Lumbar multifidus and erector spinae muscle fibre passive stiffness is lower in conjunction with greater lumbar spine neutral zone stiffness in $ENT1$-deficient mice

by

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ABSTRACT

Lumbar multifidus and erector spinae muscle fibre passive stiffness is lower in conjunction with greater lumbar spine neutral zone stiffness in ENT1-deficient mice

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Mice lacking equilibrative nucleoside transporter 1 (ENT1) develop progressive ectopic mineralization of the fibrous connective tissues of the spine. This likely results in a spine that is more resistant to movement (stiffer). The neutral zone (NZ) is the region within a spinal segment’s normal range of motion where movement occurs with minimal resistance. Experimentally induced intervertebral disc (IVD) degeneration reduces NZ stiffness and increases NZ length. This IVD degeneration has also been shown to cause an increase in the passive mechanical stiffness of the adjacent multifidus muscle. The purpose of this study was thus to determine if the lumbar spine of ENT1-deficient mice was stiffer than that of wild-type (WT), and if this was associated with a lower passive stiffness of the surrounding musculature. Lumbar spine segments (intact L2-L5) were tested uniaxially in cyclic compression and tension. Muscle fibres and bundles of fibres were continuously lengthened and held to allow for the calculation of elastic modulus. The lumbar spine NZ was shorter and stiffer in KO compared to WT while the muscle elastic modulus (passive stiffness) was lower in KO compared to WT in the lumber multifidus and erector spinae fibres, but not bundles. Tibialis anterior showed no difference in either fibres or bundles. Previous studies have shown that increases in NZ length are directly related to decreases in stiffness/stability that result from damage to the IVDs, facet joints, and ligaments. Thus, this work has confirmed that the mineralization of spinal connective tissues in the ENT1 KO mouse results in the opposite effect: a stiffened spine and a likely reduction in spinal mobility. At the same time, muscle fibre elastic modulus was lower in the paraspinal muscles thereby suggesting a direct compensatory relationship between the stiffness of the spine and the muscles that are attached to it.
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LIST OF ABBREVIATIONS

μCT – Micro Computed Tomography
ALL – Anterior longitudinal ligament
AS – Ankylosing Spondylitis
BTX – Botulinum toxin A
CP – Cerebral palsy
CSA – Cross-sectional area
DISH – Diffuse Idiopathic Skeletal Hyperostosis
ENT1 – Equilibrative Nucleoside Transporter 1
ES – Erector spinae
IS – Infraspinatus
IVD – Intervertebral disc
KO – Knock-out
M – Multifidus
NZ – Neutral zone
OA – Osteoarthritis
PCSA – Physiological cross-sectional area
ROM – Range of motion
SI – Sacro-iliac
SS – Supraspinatus
TA – Tibialis anterior
WT – Wild-type
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Chapter 1: Introduction

Low back pain is estimated to affect more than 80% of adults in their lifetime (Cassidy, Carroll, & Côté, 1998) and is the leading cause of disability worldwide (Hoy et al., 2014). These statistics, along with the fact that the world’s population is aging, highlight the need for research in this area (Hoy et al., 2014). Instability of the lumbar spine is widely believed to play a large role in the cause of low-back pain and disorder, as excessive motion of the spine can result in damage to tissues that both make up and surround the spine (Brown & McGill, 2008).

Coincidentally, the spinal column alone is inherently unstable. The vertebrae, intervertebral discs (IVDs), and ligaments that make up the spine can withstand only minimal compressive force upon the removal of supporting musculature (Lucas & Bresler, 1961; Crisco, Panjabi, Yamamoto, & Oxland, 1992). The intervertebral joint stiffness that is required to properly function under normal loading of the spine comes mainly from its muscular attachments (Brown & McGill, 2008), and it is these muscles that are responsible for keeping the spine, and its neutral zone, within certain physiological limits (Panjabi, 1992).

The neutral zone (NZ) is traditionally defined as the region within a spinal segment’s normal range of motion (ROM) where movement occurs with minimal resistance, or in other words, low stiffness (Panjabi, 1992; Brown & McGill, 2008; Smit, van Tunen, van der Veen, Kingma, & van Dieën, 2011). This NZ exists around the neutral spine posture, which is the posture in which the spine is most commonly oriented. Previous research has suggested that the NZ is a clinically important measure of spinal stability, as the mechanical behaviour of the NZ can change with disease states and injury (Panjabi, 1992). When IVDs and facet joints are destabilized due to injury, this results in an increase in the length of the NZ and/or a decrease in the stiffness of the NZ (Cannella et al., 2008; Johannessen, Clyod, O’Connell, Vresilovic, &
Elliott, 2006; Kaigle, Holm, & Hansson, 1995; Zhao, Pollintine, Hole, Dolan, & Adams, 2005). These changes mean that the spine undergoes a larger ROM over which there is little resistance to movement, and that the small amount of resistance that there is becomes even more negligible. Therefore, the spine becomes hyper-mobile, and less inherently stable (Johannessen et al., 2006).

Mice lacking equilibrative nucleoside transporter 1 (ENT1) develop progressive ectopic mineralization of the fibrous connective tissues of the spine, including that of the IVD (Warraich et al., 2013). The calcification is first detected in the cervical spine of the animal and progresses caudally (towards the tail) with age, affecting the lumbar spine within 6 months. This phenotype mimics a human condition known as diffuse idiopathic skeletal hyperostosis, or DISH (Warraich et al., 2013). This mouse phenotype, as well as DISH, may be associated with stiffening of the spine opposite to that of early disc degeneration.

Spinal musculature has also been shown to change in response to experimentally induced disc degeneration. In rabbits, early stage experimentally induced disc degeneration resulted in an increase in the passive mechanical stiffness of the adjacent lumbar multifidus muscle (muscle fibres and bundles of fibres) (Brown et al., 2011). Multifidus is well accepted as a spine stabilizing muscle (Cholewicki, Panjabi, & Khachatryan, 1997; Ward, Kim, et al., 2009; Ward, Tomiya, et al., 2009), which suggests a potential inverse, compensatory relationship between the stiffness of the muscle with the stiffness of its attachment structure, the spine. Specifically, the muscle became stiffer to compensate for the loss of stiffness of the spine. However, spine stiffness was not quantified in this study, and as a result the evidence supporting this theorized relationship is limited.

The aim of this thesis was to further examine this proposed reciprocal relationship by looking at the opposite scenario in order to determine if it still holds true, as well as to provide
direct evidence of the link in stiffness between the muscles and the spine. Therefore, this thesis investigated the relationship between the stiffness of the lumbar spine and of the lumbar paraspinal muscles in an ENT1 deficient mouse by testing the following hypotheses:

1. The NZ of the lumbar spine will be stiffer and shorter in 8 month old ENT1 KO mice compared to WT mice.

2. The lumbar paraspinal muscles (the erector spinae and multifidus), but not the lower limb tibialis anterior muscle, will have a decreased passive stiffness in 8 month ENT1 KO mice compared to WT.

3. At 2 months of age, ENT1 KO mice will not demonstrate differences in the lumbar spine NZ or in lumbar paraspinal muscle passive stiffness.
Chapter 2: Review of the Literature

In order to understand the relevance of the proposed thesis study, a review of the literature must be completed. As previously mentioned, the purpose of this thesis was to examine the relationship between the stiffness of the lumbar spine and the lumbar paraspinal muscles in an ENT1 deficient mouse. Therefore, the aims of this literature review are as follows:

1) provide background information on DISH and the ENT1 KO phenotype;
2) discuss the suitability of using animal models, specifically that of a mouse, to model the human spine;
3) discuss spine stiffness in terms of the neutral zone, including its importance and mechanisms of change; and
4) introduce the relevant spinal musculature, including its anatomy and function, as well as passive mechanical properties and their role in muscle disease/disorder.

2.1 Diffuse Idiopathic Skeletal Hyperostosis

2.1.1 Human Phenotype

Diffuse idiopathic skeletal hyperostosis (DISH) is a disease that results in the calcification of the spinal entheses: the connective tissue attaching tendons or ligaments to bone (Nascimento et al., 2014). This excessive calcification/ossification/bone growth is what is known as ‘hyperostosis’, while the term ‘idiopathic’ indicates that the cause of the disease is unknown. DISH was first described in 1950 by Forestier and Rotes-Querol, who termed the disease Senile Ankylosing Hyperostosis of the Spine, with the aim of distinguishing it from a very similar disease called ankylosing spondylitis (AS). AS consists of inflammation around the vertebral and sacroiliac joints that causes abnormal bone formation that can lead to complete fusion of adjacent
vertebrae (Slobodin, Rosner, & Odeh, 2013). The main identifying characteristic of DISH is that the bony outgrowths bulged over only the anterior of the vertebral body, which is clearly visible in lateral as opposed to anteroposterior x-rays. Additionally, these growths span the height of the entire vertebral body, whereas AS calcification occurs only at the superior and inferior ends of the vertebrae, near the intervertebral space. Another differentiating characteristic is that the sacro-iliac (SI) joints are not affected in DISH. Additionally, DISH is present only in older patients while AS is known to have an earlier age of onset, in youth or middle age, hence the term ‘senile’.

Forestier and Rotes-Querol (1950) also described differences between DISH and osteoarthritis (OA) of the spine. In the thoracic spine, OA results in small osteophyte spurs, whereas DISH has very clear calcified swelling that is continuous with the intervertebral discs and bodies. In the lumbar spine, the difference between DISH and OA can be less clear as these calcified swellings are not always continuous, and can therefore resemble osteophytes. The difference is that in DISH, these independent bone growths have a noticeably wide base and project upwards in a “candle flame” formation, and the IVDs are of normal height. These distinct anatomical features of the bony outgrowths in DISH are very important in differentiating DISH from OA, as patients with DISH often have pre-existing OA due to their older age, meaning that OA osteophytes can be present along with the bony outgrowths from DISH. Forestier and Rotes-Querol also noted the bony structure of these outgrowths, stating that the trabecular bone of the outgrowths seemed to be resemble that of vertebral bone, while the cortical bone of the outgrowths had a density similar to the femoral head.

In the years following Forestier and Rotes-Querol’s description of DISH, or Senile Ankylosing Hyperostosis of the spine, there were a several other researchers who described...
similar cases, with additional characteristics, using names such as Spondylitis ossificans ligamentosa, Spondylosis hyperostotica, Physiologic vertebral ligamentous calcification, Generalized juxtaarticular ossification of ligaments of the vertebral column, as well as a several others (Resnick et al., 1978). Resnick et al. (1978) compiled the information from these previous studies, including that of Foriester and Rotes-Querol and came up with the name DISH. The term ‘diffuse’ was used to represent the extra-spinal manifestations described by some of these studies. Resnick proposed 3 specific criteria to diagnose spinal involvement in DISH, which are still commonly used:

1. Flowing calcification and ossification along the anterolateral portion of at least four consecutive vertebral bodies
2. Preservation of disc height in the involved areas and the absence of extensive radiographic changes of degenerative disc disease
3. The absence of facet and sacral involvement (apophyseal joint body ankylosis and sacroiliac joint erosion, sclerosis, or intra-articular body fusion).

Resnick (1978) was also able to give a more detailed description of the manner in which the calcification presented, identifying two different patterns that occurred simultaneously in the spine. The first of which was the ossification of the anterior longitudinal ligament (ALL), the second of which involved the IVD, particular the annulus fibrosis (AF). The purely ligamentous pattern seemed to originate as “shaggy” and “ribbon –like” calcifications on the anterior of the vertebral body, usually near the midpoint, which would progressively enlarge and thicken to extend across the IVD space. Bony deposits were present within the fibres of the ALL, resulting in an initial radiolucency between these deposits and the underlying vertebral body. Eventually the ossified ligament and vertebra would fuse together. The discogenic pattern begins with
ossification within the AF, causing it to bulge anteriorly and grow in/around the fibres of the ALL which appears stretched over the protuberance. Hyperostosis develops on the anterior of the vertebral body, thought to originate from the periosteum (membrane covering the vertebrae) and appears adjacent to the ALL. As a result, the AF, ALL, and nearby connective tissue is calcified. In the lumbar spine, the calcification resembles that of the discogenic pattern.

Recently (2013-2014), a number of review papers have been published summarizing the findings of DISH (Mader, Verlaan, & Buskila, 2013; Mazières, 2013; Nascimento et al., 2014; Pillai & Littlejohn, 2014). While DISH was first discovered to affect only the spine, it is now known that while the spine is the primary location of this disease, there have been many reports of extraspinal ossifications in the hands, pelvis, knees, and elbows. While many patients with DISH are completely asymptomatic, common symptoms include morning stiffness, joint and/or spinal pain, reduced ROM, difficulty swallowing (dysphasia), airway obstruction, increased risk of unstable spinal fractures, as well as others (Mader et al., 2013; Mazières, 2013; Nascimento et al., 2014; Pillai & Littlejohn, 2014).

It has been suggested that because of the decreased flexibility, or increased stiffness, of the vertebral joints seen in DISH, the mechanical stresses on the vertebral column are increased (De Decker, Lam, Packer, Gielen, & Volk, 2015). When a traumatic impact occurs, the stiffening of the vertebral joints limits the mechanical energy distribution through the spine, which can lead to vertebral fractures similar to the fractures seen in long bones (Caron et al., 2010; De Decker et al., 2015; Mader et al., 2013). Further, the effective fusion of the vertebrae creates a large moment arm for traumatic forces to act on the spine, allowing relatively minor traumas to induce fractures (Mader et al., 2013; Westerveld, van Ufford, Verlaan, & Oner, 2008).
The main risk factors for DISH are sex (male) and advanced age. A study in the Netherlands reported an almost 2 to 1 odds ratio for males, with 17% of 501 males over the age of 50 having DISH (Westerveld et al., 2008). Another study, using data from 2 large metropolitan hospitals (1107 males and 1257 females) in the American Midwest estimated the average prevalence of DISH in adults over the age of 50 (up to age 90) to be 25% in males, and 15% in females (Weinfeld, Olson, Maki, & Griffiths, 1997). When divided into 10 year age groups, the prevalence was lower in the younger age groups, and increased to 44% in males between the ages of 70 and 80, and to 26% in females aged 80 to 90 (Weinfeld et al., 1997). Another study found that out of 298 men over the age of 65, 42% fit the criteria set by Resnick (Holton et al., 2011).

It has also been suggested that the true prevalence of DISH is impossible to estimate because many people who are affected don’t experience pain, or enough pain, to cause them to seek medical help (Holton et al., 2011; Mader et al., 2013). In fact, DISH is usually diagnosed when patients are seeking help for other medical conditions (Holton et al., 2011). Additionally, it has been suggested that the prevalence of DISH could also be higher due to Resnick’s criteria of a continuous bony bridge that spans 4 vertebrae (Mazières, 2013; Weinfeld et al., 1997). This criteria is very strict, meaning that only patients with advanced DISH will meet the criteria and be diagnosed (Mazières, 2013; Weinfeld et al., 1997). This has led to other studies looking into new ways to diagnose and classify DISH (Mazières, 2013).

There are also many metabolic factors that are correlated with DISH, including: obesity, waist-to-hip circumference ratio, type II diabetes, hypertension, glucose intolerance, hyperinsulinemia, dyslipidemia, as well as others (Mader et al., 2013; Mazières, 2013; Nascimento et al., 2014; Pillai & Littlejohn, 2014). It is thought that these metabolic factors may
interact with cellular targets in biomineralization pathways (Pillai & Littlejohn, 2014). For example, insulin, growth hormone, and insulin-like growth factor 1 can stimulate proliferation of osteoblasts, chondrocytes, and fibroblasts to create new bone formation (Pillai & Littlejohn, 2014).

The history of DISH (in humans) dates back far before Foreister and Rotes-Querol. DISH has been identified in a Neanderthal skeleton, known Shanidar 1, who lived in the middle paleolithic area between 35,000 and 45,000 years ago (Crubézy & Trinkaus, 1992). Saleem and Hawass (2014) took CT images of 13 royal Ancient Egyptian mummies from 1492 to 1153 BC. They found that 4 of these mummies fit the DISH criteria specified by Resnick. And more recently, several members of the Medici family, who ruled Florence, Italy between the 1430s to 1730s have also been proposed to have DISH (Weisz, Matucci-Cerinic, Lippi, & Albury, 2011).

2.1.2 Animal Phenotypes

DISH has been observed in many animal species. Similar to humans, large non-human primates, such as baboons and gorillas, develop DISH with advancing age; albeit the reported incidence is much lower at 1 to 3% (Berthelot, Le Goff, & Maugars, 2013). Many other mammals, much more distantly related to humans, have also been reported to be affected by DISH. These include bears, camels, horses, bison, and whales (Berthelot et al., 2013). Even dinosaur skeletons have shown signs of the disease, making DISH one of the oldest documented diseases on record (Berthelot et al., 2013; Rothschild, 1987).

A recent case study has discovered the disease for the first time in a domestic cat (Bossens, Bhatti, Van Soens, Gielen, & Van Ham, 2016), and made reference to a similar spinal hyperostosis described in a prehistoric Saber-toothed cat (Bjorkengren, Sartoris, Shermis, &
Resnick, 1987). However, the animal with the highest incidence of DISH is the dog. DISH in dogs presents in a very similar manner to humans, with both age and male sex being risk factors, and some reports of clinical symptoms such as stiffness and skeletal pain. Interestingly, the incidence of DISH in the Boxer breed jumps from 3.8% in clinically normal dogs to 40.6% in Boxers (Kranenburg et al., 2010). Due to this high incidence, Boxers have been suggested as a natural model for DISH, and have been used to study changes in bone mineral density (BMD) with the disease. While it is accepted that AS results in decreased BMD, there has been no consensus on the BMD status of patients with DISH. Therefore, De Decker et al. (2015), studied Boxers with DISH and confirmed that these animals did have lower vertebral BMD than healthy Boxers. The authors suggested that lower BMD may be another contributing factor to increased fracture risk in individuals affected by DISH. However, they did make note of the fact that none of the 30 affected dogs had any fractures.

A pathology that mimics DISH has also been shown to occur when mice are genetically modified for deletion of *Slc29a1*, the gene that encodes for equilibrative nucleoside transporter 1 (ENT1) (Warraich et al., 2013). These KO mice begin to develop progressive ectopic calcification of the fibrous connective tissues of the spine, similar to DISH (Warraich et al., 2013). Interestingly, an absence of ENT1 in humans also results in irregular bone homeostasis (Daniels et al., 2015). Three siblings with a homozygous null mutation of *Slc29a1* are deficient of ENT1, and as a result, exhibit periarticular and ectopic mineralization in the hands, hips, pubic symphysis and lumbar discs (although not in every sibling) (Daniels et al., 2015).
2.2 **Equilibrative Nucleoside Transporter 1**

ENT1 is a transmembrane glycoprotein that is responsible for the uptake of hydrophilic nucleosides through the plasma membrane of the cell and mitochondria. It is expressed in all mammals with 78 and 79% homology to the rat and mouse proteins, respectively (Baldwin et al., 2004). Adenosine, a purine nucleoside, is primarily transported by ENT1 (Baldwin et al., 2004), and purine metabolism has recently been identified to play a key role in the regulation of biomineralization in diseases associated with both insufficient or ectopic mineralization (Ii et al., 2016). However, the generation of an ENT1 deficient mouse model was not originally created to study bone.

Adenosine is also known to play a key role in ethanol intoxication, and *in vitro* studies have shown ENT1 is inhibited and down-regulated in response to acute and chronic ethanol exposure, respectively. In order to determine the role of ENT1 in ethanol intoxication and consumption, Choi et al. (2004) created an ENT1 KO mouse. In order to generate a mouse deficient of ENT1, a portion of the protein coding region of *Slc29a1* is deleted through Cre-Lox recombination. This is done by flanking the targeted gene, or region, with LoxP sites. These are specific DNA sequences that come from the bacteriophage P1. The enzyme Cre recombinase recognizes the bacteriophage DNA (LoxP sites) and splices them to yield a deletion of the intervening region. In the case of the ENT1 KO mouse, this recombination is performed in an embryonic stem cell line from a 129X1/SvJ mouse. The KO stem cell line is then inserted into the blastocyst of another mouse strain, C57BL/6J, to generate chimeric offspring. Chimeric animals are made up of cells from both of the two parent species. In this case, the C57BL/6J or the 129X1/SvJ which is the ENT1 KO. These offspring are bred with C57BL/6J mice (backcrossed) to generate F1 hybrids. Hybrid animals contain cells that are a combination of
both parent species. The F1 offspring that is heterozygous for ENT1 (ENT1\textsuperscript{+/−}) are bred together (intercrossed) to generate F2 hybrid offspring that have genetic material from approximately 50% C57BL/6J mice and 50% 129X1/SvJ mice. These littermates are either homozygous null (ENT1 KO or ENT1\textsuperscript{−/−}), homozygous for ENT1 (WT or ENT1\textsuperscript{+/+}) or heterozygous (ENT1\textsuperscript{+/−}). The heterozygous mice are continuously bred to maintain a colony, while the KO and WT littermates are used for experiments.

Choi and colleagues (2004) reported that ENT1 KO mice were normal with respect to: reproduction, brain anatomy, 24hr water consumption, open-field locomotor activity, and spontaneous mortality. They also noted that ENT1 KO mice had lower body mass (8.7% over the age range of 10-12 weeks). Their main finding was that ENT1 KO mice showed an increase in alcohol consumption and preference, likely due to the concurrent increases in phosphorylation of striatal cAMP element-binding protein, which is associated with diminished drug reward. Since this mouse model was introduced, other research has shown that ENT1 KO mice demonstrate behaviors suggestive of reduced anxiety (Chen et al., 2007) and seem to be protected against ischemic conditions in the heart (Rose et al., 2010) and kidney (Grenz et al., 2012).

Warraich et al. (2013) were the first to use the ENT1 KO mouse to examine effects of biomineralization. They found that in the absence of ENT1, mice developed progressive ectopic mineralization of paraspinal connective tissues. Micro computed tomography (μCT) analysis of mice at 1, 2, 4, 6, and 12+ (12-17) months of age revealed ectopic mineralization beginning at 2 months of age in the paraspinal tissues of the cervical spine as well as the rib entheses of the upper thoracic spine. This calcification progressed caudally with age, although initially bypassed the mid thoracic spine, affecting the lumbar spines within 6 months. The severity of calcification also progressed with age, involving the IVDs in both the thoracic and lumbar spine at 6 months.
of age. By 12 months of age, the caudal spine (tail) was affected, with over 30% of the IVDs showing hypermineralization. Analysis of the calcified lesions showed that they were hypermineralized, with a density equivalent to normal cortical bone. Histology of specifically the IVD lesions showed that the mineralization was localized within the AF, leading to lateral compression of the NP and bulging of the AF out of the IV space. Between 12 and 17 months of age, lesions were also found within the sternocostal articulations; however there was no evidence of ectopic mineralization in the appendicular joints. Additionally, no signs of ectopic mineralization were found in the littermate ENT1 WT mice at any age, or in the heterozygous littermates that were examined at 6 months of age.

The following year, a paper was published looking at the bone mineral density of ENT1 KO mice (Hinton et al., 2014). At 7 months of age, Hinton et al. (2014) reported increased bone density, measured through x-ray, in the cervical and upper thoracic spine, but decreased bone density in the lower thoracic and lumbar spine. Additionally, they claimed to see reductions in BMD, measured using DEXA, in the lower thoracic and lumbar spine, but did not present any data for this reduction of BMD in the spine, rather only evidence of decreased BMD in the femur at 8 months of age through 10 months of age. Hinton et al. (2014) also used µCT to quantify trabecular bone characteristics, including volume fraction, number, thickness, and separation in the L4 vertebra and the femur (distal femoral metaphysis). In the L4 vertebra, trabecular bone volume fraction showed a decreased trend in the ENT1 KO, which is likely explained by the significant decrease seen in trabecular thickness. Interestingly, the number and separation of trabeculae was not different between WT and KO. In the femur these values were all significantly different with volume fraction, thickness, and number decreasing and separation increasing.
Decreases in trabecular bone volume and thickness likely do not play a role in the stiffness properties of the lumbar vertebrae. Barak, Weiner, and Shahar (2010) et al. examined the contribution of trabecular bone to the stiffness and strength of rat lumbar vertebrae and found that even with removal of up to 42% of the trabecular bone the vertebral stiffness did not change. This suggested that there is little to no effect of trabecular bone on the deformation behaviour of the vertebral bone. Vertebral strength, which is related to the ability to withstand loads, was impaired with 22% removal of trabecular bone. It is important to note that rats, and likely mice, have a thicker cortical shell when accounting for relative size difference between humans. This implies that the cortical shell in rodent vertebrae has a larger contribution to the mechanical properties of the vertebrae than it would in a human. Regardless, in the testing of single or multiple motion segment stiffness, the IVDs, facet joints, and ligaments provide the majority of compliance during movement. Therefore, the higher stiffness of the vertebrae will not impact the stiffness of this larger unit, because the more compliant tissues are engaged first.

Hinton et al. (2014) also reported decreased body mass in the ENT1 KO mice across the 7 to 12 month timespan which supports the reduced body mass reported by Choi et al. (2004), in 10-12 week old mice. Hinton went further to quantify % body fat and found that it too was decreased in KO mice over this time period. Hinton also examined motor function in these mice, claiming that ENT1 deficient mice had severe deficits in motor coordination and locomotor ability. However, these measures were only examined in mice from 7 to 12 months of age. Mice from 3 to 6 months of age are considered mature adults, similar to humans aged 20 to 40, while a mouse 10 to 14 months is considered middle aged, similar to humans aged 38 to 47 (Fluerky, Currer, & Harrison, 2007). Keeping these age distinctions in mind, Hinton and colleagues (2014) reported the only significant locomotor deficit in ENT1 KO mice occurred at 10.5 months. This
was marked by the inability for ENT1 KO mice to remain on an accelerating rotarod treadmill for as long as their WT counterparts. There were no changes in open field locomotor activity, where distance travelled and vertical counts (representing rearing behaviour) were not significantly different at any time point. Although, vertical counts did show a slight trend (p=0.07) to be decreased in ENT1 KO compared to WT, across all age points (7 to 12 months). This impaired locomotor ability can be explained by observations made by Warraich et al. (2013). These authors noted, but did not quantify, that ENT1 KO mice at 8 months of age displayed decreased hind limb mobility, which by 12 to 17 months of age had progressed to hind limb paralysis. When these mice were analysed post-mortem (at 17 months of age), the authors noted that calcified lesions were present in the paraspinal and intervertebral tissues, and in some instances these lesions protruded into the spinal canal. This suggests that the spinal cord, and potentially also nerve roots, were impinged upon, which may explain the mobility issues noted in these two studies, especially the inability of the mice examined by Hinton et al. (2014) to remain on the accelerating rotarod. Additionally, these same measures of rotarod duration and open-field locomotor activity were examined in 2 to 4 month mice as part of a study by Chen et al. (2010) who found no differences between WT and ENT1 KO. Therefore, it remains to be determined at what age the ENT1 deficient mice become less functionally mobile.

2.3 Murine Models of Human Lumbar Spine

Examining the spine in relation to function, disease, and treatment is very difficult in humans, and as a result animal models are widely used. This solves the problems of tissue availability, variability between subjects, and the feasibility of performing \textit{in vivo} experiments to study aspects of the spine (O’Connell, Vresilovic, & Elliott, 2007). Mice in particular are easy to use, and come with the advantage of being easily genetically modified. However, not all
pertinent anatomical information is available for the lumbar spine of the mouse. Rats are also widely used and as a result have additional available research. For the purposes of this literature review, I will focus on the mouse lumbar spine when possible.

The use of any animal model comes with the question of applicability to the human situation that the animal is being used to model. Anatomically, the lumbar spine of rodents present with differences from human spines. Rodents have 6 lumbar vertebrae, whereas the majority of humans have 5 lumbar vertebrae. Additionally, the height of the vertebra (endplate to endplate) in a mouse is larger than the depth of the vertebra (anteroposterior (AP) length). This is the opposite to what is observed in humans. The spinous processes are angled cranially in a rodent, and caudally in the human. Finally, transverse processes point to the anterior of the vertebra in rodents, whereas in humans they are oriented posteriorly. However, the facet joints, as well as the mammillary processes (located posteriorly and angled superiorly) and accessory processes (located posteriorly and angled inferiorly) are located in similar locations in both rodents and humans. An image of the mouse lumbar spine osteology is depicted in Figure 2.3.1.

When examining areas of research dealing with the spine, the most common criticism is the use of quadruped animals as human models, due to the horizontal orientation of the spine. As Smit (2001) states, it is widely believed that the spine of a quadruped animal experiences different loading than that of the bipedal human, as the human spine is oriented in the upright position. However, this view is not supported by any scientific literature (Smit, 2001). In fact, Smit (2001) points to several studies that show similarities in both geometry (Cotterill, Kostuiik, D’Angelo, Fernie, & Maki, 1986; H. J. Wilke, Kettler, Wenger, & Claes, 1997) and mechanical properties (Lim, Goel, Weinstein, & Kong, 1994; H. J. Wilke, Kettler, & Claes, 1997; H.-J. Wilke, Krischak, & Claes, 1996) between quadrupedal and human spines. With a review of the
literature, Smit (2001) presents evidence that in both standing and walking, the quadruped spine is loaded primarily along its long axis, in axial compression, just as the human spine. This is due to the large bending and torsion moments that the horizontal spine is able to sustain. The spine itself is not able to resist these types of loads, but rather it is the muscle, and to a lesser extent the ligaments, that exert tensile forces to counterbalance these bending and torsion moments. Quadruped vertebrae show architecture that supports this. Because the trabeculae are oriented from endplate to endplate, just as the human spine (Augat et al., 1998), this suggests that the main loading direction is in axial compression. As Wolff’s law states, bone remolds in direct response to the loads under which it is placed. In rodents specifically, Barak et al. (2010) also shows this orientation of trabeculae. Some differences in vertebral bone architecture in rodents include thicker cortical shells (Barak et al., 2010), and higher bone densities due to the suspected larger magnitudes of axial compression stress. However, while these minor differences may somewhat limit the direct transferability of measures between rodent and human vertebrae, the mechanical behaviour of the entire lumbar spine, or even single motions segments (vertebrae-disc-vertebrae), is not largely dependent on the actual properties of the vertebrae themselves. As previously mentioned, the mechanical behavior of spinal motion segments are highly dependent on the more compliant, soft tissues of the spine, as they are engaged prior to the stiffer vertebrae. One additional difference to note is that while humans have a lordosis of the lumbar spine, mice have a slight lumbar kyphosis.
In the motion segment, the IVD plays a large role in the transfer of forces. Elliott and colleagues have done a substantial amount of research looking at the use of animal IVDs, especially those of the mouse, as models for human discs. In 2004, Elliott and Sarver validated the use of both mouse and rat discs (lumbar and caudal) as mechanical models for the human lumbar IVD. They used C57BL/6 mice at 2 and 8-9 months of age, 4 male and 4 female in each age group, with the L1/2 motion segment for axial testing and L3/4 for torsion testing. All measures of stiffness were normalized with respect to geometry to enable comparisons to both rats and humans. In axial compression, the rodent motion segments displayed a non-linear load-
displacement response which included an initial low stiffness toe region, followed by a higher stiffness linear region. They found that in rat and mouse discs both compression and torsion apparent modulus (stiffness normalized to size) compared well with human values. The mouse lumbar apparent modulus in this linear region compared very well with the human compression apparent moduli reported in the literature: 3.47 (SD 0.94) MPa compared to a range of 3 to 8.6 MPa in humans. The torsion apparent modulus in mice was 4.83 (SD 1.60) MPa compared to a range of 1.5 to 8.9 MPa in humans. Additionally, rodent lumbar, but not tail discs, showed correlations between the measured mechanical properties and body weight. In mice, the toe region length had a moderate, positive correlation with body weight ($R^2=0.33$), and in rats the toe region stiffness and normalized stiffness (apparent modulus) had strong, positive correlations with body weight ($R^2=0.69$ and $R^2=0.78$ respectively). The toe region is indicative of part of the neutral zone; however, in this study the motion segment was not brought into tension, rather only to the starting zero displacement point, and therefore the upper bounds of the neutral zone could not be determined. These correlations suggest that body weight loading is transferred to the spine in axial loading, which supports Smit’s conclusion from 2001: that the quadruped spine is loaded primarily in axial compression. Elliot and Sarver did however identify limitations with their torsion testing system and cautioned the use of their results as anything other than preliminary.

In 2007, O’Connell, Vresilovic, and Elliott compared the disc geometry of a number of different animal species to human lumbar discs. This included measurement of IVD height, IVD and NP lateral width, AP width, and area, and NP centroid offset. All of these measures were then normalized by lateral width and IVD area scaling factors to compare to human values. The mouse samples measured were the L3/4 IVD from 3 C57BL/6 mice aged 8-9 months. The other
animals examined were rats (examined lumbar and caudal in both rodent species), baboon, cow, rabbit, and sheep (lumbar). Mouse lumbar discs had the smallest average percent deviation (12%) from human measures in disc height, AP width, and NP area, followed by rat lumbar (15%), mouse tail (18%) and then baboon discs (19%).

Similarly, in 2008 Beckstein and colleagues (which included Vresilovic and Elliott) looked at axial compression mechanics and biochemical measures of glycosaminoglycan (GAG) and water content in multiple animal species compared to human IVDs. The samples examined were the calf, pig, baboon, sheep, rabbit, rat, and mouse lumbar discs, as well as the cow and rat tail. Additionally, they tested human lumbar motion segments to use for validation, as opposed to comparing to values reported in the literature using different methodologies, as in their previous studies. As Elliot and Sarver did in 2004, Beckstein et al. looked at compression stiffness and apparent modulus, and also examined mechanical behaviour in step displacement and creep. Additionally, Beckstein et al. included a small portion of tension in the axial loading protocol, which allowed them to accurately quantify the NZ. Unfortunately, no data for water and GAG content was presented for the mouse, likely due to the small size of the IVDs, so the data from the rat lumbar disc will be described. As reported by Sarver and Elliott (2004), the segments displayed a non-linear load deformation curve with an initial toe region, or NZ, followed by a higher stiffness linear region. The compressive apparent modulus was lower in mice and rats compared to humans with an apparent modulus of 2.93 (SD 0.92) MPa in the mouse compared to 9.95 (SD 3.24) MPa in humans. The authors suggested that the lower values in the rodent segments could be due to the slower loading rate (0.1 HZ compared to 0.5Hz) that was a limitation of their testing equipment. Additionally, the loading function in the rodent segments was a triangular waveform, whereas a sinusoidal wave form was used with the other species. The
rodent step displacement and creep responses, normalized to geometry, were within one order of magnitude of the human values. The rat lumbar disc had comparable NP GAG and water content, but lower GAG and water content in the inner and outer AF. However, similar differences were found in the other species as well. The authors acknowledged that further refinement of the geometry measures used to normalize the biochemical measures could be made, as well as considering the effect of age.

In 2012, Showalter et al. (which included Beckstein, Vresilovic, and Elliott) looked at the torsion mechanics and collagen content of the motion segment and IVD across different species, building on Sarver and Elliott’s work (2004) which presented preliminary torsion data in the mouse and rat. They examined the same animal species as Beckstein et al. (2008), with the addition of goat lumbar and mouse tail motion segments. Torsion mechanics were normalized to disc height and polar moment of inertia. They also measured collagen content for all except the rat and mouse samples, citing small size as the limitation. The mice samples used in this study were L3/4 motion segments from 3 male C57BL/6 mice between the ages of 9 and 12 months. Torsion apparent modulus in mice and rats was statistically similar to humans (within 10%). Once again, a limitation presented itself in the slightly altered loading protocol in the rodent spines. Mouse lumbar IVD geometry measures were consistent across all studies mentioned (Elliot and Sarver, 2004, O’Connell et al., 2007, Beckstein et al., 2009, and Showalter et al., 2012).
2.4 Spine Stiffness

2.4.1 Movement of the Spine

The methodology developed by Elliott’s laboratory is the only known methodology to examine axial or torsional loading in mouse spines. While this technology was developed in order to compare mechanical properties between various species, pure axial loading is not usually the way in which spines are loaded during movement. While the majority of loading through the spine is compressive (axial), movement of the spine results in additional, more complex directional loading. These movements are usually described with respect to three orthogonal anatomical planes or axes. The coronal/frontal plane divides the human body into anterior and posterior portions, and the quadrupedal animal into dorsal and ventral portions. As a result the AP or dorsoventral axis runs through this plane. The sagittal plane divides the body into right and left halves and has the medial-lateral axis running through it, and the transverse plane divides the body into superior and inferior portions (or cranial and caudal in an animal) with the longitudinal axis (cranial-caudal axis in animals) running through it.

In humans, movements of the spine within the coronal plane, or around the AP axis, are called lateral bending; movements in the sagittal plane, or around the ML axis, are called flexion-extension; and in the transverse plane, or around the longitudinal axis, are called axial twist or rotation. Most movement of the spine involves a bending/rotation of some sort, whereas axial loading is loading in line with the longitudinal axis of the upright human spine, or along the cranial-caudal axis of an animal. Extensive research has been done looking at the mechanical behavior of the spine in all of these loading directions, particularly at the behaviour of the neutral zone.
2.4.2 Neutral Zone

Panjabi first proposed the idea of the neutral zone in 1992, as a region within the spine’s normal ROM, where movement is met with minimal resistance, i.e. around a neutral spine posture. As the upright spine is loaded in a bending motion, the initial load-deformation response is that of low stiffness (neutral zone). As the spine moves beyond this neutral posture, the stiffness increases and reaches a linear region of high stiffness as the spine moves toward the end of its ROM. This non-linear, essentially tri-phasic, load-displacement response is very important for the stability of the spinal system. The neutral zone allows for movement of the spine with minimal energy expenditure from the muscles, while the region of higher stiffness protects the spine from damaging movement that would occur beyond the physiological ranges of motion. However, it is within the NZ that the spine is at greatest risk of buckling (due to instability) under compressive load. Therefore, Panjabi also stated that the NZ is a clinically important measure of spinal stability, as changes in NZ length were thought to occur as a response to injury and disease. For example, Panjabi hypothesized that the length of the NZ would increase with injury to the spinal column or muscles, and decrease as a result of osteophyte formation, vertebral fusion, spinal fixation, and muscle strengthening, all to keep the spine within these physiologic thresholds and avoid clinical instability. More recently, the stiffness of the NZ has also been quantified (Kaigle et al., 1995; Sarver and Elliott, 2005), and suggested as a useful measure to detect mechanical changes associated with disc degeneration.

Changes in the length and stiffness of the NZ have been quantified for multiple loading methods. Kaigle et al. (1995) used anesthetized pigs to show decreases in NZ stiffness and increases in NZ length in FE after injury to the facet joints of the lumbar spine. Increases in NZ length were also reported in FE and LB as a result of damage to the IVD or vertebral endplate in
cadaveric human lumbar spines (Zhao et al., 2005). Johannesson et al. (2006) used mouse spines to demonstrate similar NZ changes in axial loading as a result of partial and full nucleotomy. These changes correlated to the amount of denucleation. Cannella et al. (2008) also used cadaveric human spine units and examined axial loading, FE, LB, and AT in response to denucleation. Progressive denucleation correlated with measures of decreased NZ stiffness and increase NZ length in axial loading (FE, LB, and AT were not measured during this protocol). Complete nucleotomy also yielded these changes in the NZ of axial loading, as well as FE, LB, and AT movements.

2.5 Spinal Musculature

2.5.1 Anatomy and Function

The quadruped spine undergoes primarily axial loading due to high muscular demand. Specifically, it is action of the muscles that provide the bulk of the loading experienced by the spine. Therefore, it is important to consider the properties of the muscles that surround the spine. In humans, the musculature surrounding the spine is very well documented, but there is no literature documenting (deep) rodent spine musculature. In humans it is accepted that the erector spinae, and particularly the multifidus, act to stabilize the lumbar spine. Low levels of activation in these muscles are present even in an unloaded standing position, and activation levels increase with added weight in upright standing (Cholewicki et al., 1997). In humans, the lumbar multifidus muscle originates from the spinous processes and inserts mainly on the mamillary processes of inferior vertebrae (Bogduk, 2005). It can also insert on the facet joint capsules, and in the lower lumbar region, the iliac crest and sacrum (Bogduk, 2005). In mice, this appears to be similar (Figure 2.5.1). Fibres in the human and mouse multifidus muscle are oriented in the same
direction, forming a “\textsuperscript{\textdegree}” shape, regardless of the aforementioned difference in orientation of the spinous processes. The main action of the human lumbar multifidus is extension of the lumbar spine and control of movement during flexion (Bogduk, 2005).

The erector spinae muscles in humans lie lateral to the multifidus and consist of the iliocostalis, longissimus, and spinalis. The latter of these does not act through the lumbar spine. The former are termed the longissimus thoracis pars lumborum and iliocostalis lumborum in the lumbar region, and are separated by the lumbar intramuscular aponeurosis, a continuation of the erectors spinae aponeurosis (Bogduk, 2005). The longissimus consists of fascicles that originate from the accessory processes and parts of the transverse processes. The fascicles from L1-L5 converge through their tendons to form the lumbar intramuscular aponeurosis that inserts on the ilium. Fascicles from L5 also insert on the ilium, but its tendon is not a part of the aponeurosis. In the mouse lumbar spine, due to the small size, it is very hard to distinguish between longissimus and iliocostalis (Figure 2.5.1). However the general attachments and insertions appear to be very similar (Figure 2.5.1), with the exception of the longissimus’ attachment on the transverse process, as rodent transverse processes angle anteriorly, not posteriorly. The orientation of the muscle fibres appears to be similar in both species, forming a “\textsuperscript{v}” shape. The function of the erector spinae is to bilaterally extend the spine, and unilaterally to produce some lateral bending. In rodents, the actions of these muscles have not been described, but due to similar attachment sites and orientations (Figure 2.5.1), along with the knowledge that quadruped muscles generate axial compression of the spine, it is likely that the actions are very similar.

In terms of muscle architecture similarities between humans and mice, Mathewson et al. (2014) took comparative measurements of the four rotator cuff muscles between various vertebrate species: 2 primates, and 8 non-primates. These measurements included physiological
cross-sectional areas (PSCA), muscle mass, normalized fibre length, body mass of the animals, fibre length to moment arm ratio, fibre length to muscle length ratio, and the fraction of total rotator cuff PSCA contributed by each muscle. Additionally, an architectural difference ratio (Lieber and Brown, 1992) was calculated, which is a measure of similarity between species. Because ADI is a ratio, an index of 0 is perfect similarity, with similarity decreasing as the index increases. Mathewson and colleagues (2014) found that not surprisingly, chimpanzees and capuchin monkeys had the lowest ADI with scores of 2.15 and 2.16 respectively. However, of the non-primate animals, mice had the next lowest ADI (not given but visually estimated at ~2.3) followed closely by rats and dogs, with the larger mammals having the least similar ADI (pigs ~5.5, cows ~6, and sheep ~7).
Figure 2.5.1 - Images of mouse lumbar spine musculature. Images with the spine oriented vertically are posterior views of the spine with a scale shown (every line is 1mm), while images with the spine oriented horizontally are sagittal views. 1-ribs, 2-spinous processes, 3-multifidus (shown with erector spinae aponeurosis, 4-erector spinae, 5-iliac crest, 6-gluteus medius, 7-psoas major, 8-facet joint, 9-multifidus (erector spinae aponeurosis removed). A- View of multifidus and erector spinae after removal of abdominal cavity, thoracolumbar fascia, serratus posterior, and visceral fat. B) Sagittal view of A. C) Erector spinae reflected. D) Right and left erector spinae reflected and right multifidus reflected. E) – close up view of the D with the reflection of part of the erector spine aponeurosis. Yellow arrows in C and E demonstrate the orientation of the multifidus fibers.
2.5.2 Force Generation

Muscle generates force in two ways: active and passive force generation. Active muscle force is produced by cross-bridge formation and subsequent power stroke of the actin and myosin filaments (also known as the thin and thick filaments, respectively). Myosin filaments are anchored to either Z-disk through the protein titin. Due to the anisotropic (to light) nature of myosin, the region of the sarcomere consisting of myosin is known as the A-band. The actin filaments are anchored in the Z-disk and extend into the A-band in a non-stretched sarcomere. The I band is the region of the sarcomere where there is no myosin, and it is isotropic (to light) in nature. The alternating A and I bands behave as a diffraction grating under focused light (Lieber, 2002). The bands constructively and destructively interfere with the light waves to produce a diffraction grating (Lieber, 2002). Because of this, laser diffraction can be used to determine the spacing of the I-bands, which is equivalent to sarcomere length (a sarcomere is defined from Z-disk to Z-disk and the Z-disk is in the center of the I-band) (Lieber, 2002).

Passive force, or tension, is generated by the passive structures of the muscle. These structures contribute to muscle force as they begin to resist tension at longer muscle lengths. Additionally, passive structures play a large role in the transmission of force between sarcomeres, myofibrils, and muscle fibres to the external tendon (Fridén & Lieber, 2003; Purslow & Trotter, 1994). Therefore, passive muscle mechanics are an important measure of structural function (Prado et al., 2005; Shah et al., 2004; Shah & Lieber, 2003; Ward, Tomiya, et al., 2009). Additionally, passive muscle stiffness, which is a measure of the force per unit of deformation, is known to change with disease states, such as spastic muscle in cerebral palsy (CP) (de Bruin, Smeulders, Kreulen, Huijing, & Jaspers, 2014; Fridén & Lieber, 2003; Lieber, Runesson, Einarsson, & Fridén, 2003; Mathewson et al., 2014; Smith, Lee, Ward, Chambers, &
and injury, such as rotator cuff (Sato et al., 2014; Silldorff et al., 2014) or IVD injury (Brown et al., 2011).

The passive structure within the muscle fibre that is thought to contribute to the majority of passive tension is titin (Fridén & Lieber, 2003; Horowits, Kempner, Bisher, & Podolsky, 1986; Prado et al., 2005). Titin is the largest known protein in existence. It attaches from the Z-disk to the M-line, and as previously mentioned, connects the myosin filaments to the Z-disk. This connecting region, the I-band portion of titin, is very elastic and extends as the sarcomere is stretched to generate passive force (Granzier & Labeit, 2007). There are different isoforms of titin which vary greatly in their passive force generating capabilities, and are regulated by the body during normal development and disease states (Granzier & Labeit, 2007). Other potential contributors to passive force include nebulin (which is anchored in the Z line, interacts with titin, and binds along the length of actin), as well as gap filaments (located between thick and thin filaments when stretched beyond overlap), and intermediate filaments (which connect successive Z-disks) (Gautel & Djinović-Carugo, 2016; Horowits et al., 1986). Surrounding individual fibres and bundles of fibres is the extracellular matrix (ECM), which is made up largely of collagen ((Fridén & Lieber, 2003; Lieber & Ward, 2013; Purslow & Trotter, 1994)). The ECM makes up approximately 5% of the muscle cross-section (Eisenberg, 2010) but is the largest contributor to muscle passive force (Lieber et al., 2003; Lieber & Ward, 2013; Meyer & Lieber, 2011).
2.5.3 Mechanical Changes

Due to the difficulty in obtaining live human muscle tissue, animal models have often been used to look at changes in muscle properties due to various scenarios. Hodges et al. (2006) used a porcine model to examine the response of the multifidus muscle to IVD degeneration. After experimentally inducing disc injury through a large stab incision, the multifidus muscle showed significant signs of atrophy, or reduced muscle size, as well as fatty infiltration within 3 days post injury. A similar study by Hodges and colleagues (2009) also showed increased corticomotor excitability to the multifidus muscle 15 minutes post disc injury. Brown et al. (2011), using rabbits and a much milder needle-based injury to experimentally induce IVD degeneration, examined the mechanical and biochemical changes of the lumbar multifidus. At 12 weeks, but not 4 weeks post injury, multifidus muscle fibres and bundles of fibers were stiffer than control (34% and 107%, respectively). At 12 weeks there were also larger titin isoforms (which would reduce passive stiffness), and similar to Hodges et al. (2006), mild signs of fatty infiltration. There were no changes in myosin heavy chain (MHC), which signifies no obvious changes in fibre-type. Ward et al. (2009) found that multifidus bundles of fibres in low back pain patients seemed to be stiffer than longissimus and iliocostalis, while individual fibres were not different, likely as a result of its stabilizing function.

Other changes in muscle passive stiffness have been documented in studies looking at the spastic muscle of CP patients. Fridén and Lieber (2003) found that spastic muscle fibres from various upper limb muscles were almost twice as stiff, and approximately 20% shorter (had a shorter slack sarcomere length) than normal muscle (slack sarcomere length is the length at which a sample begins to resists tension during passive mechanical testing). The authors hypothesized that these changes could be due to changes in titin isoform or the ECM proteins
surrounding the individual fibres, as these components are the largest known contributors to passive muscle force. Lieber et al. (2003) expanded upon this work to look at the passive properties of spastic muscle fibre bundles, again from various upper limb muscles. In contrast to their findings at the fibre level, there was no change in slack sarcomere length, and spastic bundles were approximately 4 times less stiff than normal muscle bundles. Where normal muscle fibre bundles were approximately 16 times more stiff than individual fibres, the spastic bundle tangent modulus was only double that of the fibres. The spastic muscle also contained more ECM than the normal muscle, leading the authors to suggest that this decrease in passive stiffness in spastic muscle fibre bundles was due to compromised ECM structure/function, stating that it was of inferior mechanical strength. In 2011, Smith et al. looked at spastic muscle from hamstring contractures in CP patients. In this study, fibre stiffness and titin isoform were not altered, while bundles were stiffer and had greater collagen content in CP patients compared to controls. They also found longer in vivo sarcomere lengths in the CP patients indicating that the stiffness of the muscle was even greater than the tangent stiffness (slope as opposed to a single point) of the bundles indicated. Mathewson et al. (2014), looked at the gastrocnemius and soleus muscles of CP patients. They found that fibres were more stiff in spastic muscle, while bundles were not different. However, they also measured in vivo sarcomere lengths and compared the moduli at these lengths to predict that both fibres and bundles of fibres would be more stiff in vivo in the CP patients, as their in vivo lengths were also much longer than typically developing individuals. Titin weight and collagen content were not different between groups. In 2015, de Bruin et al. examined the flexor carpi ulnaris muscle in CP patients. They found no changes in fibre or bundle slack sarcomere length or stiffness compared to controls, but did see a
thickening of the ECM at the tertiary level of connective tissue, which the authors admit to have purposefully avoided during bundle testing.

Changes in muscle passive stiffness have also been examined in rotator cuff injuries. Silldorff et al. (2014), found that when the supraspinatus (SS) muscle was torn, both fibre and bundle stiffness was higher compared to patients with intact rotator cuff muscles. Additionally, at the bundle level, but not the fibre level, the torn muscle was stiffer than the adjacent, intact, infraspinatus (IS) muscle. The authors also saw a strong, negative correlation ($R^2 = -0.600$) with titin size and fibre elastic modulus, and a moderate, positive correlation ($R^2 = 0.465$) with collagen content and bundle elastic modulus. In 2014, Sato et al. also looked at rotator cuff tears, but used a rat model to examine the compound effect of rotator cuff tear (of both SS and IS) and chemical denervation using botulinum toxin A (BTX). Single fibre stiffness was lower in both injury groups at 18 weeks post tear in the SS muscle compared to control (no surgery), but not in the IS muscle, which was not different between control, torn, and torn + BTX. However, the SS fibres also had decreased titin weight which would be thought to cause an increase in passive stiffness. At the bundle level, the SS showed greater passive stiffness at 8 weeks only in the tear plus BTX group compared to control, along with an increase in collagen content. However, at 16 weeks the stiffness was still greater in the tear + BTX compared to control, but the collagen content was not. The IS showed greater bundle stiffness at 8 and 16 weeks with the tear plus denervation, but no differences in collagen content compared to control.

Another example of passive mechanical changes in muscle comes from a study conducted by Anderson, Li, and Goubel (2001) who looked at the effect of desmin KO in mice. Desmin is an intermediate filament that is located at the periphery of the Z disk and laterally links individual myofibrils together, as well as sarcomeres to the sarcolemma (Li et al., 1997;
Shah et al., 2004). This is thought to aid in force transmission between adjacent sarcomeres (Li et al., 1997; Shah et al., 2004). Anderson and colleagues (2001) found that in desmin KO mice, the passive, whole muscle stiffness of the soleus muscle was greater than in WT. Further study by Anderson and Goubel (2002) determined that this increase in passive stiffness was due to both the absence of desmin as well as the adaptation of connective tissue, such as the physical changes (extensions and undulations) of the sarcolemma (Li et al., 1997), or the increased composition of slow twitch fibres observed in these mice (Agbulut, Li, Mouly, & Butler-Browne, 1996; Wieneke, Stehle, Li, & Jockusch, 2000). Muscles with a larger proportion of slow twitch fibres are thought to have greater passive stiffness as a result of increased collagen in the connective tissue surrounding the muscle fibre and fascicles (Kovanen, Suominen, & Heikkinen, 1984). Meyer and Lieber (2012) further examined the passive muscle stiffness of desmin KO mice in muscle fibres and bundles of fibres and found an increased passive stiffness of the bundles along with increased total muscle collagen content at two age points (7-9 and 12-14 months), suggesting that the muscle undergoes fibrosis as a result of desmin deletion. However, individual fibre passive stiffness was decreased at 7-9 weeks and then not different at 12-14 weeks in the desmin KO compared to WT (Meyer & Lieber, 2012).

These conflicting results highlight the poor understanding of how passive structures contribute to muscle passive stiffness (Lieber & Ward, 2013; Mathewson et al., 2014), and how these structures and properties respond to injury and disease. It seems that titin is relatively poorly correlated with single muscle fibre passive stiffness, and that increases in ECM do not always result in increases in passive stiffness, as the integrity of the ECM must also be considered (Lieber et al., 2003; Lieber & Ward, 2013). Additionally, it is not clear if an increase in ECM, commonly called fibrosis, is a beneficial or detrimental adaption to injury and disease.
Chapter 3: Methods

3.1 Mice

Thirty-two male, littermate paired, ENT1<sup>-/-</sup> (KO) and ENT1<sup>+/+</sup> (WT) mice (genetic makeup ~50% C57BL/6J and ~50% 129X1/SvJ) (Choi et al., 2004) were examined: 8 KO and 8 WT at both 2 and 8 months of age. Body weight was recorded prior to euthanization. Lumbar multifidus, lumbar erector spinae, and tibialis anterior muscles were immediately excised following euthanization and placed in a storage solution at -20°C to permeabilize the sarcolemma and prevent breakdown of the sample (Shah & Lieber, 2003). Lumbar spines (L1-L6) were removed and immediately wrapped in gauze, soaked in phosphate buffer saline (PBS), and stored at -20°C.

3.2 Spine Mechanical Testing

Lumbar spines were thawed at room temperature for 2 hours, then immersed in PBS for 20 minutes while excess muscle tissue was dissected, leaving an osteoligmentous lumbar spine. Spines were tested uniaxially in cyclic compression and tension (Elliott & Sarver, 2004; Johannessen et al., 2006; Sarver & Elliott, 2005) at 0.33% displacement/second using the BioTester Biaxial Test System (CellScale Biomaterials Testing, ON, CAN), with force and displacement data sampled at 30Hz. Vice clamps were fastened to the vertebrae at either end resulting in a 3 motion segment specimen (L2/3, L3/4, and L4/5 intervertebral levels) being tested (Figure 3.2.1). Testing consisted of a preliminary assessment of approximately 5 cycles to determine the displacement limits in each of compression and tension to ensure loading did not exceed the 2.5N load cell limit. A 15 cycle test immediately followed. All analyses were completed using a custom written MATLAB code (MATLAB v8.3, MathWorks, Natick, MA,
USA). The force-displacement data of the 15th test cycle were fit with a double sigmoid (logistic) function (Smit et al., 2011), as it has been shown that 10-15 cycles are required to eliminate effects of super-hydration and to produce repeatable loading curves (Elliott & Sarver, 2004; Johannessen et al., 2006). The NZ was determined as the region between the minimum and maximum of the second derivative of the fitted function for both the top and bottom segments of the entire hysteresis loading curve (Figure 3.2.2) (Smit et al., 2011). These points were visually inspected and adjusted if needed to ensure accurate NZ quantification. The top and bottom values of both NZ length (displacement) and NZ stiffness (slope of force vs. displacement) were averaged together.

Figure 3.2.1 - Image of a mouse lumbar spine secured at either end by vice clamps to undergo mechanical testing in the CellScale BioTester. Main image is a sagittal view of the spine with anterior, posterior, inferior, and superior denoted by A, P, I, and S respectively. Inset image is an anterior view of the same spine with IVDs L3/4, L4/5, and L5/6 visible from right to left, respectively.
3.3 Muscle Passive Mechanical Testing

All muscle samples were tested within 18 days of harvest and no paired muscle (WT and KO) was tested more than 2 days apart. Testing was performed in a relaxing solution (Shah & Lieber, 2003) to ensure only passive tension was generated. Three single muscle fibres and three bundles of fibres from each muscle were dissected and tied at both ends to two separate pins: one attached to a micro-level force transducer (Model-405A, Aurora Scientific Inc., Aurora, Ontario, Canada), the other to a high-speed motor (Model-322C, Aurora Scientific Inc., Aurora, Ontario, Canada) (Figure 3.3.1). Samples were set to their slack length (length at which passive resistance to stretch is first detected), measurements of sample diameter were taken at 3 locations using a digital micromanipulator, and the sarcomere length at slack length was determined using laser
diffraction with a 5mW diode laser (Coherent, Wilsonview, Oregon, USA) (Lieber, Yeh, & Baskin, 1984). Force and sarcomere length were recorded as samples were rapidly stretched by increments of ~0.2 µm and allowed to relax for 2 minutes. The force at the end of every 2 minute period was normalized to the CSA calculated from the average of the diameter measures (assuming a circular shape) to give a measure of stress (Ward, Tomiya, et al., 2009). Initial disorganization and spacing of fibres within a bundle made diameter measurement difficult at slack length, therefore this was corrected for by taking additional diameter measurements after the third stretch and back-calculating the original CSA assuming Poisson’s ratio of 0.5 (constant volume).

A quadratic curve was fit to the resulting stress vs. sarcomere length data points and passive elastic modulus was determined by finding the tangent slope at a sarcomere length 3.2 µm (Sato et al., 2014), as this is approximately half-way down the descending limb of the predicted active force-length curve of small mammals (Buck, Tonino, Hoying, & Granzier, 2011; Burkholder & Lieber, 2001; Herzog, 1992).
Figure 3.3.1 - Experimental set-up used to test muscle samples. A) Micro-level force transducer and high speed motor. B) Pins secured to each of the former, submerged in a testing chamber filled with relaxing solution. C) Bundle of muscle fibres secured to pins shown through a dissecting microscope.

3.4 uCT Imaging

The spines of an additional 2 pairs of 8 month old mice (KO and WT littermates, 1 male pair and 1 female pair) were imaged to determine if the facet joints of the lumbar spine were affected by the calcification. This was not considered necessary for the 2 month old mice as the lumbar spines were not yet affected. After euthanization, mice were fixed in formalin and imaged using a laboratory μCT scanner (eXplore speCZT, GE Healthcare Biosciences, London, ON, Canada)
as previously described (Ii et al., 2016; Warraich et al., 2013). Briefly, 900 images were captured at angular increments of 0.4°. Multiplanar views in the sagittal, coronal, and transverse planes were examined to identify areas of hypermineralization, and were reconstructed into 3D images with high resolution isosurfacing.

3.5 Statistics

Separate statistical analyses were conducted for the 2 and 8 month age groups. One-tailed, paired T-tests (α = 0.05) were conducted (SAS 9.4, SAS Institute, Cary NC, USA) to determine differences between KO and WT for the following dependant variables: body weight, slack sarcomere length, elastic modulus of each muscle (fibres and bundles separately), lumbar spine NZ length, and lumbar spine NZ stiffness. Note: the 3 samples of fibres/bundles from each muscle were treated as independent samples. All data are reported as mean ± SEM.
Chapter 4: Results

4.1 Eight Month Old Mice

8 month old mice were 250 ± 1.8 days old at sacrifice, with the ENT1 KO mice having a significantly lower body mass than their WT counterparts (Table 4.1.1). Lumbar spine NZ length was shorter and stiffness was greater in KO compared to WT (p=0.0006, 0.0056 respectively) (Figure 4.1.1). Muscle fibre passive elastic modulus was lower in KO compared to WT in the M and ES muscles (p=0.0262 & 0.0016, respectively) (Figure 4.1.2-A), while TA showed no difference (p=0.3436). M, ES, and TA bundles were not significantly different between KO and WT (p=0.1223, 0.1641, 0.1410 respectively) (Figure 4.1.2-B). There were no significant differences in fibre slack sarcomere length between KO and WT in any muscle, however, TA and M bundles were significantly different between WT and KO, with KO TA bundles being shorter than WT, and M KO bundles being longer than WT. ES bundles were not different between genotype (Table 4.1.1).

Additionally, the supplementary KO mice imaged by µCT showed signs of calcification not only in the paraspinal and IVD areas (Figure 4.1.3), but also in the facet joints (Figure 4.1.4). Both KO animals imaged show hypermineralization at all 3 of the intervertebral levels that were tested in this study: all 3 facet levels in both animals, and 2 out of 3 and 3 out of 3 of the IVDs.

Table 4.1.1 - Mean (± SEM) body mass at time of sacrifice of ENT1 WT and KO mice at 2 and 8 months of age.

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Body Mass (g)</th>
<th>p-value$^z$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>KO</td>
</tr>
<tr>
<td>2 month</td>
<td>22.8 ± 0.41</td>
<td>21.1 ± 0.39</td>
</tr>
<tr>
<td>8 month</td>
<td>31.2 ± 2.23</td>
<td>24.9 ± 0.66</td>
</tr>
</tbody>
</table>

$^z$ Bold numbers indicate statistical significance of p<0.05.
Figure 4.1.1 - Mean (± SEM) lumbar spine neutral zone (NZ) stiffness [Panel A] and length [Panel B] in ENT1 knock-out (KO) and wild-type (WT) mice at 8 months of age. * indicates statistical significance of p<0.05. n=8 for each mean (8 mice x 1 spine).

Figure 4.1.2 - Mean (± SEM) passive elastic modulus (stiffness) of tibialis anterior (TA), lumbar erector spinae (ES), and lumbar multifidus (M) fibres [Panel A] and bundles of fibres [Panel B] in ENT1 knock-out (KO) and wild-type (WT) mice at 8 months of age. * indicates statistical significance <0.05. n=24 for each mean (8 mice x 3 fibres/bundles).
Figure 4.1.3 - uCT imaging using three-dimensional isosurface rendering of the lumbar spine from a female ENT1 knock-out (KO) and wild-type (WT) mouse at 8 months of age. An anterior view is shown with ectopic mineralization indicated with white arrows at the level of L1/2, L2/3, L3/4, and L4/5 in the KO spine. Scale bar represents 1mm.
Figure 4.1.4 - μCT images in the transverse plane of lumbar facet joints L2/3, L3/4, and L4/5 of a male ENT1 knock-out (KO) and wild-type (WT) mouse at 8 months of age. Scale bar represents 1mm.
4.2 Two Month Old Mice

2 month old mice were 63 ± 2.0 days old at sacrifice, ENT1 KO mice having a significantly lower body mass compared to WT (Table 4.1). Lumbar spine NZ length and stiffness were not statistically different between KO and WT (p=0.1679 & 0.1419, respectively) (Figure 4.2.1). TA, lumbar ES, and lumbar M muscles showed no significant difference in passive elastic modulus of fibres (p=0.2194, 0.2634, & 0.2297, respectively) or bundles (p=0.4082, 0.3885, & 0.3899, respectively) between KO and WT (Figure 4.2.2). There were no significant differences in fibre or bundle slack sarcomere length between KO and WT in any muscle (Table 4.2.1).
Figure 4.2.1 - Mean (± SEM) lumbar spine neutral zone (NZ) stiffness [Panel A] and length [Panel B] in ENT1 knock-out (KO) and wild-type (WT) mice at 2 months of age. n=8 for each mean (8 mice x 1 spine).

Figure 4.2.2 - Mean (± SEM) passive elastic modulus (stiffness) of tibialis anterior (TA), lumbar erector spinae (ES), and lumbar multifidus (M) fibres [Panel A] and bundles of fibres [Panel B] in ENT1 knock-out (KO) and wild-type (WT) mice at 2 months of age. n=24 for each mean (8 mice x 3 fibres/bundles).
Table 4.2.1 - Mean (± SEM) slack sarcomere lengths of TA, ES, and M muscle fibres and bundles at 2 and 8 months of age.

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Sample</th>
<th>Muscle</th>
<th>Slack Sarcomere Length (µm)$^a$</th>
<th>WT</th>
<th>KO</th>
<th>p-value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 Month</td>
<td>Fibres</td>
<td>TA</td>
<td>2.18 ± 0.021</td>
<td>2.18 ± 0.019</td>
<td>0.4164</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ES</td>
<td>2.23 ± 0.022</td>
<td>2.20 ± 0.023</td>
<td>0.1115</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>2.20 ± 0.025</td>
<td>2.24 ± 0.024</td>
<td>0.1627</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bundles</td>
<td>TA</td>
<td>2.21 ± 0.019</td>
<td>2.2 ± 0.021</td>
<td>0.4037</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ES</td>
<td>2.19 ± 0.029</td>
<td>2.18 ± 0.021</td>
<td>0.3955</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>2.13 ± 0.026</td>
<td>2.17 ± 0.020</td>
<td>0.1086</td>
<td></td>
</tr>
<tr>
<td>8 Month</td>
<td>Fibres</td>
<td>TA</td>
<td>2.25 ± 0.027</td>
<td>2.21 ± 0.023</td>
<td>0.0924</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ES</td>
<td>2.17 ± 0.023</td>
<td>2.17 ± 0.025</td>
<td>0.4591</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>2.27 ± 0.035</td>
<td>2.27 ± 0.025</td>
<td>0.4791</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bundles</td>
<td>TA</td>
<td>2.21 ± 0.020</td>
<td>2.13 ± 0.018</td>
<td><strong>0.0028</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ES</td>
<td>2.14 ± 0.021</td>
<td>2.16 ± 0.018</td>
<td>0.2425</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M</td>
<td>2.18 ± 0.026</td>
<td>2.24 ± 0.028</td>
<td><strong>0.0345</strong></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ n=24 for each mean (8 mice x 3 fibres/bundles).

$^b$ Bold numbers indicate statistical significance of p<0.05.
Chapter 5: Discussion

5.1 Explanation of Findings

The aim of this thesis was to further examine the relationship between the passive stiffness of muscle with that of the structure to which it attaches (i.e. the spine). Using a genetically modified mouse model that is known to induce progressive calcification of the spinal connective tissue, we hypothesized that an increase in stiffness would be measured in this calcified spine. Furthermore, we examined the surrounding musculature to determine if a reciprocal, or inverse, change in muscle passive stiffness would occur. Specifically, we hypothesized that the lumbar spines of the ENT1 KO mice at 8 months of age would have a stiffer and shorter NZ, combined with a decrease in passive stiffness of the lumbar multifidus and erector spinae muscles, when compared to WT mice. This would demonstrate an inverse, compensatory relationship between the stiffness of the muscles and that of the spine. As confirmation of these expected changes, we also hypothesised that a muscle completely separate from the spine, the tibialis anterior, would demonstrate no change in passive stiffness between KO and WT at this 8 month time point. Additionally, we hypothesized that at 2 months of age, when the lumbar spine is not yet affected by this hypermineralization, the stiffness of both the lumbar spine, as well as all three muscles would not be different in the KO mice compared to WT.

Consistent with the first hypothesis, the axial loading NZ of 3 disc lumbar spine motion segments was stiffer and shorter in the ENT1 KO mice compared to WT mice at 8 months of age. Normally, the spine experiences minimal resistance to movement around a neutral posture (neutral zone). The changes reported here indicate that in the calcified spine of the ENT1 KO mice, there is likely abnormally high resistance to this motion, as the loading behaviour of the
fibrous connective tissue is altered. The NZ is an important component of the non-linear load-displacement behaviour of the spinal system. Having an initial region of low stiffness around a neutral spine posture allows for minimum energy expenditure, as a large amount of muscle activation is not needed to overcome this resistance (Panjabi, 1992). When the spine moves beyond this neutral posture, the stiffness response increases through a short non-linear phase before reaching a linear region of high stiffness as the spine moves towards either end of its range of motion. This increased stiffness provides stability to the spinal system and prevents damaging movement from occurring beyond the ends of the physiological range of motion (Panjabi, 1992). When the stiffness of the NZ increases and the subsequent linear region remains the same, in that there is no change in the force per unit of deformation, the total force experienced by the spinal segment is much higher. It is important to note that these theories regarding the NZ were made with respect to bending motions (termed flexion-extension (FE) and lateral bend (LB) in humans). This load-displacement neutral zone is also present in axial loading, and is likely correlated with bending load-displacement responses (Cannella et al., 2008).

Previous studies have independently shown that increases in FE and LB NZ length occur with damage to the intervertebral disc and vertebral endplate (Zhao et al., 2005), and increases in FE NZ length and decreases in FE NZ stiffness occur with damage to facet joints (Kaigle et al., 1995). Additionally, partial or full removal of the NP (denucleation/nucleotomy), which causes similar structural and mechanical changes as seen in early stage disc degeneration, leads to decreased axial loading NZ stiffness and increased axial loading NZ length, and this correlates with the severity of the induced injury (Johannessen et al., 2006). Cannella et al. (2008) looked at NZ mechanics in both axial loading and movement in the three anatomical planes (FE, LB, and
axial twist (AT)) in the same specimens. Similarly to Johannessen et al. (2006), Cannella and colleagues demonstrated that changes in axial loading NZ length and stiffness correlated with the magnitude of denucleation through a progressive denucleation protocol. Additionally, in a separate group of spines (which had statistically similar mechanical and physical properties prior to denucleation), a complete nucleotomy was performed as opposed to a progressive denucleation (as well as different preconditioning protocols). In this group there was again a decrease in NZ stiffness and increase in NZ length in axial loading, as well as in movements in the three anatomical planes (Cannella et al., 2008), which suggests that changes in the axial loading NZ are representative of changes in bending and twisting NZs of the spine. These changes are directly related to decreased stiffness/stability that results from damage of spinal joints (Brown & McGill, 2008) and as a result are thought to result in hypermobility of the spine (Johannessen et al., 2006). Thus, the current work has confirmed that the mineralization of spinal connective tissues in the ENT1 KO mouse results in the opposite effect, a stiffened spine and a likely reduction in spinal mobility.

In the study conducted by Warraich et al. (2013), µCT imaging was used to quantify ectopic mineralization of the spine in ENT1 KO mice at 1, 2, 4, 6, and 12+ months of age, where the 12+ category consists of mice between the ages of 12 and 17 months. The lumbar spine was not affected until 6 months of age (n=4), where it was assigned a severity score of 1, indicating 0 to 30% of the lumbar spine “anatomical sites” contained calcified lesions, and by 12+ months (n=4) the lumbar spine had an average severity score of approximately 3.25, where 3 indicates 60-90% of sites affected. These lesions were hypermineralized, with a density equivalent to normal cortical bone, and were located in both the paraspinal ligaments and IVDs. Histology
revealed localization of the IVD lesions to within the AF, leading to lateral compression of the NP and bulging of the AF out of the IV space.

Interpolating these results to an 8 month time point would likely be unreliable, especially considering that the 12 month + group consisted of a 5 month age range. Additionally, Warraich et al. (2013) did not image the facet joints, which are known to impact the flexibility of the spine in the three anatomical planes of movement (Fujiwara et al., 2000), and alter the FE NZ length and stiffness (Kaigle et al., 1995). This could therefore also likely impact the axial loading NZ. In the current work, by μCT imaging the lumbar spines of 2 additional pairs of mice at 8 months of age it was able to be determined that the region of the lumbar spine that was mechanically tested very likely contained a large degree of hypermineralization. And, in addition to the calcification in the paraspinal tissues and IVDs that was previously reported, calcification of the facet joints was also present in the ENT1 KO mice. Quantification of the location of the lumbar spine calcification provides evidence that the stiffness of the spine in the mice used for mechanical testing were likely affected by the mineralization of the facet joints, as well as the IVDs and paraspinal ligaments. While one of these two pairs was female, Li et al. (2016) reported that females ENT1 KO mice presented with the same anatomical and temporal pattern of hypermineralization as males. It is unlikely that the 2 month old mice used in the present study had facet joint calcification, as Warraich et al. (2013) showed no involvement of the paraspinal ligaments or IVDs at this time point. However, because the facet joints have not been imaged at 2 months of age, this cannot be stated with complete certainty. Regardless, the lumbar spines at 2 months of age did not display any neutral zone mechanical differences between the KO and WT mice.
The hypothesized decrease in muscle passive stiffness (quantified as the elastic modulus) at 8 months of age in ENT1 KO mice was confirmed in the individual fibers of the multifidus and erector spine muscles; however, no significant difference in bundles of fibers was found. This partially confirms the second hypothesis of this thesis, however the latter result was unexpected, as changes in both muscle fibre and bundle elastic modulus was seen in response to experimentally induced IVD degeneration (Brown et al., 2011). Additionally, the changes reported by Brown et al. were much greater in bundles than in fibres, to an extent where the change in the modulus of the bundles could not be explained solely by the change in the fibres that comprised them. This, along with an increase in the amount of ECM, led the authors to suggest that the large increase in bundle modulus was likely due in part to reorganization and/or proliferation of the ECM, specifically that of the collagen matrix. Interestingly, changes were only seen by 12 weeks post disc injury, and not at an earlier time point of 4 weeks.

Understanding of the structural organization of extracellular collagen, as well as the physiological basis of skeletal muscle fibrosis, which is often used to describe an increase in the amount of ECM, is very limited (Lieber & Ward, 2013). Furthermore, testing of the isolated muscle ECM is currently not possible (Meyer & Lieber, 2011). However, an indirect measure of ECM stiffness, obtained by comparing the stiffness of a bundle of fibres, ensheathed in their connective tissue matrix, to a comparable sized group of individual fibres, suggests that muscle ECM can be up to 4 times more stiff than the muscle fibres themselves (Meyer & Lieber, 2011). While the difference in passive stiffness between fibres and bundles were less than 4 times in the present study, as well as in other studies (Brown et al., 2011; Brown, Carr, Ward, & Lieber, 2012; Meyer & Lieber, 2012; Sato et al., 2014; Ward, Tomiya, et al., 2009), bundles were still stiffer than fibres. This would therefore suggest that the primary driver of bundle passive
stiffness is the ECM (Meyer & Lieber, 2011), even though the ECM consists of only 5% of a muscle cross-section (Eisenberg, 2010). When mechanically testing a bundle of fibres it is impossible to determine the amount of ECM present. Some is likely lost during the separation of the bundle from the muscle. This introduces a level of variability in the elastic modulus of the muscle bundles which could have masked the changes seen in the individual fibres.

Regardless of the unexpected maintenance of stiffness in the bundles, which suggests that the connective tissue matrix of the muscle is not significantly impacted, the decrease in stiffness of the individual muscle fibres indicates a change at the cellular level. While Brown et al. (2011) hypothesized that the increase in stiffness of the ECM was initiated as a result of decreased spine stiffness/stability, the current model presents evidence for the inverse condition of this reciprocal relationship between the spine and muscle, suggesting that while an increase in muscle stiffness is driven by ECM, if a decrease is needed it occurs primarily at the level of the muscle fibre.

However, it is unclear as to which structure/s within the muscle fibre is/are responsible for this decrease in fibre stiffness. Titin is thought to contribute the majority of a muscle fibre’s passive force, as it attaches from the Z-disk to the M-line, and is very elastic in the I-band region (from Z-disk to start of the A-band) (Granzier & Labeit, 2007). This elastic portion extends as the sarcomere is stretched to generate passive force (similar to a spring) (Granzier & Labeit, 2007). There are different isoforms of titin which vary greatly in their passive force generating capabilities, and are regulated by the body during normal development and disease states (Granzier & Labeit, 2007). Other potential contributors to passive force include nebulin, (which is anchored in the Z line, interacts with titin, and binds along the length of actin), as well as gap filaments (located between thick and thin filaments when stretched beyond overlap), or intermediate filaments (which connect successive Z-disks) (Gautel & Djinović-Carugo, 2016;
Gap filaments are likely not a contributor in the present study, as there is still actin-myosin overlap at 3.2μm. An example of an intermediate filament is the protein desmin which has been shown to impact fibre passive stiffness as a result of desmin KO models (Meyer & Lieber, 2012).

The lack of difference in elastic modulus in the TA muscle of the 8 month old mice also supports the second hypothesis, and ensures that the changes seen in the multifidus and ES were not simply due to a whole-body effect of the KO. These paraspinal muscle differences seen at 8 months are further strengthened by the lack of difference at 2 months of age. As the lumbar spines are known not to be calcified at 2 months of age (Warraich et al., 2013), and the mechanical testing in this study confirmed that the stiffness of the spines were not different between KO and WT, the muscle should not have been different between the two genotypes. This was confirmed by our findings, and verified the third and final hypothesis.

An unexpected finding of this study is that the muscle at 2 months of age was stiffer than the muscle at 8 months of age, in both the WT and KO mice. This may be due to differences in the intra or extracellular structure of the muscle at these two ages, as mice are not considered skeletally mature at 2 months of age (Fluerky et al., 2007). Meyer and Lieber (2012) also showed greater muscle fibre passive stiffness in younger (7-9 weeks) compared with older (12-14 months) WT mice. These authors also observed a concurrent decrease in desmin expression in the older mice, leading them to suggest that the decrease in fibre passive stiffness may be due to the fibre cytoskeleton becoming compromised in middle to old age (Meyer & Lieber, 2012). However, this would seem less likely in the present study, as an 8 month old mouse is somewhere between a mature (3-6 months) and middle aged (10-14 months) adult, and therefore cannot be considered very old. Additionally, Meyer and Lieber (2012) did not see this age effect.
on bundle passive stiffness in the WT mice, where in the current study this was observed. The
only characteristic quantified in this thesis that could explain this difference is smaller size of the
muscle fibres at 2 months of age. The average muscle fibre diameters in WT mice at 2 months of
age vs. 8 months of age were: 0.063 ± 0.0019 mm compared to 0.067 ± 0.0021 mm in TA
(p=0.0825), 0.068 ± 0.0024 mm compared to 0.077 ± 0.0022 in ES (p=0.0058), and 0.060 ± 
0.0019 mm compared to 0.068 ± 0.0028 mm in M (p=0.0056) (T-test: 2 sample, equal variance,
one-tailed). However, normalizing the measured force to CSA (to obtain stress) should have
accounted for size differences.

It is thought that the sarcolemma does not contribute to the passive stiffness of the muscle
fibre when tested using the methodology described. This is because the glycerol utilized in the
storage solution permeabilizes the cell membrane, and is therefore thought to eliminate the
ability of sarcolemma and its associated proteins to bear load. This would suggest that changes in
the sarcolemma and to the surrounding connective tissue should not affect the passive stiffness of
the fibres. However, Fridén and Lieber (2003) made a largely overlooked statement about this
type of muscle preparation, stating that ECM proteins are still present. The differences seen in
this thesis suggest that perhaps a structure located on the sarcolemma of the muscle fibre was in
fact able to contribute to passive stiffness. Assuming a cylindrical shape, a decrease in cell
volume by 25% of the original volume with a constant length would result in a smaller
percentage decrease in lateral surface area (~11%). Therefore, if a structure on the periphery of
the muscle cell was able to bear load, its contribution to the overall stiffness of a smaller fibre
would not be reduced as much as the structures on the inside of the cell. This would result in a
larger stress than anticipated. However, this is purely speculation at this point in time, and there
are a number of characteristics that were not able to be quantified in the present study that could potentially explain this difference in passive stiffness between the 2 and 8 month old mice.

Slack sarcomere length of the muscle samples was also quantified, as changes in slack sarcomere length along with changes in passive muscle stiffness can give some insight as to the way in which the muscle is remodeled (Fridén & Lieber, 2003). The only samples which yielded a difference in slack sarcomere length between genotypes were that of the TA and M bundles at 8 months of age. However, these samples showed no differences in passive stiffness. Additionally, the TA bundles had a shorter slack sarcomere length from WT to KO, while the M bundles showed the opposite. These changes are somewhat conflicting. The single fibres from these same muscles did not demonstrate differences in slack sarcomere length between genotypes, so it is likely that the changes could be due to the way in which slack sarcomere length is measured: as the length at which a resistance stretch can first be detected in the sample. In a bundle, this occurs at the point when the fibre with the shortest resting length is engaged. It is very common for fibres within a bundle to be at different resting lengths at slack sarcomere length because of the way that the bundles are secured to the apparatus. However, as the bundle is stretched, the lengths become more similar. This can also be seen in the laser diffraction pattern where bundles often have wider and less clear peaks at slack length from which the user must choose. These two factors can introduce an amount of subjectivity and variability into the slack sarcomere length measurements of the bundles, and as a result the changes seen in this thesis are likely not representative of any structural changes within the muscle.

Body mass of the mice was quantified because ENT1 KO mice are known to have lower body mass than WT mice, which is largely due to a decrease in the percentage of body fat (Choi et al., 2004; Hinton et al., 2014). Body mass can impact the characteristics of the spine neutral
zone, as Elliott and Sarver (2004) found that the length of the NZ was moderately positively correlated \((R^2=0.33)\) with body weight in the mouse lumbar spine. This could be a contributing factor to the decrease in length of the NZ in ENT1 KO mice, as they had significantly lesser body mass than their WT counterparts. However, it is important to note that Elliot and Sarver (2004) tested single lumbar spine motion segments, had removed the transverse and posterior elements of the spine, and tested only cyclic compression, with no inclusion of tension. Additionally, an \(R^2\) of 0.33 is not very large, and together with the clear evidence of calcification of the spine, the primary reason for the large NZ changes seen in the present study is likely due to this calcification, and not to differences in body weight.

The increased spine stiffness in conjunction with decreased muscle passive stiffness found in this thesis, along with the increased muscle passive stiffness as a result of a potential decrease in spine stiffness/stability seen in the work by Brown et al. (2011) identifies an inverse, potentially compensatory relationship between the spine and its surrounding musculature. It is therefore possible that structural changes of the spine stimulate a remodeling response in the muscle, in order to maintain a set level of stiffness in the overall spinal system. This falls in line with Panjabi’s statements from 1992, that state that the spinal stabilizing system adjusts to keep the NZ within certain physiological thresholds as a means to avoid clinical instability. In the case of an over-stabilized spine, it would seem as if this spinal system decreases the passive stiffness of muscles, potentially to reduce the amount of force that the spine experiences, as it is already subjected to larger intrinsic forces. The decrease in passive muscle force at the fibre level could also impact active force generation, as many seemingly passive structures within the fibre are thought to affect the function of these active components (Gautel & Djinović-Carugo, 2016; Horowits et al., 1986).
5.2 Limitations:

A limitation present in the current study involves the methodology of measuring elastic modulus of muscle samples. While normalizing the force measurements to CSA allows for comparison between different sizes of fibres and bundles, the way in which CSA was measured can result in some error based on the assumption of a cylindrical shaped fibre. By using the measured diameter of the muscle sample to calculate CSA, the actual area of the sample can be misrepresented by a mean deviation of 21% (Blinks, 1965). However, taking the average of 3 diameter measurements along the length of the sample is a way in which we attempted to mitigate this. Ideally, an elliptical shape should be assumed (which results in a mean deviation of 4% (Blinks, 1965; de Bruin et al., 2014)); however this requires diameter measurements in two planes, which were not able to be taken due to equipment limitations in this study. However, many other published findings have used the same methodology as was used in the present study.

Another limitation of equipment presented itself during mechanical testing of the lumbar spines. We were unable to accurately quantify the higher stiffness linear region of compressive or tensile stiffness during axial loading. These measures would have added further insight as to how the calcification of the structures in the lumbar spine would affect the mechanical behavior of the spine in axial loading, as joint connective tissues (IVDs and facet joint surfaces) are thought to resist mainly compression (Cannella et al., 2008; Johannessen et al., 2006), while ligaments (including facet joint capsules) are known to resist tension. These additional stiffness measures could have also provided insight into how these structures could be damaged or broken during axial loading, providing further clinical relevance.

While spines were tested in axial compression and tension, it is not known where along the axial loading curve that a mouse spine normally acts, and therefore it is unknown if the spine
regularly transitions through compressive and tensile loading; i.e. loading through the axial NZ. However, the soft tissue structures in the spine undergo compression and tension as a result of bending and torsion (Johannessen et al., 2006). For example, during flexion of the spine, the anterior of the IVD undergoes compression while the posterior of the disc undergoes tension. As the spine is extended, the anterior of the disc moves into tension while posterior is compressed. If the spine is stiffer moving through pure axial compression to tension, it would follow that during a bending movement, when the soft tissues in the spine are undergoing varying levels of compression and tension, there would also be an increase in stiffness during this movement. So, while movement of the spine is generally discussed in terms of bending or twisting in the three anatomical planes, examining the mechanical properties of the axial loading neutral zone adds relevance, as the NZ exists within every movement plane of the spine. There is also direct evidence that suggests the NZ in axial loading is related to the NZ in these three bending dimensions (Cannella et al., 2008).

Due to the small physical size of the mouse and its musculature, fibres and bundles of fibres used for mechanical testing were unable to be isolated to a specific IV level, and therefore had to be taken from throughout the lumbar region. Additionally, it was not possible to image the specific spines that were mechanically tested to determine which levels were affected by the ectopic mineralization prior to testing the muscle. However, testing a muscle sample adjacent to the affected level was likely not essential, as the work done by Brown et al. (2011) showed that while the muscle directly adjacent to an injured IVD increased in stiffness, the muscle over 2 levels inferior to the injury site also underwent the same changes.
5.3 Future Directions

Future work to build upon this research could examine the change in the mechanical stiffness of the ENT1 KO spine in both bending and torsion, including the elastic linear portion of the force-deformation loading curves, as well as the neutral zone. Quantifying these measures would provide a complete picture of how the structural changes in the ENT1 KO spine impact the mechanical behaviour, as well as complex, three-dimensional movement of the spine.

While the current study examined the relationship between spine stiffness and muscle stiffness in a specific mouse model, there are some opportunities to expand this work into human study. The spinal calcification present in the ENT1 KO mice has been described as mimicking the human disease known as DISH (Warraich et al., 2013), which also involves calcification of the paraspinal ligaments and IVDs. DISH is also very common in the canine Boxer breed, with an incidence of 41% (Kranenburg et al., 2010). This would provide another animal model to use to study this pathology. Additionally, there are other human diseases with similarities to DISH such as ossification of the posterior longitudinal ligament (OPLL), ankylosing spondylitis (AS), and even spinal osteoarthritis (OA). All of these diseases involve abnormal bone formation in the spine and could result in a similar increase in muscle passive stiffness as was seen in the ENT1 KO mice. This could potentially be quantified in a similar manner through cadaveric studies. The muscle passive stiffness properties in patients with these diseases could also be quantified through muscle biopsies or removal of muscle tissue during spinal surgery. Although the former is very invasive, and the latter would prove difficult to validate against a control, as individuals with healthy spines would not undergo spinal surgery, unless being operated on as a result of a traumatic injury. Additionally, some of these diseases are known to have an inflammatory origin (Slobodin et al., 2013), and it is unknown if inflammatory responses in the spine would affect the
surrounding musculature. The ossification seen in the ENT1 KO mice is not thought to be due to an inflammatory response (Warraich et al., 2013). Another scenario resulting in a stiffened spine occurs in spinal fixation. Muscle biopsies could be taken during revision surgeries of patients who have undergone spinal fixation surgery to see if an increase in the stiffness of the spinal level due to fixation could result in a change in the muscle passive stiffness.

The main focus of future research should be directed at determining the cellular structures responsible for the observed decrease in muscle passive stiffness in muscle fibres, as well as the mechanism(s) that mediate(s) these changes. As mentioned previously, in the case of experimentally induced disc degeneration, the changes in fibre bundle passive stiffness were partly attributed to changes in the connective tissue matrix, but also to changes in the individual muscle fibres (Brown et al., 2011). The authors of that study were unable to provide, or speculate upon, the cause of the increased fibre passive stiffness. They reported no change in the distribution of myosin heavy chain (MHC), which is an indicator of muscle fibre type (Brown et al., 2011). Furthermore, while the authors reported an increased size of titin, this would have had the opposite effect on passive stiffness compared to what was observed (e.g. smaller titin isoforms are known to be stiffer than larger ones.) (Brown et al., 2011). Therefore, further work must be conducted to determine the mechanism(s) of change, which may include revisiting the notion that a permeabilized sarcolemma does not contribute to the load bearing properties of the muscle fibre. A related area of future research would be to examine other architectural changes in muscle that occur in scenarios with changed passive stiffness. For example, architectural measures such as PCSA or sarcomere number in series. This could be done in the ENT1 KO model and the disc degeneration model, as well as others.
5.4 Conclusion

KO of ENT1 in a mouse results in ectopic calcification of the paraspinal ligaments, IVDs, and facet joints of the spine. These changes result in a spine that is more resistant to movement (stiffer), and as a result does not experience a true neutral zone, where movement is met with minimal resistance. These stiffness increases in the spine are seen in conjunction with inverse, potentially compensatory decreases in passive stiffness of the surrounding muscle. This relationship suggests that changes in structural properties of the spine stimulate a remodeling response in the muscle in order to maintain a set level of stiffness in the overall spinal system. Paraspinal muscles, erector spinae and multifidus, experienced a decrease in the passive stiffness of single fibres, but not of bundles of fibres, suggesting that the connective tissue matrix is not significantly impacted, but rather these changes are occurring at the level of the muscle fibre. Future work will need to be conducted to identify the cellular mechanisms and structures responsible for these changes in muscle fibre passive stiffness.
Chapter 6: References


