1 year-old mice.***

	WT	KO
Soleus		
Total	1325 ± 44.43 (n=4)	1123 ± 31.04** (n=5)
Type I	1337 ± 53.28 (n=4)	1157 ± 38.45* (n=5)
Type II	1313 ± 37.23 (n=4)	1090 ± 40.62* (n=5)
EDL	1105 ± 33.59 (n=3)	1085 ± 14.89 (n=4)
TA	2015 ± 116.8 (n=3)	1915 ± 124.7 (n=3)
Plantaris	1492 ± 84.85 (n=3)	1442 ± 80.38 (n=3)

Values are means ± SEM; EDL, extensor digitorum longus; TA, tibialis anterior; WT, wild-type; KO, DNase X knockout; * The value is significantly different from the value in wild-type mice (* P <0.05; ** P <0.01).

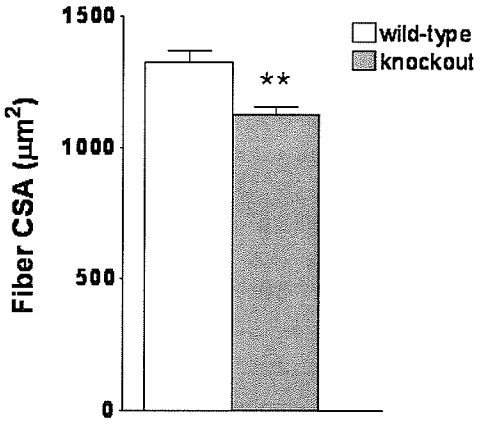


Figure 7. Cross sectional area (CSA) of single fibers in soleus of 1 year-old mice. Mean CSA was notably lower in DNase $X^{(-/-)}$ mice (solid bar) as compared with wild-types (open bar). Error bars represent SEM of a minimum of 4 muscles in each group. *The difference is mean CSA is significant between two groups (**P < 0.01).

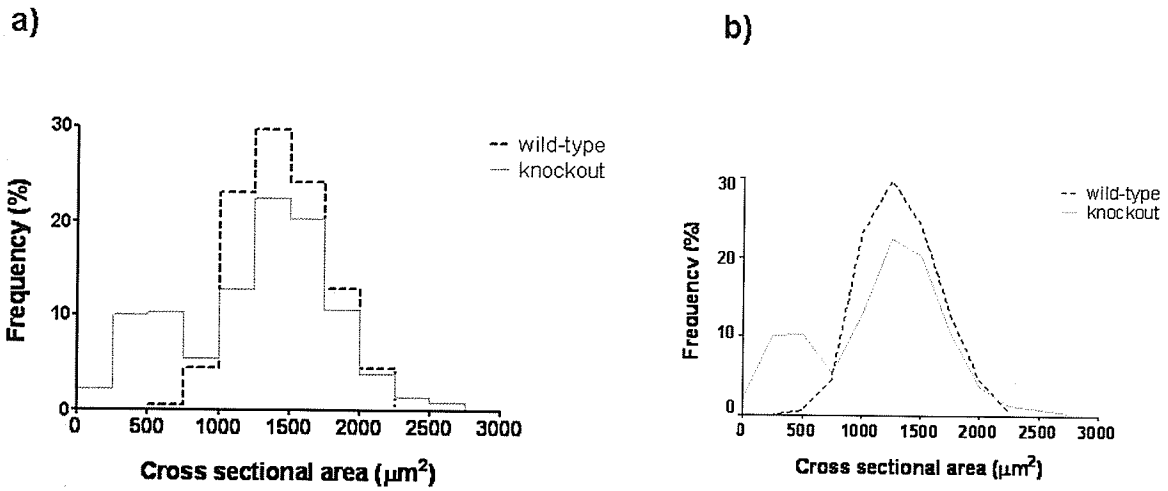


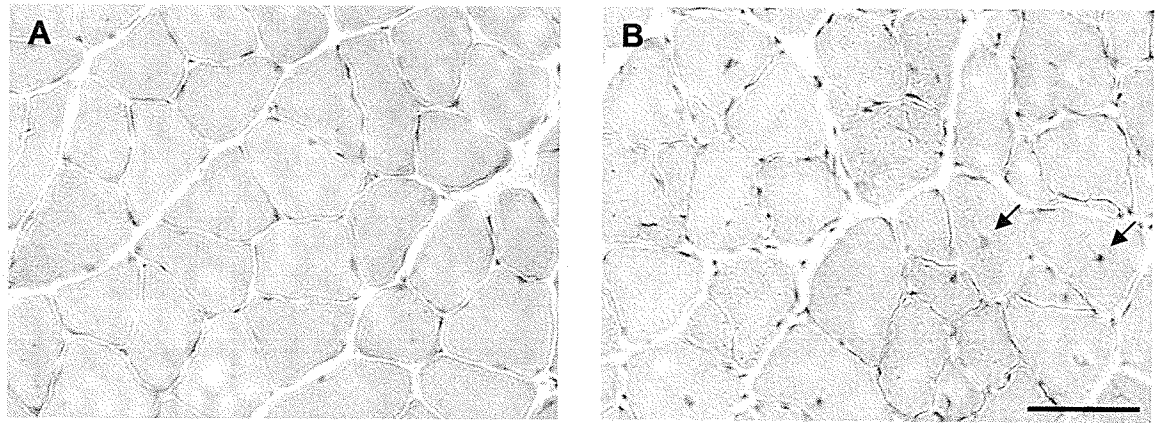
Figure 8. Frequency histogram of fiber CSA. a) Frequency distribution of fiber CSA in soleus muscle of 1 year-old mice shows a large population of fibers with small CSA in DNase X deficient muscle (solid line) as compared to wild-type muscle (dashed line). b) Bimodal distribution of fiber CSA in soleus of DNase $X^{(-/-)}$ mice.

Fibers with central nuclei

Since DNase X is expressed at varying levels in most tissues, we examined the major organs in DNase $X^{(-/-)}$ mice for any pathological changes in these tissues. Histology survey was performed at several age points and no remarkable changes in organ morphology were observed in DNase $X^{(-/-)}$ mice compared with age-matched wild-types (data not shown). In skeletal muscle, no important changes such as increase in connective tissue or mononucleated cell infiltration were found. In soleus of aged animals, however, a notable number of fibers had centrally located nuclei implying a history of damage and regeneration in these fibers [Fig. 9a]. The incidence of these fibers in knockout soleus was significantly different from that in wild-type muscle. Fibers with internal nuclei composed 8.93 ± 1.6% of fibers in knockout soleus and 2.93 ± 0.2% of fibers in wild-type muscle at the age of 1 year (P <0.05) [Fig. 9b]. In non- exercised mice, soleus was the only muscle affected, and none of the other muscles displayed a similar phenotype.

Also, damaged fibers were found in the soleus of older DNase $X^{(-/-)}$ mice (≥ 1 year-old) whereas the muscle in younger mice were unaffected.

a)



b)

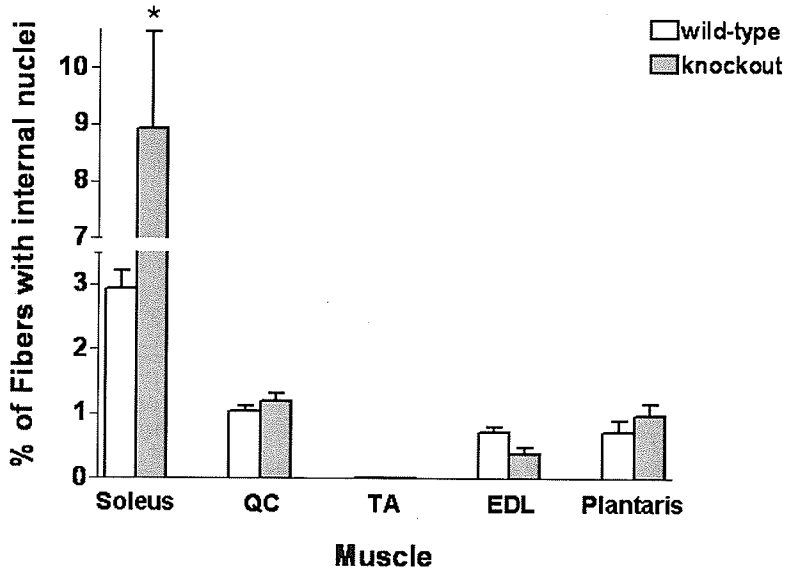


Figure 9. Histological analysis of the skeletal muscle. a) H&E staining of soleus cross section in 1 year-old non-exercised wild-type (A) and DNase $X^{(-/-)}$ (B) mice. A notable proportion of fibers carried central nuclei (two are indicated by arrows) in DNase X(-/-) mice. The fibers with internal nuclei were rarely seen in other muscles in non-exercised mice. Scale bar = 50 μm. b) Incidence of myofibers with central nuclei in 1 year-old mice in wild-type (open bars) and DNase $X^{(-/-)}$ (solid bars) groups. Values are expressed as a percentage of the total number of fibers with central nuclei. Data are obtained from 3-5 mice. Error bars represent SEM. *The difference in the frequency of fibers with internal nuclei is significant between two groups (* $P < 0.05$).

The incidence of fibers with central nuclei was also evaluated in mice after treadmill running. Mice ran an average of 269.7 ± 17.6m (5.96 ± 0.4 m/g body weight) in wild-type group, and 182.3 ± 37.5m (4.38 ± 0.8 m/g body weight) in knockout group. The incidence of fibers with central nuclei was substantially increased in soleus of knockout mice after treadmill exercise (31.44 ± 2.3%), and they also appeared in QC (38.26 ± 3.3%) and TA (5.8 ± 0.9%) in these mice [Fig. 10a,b]. The proportion of these fibers did not change in EDL and plantaris in knockout mice as well as none of the muscles in wild-type animals after exercise [Table 8]. In soleus of DNase $X^{(-/-)}$ mice , the proportion of fibers with internal nuclei was higher in slow/type I than in fast/type II fibers in exercised muscles (P <0.05), whereas the percentage of these fibers was not different in fast and slow fibers in non-exercised muscle in these mice [Fig. 11].

Table 8. ***Incidence of fibers with central nuclei.***

	Non-exercised	Exercised
Soleus		
WT	2.93 ± 0.2% (n=4)	6.31 ± 1.59% (n=4)
KO	8.93 ± 1.6%* (n=5)	31.44 ± 2.32%*** (n=4)
EDL		
WT	0.71 ± 0.09% (n=3)	0.66 ± 0.06% (n=3)
KO	0.37 ± 0.1% (n=4)	0.65 ± 0.1% (n=3)
Plantaris		
WT	0.73 ± 0.17% (n=3)	1.76 ± 0.36% (n=3)
KO	0.98 ± 0.17% (n=3)	1.53 ± 0.14% (n=3)
TA		
WT	N/O (n=3)	N/O (n=3)
KO	N/O (n=3)	5.8 ± 0.99% (n=3)
QC		
WT	1.03 ± 0.1% (n=3)	1.77 ± 0.36% (n=3)
KO	1.19 ± 0.12% (n=3)	38.26 ± 3.31%*** (n=3)

Data are from 1 year-old mice. Values are means ± SEM (expressed as a percentage of the total number of fibers); EDL, extensor digitorum longus; TA, tibialis anterior; QC, quadriceps; N/O, not observed; WT, wild-type; KO, DNase X knockout; * The value is significantly different from the value in wild-type mice (* P <0.05; ** P <0.01; *** P <0.001).

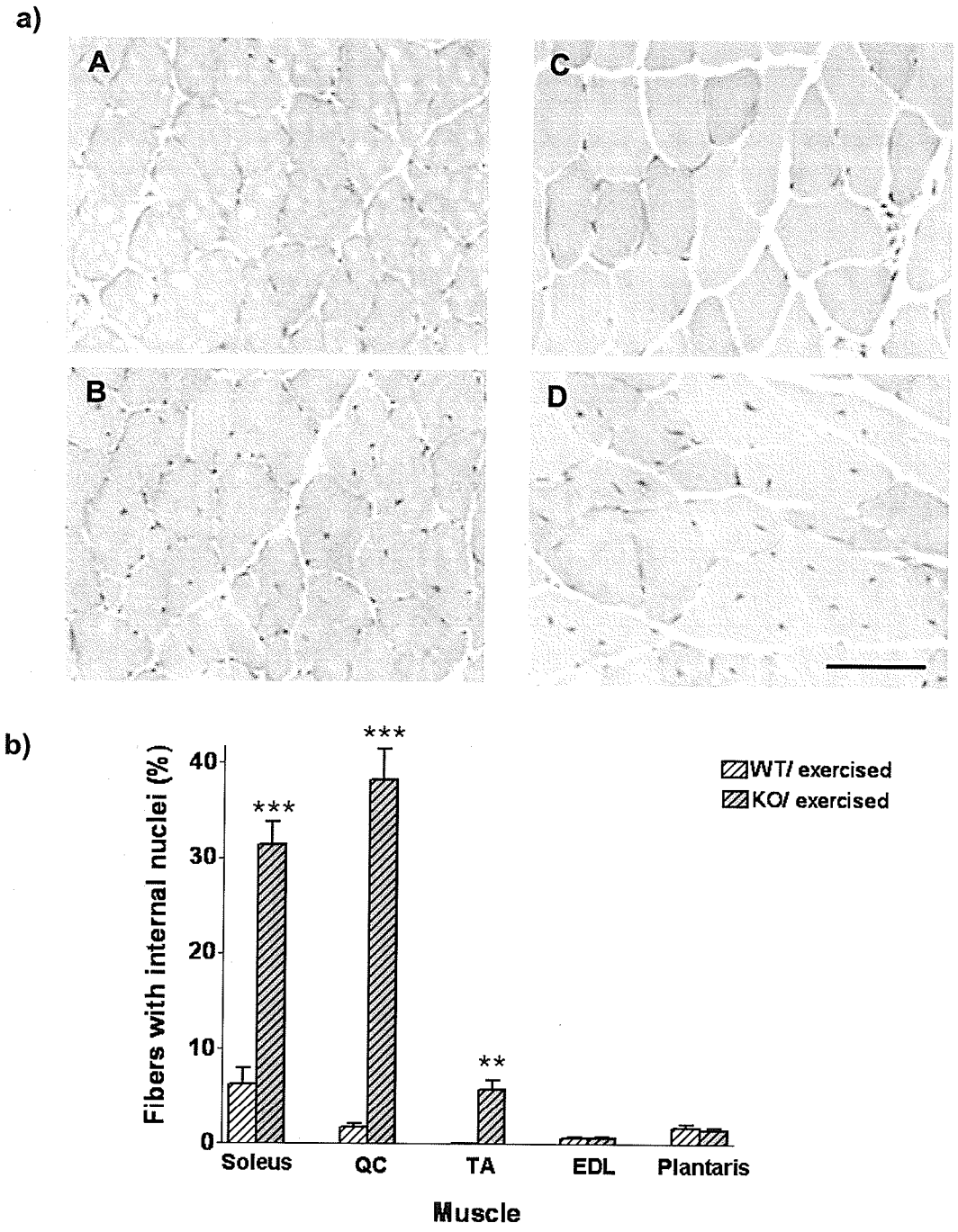


Figure 10. Histological analysis in exercised muscles. a) H&E staining of muscles cross sections in 1 year-old wild-type (A,C) and DNase $X^{(-/-)}$ (B,D) mice after treadmill running. A large population of fibers with internal nuclei are seen in DNase X deficient muscles in soleus (A,B) and quadriceps (C,D). Scale bar = 50 μm. b) The incidence of fibers with internal nuclei in muscles of the leg after treadmill exercise in wild-type (hatched open bars) and DNase $X^{(-/-)}$ (hatched solid bars) mice. Data are obtained from a minimum of 3 mice. Values are expressed as a percentage of the total number of fibers with central nuclei. Errors bars = SEM. *The difference is significant between two groups (** $P < 0.01$, *** $P < 0.001$).

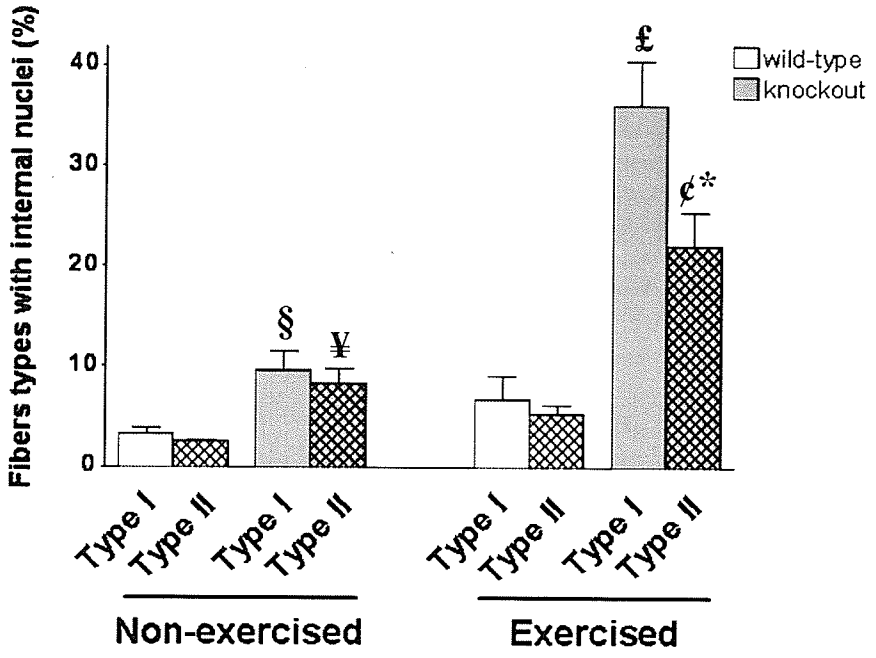


Figure 11. The incidence of fiber types with central nuclei in soleus of 1 year-old mice. Values are expressed as a percentage of the total number of slow (type I, open bars) or fast (type II, cross hatched bars) fibers with central nuclei. Data are obtained form 3-5 mice. Error bars represent SEM. §In non-exercised soleus, the frequency of fibers with internal nuclei observed in slow fibers was different between wild-type and knockout muscles. ¥In non-exercised soleus, the frequency of fibers with internal nuclei observed in fast fibers was different between wild-type and knockout muscles. No difference in the incidence of these fibers was observed between fast and slow fibers in knockout soleus in non-exercised mice. £In exercised mice, the frequency of fibers with internal nuclei observed in slow fibers was different between wild-type and knockout soleus. ¢ In exercised mice, the frequency of fibers with internal nuclei observed in fast fibers was different between wild-type and knockout soleus. *In soleus of the exercised knockout mice, the frequency of fibers with internal nuclei observed in slow fibers was different from their frequency in fast fibers. (§ ¥ £ ¢ * $P < 0.05$).

Evaluating physical activity via voluntary wheel training and treadmill running

The results of the voluntary wheel exercise showed that the performance of DNase $X^{(-/-)}$ mice was reduced in comparison with wild-type animals [Fig 12]. Mice were examined at 6, 13 and 27 weeks of age and the distance run on wheels was analyzed both prior and after normalization to body mass. Our data showed that the distance run on

wheels was significantly lower in 13 (P <0.05) and 27 week-old (P <0.001 in males, P <0.05 in females) mice in both male and female groups whereas the difference was not significant in 6 week-old animals. Running activity was reduced with age in both wild-type and knockout mice. The difference was significant for both wild-type and knockout mice between 13 and 27 weeks of age (63.8% in wild-type males vs. 73.8% in knockout males, 38.4% in wild-type females vs. 58.6% in knockout females; P <0.001), and was also significant in knockout mice between 6 and 13 weeks of age (P <0.001 in males, P <0.05 in females). There was a marked difference (P <0.001) in the voluntary wheel running activity between male and female mice, which was similar in both wild-type and knockout groups. Female mice ran an average of 2 and 1.9-fold further than male mice in wild-type and DNase X knockout groups, respectively, and the ratio was similar between wild-type and knockout mice at different ages.

The results for treadmill test are presented in Table 9. Under the same condition during the test, the average distance run by DNase $X^{(-/-)}$ mice was lower than that by wild-type mice. This difference was similarly seen in male and female groups at ages of 6 and 36 weeks, and although was not statistically significant (P >0.05), represented a 13%, 20%, 32% and 11.8% decrease in running distance in 6 week-old males and females, and 36 week-old males and females, respectively. The average time knockout mice spent on treadmill was not different from that spent by wild-type mice but DNase $X^{(-/-)}$ mice differed from wild-types with regard to their overall performance on treadmill. During the test, 5 wild-type mice were excluded from the test (1 male and 1 female aged 6 weeks, and 2 males and 1 female aged 36 weeks) whereas a total of 9 mice were removed from the knockout group during the experiment (2 male and 2 females at 6 weeks of age,

3 males and 2 female at 36 weeks of age). Besides, among those included in the test, all mice in the wild-type group ran to perceived exhaustion while 2 mice in the knockout group (8 week-old males) stopped early (total duration of the test less than 9 min). Similar to the results of voluntary training, females performed better on treadmill and ran an average of 26.2% and 32.6% farther than male mice in wild-type and knockout groups, respectively. However, unlike the voluntary wheel running, the differences in distance run on treadmill were not statistically significant ($P > 0.05$).

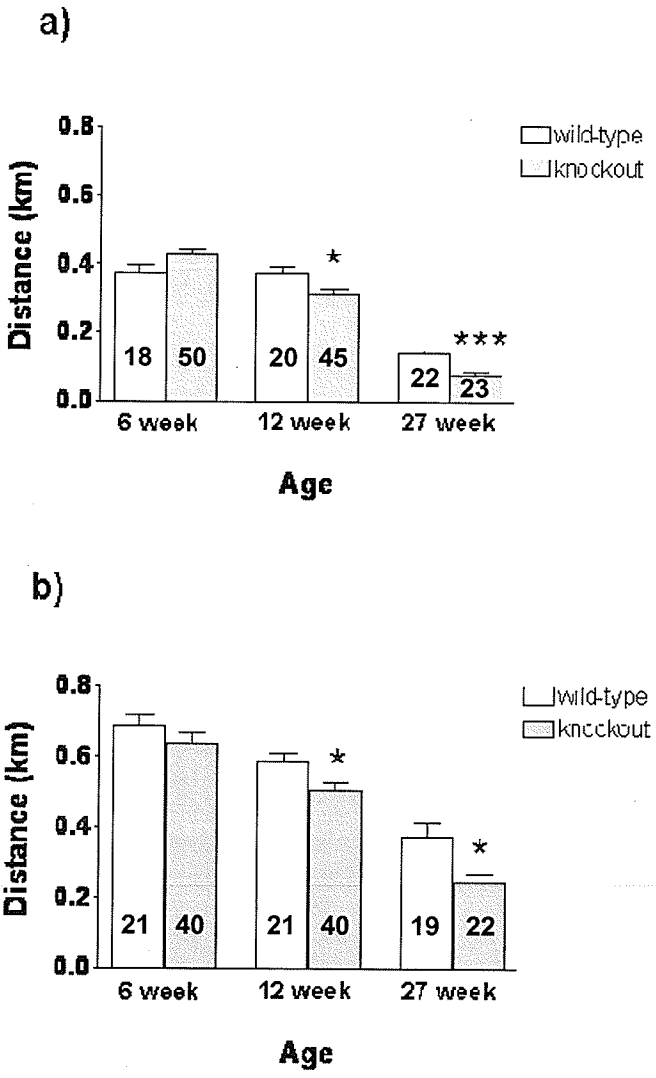


Figure 12. Voluntary wheel running. Total distance run on wheels over a 24-hour period was reduced in DNase $X^{(-/-)}$ mice (solid bars) as compared to wild-type mice (open bars) in both male (a) and female (b) groups. Distance run is normalized for body weight. The numbers inside the bars represent the number of animals examined. Error bars represent SEM. *The difference is significant between two groups (* $P < 0.05$; *** $P < 0.001$).

Table 9. ***Distance run on treadmill (m).***

	6 week-old	36 week-old
Male		
WT	418.1 ± 25.4 (n=7)	298.9 ± 29.9 (n=6)
KO	363.4 ± 36.1 (n=6)	202.9 ± 66.5 (n=5)
Female		
WT	540.0 ± 69.6 (n=7)	427.1 ± 55.4 (n=7)
KO	431.2 ± 32.8 (n=6)	380.2 ± 32.7 (n=6)

Values are means ± SEM; WT, wild-type; KO, DNase X knockout.

Gene expression analysis by quantitative real-time RT-PCR

We looked at the expression profile of DNase X mRNA in several muscles at different age points in both male and female mice. Each primer set, except for the pair designed for DNaseIl2, successfully amplified a unique product [Fig.13a], hence DNaseIl2 was excluded from the study. Samples were run in duplicates and the average values of wells were used for the analysis. In all cases, the variations of *Ct* values between duplicate samples were less than one cycle. The amount of the endogenous control transcripts, cyclophilin C, did not show significant changes in different tissues and between knockout and wild-type samples ($P > 0.05$). Our data showed that DNase X mRNA was 13-18 times more abundant in skeletal muscle than in liver. Also, DNase X mRNA expression was ~5.5- and ~3-fold higher in cardiac muscle and diaphragm, respectively, compared with liver [Fig. 13b]. There was no difference in the expression of DNase X transcript between different muscles of the leg examined (TA, QC and soleus; $P > 0.05$). The results obtained from mice at 6 weeks and 1 year of age showed no alterations in the expression of DNase X with age. Similarly, the expression profile of DNase X mRNA was not different between tissues in male and female mice (data not

shown). To examine whether DNase X ablation triggers the compensatory upregulation of homologous genes, we extended the analysis to compare the expression of other members in DNase I family. Our data showed a moderate and non-significant upregulation of about 2.2-3.6 fold in the expression of DNase I and DNaseII3 transcripts in myocytes of skeletal muscle in DNase $X^{(-/-)}$ mice. Similarly, the average amounts of DNase I and DNaseII3 mRNAs were increased by a magnitude of 1.5-2, 1.6-1.9, and 1.2-1.5 fold in heart, diaphragm and liver of knockout mice [Fig. 13c]. None of the fold inductions in DNase I and DNaseII3 expression were statistically significant ($P > 0.05$).

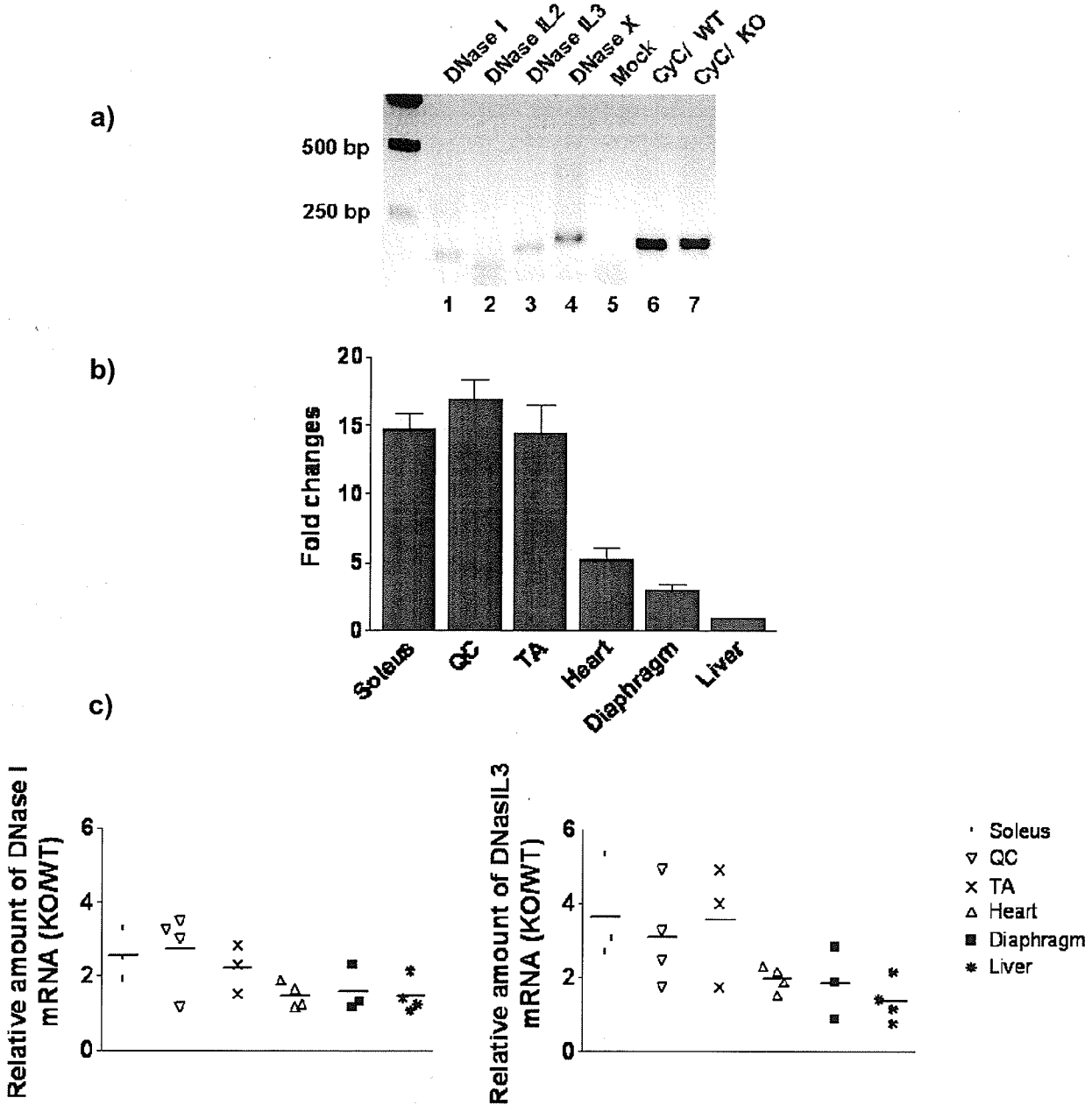


Figure 13. Real-time PCR analysis of the expression of DNase I family transcripts. a) Gel electrophoresis of amplicons. Single bands in lane 1, 3 and 4 indicate the successful amplification of target mRNAs. The band in lane 2 corresponds to non-specific amplification and/or formation of primer dimer. Mock reaction contains no template. The RT-PCR products of internal control, cyclophilin C (CyC), in wild-type and DNase X mutant samples are shown in lane 6 and 7, respectively. b) The relative amounts of DNase X mRNA in different tissues compared to liver in 1 year old wild-type mice. c) Quantification of DNase I (left) and DNaseII3 (right) mRNAs in 1 year-old DNase $X^{(-/-)}$ mice. The mild to moderate induction in DNase I and DNaseII3 mRNAs is not specific to skeletal muscles. Data are obtained form a minimum number of 3 mice. Error bars represent SEM. QC, quadriceps; TA, tibialis anterior.

Measuring the nuclease activity in leg muscles

We next examined the nuclease activity of DNase X deficient tissues by semi-quantifying the degradation of plasmid DNA in the presence of tissue protein extract. Our results showed that the DNase activity was significantly lower (P <0.05) in knockout muscles as compared to wild-type muscles [Fig. 14]. The mean DNase activity in knockout muscles was decreased by 53.8%, 29.1%, 44.1% and 32.8% in TA, soleus, QC and GN, respectively. In diaphragm, liver, kidney and heart, the differences in nuclease activity were not different between knockout and wild-type samples (P >0.05). The measures of nuclease activity for individual DNases showed a DNA-degrading activity in the presence of DNase X-specific reaction buffer in knockout tissues which was attributable to the suboptimal activity of other DNases in this buffer. The activity of each DNase showed a decrease in knockout muscles whereas in other tissues there were variations in the activity of these nucleases. Nevertheless, the differences in nuclease activity were only significant for DNase X in skeletal muscles.

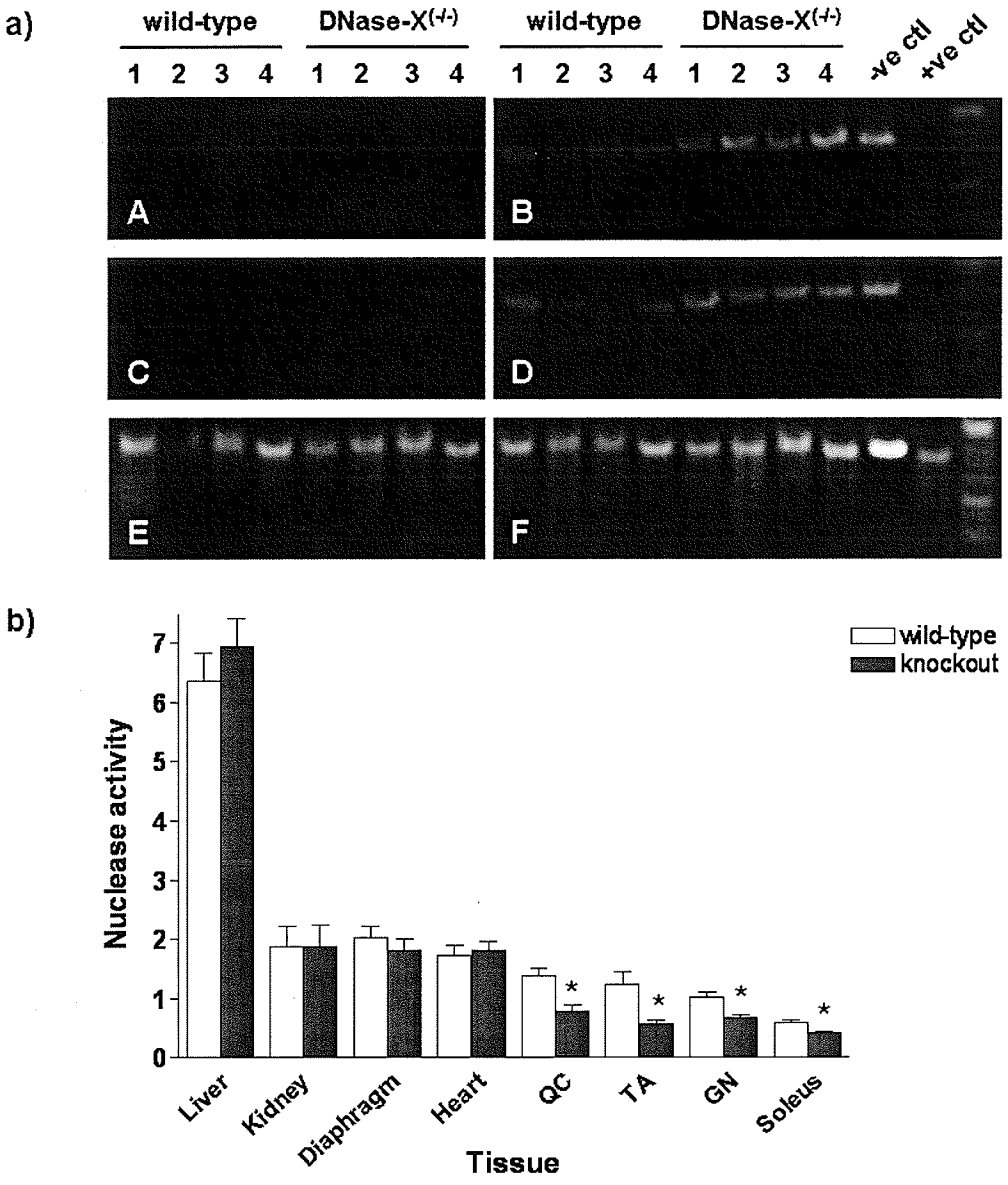


Figure 14. Nuclease activity of the DNase I family. a) The activity of tissue protein extract to degrade plasmid DNA was compared in kidney (A), tibialis muscle (B), liver (C), quadriceps (D), heart (E), and diaphragm (F) in 8 months-old wild-type and DNase $X^{(-/-)}$ mice. Nuclease activity of DNase I (1), DNaseII2 (2), DNaseII3 (3), and DNase X (4) was assayed in the presence of specific buffers for each DNase, as reported previously (see the text). Semiquantification was performed based on the band densities in each lane compared to those of negative (no enzyme in the reaction) and positive (containing commercial DNase I) controls. Nuclease activity in skeletal muscle (B,D) was reduced in DNase X deficient samples compared with wild-type muscles. b) Semiquantification of DNA-degrading activity in wild-type (open bars) and DNase X deficient (solid bars) tissues. Data are obtained form a minimum of 3 mice. Error bars represent SEM. *The difference in nuclease activity is significant between two groups (* $P < 0.05$).

Discussion

Whole body weight is lower in DNase $X^{(-/-)}$ mice

We have generated DNase $X^{(-/-)}$ mice by the targeted replacement of the first two coding exons and a preceding non-coding exon in wild-type allele by a neomycin-resistance cassette. The inactivation was confirmed by RT-PCR showing that no DNase X mRNA is produced in knockout mice. Survival and fertility was not affected in DNase $X^{(-/-)}$ mice. However, we weighed these mice over the first 6 months of their postnatal life and found that DNase X deficient mice weighed less than age-matched wild-types. The reduction in body mass was significant in younger animals (aged 6 and 13 weeks) and was more dramatic in male mice [Fig. 4]. The reduction in average body weight does not seem to be a result of reduced feeding, though the food and water intake was not studied in the present work. Firstly this difference was larger at early ages and secondly the analysis of the growth curve was not indicative of any progressive growth retardation in knockout mice over time. The reduction in body mass in knockout mice cannot be a result of higher energy expenditure due to increased locomotor activity either. This is supported by data on voluntary wheel training that showed DNase $X^{(-/-)}$ mice were less active than their wild-type counterparts when put in cages equipped with training wheels over a 24-hour period. Increased level of energy expenditure may also occur as a result of an increased basal metabolic rate (BMR). If elevated, BMR could reflect an increase in sympathetic nervous system activity which impacts whole body thermogenesis. The resting metabolic status and autonomic activity could be evaluated in these mice by basic measurements of heart rate, body temperature and O_2 consumption/CO_2 production. Nevertheless, body mass is influenced by multiple genetic and environmental factors and

the assessment of metabolic status and involvement of neuroendocrine system in regulating body mass is known to be complex. Furthermore, it has been argued that in addition to the targeted deletion of specific genes directly involved in metabolic properties, the reduction in body weight might be a mere consequence of introducing null mutations in animal models (Reed et al., 2008).

To determine whether the reduced body weight related to any alteration in muscle mass, we examined the 5 muscle groups of the leg as well as major organs in adult mice, and compared the results with those of age- and sex-matched wild-types. Our data showed no significant difference between the wet weights of major muscles of the hindlimb corrected to the whole body weight between wild-type and DNase $X^{(-/-)}$ mice. However, muscles uniformly weighed less in knockout mice. Given that skeletal muscle comprises 40-50% of body weight in mammals, the reduction of whole-body weight can be explained, in part, by combined effects of modest loss of muscle mass in each knockout muscle. Indeed, the lower weight in examined muscles (QC, GN, EDL, TA and soleus) accounted for 55.6% of the difference in mean body weight between wild-type and DNase $X^{(-/-)}$ mice. This may be further confirmed by the observation that the difference in body mass was more substantial in male mice in which skeletal muscle accounts for a higher proportion of body mass. The difference in body mass was not related to the developmental defects in bone compartment. In fact, our data showed a nearly identical femoral mass and length in wild-type and knockout mice (data not shown). These findings suggest that lack of DNase X has no major impact on muscle growth, and the modest reduction in muscle mass in DNase $X^{(-/-)}$ mice might be due to a rather systemic than a specific effect of DNase X absence in this tissue.

DNase X is not essential for muscle development

We examined the morphologic characteristics of muscles by determining the number of fibers and the cross sectional area of single fibers. We performed fiber enumeration using a previously described method to estimate the number of fibers based on the determination of mean fiber dry weight and total muscle dry weight after acid digestion (Timson and Dudenhoeffer, 1984). This method has been shown to be a fast and accurate approach to estimate the fiber number in muscles in which fibers run parallel to the longitudinal axis of the muscle. It has been used with different modifications to study the fiber numbers in skeletal muscles (Antonio and Gonyea, 1994; Soffler and Hermanson, 2006). Examination of soleus, EDL and TA revealed no significant variation in the number of fibers between knockout and wild-type muscles. Our findings of no difference between the number of fibers between limbs in an animal, or between animals at different ages were in agreement with the previous reports (Gollnick et al., 1981; Matoba and Murakami, 1986; Swatland, 1976). Also, the increase in mean CSA of both type I and type II fibers in soleus between 6 weeks and 1 year of age, which was similar to wild-type muscle, was consistent with the literature (Matoba and Murakami, 1986; Sakakima et al., 2004). The findings that CSA of fibers was comparable in wild-type and knockout muscles, did not change by age, and showed a constant ratio in fast and slow fibers in young and older animals imply that the lack of DNase X is not associated with atrophic or hypertrophic changes in skeletal muscles.

The marked reduction in fiber CSA in soleus of older DNase $X^{(-/-)}$ mice was mainly due to an increase in the frequency of small fibers in this muscle (9.5-28.4% in knockout vs. 2.3% in wild-type). The increased frequency of small-sized fibers in soleus

is not related to any phenotype variation in fiber types as both wild-type and knockout muscles exhibit similar fiber type composition. The higher number of small fibers in knockout soleus is most likely a result of increased regeneration of myofibers. This fiber formation; however, is not associated with hyperplasia in knockout soleus since our enumeration results showed no increase in the number of fibers in this muscle. The phenotype of these small fibers could be evaluated by the expression of embryonic MHC isoforms and other muscle specific genes such as MyoD and Myf5, which are upregulated in newly-formed myofibers (Maier et al., 1986; Winchester and Gonyea, 1992). The increase in CSA variability in knockout soleus [Fig. 8], and the appearance of newly formed fibers might have been a result of regeneration process in knockout soleus, as previously described for *mdx* mice (De la Porte et al., 1999), and be related to a higher incidence of degeneration/repair events in this muscle in DNase $X^{(-/-)}$ mice.

In conclusion, these data show that no important morphologic variations exist between wild-type and knockout muscles. The fact that DNase $X^{(-/-)}$ mice have normal muscles indicates that DNase X does not play an important role in muscle development during embryonic and postnatal life.

DNase X deficiency has no effect on fiber type composition

We examined fiber type composition using a mAb against slow MHC, and found no significant difference between EDL, plantaris and soleus of wild-type and DNase $X^{(-/-)}$ mice. The results of this study are consistent with the previous reports that showed EDL and plantaris are exclusively composed of fast fibers, and slow fibers are outnumbered by fast fibers in mouse soleus (Hitomi et al., 2005; Matsuura et al., 2007; Schuenke et al.,

2008; Timson et al., 1985; Totsuka et al., 2003; Wigston and English, 1992). Also, the proportion of type I and type II fibers, and the changes in fiber type composition during the first year of postnatal life in soleus is in agreement with the previous studies (Matoba and Murakami, 1986; Matsuura et al., 2007; Schuenke et al., 2008; Timson et al., 1985; Wigston and English, 1992).

It has been demonstrated that during the first few weeks after birth, a percentage (10-12%) of fibers in soleus express two MHC isoforms simultaneously (Matsuura et al., 2007; Wigston and English, 1992). Soon afterwards, the number of these hybrid fibers decreases and myofibers exclusively express either one of slow or fast MHC isozymes (Wigston and English, 1992). Subsequently, fiber typing based on single staining with antibody specific to either fiber type provides accurate results in phenotyping fast and slow fibers. Fast-twitch fibers; however, comprise several subtypes variably expressed in different muscles. For instance, in addition to type I fibers, mouse soleus contains a high percentage of type IIA fibers followed by a small percentage of type IIA/IID and type IID fibers (Stelzer and Widrick, 2003; Totsuka et al., 2003). Also, mouse plantaris contains a similar proportion of type IIA, type IID, type IID/IIB and type IIB whereas EDL is mainly composed of type IID and type IIB fibers (Schuenke et al., 2008; Zardini and Parry, 1994). Fiber typing and the relative expression of MHC isoforms could be further investigated by qRT-PCR or immunoblotting. In the present study, we did not examine the subtype composition of fast fibers and the hybrid fibers coexpressing two MHC isoforms in these muscles. Yet, based on our data on two main types of fast and slow fiber population in these mice, we suggest that DNase X deficiency has no major effect on fiber type composition in skeletal muscle.

DNase X deficiency results in muscle fiber damage

The incidence of fibers with central nuclei which appear when damaged fibers are regenerated by satellite cells was low in muscles of non-exercised wild-type and knockout mice at the age of 6 weeks as well as in wild-type animals at 1 year of age. Among the hindlimb muscles of the older DNase $X^{(-/-)}$ mice, the soleus was the only muscle that exhibited a marked histological change. Soleus in these mice contained a notable population of fibers with internal nuclei (8.9% in knockout vs. 2.9% in wild-type). Fibers with central nuclei were present, although in varying degrees (4.2-14.2%), in all soleus muscles examined in older DNase $X^{(-/-)}$ mice, and composed a similar proportion of type I and type II fibers. The variability in the proportion of fibers affected implied that interindividual variations might be involved and determine the extent of damage. To test whether physical activity is a factor contributing to this phenotype, we examined the muscles of the hindlimb in these mice after uphill treadmill running. Unlike wild-type muscles which did not show any dramatic changes in the frequency of damaged fibers after exercise, several muscles of the leg in DNase $X^{(-/-)}$ mice displayed an increase in the number of fibers with internal nuclei [Fig. 10]. Among these muscles, QC and soleus had the largest population of regenerated fibers followed by TA, with 38.2%, 31.4% and 5.8% of fibers carrying internal nuclei, respectively. Additionally, in soleus, type I fibers comprised a larger proportion of fibers with internal nuclei.

The difference in the incidence of damaged/regenerated fibers in wild-type and knockout mice after exercise could not be related to the difference in the intensity of their running activity on treadmill. Mice were trained under the same condition and ran similar

distances corrected to their body weights. The appearance of these fibers in certain muscles of DNase $X^{(-/-)}$ mice after exercise (QC, soleus, TA) might be explained by the preferential recruitment of these muscles in running uphill. Similarly, the observation that muscle pathology in DNase $X^{(-/-)}$ mice was limited to soleus in non-exercised mice can be attributed, in part, to the continuous activation and major function of this muscle in weight-bearing and stabilization. Nonetheless, the physical stress and workload cannot explain all the difference in the rate of fiber damage in wild-type and knockout muscles. This is because the number of fibers with central nuclei in plantaris which is also involved in uphill running was low in knockout mice after training. The finding that in DNase $X^{(-/-)}$ mice the incidence of damaged fibers was low in EDL and plantaris, and jumped up only to 5.8% in TA after exercise raises the possibility that fiber type composition of these muscles may account for the variability in their exhibition of the phenotype. These muscles in mouse are mainly composed of type IIB, type IIB/IID and type IID fibers which are the most glycolytic and the least oxidative/vascularized fibers. In contrary, soleus which constantly displayed moderate to high degree of fiber damage in older knockout mice, is rich in oxidative (type I and IIA) fibers. Accordingly, it could be assumed that the oxidative phenotype is associated with the pathogenesis of muscle damage in DNase X deficient mice. This is in line with the rate of fiber damage in exercised QC group which contains a heterogeneous population of fibers from pure slow/oxidative to fast/glycolytic fibers, and TA which contains a small population of type IIA fibers with oxidative/glycolytic metabolism. Therefore, it seems possible that the oxidative metabolism of muscle/fiber in the context of muscle activity and physiological demands may contribute to the pathogenesis of muscle injury observed in DNase $X^{(-/-)}$

mice. Yet, this hypothesis does not explain the low incidence of fiber damage in plantaris which also contains a modest population of type IIA fibers. This may relate to other functional and metabolic properties in these muscles, and indicates the involvement of other factors in the process, which are specific to different muscles. For instance, the kinetics of muscle function differ in soleus which crosses one joint and has a major role in standing and walking, and plantaris which crosses two joints and contributes only weakly to the function of muscles in the posterior compartment of the leg. The late onset of fiber damage in DNase X deficient muscles suggests that this phenotype is not a mere consequence of DNase X ablation in these muscles and there are most likely some senescence-related factors in muscle cells that also contribute to the pathogenesis of muscle damage in DNase $X^{(-/-)}$ mice. Nevertheless, evaluating the measures of fibers with internal nuclei in different subtypes of fast fibers in DNase X deficient muscles would provide important clues for eliciting the underlying mechanism/s contributing to muscle damage in these mice.

A proposed role for DNase X in muscle physiology

It is unlikely that the fiber damage in DNase X deficient muscles is a result of systemic hypoxic condition due to a deficiency in blood flow and oxygenation. This is because no significant morphological changes such as a frequency shift toward type II fibers and reduction in fiber CSA that are associated with chronic hypoxia (Luedeke et al., 2004) were observed in DNase X deficient muscles. The findings that aging, physical activity, and oxidative phenotype were associated with muscle damage in DNase $X^{(-/-)}$ mice raises the possibility that metabolic alterations in the absence of DNase X might be

involved in muscle damage in DNase X deficient mice. In this view, intracellular ionic disturbances and/or enhanced production of free reactive species may contribute to fiber damage in these mice. Free reactive species are mainly generated during the electron transport in mitochondrial respiratory chain, and can cause muscle injury and muscle cell degeneration by promoting intracellular and membrane protein damage. Mitochondrial dysfunction and reduced respiratory capacity has been shown to be associated with muscle damage (Glesby et al., 1988; Kuznetsov et al., 1998; Timmons et al., 2005). It has been hypothesized that the alteration in mitochondrial function involves ionic disturbances and is mainly due to altered intracellular calcium homeostasis (Kuznetsov et al., 1998; Yeung et al., 2003). Slow muscles/fibers have a high content of mitochondria, and consume oxygen to produce energy from different substrates including fatty acids. This makes slow fibers more vulnerable to oxidative damage as a result of any compromised mitochondrial function. In DNase $X^{(-/-)}$ mice, the finding that muscle damage was primarily seen in the soleus which contains a large number of oxidative fibers supports this idea that susceptibility to damage might be a result of excessive exposure to reactive species in these muscles. Additionally, in DNase X mutants, damaged fibers appeared in muscles of aged animals (and not in young mice) in which the rate of mtDNA damage and oxidant production is higher (Esposito et al., 1999; Mansouri et al., 2006; Tanaka et al., 1996). Moreover, this idea is consistent with the observed increase in the rate of fiber damage in soleus and QC by running activity during which the rate of free radical generation is increased (Ji et al., 1992; Perez et al., 2002). Both aging and acute exercise induce the generation of free radicals that render muscles susceptible to oxidative damage. However, the incidence of fiber damage was lower in

aged wild-type muscles even after exercise. This indicates that the susceptibility to (oxidative) damage is somehow increased in the absence of DNase X.

DNase X has been reported to be associated with the membrane by a GPI anchor (Shiokawa et al., 2007). GPI-anchored proteins are preferentially localized in lipid raft domains (caveola) of the membrane and are involved in various biological functions including signal transduction across the membrane and endocytosis. Shiokawa et al. have proposed that DNase X functions as a barrier for the transfer of foreign genes through endocytosis-mediated destruction of foreign DNA (Shiokawa et al., 2007). However, a large body of evidence suggests that the presence of GPI anchor affects the conformational and functional properties of the protein attached to it conferring novel properties to the modified protein (Hooper, 1997; Sharom et al., 1996). In this view, DNase X might be involved in pathways mediating intracellular events that regulate mitochondrial function in response to extracellular signals such as hormones, cytokines and growth factors. The presence of an HLH motif in the structure of DNase X may contribute to the formation of homo/heterodimers, which may induce catalytic/non catalytic activation of a signaling cascade across the membrane through ion channels or adaptor molecules. Indeed, the presence of a dimeric form of DNase X has been previously reported (Los et al., 2000). It has been suggested that signaling via GPI-anchored proteins proceeds through Src kinases and downstream pathways (Cinek and Horejsi, 1992; Draberova and Draber, 1993; Wang et al., 2005). AKAP121 is one of the downstream mediators that link cAMP and Src to oxidative metabolism and mitochondrial function. AKAP121 is a member of anchor proteins which tethers Src tyrosine kinase as well as cAMP-dependent protein kinase-A to mitochondria, and

enhances mitochondrial metabolism through increasing phosphorylation of mitochondrial substrates and cytochrome c oxidase activity (Cardone et al., 2002; Livigni et al., 2006). Similarly, it could be assumed that DNase X might be involved in the signaling to regulate mitochondrial function and hence its absence would affect the oxidative capacity of myocytes leading to enhanced production of free radicals which subsequently cause muscle damage in DNase X deficient muscles. Moreover, DNase X may function as part of the machinery to repair mtDNA damages which are associated with aging and are important in postmitotic tissues such as skeletal muscle. In this context, lack of DNase X may result in accelerated accumulation of mtDNA mutation/damage over time leading to depletion of mitochondrial function.

In relation to and to test this idea, the oxidative capacity of DNase $X^{(-/-)}$ muscles could be evaluated by measuring various biomarkers of oxidative stress such as lipid peroxidation, protein oxidation and oxidative DNA damage. Also, the levels of endogenous antioxidants including glutathione, ubiquinol and cysteine in different DNase X deficient muscles may provide useful information on the susceptibility of these muscles to damage as well as the variability in the extent of damage in muscles such as plantaris in DNase $X^{(-/-)}$ mice. The rate of mtDNA damage and repair capacity can also be studied by several methods described previously (Barja and Herrero, 2000; Driggers et al., 1993). Furthermore, the susceptibility to oxidative stress in these mice can be evaluated by comparing the measures of fiber damage in mice exposed to hypoxic conditions. Also, the alteration in damage following the administration of antioxidants can be tested in these mice. Changes in myoplasmic Ca^{2+} concentration can be measured by several available methods, and the role of calcium as primary or secondary cause of

damage could be examined *in vitro* in the presence of different [Ca^{2+}], calcium antagonists and oxidative stress. Changes in sarcolemmal permeability could be assessed by measuring the leakage of cytosolic contents or reciprocally by measuring the uptake of membrane-impermeant dyes such as Evans blue or procion orange as an indicative of myofiber membrane damage (McNeil and Khakee, 1992). Further assessment could be performed *in vitro* either on cells derived form DNase $X^{(-/-)}$ muscles or by knock down assays to examine the behavior of DNase X deficient cells in normoxic or hypoxic conditions. The induction of muscle damage in DNase $X^{(-/-)}$ mice could also be examined by using various myotoxins or more physiologically-relevant approaches such as denervaion/reinnervation and ischemia/reperfusion.

Based on our observations, we propose that DNase X is involved in signaling to regulate myocyte oxidative capacity and its ablation might primarily affect fibers with higher oxidative metabolism. Further assessments are required to verify this hypothesis and to elucidate the mechanism by which lack of DNase X may contribute to this process. We cannot rule out the possibility of a more systemic effect of DNase X ablation which affects specific muscles. This could be addressed by a conditional knockout in which the expression of DNase X is abolished in skeletal muscle. The role of DNase X in muscle damage can be further analyzed by examining the effect of DNase X gene transfer in rescuing fiber damage in knockout muscles.

DNase X deficiency diminishes running performance

DNase $X^{(-/-)}$ mice showed a markedly reduced running performance on training wheels over a 24-hour period, and also fatigued faster during treadmill training.

However, the results of voluntary wheel exercise cannot be used to assess the bio-motor components of exercise, and should be used with caution to quantify the parameters of physical activity. This is, in part, because of the nature of the test which is composed of intermittent running periods followed by rest intervals. In spite of this, the cumulative distance run on wheels is commonly used as an indicator of physiological capabilities and general endurance in tested animals (Chappell et al., 2004; Golumbek et al., 2007; Rezende et al., 2005). We did not determine the running speed and the number and duration of running bouts, and subsequently do not know what fraction of the computed distance relate to the so-called "coasting" run of these wheels (the continuation of rotation caused by inertia after mice cease running). Coasting has been shown to account for approximately one third of the total wheel rotations in mice running on identical wheels (Girard et al., 2001; Koteja et al., 1999). However, in our study the test condition was identical for both wild-type and knockout groups and therefore "coasting" was expected to affect the results of both groups in a similar fashion. Additionally, it has been reported that voluntary running activity varies between individuals and from day to day in individual laboratory animals (De Bono et al., 2006; Friedman et al., 1992). The relatively large number of mice tested on wheels in this study (a total of 220 knockout and 121 wild-type mice) can attenuate this variability and provide a good reflection of the true difference between the two groups. We believe that the differences in distance run on wheel most likely relate to the physiological differences that affect physical endurance rather than the behavioral variations in running activity in these mice. Yet, the latter remains a possibility and cannot be ruled out in this study.

The finding that female mice performed better on both voluntary wheel training and forced treadmill running is in agreement with the previous reports that showed a similar difference between male and female groups (De Bono et al., 2006; Konhilas et al., 2004; Swallow et al., 1998). The difference in distance run by male and female mice was similar in both wild-type and knockout mice, which indicates that the lack of DNase X equally affects the running capacity in both males and females. The distances run in either wheel or treadmill test were reduced in both wild-type and knockout mice by age but the differences, except for the result of treadmill running in knockout females, were more substantial for knockout mice. This may relate to age-associated changes in these mice that may alter fatigue susceptibility in a synergistic manner with the absence of DNase X. However, the age differences in running performance are less likely to be due to age-related alterations in DNase X expression as indicated by our data for real-time PCR results of DNase X mRNA. The exercise capacity of DNase $X^{(-/-)}$ mice was less than age-matched wild-types. This impaired activity was observed in both voluntary training and forced running tests. There was an exception in 6-week old male group in which the performance of wild-type mice was slightly and non-significantly more compromised than knockouts. The only explanation we can offer is that wild-type mice which were relocated from another facility were not acclimated to the new environment before being introduced to the running wheels.

The ability to perform endurance exercise, as is tested in treadmill running to exhaustion, depends on the efficiency of several physiological systems including cardiovascular, respiratory, musculoskeletal and autonomous nervous system. Also, impaired physical activity has been shown to be associated with the abnormalities in

other organs such as immune system (Golumbek et al., 2007), although the underlying mechanisms remain to be elucidated. Accordingly, the cause for lower fatigue resistance in DNase $X^{(-/-)}$ mice most likely relates to multiple factors, as DNase X is expressed at moderate levels in several tissues including lung and diaphragm. The high expression of DNase X in cardiac muscle raises the possibility that the heart performance may play a factor in reduced capacity for physical exercise in DNase X deficient mice. We did not evaluate the functional capacity of heart and major respiratory organs, lung and diaphragm, in these mice, and therefore have no insight how the impaired running activity in DNase $X^{(-/-)}$ mice may relate to the alteration in cardiorespiratory capacity in these mice. In relation to musculoskeletal system, muscle fatigue in DNase $X^{(-/-)}$ mice is not a result of variations in muscle fiber composition. This is because no changes toward decreased proportion of fatigue-resistant/type I fibers and the concurrent increase in the relative number of fatigable/type II fibers were seen in knockout muscles. In muscles of the leg examined, the type I and type II fiber content was similar in both wild-type and DNase $X^{(-/-)}$ mice, and no significant variation was found in fiber type transformation over time in these muscles. The reduced exercise tolerance may not be a result of muscle damage in DNase $X^{(-/-)}$ mice either. DNase $X^{(-/-)}$ mice tested for running activity were younger than 9 months-old by which age these mice did not exhibit pathological changes in their muscles (soleus). In this context, it is possible to assume that a similar mechanism may underlie and contribute to both pathological changes and fatigue progression in these muscles.

Muscle fatigue may be a result of changes in central nervous system to excite motoneurons (central fatigue), or involve the peripheral components of muscle function

which are mostly related to alteration in excitation and muscle cell metabolism (Allen et al., 2008; Fitts, 1994). The etiology of muscle fatigue appears to involve the impaired release of calcium from the sarcoplasmic reticulum (SR); however, the contributing factors vary depending on the muscle fiber type and metabolic contents, and exercise intensity. In prolonged submaximal exercise, muscle cell energy is primarily derived from the aerobic metabolism which may also serve as a contributor to fatigue development. Free radicals have been proposed to be involved in fatigue process in skeletal muscle (Barclay and Hansel, 1991; Moopanar and Allen, 2005; Reid et al., 1992). The exact mechanism by which reactive radicals may contribute to muscle fatigue is not known though it is proposed that the loss of SR integrity as a result of structural protein/lipid modifications might be the causative event. ROS may affect Ca^{2+} release from SR by oxidative modification of residues in SR calcium-release channels (Aracena-Parks et al., 2006; Favero, 1999). Fatigue progression and impaired SR Ca^{2+} handling may also occur in the presence of ionic changes such as extracellular $[K^+]$ accumulation, increased intracellular $[Mg^{2+}]$, and entrance of inorganic phosphate into SR (Allen et al., 2008).

Central mechanisms are unlikely to play a factor in fatigue development in DNase $X^{(-/-)}$ mice. This is because the protein is expressed at a very low level in the central nervous system (Los et al., 2000; Shiokawa and Tanuma, 2001). However, mice in this study were subjected to a submaximal workload during treadmill running with final running speed of 17 m/min (10° incline) which is less than the maximum running speed previously reported in the C57BL/6 strain (23-40 m/min at 7°-15° incline) or the intensity used for evaluating the endurance capacity in these mice (Faldt et al., 2004; Lerman et

al., 2002; Massett and Berk, 2005; Milner et al., 2000). Given that the main source of ATP production during this activity would be through mitochondrial respiration, increased ROS generation might explain muscle fatigue in DNase $X^{(-/-)}$ mice. In this view, it can be postulated that the proposed involvement of DNase X in mitochondrial function which is absent in DNase X deficient muscles results in higher rate of ROS generation in these muscles, leading to development of muscle fatigue and impaired running performance in DNase $X^{(-/-)}$ mice. Similarly, lack of DNase X might initially impair ion traffic across sarcolemma which is more prominent during physical exercise. This would subsequently affect excitation-contraction coupling in muscle, leading to less force generation and fatigue process.

The contribution of muscle fatigue to impaired running performance in DNase X deficient mice can be tested via both *in vitro* and *in vivo* evaluation of contractile properties of individual muscles. Fatigue progression in these muscles can be examined by measuring force output during repetitive stimulation of muscle in a fatigue protocol. The strength and endurance capacity of limb muscles can also be examined using various tests designed for this assessment in animal models (Kong and Xu, 1998; Smith et al., 1995). Furthermore, the likely causative association of oxidative damage with increased fatigability in DNase X deficient muscles can be tested by evaluating contractile performance of muscles in these mice taking a diet containing antioxidants.

DNase X expression is independent of age and gender

We evaluated gene expression by a semiquantitative measurement of the relative number of transcripts in the real-time PCR assay. To improve the reliability of the results,

we controlled several factors that may affect the accuracy of real-time QRT-PCR in our experiments. (a) The primer sets were designed to amplify a single product in the corresponding sequence; this was confirmed by melting curve analysis and gel electrophoresis. (b) To eliminate genomic DNA contamination, one of the primers in each set spanned an exon/exon junction. Additionally, the RNA samples were treated with DNase. (c) We ran the samples in duplicates and used the average values in at least three independent experiments for the analysis. (d) We examined the *Ct* values of the internal control, cyclophilin c, in our experiments which were not different between samples ($P > 0.05$). The comparative *Ct* method used in this study is extensively used to approximate the mRNA expression level in real-time PCR, and is based on the assumption that the rate of target copy change is identical for both the gene of interest and reference gene, and is equal to 2 for all amplicons (doubling to target sequence in each PCR cycle) (Livak and Schmittgen, 2001). Alternatively, since these assumptions are rarely met in reactions, there are several methods developed to increase the accuracy of quantification by calculating the amplification efficiency from each reaction separately (Meijerink et al., 2001; Peirson et al., 2003; Schefe et al., 2006). In this study, we did not calculate the amplification efficiency of samples and performed the quantification based on the presumed constant efficiency of 2 for the PCR amplification. Although this may affect the accuracy of the PCR results, it has been reported that this method is able to closely estimate the relative gene expression and yields reproducible results even though the assumptions are not exactly met (Winer et al., 1999).

Real-time PCR results showed that the expression of DNase X mRNA was approximately 16 and 5.5-fold higher in skeletal muscles and heart, respectively,

compared with that in liver. This is in agreement with the previous work by Shiokawa et al. that reported DNase X expression about 21 and 4-fold higher in skeletal and cardiac muscles, respectively (Shiokawa et al., 2005). The finding that DNase X mRNA was not different in fast-twitch (TA) and slow-twitch (soleus) muscles suggests that DNase X expression is not specific to either type I or type II fibers. This argues against the possibility that fiber type-specific expression of DNase X might account for the selective fiber damage in DNase $X^{(-/-)}$ mice discussed earlier. Yet, examining DNase X expression in isolated fibers can provide more accurate information on the expression pattern in adult myocytes. The finding that the expression of DNase X mRNA does not go through significant alteration during postnatal life also implies that the pathologic changes in older DNase $X^{(-/-)}$ animals may relate to the combinatorial effect of several factors rather than simply the absence of DNase X alone. Similarly, the sex-independent expression of DNase X mRNA is supported by the fact that the phenotype in DNase $X^{(-/-)}$ mice was similar in males and females, and indicates that any possible differences between two groups may relate to other gender-related variations.

We examined the expression profile of other members of DNase I family to investigate the existence of any possible compensation in DNase $X^{(-/-)}$ mice. DNaseII2 was excluded from our analysis because corresponding mRNA sequence was not amplified in our experiments. This might be either due to the low expression level of the gene in most of the organs including skeletal muscle (Rodriguez et al., 1997; Shiokawa et al., 2004) and/or the optimization of the PCR reaction in our experiments. The non-significant fold induction in the expression of DNase I and DNaseII3 in DNase X deficient muscles (2-3.6 folds) suggests that there is not functional redundancy within

DNase I family in skeletal muscle. This may be supported by a similar finding in heart and diaphragm with a mild increase (1.5-2 folds) in DNase I and DNaseII3 mRNAs, and may reflect a partial cooperation within this family only related to their involvement in general cellular processes. In this view, establishing the double knockout line null for both DNase X and either one of DNase I or DNaseII3 (DNase I deficient mice is already generated and investigated), and characterizing the phenotype of these mice will provide helpful information on the aspects of the functional redundancy among these DNases. At this time, there is no specific antibody available to recognize murine DNase X; however, the gene expression results of this study can be reevaluated, in part, by blotting and/or *in situ* assays using antibodies that are available against DNase I or have been developed to react with murine DNaseII3 (Shiokawa et al., 2000).

Nuclease activity is reduced in DNase X deficient muscles

Nuclease activity was assessed by incubating tissue protein extract with plasmid DNA followed by visualizing on a gel system. Gel electrophoresis-based methods are the most commonly used assays to evaluate nuclease activity. The nuclease enzyme degrades substrate DNA (usually plasmid), yielding products of various sizes that are separated on a gel, which is further visualized and examined for quantification analyses. In this study, we ran positive and negative controls for each experiment, and semi-quantitatively compared the band densities between wild-type and knockout samples normalized to the difference observed between bands from two control reactions. We used separate buffers for each DNase, which were shown in a similar assay to yield the maximal activity of the corresponding nuclease (Shiokawa and Tanuma, 2001). However, except for DNaseII2,

which is most active in acid pH, DNase I-like enzymes appear to be active over a range of ionic strength and pH. Therefore, the subtle variations in the reaction buffers used in this experiment could not affect the suboptimal activity of these DNases and subsequently, the nuclease activity of other members of this family would not be fully eliminated in the presence of buffers specific for either of them. This may explain the residual nuclease activity we observed in DNase X specific reactions in DNase X deficient tissues. Nevertheless, it should be considered that our data are obtained from a semi-quantitative analysis and have to be interpreted cautiously.

The significant decrease in nuclease activity in muscles of DNase $X^{(-/-)}$ mice, which was not observed in other tissues such as kidney and liver, suggests that DNase X is the major nuclease found in skeletal muscle. This is in parallel with DNase X being abundant in skeletal muscle and its function as an active enzyme with DNA-degrading activity (Coy et al., 1996; Los et al., 2000; Parrish et al., 1995; Shiokawa and Tanuma, 2001). However, the physiological significance of this finding depends on the biological function of DNase X as a nuclease in skeletal muscle. Adult skeletal muscle is a stable tissue with a low rate of nuclear turnover (Decary et al., 1997; Schmalbruch and Lewis, 2000), and hence the endonucleolytic activity may not play a critical role in this tissue. Accordingly, although DNase X seems to be the major nuclease in skeletal muscle and its knockout reduces DNase activity in this tissue, the causal relationship between the nuclease capacity of muscle and muscle pathology such as what is seen in DNase$X^{(-/-)}$ mice can not be definitely concluded and requires further investigation. The abundance of DNase X in skeletal muscle and its ability to degrade substrate DNA *in vitro* may not necessarily mean that the main function of DNase X in muscle relates to its nuclease

property. In this context, it could be helpful to investigate the role of DNase X in muscle cell apoptosis *in vitro* in myoblast cell lines as well as in primary culture of skeletal myocytes. Also, *in vivo* analysis of apoptotic events can be pursued by *in situ* assays in wild-type and DNase X deficient muscles. Our finding of the similar cytoplasm area per nucleus ratio in wild-type and DNase X knockout muscles may imply that nuclei turnover is not affected in the absence of DNase X. Nevertheless, we performed the analysis on the cross sections of muscle tissues while isolating single fibers, and staining and measuring nuclei and entire length (size) of myofibers would yield more accurate results.

The reduced activity of DNase I and DNaseII3 in knockout samples was not in line with our expectation for a compensatory increase in their activity in muscle but can be supported by the result of real-time PCR that showed no significant induction in their expression in DNase $X^{(-/-)}$ muscles. However, it should also be considered that the lower activity of DNase I and DNaseII3 in muscle samples of knockout mice might be a relative effect due to the presence of DNase X activity in wild-type samples (the suboptimal activity in non-specific buffer condition, as discussed earlier) which is eliminated from knockout samples.

Conclusions

The major findings of this study include:

1) The soleus muscle in DNase $X^{(-/-)}$ mice exhibited a high incidence of regenerated fibers with central nuclei at older ages. Also, these fibers with a small diameter composed a notable proportion of fibers in this muscle.

2) Muscle pathology was limited to the soleus in non-exercised DNase $X^{(-/-)}$ mice. Muscle damage was intensified in soleus after treadmill exercise and was also seen in the quadriceps and tibialis anterior.

3) Muscle morphology was comparable in wild-type and DNase $X^{(-/-)}$ mice, and no important variation was found in the number of fibers, fiber type composition and mean cross sectional area of single fibers.

4) DNase $X^{(-/-)}$ mice displayed an impaired running capacity compared with wild-type counterparts which was prominent in voluntary activity on running wheel.

5) Body mass was reduced in DNase $X^{(-/-)}$ mice and the difference was more dramatic at younger ages.

6) The nuclease activity was lower in DNase X deficient muscles, whereas no such difference was found in other tissues examined.

7) Expression of DNase X did not change with age and was similar in both sexes.

8) No significant changes in the expression profile of DNase I family members were found in DNase $X^{(-/-)}$ mice.

Based on our findings that pathology was prominent in muscles which contain high proportion of oxidative fibers, either in exercised or non-exercised mice, we propose that the underlying mechanism of fiber damage may relate to the oxidative damage in

these muscles, which is exacerbated in the absence of DNase X and surpassing the buffering capacity of the antioxidant system [Fig. 15]. The subcellular localization of DNase X in muscle cell membrane and its high expression as a GPI-anchored protein in muscle make it a good candidate to be involved in signaling in muscle cells. We suggested that DNase X might be involved in pathways mediating mitochondrial function, a role that may not directly relate to its nuclease function. Nevertheless, our data were not sufficient to draw any compelling connection between the absence of DNase X and the pathogenesis of muscle injury. In conclusion, our results have demonstrated that DNase X is dispensable for the normal development of skeletal muscle, but is involved in preventing muscle damage associated with aging and physical stress. Further investigation, however, is required to unravel the precise function of DNase X in skeletal muscle.

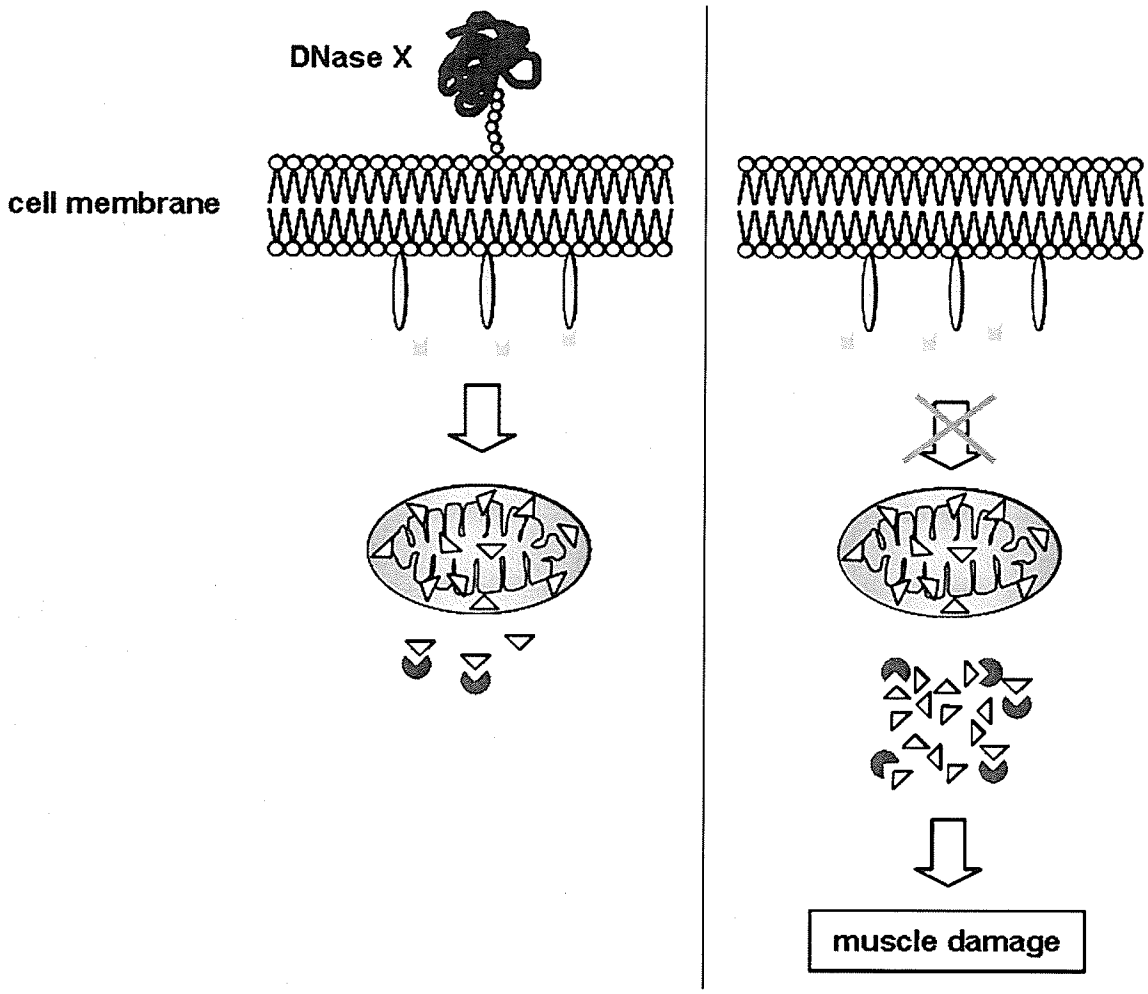


Figure 15. Schematic presentation of a proposed role for DNase X in skeletal muscle. Left: DNase X, as a GPI-anchored protein, might be involved in regulation of mitochondrial function through transmembrane signaling which is mediated in association with adaptor molecules (blue ovals) and second messengers (green blocks). Reactive species (yellow triangles) are mostly produced in mitochondria in electron transport chain. These oxidants are predominantly sequestered in mitochondria and are mainly neutralized by the antioxidants of the tissue (shown in purple circles) found in mitochondria and cytosol. Right: in the absence of DNase X and its presumed signaling, the generation of oxidants surpasses the buffering capacity of the cell resulting in muscle cell damage as seen in DNase $X^{(-/-)}$ mice.

Future directions

In addition to the remarks made earlier under the discussion, the major future directions for this study may include:

1) Investigating the activity-dependent expression of DNase X in skeletal muscle. This may help to find out whether the activity-associated induction of muscle damage in mutant muscles is a result of absence of the protein function when it is normally required.

2) Evaluating the measures of apoptosis in mutant muscles via both immunoblotting and *in situ* staining (TUNEL assay) as well as in primary cultured myocytes from DNase $X^{(-/-)}$ mice. This will aid us to see how the nuclease function of DNase X may contribute to the apoptotic death process in skeletal muscle.

3) Developing mouse DNase X-specific antibody. This will greatly facilitate the study of protein expression and localization during tissue development and cellular processes.

4) Using screening approaches (e.g. Y2H) to find the interacting partners for DNase X. This may improve our understanding of the involvement of DNase X in the signaling that results in muscle fiber damage.

Literature

Abe, A. and Liao, T.H. (1983) The immunological and structural comparisons of deoxyribonucleases I. Glycosylation differences between bovine pancreatic and parotid deoxyribonucleases. *J Biol Chem*, 258, 10283-10288.

Allen, D.G., Lamb, G.D. and Westerblad, H. (2008) Impaired calcium release during fatigue. *J Appl Physiol*, 104, 296-305.

Antonio, J. and Gonyea, W.J. (1994) Muscle fiber splitting in stretch-enlarged avian muscle. *Med Sci Sports Exerc*, 26, 973-977.

Apostolov, E.O., Wang, X., Shah, S.V. and Basnakian, A.G. (2007) Role of EndoG in development and cell injury. *Cell Death Differ*, 14, 1971-1974.

Aracena-Parks, P., Goonasekera, S.A., Gilman, C.P., Dirksen, R.T., Hidalgo, C. and Hamilton, S.L. (2006) Identification of cysteines involved in S-nitrosylation, S-glutathionylation, and oxidation to disulfides in ryanodine receptor type 1. *J Biol Chem*, 281, 40354-40368.

Archer, J.D., Vargas, C.C. and Anderson, J.E. (2006) Persistent and improved functional gain in mdx dystrophic mice after treatment with L-arginine and deflazacort. *Faseb J*, 20, 738-740.

Bansal, D., Miyake, K., Vogel, S.S., Groh, S., Chen, C.C., Williamson, R., McNeil, P.L. and Campbell, K.P. (2003) Defective membrane repair in dysferlin-deficient muscular dystrophy. *Nature*, 423, 168-172.

Bar, A. and Pette, D. (1988) Three fast myosin heavy chains in adult rat skeletal muscle. *FEBS Lett*, 235, 153-155.

Barclay, J.K. and Hansel, M. (1991) Free radicals may contribute to oxidative skeletal muscle fatigue. *Can J Physiol Pharmacol*, 69, 279-284.

Barja, G. and Herrero, A. (2000) Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals. *Faseb J*, 14, 312-318.

Baron, W.F., Pan, C.Q., Spencer, S.A., Ryan, A.M., Lazarus, R.A. and Baker, K.P. (1998) Cloning and characterization of an actin-resistant DNase I-like endonuclease secreted by macrophages. *Gene*, 215, 291-301.

Barry, M.A. and Eastman, A. (1993) Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. *Arch Biochem Biophys*, 300, 440-450.

Basso, D., Fabris, C., Meani, A., Del Favero, G., Panucci, A., Vianello, D., Piccoli, A. and Naccarato, R. (1985) Serum deoxyribonuclease and ribonuclease in pancreatic cancer and chronic pancreatitis. *Tumori*, 71, 529-532.

Belcastro, A.N. (1993) Skeletal muscle calcium-activated neutral protease (calpain) with exercise. *J Appl Physiol*, 74, 1381-1386.

Billeter, R., Weber, H., Lutz, H., Howald, H., Eppenberger, H.M. and Jenny, E. (1980) Myosin types in human skeletal muscle fibers. *Histochemistry*, 65, 249-259.

Biressi, S., Molinaro, M. and Cossu, G. (2007a) Cellular heterogeneity during vertebrate skeletal muscle development. *Dev Biol*, 308, 281-293.

Biressi, S., Tagliafico, E., Lamorte, G., Monteverde, S., Tenedini, E., Roncaglia, E., Ferrari, S., Ferrari, S., Cusella-De Angelis, M.G., Tajbakhsh, S. and Cossu, G. (2007b) Intrinsic phenotypic diversity of embryonic and fetal myoblasts is revealed by genome-wide gene expression analysis on purified cells. *Dev Biol*, 304, 633-651.

Black, B.L. and Olson, E.N. (1998) Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. *Annu Rev Cell Dev Biol*, 14, 167-196.

Blaivas, M. and Carlson, B.M. (1991) Muscle fiber branching--difference between grafts in old and young rats. *Mech Ageing Dev*, 60, 43-53.

Blaveri, K., Heslop, L., Yu, D.S., Rosenblatt, J.D., Gross, J.G., Partridge, T.A. and Morgan, J.E. (1999) Patterns of repair of dystrophic mouse muscle: studies on isolated fibers. *Dev Dyn*, 216, 244-256.

Bodano, A., Gonzalez, A., Ferreiros-Vidal, I., Balada, E., Ordi, J., Carreira, P., Gomez-Reino, J.J. and Conde, C. (2006) Association of a non-synonymous single-nucleotide polymorphism of DNASEI with SLE susceptibility. *Rheumatology (Oxford)*, 45, 819-823.

Borisov, A.B. and Carlson, B.M. (2000) Cell death in denervated skeletal muscle is distinct from classical apoptosis. *Anat Rec*, 258, 305-318.

Bottinelli, R., Betto, R., Schiaffino, S. and Reggiani, C. (1994) Maximum shortening velocity and coexistence of myosin heavy chain isoforms in single skinned fast fibres of rat skeletal muscle. *J Muscle Res Cell Motil*, 15, 413-419.

Braun, T., Bober, E., Buschhausen-Denker, G., Kohtz, S., Grzeschik, K.H. and Arnold, H.H. (1989) Differential expression of myogenic determination genes in muscle cells: possible autoactivation by the Myf gene products. *Embo J*, 8, 3617-3625.

Brent, A.E. and Tabin, C.J. (2002) Developmental regulation of somite derivatives: muscle, cartilage and tendon. *Curr Opin Genet Dev*, 12, 548-557.

Brooke, M.H. and Kaiser, K.K. (1970) Muscle fiber types: how many and what kind? *Arch Neurol*, 23, 369-379.

Brookes, P.S., Yoon, Y., Robotham, J.L., Anders, M.W. and Sheu, S.S. (2004) Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J Physiol Cell Physiol*, 287, C817-833.

Buttin, G. and Kornberg, A. (1966) Enzymatic synthesis of deoxyribonucleic acid. XXI. Utilization of deoxyribonucleoside triphosphates by Escherichia coli cells. *J Biol Chem*, 241, 5419-5427.

Cande, C., Vahsen, N., Kouranti, I., Schmitt, E., Daugas, E., Spahr, C., Luban, J., Kroemer, R.T., Giordanetto, F., Garrido, C., Penninger, J.M. and Kroemer, G. (2004) AIF and

cyclophilin A cooperate in apoptosis-associated chromatinolysis. *Oncogene*, 23, 1514-1521.

Carafoli, E. and Molinari, M. (1998) Calpain: a protease in search of a function? *Biochem Biophys Res Commun*, 247, 193-203.

Cardone, L., de Cristofaro, T., Affaitati, A., Garbi, C., Ginsberg, M.D., Saviano, M., Varrone, S., Rubin, C.S., Gottesman, M.E., Avvedimento, E.V. and Feliciello, A. (2002) A-kinase anchor protein 84/121 are targeted to mitochondria and mitotic spindles by overlapping amino-terminal motifs. *J Mol Biol*, 320, 663-675.

Carlson, B.M. and Gutmann, E. (1975) Regneration in free grafts of normal and denervated muscles in the rat: morphology and histochemistry. *Anat Rec*, 183, 47-62.

Chappell, M.A., Garland, T., Jr., Rezende, E.L. and Gomes, F.R. (2004) Voluntary running in deer mice: speed, distance, energy costs and temperature effects. *J Exp Biol*, 207, 3839-3854.

Charge, S.B. and Rudnicki, M.A. (2004) Cellular and molecular regulation of muscle regeneration. *Physiol Rev*, 84, 209-238.

Chin, E.R., Olson, E.N., Richardson, J.A., Yang, Q., Humphries, C., Shelton, J.M., Wu, H., Zhu, W., Bassel-Duby, R. and Williams, R.S. (1998) A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes Dev*, 12, 2499-2509.

Chitrabamrung, S., Rubin, R.L. and Tan, E.M. (1981) Serum deoxyribonuclease I and clinical activity in systemic lupus erythematosus. *Rheumatol Int*, 1, 55-60.

Chung, L. and Ng, Y.C. (2006) Age-related alterations in expression of apoptosis regulatory proteins and heat shock proteins in rat skeletal muscle. *Biochim Biophys Acta*, 1762, 103-109.

Cinek, T. and Horejsi, V. (1992) The nature of large noncovalent complexes containing glycosyl-phosphatidylinositol-anchored membrane glycoproteins and protein tyrosine kinases. *J Immunol*, 149, 2262-2270.

Compton, M.M. and Cidlowski, J.A. (1987) Identification of a glucocorticoid-induced nuclease in thymocytes. A potential "lysis gene" product. *J Biol Chem*, 262, 8288-8292.

Cornelison, D.D. and Wold, B.J. (1997) Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev Biol*, 191, 270-283.

Cossu, G. and Borello, U. (1999) Wnt signaling and the activation of myogenesis in mammals. *Embo J*, 18, 6867-6872.

Cote, J. and Ruiz-Carrillo, A. (1993) Primers for mitochondrial DNA replication generated by endonuclease G. *Science*, 261, 765-769.

Counis, M.F. and Torriglia, A. (2006) Acid DNases and their interest among apoptotic endonucleases. *Biochimie*, 88, 1851-1858.

Coy, J.F., Velhagen, I., Himmele, R., Delius, H., Poustka, A. and Zentgraf, H. (1996) Isolation, differential splicing and protein expression of a DNase on the human X chromosome. *Cell Death Differ*, 3, 199-206.

David, K.K., Sasaki, M., Yu, S.W., Dawson, T.M. and Dawson, V.L. (2006) EndoG is dispensable in embryogenesis and apoptosis. *Cell Death Differ*, 13, 1147-1155.

Davies, K.J., Quintanilha, A.T., Brooks, G.A. and Packer, L. (1982) Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Commun*, 107, 1198-1205.

Davis, R.L., Weintraub, H. and Lassar, A.B. (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell*, 51, 987-1000.

De Bono, J.P., Adlam, D., Paterson, D.J. and Channon, K.M. (2006) Novel quantitative phenotypes of exercise training in mouse models. *Am J Physiol Regul Integr Comp Physiol*, 290, R926-934.

De la Porte, S., Morin, S. and Koenig, J. (1999) Characteristics of skeletal muscle in mdx mutant mice. *Int Rev Cytol*, 191, 99-148.

De Maria, A. and Bassnett, S. (2007) DNase IIbeta distribution and activity in the mouse lens. *Invest Ophthalmol Vis Sci*, 48, 5638-5646.

Decary, S., Mouly, V., Hamida, C.B., Sautet, A., Barbet, J.P. and Butler-Browne, G.S. (1997) Replicative potential and telomere length in human skeletal muscle: implications for satellite cell-mediated gene therapy. *Hum Gene Ther*, 8, 1429-1438.

Demple, B., Herman, T. and Chen, D.S. (1991) Cloning and expression of APE, the cDNA encoding the major human apurinic endonuclease: definition of a family of DNA repair enzymes. *Proc Natl Acad Sci U S A*, 88, 11450-11454.

Deng, G. and Podack, E.R. (1995) Deoxyribonuclease induction in apoptotic cytotoxic T lymphocytes. *Faseb J*, 9, 665-669.

Dirks, A. and Leeuwenburgh, C. (2002) Apoptosis in skeletal muscle with aging. *Am J Physiol Regul Integr Comp Physiol*, 282, R519-527.

Dolmetsch, R.E., Lewis, R.S., Goodnow, C.C. and Healy, J.I. (1997) Differential activation of transcription factors induced by Ca2+ response amplitude and duration. *Nature*, 386, 855-858.

Draberova, L. and Draber, P. (1993) Thy-1 glycoprotein and src-like protein-tyrosine kinase p53/p56lyn are associated in large detergent-resistant complexes in rat basophilic leukemia cells. *Proc Natl Acad Sci U S A*, 90, 3611-3615.

Driggers, W.J., LeDoux, S.P. and Wilson, G.L. (1993) Repair of oxidative damage within the mitochondrial DNA of RINr 38 cells. *J Biol Chem*, 268, 22042-22045.

Duncan, C.J. (1978) Role of intracellular calcium in promoting muscle damage: a strategy for controlling the dystrophic condition. *Experientia*, 34, 1531-1535.

Duncan, C.J. and Jackson, M.J. (1987) Different mechanisms mediate structural changes and intracellular enzyme efflux following damage to skeletal muscle. *J Cell Sci*, 87 (Pt 1), 183-188.

Dupont-Versteegden, E.E. (2005) Apoptosis in muscle atrophy: relevance to sarcopenia. *Exp Gerontol*, 40, 473-481.

Earnshaw, W.C. (1995) Nuclear changes in apoptosis. *Curr Opin Cell Biol*, 7, 337-343.

Edmondson, D.G. and Olson, E.N. (1989) A gene with homology to the myc similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. *Genes Dev*, 3, 628-640.

El-Shemerly, M., Janscak, P., Hess, D., Jiricny, J. and Ferrari, S. (2005) Degradation of human exonuclease 1b upon DNA synthesis inhibition. *Cancer Res*, 65, 3604-3609.

Enari, M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, A. and Nagata, S. (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature*, 391, 43-50.

Esposito, L.A., Melov, S., Panov, A., Cottrell, B.A. and Wallace, D.C. (1999) Mitochondrial disease in mouse results in increased oxidative stress. *Proc Natl Acad Sci U S A*, 96, 4820-4825.

Evans, C.J. and Aguilera, R.J. (2003) DNase II: genes, enzymes and function. *Gene*, 322, 1-15.

Faldt, J., Wernstedt, I., Fitzgerald, S.M., Wallenius, K., Bergstrom, G. and Jansson, J.O. (2004) Reduced exercise endurance in interleukin-6-deficient mice. *Endocrinology*, 145, 2680-2686.

Fan, C.M., Porter, J.A., Chiang, C., Chang, D.T., Beachy, P.A. and Tessier-Lavigne, M. (1995) Long-range sclerotome induction by sonic hedgehog: direct role of the amino-terminal

cleavage product and modulation by the cyclic AMP signaling pathway. *Cell*, 81, 457-465.

Fan, Z., Beresford, P.J., Oh, D.Y., Zhang, D. and Lieberman, J. (2003) Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell*, 112, 659-672.

Favero, T.G. (1999) Sarcoplasmic reticulum Ca(2+) release and muscle fatigue. *J Appl Physiol*, 87, 471-483.

Fimia, G.M., Gottifredi, V., Passananti, C. and Maione, R. (1996) Double-stranded internucleosomal cleavage of apoptotic DNA is dependent on the degree of differentiation in muscle cells. *J Biol Chem*, 271, 15575-15579.

Friden, J., Sjostrom, M. and Ekblom, B. (1983) Myofibrillar damage following intense eccentric exercise in man. *Int J Sports Med*, 4, 170-176.

Fitts, R.H. (1994) Cellular mechanisms of muscle fatigue. *Physiol Rev*, 74, 49-94.

Friedman, W.A., Garland, T., Jr. and Dohm, M.R. (1992) Individual variation in locomotor behavior and maximal oxygen consumption in mice. *Physiol Behav*, 52, 97-104.

Fuchtbauer, E.M. and Westphal, H. (1992) MyoD and myogenin are coexpressed in regenerating skeletal muscle of the mouse. *Dev Dyn*, 193, 34-39.

Funakoshi, A., Tsubota, Y., Wakasugi, H., Ibayashi, H. and Takagi, Y. (1979a) Clinical studies on human pancreatic deoxyribonuclease I. *Gastroenterol Jpn*, 14, 48-54.

Funakoshi, A., Wakasugi, H. and Ibayashi, H. (1979b) Clinical investigation of serum deoxyribonuclease: II. Clinical studies of serum deoxyribonuclease activity in pancreatic disease. *Gastroenterol Jpn*, 14, 436-440.

Gaido, M.L. and Cidlowski, J.A. (1991) Identification, purification, and characterization of a calcium-dependent endonuclease (NUC18) from apoptotic rat thymocytes. NUC18 is not histone H2B. *J Biol Chem*, 266, 18580-18585.

Gardiner, P.F. (2001) *Neuromuscular aspects of physical activity*. Human Kinetics, Champaign, IL.

Garry, D.J., Bassel-Duby, R.S., Richardson, J.A., Grayson, J., Neufer, P.D. and Williams, R.S. (1996) Postnatal development and plasticity of specialized muscle fiber characteristics in the hindlimb. *Dev Genet*, 19, 146-156.

Girard, I., McAleer, M.W., Rhodes, J.S. and Garland, T., Jr. (2001) Selection for high voluntary wheel-running increases speed and intermittency in house mice (Mus domesticus). *J Exp Biol*, 204, 4311-4320.

Glesby, M.J., Rosenmann, E., Nylen, E.G. and Wrogemann, K. (1988) Serum CK, calcium, magnesium, and oxidative phosphorylation in mdx mouse muscular dystrophy. *Muscle Nerve*, 11, 852-856.

Gollnick, P.D., Timson, B.F., Moore, R.L. and Riedy, M. (1981) Muscular enlargement and number of fibers in skeletal muscles of rats. *J Appl Physiol*, 50, 936-943.

Golumbek, P.T., Keeling, R.M. and Connolly, A.M. (2007) RAG2 gene knockout in mice causes fatigue. *Muscle Nerve*, 36, 471-476.

Grounds, M.D., Garrett, K.L., Lai, M.C., Wright, W.E. and Beilharz, M.W. (1992) Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. *Cell Tissue Res*, 267, 99-104.

Harman, D. (1956) Aging: a theory based on free radical and radiation chemistry. *J Gerontol*, 11, 298-300.

Harosh, I., Binninger, D.M., Harris, P.V., Mezzina, M. and Boyd, J.B. (1991) Mechanism of action of deoxyribonuclease II from human lymphoblasts. *Eur J Biochem*, 202, 479-484.

Harris, J.B. and Johnson, M.A. (1978) Further observations on the pathological responses of rat skeletal muscle to toxins isolated from the venom of the Australian tiger snake, Notechis scutatus scutatus. *Clin Exp Pharmacol Physiol*, 5, 587-600.

Hasty, P., Bradley, A., Morris, J.H., Edmondson, D.G., Venuti, J.M., Olson, E.N. and Klein, W.H. (1993) Muscle deficiency and neonatal death in mice with a targeted mutation in the myogenin gene. *Nature*, 364, 501-506.

Hawke, T.J. and Garry, D.J. (2001) Myogenic satellite cells: physiology to molecular biology. *J Appl Physiol*, 91, 534-551.

He, Z.H., Bottinelli, R., Pellegrino, M.A., Ferenczi, M.A. and Reggiani, C. (2000) ATP consumption and efficiency of human single muscle fibers with different myosin isoform composition. *Biophys J*, 79, 945-961.

Hitomi, Y., Kizaki, T., Watanabe, S., Matsumura, G., Fujioka, Y., Haga, S., Izawa, T., Taniguchi, N. and Ohno, H. (2005) Seven skeletal muscles rich in slow muscle fibers may function to sustain neutral position in the rodent hindlimb. *Comp Biochem Physiol B Biochem Mol Biol*, 140, 45-50.

Hooper, N.M. (1997) Glycosyl-phosphatidylinositol anchored membrane enzymes. *Clin Chim Acta*, 266, 3-12.

Howl, J.D. and Publicover, S.J. (1990) Permeabilisation of the sarcolemma in mouse diaphragm exposed to Bay K 8644 in vitro: time course, dependence on Ca2+ and effects of enzyme inhibitors. *Acta Neuropathol*, 79, 438-443.

Huang, K.J., Ku, C.C. and Lehman, I.R. (2006) Endonuclease G: a role for the enzyme in recombination and cellular proliferation. *Proc Natl Acad Sci U S A*, 103, 8995-9000.

Huang, K.J., Zemelman, B.V. and Lehman, I.R. (2002) Endonuclease G, a candidate human enzyme for the initiation of genomic inversion in herpes simplex type 1 virus. *J Biol Chem*, 277, 21071-21079.

Iida, R., Yasuda, T., Aoyama, M., Tsubota, E., Kobayashi, M., Yuasa, I., Matsuki, T. and Kishi, K. (1997) The fifth allele of the human deoxyribonuclease I (DNase I) polymorphism. *Electrophoresis*, 18, 1936-1939.

Inohara, N., Koseki, T., Chen, S., Wu, X. and Nunez, G. (1998) CIDE, a novel family of cell death activators with homology to the 45 kDa subunit of the DNA fragmentation factor. *Embo J*, 17, 2526-2533.

Irintchev, A. and Wernig, A. (1987) Muscle damage and repair in voluntarily running mice: strain and muscle differences. *Cell Tissue Res*, 249, 509-521.

Irvine, R.A., Adachi, N., Shibata, D.K., Cassell, G.D., Yu, K., Karanjawala, Z.E., Hsieh, C.L. and Lieber, M.R. (2005) Generation and characterization of endonuclease G null mice. *Mol Cell Biol*, 25, 294-302.

Jacobson, M.D., Weil, M. and Raff, M.C. (1997) Programmed cell death in animal development. *Cell*, 88, 347-354.

Jenkins, R.R. and Goldfarb, A. (1993) Introduction: oxidant stress, aging, and exercise. *Med Sci Sports Exerc*, 25, 210-212.

Ji, L.L., Fu, R. and Mitchell, E.W. (1992) Glutathione and antioxidant enzymes in skeletal muscle: effects of fiber type and exercise intensity. *J Appl Physiol*, 73, 1854-1859.

Jones, D.A., Jackson, M.J. and Edwards, R.H. (1983) Release of intracellular enzymes from an isolated mammalian skeletal muscle preparation. *Clin Sci (Lond)*, 65, 193-201.

Jostes, B., Walther, C. and Gruss, P. (1990) The murine paired box gene, Pax7, is expressed specifically during the development of the nervous and muscular system. *Mech Dev*, 33, 27-37.

Kawai, Y., Yoshida, M., Arakawa, K., Kumamoto, T., Morikawa, N., Masamura, K., Tada, H., Ito, S., Hoshizaki, H., Oshima, S., Taniguchi, K., Terasawa, H., Miyamori, I., Kishi, K. and Yasuda, T. (2004) Diagnostic use of serum deoxyribonuclease I activity as a novel early-phase marker in acute myocardial infarction. *Circulation*, 109, 2398-2400.

Kawane, K., Fukuyama, H., Kondoh, G., Takeda, J., Ohsawa, Y., Uchiyama, Y. and Nagata, S. (2001) Requirement of DNase II for definitive erythropoiesis in the mouse fetal liver. *Science*, 292, 1546-1549.

Kawane, K., Fukuyama, H., Yoshida, H., Nagase, H., Ohsawa, Y., Uchiyama, Y., Okada, K., Iida, T. and Nagata, S. (2003) Impaired thymic development in mouse embryos deficient in apoptotic DNA degradation. *Nat Immunol*, 4, 138-144.

Kawane, K., Ohtani, M., Miwa, K., Kizawa, T., Kanbara, Y., Yoshioka, Y., Yoshikawa, H. and Nagata, S. (2006) Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. *Nature*, 443, 998-1002.

Kishi, K., Yasuda, T., Ikehara, Y., Sawazaki, K., Sato, W. and Iida, R. (1990a) Human serum deoxyribonuclease I (DNase I) polymorphism: pattern similarities among isozymes from serum, urine, kidney, liver, and pancreas. *Am J Hum Genet*, 47, 121-126.

Kishi, K., Yasuda, T. and Mizuta, K. (1990b) New genetic markers detected in human urine: DNase I, RNase 1, and 43-kDa glycoprotein. *Prog Clin Biol Res*, 344, 891-913.

Kishi, K., Yasuda, T., Nadano, D., Iida, R. and Takeshita, H. (1995) [Isozymes of DNase I]. *Nippon Rinsho*, 53 Su Pt 1, 450-453.

Kishi, K., Yasuda, T. and Takeshita, H. (2001) DNase I: structure, function, and use in medicine and forensic science. *Leg Med (Tokyo)*, 3, 69-83.

Klein, J.A., Longo-Guess, C.M., Rossmann, M.P., Seburn, K.L., Hurd, R.E., Frankel, W.N., Bronson, R.T. and Ackerman, S.L. (2002) The harlequin mouse mutation downregulates apoptosis-inducing factor. *Nature*, 419, 367-374.

Kominato, Y., Ueki, M., Iida, R., Kawai, Y., Nakajima, T., Makita, C., Itoi, M., Tajima, Y., Kishi, K. and Yasuda, T. (2006) Characterization of human deoxyribonuclease I gene (DNASE1) promoters reveals the utilization of two transcription-starting exons and the involvement of Sp1 in its transcriptional regulation. *Febs J*, 273, 3094-3105.

Kong, J. and Xu, Z. (1998) Massive mitochondrial degeneration in motor neurons triggers the onset of amyotrophic lateral sclerosis in mice expressing a mutant SOD1. *J Neurosci*, 18, 3241-3250.

Konhilas, J.P., Maass, A.H., Luckey, S.W., Stauffer, B.L., Olson, E.N. and Leinwand, L.A. (2004) Sex modifies exercise and cardiac adaptation in mice. *Am J Physiol Heart Circ Physiol*, 287, H2768-2776.

Koteja, P., Garland, T., Jr., Sax, J.K., Swallow, J.G. and Carter, P.A. (1999) Behaviour of house mice artificially selected for high levels of voluntary wheel running. *Anim Behav*, 58, 1307-1318.

Krieser, R.J., MacLea, K.S., Longnecker, D.S., Fields, J.L., Fiering, S. and Eastman, A. (2002) Deoxyribonuclease IIalpha is required during the phagocytic phase of apoptosis and its loss causes perinatal lethality. *Cell Death Differ*, 9, 956-962.

Krieser, R.J., MacLea, K.S., Park, J.P. and Eastman, A. (2001) The cloning, genomic structure, localization, and expression of human deoxyribonuclease IIbeta. *Gene*, 269, 205-216.

Kumamoto, T., Kawai, Y., Arakawa, K., Morikawa, N., Kuribara, J., Tada, H., Taniguchi, K., Tatami, R., Miyamori, I., Kominato, Y., Kishi, K. and Yasuda, T. (2006) Association of Gln222Arg polymorphism in the deoxyribonuclease I (DNase I) gene with myocardial infarction in Japanese patients. *Eur Heart J*, 27, 2081-2087.

Kuznetsov, A.V., Winkler, K., Wiedemann, F.R., von Bossanyi, P., Dietzmann, K. and Kunz, W.S. (1998) Impaired mitochondrial oxidative phosphorylation in skeletal muscle of the dystrophin-deficient mdx mouse. *Mol Cell Biochem*, 183, 87-96.

Lancut, M., Godlewski, P., Lis-Sochocka, M., Visconti, J. and Czerny, K. (2004) Investigations of ultrastructure of damaged and regenerated skeletal muscle fibers. *Ann Univ Mariae Curie Sklodowska [Med]*, 59, 80-85.

Lassar, A.B., Davis, R.L., Wright, W.E., Kadesch, T., Murre, C., Voronova, A., Baltimore, D. and Weintraub, H. (1991) Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins in vivo. *Cell*, 66, 305-315.

Lassar, A.B., Skapek, S.X. and Novitch, B. (1994) Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. *Curr Opin Cell Biol*, 6, 788-794.

Laube, B.L., Auci, R.M., Shields, D.E., Christiansen, D.H., Lucas, M.K., Fuchs, H.J. and Rosenstein, B.J. (1996) Effect of rhDNase on airflow obstruction and mucociliary clearance in cystic fibrosis. *Am J Respir Crit Care Med*, 153, 752-760.

Lazarides, E. and Lindberg, U. (1974) Actin is the naturally occurring inhibitor of deoxyribonuclease I. *Proc Natl Acad Sci U S A*, 71, 4742-4746.

Leeuwenburgh, C., Gurley, C.M., Strotman, B.A. and Dupont-Versteegden, E.E. (2005) Age-related differences in apoptosis with disuse atrophy in soleus muscle. *Am J Physiol Regul Integr Comp Physiol*, 288, R1288-1296.

Lefaucheur, J.P. and Sebille, A. (1995) The cellular events of injured muscle regeneration depend on the nature of the injury. *Neuromuscul Disord*, 5, 501-509.

Lehman, I.R. (1967) Deoxyribonucleases: their relationship to deoxyribonucleic Acid synthesis. *Annu Rev Biochem*, 36, 645-668.

Lerman, I., Harrison, B.C., Freeman, K., Hewett, T.E., Allen, D.L., Robbins, J. and Leinwand, L.A. (2002) Genetic variability in forced and voluntary endurance exercise performance in seven inbred mouse strains. *J Appl Physiol*, 92, 2245-2255.

Li, L.Y., Luo, X. and Wang, X. (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature*, 412, 95-99.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, 91, 479-489.

Liao, T.H. (1985) The subunit structure and active site sequence of porcine spleen deoxyribonuclease. *J Biol Chem*, 260, 10708-10713.

Liao, T.H., Liao, W.C., Chang, H.C. and Lu, K.S. (1989) Deoxyribonuclease II purified from the isolated lysosomes of porcine spleen and from porcine liver homogenates. Comparison with deoxyribonuclease II purified from porcine spleen homogenates. *Biochim Biophys Acta*, 1007, 15-22.

Lieberman, J. and Fan, Z. (2003) Nuclear war: the granzyme A-bomb. *Curr Opin Immunol*, 15, 553-559.

Linker, C., Lesbros, C., Stark, M.R. and Marcelle, C. (2003) Intrinsic signals regulate the initial steps of myogenesis in vertebrates. *Development*, 130, 4797-4807.

Liu, Q.Y., Pandey, S., Singh, R.K., Lin, W., Ribecco, M., Borowy-Borowski, H., Smith, B., LeBlanc, J., Walker, P.R. and Sikorska, M. (1998a) DNaseY: a rat DNaseI-like gene coding for a constitutively expressed chromatin-bound endonuclease. *Biochemistry*, 37, 10134-10143.

Liu, Q.Y., Ribecco, M., Pandey, S., Walker, P.R. and Sikorska, M. (1999) Apoptosis-related functional features of the DNaseI-like family of nucleases. *Ann N Y Acad Sci*, 887, 60-76.

Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W.T. and Wang, X. (1998b) The 40-kDa subunit of DNA fragmentation factor induces DNA fragmentation and chromatin condensation during apoptosis. *Proc Natl Acad Sci U S A*, 95, 8461-8466.

Liu, X., Zou, H., Slaughter, C. and Wang, X. (1997) DFF, a heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*, 89, 175-184.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, 25, 402-408.

Livigni, A., Scorziello, A., Agnese, S., Adornetto, A., Carlucci, A., Garbi, C., Castaldo, I., Annunziato, L., Avvedimento, E.V. and Feliciello, A. (2006) Mitochondrial AKAP121 links cAMP and src signaling to oxidative metabolism. *Mol Biol Cell*, 17, 263-271.

Lomonossoff, G.P., Butler, P.J. and Klug, A. (1981) Sequence-dependent variation in the conformation of DNA. *J Mol Biol*, 149, 745-760.

Los, M., Neubuser, D., Coy, J.F., Mozoluk, M., Poustka, A. and Schulze-Osthoff, K. (2000) Functional characterization of DNase X, a novel endonuclease expressed in muscle cells. *Biochemistry*, 39, 7365-7373.

Low, R.L., Buzan, J.M. and Couper, C.L. (1988) The preference of the mitochondrial endonuclease for a conserved sequence block in mitochondrial DNA is highly conserved during mammalian evolution. *Nucleic Acids Res*, 16, 6427-6445.

Lu, J., McKinsey, T.A., Zhang, C.L. and Olson, E.N. (2000) Regulation of skeletal myogenesis by association of the MEF2 transcription factor with class II histone deacetylases. *Mol Cell*, 6, 233-244.

Luedeke, J.D., McCall, R.D., Dillaman, R.M. and Kinsey, S.T. (2004) Properties of slow- and fast-twitch skeletal muscle from mice with an inherited capacity for hypoxic exercise. *Comp Biochem Physiol A Mol Integr Physiol*, 138, 373-382.

Ma, D., Xing, Z., Liu, B., Pedigo, N.G., Zimmer, S.G., Bai, Z., Postel, E.H. and Kaetzel, D.M. (2002) NM23-H1 and NM23-H2 repress transcriptional activities of nuclease-hypersensitive elements in the platelet-derived growth factor-A promoter. *J Biol Chem*, 277, 1560-1567.

Maier, A., Gambke, B. and Pette, D. (1986) Degeneration-regeneration as a mechanism contributing to the fast to slow conversion of chronically stimulated fast-twitch rabbit muscle. *Cell Tissue Res*, 244, 635-643.

Mannherz, H.G., Leigh, J.B., Leberman, R. and Pfrang, H. (1975) A specific 1:1 G-actin:DNAase i complex formed by the action of DNAase I on F-actin. *FEBS Lett*, 60, 34-38.

Mansouri, A., Muller, F.L., Liu, Y., Ng, R., Faulkner, J., Hamilton, M., Richardson, A., Huang, T.T., Epstein, C.J. and Van Remmen, H. (2006) Alterations in mitochondrial function, hydrogen peroxide release and oxidative damage in mouse hind-limb skeletal muscle during aging. *Mech Ageing Dev*, 127, 298-306.

Marzani, B., Felzani, G., Bellomo, R.G., Vecchiet, J. and Marzatico, F. (2005) Human muscle aging: ROS-mediated alterations in rectus abdominis and vastus lateralis muscles. *Exp Gerontol*, 40, 959-965.

Mason, R.P., Walter, M.F. and Mason, P.E. (1997) Effect of oxidative stress on membrane structure: small-angle X-ray diffraction analysis. *Free Radic Biol Med*, 23, 419-425.

Massett, M.P. and Berk, B.C. (2005) Strain-dependent differences in responses to exercise training in inbred and hybrid mice. *Am J Physiol Regul Integr Comp Physiol*, 288, R1006-1013.

Matoba, H. and Murakami, N. (1986) [A histochemical study on postnatal growth of the soleus muscles of male and female mice]. *Nippon Seirigaku Zasshi*, 48, 466-473.

Matsuura, T., Li, Y., Giacobino, J.P., Fu, F.H. and Huard, J. (2007) Skeletal muscle fiber type conversion during the repair of mouse soleus: potential implications for muscle healing after injury. *J Orthop Res*, 25, 1534-1540.

Mauro, A. (1961) Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol*, 9, 493-495.

McArdle, A., Maglara, A., Appleton, P., Watson, A.J., Grierson, I. and Jackson, M.J. (1999) Apoptosis in multinucleated skeletal muscle myotubes. *Lab Invest*, 79, 1069-1076.

McCarty, J.S., Toh, S.Y. and Li, P. (1999) Multiple domains of DFF45 bind synergistically to DFF40: roles of caspase cleavage and sequestration of activator domain of DFF40. *Biochem Biophys Res Commun*, 264, 181-185.

McIlroy, D., Tanaka, M., Sakahira, H., Fukuyama, H., Suzuki, M., Yamamura, K., Ohsawa, Y., Uchiyama, Y. and Nagata, S. (2000) An auxiliary mode of apoptotic DNA fragmentation provided by phagocytes. *Genes Dev*, 14, 549-558.

McKinsey, T.A., Zhang, C.L., Lu, J. and Olson, E.N. (2000a) Signal-dependent nuclear export of a histone deacetylase regulates muscle differentiation. *Nature*, 408, 106-111.

McKinsey, T.A., Zhang, C.L. and Olson, E.N. (2000b) Activation of the myocyte enhancer factor-2 transcription factor by calcium/calmodulin-dependent protein kinase-stimulated binding of 14-3-3 to histone deacetylase 5. *Proc Natl Acad Sci U S A*, 97, 14400-14405.

McNeil, P.L. and Khakee, R. (1992) Disruptions of muscle fiber plasma membranes. Role in exercise-induced damage. *Am J Pathol*, 140, 1097-1109.

Miquel, J., Economos, A.C., Fleming, J. and Johnson, J.E., Jr. (1980) Mitochondrial role in cell aging. *Exp Gerontol*, 15, 575-591.

Miramar, M.D., Costantini, P., Ravagnan, L., Saraiva, L.M., Haouzi, D., Brothers, G., Penninger, J.M., Peleato, M.L., Kroemer, G. and Susin, S.A. (2001) NADH oxidase activity of mitochondrial apoptosis-inducing factor. *J Biol Chem*, 276, 16391-16398.

Montague, J.W., Hughes, F.M., Jr. and Cidlowski, J.A. (1997) Native recombinant cyclophilins A, B, and C degrade DNA independently of peptidylprolyl cis-trans-isomerase activity. Potential roles of cyclophilins in apoptosis. *J Biol Chem*, 272, 6677-6684.

Mu, X., Brown, L.D., Liu, Y. and Schneider, M.F. (2007) Roles of the calcineurin and CaMK signaling pathways in fast-to-slow fiber type transformation of cultured adult mouse skeletal muscle fibers. *Physiol Genomics*, 30, 300-312.

Mukae, N., Enari, M., Sakahira, H., Fukuda, Y., Inazawa, J., Toh, H. and Nagata, S. (1998) Molecular cloning and characterization of human caspase-activated DNase. *Proc Natl Acad Sci U S A*, 95, 9123-9128.

Munsterberg, A.E., Kitajewski, J., Bumcrot, D.A., McMahon, A.P. and Lassar, A.B. (1995) Combinatorial signaling by Sonic hedgehog and Wnt family members induces myogenic bHLH gene expression in the somite. *Genes Dev*, 9, 2911-2922.

Munsterberg, A.E. and Lassar, A.B. (1995) Combinatorial signals from the neural tube, floor plate and notochord induce myogenic bHLH gene expression in the somite. *Development*, 121, 651-660.

Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I. and Nabeshima, Y. (1993) Myogenin gene disruption results in perinatal lethality because of severe muscle defect. *Nature*, 364, 532-535.

Nadano, D., Yasuda, T. and Kishi, K. (1993) Measurement of deoxyribonuclease I activity in human tissues and body fluids by a single radial enzyme-diffusion method. *Clin Chem*, 39, 448-452.

Naidu, P.S., Ludolph, D.C., To, R.Q., Hinterberger, T.J. and Konieczny, S.F. (1995) Myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenesis. *Mol Cell Biol*, 15, 2707-2718.

Napirei, M., Karsunky, H., Zevnik, B., Stephan, H., Mannherz, H.G. and Moroy, T. (2000) Features of systemic lupus erythematosus in Dnase1-deficient mice. *Nat Genet*, 25, 177-181.

Neale, M.J., Pan, J. and Keeney, S. (2005) Endonucleolytic processing of covalent protein-linked DNA double-strand breaks. *Nature*, 436, 1053-1057.

Nelson, H.C., Finch, J.T., Luisi, B.F. and Klug, A. (1987) The structure of an oligo(dA).oligo(dT) tract and its biological implications. *Nature*, 330, 221-226.

Nemeth, P., Hofer, H.W. and Pette, D. (1979) Metabolic heterogeneity of muscle fibers classified by myosin ATPase. *Histochemistry*, 63, 191-201.

Nishimoto, S., Kawane, K., Watanabe-Fukunaga, R., Fukuyama, H., Ohsawa, Y., Uchiyama, Y., Hashida, N., Ohguro, N., Tano, Y., Morimoto, T., Fukuda, Y. and Nagata, S. (2003)

Nuclear cataract caused by a lack of DNA degradation in the mouse eye lens. *Nature*, 424, 1071-1074.

Odaka, C. and Mizuochi, T. (1999) Role of macrophage lysosomal enzymes in the degradation of nucleosomes of apoptotic cells. *J Immunol*, 163, 5346-5352.

Oefner, C. and Suck, D. (1986) Crystallographic refinement and structure of DNase I at 2 A resolution. *J Mol Biol*, 192, 605-632.

Ohiro, Y., Garkavtsev, I., Kobayashi, S., Sreekumar, K.R., Nantz, R., Higashikubo, B.T., Duffy, S.L., Higashikubo, R., Usheva, A., Gius, D., Kley, N. and Horikoshi, N. (2002) A novel p53-inducible apoptogenic gene, PRG3, encodes a homologue of the apoptosis-inducing factor (AIF). *FEBS Lett*, 524, 163-171.

Ohsato, T., Ishihara, N., Muta, T., Umeda, S., Ikeda, S., Mihara, K., Hamasaki, N. and Kang, D. (2002) Mammalian mitochondrial endonuclease G. Digestion of R-loops and localization in intermembrane space. *Eur J Biochem*, 269, 5765-5770.

Okamoto, M., Okamoto, N., Yashiro, H., Shiokawa, D., Sunaga, S., Yoshimori, A., Tanuma, S. and Kitamura, D. (2005) Involvement of DNase gamma in the resected double-strand DNA breaks in immunoglobulin genes. *Biochem Biophys Res Commun*, 327, 76-83.

Okumura, N., Hashida-Okumura, A., Kita, K., Matsubae, M., Matsubara, T., Takao, T. and Nagai, K. (2005) Proteomic analysis of slow- and fast-twitch skeletal muscles. *Proteomics*, 5, 2896-2906.

Olson, E.N., Arnold, H.H., Rigby, P.W. and Wold, B.J. (1996) Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. *Cell*, 85, 1-4.

Padron-Barthe, L., Lepretre, C., Martin, E., Counis, M.F. and Torriglia, A. (2007) Conformational modification of serpins transforms leukocyte elastase inhibitor into an endonuclease involved in apoptosis. *Mol Cell Biol*, 27, 4028-4036.

Pandey, S., Walker, P.R. and Sikorska, M. (1997) Identification of a novel 97 kDa endonuclease capable of internucleosomal DNA cleavage. *Biochemistry*, 36, 711-720.

Parrish, J.E., Ciccodicola, A., Wehhert, M., Cox, G.F., Chen, E. and Nelson, D.L. (1995) A muscle-specific DNase I-like gene in human Xq28. *Hum Mol Genet*, 4, 1557-1564.

Parrish, J.Z., Yang, C., Shen, B. and Xue, D. (2003) CRN-1, a Caenorhabditis elegans FEN-1 homologue, cooperates with CPS-6/EndoG to promote apoptotic DNA degradation. *Embo J*, 22, 3451-3460.

Patel, A., Harrison, E., Durward, A. and Murdoch, I.A. (2000) Intratracheal recombinant human deoxyribonuclease in acute life-threatening asthma refractory to conventional treatment. *Br J Anaesth*, 84, 505-507.

Peitsch, M.C., Polzar, B., Stephan, H., Crompton, T., MacDonald, H.R., Mannherz, H.G. and Tschopp, J. (1993) Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). *Embo J*, 12, 371-377.

Perez, A.C., de Oliveira, C.C., Prieto, J.G., Ferrando, A., Vila, L. and Alvarez, A.I. (2002) Quantitative assessment of nitric oxide in rat skeletal muscle and plasma after exercise. *Eur J Appl Physiol*, 88, 189-191.

Pergolizzi, R., Appierto, V., Bosetti, A., DeBellis, G.L., Rovida, E. and Biunno, I. (1996) Cloning of a gene encoding a DNase I-like endonuclease in the human Xq28 region. *Gene*, 168, 267-270.

Pette, D. and Staron, R.S. (1997) Mammalian skeletal muscle fiber type transitions. *Int Rev Cytol*, 170, 143-223.

Pette, D. and Staron, R.S. (2000) Myosin isoforms, muscle fiber types, and transitions. *Microsc Res Tech*, 50, 500-509.

Pette, D., Staudte, H.W. and Vrbova, G. (1972) Physiological and biochemical changes induced by long-term stimulation of fast muscle. *Naturwissenschaften*, 59, 469-470.

Pette, D. and Vrbova, G. (1985) Neural control of phenotypic expression in mammalian muscle fibers. *Muscle Nerve*, 8, 676-689.

Podhorska-Okolow, M., Sandri, M., Zampieri, S., Brun, B., Rossini, K. and Carraro, U. (1998) Apoptosis of myofibres and satellite cells: exercise-induced damage in skeletal muscle of the mouse. *Neuropathol Appl Neurobiol*, 24, 518-531.

Pollack, M., Phaneuf, S., Dirks, A. and Leeuwenburgh, C. (2002) The role of apoptosis in the normal aging brain, skeletal muscle, and heart. *Ann N Y Acad Sci*, 959, 93-107.

Polzar, B., Zanotti, S., Stephan, H., Rauch, F., Peitsch, M.C., Irmler, M., Tschopp, J. and Mannherz, H.G. (1994) Distribution of deoxyribonuclease I in rat tissues and its correlation to cellular turnover and apoptosis (programmed cell death). *Eur J Cell Biol*, 64, 200-210.

Prats, E., Noel, M., Letourneau, J., Tiranti, V., Vaque, J., Debon, R., Zeviani, M., Cornudella, L. and Ruiz-Carrillo, A. (1997) Characterization and expression of the mouse endonuclease G gene. *DNA Cell Biol*, 16, 1111-1122.

Price, P.A. (1975) The essential role of Ca2+ in the activity of bovine pancreatic deoxyribonuclease. *J Biol Chem*, 250, 1981-1986.

Price, P.A., Liu, T.Y., Stein, W.H. and Moore, S. (1969a) Properties of chromatographically purified bovine pancreatic deoxyribonuclease. *J Biol Chem*, 244, 917-923.

Price, P.A., Stein, W.H. and Moore, S. (1969b) Effect of divalent cations on the reduction and re-formation of the disulfide bonds of deoxyribonuclease. *J Biol Chem*, 244, 929-932.

Ramandanis, G., Agnantis, N., Garas, J. and Spandidos, D.A. (1982) Correlation between serum and tissue deoxyribonuclease levels in breast cancer patients. *Anticancer Res*, 2, 213-218.

Rawls, A., Morris, J.H., Rudnicki, M., Braun, T., Arnold, H.H., Klein, W.H. and Olson, E.N. (1995) Myogenin's functions do not overlap with those of MyoD or Myf-5 during mouse embryogenesis. *Dev Biol*, 172, 37-50.

Reid, M.B. and Durham, W.J. (2002) Generation of reactive oxygen and nitrogen species in contracting skeletal muscle: potential impact on aging. *Ann N Y Acad Sci*, 959, 108-116.

Relaix, F., Montarras, D., Zaffran, S., Gayraud-Morel, B., Rocancourt, D., Tajbakhsh, S., Mansouri, A., Cumano, A. and Buckingham, M. (2006) Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J Cell Biol*, 172, 91-102.

Reshef, R., Maroto, M. and Lassar, A.B. (1998) Regulation of dorsal somitic cell fates: BMPs and Noggin control the timing and pattern of myogenic regulator expression. *Genes Dev*, 12, 290-303.

Reynolds, J.E., Li, J. and Eastman, A. (1996) Detection of apoptosis by flow cytometry of cells simultaneously stained for intracellular pH (carboxy SNARF-1) and membrane permeability (Hoechst 33342). *Cytometry*, 25, 349-357.

Rhodes, S.J. and Konieczny, S.F. (1989) Identification of MRF4: a new member of the muscle regulatory factor gene family. *Genes Dev*, 3, 2050-2061.

Ribeiro, J.M. and Carson, D.A. (1993) Ca2+/Mg(2+)-dependent endonuclease from human spleen: purification, properties, and role in apoptosis. *Biochemistry*, 32, 9129-9136.

Roberts, P., McGeachie, J.K. and Grounds, M.D. (1997) The host environment determines strain-specific differences in the timing of skeletal muscle regeneration: cross-transplantation studies between SJL/J and BALB/c mice. *J Anat*, 191 (Pt 4), 585-594.

Robson, C.N. and Hickson, I.D. (1991) Isolation of cDNA clones encoding a human apurinic/apyrimidinic endonuclease that corrects DNA repair and mutagenesis defects in E. coli xth (exonuclease III) mutants. *Nucleic Acids Res*, 19, 5519-5523.

Rodriguez, A.M., Rodin, D., Nomura, H., Morton, C.C., Weremowicz, S. and Schneider, M.C. (1997) Identification, localization, and expression of two novel human genes similar to deoxyribonuclease I. *Genomics*, 42, 507-513.

Rudnicki, M.A., Schnegelsberg, P.N., Stead, R.H., Braun, T., Arnold, H.H. and Jaenisch, R. (1993) MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell*, 75, 1351-1359.

Ruiz-Carrillo, A. and Renaud, J. (1987) Endonuclease G: a (dG)n X (dC)n-specific DNase from higher eukaryotes. *Embo J*, 6, 401-407.

Russo, S., Tomatis, D., Collo, G., Tarone, G. and Tato, F. (1998) Myogenic conversion of NIH3T3 cells by exogenous MyoD family members: dissociation of terminal differentiation from myotube formation. *J Cell Sci*, 111 (Pt 6), 691-700.

Sacks, L.D., Cann, G.M., Nikovits, W., Jr., Conlon, S., Espinoza, N.R. and Stockdale, F.E. (2003) Regulation of myosin expression during myotome formation. *Development*, 130, 3391-3402.

Sakahira, H., Enari, M. and Nagata, S. (1998) Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature*, 391, 96-99.

Sakahira, H., Takemura, Y. and Nagata, S. (2001) Enzymatic active site of caspase-activated DNase (CAD) and its inhibition by inhibitor of CAD. *Arch Biochem Biophys*, 388, 91-99.

Salnikow, J., Moore, S. and Stein, W.H. (1970) Comparison of the multiple forms of bovine pancreatic deoxyribonuclease. *J Biol Chem*, 245, 5685-5690.

Samejima, K. and Earnshaw, W.C. (1998) ICAD/DFF regulator of apoptotic nuclease is nuclear. *Exp Cell Res*, 243, 453-459.

Schiaffino, S., Gorza, L., Sartore, S., Saggin, L., Ausoni, S., Vianello, M., Gundersen, K. and Lomo, T. (1989) Three myosin heavy chain isoforms in type 2 skeletal muscle fibres. *J Muscle Res Cell Motil*, 10, 197-205.

Schiaffino, S. and Reggiani, C. (1996) Molecular diversity of myofibrillar proteins: gene regulation and functional significance. *Physiol Rev*, 76, 371-423.

Scholz, S.R., Korn, C., Gimadutdinow, O., Knoblauch, M., Pingoud, A. and Meiss, G. (2002) The effect of ICAD-S on the formation and intracellular distribution of a nucleolytically active caspase-activated DNase. *Nucleic Acids Res*, 30, 3045-3051.

Schuenke, M.D., Kopchick, J.J., Hikida, R.S., Kraemer, W.J. and Staron, R.S. (2008) Effects of growth hormone overexpression vs. growth hormone receptor gene disruption on mouse hindlimb muscle fiber type composition. *Growth Horm IGF Res*.

Seale, P. and Rudnicki, M.A. (2000) A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Dev Biol*, 218, 115-124.

Sharom, F.J., McNeil, G.L., Glover, J.R. and Seier, S. (1996) Modulation of the cleavage of glycosylphosphatidylinositol-anchored proteins by specific bacterial phospholipases. *Biochem Cell Biol*, 74, 701-713.

Shi, H., Scheffler, J.M., Pleitner, J.M., Zeng, C., Park, S., Hannon, K.M., Grant, A.L. and Gerrard, D.E. (2008) Modulation of skeletal muscle fiber type by mitogen-activated protein kinase signaling. *Faseb J*.

Shin, H.D., Park, B.L., Kim, L.H., Lee, H.S., Kim, T.Y. and Bae, S.C. (2004) Common DNase I polymorphism associated with autoantibody production among systemic lupus erythematosus patients. *Hum Mol Genet*, 13, 2343-2350.

Shiokawa, D., Kobayashi, T. and Tanuma, S. (2002) Involvement of DNase gamma in apoptosis associated with myogenic differentiation of C2C12 cells. *J Biol Chem*, 277, 31031-31037.

Shiokawa, D., Matsushita, T., Kobayashi, T., Matsumoto, Y. and Tanuma, S. (2004) Characterization of the human DNAS1L2 gene and the molecular mechanism for its transcriptional activation induced by inflammatory cytokines. *Genomics*, 84, 95-105.

Shiokawa, D., Matsushita, T., Shika, Y., Shimizu, M., Maeda, M. and Tanuma, S. (2007) DNase X is a glycosylphosphatidylinositol-anchored membrane enzyme that provides a barrier to endocytosis-mediated transfer of a foreign gene. *J Biol Chem*, 282, 17132-17140.

Shiokawa, D., Shika, Y., Saito, K., Yamazaki, K. and Tanuma, S. (2005) Physical and biochemical properties of mammalian DNase X proteins: non-AUG translation initiation of porcine and bovine mRNAs for DNase X. *Biochem J*, 392, 511-517.

Shiokawa, D., Shika, Y. and Tanuma, S. (2003) Identification of two functional nuclear localization signals in DNase gamma and their roles in its apoptotic DNase activity. *Biochem J*, 376, 377-381.

Shiokawa, D. and Tanuma, S. (2001) Characterization of human DNase I family endonucleases and activation of DNase gamma during apoptosis. *Biochemistry*, 40, 143-152.

Siu, P.M., Pistilli, E.E., Butler, D.C. and Alway, S.E. (2005) Aging influences cellular and molecular responses of apoptosis to skeletal muscle unloading. *Am J Physiol Cell Physiol*, 288, C338-349.

Smith, H.K., Maxwell, L., Martyn, J.A. and Bass, J.J. (2000) Nuclear DNA fragmentation and morphological alterations in adult rabbit skeletal muscle after short-term immobilization. *Cell Tissue Res*, 302, 235-241.

Soffler, C. and Hermanson, J.W. (2006) Muscular design in the equine interosseus muscle. *J Morphol*, 267, 696-704.

Steeg, P.S., Bevilacqua, G., Kopper, L., Thorgeirsson, U.P., Talmadge, J.E., Liotta, L.A. and Sobel, M.E. (1988) Evidence for a novel gene associated with low tumor metastatic potential. *J Natl Cancer Inst*, 80, 200-204.

Suck, D. and Oefner, C. (1986) Structure of DNase I at 2.0 A resolution suggests a mechanism for binding to and cutting DNA. *Nature*, 321, 620-625.

Suck, D., Oefner, C. and Kabsch, W. (1984) Three-dimensional structure of bovine pancreatic DNase I at 2.5 A resolution. *Embo J*, 3, 2423-2430.

Susin, S.A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K.F., Irinopoulou, T., Prevost, M.C., Brothers, G., Mak, T.W., Penninger, J., Earnshaw, W.C. and Kroemer, G. (2000) Two distinct pathways leading to nuclear apoptosis. *J Exp Med*, 192, 571-580.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. and Kroemer, G. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature*, 397, 441-446.

Tajbakhsh, S., Borello, U., Vivarelli, E., Kelly, R., Papkoff, J., Duprez, D., Buckingham, M. and Cossu, G. (1998) Differential activation of Myf5 and MyoD by different Wnts in explants of mouse paraxial mesoderm and the later activation of myogenesis in the absence of Myf5. *Development*, 125, 4155-4162.

Takekura, H. and Yoshioka, T. (1987) Determination of metabolic profiles on single muscle fibres of different types. *J Muscle Res Cell Motil*, 8, 342-348.

Takeshita, H., Yasuda, T., Nakajima, T., Hosomi, O., Nakashima, Y. and Kishi, K. (1997) Mouse deoxyribonuclease I (DNase I): biochemical and immunological characterization, cDNA structure and tissue distribution. *Biochem Mol Biol Int*, 42, 65-75.

Tanaka, M., Kovalenko, S.A., Gong, J.S., Borgeld, H.J., Katsumata, K., Hayakawa, M., Yoneda, M. and Ozawa, T. (1996) Accumulation of deletions and point mutations in mitochondrial genome in degenerative diseases. *Ann N Y Acad Sci*, 786, 102-111.

Tanuma, S. and Shiokawa, D. (1999) Cloning of a cDNA encoding a rat DNase II-like acid DNase. *Biochem Biophys Res Commun*, 265, 395-399.

Tews, D.S. and Goebel, H.H. (1997) DNA-fragmentation and expression of apoptosis-related proteins in muscular dystrophies. *Neuropathol Appl Neurobiol*, 23, 331-338.

Tews, D.S., Goebel, H.H. and Meinck, H.M. (1997) DNA-fragmentation and apoptosis-related proteins of muscle cells in motor neuron disorders. *Acta Neurol Scand*, 96, 380-386.

Tidball, J.G. (1995) Inflammatory cell response to acute muscle injury. *Med Sci Sports Exerc*, 27, 1022-1032.

Timson, B.F. (1982) The effect of varying postnatal growth rate on skeletal muscle fiber number in the mouse. *Growth*, 46, 36-45.

Torriglia, A., Perani, P., Brossas, J.Y., Chaudun, E., Treton, J., Courtois, Y. and Counis, M.F. (1998) L-DNase II, a molecule that links proteases and endonucleases in apoptosis, derives from the ubiquitous serpin leukocyte elastase inhibitor. *Mol Cell Biol*, 18, 3612-3619.

Totsuka, Y., Nagao, Y., Horii, T., Yonekawa, H., Imai, H., Hatta, H., Izaike, Y., Tokunaga, T. and Atomi, Y. (2003) Physical performance and soleus muscle fiber composition in wild-derived and laboratory inbred mouse strains. *J Appl Physiol*, 95, 720-727.

Tsutsumi, S., Asao, T., Nagamachi, Y., Nakajima, T., Yasuda, T. and Kishi, K. (1998) Phenotype 2 of deoxyribonuclease I may be used as a risk factor for gastric carcinoma. *Cancer*, 82, 1621-1625.

Tsutsumi, S., Takeshita, H., Yasuda, T., Kuwano, H. and Kishi, K. (2000) Association of DNase I phenotype 2 with colorectal carcinoma risk in Japanese populations. *Cancer Lett*, 159, 109-112.

Urbano, A., McCaffrey, R. and Foss, F. (1998) Isolation and characterization of NUC70, a cytoplasmic, hematopoietic apoptotic endonuclease. *J Biol Chem*, 273, 34820-34827.

Vahsen, N., Cande, C., Dupaigne, P., Giordanetto, F., Kroemer, R.T., Herker, E., Scholz, S., Modjtahedi, N., Madeo, F., Le Cam, E. and Kroemer, G. (2006) Physical interaction of apoptosis-inducing factor with DNA and RNA. *Oncogene*, 25, 1763-1774.

van Loo, G., Schotte, P., van Gurp, M., Demol, H., Hoorelbeke, B., Gevaert, K., Rodriguez, I., Ruiz-Carrillo, A., Vandekerckhove, J., Declercq, W., Beyaert, R. and Vandenabeele, P. (2001) Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. *Cell Death Differ*, 8, 1136-1142.

Venuti, J.M., Morris, J.H., Vivian, J.L., Olson, E.N. and Klein, W.H. (1995) Myogenin is required for late but not early aspects of myogenesis during mouse development. *J Cell Biol*, 128, 563-576.

Weston, S.A., Lahm, A. and Suck, D. (1992) X-ray structure of the DNase I-d(GGTATACC)2 complex at 2.3 A resolution. *J Mol Biol*, 226, 1237-1256.

Widlak, P., Li, P., Wang, X. and Garrard, W.T. (2000) Cleavage preferences of the apoptotic endonuclease DFF40 (caspase-activated DNase or nuclease) on naked DNA and chromatin substrates. *J Biol Chem*, 275, 8226-8232.

Wigston, D.J. and English, A.W. (1992) Fiber-type proportions in mammalian soleus muscle during postnatal development. *J Neurobiol*, 23, 61-70.

Wilmott, R.W., Amin, R.S., Colin, A.A., DeVault, A., Dozor, A.J., Eigen, H., Johnson, C., Lester, L.A., McCoy, K., McKean, L.P., Moss, R., Nash, M.L., Jue, C.P., Regelmann, W., Stokes, D.C. and Fuchs, H.J. (1996) Aerosolized recombinant human DNase in hospitalized cystic fibrosis patients with acute pulmonary exacerbations. *Am J Respir Crit Care Med*, 153, 1914-1917.

Winchester, P.K. and Gonyea, W.J. (1992) A quantitative study of satellite cells and myonuclei in stretched avian slow tonic muscle. *Anat Rec*, 232, 369-377.

Windisch, A., Gundersen, K., Szabolcs, M.J., Gruber, H. and Lomo, T. (1998) Fast to slow transformation of denervated and electrically stimulated rat muscle. *J Physiol*, 510 (Pt 2), 623-632.

Wood, S.A., Morgan, D.L. and Proske, U. (1993) Effects of repeated eccentric contractions on structure and mechanical properties of toad sartorius muscle. *Am J Physiol*, 265, C792-800.

Wright, W.E., Sassoon, D.A. and Lin, V.K. (1989) Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell*, 56, 607-617.

Wu, M., Xu, L.G., Li, X., Zhai, Z. and Shu, H.B. (2002) AMID, an apoptosis-inducing factor-homologous mitochondrion-associated protein, induces caspase-independent apoptosis. *J Biol Chem*, 277, 25617-25623.

Yablonka-Reuveni, Z. and Rivera, A.J. (1994) Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev Biol*, 164, 588-603.

Yakovlev, A.G., Wang, G., Stoica, B.A., Simbulan-Rosenthal, C.M., Yoshihara, K. and Smulson, M.E. (1999) Role of DNAS1L3 in Ca2+- and Mg2+-dependent cleavage of DNA into oligonucleosomal and high molecular mass fragments. *Nucleic Acids Res*, 27, 1999-2005.

Yan, B., Wang, H., Peng, Y., Hu, Y., Wang, H., Zhang, X., Chen, Q., Bedford, J.S., Dewhirst, M.W. and Li, C.Y. (2006) A unique role of the DNA fragmentation factor in maintaining genomic stability. *Proc Natl Acad Sci U S A*, 103, 1504-1509.

Yasuda, T., Nadano, D., Awazu, S. and Kishi, K. (1992) Human urine deoxyribonuclease II (DNase II) isoenzymes: a novel immunoaffinity purification, biochemical multiplicity, genetic heterogeneity and broad distribution among tissues and body fluids. *Biochim Biophys Acta*, 1119, 185-193.

Yasuda, T., Nadano, D., Iida, R., Takeshita, H., Lane, S.A., Callen, D.F. and Kishi, K. (1995a) Chromosomal assignment of the human deoxyribonuclease I gene, DNASE 1 (DNL1), to band 16p13.3 using the polymerase chain reaction. *Cytogenet Cell Genet*, 70, 221-223.

Yasuda, T., Nadano, D., Takeshita, H., Tenjo, E., Sawazaki, K., Ootani, M. and Kishi, K. (1995b) The molecular basis for genetic polymorphism of human deoxyribonuclease I: identification of the nucleotide substitution that generates the fourth allele. *FEBS Lett*, 359, 211-214.

Yasuda, T., Nadano, D., Tenjo, E., Takeshita, H., Sawazaki, K., Nakanaga, M. and Kishi, K. (1995c) Genotyping of human deoxyribonuclease I polymorphism by the polymerase chain reaction. *Electrophoresis*, 16, 1889-1893.

Yasuda, T., Takeshita, H., Iida, R., Nakajima, T., Hosomi, O., Nakashima, Y. and Kishi, K. (1998) Molecular cloning of the cDNA encoding human deoxyribonuclease II. *J Biol Chem*, 273, 2610-2616.

Yasutomo, K., Horiuchi, T., Kagami, S., Tsukamoto, H., Hashimura, C., Urushihara, M. and Kuroda, Y. (2001) Mutation of DNASE1 in people with systemic lupus erythematosus. *Nat Genet*, 28, 313-314.

Ye, H., Cande, C., Stephanou, N.C., Jiang, S., Gurbuxani, S., Larochette, N., Daugas, E., Garrido, C., Kroemer, G. and Wu, H. (2002) DNA binding is required for the apoptogenic action of apoptosis inducing factor. *Nat Struct Biol*, 9, 680-684.

Yeung, E.W., Whitehead, N.P., Suchyna, T.M., Gottlieb, P.A., Sachs, F. and Allen, D.G. (2005) Effects of stretch-activated channel blockers on [Ca2+]i and muscle damage in the mdx mouse. *J Physiol*, 562, 367-380.

Yoshida, A., Urasaki, Y., Waltham, M., Bergman, A.C., Pourquier, P., Rothwell, D.G., Inuzuka, M., Weinstein, J.N., Ueda, T., Appella, E., Hickson, I.D. and Pommier, Y. (2003) Human apurinic/apyrimidinic endonuclease (Ape1) and its N-terminal truncated form (AN34) are involved in DNA fragmentation during apoptosis. *J Biol Chem*, 278, 37768-37776.

Yoshida, H., Okabe, Y., Kawane, K., Fukuyama, H. and Nagata, S. (2005) Lethal anemia caused by interferon-beta produced in mouse embryos carrying undigested DNA. *Nat Immunol*, 6, 49-56.

Young, H.E., Rogers, J.J., Adkison, L.R., Lucas, P.A. and Black, A.C. (1998) Muscle morphogenetic protein induces myogenic gene expression in Swiss-3T3 cells. *Wound Repair Regen*, 6, 543-554.

Yu, S.W., Wang, H., Poitras, M.F., Coombs, C., Bowers, W.J., Federoff, H.J., Poirier, G.G., Dawson, T.M. and Dawson, V.L. (2002) Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor. *Science*, 297, 259-263.

Zardini, D.M. and Parry, D.J. (1994) Identification, distribution, and myosin subunit composition of type IIX fibers in mouse muscles. *Muscle Nerve*, 17, 1308-1316.

Zeng, Z., Parmelee, D., Hyaw, H., Coleman, T.A., Su, K., Zhang, J., Gentz, R., Ruben, S., Rosen, C. and Li, Y. (1997) Cloning and characterization of a novel human DNase. *Biochem Biophys Res Commun*, 231, 499-504.

Zhang, J., Dong, M., Li, L., Fan, Y., Pathre, P., Dong, J., Lou, D., Wells, J.M., Olivares-Villagomez, D., Van Kaer, L., Wang, X. and Xu, M. (2003) Endonuclease G is required for early embryogenesis and normal apoptosis in mice. *Proc Natl Acad Sci U S A*, 100, 15782-15787.

Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J.W. and Elledge, S.J. (1999) p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. *Genes Dev*, 13, 213-224.

Zhao, M., New, L., Kravchenko, V.V., Kato, Y., Gram, H., di Padova, F., Olson, E.N., Ulevitch, R.J. and Han, J. (1999) Regulation of the MEF2 family of transcription factors by p38. *Mol Cell Biol*, 19, 21-30.