Role of cIAP1 and cIAP2 in Skeletal Muscle

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A thesis submitted in partial fulfillment of the requirements for the
Master's degree in Cellular and Molecular Medicine

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ABSTRACT

The cellular inhibitor of apoptosis 1 and 2 (cIAP1 and cIAP2) proteins are essential regulators of the classical and alternative NF-κB pathways. The NF-κB pathway has been shown to be an important regulator of myogenesis and plays a role in skeletal muscle disease, but the involvement of cIAP1 and cIAP2 has not been examined in healthy skeletal muscle. I sought to characterize skeletal muscle of the cIAP1-null and cIAP2-null mice. We show mice lacking cIAP1 exhibit decreased satellite cell numbers in the TA following cardiotoxin-induced injury and in the uninjured soleus muscle, suggesting cIAP1 may be important for satellite cell expansion. cIAP2 may play a role in fiber maintenance and homeostasis as we show cross-sectional are of cIAP2-null uninjured tibialis anterior fibers at 7 and 10 weeks of age were significantly smaller than wild-type fibers. Furthermore, cIAP1- and cIAP2-null mice subjected to *in situ* force experiments demonstrated altered twitch kinetics compared to wild-type controls in the soleus and EDL, suggesting fast and slow-twitch fibers are affected differently by loss of cIAP1 and cIAP2. Further work elucidating the downstream mechanisms by which cIAP1 and cIAP2 regulate skeletal muscle development and regeneration will be beneficial to the development of treatments for muscular disorders. In this regard, Smac mimetic compounds (SMCs) are small molecule inhibitors that target cIAP1/2 for degradation, thus provide a potential therapeutic treatment for muscular disorders.

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ATP	Adenosine triphosphate
$BaCl_2$	Barium Chloride
BAFFR	B-cell Activating Factor Receptor
BIR	Baculovirus IAP repeat
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium
CD40	Cluster of Differentiation 40
cKO	Conditional Knockout
CSA	Cross-sectional Area
CTX	Cardiotoxin
DIA	Denervation Induced Atrophy
DMD	Duchenne's Muscular Dystrophy
DPI	Days Post-Injury
EDL	Extensor Digitorum Longus
ER	Endoplasmic Reticulum
Fn14	Fibroblast Growth Factor-Inducible 14
F50	Frequency 50
IκB	Inhibitors of κB
IKKα	Inhibitor of IκB kinase-α
IKKβ	Inhibitor of IκB kinase-β
IAP	Inhibitor of Apoptosis
I.P.	Intraperitoneal
LPS	Lipopolysaccharide
LTβR	Lymphotoxin β Receptor
MHC	Myosin Heavy Chain
MRFs	Myogenic Regulatory Factors
MRF4	Myogenic Regulatory Factor 4
MTCO1	Mitochondrial Marker Gene Cytochrome Oxidase 1
Myf5	Myogenic Factor 5
MyoD	Myoblast Determination Protein
NEMO	NF-κB Essential Modulator
NF-κB	Nuclear Factor-κB
NIK	NF-κB Inducing Kinase
OCT	Optimal Cutting Temperature Compound
Pax7	Paired box 7
PBS	Phosphate Buffered Saline
PGC1α	Peroxisome Proliferator-Activated Receptor-gamma Coactivator 1α
RANK	Receptor Activator of Nuclear Factor κB
RFD	Rate of Force Development

RHD	Rel Homology Domain
RING	Really Interesting New Gene
RIP1	Receptor Interacting Kinase 1
RyR	Ryanodine Receptor
SDH	Succinate Dehydrogenase
SERCA	Sarcoendoplasmic Reticulum Ca^{2+} ATPase
SMAC	Second Mitochondrial-Derived Activator of Caspases
SMCs	Smac Mimetic Compounds
Sol	Soleus
SR	Sarcoplasmic Reticulum
TA	Tibialis Anterior
TAM	Tamoxifen
TNFα	Tumour Necrosis Factor-α
TNFR	Tumour Necrosis Factor Receptor
TRAF	TNF Receptor-Associated Factor 2
TWEAK	TNF-Like Weak Inducer of Apoptosis
UI	Uninjured
WT	Wild-Type

ACKNOWLEDGEMENTS

First and foremost, I'd like to thank my supervisors, Dr. Robert Korneluk and Dr. Shawn Beug, for accepting me into their lab. I am appreciative of your friendly supervision and for providing me with the guidance I needed to succeed in the Master's program.

The most sincere thank you goes to Dr. Neena Lala-Tabbert, for taking me under her wing and teaching me everything I know. You were an amazing mentor and I am so lucky to have had the opportunity to work with you over the last two years. You have helped me in so many ways, and for that I am so thankful. I don't think I can ever express enough gratitude towards everything you have done for me.

Thanks goes to my thesis advisory committee members, Dr. Alex MacKenzie and Dr. Nadine Wiper-Bergeron for all of your help, support and advice.

I'd also like to thank my lab members for making my time in the lab so enjoyable. A special thanks to Nathalie Earl for all of your animal expertise and assistance with my work. Additionally, to Allan Humphrey – you deserve an award for answering my endless questions and listening to everything I always had to say.

I would like to thank my parents, Julie and David Whitney. You have always supported me in everything I do and helped me get to where I am today. I'd also like to thank my boyfriend, Bryce Norman, for supporting me through everything the last two years in science has put me through. You have been there through the ups and the downs, helping me get through it all. I could not have done it without you.

CHAPTER ONE: INTRODUCTION

1.1 Muscle Regeneration and Satellite Cells

Skeletal muscle is a dynamic tissue that is characterized by muscle fibers arranged into contractile units known as sarcomeres (Frontera & Ochala, 2015). Skeletal muscle plays a significant role in various bodily functions both mechanically and physiologically. Importantly, skeletal muscle functions to convert chemical energy into mechanical energy to allow for force generation and postural maintenance, as well as acting to maintain body temperature and homeostasis (Frontera & Ochala, 2015).

A defining feature of skeletal muscle is its impeccable ability to regenerate. This phenomenon can be attributed to resident muscle stem cells known as satellite cells, named for their location between the basal lamina and sarcolemma (Bischoff, 1975; Mauro, 1961). Under homeostatic conditions, satellite cells are quiescent and express the transcription factor Pax7 (Chakkalakal et al., 2012; Collins et al., 2005, Seale et al., 2000). In response to physical stimuli such as injury, satellite cells become activated and proliferate as myoblasts (Cooper et al., 1999; Hawke & Garry, 2005; Zammit et al., 2004). Myoblasts express the myogenic regulatory factors (MRFs) MyoD and Myf5 (Cooper et al., 1999; Grounds et al., 1992; Smith et al., 1994). Myoblasts will then downregulate Pax7, exit the cell cycle, upregulate the expression of myogenin and MRF4, and terminally differentiate to become myocytes (Chen & Goldhamer, 1999; Hasty et al., 1993; Zammit et al., 2004). To repair damaged muscle or form new multinucleated fibers, myocytes undergo cell-to-cell fusion (Robertson et al., 1990; Robertson et al., 1993). In addition to their ability to differentiate, satellite cells also have the capacity to self-renew to maintain the satellite cell population (Figure 1). Self-renewal occurs in select myoblasts that maintain Pax7 expression, downregulate MyoD expression and return to quiescence

(Zammit et al., 2004, 2006). Satellite cell self-renewal and repopulation of the satellite cell niche is essential to sustain multiple rounds of muscle regeneration.

Successful muscle regeneration is dependent on satellite cells, thus, in the absence of $Pax7^{+}$ cells, there is a profound failure of regeneration. Using $Pax7^{+/CE};R26R^{eGFP\text{-}DTA/lacZ}$ mice, Pax7-DTA mice, and $Pax7^{CreERT2}$ mice, ablation of satellite cells was achieved via tamoxifen administration (Lepper et al., 2011; Mccarthy et al., 2011; Murphy et al., 2011). Following cardiotoxin- or $BaCl_2$-induced muscle injury, little to no myotubes were formed 5-7 days post injury in Pax7 mutant mice (Lepper et al., 2011; Mccarthy et al., 2011; Murphy et al., 2011). Of note, muscle regeneration was shown to not be delayed, but rather prevented, upon ablation of Pax7-expressing cells (Murphy et al., 2011). Indeed, up to 56 days after injury, no visible muscle fibers were formed in the tibialis anterior of $Pax7^{iCreERT2/+}$: $R26R^{DTA/+}$ mice (Murphy et al., 2011). Furthermore, ablation of the satellite cell population in these transgenic mice prevented their repopulation. For example, no satellite cells were found in $Pax7^{CE/+}$: $Rosa^{eGFP\text{-}DTA/+}$ mice 6.5 days after the last dose of tamoxifen (Lepper et al., 2011). Furthermore, by the 5^{th} day of regeneration, there were very few satellite cells in $Pax7^{iCreERT2/+}$: $R26R^{DTA/+}$ mice treated with tamoxifen (Murphy et al., 2011). Thus, satellite cells are absolutely required for successful muscle regeneration.

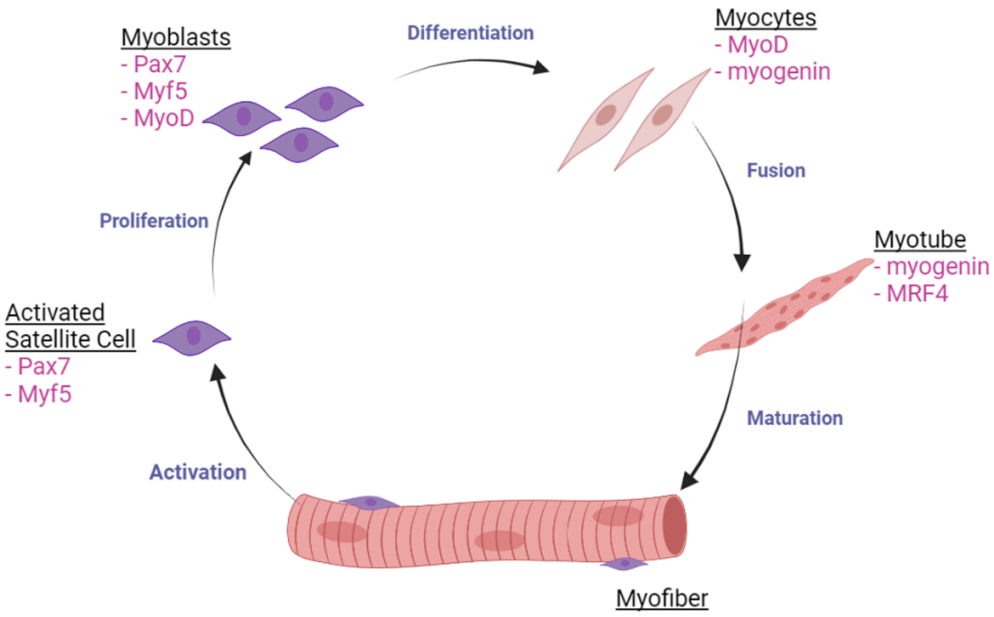

Figure 1. Satellite Cells During Adult Myogenesis. Quiescent satellite cells express Pax7. Upon muscle injury, satellite cells become activated and upregulate Myf5 expression. Satellite cells undergo proliferation to produce myoblasts expressing MyoD. Myoblasts will upregulate myogenin and differentiate into myocytes which in turn will fuse to form myogenin and MRF4 expressing myotubes. These myotubes will mature to form multinucleated myofibers. In addition to differentiation, satellite cells have the capacity to maintain the satellite cell population. Satellite cells will downregulate MyoD expression after activation and maintain Pax7 expression to undergo self-renewal.

1.1.1 Requirement of Pax7 for Satellite Cell Function

The Pax transcription factors play an important role in regulation of developmental processes. For example, Pax7 is important for muscle development and post-natal muscle repair (Kuang et al., 2006; Seale et al., 2000; Von Maltzahn et al., 2013). Mansouri et al. (1996) generated Pax7-null mice and found that while they appeared normal at birth, they failed to survive past 2-3 weeks of age. The cause of premature death is unknown, and those that survived to adulthood are unhealthy, displaying abnormalities in their appendix and small intestine (Mansouri et al., 1996). After birth, Pax7-null mice failed to maintain normal growth, and by post-natal day 10 they weighed less than half the weight of wild-type littermates (Kuang et al., 2006). Decreased skeletal muscle growth, decreased myofiber size and decreased number of myonuclei per fiber have been reported in Pax7-null mice (Kuang et al., 2006; Relaix et al., 2006; Seale et al., 2000). In contrast, one study reported that despite low satellite cell numbers, increased number of smaller myofibers, and reduced body weight in Pax7-null mice, post-natal growth appeared normal and unaffected (Oustanina et al., 2004).

Several studies have reported impaired muscle regeneration following cardiotoxin injection in Pax7-null mice. Smaller number of regenerating fibers, rare centronucleation of fibers, and replacement of muscle fibers with adipose tissue, fibrotic tissue and calcium deposits have been reported in Pax7-null mice, suggesting a limited capacity for regeneration due to a loss of satellite cell proliferative capacity (Kuang et al., 2006; Von Maltzahn et al., 2013).

Using conditional knockout $Pax7^{CreERT2/flox}$ mice, several studies have examined the role of Pax7 in adult satellite cells (Lepper et al., 2009; Von Maltzahn et al., 2013). When Pax7 is specifically excised in the $Pax7^{CreERT2/flox}$ mice through tamoxifen administration, Lepper et al. (2009) determined Pax7 function is not required for myogenesis following injury in adulthood,

and thus, Pax7 is dispensable in adult regenerative myogenesis. However, these results contrast with those obtained using Pax7-null mice (Kuang et al., 2006; Relaix et al., 2006). When the same $Pax7^{CreERT2/flox}$ mice used in the study done by Lepper et al. (2009) were put on a tamoxifen diet in addition to tamoxifen I.P. injections, satellite cells and myoblasts exhibited dysregulation of satellite cell expansion and differentiation leading to severely impaired muscle regeneration (Von Maltzahn et al., 2013). These findings confirm that Pax7 is required for the normal function of satellite cells during post-natal muscle repair.

1.2 Fiber Type Heterogeneity

In adult muscle, fibers can be classified into different fiber types based on four properties: (1) the speed of contraction, (2) fatigue characteristics, (3) metabolic characteristics, and (4) morphological characteristics. Based on the speed of contraction, fibers are termed either fast (type II) or slow (type I), where the myosin heavy chain isoform determines the ATPase activity. Fibers are either fast-fatigable, fast-resistant, or slow oxidative based on their fatigue characteristics. Metabolic characteristics divide the fibers into either oxidative or glycolytic, with further sub-categories classified as fast-glycolytic, fast-oxidative-glycolytic, or slow-oxidative. The metabolic characteristics can be identified through succinate dehydrogenase (SDH) content, with increased SDH content as an indicator of high oxidative capacity. Finally, fibers can be classified based on their size, capillary density, and myoglobin and organelle content. Within a single muscle group, there is often a mix of fiber-types, however, certain muscles can be quite concentrated or have varying proportions of a single fiber-type (Johnson et al., 1973; Schiaffino & Reggiani, 2011; Tajsharghi, 2008). The proportions of fiber-types may change in response to hormonal influences, varying nerve-activity, and disease states altering how that muscle is used

(Schiaffino & Reggiani, 2011; Tajsharghi, 2008; Talbot & Mavez, 2016). The ability of the phenotype of fibers to change has been termed plasticity, or fiber malleability.

The diversity of different fiber types allows muscles to perform specific functional tasks. For example, type 1 oxidative fibers are more suited for endurance exercise whereas type II glycolytic fibers are better suited for short term resistance exercises (Schiaffino & Reggiani, 2011; Talbot & Mavez, 2016). Muscle fiber type diversity can also affect the susceptibility of different muscles to disease. For example, Duchenne Muscular Dystrophy (DMD) preferentially affects type II fibers, causing them to be the first fibers to degenerate. Conversely, Type I Myotonic Dystrophy causes atrophy and degeneration of type I fibers (Vihola et al., 2003). Other muscle wasting disorders that are not genetically inherited also preferentially affect fiber-types. For example, metabolic disorders such as diabetes and obesity cause a shift in the proportion of type I fibers, resulting in an increased presence of type II fibers (Oberbach et al., 2006; Tanner et al., 2002). Similarly, physical inactivity and muscle disuse as a result of conditions such as bed rest, spinal cord injury, and limb immobilization cause type I fiber atrophy, and a fiber-type shift from type I to type II (Burnham et al., 1997; Gallagher et al., 2005; Trappe et al., 2007). Meanwhile, age-related muscle loss, known as sarcopenia, results in type II fibers with a greater susceptibility to atrophy, while type I fibers appear unaffected (Nilwik et al., 2013). Although it is well established that different fiber-types are preferentially affected by different disease states, the mechanism behind why a particular fiber-type is affected remains largely unknown. Thus, continuing research into the fiber-type specific effects of muscle and metabolic disorders will help to provide new therapeutic routes for muscular disorders.

1.3 NF-κB Pathway

Nuclear factor binding near the κ light-chain gene in B cells (NF-κB) transcription factors are widely expressed and act as master regulators of immune and inflammatory processes in response to both injury and infection. The NF- κB family members include RelA/p65, RelB, c-Rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2) which are characterized by a Rel homology domain (RHD) containing a highly conserved 300 amino acid N-terminal domain which functions to mediate subunit dimerization and interaction with inhibitory proteins (Mourkioti & Rosenthal, 2008). Under normal conditions, NF-κB heterodimers remain sequestered in the cytoplasm, bound by inhibitors of κB (IκB) proteins. However, activation of the NF-κB pathway occurs in response to various stimuli including bacterial products, inflammatory cytokines, mitogens, as well as oxidative stress (Mourkioti & Rosenthal, 2008; Peterson & Guttridge, 2008). Various downstream signaling pathways may be initiated depending on the stimuli presented, however, many of these pathways will congregate on the inhibitor of κB kinase (IKK) complex which is composed of two catalytic subunits (IKKα and IKKβ), as well as a regulatory subunit (IKKγ/NEMO) (Li et al., 2008). The NF-κB pathway consists of two different arms: the classical and alternative pathway (Li et al., 2008).

Activation of the classical pathway requires IKKβ and NEMO, whereas IKKα is involved in activation of the alternative pathway (Peterson & Guttridge, 2008). Activation of the classical pathway occurs upon inflammatory stimuli including TNFα binding to TNF receptors. Upon activation, IκB is phosphorylated by an IKK complex consisting of IKKβ, IKKα and NEMO. IκB phosphorylation leads to its polyubiquitination and subsequent proteasomal degradation thus allowing the translocation of p50/p65 to the nucleus (Peterson & Guttridge, 2008). In contrast, the alternative NF-κB pathway is activated by a specific group of stimuli, including a subset of TNFR superfamily members such as B-cell activating factor receptor (BAFFR), Lymphotoxin β

receptor (LTβR), Receptor activator of nuclear factor κ B (RANK) and cluster of differentiation 40 (CD40) (Mourkioti & Rosenthal, 2008). These stimuli result in accumulation of NF-κB inducing kinase (NIK), which activates and cooperates with IKKα to mediate p100 phosphorylation. Phosphorylation of p100 leads to its partial degradation resulting in the generation of p52 which translocated to the nucleus with RelB (Bakkar & Guttridge, 2010; Li et al., 2008; Mourkioti & Rosenthal, 2008).

1.3.1 NF-κB in Muscle

NF-κB has been shown to be an important regulator of skeletal muscle regeneration and diseases. During muscle regeneration, the classical NF-κB pathway is important for promoting proliferation while simultaneously inhibiting differentiation (Bakkar et al., 2008), while the alternative pathway is involved in fusion (Enwere et al., 2013), myotube maintenance and mitochondrial biogenesis (Bakkar et al., 2008).

The classical pathway has been shown to function as a negative regulator of myogenesis, specifically through the transcriptional activity of p65 (Bakkar et al., 2008; Guttridge et al., 2000; Guttridge et al., 1999; Wang et al., 2007). A temporal switch occurs whereby a decline in the classical pathway coincides with the upregulation and activation of the alternative pathway (Bakkar et al., 2008). While the classical pathway has been extensively studied in relation to myogenesis, the function of the alternative pathway is less understood. The alternative pathway has been shown to play an important role in regulation of myoblast fusion, such that when key components (IKKα, RelB) of the alternative pathway were knocked down via siRNA, the diameter of myoblasts and number of hypernucleated myotubes were reduced (Enwere et al., 2013). In addition, the percentage of hypernucleated myotubes and average myotube diameter

increased upon overexpression of p52 and RelB (Enwere et al., 2013). Furthermore, Bakkar et al. (2008) demonstrated a resistance to damage induced by metabolic stress upon activation of the NF-κB pathway, resulting in myotube maintenance and homeostasis. Overexpression of key components of the alternative pathway resulted in myotubes displaying increased levels of the mitochondrial marker gene cytochrome oxidase 1 (MTCO1), enriched in cytochrome *c*, increased activity of citrate synthase and dehydrogenase enzymes, and had elongated mitochondria, a sign of extensive mitochondrial proliferation, thus supporting the notion that the alternative NF-κB pathway is crucial for mitochondrial biogenesis (Bakkar et al., 2008).

Under pathological conditions, constitutive activation of NF-κB signalling has been reported in various muscle pathologies including atrophy (Cai et al., 2004; Hunter and Kandarian., 2004; Mourkioti et al., 2006), cachexia (He et al, 2013; Cai et al, 2004), rhabdomyosarcomas (Wang et al., 2008) and muscular dystrophies (Acharyya et al., 2007; Carlson et al., 2005; Messina et al., 2006). For example, high levels of NF-κB activity was found in *mdx* mice, thus leading to the accumulation of pro-inflammatory cytokines such as TNFα and IL-1β (Acharyya et al., 2007; Kumar & Boriek, 2003). In addition, NF-κB activity has been shown to be increased in skeletal muscle as well as infiltrating cells, such as macrophages in *mdx* mice (Acharyya et al., 2007; Li et al., 2008). Indeed, inhibition of components of the classical pathway reduced the severity of the disease while improving muscle regeneration in *mdx* mice (Acharyya et al., 2007; Lu et al., 2012; Tang et al., 2010; Yang et al., 2012). Furthermore, elevated NF-κB activity was demonstrated in cancer cachexia which was likely to be attributed to the tumor-derived circulating factors (Cai et al., 2004). Taken together, these findings point to chronic classical NF-κB pathway activation as a molecular mechanism underlying muscle

atrophy. Thus, classical, and alternative NF-κB signalling is a critical and complex regulator of skeletal muscle biology and disease.

1.4 Cellular Inhibitor of Apoptosis 1 and 2 (cIAP1, cIAP2)

The Inhibitor of Apoptosis (IAP) proteins are a family of proteins that are involved in apoptotic control, mitosis, and the regulation of numerous signal transduction pathways (Liston et al., 1996; Mahoney et al., 2008; Samuel et al., 2006; Zarnegar et al., 2008). The IAPs are characterized by the presence of at least one baculovirus IAP repeat (BIR) domain located near their N termini (Samuel et al., 2008). The best studied IAPs include cellular IAP1 and cellular IAP2 (cIAP1, cIAP2). These IAPs are characterized by the presence of three BIR domains (Liston et al., 1996) and a C-terminal really interesting new gene (RING) zinc finger which acts as an E3 ubiquitin ligase (Figure 2) (Yang et al., 2000).

Importantly, it has been discovered that cIAP1 and cIAP2 act as positive and negative regulators of the classical and alternative arms of the NF-κB pathway, respectively (Bakkar et al., 2008; Enwere et al., 2012; Enwere et al., 2013; Mahoney et al., 2008; Zarnegar et al., 2008). As stated previously, the cIAP proteins act as E3 ubiquitin ligases, and target key adaptor proteins for degradation (Mahoney et al., 2008; Zarnegar et al., 2008). Upon activation of the classical NF-κB pathway, cIAP1 and cIAP2, recruited by TNF receptor-associated factor 2 (TRAF2) functions to assemble a complex required for signal transduction by catalyzing the K63 ubiquitination of receptor interacting kinase 1 (RIP1), leading to NF-κB activation (Mahoney et al., 2008; Zarnegar et al., 2008). In the presence of cIAP1/2, ubiquitinated RIP1 acts as an adaptor molecule; however, in the absence of cIAP1/2, unubiquitinated RIP1 triggers apoptosis through activation of caspase-8 (Mahoney et al., 2008).

In contrast, cIAP1 and cIAP2 act as negative regulators of the alternative NF-κB pathway. In the presence of cIAP1 and cIAP2, NIK is targeted for proteosomal degradation as the adaptor proteins (TRAF2 and TRAF3) along with cIAP1/2 form a complex to ubiquitinate NIK (Zarnegar et al., 2008). Thus, the presence of cIAP1/2 inhibits the degradation of p100 into its functional p52 subunit and prevents p52:RelB translocation to the nucleus (Figure 3) (Enwere et al., 2013).

To determine the role of cIAP1 in a physiologic setting, mice lacking cIAP1 were generated by the Ashwell lab (Conze et al., 2005). cIAP1$^{-/-}$ mice showed no overt phenotype, with no physical or behavioural abnormalities and had a normal lifespan (Conze et al., 2005). Despite no differences in distribution of thymocyte and peripheral lymphocyte populations found, cIAP1$^{-/-}$ mice continued to show a reduction in the number of lymphocytes (Conze et al., 2005). As cIAP1 and cIAP2 are homologous, changes in one IAP protein have the potential to affect the expression of other IAP proteins. Indeed, loss of cIAP1 resulted in higher expression levels of cIAP2 in splenocytes, thymocytes and murine embryonic fibroblasts (Conze et al., 2005). Interestingly, little cIAP2 expression was found in the tibialis anterior and soleus muscle of cIAP1$^{-/-}$ mice, and no expression was found in the brain (Mahoney et al., 2008). In addition, mice with whole body deletion of cIAP2 are asymptomatic, showing no physical or behavioural differences (Conte et al., 2006). However, cIAP2$^{-/-}$ mice show resistance to LPS-induced endotoxic shock, demonstrate an attenuated inflammatory response, and their macrophages are sensitive to apoptosis (Conte et al., 2006; Mahoney et al., 2008).

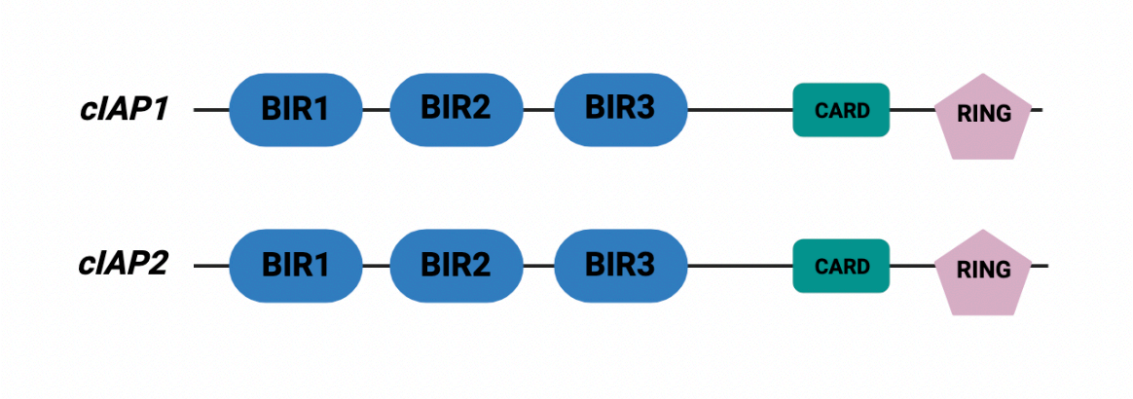

Figure 2. Domain structure of the inhibitor of apoptosis (IAP) family, specifically cIAP1 and cIAP2. cIAP1 and cIAP2 are both characterized by the presence of three BIR domains, a CARD domain located in the linker region, as well as the RING zinc finger domain, located at the carboxy terminus.

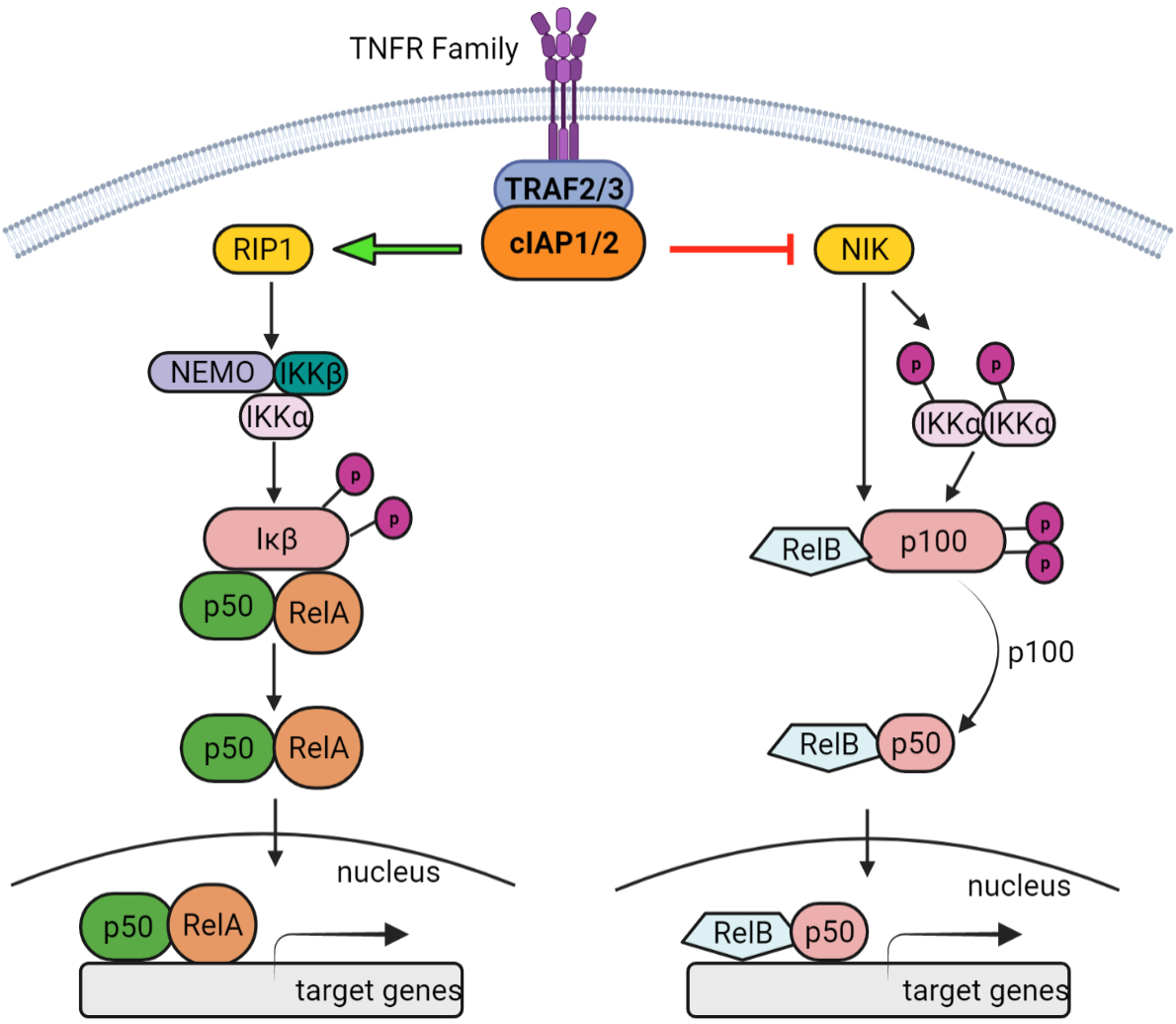

Figure 3. Schematic of the classical and alternative NF-κB pathways. Binding of ligands such as TNFα results in recruitment of several proteins in a receptor signalling complex. In the classical pathway (left), cIAP1/2 interact with RIP1, which will serve as an adaptor for the binding of the IKK complex. In turn, the IKK complex phosphorylates IκB, targeting it for degradation, thus releasing the p50:RelA dimer for nuclear translocation. In the alternative pathway (right), cIAP1/2 interact with NIK, causing its degradation, resulting in impaired processing of p100 into p52.

1.4.1 cIAP1 and SMAC-mimetic compounds in skeletal muscle regeneration and disease.

Given the central roles of cIAPs in regulating NF-κB signaling, the Korneluk lab has investigated the role of cIAP1 in skeletal muscle biology. In relation to skeletal muscle, loss of cIAP1 resulted in increased myoblast fusion through activation of the alternative NF-κB pathway in vitro and improved muscle fiber formation *in vivo* after cardiotoxin induced muscle regeneration (Enwere et al., 2012). Since classical NF-κB signaling has been shown to play a role in Duchenne's Muscular Dystrophy (Acharyya et al., 2007; Acharyya et al., 2010; Messina et al., 2006), the Korneluk lab also examined the role of cIAP1 in the pathology of skeletal muscle in mdx mice. Using mutant whole body *cIAP1*$^{-/-}$*;mdx* mice, Enwere et al. (2013) determined a reduction in centronucleation in the soleus muscle of double mutant mice, however, the EDL showed no difference compared *to cIAP1*$^{+/+}$*;mdx* mice. In addition, loss of cIAP1 resulted in reduced muscle damage through a reduction in necrotic tissue and immune cell infiltration in the solei of double mutant mice (Enwere et al., 2013). As *mdx* mice have previously been shown to possess contractile deficits, the effect loss of cIAP1 has on contractile properties in *mdx* was investigated. As a result, the force frequency curve produced by the soleus of *cIAP1*$^{-/-}$*;mdx* mice was indistinguishable from the curve produced by wild-type mice (Enwere et al., 2013). These results suggest loss of cIAP1 improved muscle histology and function of solei of mdx mice (Enwere et al., 2013). Furthermore, a study published in 2019 examined the function of cIAP1 in skeletal muscle atrophy and demonstrated cIAP1 acts as a positive regulator of the classical NF-κB pathway (Lala-Tabbert et al., 2019). Using a model of denervation-induced atrophy, results showed that cIAP1 was upregulated in denervated muscle (Lala-Tabbert et al., 2019). Interestingly, increased cIAP1 levels in atrophying muscle led to increased phosphorylation of p65 (Lala-Tabbert et al., 2019). In addition, denervation-induced muscle

atrophy was attenuated in cIAP1-null mice suggesting that cIAP1 is an important mediator of NF-κB signaling in skeletal muscle atrophy. These studies demonstrate that cIAP1 is a promising therapeutic target of muscle disorders (Enwere et al., 2013; Lala-Tabbert et al., 2019).

1.4.2 SMAC mimetic compounds

Second mitochondrial-derived activator of caspases (SMAC) is a pro-apoptotic protein that is released from the mitochondria in the presence of a proapoptotic stimulus (Du et al., 2000). Upon release from the mitochondria, SMAC antagonizes the IAPs, resulting in inhibition of the classical NF-kB pathway. The BIR3 domain is where SMAC will interact with cIAP1 and cIAP2, and this binding interaction prevents the caspase inhibition functions of cIAP1 and cIAP2 (Du et al., 2000). In this regard, Smac Mimetic Compounds, or SMCs, are potent cIAP1/2 small molecule inhibitors. SMCs cause auto-ubiquination of cIAP1 and cIAP2, resulting in their rapid proteasomal degradation and are currently undergoing clinical trials for the treatment of cancer (Bertrand et al., 2008; Chang & Cheung, 2021). In skeletal muscle, SMCs have been shown to improve myofiber formation as myoblasts differentiated in SMC displayed an increase in myonuclear number and myotube size (Enwere et al., 2012). In addition, the use of SMCs has been shown to prevent myotube atrophy *in vitro* and attenuate denervation-induced atrophy *in vivo* (Lala-Tabbert et al., 2019). The use of SMCs to target cIAP1 and cIAP2 for proteasomal degradation is a novel and promising therapeutic approach to treat muscular disorders.

1.5 Rationale and Hypothesis

Since loss of cIAP1 has been shown to increase myoblast fusion *in vitro* and improve muscle function and structure in diseased muscle (DMD and DIA), I hypothesized that **cIAP1 is**

a negative regulator of muscle regeneration. Furthermore, since macrophages play a crucial role in muscle regeneration and cIAP2 is an important regulator of macrophage biology (Conte, 2005), I will concurrently examine the role of cIAP2 in muscle regenerating using the cIAP2 knockout mouse.

The ***specific aims*** of this study were to characterize skeletal muscle of **(1)** the cIAP1-null and **(2)** the cIAP2-null mice.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Mice and Animal Care

Mice were bred and handled in accordance with the guidelines established by the University of Ottawa Animal Care Veterinary Service and the Canadian Council on Animal Care. C57BL/6 mice (age 6 to 8 weeks) were obtained from Charles River Laboratories. The cIAP1-null and cIAP2-null were generated by the Ashwell and Korneluk lab, respectively (Conte et al., 2006; Conze et al., 2005). Mice were housed in a controlled facility at the University of Ottawa (22°C on a 12-h light/dark cycle) and had *ad libitum* access to food and water.

To generate cIAP1 conditional knockout (cKO) mice, $cIAP1^{loxP/loxP}$;$cIAP2^{FRT/FRT}$ mice (on a C57BL/6 background) (Gardam et al., 2011) were bred with the $Pax7CreER^{TM}$ mouse (Nishijo et al., 2009) to create a temporally-controlled conditional knockout of cIAP1 in $Pax7^{+}$ cells ($cIAP1^{-/-}$;$cIAP2^{FRT/FRT}$;$Pax7^{CreER+}$). cIAP1 cKO mice aged 6-8 weeks were given give intraperitoneal (i.p.) tamoxifen (TAM) injections (1.5mg/20g body weight) and maintained on TAM-containing standard chow to prevent recombination escaping satellite cells from taking over (1 mg TAM/day/20 g body weight).

2.2 Cardiotoxin Injury

For cardiotoxin (CTX) injury, the mice were anesthetized with isoflurane and the hindlimb was shaved before the procedure. C57BL/6, cIAP1-null and cIAP2-null mice were injected with 30ul of 10mM CTX into the left tibialis anterior (TA) muscle. The mice were sacrificed at various time points (7 and 28 days) following CTX injury and the left and right TA were collected, weighed, embedded in OCT and flash frozen in isopentane cooled liquid nitrogen. Muscle was sectioned into 8um thick cross-sections using a cryostat (Leica CM1860 UV).

2.3 Grip Strength

To determine grip strength, C57BL/6, cIAP1-null and cIAP2-null mice were subjected to five forelimb pulls, given a break, followed by another 5 forelimb pulls. They were subsequently pulled five times but allowed to grip using all limbs, given a break, then repeated five more all-limb pulls. The pulls were performed by the same experimenter at the same time of day each time.

2.4 *In situ* Force Measurements

To determine force-frequency relationship in soleus and EDL muscles in C57BL/6, cIAP1-null and cIAP2-null mice, mice were anesthetized with a single intraperitoneal injection of 65 mg ketamine/13 mg xylazine/2 mg acepromazine per kg body weight and sacrificed by cervical dislocation. The soleus and EDL were dissected out and tendons were tied with surgical silk, with one end attached to the force transducer (model no. 400A; Aurora Scientific Canada) and the other attached to a fixed metal pin. A physiological saline solution, made as previously described (Albadrani et al., 2021) immersed the muscles and was maintained at 37°C. Muscle length was adjusted to elicit maximum isometric tetanic force. The force frequency relationship was measured after a 30-minute equilibrium period. Beginning at 1V, the soleus and EDL were stimulated every 5 minutes and the frequency was increased by 10V each time until 140V, where it began to increase by 20V every 5 minutes to a maximum of 200V. At the end of each experiment, muscle weight was measured.

2.5 Immunofluorescence

For Pax7 and Laminin immunofluorescence, cryosections were processed for antigen retrieval in 10mM citrate buffer (pH 6.0) in an antigen retriever (Electron microscopy sciences). Following antigen retrieval, sections were rinsed briefly with phosphate buffered saline (PBS) containing 0.1% Triton X-100, followed by permeabilization with 0.5% Triton X-100 PBS for 10 minutes. The sections were blocked with 3% BSA in 0.1% Triton X-100 PBS for one hour at room temperature. Sections were further blocked with Donkey anti-mouse Fab fragment (Jackson ImmunoResearch) in blocking solution for one hour at room temperature. Sections were rinsed briefly three times with 0.1% PBS before incubating with primary antibodies: Pax7 (DSHB, 1:20) and Laminin (Abcam, 1:100) overnight at 4°C. The following day, sections were washed with 0.1% Triton X-100 PBS three times for five minutes before incubating with biotin anti-mouse IgG (Jackson ImmunoResearch, 1:200) for one hour at room temperature. Sections were washed with 0.1%Triton X-100 three times for five minutes and incubated in secondary antibodies (Cy3-streptavidin and goat anti-rabbit Alexa Flour 488 (Thermofischer, 1:500)) for one hour at room temperature. Sections were washed with 0.1%Triton X-100 three times for five minutes before counterstaining nuclei with DAPI for five minutes. Slides were mounted with fluorescent mounting medium (DAKO).

For myosin heavy chain staining, sections were incubated with blocking solution (PBS containing 10% goat serum) for 30 minutes at room temperature prior to primary antibody incubation in blocking solution for 1 hour at 37°C. Sections were washed with PBS three times for five minutes each and incubated with secondary antibodies for 30 minutes at 37°C. Sections were washed three times for five minutes with PBS prior to mounting with a fluorescent mounting medium. Primary antibodies used: BA-F8 (1:100), Sc-71 (1:100), BF-F3 (1:100), and

Laminin (1:100). Secondary antibodies used: Alexa Fluor 350, Alexa Fluor 594, Alexa Fluor 488, and Alexa Fluor 647 (all 1:250 from Invitrogen).

2.6 Image Acquisition and Analysis

Images were attained at room temperature using (Zeiss Axio Imager M2) and Zeiss Zen imaging software. Images were composed and edited in Paint.net. To determine the size of regenerating fibers, the minimum Feret diameter of a minimum of 200 fibers with centrally located nuclei was manually analysed using ImageJ. Pax7 and fiber-type distribution was also performed manually using ImageJ. Broken sections and samples with significant staining artifacts were excluded from analysis. For cross-sectional area of uninjured soleus and EDL, the minimum Feret diameter of fibers in the entire muscle cross-section was analyzed using openCSAM (Desgeorges et al., 2019) with manual correction as necessary.

CHAPTER THREE: RESULTS

3.1. Objective One: To characterize the muscle phenotype of cIAP1 knockout mice

Loss of cIAP1 decreases the number of satellite cells but has no effect on muscle fiber size following regeneration 7 days after injury

Previous reports demonstrated that loss of cIAP1 resulted in larger regenerated fibers in the gastrocnemius 7 days after cardiotoxin (CTX) injection (Enwere et al., 2012). Given this finding, I sought to validate and further characterize the role of cIAP1 in muscle regeneration. Contrary to previous findings, I found no change in muscle fiber size between wild-type and cIAP1-null TA muscle 7 days post-injury (dpi) (Figure 4A,C,D). When quantifying the number of $Pax7^+$ cells, I found that loss of cIAP1 led to significantly fewer satellite cells at 7dpi (Figure 4B), suggesting that cIAP1 may be required for satellite cell expansion after CTX-induced muscle regeneration. Interestingly, when I analyzed the contralateral uninjured TA, I found that cIAP1-null mice had significantly fewer small to mid-sized fibers (10-30μm) (Figure 4E).

Loss of cIAP1 results in larger muscle fibers 28 days following CTX-induced injury

Since satellite cell numbers were decreased in regenerating cIAP1-null muscle, I examined the muscle fiber cross-sectional area and satellite cell numbers at 28dpi, a timepoint that corresponds to complete muscle regeneration. In contrast to my findings at 7dpi, I found that at 28dpi regenerated fibers from cIAP1-null mice trended towards a larger average cross-sectional area compared to wild-type muscle (Figure 5A,C,D). Interestingly, at 28dpi there was no difference in satellite cell numbers in injured muscle of wild-type and cIAP1-null mice following injury (Figure 5B).

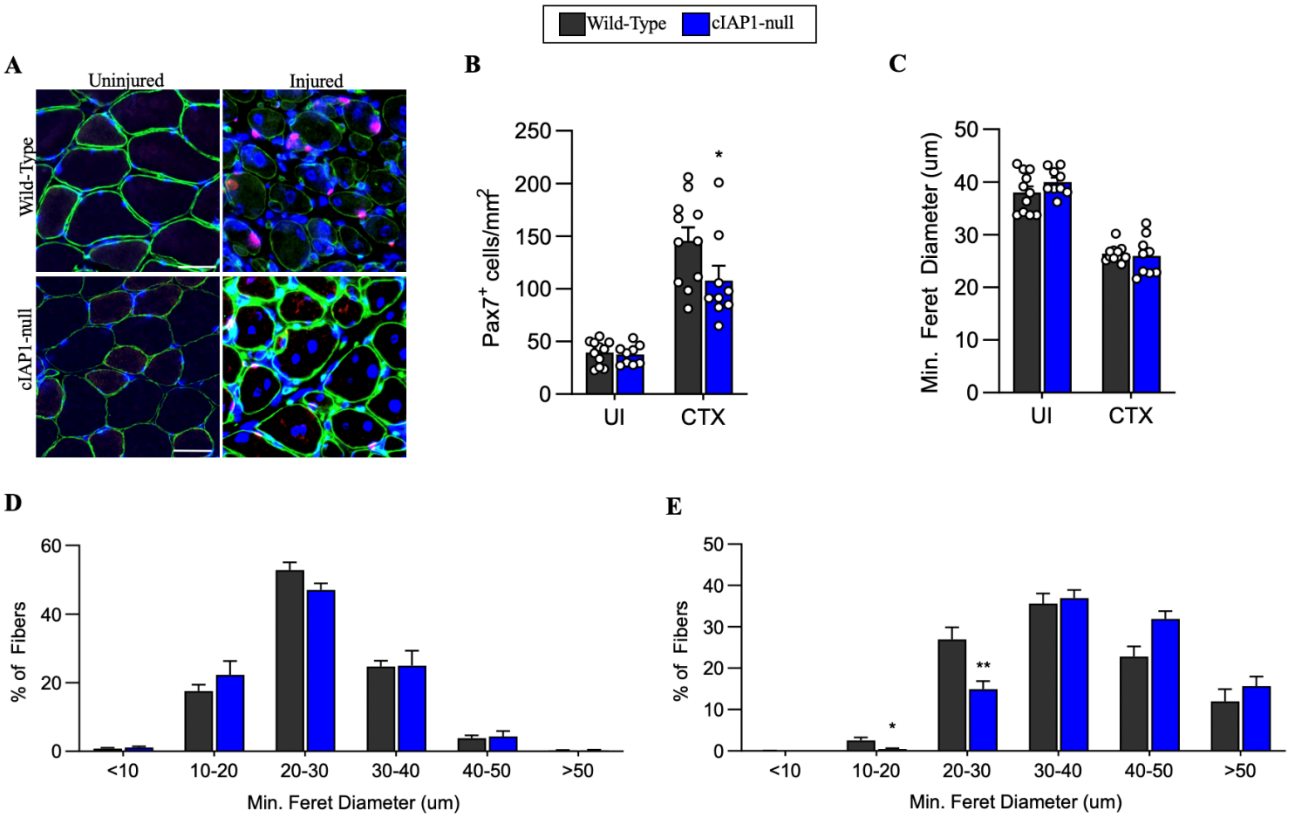

Figure 4. Satellite cell number in the tibialis anterior is affected by loss of cIAP1. The left tibialis anterior of wild-type and cIAP1-null mice were injured with 30µL of 10µM cardiotoxin to induce muscle regeneration. Uninjured (right) and injured (left) TAs were collected 7 days post-injury. *(A)* Representative images of muscle sections stained with laminin (green) and Pax7 (pink). Nuclei were counterstained with DAPI (blue). Bar, 50 µm. Quantification of *(B)* $Pax7^+$ cells per area (mm^2), *(C)* min. feret diameter (um) of uninjured (UI) and regenerated (CTX) muscle fibers, *(D)* fiber size distribution of injured fibers, and *(E)* fiber size distribution of uninjured fibers. Data are presented as mean + SEM. *p<0.05, **p<0.01 with student's t-test compared with uninjured.

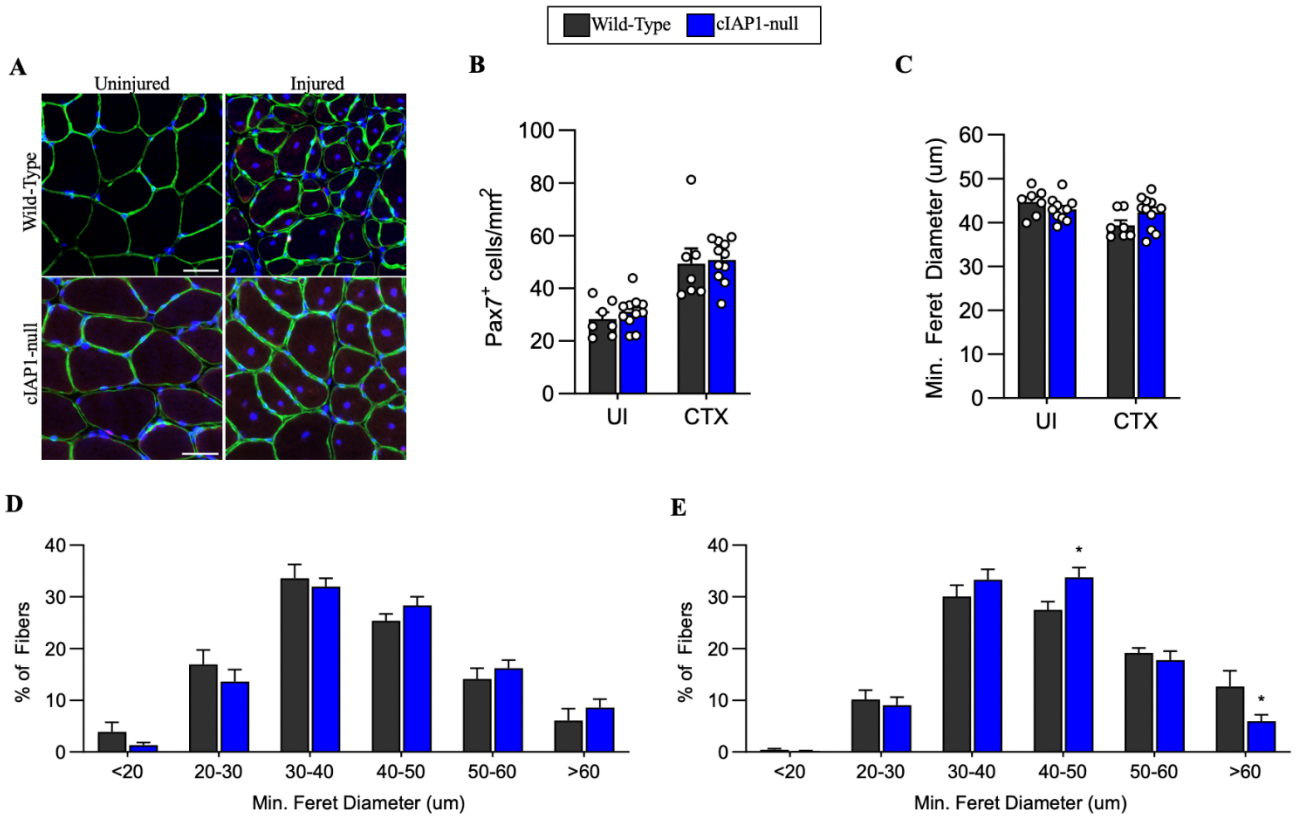

Figure 5. Fibers regenerated in cIAP1-null mice at 28 days post-injury are larger than wild-type fibers. The left tibialis anterior of wild-type and cIAP1-null mice was injured with 30µL of 10µM cardiotoxin to induce muscle regeneration. Uninjured (right) and injured (left) TAs were collected 28 days post-injury (dpi). *(A)* Representative images of muscle sections stained with laminin (green) and Pax7 (pink). Nuclei were counterstained with DAPI (blue). Bar, 50 µm. Quantification of *(B)* $Pax7^+$ cells per area (mm^2), *(C)* min. feret diameter (um) of uninjured (UI) and regenerated (CTX) muscle fibers, *(D)* fiber size distribution of injured fibers, and *(E)* fiber size distribution of uninjured fibers. Data are presented as mean + SEM. *$p<0.05$, with student's t-test compared with uninjured.

Conditional knockout of cIAP1 from Pax7$^+$ cells results in smaller regenerating fibers

Since I saw a defect in CTX-induced satellite cell expansion in cIAP1-null mice, I sought to examine the effect of cIAP1 in satellite cell biology using a conditional knockout mouse in which cIAP1 is specifically excised from Pax7$^+$ cells following tamoxifen administration. I found that at 7dpi regenerated fibers from the TA of conditional knockout mice were significantly smaller than wild-type fibers (Figure 6A,C,D), with significantly more small fibers (10-20μm) and significantly fewer mid-size fibers (30-40μm) (Figure 6D). Quantification of Pax7$^+$ cells showed a trend towards an increased number of satellite cells in cIAP1 conditional knockout mice compared to wild-type (Figure 6B). Analysis of the contralateral uninjured TA showed cross-sectional area and satellite cell number were indistinguishable between cIAP1 conditional knockout mice and wild-type mice (Figure 6A,B,C,E).

Loss of cIAP1 results in fewer satellite cells and smaller MHCIIa fibers in the soleus

To examine the effect loss of cIAP1 has on uninjured muscle, I collected the soleus at approximately 7 weeks of age to determine any changes in muscle phenotype compared to wild-type mice. I found loss of cIAP1 did not affect the size or the number of myofibers (Figure 7A-C,E). However, the number of satellite cells in the soleus muscle were significantly reduced in mice lacking cIAP1 (Figure 7D). To investigate if loss of cIAP1 alters fiber-type distribution in the soleus, I analyzed the percentage of each fiber type and found no differences between the fiber-type distribution of wild-type and cIAP1-null solei (Figure 7G). However, when fiber-type specific cross-sectional area was examined, I found that loss of cIAP1 resulted in significantly larger average cross-sectional area of MHCIIa (Figure 7F), suggesting that cIAP1 may play a role in specific fiber-type hypertrophy.

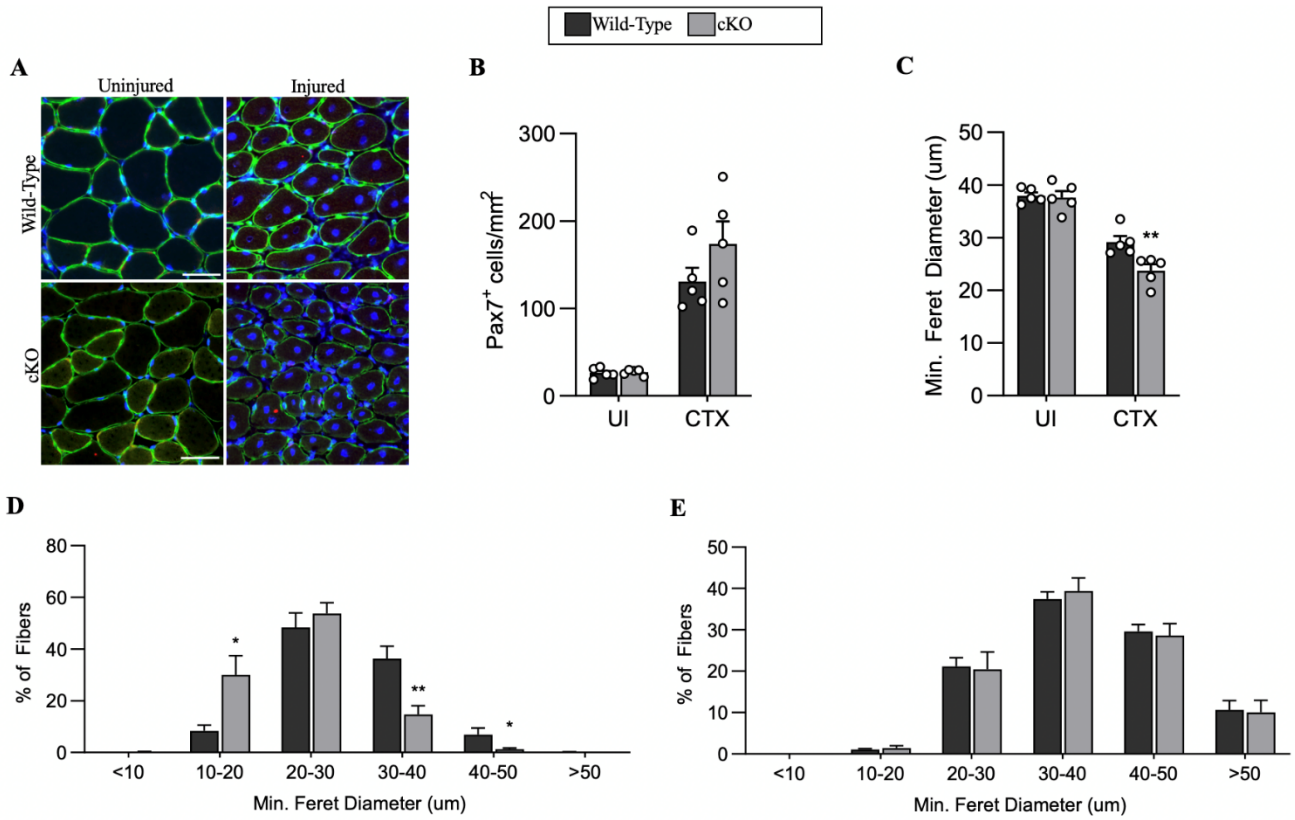

Figure 6. Excision of cIAP1 from Pax7$^+$ cells results in smaller regenerating fibers 7 days post-injury. The left tibialis anterior of wild-type and cIAP1 conditional knockout mice were injured with 30µL of 10µM cardiotoxin to induce muscle regeneration. Uninjured (right) and injured (left) TAs were collected 7 days post-injury (dpi). *(A)* Representative images of muscle sections stained with laminin (green) and Pax7 (pink). Nuclei were counterstained with DAPI (blue). Bar, 50 µm. Quantification of *(B)* Pax7$^+$ cells per area (mm^2), *(C)* min. feret diameter (um) of uninjured (UI) and regenerated (CTX) muscle fibers, *(D)* fiber size distribution of injured fibers, and *(E)* fiber size distribution of uninjured fibers. Data are presented as mean + SEM. *p<0.05, **p<0.005 with student's t-test compared with uninjured.

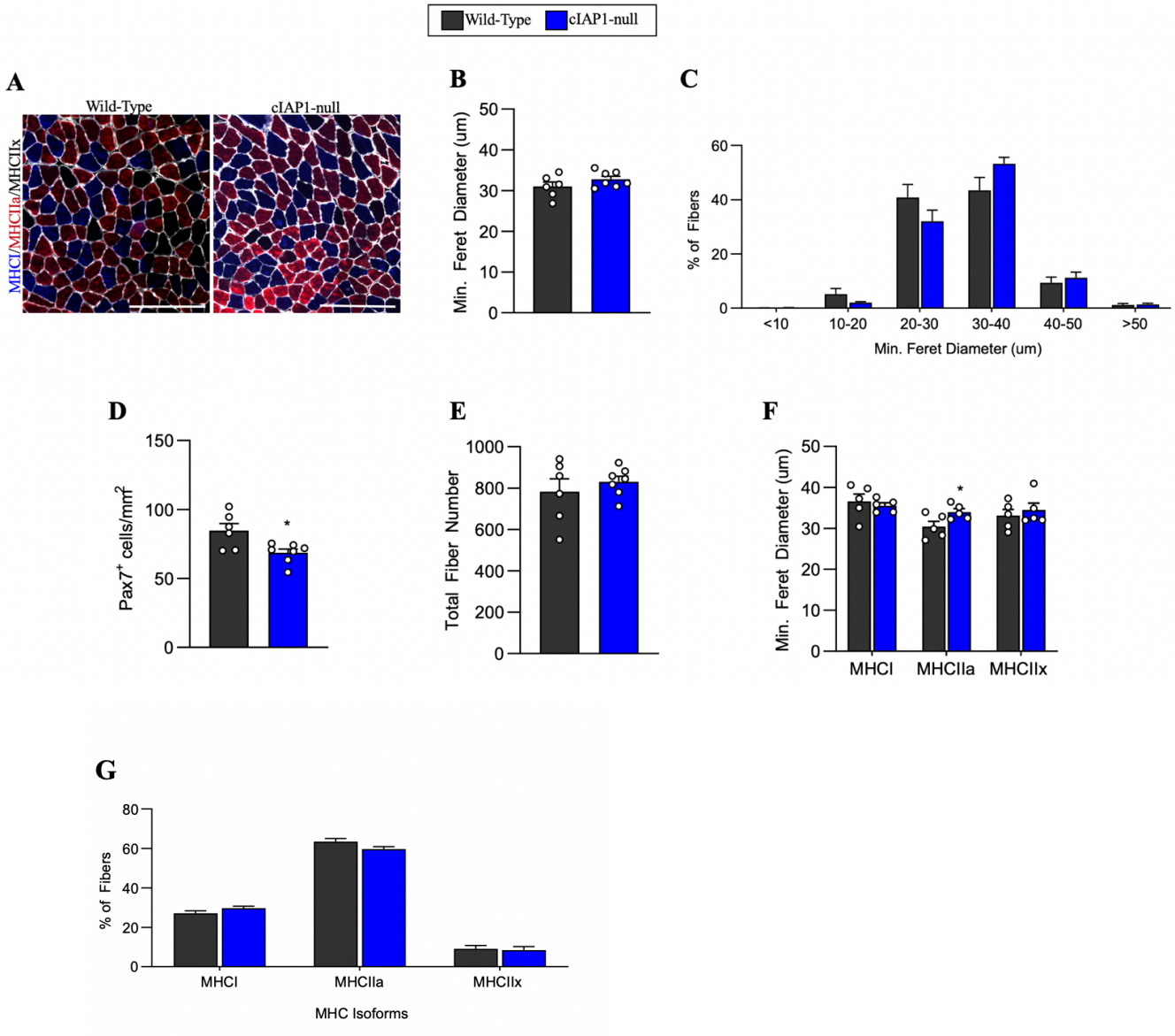

Figure 7. Satellite cell number in the soleus is reduced by the loss of cIAP1. The soleus of wild-type and cIAP1-null mice was collected at approximately 7 weeks of age and stained with laminin and Pax7 to determine cross-sectional area, and various myosin heavy chain isoforms to determine fiber-type distribution. *(A)* Representative images of muscle section stained with MHCI (blue), MHCIIa (red) and laminin (white). Bar, 200 µm. Quantification of *(B)* min. feret diameter (um), *(C)* fiber size distribution, *(D)* $Pax7^+$ cells per area (mm^2), *(E)* total fiber number, *(F)* fiber-type specific cross-sectional area, and *(G)* percentage of myosin heavy chain isoforms. Data are presented as mean + SEM. *$p<0.05$, with student's t-test compared with wild-type.

Loss of cIAP1 has no effect on fiber size or satellite cell number but increases the percentage of MHCIIx fibers in the EDL muscle

Next, I sought to evaluate the consequences of EDL muscle lacking cIAP1. The EDLs of cIAP1-null mice were indistinguishable from wild-type controls in regard to fiber size, number of fibers and satellite cells (Figure 8A-E). Despite no differences in cross-sectional area and satellite cell number, cIAP1-null EDLs possess significantly increased percentage of the fast isoform, MHCIIx. compared to wild-type EDLs (Figure 8G); however, the cross-sectional area of the various MHC isoforms was unaffected by loss of cIAP1 (Figure 8F).

Loss of cIAP1 has no effect on forelimb or all limb grip strength

Given the fact that loss of cIAP1 had differing effects in the soleus and EDL, I wanted to further evaluate the physiological and behavioural significance of cIAP1 loss. I examined muscle strength by performing a forelimb and all limb grip strength test. I found no significant differences between wild-type mice compared to cIAP1-null mice in both the forelimb and all limb normalized grip strength (Figure 9A,B). In addition, I wanted to look at fatigability during the grip strength test. Again, I found there was no change in fatigability in mice lacking cIAP1 (Figure 9C,D). Taken together, these results suggest that loss of cIAP1 has no effect on muscle strength or endurance.

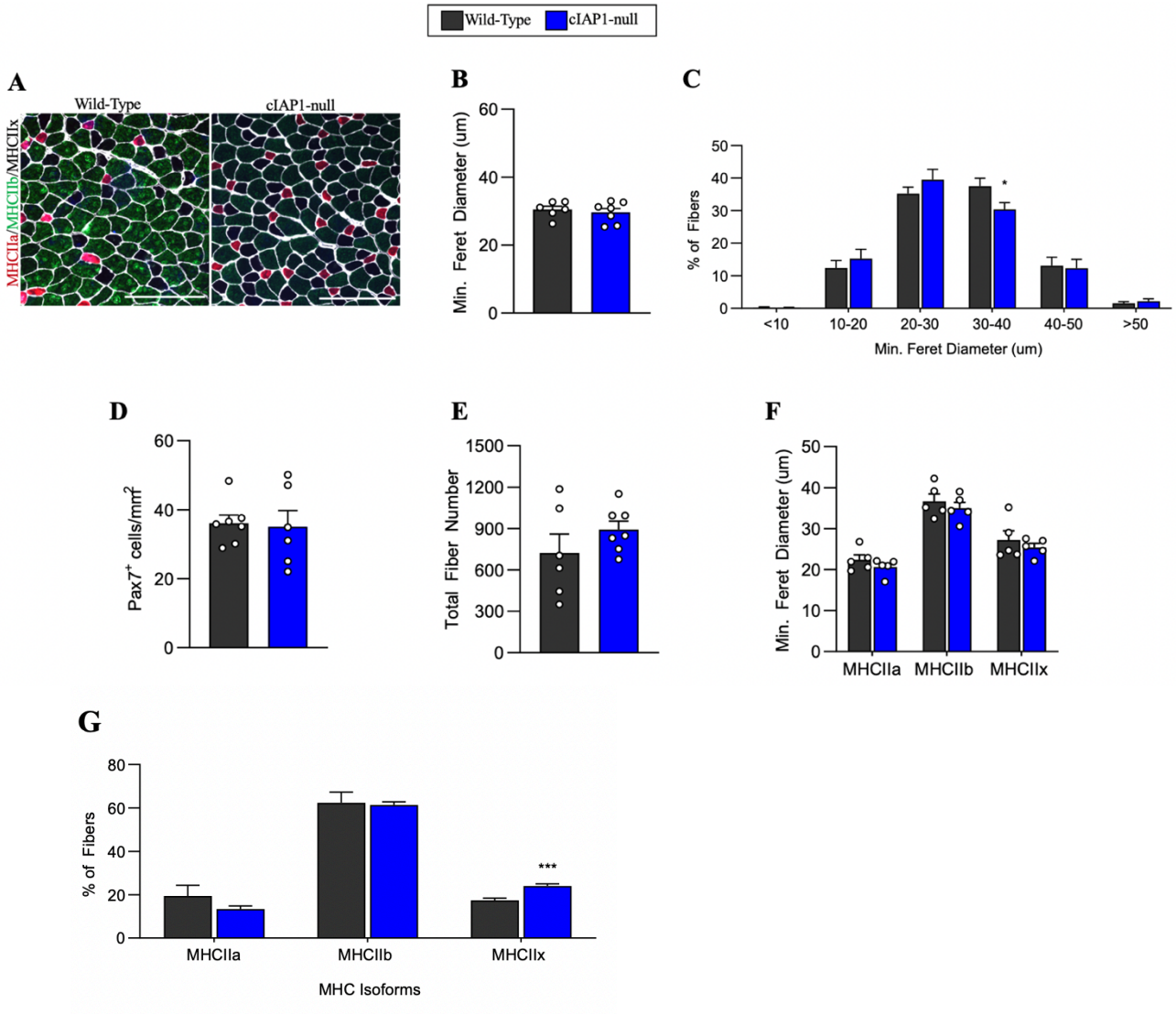

Figure 8. Loss of cIAP1 results in an increase in the fast MHC isoform in the EDL. The EDL of wild-type (white bars) and cIAP1-null mice (grey bars) was collected at approximately 7 weeks of age and stained with laminin and Pax7 to determine cross-sectional area, and various myosin heavy chain isoforms to determine fiber-type distribution. *(A)* Representative images of muscle section stained with MHCIIa (red), MHCIIb (green) and laminin (white). Bar, 200 µm. Quantification of *(B)* min. feret diameter (um), *(C)* fiber size distribution, *(D)* $Pax7^+$ cells per area (mm^2), *(E)* total fiber number, *(F)* fiber-type specific cross-sectional area, and *(G)* percentage of myosin heavy chain isoforms. Data are presented as mean + SEM. *$p<0.05$, with student's t-test compared with wild-type.

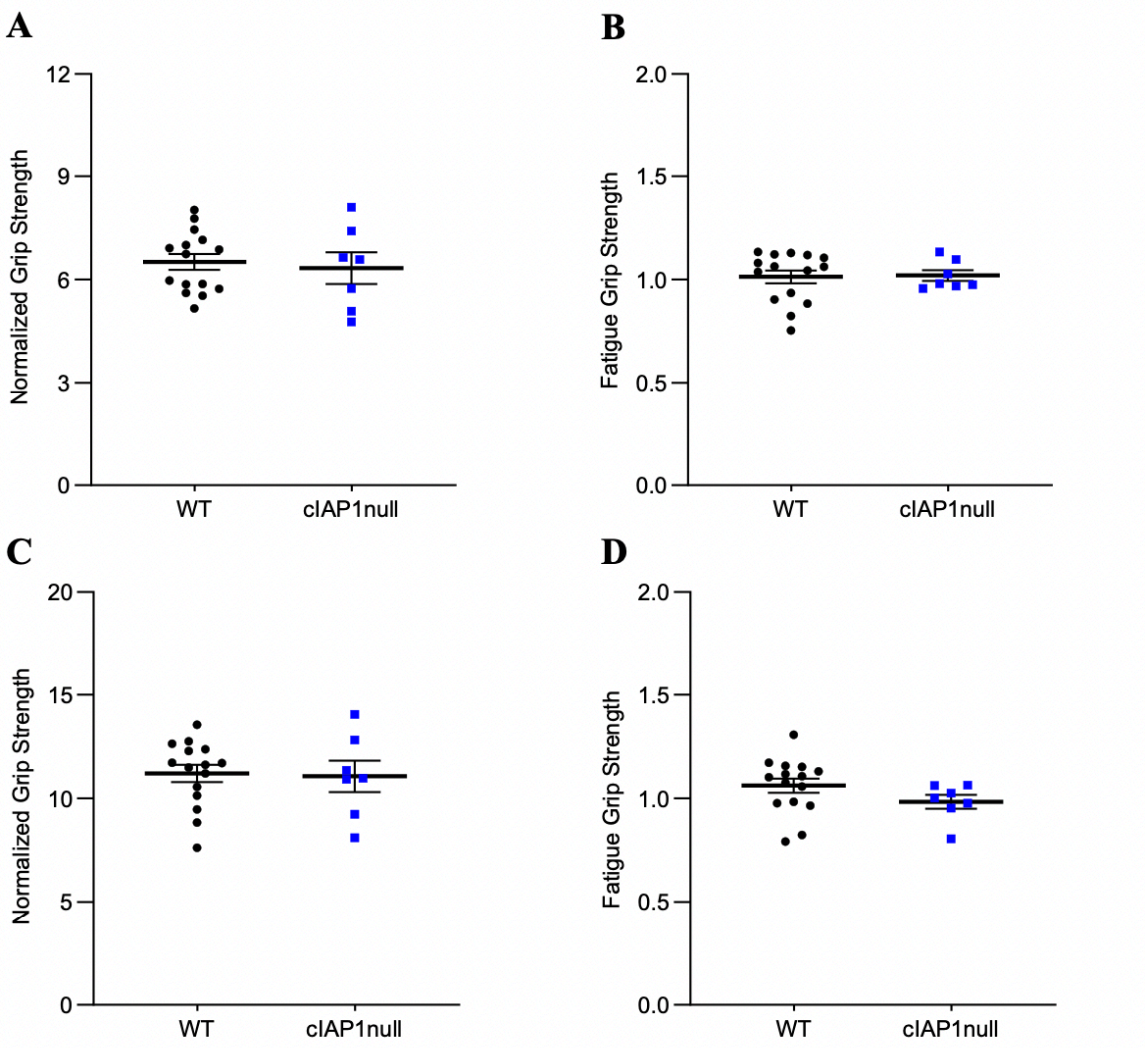

Figure 9. Grip strength is unaffected by loss of cIAP1. Wild-type and cIAP1-null mice were subjected to a forelimb grip and all limb grip strength test. Pulls were performed 5 times followed by a brief rest period and then repeated. *(A)* Normalized forelimb grip strength, *(B)* forelimb fatigue grip strength, *(C)* normalized all limb grip strength, and *(D)* fatigue all limb grip strength. Data presented as mean ± SEM, with student's t-test compared with wild-type.

Loss of cIAP1 has no effect on soleus contractile properties

To investigate the effect that cIAP1 loss has on muscle contraction kinetics in uninjured tissues, I subjected the mice to *in situ* force experiments. I determined that loss of cIAP1 has no effect on the twitch kinetics (Table 1), peak force, frequency 50 (F50), or the force-frequency curve (Figure 10A-C) in the solei muscle of mutant mice compared to wild-type.

Peak force and Maximum Rate of Force Development is significantly reduced in EDLs lacking cIAP1

Interestingly, the loss of cIAP1 negatively affected muscle contraction kinetics in the EDL. The maximum rate of force development was significantly reduced in the EDL of mice lacking cIAP1 (Table 1). In addition, EDLs from mutant mice produced significantly less force than wild-type mice (Figure 11A). However, F50 and the force-frequency curve were unaffected (Figure 11B,C). Together, reduced maximum rate of force development and reduced peak force characterize a muscle that takes longer to produce a smaller force.

Table 1. Loss of cIAP1 does not alter twitch kinetic properties in the soleus, but decreases maximum rate of force development in the EDL.

	Wild-Type		cIAP1-null	
	Soleus	**EDL**	**Soleus**	**EDL**
Time to Peak (ms)	15.44 ± 0.52	6.84 ± 0.51	14.64 ± 0.12	6.96 ± 0.54
½-Rise Time (ms)	4.04 ± 0.13	1.76 ± 0.04	3.84 ± 0.19	1.92 ± 0.19
Max Rate of Force Development ($N/cm^2 \cdot sec$)	388 ± 41	1,411 ± 85	389 ± 57	988 ± 159*
½-Relaxation Time (ms)	14.28 ± 1.08	8.4 ± 0.5	15.96 ± 0.73	9.0 ± 1.2

Data are presented as mean ± SEM. *$p<0.05$, with student's t-test compared to wild-type.

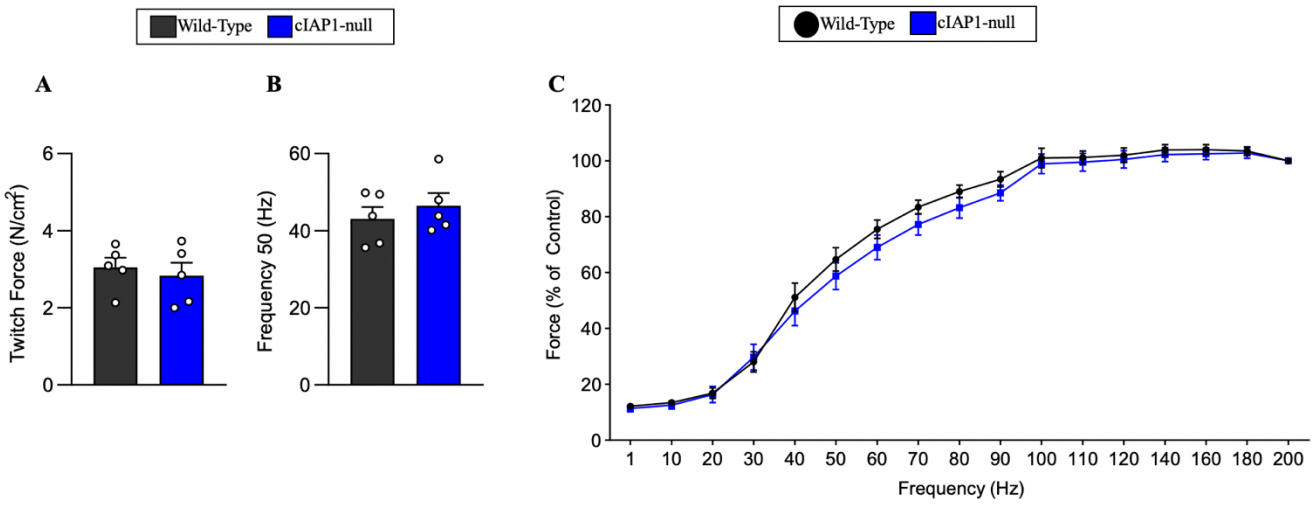

Figure 10. Loss of cIAP1 did not alter peak force, F50 or cause a shift in the force-frequency curve in the soleus muscle. *(A)* Twitch force in N/cm^2 in soleus, *(B)* Frequency 50 (stimulation frequency at which force was 50% of the maximum tetanic force), and *(C)* force-frequency relationship expressed as a percentage of the control (200Hz). Data presented as mean ± SEM, with student's t-test compared with wild-type.

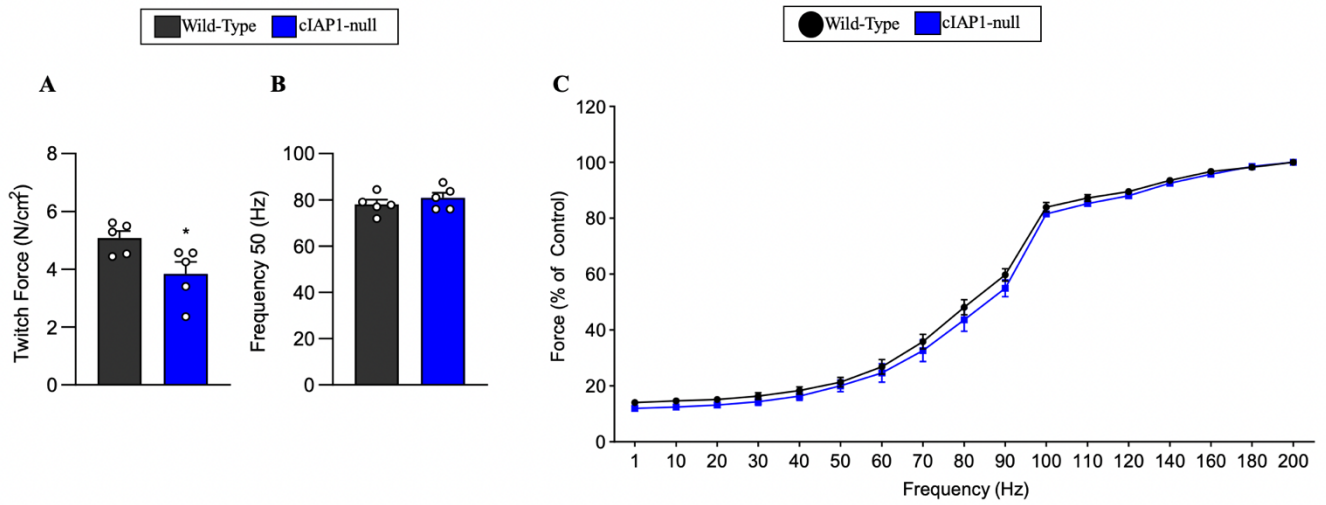

Figure 11. Loss of cIAP1 decreases peak force in the EDL. *(A)* Twitch force in N/cm^2 in soleus, *(B)* Frequency 50 (stimulation frequency at which force was 50% of the maximum tetanic force), and *(C)* force-frequency relationship expressed as a percentage of the control (200Hz). Data presented as mean ± SEM. *p<0.05, with student's t-test compared with wild-type.

3.2. Objective Two: To characterize the muscle phenotype of cIAP2 knockout mice

Loss of cIAP2 leads to smaller muscle fibers at 7 weeks of age

While cIAP2 is not expressed in muscle fibers, it is expressed in cells that surround and infiltrate the area. Using a whole body cIAP2 knock-out, I sought to characterize the role of cIAP2 in muscle development and regeneration. After CTX-induced injury, I found that cross sectional area was significantly decreased in cIAP2-null TA muscle compared to wild-type muscle (Figure 12A,C,D). A shift in fiber size distribution confirms this result, such that mice lacking cIAP2 had significantly more small fibers (10-20μm), and significantly fewer mid to large size fibers (30 - >50μm) (Figure 12D). When quantifying the number of $Pax7^+$ cells, I found that loss of cIAP2 had no effect on satellite cell number (Figure 12B). Interestingly, when I analyzed the contralateral uninjured TA, I found that cIAP2-null mice had significantly smaller cross-sectional area compared to wild-type muscle (Figure 12A,C,E). These results suggest that cIAP2 may be required for muscle growth and regeneration.

Loss of cIAP2 results in larger muscle fibers 28 days post injury and decreased fiber size in uninjured tissue

Since I saw a decrease in average cross-sectional area at 7dpi, I examined fiber cross-sectional area and satellite cell numbers at 28dpi. I found that at 28dpi, fibers regenerated from cIAP2-null mice trended towards a larger average cross-sectional area compared to wild-type mice (Figure 13A,C,E). Quantification of $Pax7^+$ cells showed no differences between wild-type mice and cIAP2-null mice (Figure 13B). The contralateral uninjured TA muscles from cIAP2-null mice had a significantly smaller average cross-sectional area compared to wild-type mice (Figure 13A,C,E). As demonstrated by a shift in fiber size distribution, cIAP2-null mice had

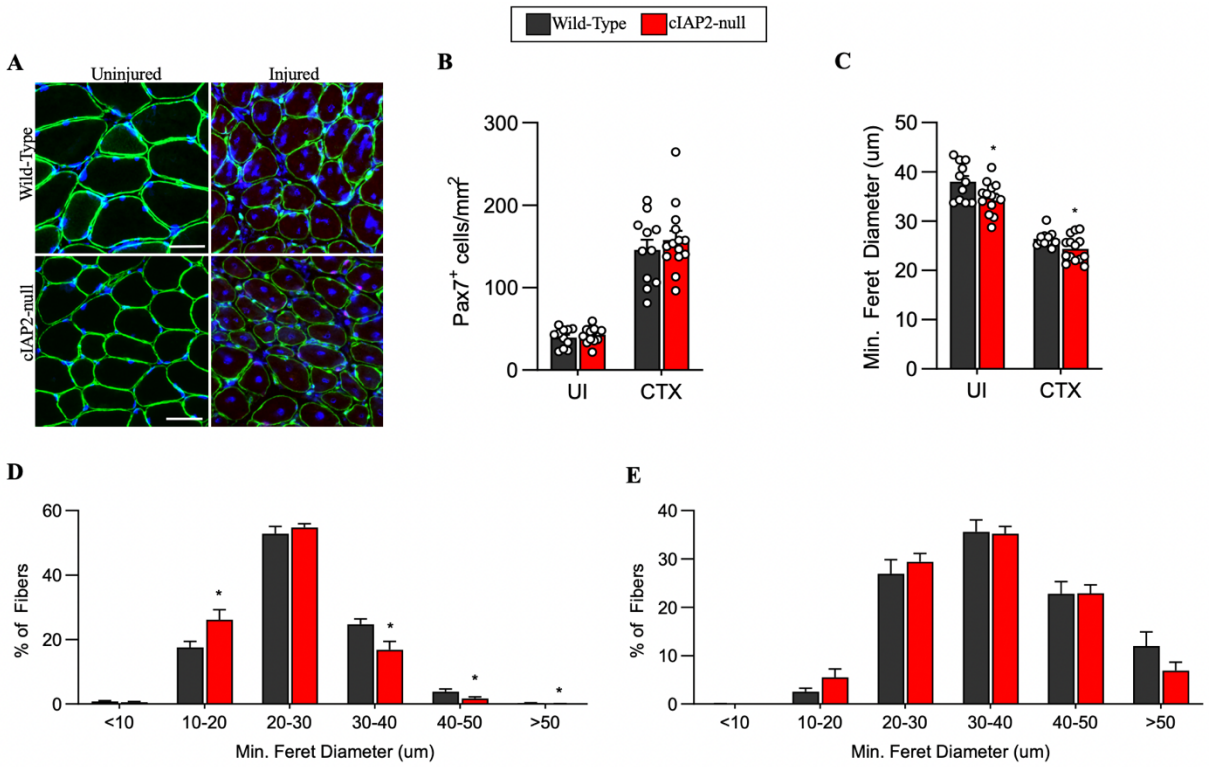

Figure 12. Cross-sectional area of muscle fibers is reduced in mice lacking cIAP2. The left tibialis anterior of wild-type (white bars) and cIAP2-null mice (black bars) was injured with 30µL of 10µM cardiotoxin to induce muscle regeneration. Uninjured (right) and injured (left) TAs were collected 7 days post-injury (dpi). *(A)* Representative images of muscle sections stained with laminin (green) and Pax7 (pink). Nuclei were counterstained with DAPI (blue). Bar, 50 µm. Quantification of *(B)* $Pax7^+$ cells per area (mm^2), *(C)* min. feret diameter (um) of uninjured (UI) and regenerated (CTX) muscle fibers, *(D)* fiber size distribution of injured fibers, and *(E)* fiber size distribution of uninjured fibers. Data are presented as mean + SEM. *$p<0.05$, with student's t-test compared with wild-type.

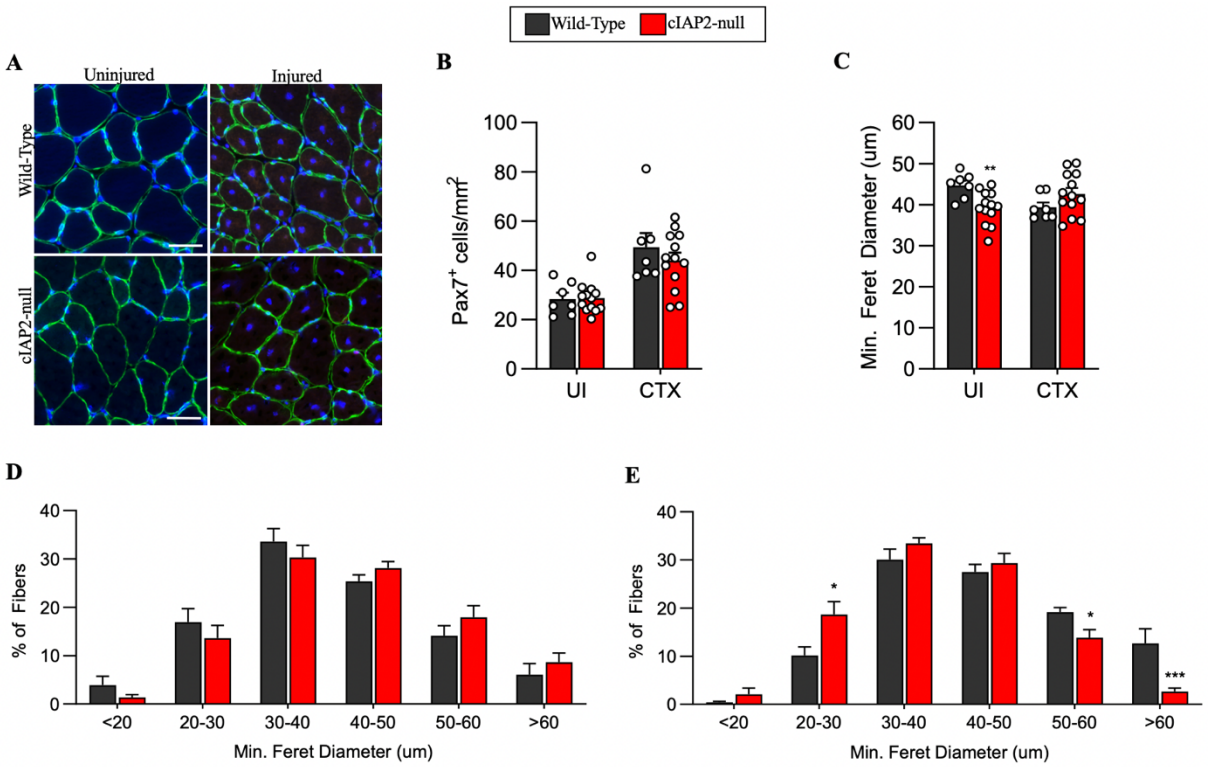

Figure 13. Cross-sectional area of muscle fibers is altered in mice lacking cIAP2. The left tibialis anterior of wild-type (white bars) and cIAP2-null mice (black bars) was injured with 30µL of 10µM cardiotoxin to induce muscle regeneration. Uninjured (right) and injured (left) TAs were collected 28 days post-injury (dpi). *(A)* Representative images of muscle sections stained with laminin (green) and Pax7 (pink). Nuclei were counterstained with DAPI (blue). Bar, 50 µm. Quantification of *(B)* $Pax7^+$ cells per area (mm^2), *(C)* min. feret diameter (um) of uninjured (UI) and regenerated (CTX) muscle fibers, *(D)* fiber size distribution of injured fibers, and *(E)* fiber size distribution of uninjured fibers. Data are presented as mean + SEM. *p<0.05, **p<0.01, with student's t-test compared with wild-type.

significantly more small fibers (20-30μm), and significantly fewer large fibers (>60μm) (Figure 13E).

Loss of cIAP2 results in larger muscle fibers and increases the percentage of MHCI fibers in the soleus

To examine the effect loss of cIAP2 has on uninjured muscle, I collected the soleus at approximately 7 weeks of age to determine any changes in muscle phenotype compared to wild-type mice. I found cIAP2-null mice had significantly larger average cross-sectional area compared to wild-type mice (Figure 14A-C). Quantification of $Pax7^{+}$ cells showed a trend towards fewer satellite cells in cIAP2-null mice (Figure 14D).

To investigate if loss of cIAP2 alters fiber-type distribution in the soleus, I analyzed the percentage of each fiber type and found an increased percentage of MHCI fibers in the solei of cIAP2-null mice (Figure 14G). In addition, fiber-type specific cross-sectional area analysis showed a trend towards larger cross-sectional area of MHCIIa and MHCIIx fibers in the solei of cIAP2-null mice (Figure 14F), suggesting that cIAP2 may play a role in specific fiber-type hypertrophy.

Loss of cIAP2 has no effect on fiber size or satellite cell number, but increases the percentage of MHCIIx fibers in the EDL

Next, I sought to examine the muscle phenotype of EDL muscles lacking cIAP2. The EDLs of cIAP2-null mice were indistinguishable from wild-type controls in regard to fiber size, satellite cell number, and number of fibers (Figure 15A-D). Loss of cIAP2 resulted in the EDL possessing significantly increased percentage of the fast MHC isoform, MHCIIx, compared to

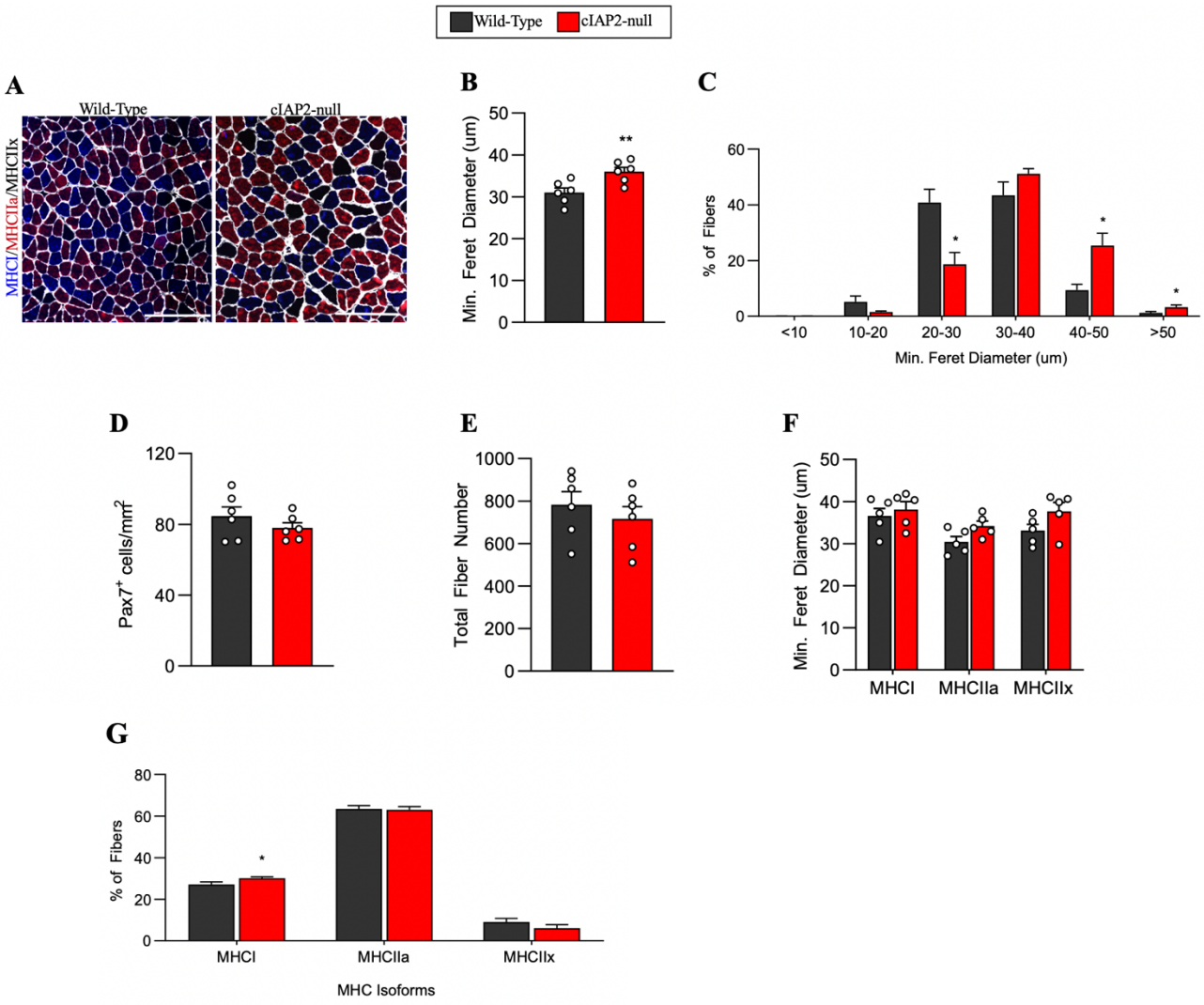

Figure 14. Loss of cIAP2 increases cross-sectional area of muscle fibers in the soleus. The soleus of wild-type and cIAP2-null mice was collected at approximately 7 weeks of age and stained with laminin and Pax7 to determine cross-sectional area, and various myosin heavy chain isoforms to determine fiber-type distribution. *(A)* Representative images of muscle section stained with MHCI (blue), MHCIIa (red) and laminin (white). Bar, 200 µm. Quantification of *(B)* min. feret diameter (um), *(C)* fiber size distribution, *(D)* $Pax7^+$ cells per area (mm^2), *(E)* total fiber number, *(F)* fiber-type specific cross-sectional area, and *(G)* percentage of myosin heavy chain isoforms. Data are presented as mean + SEM. *p<0.05, **p<0.01, with student's t-test compared with wild-type.

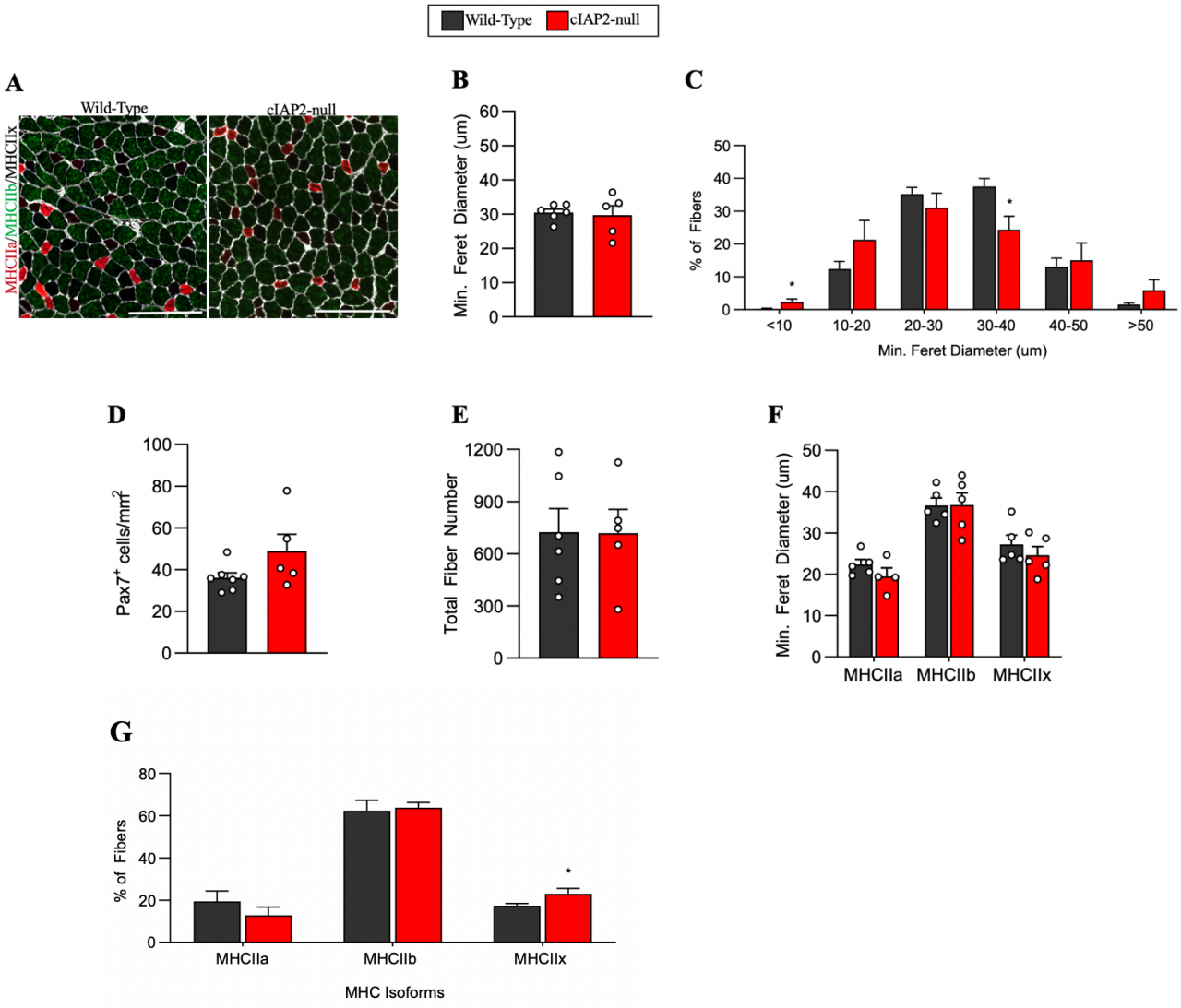

Figure 15. The percentage of the fast MHC isoform is increased in the EDL of cIAP2-null mice. The EDL of wild-type and cIAP2-null mice was collected at approximately 7 weeks of age and stained with laminin and Pax7 to determine cross-sectional area, and various myosin heavy chain isoforms to determine fiber-type distribution. *(A)* Representative images of muscle section stained with MHCIIa (red), MHCIIb (green) and laminin (white). Bar, 200 µm. Quantification of *(B)* min. feret diameter (um), *(C)* fiber size distribution, *(D)* $Pax7^{+}$ cells per area (mm^2), *(E)* total fiber number, *(F)* fiber-type specific cross-sectional area, and *(G)* percentage of myosin heavy chain isoforms. Data are presented as mean + SEM. *$p<0.05$, with student's t-test compared with wild-type.

wild-type controls (Figure 15G); however, fiber-type specific cross-sectional area was unaffected by loss of cIAP2 (Figure 15F).

Loss of cIAP2 has no effect on forelimb or all limb grip strength

To evaluate the consequences of muscle strength and endurance in mice lacking cIAP2, I performed a forelimb and all limb grip strength test. I found cIAP2-null mice were indistinguishable from wild-type controls in both the forelimb and all limb grip strength (Figure 16A,B). In addition, there were no differences in fatigability in mice lacking cIAP2 compared to wild-type mice (Figure 16C,D). Taken together, these results suggest that loss of cIAP2 has no effect on muscle strength or endurance.

Solei from mice lacking cIAP2 have a force-frequency curve that is shifted to the right of wild-type mice

Given the fact that loss of cIAP2 resulted in larger fibers in the soleus, I subjected the mice to *in situ* force experiments to evaluate the consequences of cIAP2 loss on muscle contraction kinetics. I determined that loss of cIAP2 had no effect on twitch kinetics (Table 2), peak force or F50 (Figure 17A,B). However, loss of cIAP2 resulted in a significant shift of the force-frequency curve to the right of wild-type solei (Figure 17C). Interestingly, the force-frequency curve was unchanged for majority of the stimulation (1-120Hz), and began to shift at higher frequencies, beginning at 140Hz (Figure 17C).

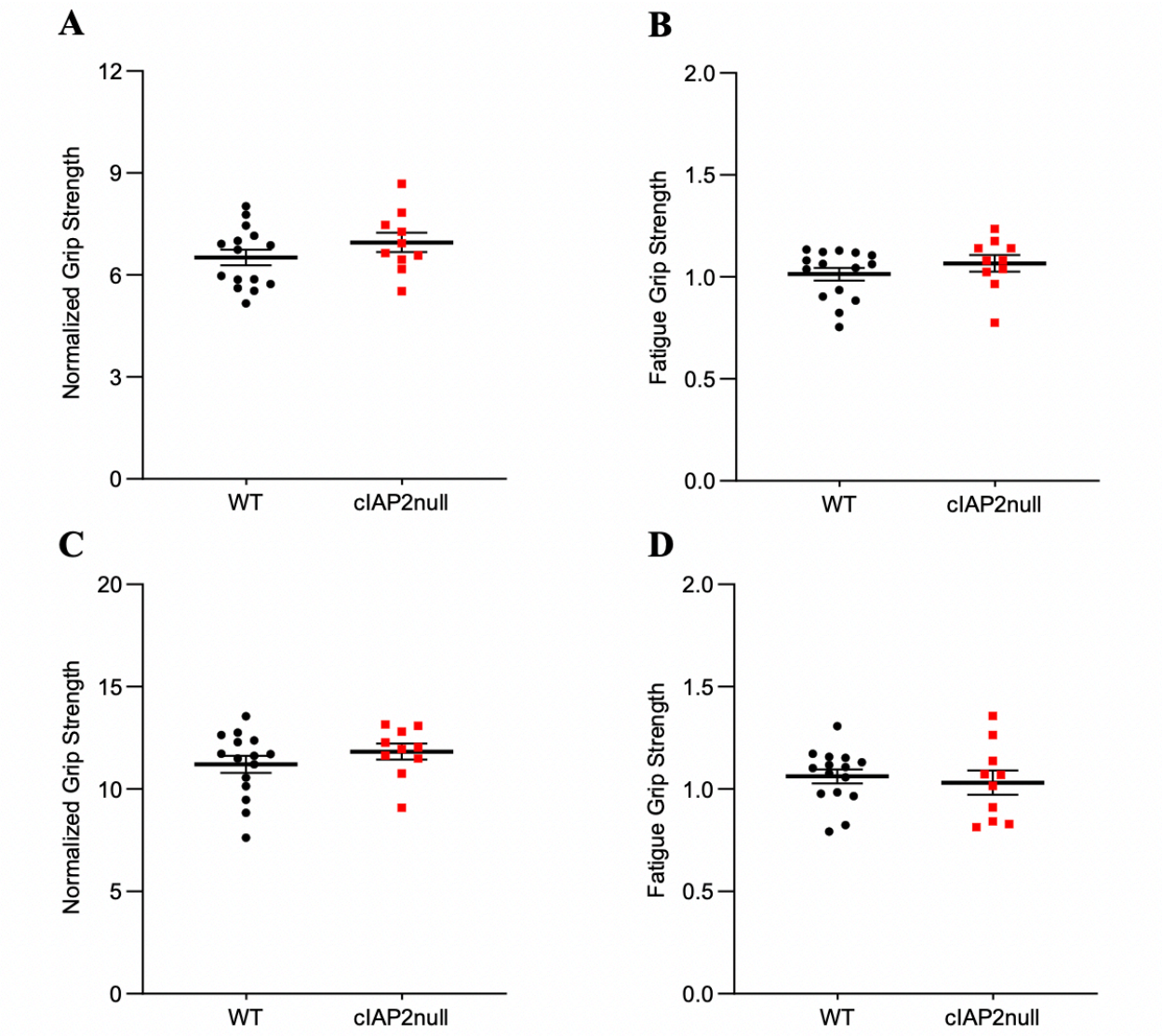

Figure 16. Grip strength is unaffected by loss of cIAP2. Wild-type and cIAP2-null mice were subjected to a forelimb grip and all limb grip strength test. Pulls were performed 5 times followed by a brief rest period and then repeated. *(A)* Normalized forelimb grip strength, *(B)* forelimb fatigue grip strength, *(C)* normalized all limb grip strength, and *(D)* fatigue all limb grip strength. Data presented as mean ± SEM, with student's t-test compared with wild-type.

Table 2. Twitch kinetics of the soleus and EDL are unaffected by loss of cIAP2.

	Wild-Type		cIAP2-null	
	Soleus	**EDL**	**Soleus**	**EDL**
Time to Peak (ms)	15.44 ± 0.52	6.84 ± 0.51	15.16 ± 0.24	8.00 ± 0.24
½-Rise Time (ms)	4.04 ± 0.13	1.76 ± 0.04	3.92 ± 0.10	2.76 ± 0.48
Max Rate of Force Development ($N/cm^2 \cdot sec$)	388 ± 41	1,411 ± 85	445 ± 78	1,493 ± 181
½-Relaxation Time (ms)	14.28 ± 1.08	8.4 ± 0.5	16.44 ± 0.95	8.6 ± 0.6

Data are presented as mean ± SEM.

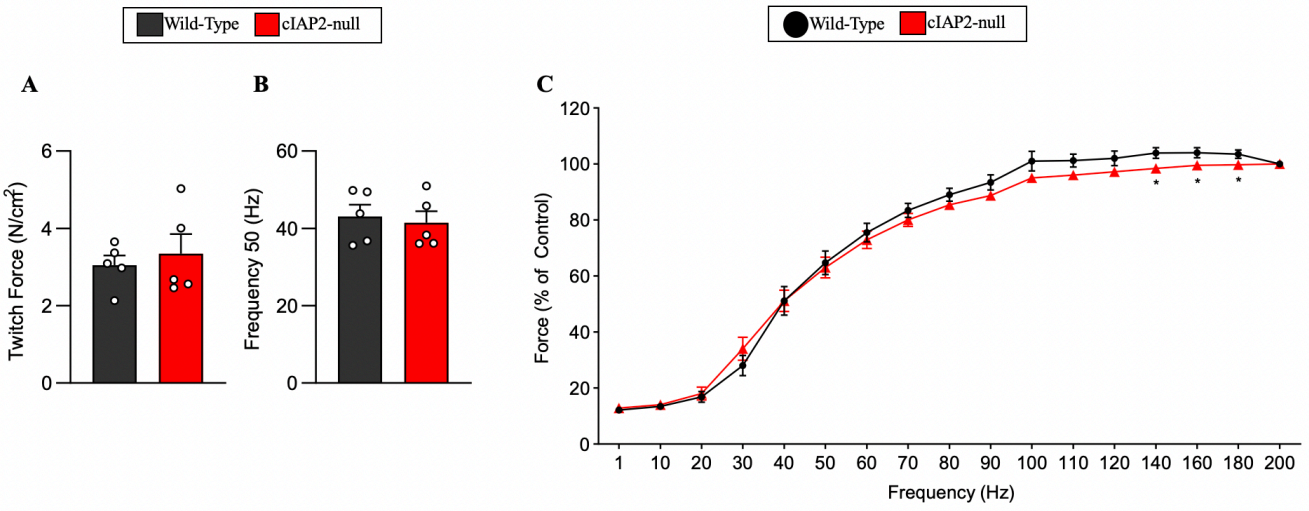

Figure 17. Loss of cIAP2 causes a shift in the force-frequency curve of the soleus. *(A)* Twitch force in N/cm^2 in soleus, *(B)* Frequency 50 (stimulation frequency at which force was 50% of the maximum tetanic force), and *(C)* force-frequency relationship expressed as a percentage of the control (200Hz). Data presented as mean ± SEM. *p<0.05, with student's t-test compared with wild-type.

Loss of cIAP2 alters muscle fiber kinetics in the EDL

Next, I sought to evaluate the consequences of EDL muscle lacking cIAP2. The EDLs of cIAP2-null mice were indistinguishable from wild-type controls regarding twitch kinetics (Table 2) and peak force (Figure 18A); however, the F50 of cIAP2-null mice was significantly reduced compared to wild-type (Figure 18B). In addition, the force-frequency curve of cIAP2-null mice was significantly shifted to the left of the wild-type curve from 40-90Hz (Figure 18C).

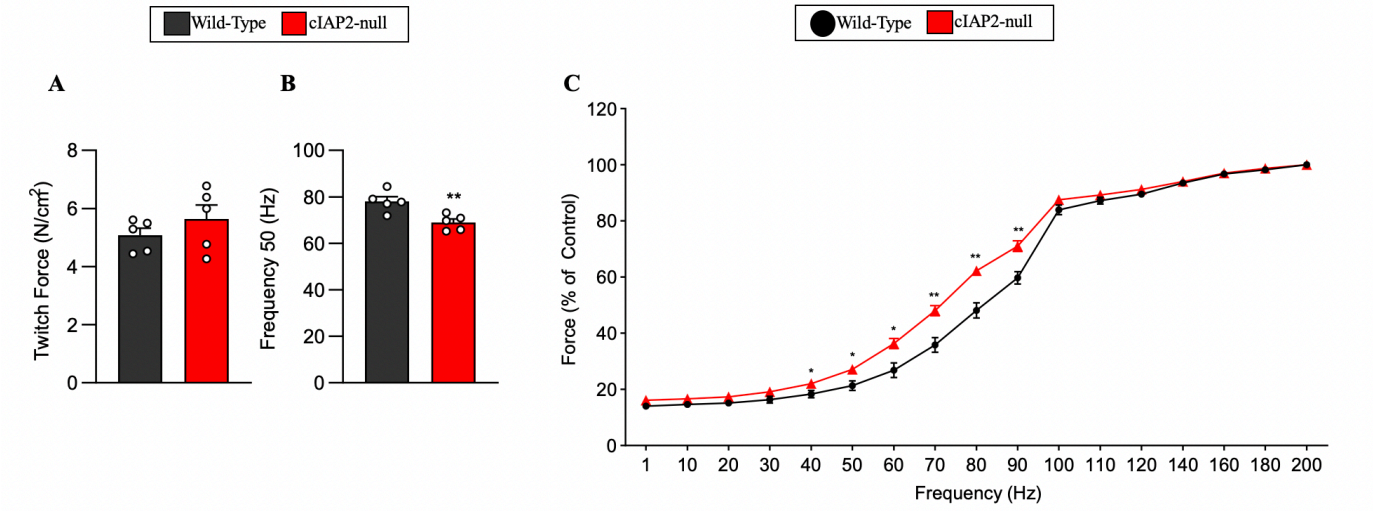

Figure 18. EDL muscle fiber kinetics are altered in mice lacking cIAP2. *(A)* Twitch force in N/cm^2 in soleus, *(B)* Frequency 50 (stimulation frequency at which force was 50% of the maximum tetanic force), and *(C)* force-frequency relationship expressed as a percentage of the control (200Hz). Data presented as mean ± SEM. *p<0.05, **p<0.01 with student's t-test compared with wild-type.

CHAPTER FOUR: DISCUSSION

Several studies have examined the role of cIAP1 and cIAP2 in diseased muscle, however, little work has been done to elucidate their importance in skeletal muscle regeneration and homeostasis. In this study, I have found that loss of cIAP1/2 has a greater impact on muscle growth compared to muscle regeneration. Further studies need to be done to understand the cellular and molecular mechanisms by which cIAP1/2 regulate muscle growth, fiber type plasticity and the contractile properties of different muscle groups.

4.1 A potential role of cIAP1 in satellite cell expansion

Although the effect of cIAP1 loss on muscle size after regeneration has been studied (Enwere et al., 2012; Enwere et al., 2013), little work has been done examining the role of cIAP1 in satellite cell function during muscle regeneration *in vivo*. Interestingly, we show a reduction in satellite cell numbers 7 days after CTX-induced injury in the TA (Figure 4B) and in the non-injured soleus muscle of cIAP1-null mice (Figure 7D). A reduction in satellite cell number could be attributed to increased satellite cell apoptosis, impaired proliferation, or premature differentiation of satellite cells.

Previous work has implicated the role of cIAP1 in proliferation of cancerous cells. Specifically, cIAP1 is upregulated in gallbladder cancer tissues, and knockdown of cIAP1 in stably transfected gallbladder cancer cell lines limited proliferation rate (Su et al., 2019). In addition, research examining the role of cIAP1 in proliferation of squamous cell carcinoma demonstrated TNF-like weak inducer of apoptosis (TWEAK) and its interaction with its receptor fibroblast growth factor-inducible 14 (Fn14) activates cIAP1 signals and results in enhanced proliferation of squamous cell carcinoma cells (Liang et al., 2020). Moreover, the role of cIAP1 in the progression of ovarian cancer has been demonstrated (Psyrri et al., 2006). Specifically,

cIAP1 was shown to be involved in cell proliferation such that knockdown of cIAP1 in a human ovarian carcinoma cell line showed significantly reduced proliferation (Jin et al., 2014). Thus, loss of cIAP1 in the muscle may be attenuating satellite cell expansion by inhibiting their proliferation.

The involvement of the NF-κB pathway in myogenesis and muscle regeneration has been examined, and distinct roles of the canonical and alternative pathways have been established (Bakkar & Guttridge, 2010). While the canonical pathway is required for myoblast proliferation (He et al., 2013), the alternative pathway promotes mitochondrial biogenesis (Bakkar et al., 2012), and myoblast fusion during myogenesis (Enwere et al., 2012). While the role of the NF-κB pathway has been elucidated in myogenesis, it's role in regulation of satellite cell function remains enigmatic. A study using a mouse model of cancer cachexia reported reduced differentiation of satellite cells into the myogenic lineage following activation of IKKβ in satellite cells (He et al., 2013). In contrast, Ogura et al. (2013) reported the number of $Pax7^{+}$ cells was increased in canonical NF-κB inhibited cultures that were treated with TWEAK. In addition, targeted ablation of upstream regulators of the canonical pathway in satellite cells, TAK1 and TRAF6, resulted in defective muscle regeneration, and therefore are essential for the self-renewal, survival and proliferation of satellite cells (Hindi & Kumar, 2016; Ogura et al., 2015). Together, these studies have implicated a role of the NF-κB pathway in regulation of satellite cell function during myogenesis; although it remains unknown exactly what that role is. Recently, it has been shown that canonical NF-κB signaling is activated in satellite cells after injury, following injection of $BaCl_2$ solution into the TA of wild-type mice (Straughn et al., 2018). Furthermore, satellite cell number was reduced in satellite cell-specific IKKβ knockout mice following injury, and proliferation was significantly decreased (Straughn et al., 2018). Since the

canonical pathway is known to play a role in cell survival (Hayden & Ghosh, 2012; Kumar et al., 2004), Straughn et al. (2018) wanted to examine the role of the canonical NF-κB pathway in satellite cell survival. Satellite cell-specific IKKβ knockout mice showed significantly more $TUNEL^+$ cells, and levels of lactate dehydrogenase (LDH), an enzyme that accumulates following cell death, were significantly higher in the supernatants of cells transfected with IKKβ and p65 siRNA; therefore suggesting the canonical pathway promotes the survival of satellite cells (Straughn et al., 2018). Furthermore, *ex vivo* suspension cultures demonstrated a significant increase in $Pax7^-/MyoD^+$ cells in satellite cell-specific IKKβ knockout mice EDLs at 72 hours compared to control mice, suggesting the activation of the NF-κB pathway prevents precocious differentiation of satellite cells (Straughn et al., 2018). Together, these results demonstrate the importance of the canonical NF-κB pathway in regulation of satellite cell function during muscle regeneration. Importantly, the canonical pathway functions to promote proliferation and survival, while preventing precocious differentiation of satellite cells. Since cIAP1 has been shown to be an important positive regulator of the canonical pathway, loss of cIAP1 in satellite cells could inhibit proliferation and promote cell death through decreased canonical NF-kB signaling. To determine the effect loss of cIAP1 has on satellite cells specifically, Pax7/TUNEL staining would be beneficial to examine satellite cell apoptosis, injection of mice with EdU to examine proliferation, and myogenin staining for differentiation are all future work that should be completed.

It is well established that skeletal muscle regeneration is dependent on satellite cell function. Since there was a significant reduction seen in satellite cell number 7 days post-injury in cIAP1-null mice, I used a cIAP1 conditional knockout mouse in which cIAP1 was specifically excised from $Pax7^+$ cells to determine the role of cIAP1 in satellite cell biology. The results

demonstrate a significant reduction in cross-sectional area in cIAP1 conditional knockout mice 7 days after CTX-induced injury (Figure 6C). Straughn et al. (2018) showed that inhibition of the canonical pathway in satellite cells delayed skeletal muscle regeneration; however, previous studies suggest that activation of the canonical pathway impairs muscle regeneration (He et al., 2013; Li et al., 2008). Perhaps the satellite cell specific excision of cIAP1 blunts the classical signaling pathway, leading to less proliferation and a reduction in the availability of satellite cells to fuse and regenerate muscle. This mechanism of reduced activation of the canonical NF-κB pathway upon cIAP1 loss in satellite cells and thus less satellite cell proliferation could be a possible explanation as to the cause of significantly reduced cross-sectional area after CTX-induced injury. Further studies will need to be done looking at later timepoints after CTX injury to see if muscle regeneration in cIAP1 conditional mice is inhibited or just delayed.

4.2 Loss of cIAP1 affects fast and slow twitch fibers differently

Previous studies from our lab have shown that loss of cIAP1 protects different muscles in dystrophic and denervated muscle (Enwere et al., 2013; Lala-Tabbert et al., 2019). For example, loss of cIAP1 protected the soleus, but not the EDL from dystrophic damage (Enwere et al., 2013). Specifically, the soleus from *mdx* mice showed reduced centronucleated fibers, decreased immune cell infiltration, improved contractile properties, increased exercise endurance and increased myoblast fusion upon loss of cIAP1 (Enwere et al., 2013). The soleus is predominantly a slow-twitch muscle while the EDL is predominantly fast-twitch. The demands placed on fast-twitch fibers are vastly different than the demands placed on slow-twitch, and this is likely the reason for the discrepancies demonstrated. Fast-twitch fibers are more susceptible to activity-induced damage, and in DMD they are the fibers that are primarily affected (Webster et al.,

1988). The improvements seen in exercise capacity of *mdx* mice is likely due to the improvements in the soleus and diaphragm as per the loss of cIAP1. In contrast, slow-twitch fibers are primarily affected by damage following sciatic nerve transection (Wang & Pessin, 2013). Indeed, previous work from our lab demonstrated the EDL, but not the soleus, was protected from denervation-induced atrophy (Lala-Tabbert et al., 2019). Loss of cIAP1 resulted in significantly reduced muscle mass in the EDL, as well as reduction in cross-sectional area in the EDLs of cIAP1-null mice (Lala-Tabbert et al., 2019). Further studies will need to be done to examine the mechanisms underlying fiber-type specific protection of cIAP1 loss in various muscle diseases.

Rate of force development (RFD), is the rate of rise during an explosive contraction (Aagaard et al., 2002), and has been shown to be faster in type II muscles than type I (Buchthal & Schmalbruch, 1970; Harridge et al., 1996). The reasons for type II fibers having a faster RFD have been shown to be as a result of type II fibers having greater proportions of Ca^{2+} release during action potentials (Baylor & Hollingworth, 1988), and fast isoforms of myosin, troponin and tropomyosin (Schiaffino & Reggiani, 1996), resulting in faster cross-bridge cycling rates (Bottinelli et al., 1996). While the role of cIAP1 in Ca^{2+} homeostasis remains unknown, previous studies have suggested a role of Ca^{2+} in regulation of cell death; as the influx of Ca^{2+} through Ca^{2+} activated-Ca^{2+} release channels, and release from the ER have been suggested to be apoptogenic (Pinton & Rizzuto, 2006). Mitochondria are conveniently located to allow the flux of Ca^{2+} across their membranes, thus prompting ATP production (Pinton et al., 1998), but also function as apoptotic pathway checkpoints (Kroemer et al., 2007). Thus, the question arises if Ca^{2+} is involved in regulating the release of proapoptotic proteins, such as Smac, which interacts and inhibits cIAP1. If such is the case, loss of cIAP1 could possibly alter the function of the

mitochondria in Ca^{2+} regulation. While the release of Ca^{2+} required to cause a muscle contraction is not released from the mitochondria, the loss of cIAP1 could possibly alter Ca^{2+} regulation in other organelles, such as the ryanodine receptors which are responsible for Ca^{2+} release in muscle tissues.

Moreover, previous work demonstrated murine embryonic fibroblasts (MEFs) void of cIAP1 exhibited altered morphology in their cytoskeleton, such that the appearance and function of actin filaments, specifically filopodia, was negatively affected (Marivin et al., 2014; Oberoi et al., 2012). In both macrophages and fibroblasts, the formation of actin-rich thin membrane protrusions known as filopodia is activated by TNF induced induction of cdc42 (Mathew et al., 2009; Peppelenbosch et al., 1999; Puls et al., 1999). When TNF binds to its receptor, TNFR1, cIAP1 is recruited and thus is involved in TNF mediated regulation of cdc42 (Marivin et al., 2014). As a result, Marivin et al. (2014) suggested cIAP1 is involved with filopodia formation and actin rearrangement. As actin in a critical protein in the sarcomere that is involved in cross bridge formation and muscle contraction, rearrangement of the actin bundles may influence contractile properties. Thus, the rate of force development and peak force would be decreased as the cross-bridges and contractile machinery are affected.

Future work needs to be done to assess calcium concentration and regulation by cIAP1/2, as well examining the content and morphology of actin to determine if fiber contraction kinetics are altered because of these possibilities upon loss of cIAP1 or cIAP2.

4.3 Loss of cIAP2 decreases fiber size 7 days-post injury

In this study, I also examined the effect of cIAP2 loss on muscle histology and function. cIAP2 has been largely ignored in muscle biology because it is not expressed in skeletal muscle

or myoblasts. However, since cIAP2 is expressed in macrophages which are necessary for successful muscle regeneration, I examined the role of cIAP2 in muscle development and regeneration using a full body cIAP2 knockout mouse. Interestingly, I found that mice lacking cIAP2 did not regenerate as well as wild-type mice as demonstrated by their smaller fiber cross-sectional area (Figure 12C). This finding suggests that cIAP2 may be affecting the function of infiltrating immune cells. Furthermore, cIAP2 has been shown to be an essential component involved in maintaining a properly functioning inflammatory response (Conte et al., 2006). A more thorough investigation of the role of cIAP2 in muscle immune cell biology needs to be conducted in order to fully elucidate the role of cIAP2 in muscle regeneration.

By 28 days post-injury, injured fibers have returned to homeostasis, there is central nucleation of fibers, and satellite cells have returned to quiescence. Interestingly, I found the TA fibers of cIAP2-null mice show no significant differences 28 days after CTX-induced injury, despite cIAP2 not being expressed in muscle fibers or myoblasts (Figure 13C). It appears cIAP2 has an effect at early differentiation, but regeneration catches up to wild-type levels later. Indeed, the role of TWEAK, an activator of the NF-κB pathway, has been shown to affect muscle regeneration (Girgenrath et al., 2006). The delay in muscle regeneration seen in Fn14-knockout mice was attributed to an impaired immune response after CTX-induced injury (Girgenrath et al., 2006). As cIAP2 has been shown to be a regulator of the NF-κB pathway and is expressed in macrophages, targeted loss of cIAP2 is likely delaying regeneration through decreased concentrations of macrophages, but the fibers are eventually able to repair and catch up to wild-type levels. Examining immune cell infiltration in injured skeletal muscle sections may provide insight on macrophage infiltration in cIAP2-null mice.

4.4 Potential involvement of cIAP2 in muscle homeostasis and fiber maintenance

Previous studies have focused on the role of cIAP1/2 during muscle regeneration and diseased states, with little work done on healthy, uninjured muscle. Thus, I examined the uninjured TA, soleus and EDL from healthy uninjured wild-type and cIAP2-null mice. Of note, I observed that fast-twitch muscles, such as the TA, from cIAP2-null mice had significantly smaller fibers compared to muscles from wild-type mice (Figure 12C, 13C). By approximately 7 and 10 weeks of age, the fibers of cIAP2-null TAs were significantly smaller than age-matched wild-type mice, suggesting cIAP2 may play a role in muscle development or postnatal muscle growth. An increase in the number of myofibers (hyperplasia) and increase in size (hypertrophy) is how postnatal growth occurs (Ross et al., 1987). A previous study by White et al. (2010) demonstrated the number of fibers in the mouse EDL does not change between postnatal day 7 and postnatal day 56, suggesting changes in muscle growth can be attributed to an increase in cross-sectional area of postnatal myofibers. Little work has been done to examine the role of cIAP2 in healthy muscle development and myogenesis, but perhaps cIAP2 is important for postnatal muscle growth. Since the most crucial time period of postnatal muscle growth is within the first two weeks, future work should examine uninjured TA of mice at postnatal day 7 to determine if cIAP2 is required for myonuclear accretion or myofiber growth.

Additionally, several studies have reported that muscle fiber size is positively correlated to capillary density, such that an increase in fiber size is paired with extensive capillary proliferation as demonstrated in rodents (Egginton et al., 1998). Perhaps cIAP2 is involved in angiogenesis and muscle fiber maintenance and, upon loss of cIAP2, fiber growth is limited and oxidative metabolism is altered. Indeed, reduced cIAP2 led to a decrease in capillary density in a murine myocardial infarction model (Selvaraju et al., 2020). Furthermore, the decrease in cIAP2

resulted in a decrease in the number of capillaries per myocyte, decreased capillary density in the gastrocnemius muscle, and an increase in fibrosis (Selvaraju et al., 2020). In addition, cIAP2 has been shown to affect angiogenesis through interactions with the protein ARIA using human umbilical vein endothelial cells (HUVECs) (Ikeda et al., 2009). This hypothesis regarding the involvement of cIAP2 in altering angiogenesis, oxidative metabolism and fiber growth would need further investigation by evaluating the degree of angiogenesis by measuring the number of $CD31^+$ cells and performing fatigue experiments such as treadmill running.

4.5 Possible role of cIAP2 in glucose metabolism and involvement with slow-twitch fibers

A further finding of this study was the interesting effects that the loss of cIAP2 had on the soleus muscle. I determined loss of cIAP2 led to a significantly larger cross-sectional area compared to wild-type mice (Figure 14B). The soleus is a slow-twitch, oxidative, Type I fiber that is composed primarily of MHCI and MHCIIa fibers. Previous studies have reported a role for NF-κB in cardiac hypertrophy (Gupta et al., 2008), specifically as TNFα mediated activation of NF-κB results in a downregulation of PGC1α (Álvarez-Guardia et al., 2010; Palomer et al., 2009). PGC1α has functions in mitochondrial biogenesis and oxidative phosphorylation in the cardiovascular system (Finck & Kelly, 2007; Ventura-Clapier et al., 2008). Activation of the NF-κB pathway and subsequent downregulation of PGC1α resulted in cardiomyocyte hypertrophy and a shift from oxidative metabolism to glycolysis in a murine model (Palomer et al., 2009). While hypertrophy of cardiomyocytes is a negative result, hypertrophy of skeletal muscle can be beneficial. As cIAP2 is a key regulator of the NF-κB pathway, the role of the NF-κB pathway in skeletal muscle hypertrophy should be further investigated. Furthermore, the activation of the NF-κB pathway and subsequent shift towards glycolysis supports the results demonstrated in this

study, such that the increase in fiber size of the soleus and rightward shift of the force-frequency curve demonstrates fast-twitch fiber tendencies, and these fibers rely on glycolysis for ATP production. Thus, the role of the NF-κB pathway in skeletal muscle fiber hypertrophy would be an interesting path to investigate.

I had also found that cIAP2-null mice solei had a force-frequency curve that was significantly shifted to the right of the wild-type solei curve (Figure 17C). An increased cross-sectional area and a rightward shift on the force-frequency curve suggest the soleus is taking on more fast-twitch characteristics. There are two causes for a shift in the force-frequency relationship; (1) a change or shift in fiber-type composition and/or (2) a change in the capacity of the Ca^{2+}ATPase pump to transport Ca^{2+} back into the sarcoplasmic reticulum (SR). For the rightward shift seen in this study, there would need to be an increase in the fast fibers in the soleus; however, I did not see a change in MHC isoforms in cIAP2-null solei compared to wild-type solei (Fig. 14G). Therefore, it is unlikely the shift in the curve is a result of differences in fiber-type composition. Instead, this observation could be due to changes in the function of the Ca^{2+}ATPase pump, known as the Sarcoendoplasmic Reticulum Calcium ATPase (SERCA). Paul et al. (2017) demonstrated inhibition of NF-κB resulted in increased intracellular Ca^{2+} and a decrease in SERCA activity in platelets, thus suggesting the NF-κB pathway has a role in regulation of SERCA activity and is important for Ca^{2+} homeostasis. Although the study by Paul et al. (2017) implicating the NF-κB pathway in SERCA regulation and Ca^{2+} homeostasis was performed using platelets, it suggests the possibility SERCA and Ca^{2+} regulation can be affected in other tissues by NF-κB, including skeletal muscle. While there is a rightward shift on the force-frequency curve in the soleus upon loss of cIAP2, it is unknown whether this is a result of a change in the SERCA isoform resulting in the differences, or if the usual isoform of SERCA

present in slow-twitch fibers has a different morphology or function, altering its Ca^{2+} release and uptake abilities. Thus, the role of cIAP2 on regulation of SERCA should be investigated further in the context of skeletal muscle.

4.6 Loss of cIAP2 affects fast-twitch fiber contraction kinetics

In contrast to the soleus muscle, the EDL muscle is composed primarily of fast-twitch MHC isoforms. Perhaps the most interesting result found with the EDL is the fact that cIAP2-null mice displayed a significant shift to the left of the wild-type on the force-frequency curve (Figure 18C), paired with a significantly reduced Frequency 50 value (Figure 18B). Together, these results suggest a fiber-type switch in the EDL are because of the same two possible reasons as mentioned with the soleus; (1) a change in fiber-type composition, and/or (2) a change in the capacity of the SERCA pump to transport Ca^{2+} back into the SR. Similar to the soleus, there was no fiber type shift seen in the cIAP2-null mice (Figure 15G), leading us to believe there is altered function of SERCA. Currently, the effect of cIAP1 and cIAP2 loss on SERCA in skeletal muscle remains unstudied, and so further investigation into the mechanisms behind this will help to further our understanding of the role that cIAP1 and cIAP2 play in muscle homeostasis and function.

The reason behind what appears to be the soleus behaving as a fast-twitch muscle and the EDL behaving as a slow-twitch muscle upon loss of cIAP2 is unknown, and quite frankly perplexing. The loss of cIAP1 and cIAP2 affects fast and slow-twitch fibers differently, but the cause of each muscle taking on characteristics of the other type is interesting. The mechanisms underlying these findings need to be elucidated to fully understand the role cIAP1 and cIAP2

play in skeletal muscle homeostasis and what therapeutic approaches would be appropriate to treat various muscular disorders.

4.7 Significance

Notably, cIAP1 and cIAP2 have been identified as key regulators of the NF-κB pathway (Mahoney et al., 2008; Varfolomeev et al., 2007; Vince et al., 2007; Zarnegar et al., 2008), and the NF-κB pathway has been shown to be important in skeletal muscle biology (Mahoney et al., 2008). Hence, the potential of cIAP1 and cIAP2 as therapeutic targets for treating muscular disorders has emerged. cIAP1 has been shown to be a negative regulator of myoblast fusion (Enwere et al., 2013; Enwere et al., 2012; Lala-Tabbert et al., 2019), and expression of cIAP1 is upregulated in denervated and dystrophic muscle (Enwere et al., 2013; Lala-Tabbert et al., 2019). In addition, genetic ablation attenuated denervation-induced atrophy and improved exercise capacity and resulted in improved muscle histology in *mdx* mice (Enwere et al., 2013; Lala-Tabbert et al., 2019). Thus, targeted pharmacological ablation of cIAP1 and cIAP2 is an attractive approach for treatment of muscular disorders. In this regard, potent small molecular inhibitors called Smac mimetic compounds (SMCs) can potently target cIAP1/2 for proteasomal degradation, thus inhibiting constitutive activation of the NF-kB pathway and possibly ameliorating disease pathology in muscle wasting disorders. Indeed, preliminary studies examining the use of SMCs in denervation induced atrophy have shown that treatment with SMCs can reduce the loss of fiber size in the EDL. Furthermore, SMCs are currently undergoing clinical trials for the treatment of cancer, and their use in treatment of muscular disorders may form a relatively direct path from bench to bedside.

4.8 Conclusion

In this study I have characterized the skeletal muscle of cIAP1- and cIAP2-null mice. Specifically, I found that loss of cIAP1 is important for satellite cell expansion and affects the twitch kinetics of fast and slow-twitch fibers differently. Furthermore, I found that loss of cIAP2 is important for muscle fiber homeostasis, oxidative metabolism, and alters muscle contraction kinetics in the soleus and EDL. Further work needs to be done to elucidate the downstream mechanisms by which cIAP1 and cIAP2 regulates these functions which will be beneficial to the development of novel therapeutic approaches for the treatment of muscle diseases.

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CONTRIBUTIONS OF COLLABORATORS

I would like to thank Dr. Jean-Marc Renaud (University of Ottawa) and Dr. Tarek Ammar (University of Ottawa) for their support, equipment, and expert advice needed to perform the *in situ* force experiments.