REGULATION OF SKELETAL MUSCLE FORMATION AND REGENERATION BY THE CELLULAR INHIBITOR OF APOPTOSIS 1 (cIAP1) PROTEIN

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Abstract

The inhibitor of apoptosis (IAP) proteins traditionally regulate programmed cell death by binding to and inhibiting caspases. Recent studies have uncovered a variety of alternate cellular roles for several IAP family members. The cellular inhibitor of apoptosis 1 (cIAP1) protein, for instance, regulates different axes of the NF-κB signalling pathway. Given the extensive functions of NF-κB signalling in muscle differentiation and regeneration, I asked if cIAP1 also plays critical roles in skeletal muscle myogenesis. In a primary myoblast cell-culture system, genetic and pharmacological approaches revealed that loss of cIAP1 dramatically increases the fusion of myoblasts into myotubes. Remarkably, this increase in fusion is associated with a delay in differentiation, as evidenced by reduced expression of markers such as myosin heavy chain, myogenin, and α-actin, as well as a delay in cell cycle withdrawal. NF-κB signalling occurs along a classical and an alternative pathway, both of which are active in wildtype primary myoblasts. Differentiation normally leads to gradual inactivation of NF-κB signalling in myoblasts. However, differentiating *cIAP1*$^{-/-}$ myoblasts exhibit sustained activation of the classical NF-κB signalling, which delays differentiation, as well as of the alternative pathway. Suppression of the alternative pathway attenuates myotube fusion in wildtype and *cIAP1*$^{-/-}$ myoblasts. Conversely, constitutive activation of the alternative pathway is sufficient to increase myoblast fusion in wildtype myoblasts. *cIAP1*$^{-/-}$ mice exhibit increased muscle size and mass as compared to wildtypes, as well as an increased number of muscle stem cells known as "satellite" cells. These results identify cIAP1 as a regulator of myogenesis through its modulation of classical and alternative NF-κB signalling pathways.

The absence of the structural protein dystrophin in the *mdx* mouse model of Duchenne muscular dystrophy leads to chronic degeneration and regeneration of skeletal muscle. These processes (muscle degeneration and regeneration) are also strongly influenced by NF-κB signaling, both in the muscle tissue itself and in macrophages that abound in the dystrophic milieu. Given the roles demonstrated for cIAP1 in cell culture and *in vivo*, I asked whether loss of cIAP1 would influence muscle pathology in the *mdx* mouse. To address this question, double-mutant mice were bred lacking both cIAP1 and dystrophin (*cIAP1*$^{-/-}$*;mdx*). Histological analyses revealed that double-mutant mice exhibited reduced indications of damage on several measures, as compared to single-mutant (*cIAP1*$^{+/+}$*;mdx*) controls. Unexpectedly, these reductions were seen in the "slow-twitch" soleus muscle but not in the "fast-twitch" extensor digitorum longus (EDL) muscle. The improvements in pathology of double-mutant solei were associated with reductions in muscle infiltration by CD68-expressing macrophages. On a test of physiological responsiveness, double-mutant solei performances were indistinguishable from non-dystrophic C57BL/6 mouse solei, whereas dysfunction was evident in single-mutant solei. Finally, the double-mutant mice exhibited improved endurance and resistance to damage during treadmill-running exercise. Taken together, these results suggest that loss of cIAP1, through its multiple regulatory functions, acts to improve myogenesis and increase muscle resistance to damage.

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List of Abbreviations

ATM	Ataxia telangiectasia mutated
bFGF	Basic fibroblast growth factor
bHLH	Basic helix-loop-helix
BIR	Baculoviral IAP repeat
BMP	Bone morphogenic protein
BrdU	Bromodeoxyuridine
cIAP1/2	Cellular inhibitor of apoptosis-1/2
DD	Death domain
DIABLO	Direct IAP binding protein with low pH
DISC	Death-inducing signalling complex
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
EDL	Extensor digitorum longus
ELISA	Enzyme-linked immunosorbent assay
FADD	Fas-associated Death Domain
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde phosphate dehydrogenase
GFP	Green fluorescent protein
HGF	Hepatocyte growth factor
IAP	Inhibitor of apoptosis
IKKα	Inhibitor of IκB kinase-α
IKKβ	Inhibitor of IκB kinase-β
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MHC	Myosin heavy chain
NEMO	NF-κB essential modulator
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-κB
NIK	NF-κB interacting protein
NOD	Nucleotide oligomerization domain
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
RING	Really Interesting New Gene
RIP1	Receptor-interacting protein-1
SMC	Smac-mimetic compound
TA	Tibialis anterior
TNFα	Tumor necrosis factor-α
TRADD	TNFR1-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TWEAK	TNF-related weak inducer of apoptosis
XAF1	XIAP-associated factor-1
XIAP	X-linked inhibitor of apoptosis

Chapter 1: General Introduction

Skeletal or striated muscle tissue plays roles that include, but are not limited to, maintenance of posture, locomotion, respiration, and circulation. It is consequently subject to extreme physical stress and damage, and requires a rapid and efficient regeneration process. A population of stem cells known as "satellite cells" (Mauro, 1961; Zammit and Beauchamp, 2001; Collins *et al.*, 2005) is responsible for regeneration of skeletal muscle. These cells emerge from mitotic quiescence to proliferate, differentiate, and fuse into new muscle, and thereinafter revert again to the quiescent state. Our understanding of the biochemical and physiological specifics of skeletal muscle regeneration has improved measurably over the past two decades, but essential questions remain unanswered about all steps of the regeneration process. This introductory chapter will address some of these questions, and examine evidence for the atypical roles of regulators of apoptosis, in particular the inhibitor of apoptosis (IAP) proteins, in muscle development and regeneration.

1. Skeletal muscle development and regeneration

Skeletal muscle is unusual in that when mature, it consists of multinucleated fibres, or myofibers, rather than individual cells. It develops from mononucleated cells called myoblasts, which differentiate and fuse to form muscle fibres. In the mammalian embryo, most skeletal muscle originates in the somites (Brand-Saberi and Christ, 1999), which are spherical units of mesoderm that form adjacent to the neural tube, along the rostro-caudal axis of the embryo. The somites produce diverse cell types in a number of distinct source or "germinal" zones. Specific to a discussion of myogenesis, the germinal zone in the dorsal somite is the dermomyotome, and produces progenitors for both skin and skeletal muscle. The dermomyotome consists of the epaxial dermomyotome, which produces muscle of the

back; and the hypaxial dermomyotome, which gives rise to musculature of the trunk and limbs. Muscle progenitors in the dermomyotome respond to signalling molecules from the overlying ectoderm to either migrate out to distal (limb) locations, or differentiate *in situ* to form the first muscle fibres. Characteristic of these progenitors is the expression of markers that guide them down a genetic hierarchy from relatively plastic precursors to committed progenitors. It is still unclear, however, exactly how the process starts or progresses to produce muscle myoblasts of different fates. Among the earliest known markers are transcription factors including *Six1/4, Eya1/2,* and *Pax3*. *Six1* and *Six4* are required for formation of muscles in the limbs, diaphragm, and abdomen (Laclef et al., 2003; Grifone et al., 2005), as are *Eya1* and *Eya2* (Grifone et al., 2007). Genes such as *Lbx1* and *c-Met* are necessary for migration of hypaxial muscle progenitors to their final locations in the limbs (Bladt et al., 1995; Schafer and Braun, 1999). *Pax3* is expressed downstream of the aforementioned transcription factors in the commitment hierarchy, and along with *Pax7* acts upstream of the muscle regulatory factors to specify

premyogenic precursors from the mesoderm as skeletal muscle progenitors.

Muscle regulatory factors are basic helix-loop-helix (bHLH) transcription factors expressed early in development, and are crucial determinants of myogenic fate. They include MyoD, Myf5, myogenin and MRF4 (Brand-Saberi, 2005). *In vitro*, MyoD and Myf5 are sufficient to convert non-myogenic cell types, such as fibroblasts and adipocytes, to muscle (Tapscott *et al.*, 1988; Edmondson and Olson, 1989; Montarras *et al.*, 1989; Sorrentino *et al.*, 1990). *In vivo*, a number of factors suggest that MyoD and Myf5 act redundantly to specify

the muscle progenitor fate. Loss of MyoD or Myf5 alone has relatively minimal effect on myogenesis, and either one can substitute for the absence of the other (Rudnicki et al., 1993). Loss of Myf5 leads to the normal development of muscle under the control of MyoD (Gensch et al., 2008; Haldar et al., 2008). An early study of mice lacking both MyoD and Myf5 found that they do not form skeletal muscle at all (Rudnicki *et al.*, 1993). Further studies of the presumed *MyoD*$^{-/-}$;*Myf5*$^{-/-}$ mice revealed that the placement of the *Mrf4* gene adjacent to *MyoD* actually resulted in a triple knockout which aggravates the phenotype of the mouse; restoring Mrf4 expression results in production of some muscle (Kassar-Duchossoy et al., 2004), and conditional ablation of Mrf4-expressing muscle impairs formation of mature fibres (Gensch et al., 2008). Both MyoD and Myf5 function by forming heterodimers with the ubiquitously expressed immunoglobulin enhancers E12, HEBβ, and E47, whereupon they bind to conserved sequences, known as E-boxes, in the promoter regions of genes necessary for muscle development (Olson, 1992; Lu et al., 1999). E-boxes are fairly common in the genome (CANNTG), however, and considerable efforts are directed towards identifying co-factors that recruit MyoD/Myf5 to particular sites. Furthermore, MyoD/Myf5 may act not just as transcription factors, but also as enhancers, as many of the binding sites identified in ChIP/seq experiments are on putative enhancers situated far from any known genes (Cao et al., 2010).

As mentioned previously, adult muscle maintains a robust ability to regenerate in response to physical damage. Satellite cells surround individual muscle fibres, and are contained within a sheath of laminin known as the basal lamina (Muir *et al.*, 1965; Church, 1970; Maltin, 1986). These cells express markers such as c-met (Cornelison and Wold, 1997; Tatsumi *et al.*, 1998), M-cadherin (Irintchev *et al.*, 1994; Maier and Bornemann,

2004), CD34 (Beauchamp *et al.*, 2000; Montarras *et al.*, 2005), and the transcription factor Pax7 (Seale *et al.*, 2000). Satellite cells are normally mitotically quiescent, but proliferate and migrate extensively in response to muscular injury. The proliferating satellite cells are known as myoblasts; some of these eventually differentiate and fuse into myofibers, while others down-regulate muscle markers and exit the cell cycle, once again becoming satellite cells. The paired-box transcription factors Pax3 and Pax7 are most commonly associated with *adult* satellite cell specification and function: the embryonic (somitic) progenitors described earlier are considered to have distinct origins and may or may not continue to adulthood in their progenitor form. Recent studies support a developmental role for Pax3, and an anti-apoptotic role for Pax7 later in myogenesis. For instance, in a fate mapping experiment, Gabrielle Kardon's group showed that while cells of the *Pax3* lineage developed into both embryonic and adult limb muscle, *Pax7*- and not *Pax3*-derived cells predominated in adult muscle. Targeting of a diphtheria toxin to the *Pax7* lineage permitted normal formation of embryonic muscle, but there was widespread loss of mature fibres by P0 (Hutcheson et al., 2009). These results were consistent with a model of developmental myogenesis in which embryonic muscle fibres form from one progenitor population (Pax3-expressing), and fuse with fetal fibres from a secondary, Pax7-expressing population. A Pax7 knockout produced by Michael Rudnicki's lab appeared to completely lack satellite cells (Seale et al., 2000); however, further examination revealed that these cells initially formed, but are progressively lost to apoptosis (Oustanina et al., 2004; Relaix et al., 2006). The protection accorded to satellite cells from apoptosis is temporary: conditional knockout of Pax7 in adult mice has no effect on the satellite cell population's survival, self-renewal, or ability to regenerate muscle (Lepper et al., 2009). Pax7 nevertheless identifies a lineage that

may be a bona fide stem cell: Pax7-expressing satellite cells that upregulate Myf5 become committed myogenic progenitors, while those that remain Myf5-negative can regenerate the satellite cell niche (Kuang et al., 2007).

2. Myoblast fusion

The process of differentiation includes a variety of alterations to the cytoarchitecture of the myoblast, and these include the fusion of myoblasts into the multinucleated syncytium known as a myotube (*in vitro*) or muscle fibre (*in vivo*). While differentiation is required for myoblast fusion, a myoblast may differentiate without undergoing fusion, suggesting, along with other evidence, that fusion has regulatory mechanisms independent of differentiation. Extensive insight into fusion regulatory pathways emerged from genetic studies in *Drosophila*, where myoblasts fuse to form exactly 30 muscles in the body wall of the developing embryo (Bate, 1990). In *Drosophila*, muscle formation results from the fusion of two cell types: a "founder cell" that starts the process, and a host of fusion-competent myoblasts (FCMs) that add mass to the resulting muscle. Subsequently, a host of molecules involved in cell-cell recognition and adhesion, trans- and intracellular signalling, cytoskeletal reorganization, and vesicular fusion act to control both the extent of fusion and the type of muscle that emerges.

The *Drosophila* system has the advantage of considerable simplicity over vertebrate muscle myogenesis. Unlike in *Drosophila*, vertebrate muscles form in bundles and often exhibit wider ranges of function and adaptive response. Furthermore, vertebrate myogenesis lacks the organization of a single founder cell-like locus about which myoblast fusion proceeds. These differences may suggest that *Drosophila* and vertebrates vary markedly in

their fusion mechanisms and genetic requirements; however, more studies are emerging that prove otherwise. For instance, cadherins serve to forge cytoskeletal points of contact between myoblasts and initiate cell contact-mediated signalling, both of which are essential in mammalian myoblast fusion (Takaesu et al., 2006; Kang et al., 2008). These serve analogous functions to *Drosophila* Kirrel (Srinivas et al., 2007). *Drosophila* Sticks and Stones (sns) is an Ig-containing cell-adhesion molecule that is essential for fusion in the fruit fly (Bour et al., 2000); its mammalian homolog, nephrin, is best known for its roles in blood filtration in the kidney (Kestila et al., 1998), but is also essential for late-stage fusion of myoblasts (Sohn et al., 2009). Nap1 is the vertebrate ortholog of *Drosophila* kette, and both are crucial members of the actin-remodeling complex that reorganizes the myoblast cytoskeleton during fusion (Nowak et al., 2009). Our understanding of fusion in mammals lags far behind the state of knowledge in *Drosophila*, and only time and further studies will offer a greater reconciliation of the mechanistic underpinnings behind myoblast fusion in both systems.

Fundamentally, there are two stages to mammalian myoblast fusion. First, myoblasts fuse with other myoblasts to form a nascent muscle fibre (myoblast-myoblast fusion). Second, other myoblasts are recruited into the developing fibre to form the resulting extensively multinucleated structure (myoblast-myotube fusion) (Horsley and Pavlath, 2004). Several proteins have been identified that specifically affect one or both stages of myoblast fusion. The best-characterized examples occur along the Ca^{2+}-calcineurin signalling axis. The influx of Ca^{2+} into the myoblast during differentiation has long been established as a triggering factor for myoblast fusion (Wakelam, 1985; Entwistle et al., 1988; Rapuano et al., 1989; Cooper, 2001). The source of calcium is usually extracellular, though

disruption of the sarcoplasmic and endoplasmic reticula using thapsigargin and tunicamycin release intracellular calcium stores that has the same fusion-promoting effect (Nakanishi et al., 2007). Ca^{2+} signalling converges on activation of calcineurin, a calcium-calmodulin-dependent protein phosphatase, which in turn dephosphorylates a group of transcription factors of the nuclear factor of activated T cells (NFAT) family. Dephosphorylation relieves NFAT proteins of their cytosolic sequestration, allowing translocation to the nucleus and transcription of downstream effectors (Michel et al., 2004). In spite of the similarities in regulation, the NFAT proteins appear to play different roles in myoblast fusion. Studies performed in Grace Pavlath's lab identified NFATc2 as a key regulator of the late myoblast-myotube stage of fusion (Horsley et al., 2001). This aspect of the pathway is probably aided by prostaglandins E1 and F2α that control potassium and calcium influx into the cells (Entwistle et al., 1988; Horsley and Pavlath, 2003). NFATc2 activates transcription of interleukin-4, which operates by unknown mechanisms to recruit myoblasts to nascent myotubes. The four-and-a-half-LIM-1 (FHL1) protein drives the early myoblast-to-myoblast stage of fusion by binding to and increasing transcriptional activity of NFATc1 (Cowling et al., 2008). Most of the NFAT transcriptional targets relevant to myoblast fusion remain unidentified, but it is anticipated that future approaches using genetic screens and other tools will clarify the identities and functions of the downstream targets.

3. Apoptosis and skeletal muscle regeneration

Apoptosis is an active process, involving protein synthesis and energy consumption, which leads to a cell's rapid and efficient death (Jacobson *et al.*, 1997). It was first differentiated from death resulting from necrosis or direct physical damage by Kerr and colleagues (Kerr *et al.*, 1972), who observed that certain incidents of cell death shared morphological features, such as cell shrinkage and rapid phagocytosis. There are many other hallmarks of apoptosis, such as chromatin condensation, DNA degradation (into ~100bp fragments which, when observed on a gel, is known as "DNA laddering") and plasma membrane blebbing; however, these features are not necessarily *all* present in every instance of apoptosis. This observation led to the definition of apoptosis as "any cell death that is mediated by the intracellular death program, no matter what triggers it, and whether or not it displays all the characteristic features of apoptosis" (Jacobson *et al.*, 1997). Apoptosis plays many critical roles in development, such as elimination of unneeded cells and tissue, creation and patterning of organs and structures, control of cell number, and control of infection by elimination of dead or compromised cells (Weil *et al.*, 1999; Bangs and White, 2000; Yeo and Gautier, 2004; Leevers and McNeill, 2005).

While the mechanisms that initiate apoptosis are varied and extensive, a superfamily of evolutionarily conserved cysteine proteases, known as caspases, carry out the actual cellular degradation. Upon activation, these proteases cleave various proteins, at conserved sites after aspartic acid residues, in a proteolytic cascade that results in the death of the cell. The first caspases identified were products of the *ced* genes in the *C. elegans* nematode: *ced-3* and *-4* are required for apoptosis, while *ced-9* antagonizes the process (Yuan *et al.*, 1993; Hengartner and Horvitz, 1994). There are many more homologues since identified in

organisms ranging from fruit flies to humans. To date there are 18 known mammalian caspases. Twelve of these are expressed in humans (Twomey and McCarthy, 2005; Eckhart et al., 2008) and ten in mice (Reed *et al.*, 2003), while such caspases as caspase-13 and -15 are found in cattle and other mammals (Eckhart *et al.*, 2005). Caspases fall into three general categories based on function. Inflammatory caspases (1, 4, 5 and 11 in humans) initiate inflammatory responses rather than cell death; the initiator caspases (2, 8, 9 and 10) function by cleavage-activating effector caspases (3, 6 and 7), which in turn carry out cell-wide proteolytic activity (Shi, 2002). All caspases are produced as inactive zymogens: cleavage results in formation of p10 and p20 fragments, which in close association constitute one caspase monomer (Riedl and Shi, 2004).

Recent evidence suggests that apoptosis and cellular differentiation are intricately linked processes, with precise and occasionally identical control mechanisms. Caspases are essential in differentiation of keratinocytes (Weil *et al.*, 1999; Okuyama *et al.*, 2004), erythrocytes (Testa, 2004), lens cells (Weber and Menko, 2005), osteoblasts (Mogi and Togari, 2003), and monocytes (Sordet *et al.*, 2002). Other studies identified various caspase-3 cleavage substrates important in myoblast differentiation (Sabourin *et al.*, 2000; Hilder *et al.*, 2005). Accordingly, separate groups demonstrated that caspase-3 (Fernando *et al.*, 2002) and caspase-9 (Murray et al., 2008) are essential for differentiation of myoblasts. Caspase-3 is cleavage-activated upon myoblast differentiation. Fernando and colleagues showed that differentiation is markedly impaired in caspase-3-null myoblasts, as well as in wildtype myoblasts differentiated in the presence of caspase-3 inhibitors. These data suggest, first, that caspase-3 activity is necessary for normal differentiation; and second, that the expected DNA damage and apoptosis are somehow blocked.

The normally terminal nature of caspase activation must require a control mechanism to prevent accidental apoptosis and ensuing cell death. This control is provided by various anti-apoptotic proteins, such as members of the Bcl-2 family, but also by the IAPs. IAPs constitute a group of evolutionarily conserved proteins with two characteristic features: the presence of a baculoviral IAP repeat (BIR) motif (Miller, 1999), and the ability to inhibit apoptosis by hindering caspase activity. BIR motifs are zinc-finger-like domains first identified in baculoviruses that could prevent the apoptosis that normally results due to infection (Crook *et al.*, 1993; Birnbaum *et al.*, 1994). While not all BIR-bearing proteins are IAPs, all IAPs have at least one BIR. Furthermore, while all IAPs can inhibit apoptosis, this ability is not necessarily their primary function. The IAP survivin, for instance, is part of a complex required for spindle formation during meiosis (Sampath *et al.*, 2004). IAPs are competitive inhibitors of caspases, and recent structural studies have made significant headway in improving our understanding of the mechanisms involved. The X-linked IAP (XIAP), for instance, has three BIR domains and a ubiquitin ligase moiety known as a "Really Interesting New Gene" (RING), and inhibits caspases-3, -7 and -9 (Liston *et al.*, 1997) by separate mechanisms (Tenev *et al.*, 2005). The region between BIR1 and BIR2, when bound to the BIR2 domain itself, binds across the substrate binding site of activated caspases-3 and -7 (Chai et al., 2001; Huang et al., 2001; Riedl et al., 2001). Caspase-9, on the other hand, is inhibited by the BIR3 domain (Riedl *et al.*, 2001). Furthermore, evidence from *in vitro* studies suggests that XIAP (Suzuki et al., 2001a; Morizane et al., 2005) and cellular IAP-1 (cIAP-1) (Huang *et al.*, 2000) can ubiquitinate caspases and thus target them for proteasomal degradation.

The ubiquitous presence of IAPs in many cells might pose difficulty when a cell must apoptose. This problem is resolved by the presence of endogenous IAP inhibitors, which, following an apoptotic stimulus, antagonize the IAPs to permit function of the caspases. Known IAP inhibitors include Smac/DIABLO, Omi/HtrA2, and the XIAP-associated factor-1 (XAF1). Smac and Omi are proteins containing an N-terminal motif that localizes them to the mitochondria. Following an apoptotic stimulus, the N-terminal domain is proteolytically cleaved, with two effects. First, both proteins are released from the mitochondria into the cytosol. Second, the cytosolic protein now has an N-terminal tetrapeptide motif (AVPI) that binds to the caspase 9-binding domain in BIR3 of XIAP and cIAP1/2, thus relieving the IAP inhibition of this caspase (Wu et al., 2000; Kulathila et al., 2009). In the process, Omi, which is a serine protease, can also trigger proteolytic degradation of the bound IAPs (Jones et al., 2003; Yang et al., 2003). It is worth noting that while there is little sequence or structural homology among the various IAP inhibitors, they all contain the N-terminal motif for IAP binding. In recent years, our knowledge of the N-terminal motif has extended to production of various synthetic IAP antagonists, or Smac mimetic compounds (SMC), for use as cancer therapeutics.

4. Initiation of apoptosis – Intrinsic and extrinsic pathways

A wide variety of stimuli can trigger apoptosis, but in general the signal to die filters down to a limited number of pathways. There are two such pathways, based on the source of the death trigger. The binding of ligands to cell surface receptors triggers the “extrinsic” death pathway. Such ligands include several members of the TNF gene superfamily such as TNFα, TRAIL, FasL, and TWEAK. Binding of ligand causes the receptors to trimerize, which

aggregates 70-amino-acid motifs at their C-termini called “death domains” (DD). Receptor aggregation facilitates recruitment of other molecules such as Fas-associated death domain (FADD) and caspase-8, forming a death-inducing signalling complex (DISC) (Chan et al., 2000; Sprick and Walczak, 2004). Formation of the DISC results in auto-activation of caspase-8, which goes on to activate effector caspases that carry out the downstream aspects of the cell death program. The anti-apoptotic protein cFLIP (for cellular FLICE-inhibitory protein) can replace caspase-8 in the DISC to form an inactive complex.

Certain cytokines, such as TNFα, can either trigger or inhibit apoptosis, and research into events downstream of TNFα binding to its receptor in different cell types has uncovered diversity in signalling options. TNFα recruits one of two possible complexes to the TNF receptor-1 (TNF-R1), depending on the platform adaptor molecule engaged. In addition to the FADD-based complex indicated above, the TNF receptor-associated death domain (TRADD) protein can engage both apoptotic and non-apoptotic signalling pathways at the TNF receptor, explaining in part the diversity of cellular responses seen with TNFα stimulation. TRADD recruits the kinase RIP1, as well as TRAF2/5, cIAP1/2, and a linear ubiquitin chain assembly complex consisting of ubiquitin ligase HOIL-1 and its partner HOIP (Haas et al., 2009; Tokunaga et al., 2009). RIP1 here serves as a pivotal molecule for initiation of survival or apoptosis signalling. cIAP1 and cIAP2 act as E3 ubiquitin ligases catalyzing both K48- and K63-linked ubiquitination of RIP1. While RIP1 is K63-ubiquitinated, it serves as a scaffold for binding of the kinases NEMO and IKKβ, forming what is referred to as “Complex 1”. These kinases subsequently activate pro-survival and pro-inflammatory cellular pathways via NF-κB. In the absence of cIAP1/2, a deubiquitinated RIP1 binds to caspase-8, forms a DISC, and triggers apoptosis (Bertrand et al., 2008;

Mahoney et al., 2008; Geserick et al., 2009). cIAP1/2 thus regulate cell survival in response to TNFα stimulation. In certain cancer cells, the loss of cIAP1/2 is sufficient to trigger apoptosis, without addition of exogenous TNFα. This occurs because, in a process not yet understood, the loss of cIAP1 can *activate* the NF-κB signalling pathway. This leads to autocrine production of TNFα, which is a transcriptional target of NF-κB, and subsequent caspase-8-dependent apoptosis.

The intrinsic apoptosis pathway is ligand-independent, and results from diverse intracellular stimuli such as DNA damage and ER-stress. It is distinct from the extrinsic pathway by involving the mitochondria and activation of caspase-9. The key initiators of the intrinsic pathway are Bax and Bak, members of the BCL-2 family of proapoptotic (and antiapoptotic) proteins. In response to a death stimulus, other proapoptotic members of the Bcl-2 family such as BIM, tBID and PUMA trigger mitochondrial targeting (of Bax) and homo-oligomerization of Bax and Bak. This process leads to eventual permeabilization of the outer mitochondrial membrane, releasing various apoptogenic factors into the cytosol including AIF, Apaf-1, and the IAP antagonist Smac/DIABLO (Breckenridge et al., 2003; Scorrano et al., 2003; Wong and Puthalakath, 2008). In the presence of dATP, several of these released factors form a holoenzyme complex with caspase-9 called the "apoptosome", in which caspase-9 is activated and, in turn, activates the effector caspases and causes cell death (Zou et al., 1999). The associated release of Smac into the cytoplasm serves to sequester/inhibit the IAPs (XIAP, cIAP1/2) and prevent their inhibition of apoptosis. The intrinsic pathway just described is of particular importance for apoptosis in what are classified as Type II cells. In these, the extrinsic pathway leading to activation of caspase-8 can only trigger apoptosis with subsequent engagement of the mitochondrial pathway. In

Type I cells, the extrinsic pathway is sufficient to cause cell death. Nevertheless, in both apoptosis pathways and in both cell types, IAPs play key roles, and their inhibition and targeted degradation thus presents a robust means to promote cell death in a variety of physiological situations.

5. The case for IAPs in muscle biology

While the study of caspases and associated proteins in muscle biology is an emerging area of research, the possibilities offered by IAPs in myogenic and developmental signalling pathways are equally intriguing but far less studied. The IAPs are present at key junctures in several pathways relevant to muscle formation and homeostasis. For instance, the NF-κB signalling pathways are involved in muscle differentiation (Bakkar et al., 2008) and atrophy (Cai et al., 2004); several studies place XIAP as a mediator of interaction between TAB1 and TAK1, which are effectors of NF-κB as well as transforming growth factor superfamily signalling pathways (Yamaguchi et al., 1999); cIAP2 protects peritoneal macrophages from the cytokine storm that results from sepsis (Conte et al., 2006) and permits normal NOD-dependent immunological signalling, again in macrophages (Bertrand et al., 2009). This last association of IAPs with macrophage biology applies to muscle regeneration as well, since macrophage infiltration of muscle becomes a major factor during injury and degenerative disease states. Considering that cIAP1/2 are often co-expressed in many tissues and function redundantly, the absence of cIAP2 from skeletal muscle (Mahoney et al., 2008) offers a rare opportunity to examine the function of cIAP1 alone in this milieu.

A closer inspection of the literature on cIAP1 allows a number of predictions about the functions of cIAP1 in skeletal muscle biology. The first prediction is that cIAP1 would

live up to its name and serve as an inhibitor of apoptosis. Earlier studies suggest that myoblasts undergo considerable apoptosis in the process of differentiation (Dee et al., 2002; Schwartz et al., 2009; Smith et al., 2009). The reason for this apoptosis is not known; however, it may relate to the autocrine release of TNFα from proliferating myoblasts (Langen et al., 2004). Since cIAP1 is required for TNFα-dependent NF-κB activation and consequent inhibition of the parallel pro-apoptotic pathway (Mahoney et al., 2008), this hypothesis is not without merit. Furthermore, as a positive regulator of NF-κB signalling, on this premise, cIAP1 would also inhibit myoblast differentiation. A second possibility is that cIAP1 would operate in a ligand-independent fashion (assuming the autocrine TNFα is insufficient to trigger ligand-dependent signalling) and *inhibit* the classical NF-κB pathway. In this case, we would hypothesize that cIAP1 would sensitize myoblasts to differentiation-associated apoptosis. A third possibility relies on recent evidence that cIAP1 is a negative regulator of the alternative NF-κB pathway (Zarnegar et al., 2008). On this premise, we would hypothesize that cIAP1 would inhibit myotube survival and mitochondrial biogenesis (Bakkar et al., 2008). A fourth possibility is that cIAP1 may play hitherto unforeseen roles in myogenesis, based on its unknown range of protein-protein interactions and targets as an E3 ubiquitin ligase. I conclude this chapter with a "best guess" statement of hypotheses based on the tools available and the experiments described later.

6. Hypotheses

1) Loss of cIAP1 inhibits differentiation of adult myoblasts *in vitro* and *in vivo*.

2) Downregulation of cIAP1 triggers ligand-independent activation of the NF-κB signalling pathway.

3) Loss of cIAP1 increases the resistance of muscle fibres to damage in the *mdx* model of Duchenne muscular dystrophy.

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Chapter 2: Loss of cellular inhibitor of apoptosis 1 (cIAP1) increases myoblast fusion by activating the alternative NF-κB signalling pathway

Abstract

The fusion of myoblasts into muscle fibres is a critical aspect of muscle development and regeneration. While fusion is generally categorized as an aspect of myoblast differentiation, the specific mechanisms that distinctly regulate the process are still poorly understood. Many studies along these lines are successfully conducted using cell lines such as the murine C2C12 (Yaffe and Saxel, 1977) and MM14 (Linkhart et al., 1981), and rat L6 (Yaffe, 1968). These, however, are transformed, often tumorigenic cell lines, and do not always accurately represent the behaviour of myoblasts and satellite cells *in vivo*. This often necessitates the use of primary myoblasts isolated from intact skeletal muscle. The existing procedures for myoblast isolation are laborious, time-consuming, and involve extensive steps to remove fibroblast contamination. Here I present a method for myoblast isolation that produces viable myoblasts in 4 – 6 days with 95 – 99% purity. Using these cells, and genetic and pharmacological approaches, I show that loss of the cellular inhibitor of apoptosis 1 (cIAP1) from primary myoblasts dramatically increases their fusion into myotubes. Remarkably, this increase in fusion is associated with a delay in differentiation, as evidenced by reduced expression of myosin heavy chain, myogenin, and α-actin, as well as a delay in cell cycle withdrawal. In accordance with this result, differentiating *cIAP1*$^{-/-}$ myoblasts exhibit sustained activation of the classical NF-κB signalling, which is known to delay differentiation (Guttridge et al., 1999; Wang et al., 2007). Moreover, *cIAP1*$^{-/-}$ myoblasts also exhibit elevated activation of the alternative NF-κB pathway. I show that suppression of the alternative pathway attenuates myotube fusion in wildtype and *cIAP1*$^{-/-}$ myoblasts. Conversely, constitutive activation of the alternative pathway is sufficient to increase

myoblast fusion in wildtype myoblasts. Notably, *cIAP1*$^{-/-}$ mice had increased muscle size and mass, as well as an increased number of Pax7-expressing satellite cells. These results demonstrate that cIAP1 regulates myoblast fusion and myogenesis through its modulation of classical and alternative NF-κB signalling pathways.

Introduction

The processes of skeletal muscle development and regeneration are under tight control by external cues such as hormones, cytokines, and growth factors (Yablonka-Reuveni, 1995; Forcales and Puri, 2005). Such cues are of particular importance at key points in the process of muscle formation, such as during myoblast proliferation, differentiation, or fusion of myoblasts into mature muscle fibres. Considerable research focuses on discerning the nature and actions of both the controlling stimuli and the associated responses, as both are actively involved in disease situations such as muscle dystrophy and atrophy. Nevertheless, these processes remain poorly understood in the context of muscle biology.

The IAPs were originally identified based on their ability to prevent apoptosis by binding to and inhibiting caspases (Crook et al., 1993; Roy et al., 1995; Liston et al., 1996; Roy et al., 1997). They are characterized by the presence of one or more baculoviral IAP repeat (BIR) domains near their N-termini, which participate in caspase inhibition. The best-studied IAPs include the cellular IAP-1 (cIAP1), cIAP2, and X-chromosome-linked IAP (XIAP). Each of these has three BIR domains (Rothe et al., 1995; Duckett et al., 1996) as well as a C-terminal RING zinc finger that acts as an E3 ubiquitin ligase. The IAPs themselves are inhibited by mitochondrial proteins Smac and Omi/HtrA2 (Du et al., 2000; Liu et al., 2000; Suzuki et al., 2001b; Martins et al., 2002). Upon apoptotic stimulus, Smac is released from the mitochondria and is cleaved to produce a truncated protein. The N-terminal tetrapeptide sequence of truncated Smac binds across a surface groove on BIR3 of cIAP1, cIAP2, and XIAP. This Smac-BIR3 interaction completely prevents inhibition of caspase-9 by XIAP, cIAP1 or cIAP2 (Du et al., 2000; Liu et al., 2000), and forms the basis

of Smac mimetic compounds (SMCs) currently in development as putative cancer therapeutics.

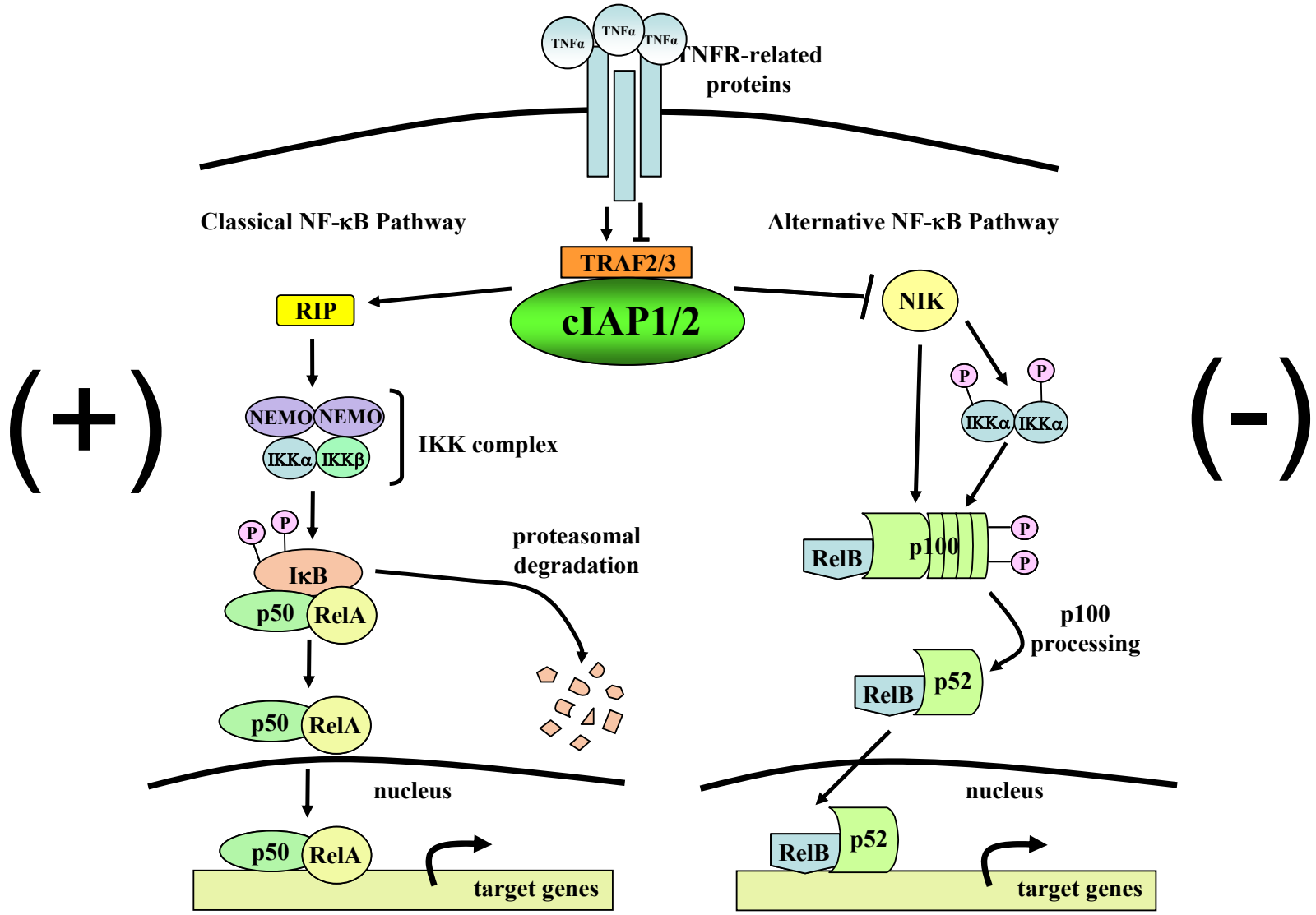
Several studies have identified cIAP1 and cIAP2 as regulators of the nuclear factor κB (NF-κB) signalling pathway (Vince et al., 2007; Bertrand et al., 2008; Mahoney et al., 2008; Wang et al., 2008). The NF-κB transcription factors are key effectors of skeletal muscle atrophy induced by various triggers (Hunter et al., 2002; Cai et al., 2004; Hunter and Kandarian, 2004; Judge et al., 2007; Mittal et al., 2010), as well as in the pathology of muscle diseases (Baghdiguian et al., 1999; Acharyya and Guttridge, 2007). The NF-κB family consists of five closely related proteins: RelA, RelB, c-Rel, p50/105 and p52/100. The most common arm of NF-κB signalling, referred to as the "classical" pathway, involves gene expression driven by the p50:RelA dimer (Fig. 2.1). This dimer normally remains sequestered in the cytoplasm by one of a family of inhibitor of κB (IκB) proteins, until a stimulus, such as treatment with TNFα or bacterial lipopolysaccharide (LPS) (Bohrer et al., 1997; Coletti et al., 2002) leads to activation of IκB kinase-β (IKKβ). Phosphorylation of IκB by IKKβ triggers polyubiquitination and degradation of IκB, and allows the NF-κB dimers to translocate to the nucleus. Upstream of IKKβ activation, cIAP1 or cIAP2 are recruited to the receptor-signalling complex to ubiquitinate the receptor interacting kinase 1 (RIP1) by a lysine-63-mediated linkage. In the absence of cIAP1/2, RIP1 becomes ubiquitinated by lysine-48-mediated linkage, which leads to its degradation and the activation of a cell death pathway (Mahoney et al., 2008). cIAP1/2 also regulate NF-κB signalling through a non-canonical or "alternative" pathway. In the alternative pathway, an NF-κB inducing kinase (NIK) activates IKKα, which phosphorylates p100 and leads to its partial degradation into p52 (Senftleben et al., 2001). p52 forms a heterodimer with RelB,

both of which translocate to the nucleus and activate transcription of target genes. cIAP1/2 catalyze lysine-48-mediated ubiquitination of NIK, targeting it for proteasomal degradation and thus repressing the alternative pathway (Varfolomeev et al., 2007). A consequence of the placement of the cIAPs into the NF-κB signalling cascade is the concept of ligand-dependent and ligand-independent pathway activation. Ligands such as TNFα activate the classical NF-κB pathway; this mechanism requires cIAP1/2, as the pathway is attenuated in the absence of at least one cIAP (Fig. 2.1). Paradoxically, in the absence of a ligand, cIAP autoubiquitination and degradation with a SMC also activates the classical pathway (Varfolomeev et al., 2007). Thus, cIAP1/2 can act as both positive (ligand-dependent) and negative (ligand-independent) regulators of NF-κB signalling.

In recent years, a considerable number of studies have addressed the role of NF-κB signalling in myogenesis, with or without pathway activation by an exogenous ligand. Most of these studies indicate that the classical pathway, which is active in myoblasts but inactivated during differentiation (Bakkar et al., 2008), inhibits myogenesis. This is the outcome when myoblasts are differentiated in the presence of NF-κB activators such as TNFα (Coletti et al., 2002), IL-1β (Broussard et al., 2004), and the Ras GTPase (Mitin et al., 2001). However, the alternative pathway may produce separate effects on myogenesis. For instance, activation of the alternative pathway with constitutively active NIK is sufficient to promote myogenesis in L6 rat myoblasts (Canicio et al., 2001). A more recent study suggests that the alternative pathway is necessary for myotube maintenance past the early phases of differentiation (Bakkar et al., 2008). Given that the loss of cIAP1/2 is sufficient to activate

Fig. 2.1. Schematic of the classical and alternative NF-κB signalling pathways.

Receptor trimerization by a ligand such as TNFα leads to recruitment of several proteins in a receptor-signalling complex (blue). On the left, cIAP1/2 catalyze the lysine-63-mediated ubiquitination of RIP1, which then serves as an adaptor for binding of the IKK complex. These latter proteins phosphorylate IκB, targeting it for degradation, and releasing p50:RelA for nuclear translocation and signalling. On the right, cIAP1/2 catalyze the lysine-48-mediated ubiquitination and subsequent proteasomal degradation of NIK. This impairs processing of p100 into p52, which would otherwise translocate into the nucleus in a p52:RelB dimer.


TNFα
TNFα
TNFα
TNFR-related proteins
Classical NF-κB Pathway
Alternative NF-κB Pathway
TRAF2/3
cIAP1/2
RIP
NIK
(+)
(-)
NEMO
NEMO
IKKα
IKKβ
IKK complex
P
P
IκB
p50
RelA
proteasomal degradation
p50
RelA
nucleus
p50
RelA
target genes
P
P
IKKα
IKKα
RelB
p100
P
P
p100 processing
RelB
p52
nucleus
RelB
p52
target genes

both classical and alternative NF-κB pathways independently of ligand stimulation (Petersen et al., 2007; Varfolomeev et al., 2007; Wang et al., 2008), the result of cIAP1 loss (since cIAP2 is absent in muscle (Mahoney et al., 2008)) on muscle differentiation may reflect a combination of effects.

Myoblasts proliferate extensively *in vitro* and can transdifferentiate into different cell types such as adipocytes (Li et al., 2005), osteoblasts (Katagiri et al., 1994; Watanabe-Takano et al., 2010), chondrocytes (Bettex-Galland and Wiesmann, 1987), and brown fat cells (Seale et al., 2008; Kajimura et al., 2009). In skeletal muscle biology, while cell lines such as C2C12 and L6 are commonly employed due to their relative ease of handling, primary myoblasts have the advantage of relatively rapid differentiation (two days for primary cells, versus four to five days for C2C12). Furthermore, primary cells invariably offer a more physiologically relevant model to address questions of biology and function. Recent developments in muscle biology have confirmed the observations that not all satellite cells are equal, but instead have properties that vary with both anatomical location and age (Conboy and Rando, 2002; Relaix et al., 2004). Such spatiotemporal questions cannot be addressed with cell lines of any sort, and particularly require myoblasts isolated with high yield and low passage number. Another advantage that becomes apparent in both physiological and clinical applications is that, unlike cell lines such as C2C12s, primary myoblasts do not form tumours upon transplantation (Zhang et al., 2009). These reasons offer suitable justification for the use of primary myoblasts wherever possible in the cell biology of myogenesis.

While primary myoblasts have been used in experimentation for decades, their use is limited because they are difficult to isolate, purify and maintain. Myoblasts exist in a milieu

shared with considerably more fibroblasts, the latter of which proliferate more rapidly and are more tolerant of variable culture conditions. Methods used to separate fibroblasts from myoblasts in culture include tissue "pre-plating", which relies on the cells' differential abilities to adhere to tissue culture plastic; magnetic activated cell sorting (MACS), which use antibody-labelled magnetic beads to separate cells by expression of cell surface antigens (Park et al., 2006); and single fibre cultures, in which individual muscle fibres are isolated, cleaned and plated to allow expansion of a pure population of myoblasts. While all these methods are viable and have their advantages, they suffer from disadvantages that limit their use in the laboratory. The conventional pre-plating approaches suffer from low yields and very high fibroblast contamination, requiring as much as four weeks between myoblast isolation and experimental use. MACS, while offering highly pure populations of myoblast, require considerable start-up and per-use costs, as well as low cell yields (Park et al., 2006). The single fibre cultures probably offer the purest populations of myoblasts; however, the technique is extremely time-consuming and error-prone, as single muscle fibres are very delicate in culture, and failure rates of single fibre seedings are high. There is therefore a need for a rapid, high efficiency, low-cost method to isolate primary myoblasts for use in laboratory experiments and transplantation studies.

In this study, I investigated the role played by cIAP1 in myoblast differentiation and muscle development. First, I present a rapid and efficient method for isolation of primary myoblasts from mouse skeletal muscle. This method has the advantage of speed (isolation to experiment-ready cells in four to six days), efficiency (a single muscle is sufficient to produce millions of myoblasts), and purity (90 – 95% fibroblast-free). Second, I demonstrate that loss of cIAP1 leads to an increase in muscle mass, both *in vitro* and *in vivo*. Our data

suggest that loss of cIAP1 promotes muscle formation by activating the alternative NF-κB pathway, in spite of a delay in differentiation caused by a corresponding activation of the classical NF-κB pathway. These studies identify cIAP1 as a key mediator of muscle formation, and as a potential therapeutic target in degenerative muscle diseases.

Methods and Materials

Materials

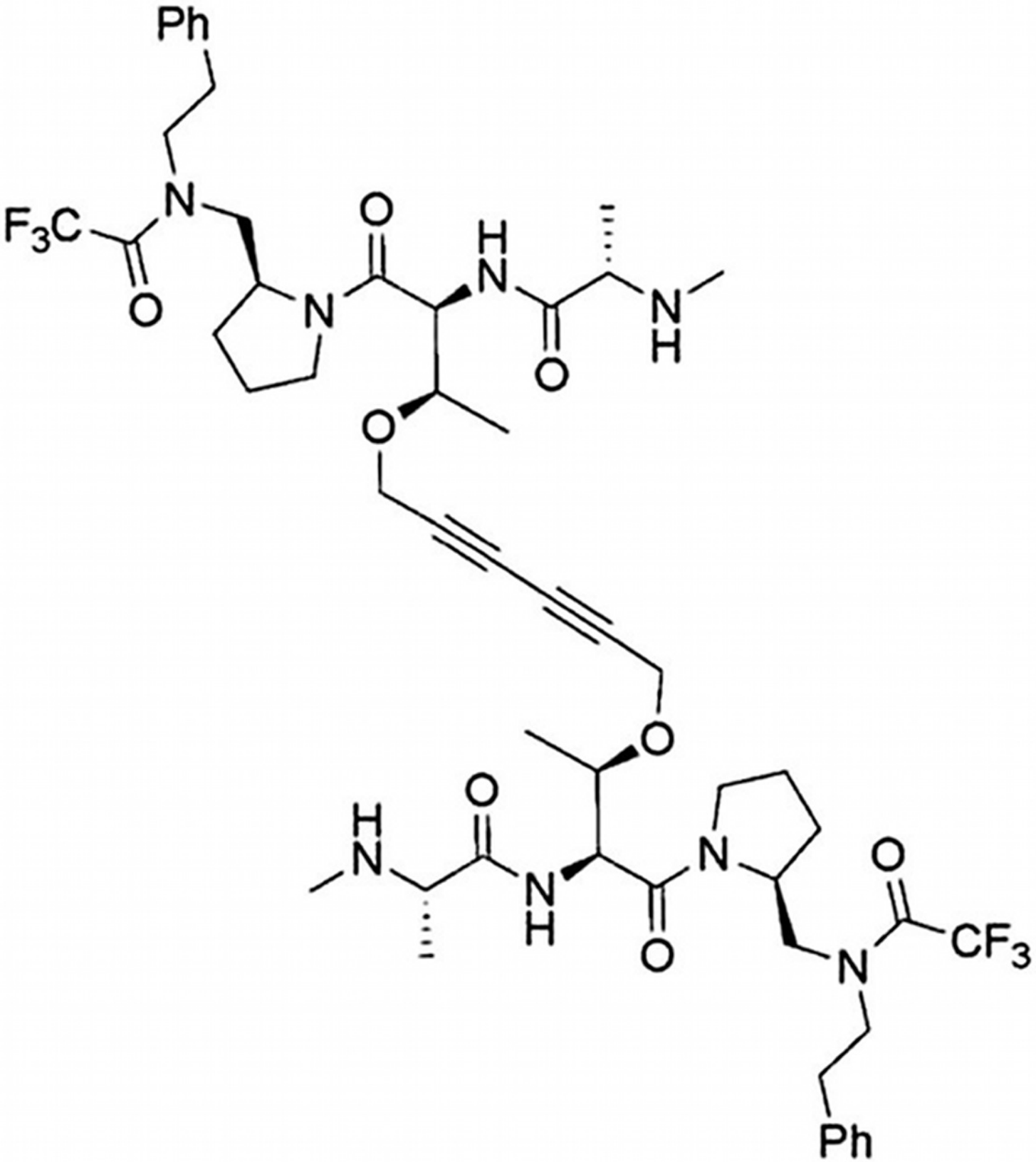
Rabbit polyclonal antibodies to p100, RelB, IKKα, Akt1 and phospho-Akt (T308) were obtained from Cell Signalling Technology. The polyclonal antibodies to laminin and desmin were obtained from Sigma-Aldrich. Antibodies to MHC (MF20), Pax7 and myogenin (F5D) were obtained from the Developmental Studies Hybridoma Bank (Iowa City, Iowa). The antibodies to α-actin and NFATc3 were obtained from Santa Cruz Biotechnology, Inc. Our rabbit anti-rat cIAP2 (RIAP1) polyclonal antibody was used to detect mouse cIAP1 (Holcik et al., 2002). Growth factor-reduced Matrigel was obtained from BD Biosciences, and collagenase A and dispase were obtained from Roche Applied Sciences. Basic human fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF) were obtained from PeproTech Inc, and all fluorescent secondary antibodies were from Invitrogen. The p52 and RelB expression plasmids were generous gifts from Dr. Denis Guttridge of Ohio State University (Columbus, Ohio). The bivalent Smac-mimetic compound (SMC) OICR-720A was a gift from Dr. Rima Al-Awar of the Ontario Institute of Cancer Research (Toronto, Ontario, Canada). The other bivalent SMC used in the studies, AEG40730 (Bertrand et al., 2008) was synthesized by Vibrant Pharma (Cambridge, ON, Canada). Structure for AEG40730 is also disclosed in Fig. 2.2.

Transfections

Primary myoblasts were transfected in antibiotic-free Opti-MEM using Lipofectamine LTX and Lipofectamine Plus (for DNA) and Lipofectamine RNAiMAX (for siRNA). All

Fig. 2.2. Structure of the Smac-mimetic compound AEG40730.

AEG40730 exists as a cross-linked dimer of two modified peptides. Each monomer binds a single IAP; both IAPs bound to the SMC will cross-ubiquitinate each other leading to proteasomal degradation. (Taken from Bertrand, 2008.)



AEG40730

siRNAs and transfection reagents were obtained from Invitrogen, and used according to the manufacturer's instructions.

Mice and genotyping

Animals were housed at the animal care facility of the University of Ottawa Faculty of Medicine, in temperature-controlled rooms (19 – 22°C) with a 12:12 hour light:dark cycle. All *in vivo* experiments were performed using female wildtype and/or C57BL/6 and *cIAP1*$^{-/-}$ mice between 6 and 12 weeks of age. Treatment of mice was in accordance with institutional guidelines of the University of Ottawa Animal Care Committee. *cIAP1*$^{-/-}$ mice were obtained from Dr. Tak Mak (Toronto, ON, Canada) (Conze et al., 2005). Genotype was confirmed by PCR analysis from ear notch DNA, and by Western blotting using the RIAP1 antibody to detect cIAP1.

Cell culture

Murine C2C12 myoblasts (ATCC) were cultured in complete media (DMEM [ATCC] supplemented with 10% FBS, penicillin, and streptomycin [all Invitrogen]). They were differentiated in DMEM containing 2% equine serum (HyClone). Primary myoblasts were prepared from female mice killed by cervical dislocation, and lower limb muscles were carefully dissected away from the bone. Muscle samples were digested with collagenase-Dispase solution (Roche) for 2 – 3 hours at 37°C, before being triturated, washed by centrifugation twice at 300 x g for 5 minutes, and resuspended in high-glucose DMEM containing 10% equine serum and 5 ng/ml basic FGF. Fibroblasts were separated out by "preplating" on a regular tissue culture coated-plate for an hour before the cell suspension

was seeded into a six-well plate coated with Matrigel. Two days later, the myoblasts were switched to and maintained in growth media containing 20% FBS, 10% equine serum, 10 ng/ml bFGF, and 2 ng/ml HGF.

Differentiation and immunofluorescence of myoblasts

For differentiation assays, primary myoblasts were seeded on Matrigel-coated cover slips in 12-well plates (Fisher Scientific) at 7.5 x 10^4 cells per well. They were differentiated in DMEM containing 10% equine serum and then washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 5 minutes. They were then incubated overnight at 4°C with the desired primary antibody, diluted in PBS containing 0.3% Triton X-100 (Sigma) and 0.5% bovine serum albumin (Invitrogen). After washing, cover slips were incubated with the secondary antibody, diluted in PBS with Hoechst 33258 (Sigma) as a nuclear counterstain, and incubated for one hour at room temperature. This was followed by washing, mounting on glass slides, and visualization. Cover slips were photographed using a Zeiss Axiophot microscope equipped with a Zeiss digital camera and Northern Eclipse image analysis software, version 6.0 (Empix Imaging, Mississauga, Ontario). For morphometry, 5 – 10 fields at 10X were photographed and analyzed for number of nuclei per myotube, or for myotube diameter. In the latter instance, individual myotubes were traced along to either the smallest contiguous point (along an entirely linear myotube) or the smallest region between branches (where branching occurred).

NF-κB activity assay

Binding of p65- and p52-containing complexes to DNA were measured using an NF-κB DNA binding ELISA (Active Motif, Carlsbad, CA) following the manufacturer's instructions. Briefly, cellular NF-κB complexes from cell lysates were captured on ELISA plates containing immobilized NF-κB consensus DNA duplexes. The complexes were washed, probed with either a p65- or a p52-specific antibody, and visualized using a luminescent antibody conjugate.

BrdU labelling

Cells differentiating on cover slips in 12-well plates were pulsed with 100 µl of 10 µM BrdU (Sigma) for 15 minutes, before being processed for immunofluorescent detection as previously described (Enwere et al., 2004).

Immunoblotting

Western blotting was performed as previously described(Mahoney et al., 2008).

Histology

Fresh frozen muscles were sectioned at 10 µm thickness on a cryostat (Leica) and stained with haematoxylin and eosin (Sigma). Random fields were photographed using a Zeiss Axiophot microscope equipped with a digital camera and Northern Eclipse image analysis software, version 6.0 (Empix Imaging, Mississauga, Ontario). Muscle sections selected for

analysis were derived from the centre of each muscle. Cross-sectional areas of 500 – 1000 fibres per muscle were measured using Northern Eclipse or ImageJ (version 1.43, NIH).

Statistical analyses

All quantitative data are represented as mean ± SEM. Statistical analyses were performed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA) using a two-tailed t-test with significance set at $p < 0.05$.

Reagents for myoblast isolation

A mouse (C57BL/6, preferably female, preferably as young as possible)

DMEM (ATCC, hi carbonate content) + Pen/Strep

PBS + Pen/Strep

Collagenase Type I (Invitrogen)

Dispase (Roche)

10ml syringe

Syringe filter (0.2 μm)

Scissors, forceps, hemostats

Spray bottle of 70% EtOH

Nitex 74 μm mesh filter

Matrigel (Sigma), diluted 1:10 in ice-cold DMEM

35mm plate

Six-well plate

Donor Equine Serum (HyClone, cat #SH30074.03), *not* heat-inactivated

bFGF (basic Fibroblast Growth Factor, a.k.a. FGF-2) (Peprotech via Cedarlane)

HGF (Hepatocyte Growth Factor) (Peprotech via Cedarlane)

Fetal bovine serum

Shaker @ 37°C

Cell culture incubator (5% CO_2 @ 37°C)

Plating media:

10% Donor Equine Serum

5 ng/ml bFGF

DMEM (ATCC)

Growth media:

10% Donor Equine Serum

20% Fetal Bovine Serum

10 ng/ml bFGF

2 ng/ml HGF

DMEM (ATCC)

Myoblast isolation protocol

1) Prepare a fresh solution of 0.2% **collagenase** in DMEM (8ml per mouse). Add 500µl of **dispase**. Mix, warm to 37°C, and sterilize by **syringe filtration**.
2) Prepare a 15ml Falcon tube with ~10ml of **PBS** + pen/strep.
3) Euthanize the mouse by overdose of Somnotol. Spray down fur with **70% EtOH** to keep the fur down.
4) Isolate desired muscle groups, and peel away the fascia. Cut the muscles longitudinally, to about halfway down their length. Place muscle fragments in PBS.
5) Transfer muscle to a 35mm dish with the collagenase/dispase solution. Seal the plate with autoclave tape.
6) Place in shaker at 37°C. Shake at 75 RPM for 2 – 2.5 hours.
7) Triturate the muscle with a 10ml pipette until the muscle fibres are completely dissociated (as verified under the microscope).

8) Transfer the digested muscle to a 50ml Falcon tube. Add DMEM to a total of 20ml. Pass through the **Nitex** filter to remove undigested connective tissue. There should be no large muscle fragments at this point.
9) Centrifuge at 1200 RPM for 5 minutes at room temperature.
10) Carefully remove the supernatant, leaving 2 – 3ml of medium above the pellet.
11) Resuspend the pellet in fresh DMEM.
12) Centrifuge again at 1200 RPM for 5 minutes. Remove the supernatant.
13) Resuspend the pellet in plating media: **10ml of DMEM** containing **10% equine serum** and **5 ng/ml bFGF**. Transfer to a regular tissue culture-coated (no Matrigel) **10cm plate**.
14) Pre-plate by leaving cell suspension in cell culture incubator for 1 hour.
15) While the cells are preplating, prepare the **six-well plate** with Matrigel. *On ice* (or in the cold room), briefly rinse the wells (one well per mouse) with the diluted **Matrigel**. Leave the plate to set in a cell culture incubator for at least 15 minutes.
16) Transfer the entire cell suspension to the six-well plate. Rinse the 10cm plate carefully to ensure that all loose cells are transferred. The entire 10ml suspension should be contained in a single well.
17) Leave the cells to attach and grow for 2 days. Do not change the media in this time.
18) Examine the cells. If they are confluent or approaching confluence, passage; otherwise, simply switch to growth media. They should not be allowed to grow past 80%. Conversely, they should not be seeded too sparsely (below ~30%). As the cells should now grow very rapidly, daily passaging may be necessary.

19) **Change media daily past this point** (growth factors are only good for 24 hours in culture). Periodically pre-plate (once every 2 passages, or as necessary) to remove fibroblasts.

20) By 5 – 6 days after isolation, with proper maintenance, you should have 20 – 25 million cells to freeze down. This can be successfully accomplished with 1 million cells/ml, in growth medium and 10% sterile **DMSO**.

A number of existing protocols were adapted and modified (Springer et al., 2002; Shefer and Yablonka-Reuveni, 2005; Kuang et al., 2007). Several key elements were changed to improve and streamline the protocol. First, F10 medium was replaced with DMEM, which proved less supportive of fibroblast growth. Second, early homogenization of the muscle drastically increased the release of fibroblasts into the culture preparation and decreased myoblast viability. Instead, slicing the muscle lengthwise allowed for gentle digestion of the muscle and removal of fibroblast-containing connective tissue. Third, the introduction of HGF into the growth media allowed growth of myoblasts to relatively high densities without precocious differentiation. HGF is a key mitogen of satellite cells *in vitro* and *in vivo* (Tatsumi et al., 1998).

Results

To determine the facility of the myoblast isolation protocol, primary myoblast cultures were derived from individual pairs of tibialis anterior (TA) and quadriceps muscle from three-week-old C57BL/6 mice. Each of these cultures produced ~2 x 10^6 myoblasts within four days of extraction, which quickly expanded to 25 x 10^6 by six days *in vitro* (DIV). To determine the identity and purity of the isolated cells, they were seeded onto Matrigel-coated cover slips and immunolabeled for Pax7 and desmin. 90 ± 5% (n = 3) of cells expressed Pax7 (Fig. 2.3); 97 ± 2% expressed desmin (Fig. 2.4), indicating that the cells isolated were principally myoblasts.

I next asked if the cells would differentiate to form myotubes in culture. TA and quadriceps myoblasts were seeded in DMEM containing 10% horse serum for two days, and immunolabeled for myosin heavy chain (MHC). Extensive MHC-expressing myotubes were present in both TA and quadriceps cultures (Fig. 2.5). The myotubes remained viable for up to seven days with daily changing of media (data not shown). In large myotube clusters, extensive contractility was evident, indicating that the mature muscle apparatus formed normally under the differentiation conditions. In experiments described subsequently, the cells were maintained for approximately four passages, and with routine pre-plating, they maintained their ability to differentiate efficiently. A secondary measure of the "cleanliness" of a culture was performed by Western blotting: since myoblasts do not express cIAP2 (Mahoney et al., 2008), the RIAP1 antibody, which detects both cIAP1 and cIAP2, should only detect a band corresponding to cIAP1 in wildtype myoblast samples. In the absence of

Fig. 2.3: Expression of Pax7 in primary myoblasts.

Primary myoblasts were isolated from quadriceps and TA muscles of three week-old C57BL/6 female mice, following the protocol described in Methods and Materials. The cells were seeded on Matrigel-coated cover slips, fixed, and processed for immunohistochemistry. Nuclei are counterstained with Hoechst (blue). As expected, the great majority of nuclei express the satellite cell marker Pax7. Scale bar represents 50 μm.

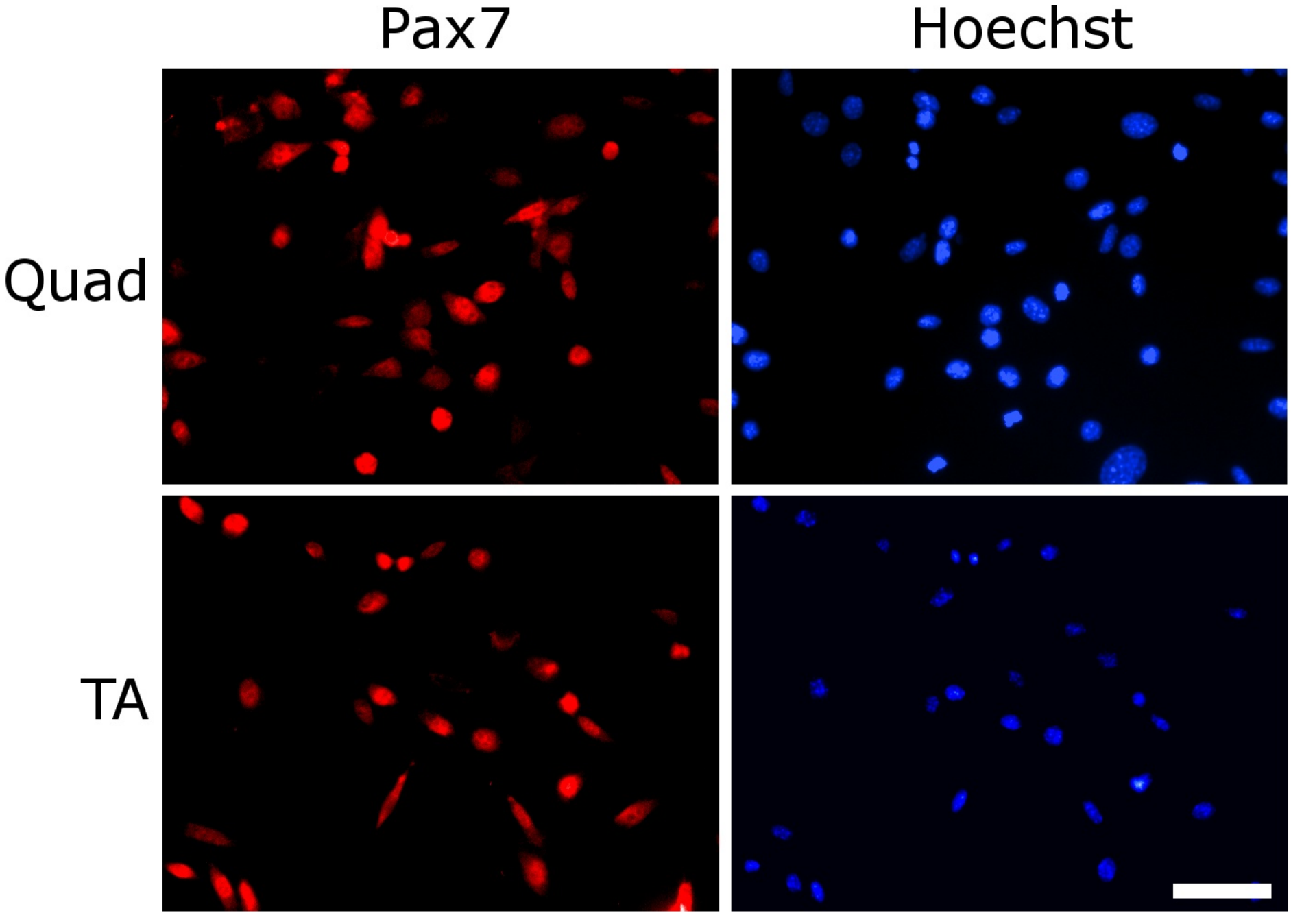

Pax7
Hoechst
Quad
TA

Fig. 2.4: Expression of desmin in primary myoblasts

Primary myoblasts were isolated from quadriceps and TA muscles. The cells were seeded on Matrigel-coated cover slips, fixed, and processed for immunohistochemistry. Nuclei are counterstained with Hoechst (blue). As with Pax7, the great majority of nuclei are present in desmin-positive cells, indicating their myogenic identity. Scale bar represents 50 μm.

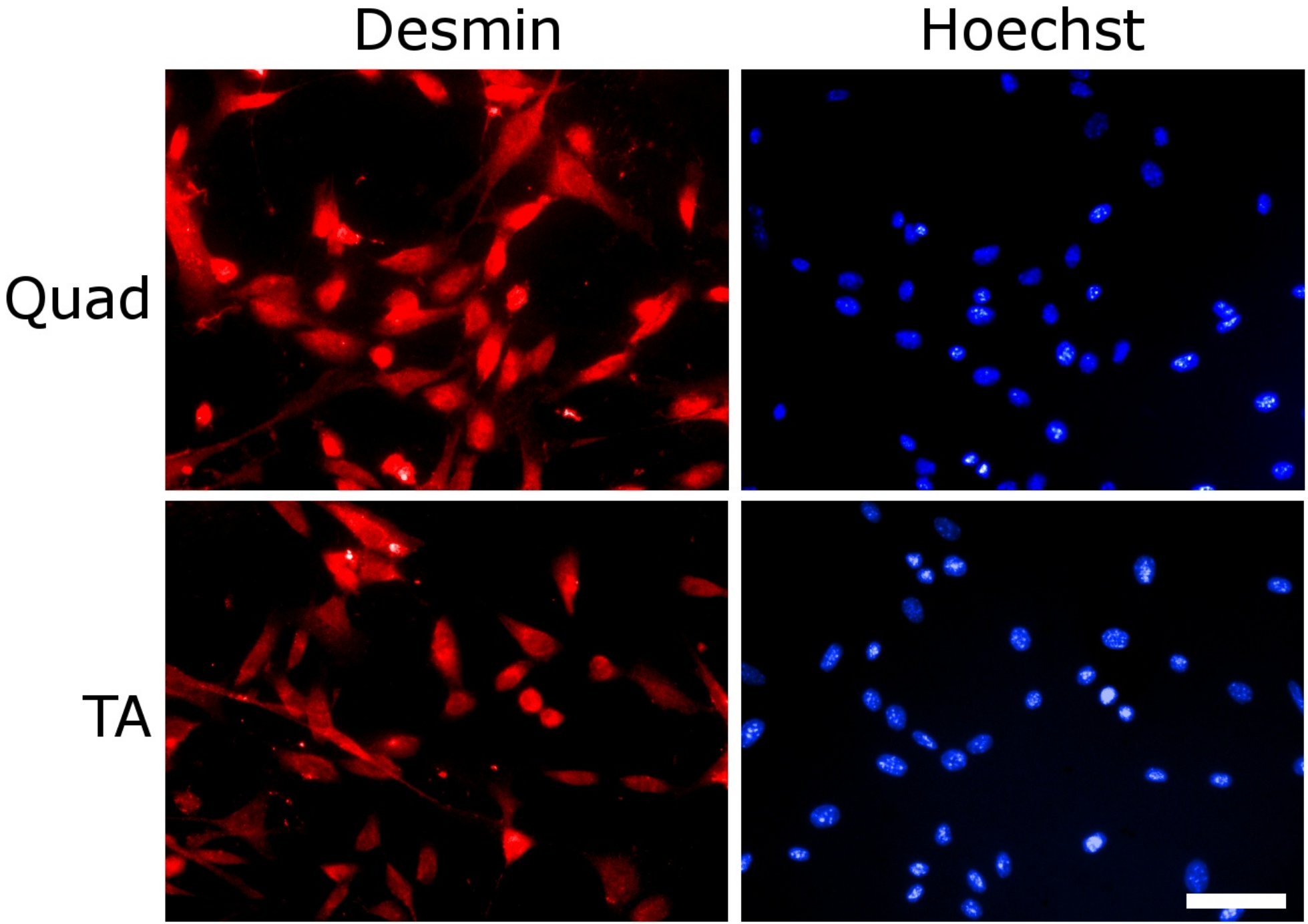
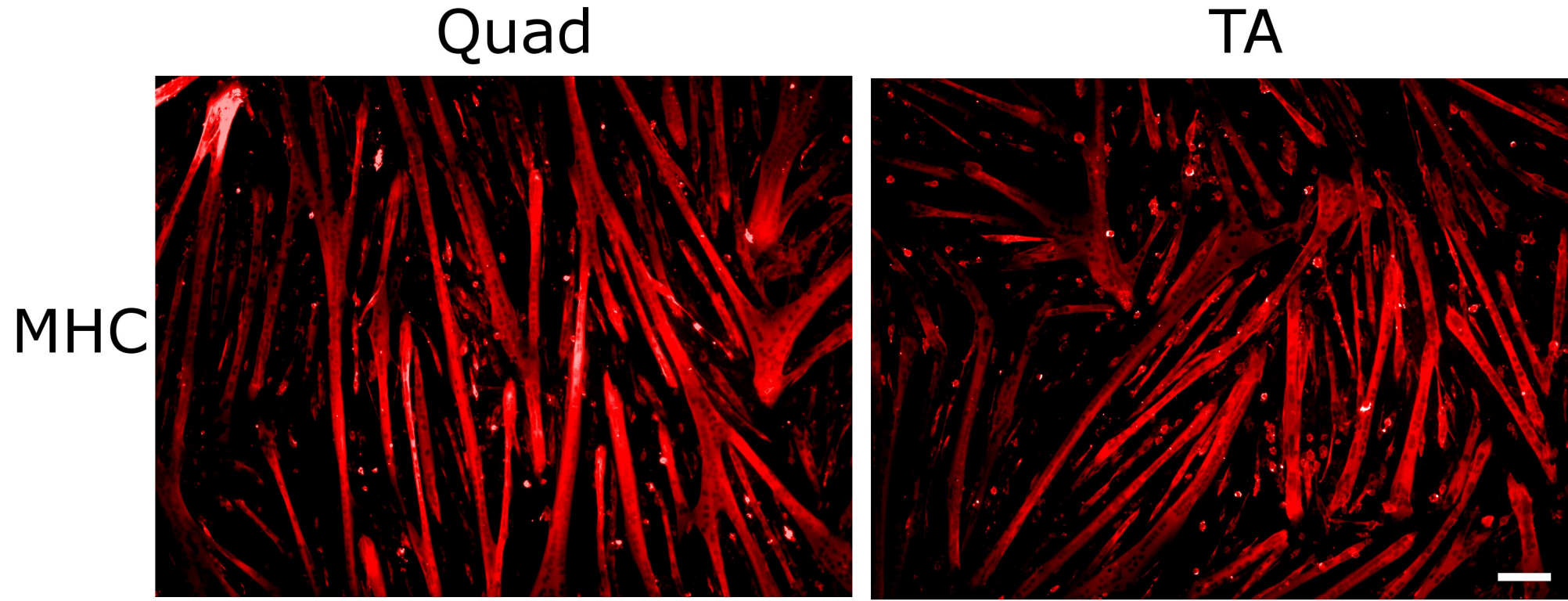

Desmin
Hoechst
Quad
TA

Fig. 2.5: Myoblasts from quadriceps and TA myoblasts differentiate appropriately to form myotubes.

Primary myoblasts isolated from quadriceps and TA muscles were seeded on cover slips and differentiated for two days by serum withdrawal. The cells were then fixed and processed for immunohistochemistry. The majority of cells from both quadriceps and TA fused into MHC-positive myotubes, indicating that the cells were myoblasts. Scale bar represents 125 μm.


Quad
TA
MHC

cIAP1, no bands should be visible at all (for example, see Fig. 2.11). *cIAP1*$^{-/-}$ fibroblasts, however, do express cIAP2, so if a blot of *cIAP1*$^{-/-}$ myoblasts indicates the presence of cIAP2, it is likely due to the presence of contaminating fibroblasts in the culture.

Loss of cIAP1 increases myotube size

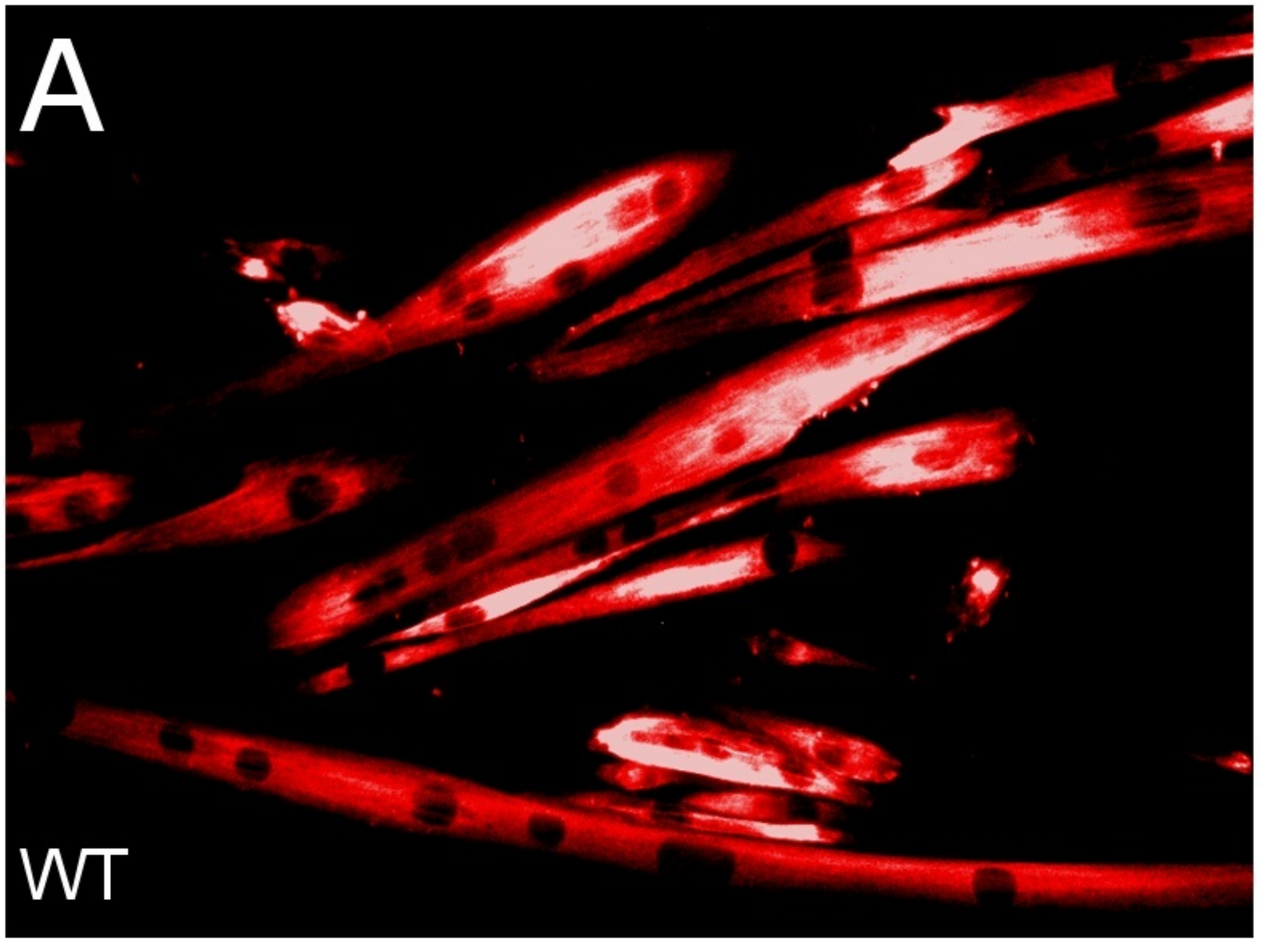
To analyze the function of cIAP1 in skeletal myogenesis, I used primary myoblasts from wildtype and *cIAP1*$^{-/-}$ mice, which readily differentiate to form myotubes in reduced-serum differentiation media (Rando and Blau, 1994). After 48 hours of differentiation, the myotubes formed from the *cIAP1*$^{-/-}$ myoblasts were considerably larger than their wildtype counterparts (Fig. 2.6A,B). These myotubes were otherwise normal, and exhibited robust contractile activity. The increased size was at least in part due to an increase in myonuclear number: while wildtype myotubes in culture have an average of five nuclei each ((Horsley et al., 2003) and data not shown), 40% of the *cIAP1*$^{-/-}$ myotubes contained 20 or more nuclei (Fig. 2.6C). The increased fusion also resulted in an increase in myotube size, as *cIAP1*$^{-/-}$ myotubes had twice the diameter of wildtype myotubes (Fig. 2.6D). The siRNA knockdown of cIAP1 in wildtype myoblasts also increased myotube size and myonuclear number upon differentiation (Fig. 2.7A-D). Next, I took advantage of a small-molecule antagonist of the IAPs that mimics their interaction with the endogenous inhibmitor, Smac (Wu et al., 2000; Li et al., 2004; Mahoney et al., 2008; Sun et al., 2008; Cheung et al., 2009). These "Smac-mimetic" compounds (SMC) cause the rapid inhibition and proteasomal degradation of

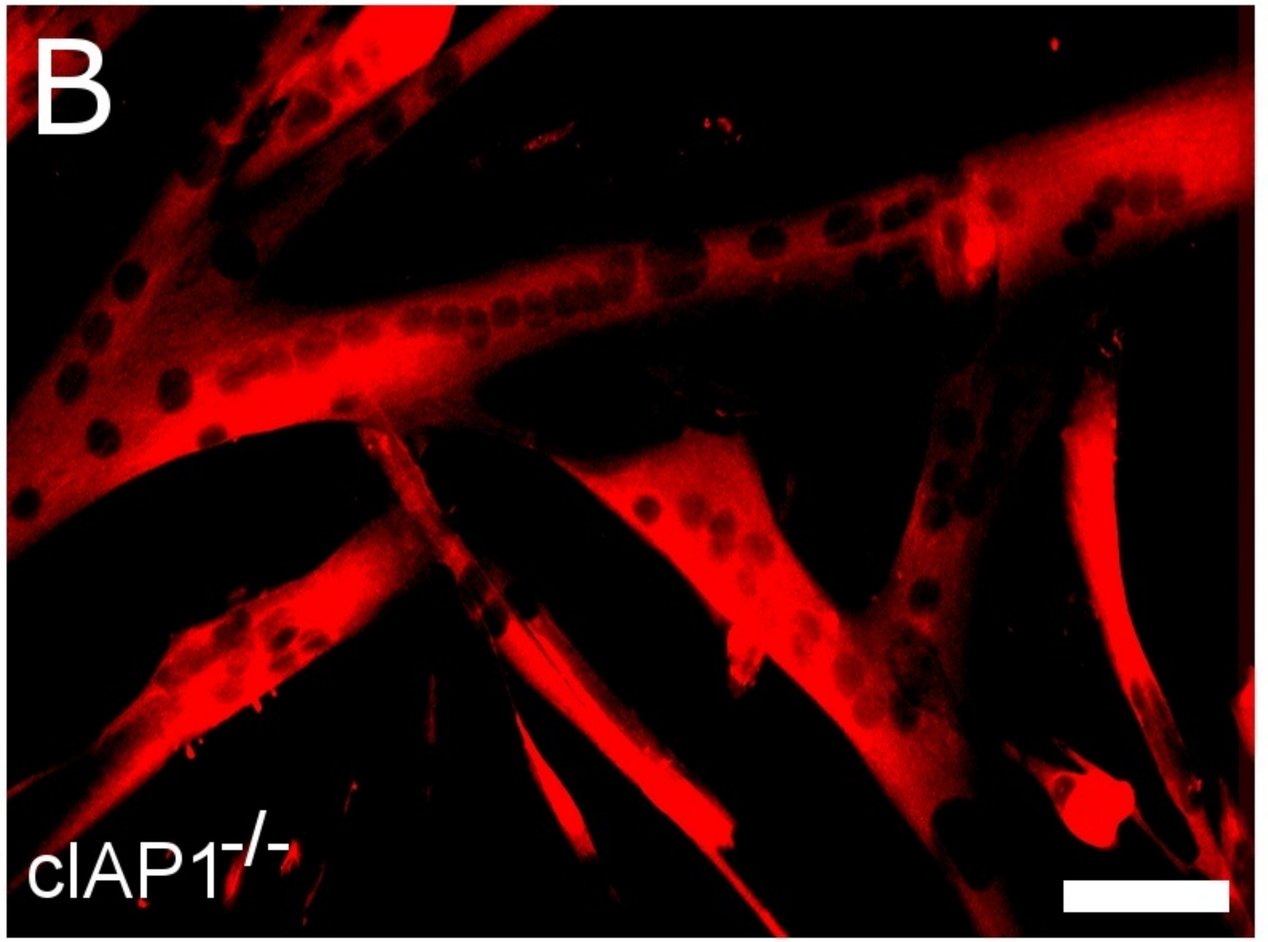
Fig. 2.6: Loss of cIAP1 results in increased myoblast fusion.

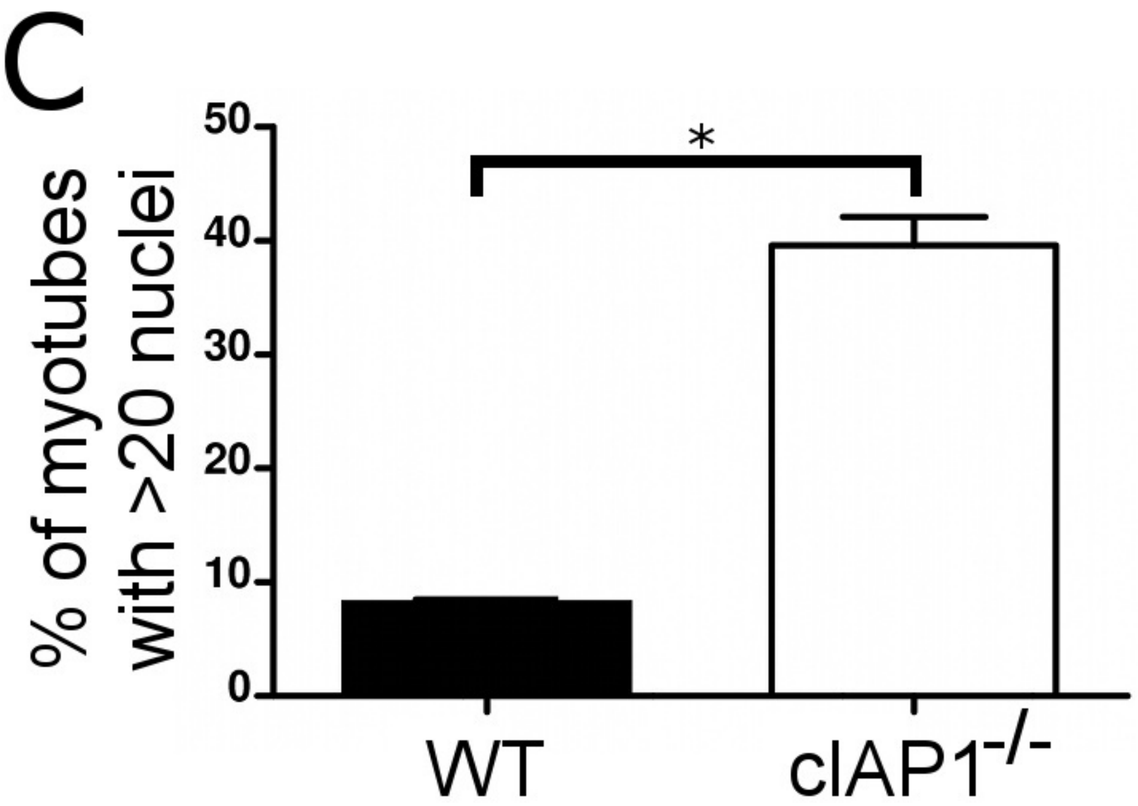
(A, B) To determine the effect of cIAP1 on myoblast differentiation, wildtype and *cIAP1*$^{-/-}$ primary myoblasts were differentiated for 48 hours. The myotubes were washed, fixed, and immunolabeled for MHC (red) as described in Methods and Materials. The *cIAP1*$^{-/-}$ myotubes (B) are distinctively larger than their wildtype (A) counterparts, suggesting that cIAP1 limits myotube size.

(C) The presence of a large number of nuclei (>20) per myotube is an indicator of increased myoblast fusion. To determine the effect of cIAP1 on fusion, the percentage of myotubes with more than 20 nuclei each was determined from photomicrographs taken in Fig. 2.6A, using Hoechst 33258 to reveal nuclei. Significantly, more *cIAP1*$^{-/-}$ myotubes contained over 20 nuclei, again indicating that the loss of cIAP1 increases myoblast fusion.

(D) Increased diameter is another indication of myotube size, though it is a specific indicator of increased myoblast fusion. The diameters of myotubes labelled as in Fig. 2.6A were measured using Northern Eclipse or ImageJ software, with the diameter measured between myotube branch points for greatest consistency. Again, *cIAP1*$^{-/-}$ myotubes demonstrated increased diameter over the wildtypes. Data are mean ± standard error of three independent experiments. * indicates significantly different, with $p < 0.05$. ** indicates $p < 0.01$. Scale bar represents 50 μm.


A
WT


B
cIAP1-/-


C
% of myotubes with >20 nuclei
50
40
30
20
10
0
*
WT
cIAP1-/-

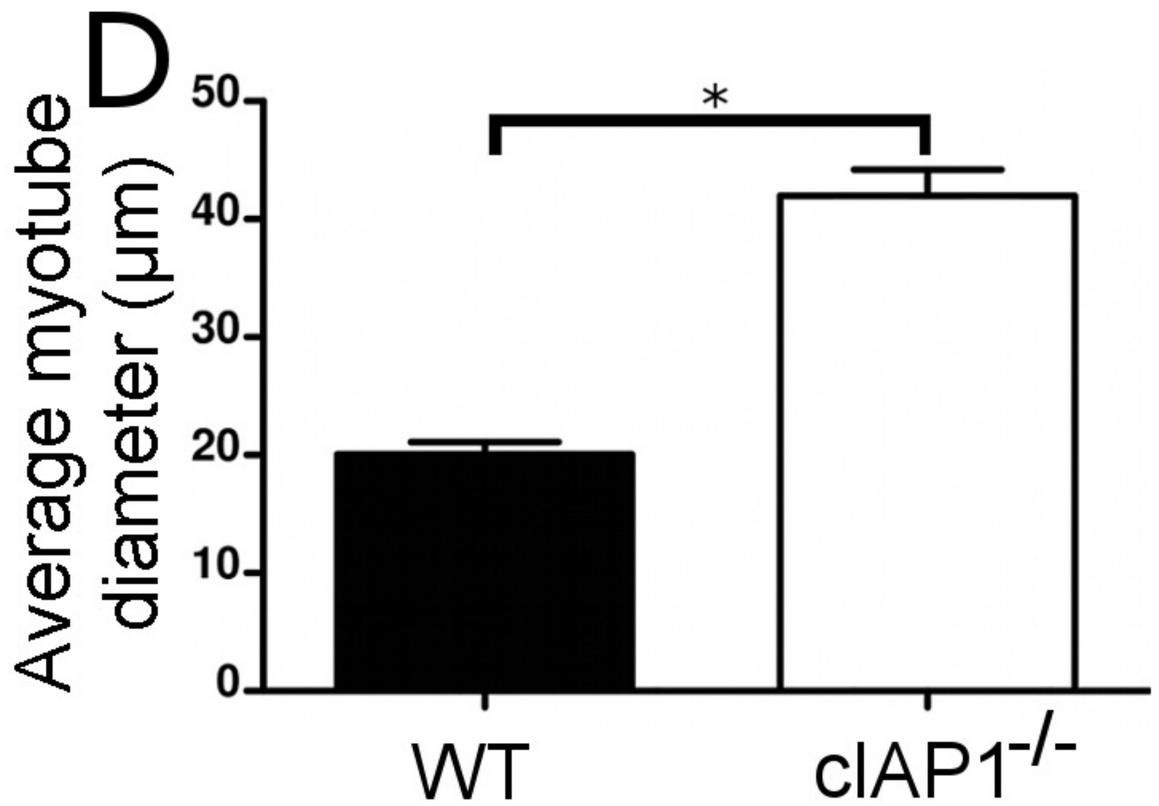

D
Average myotube diameter (µm)
50
40
30
20
10
0
*
WT
cIAP1-/-

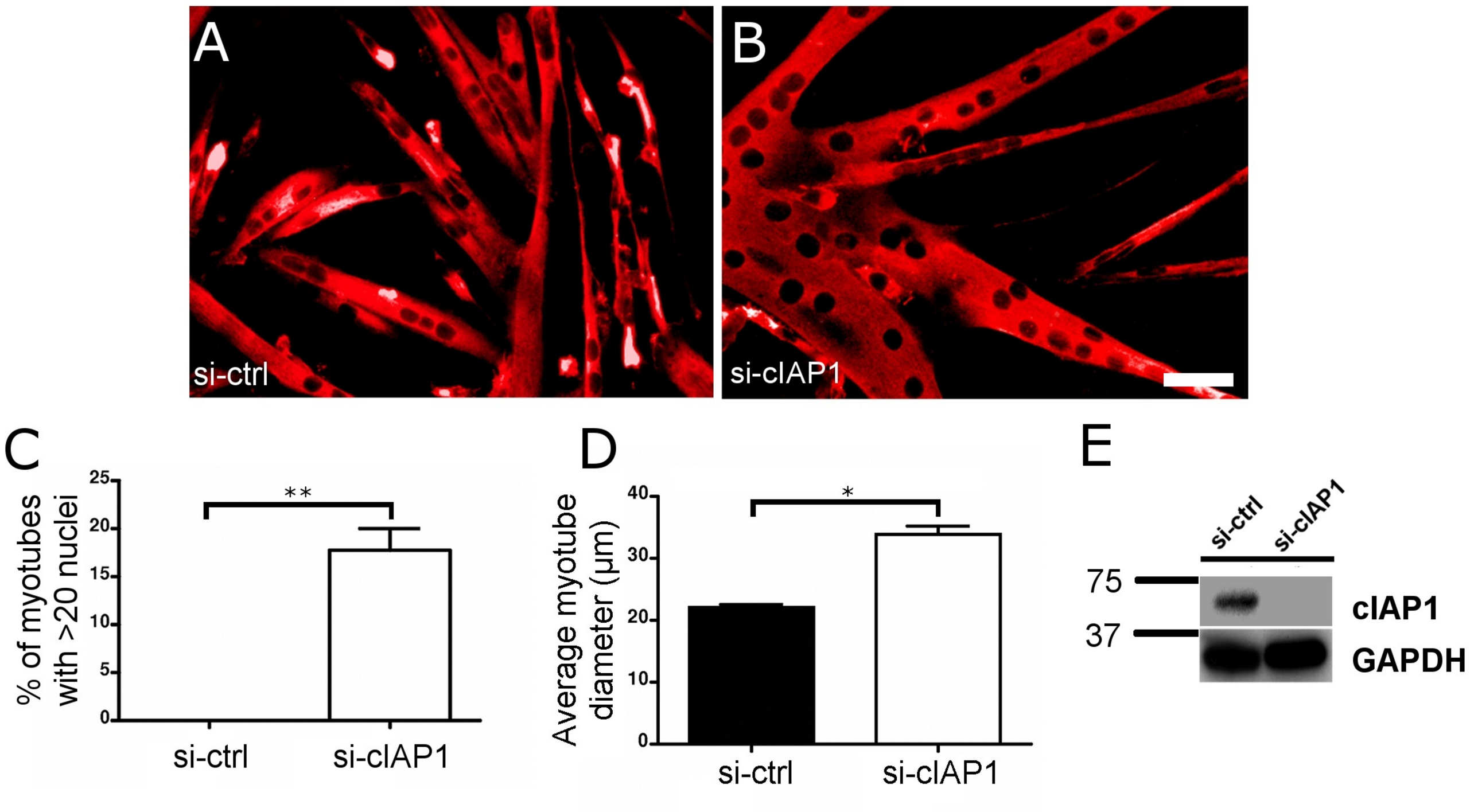
Fig. 2.7: siRNA knockdown of cIAP1 increases myotube size.

(A, B) To confirm the effect of cIAP1 loss on myotube fusion seen in the *cIAP1*$^{-/-}$ myotubes, wildtype myoblasts were transfected with 120 nM of non-targeting siRNAs or siRNAs targeting cIAP1 for 24 hours, before being differentiated for a further 48 hours. Myotubes were immunolabelled for MHC (red) as in Fig. 2.6A. siRNA knockdown of cIAP1 was sufficient to increase myoblast fusion in wildtype cells.

(C) As described previously, the presence of a large number of nuclei (>20) per myotube is an indicator of increased myoblast fusion. To determine the effect of cIAP1 on fusion, the percentage of myotubes with more than 20 nuclei each was determined from photomicrographs taken in Fig. 2.7A&B, using Hoechst 33258 to reveal nuclei.

(D) Increased diameter is another indication of myotube size, though it is a specific indicator of increased myoblast fusion. The diameters of myotubes labelled as in Fig. 2.7A&B were measured using Northern Eclipse or ImageJ software, with the diameter measured between myotube branch points for greatest consistency. Again, knockdown of cIAP1 in wildtype myotubes increased myotube diameter.

(E) To confirm efficacy of siRNA transfections, Western blotting was performed on myotubes transfected as myoblasts with siRNAs targeting cIAP1 or non-targeting controls. Data are mean ± standard error of three independent experiments. * indicates significantly different, with $p < 0.05$. ** indicates $p < 0.01$. Scale bar represents 50 μm.


A
si-ctrl
B
si-cIAP1
C
% of myotubes with >20 nuclei
25
20
15
10
5
0
**
si-ctrl
si-cIAP1
D
Average myotube diameter (µm)
40
30
20
10
0
*
si-ctrl
si-cIAP1
E
si-ctrl
si-cIAP1
75
37
cIAP1
GAPDH

cIAP1 and its paralogue cIAP2 (which is absent in skeletal muscle (Mahoney et al., 2008)). Differentiation of wildtype myoblasts for 48 hours in the presence of 500 nM SMC, a concentration that effectively eliminates cIAP1 (Fig. 2.8D and data not shown) also increased myonuclear number and myotube size (Fig. 2.8A-D).

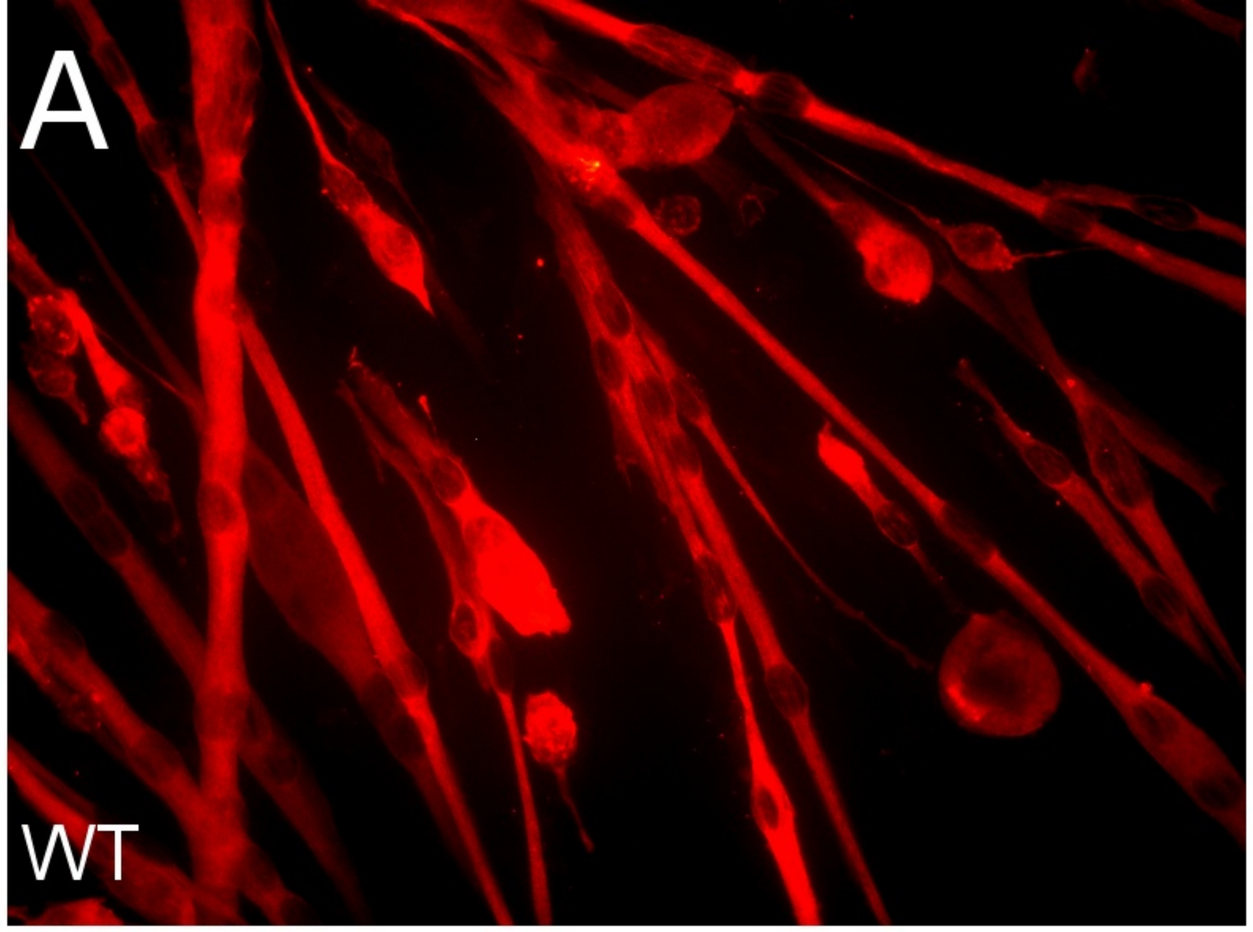
Myoblast fusion occurs in two temporally distinct stages: an early stage (during the first 24 hours of differentiation), and a late stage (in the second 24 hours) (Pavlath and Horsley, 2003). I asked whether the loss of cIAP1 was critical to early or late stages of fusion. To this end, I used the SMC, which led to complete loss of cIAP1 within 30 minutes of addition to the culture media (Fig. 2.9A). Myoblasts treated with SMC upon serum withdrawal developed into hypernucleated myotubes 48 hours later. However, when SMC addition was delayed until 24 hours after serum withdrawal, myotube size remained the same as in controls (Fig. 2.9B). The IGF-PI3K-Akt pathway may produce similar effects by promoting myotube hypertrophy (Rommel et al., 2001; Stitt et al., 2004); to determine its involvement in this context, I examined the phosphorylation of Akt by immunoblotting samples from differentiating wildtype and *cIAP1*$^{-/-}$ myoblasts. While total Akt levels remained unchanged between both myoblast populations, Akt phosphorylation was, if anything, slightly depressed in the *cIAP1*$^{-/-}$ cells (Fig. 2.10). Conversely, expression of NFATc3, which is associated with cardiac and skeletal muscle cell fusion(Delling et al., 2000; van der Velden et al., 2008; Pansters et al., 2010) was considerably elevated in *cIAP1*$^{-/-}$ myoblasts, particularly in the first 24 hours following serum withdrawal. These results suggest that loss of cIAP1 promotes early stages of myoblast fusion.

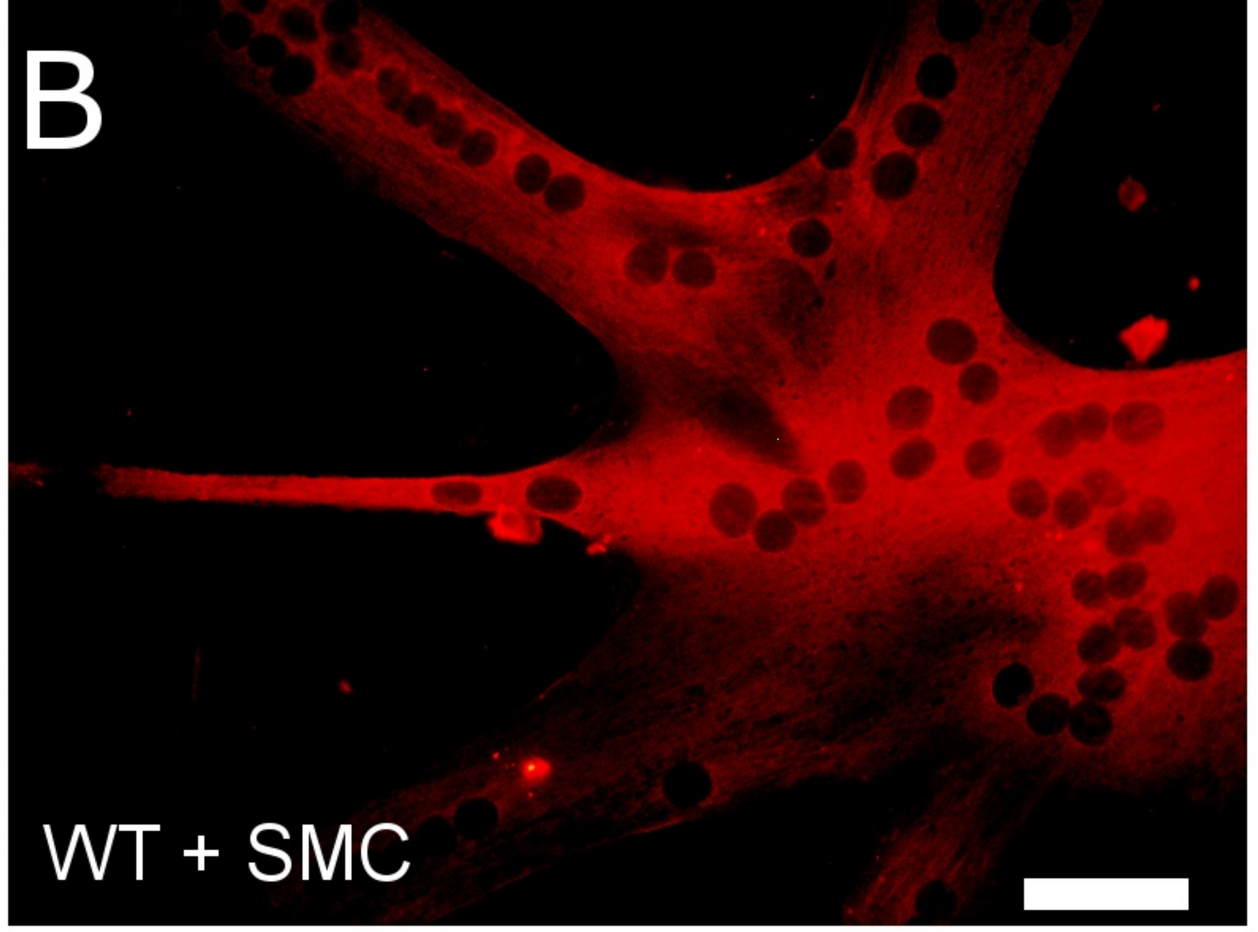
Fig. 2.8: Pharmacological depletion of cIAP1 increases myotube size.

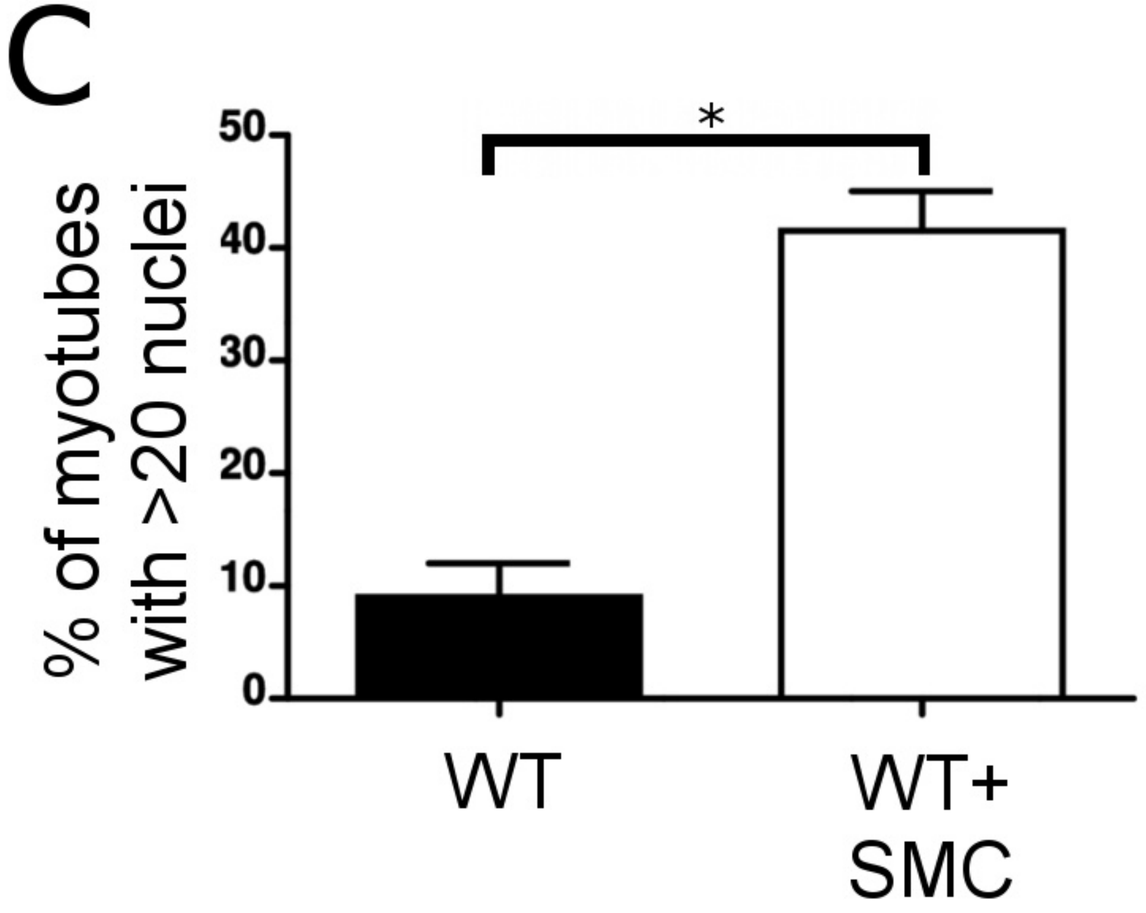
(A, B) To confirm the effect of cIAP1 loss on myoblast fusion, wildtype myoblasts were differentiated for 48 hours in the presence of DMSO (A) or 500nM SMC (B). The resulting myotubes were immunolabeled for MHC (red) as in Fig. 1A. SMC treatment during differentiation was sufficient to increase myoblast fusion.

(C) As described previously, the presence of a large number of nuclei (>20) per myotube is an indicator of increased myoblast fusion. To confirm the effect of cIAP1 on fusion, the percentage of DMSO- or SMC-treated myotubes with more than 20 nuclei was determined from photomicrographs taken in Fig. 2.8A&B, using Hoechst 33258 to reveal nuclei.

(D) Protein lysates from myotubes differentiated with or without SMC for 48 hours were analyzed by Western blotting for expression of cIAP1. GAPDH was used as a loading control. Data are mean ± standard error of three independent experiments. * indicates significantly different, with $p < 0.05$. ** indicates $p < 0.01$. Scale bar represents 50 μm.


A
WT


B
WT + SMC


C
% of myotubes with >20 nuclei
50
40
30
20
10
0
*
WT
WT+ SMC

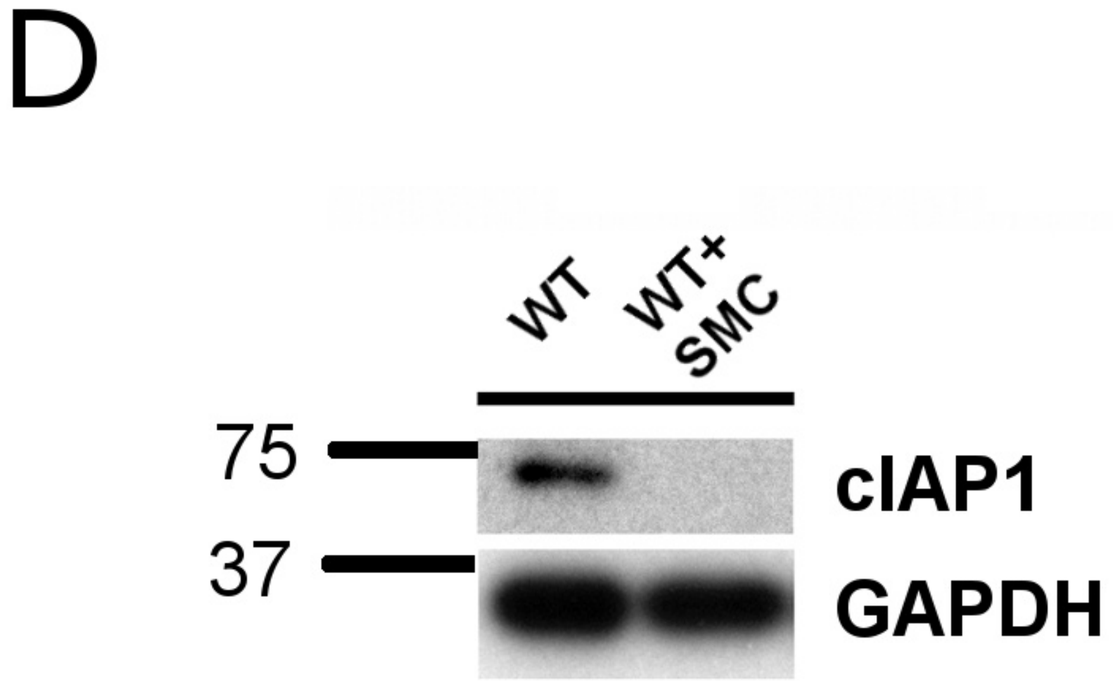

D
WT
WT+ SMC
75
37
cIAP1
GAPDH

Fig. 2.9: Loss of cIAP1 does not cause muscle hypertrophy.

(A) To determine the rate of action of the SMC, wildtype primary myoblasts were treated with 500 nM SMC, and lysates were harvested after the indicated durations of treatment. Samples were collected and analyzed by immunoblotting for expression of cIAP1 and GAPDH. SMC caused complete loss of cIAP1 within half an hour of addition to the myoblasts.

(B) To determine if cIAP1 influenced early or late stages of myoblast fusion, differentiating wildtype myoblasts were treated with SMC during the entire differentiation time course, for the latter 24 hours, or not at all. The resulting myotubes were immunolabeled for MHC for analysis of myotube diameter. The results indicate that SMC increased myotube diameter only when present in the first 24 hours of differentiation, suggesting that loss of cIAP1 increases the early stage of myoblast fusion. Furthermore, myotubes treated with SMC for the last 24 hours of differentiation were not different in diameter from untreated myotubes, indicating that SMC does not cause myotube hypertrophy. * indicates significantly different, with $p < 0.05$.

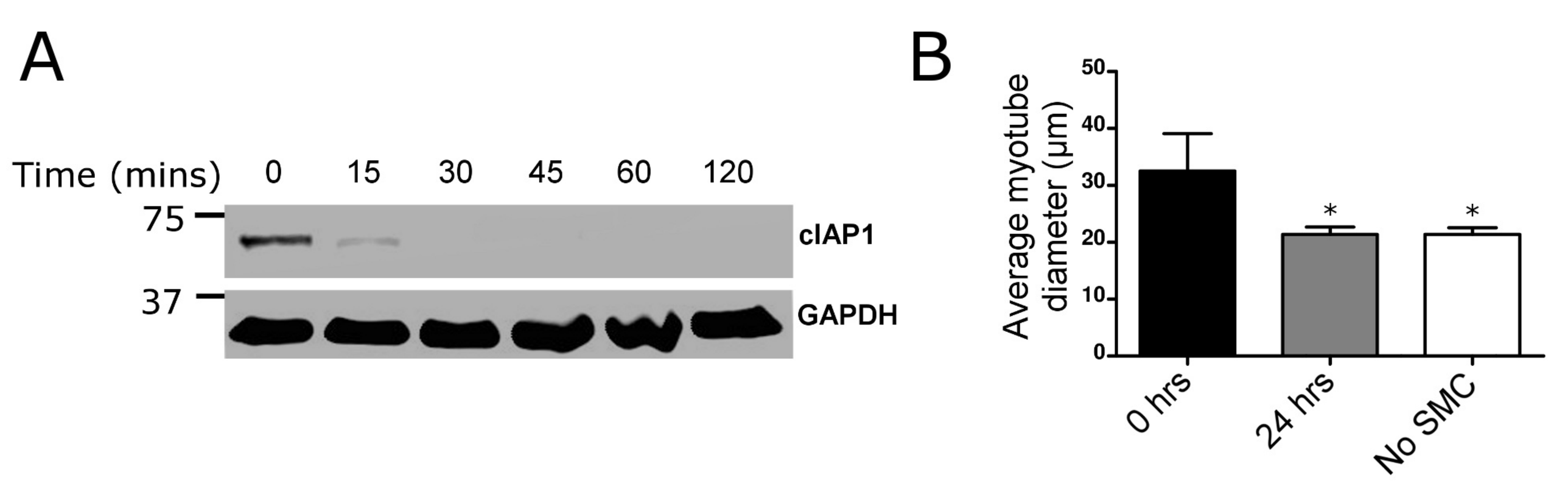
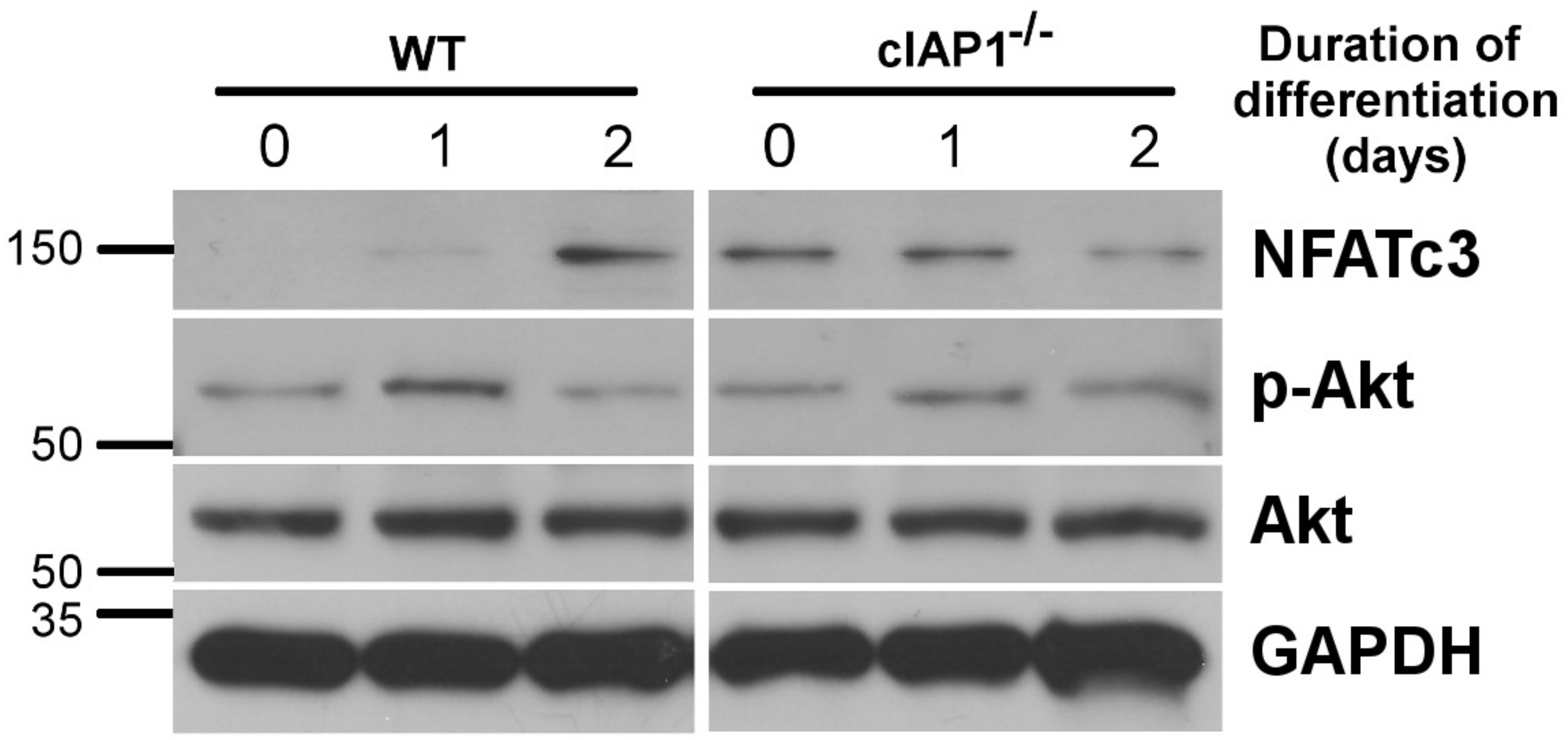

A
Time (mins)
0
15
30
45
60
120
75
37
cIAP1
GAPDH
B
Average myotube diameter (µm)
50
40
30
20
10
0
*
*
0 hrs
24 hrs
No SMC

Fig. 2.10: Loss of cIAP1 increases expression of markers of fusion but not hypertrophy. To determine if the loss of cIAP1 increased myoblast fusion or myotube hypertrophy, wildtype primary and *cIAP1*$^{-/-}$ myoblasts were differentiated for the indicated durations, and samples were collected and analyzed by immunoblotting for markers of hypertrophy and fusion. NFATc3 is involved in early (myoblast-myoblast) stages of fusion, and its expression was elevated in the absence of cIAP1. Conversely, the phosphorylation of Akt is a significant indicator of hypertrophy; expression of phospho-Akt actually diminished in the absence of cIAP1. These results suggest that loss of cIAP1 increases myoblast fusion and not myotube hypertrophy.


WT
cIAP1-/-
Duration of differentiation (days)
0
1
2
0
1
2
150
50
50
35
NFATc3
p-Akt
Akt
GAPDH

cIAP1 negatively regulates the classical and alternative NF-κB pathways

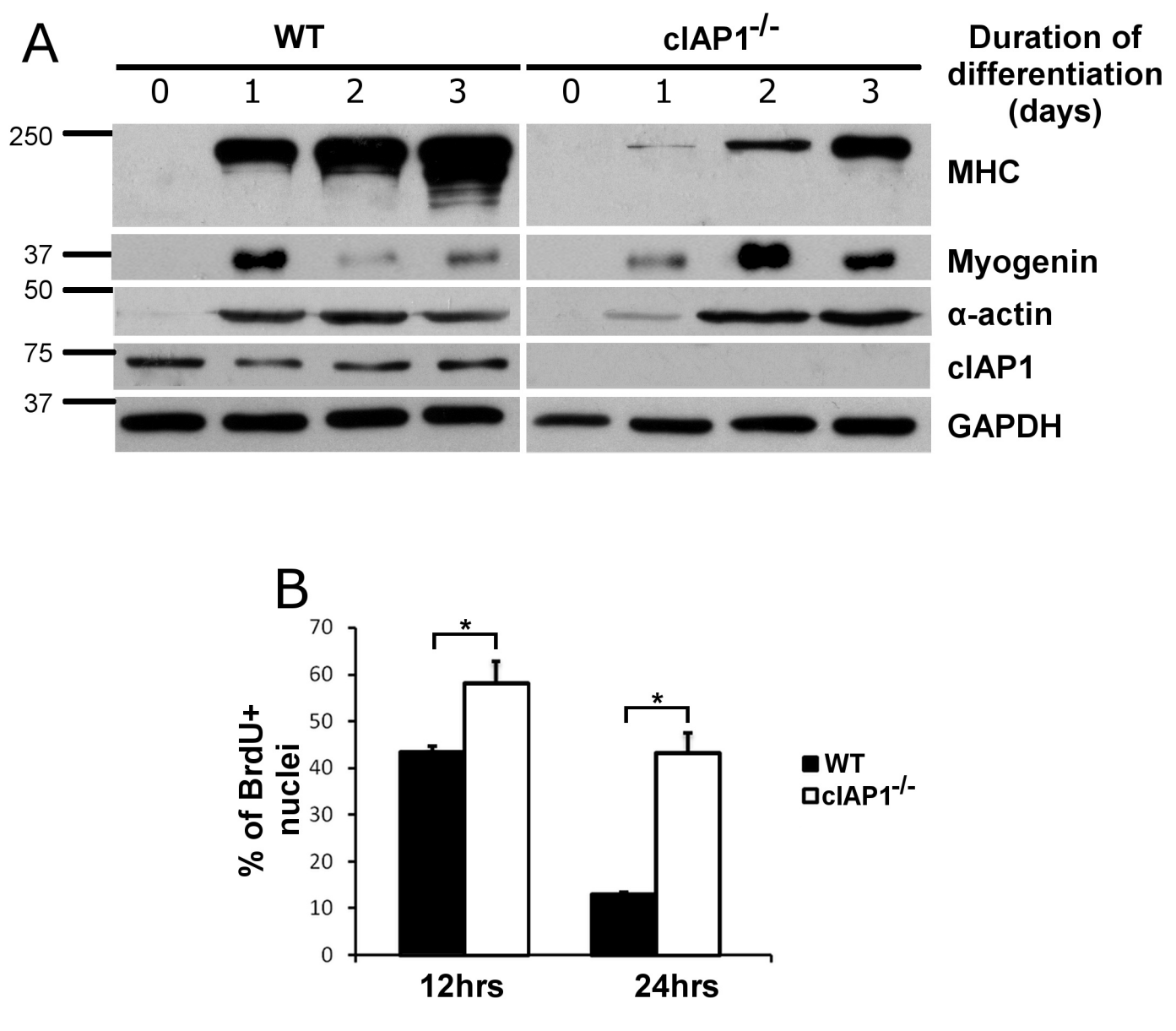
To address the mechanisms behind the regulation of fusion by cIAP1, I differentiated wildtype and *cIAP1*$^{-/-}$ myoblasts for between 0 and 3 days, and examined expression of differentiation markers by immunoblotting. Expression of myogenin, myosin heavy chain (MHC), and α-actin were significantly delayed in *cIAP1*$^{-/-}$ myoblasts as compared to wildtype cells (Fig. 2.11A). I asked whether cell cycle exit, another indicator of myoblast differentiation (Shen et al., 2003; Zammit et al., 2006), was also affected by the loss of cIAP1. Wildtype and *cIAP1*$^{-/-}$ myoblasts were pulsed with BrdU for 15 minutes at 12 and 24 hours after serum withdrawal. At each time point, a greater percentage of nuclei incorporated BrdU in the *cIAP1*$^{-/-}$ myoblasts than in wildtype cells (Fig. 2.11B), further suggesting that loss of cIAP1 delays differentiation. This delay occurred in spite of the timely and robust formation of hyperfused *cIAP1*$^{-/-}$ myotubes. The seeming contrast between inhibition of differentiation and promotion of fusion suggested that divergent processes were involved in the phenotype observed in *cIAP1*$^{-/-}$ myotubes.

The classical NF-κB pathway is active in proliferating myoblasts, and several studies suggest that it inhibits differentiation through multiple mechanisms (Guttridge et al., 1999; Canicio et al., 2001; Wang et al., 2007; Bakkar and Guttridge, 2010). Recent studies (Varfolomeev et al., 2007; Vince et al., 2007; Varfolomeev and Vucic, 2008) reveal that the loss of cIAP1, such as is triggered using SMC, leads to ligand-autonomous activation of both classical and alternative NF-κB pathways in various cell lines. It is thus possible that the loss of cIAP1 inhibits differentiation by activation of NF-κB. To address this question, I

Fig. 2.11: Myoblast differentiation is delayed in the absence of cIAP1.

(A) To determine the effect of cIAP1 on differentiation, wildtype and *cIAP1*$^{-/-}$ myoblasts were differentiated for the indicated number of days, and samples were analyzed by Western blotting. *cIAP1*$^{-/-}$ myoblasts exhibited a delay in differentiation, as is evident from the reduced expression of markers MHC, myogenin, and α-actin.

(B) Cell cycle withdrawal is a hallmark of myoblast differentiation. To determine if loss of cIAP1 affected this aspect of differentiation, primary myoblasts were differentiated for 12 or 24 hours and pulsed with 100 μl of 10 μM BrdU for 15 minutes before being fixed and immunolabeled for BrdU. Hoechst 33258 was used as a counterstain to identify nuclei, and a minimum of 200 nuclei were counted per condition. At each time point, a greater percentage of *cIAP1*$^{-/-}$ myoblasts incorporated BrdU, indicating that loss of cIAP1 delays differentiation-associated cell cycle exit. * indicates significantly different, with $p < 0.05$.


A
WT
cIAP1-/-
Duration of differentiation (days)
0
1
2
3
0
1
2
3
250
37
50
75
37
MHC
Myogenin
α-actin
cIAP1
GAPDH
B
% of BrdU+ nuclei
70
60
50
40
30
20
10
0
*
*
12hrs
24hrs
WT
cIAP1-/-

differentiated wildtype and *cIAP1*$^{-/-}$ myoblasts for between 0 and 2 days and measured activity of the classical and alternative NF-κB pathways, using an NF-κB ELISA, and antibodies directed against p65 (classical pathway) or p100/p52 (alternative pathway). In agreement with previous reports (Bakkar et al., 2008), activity of the classical pathway decreased over the course of differentiation in wildtype myoblasts (Fig. 2.12A). Strikingly, however, activity remained elevated in the *cIAP1*$^{-/-}$ cells after 24 and 48 hours of differentiation. While *cIAP1*$^{-/-}$ myoblasts also demonstrated three-fold greater activation of the alternative NF-κB pathway as compared to wildtypes, the activity decreased over the course of differentiation in both groups (Fig. 2.12B). Nevertheless, alternative pathway activity remained considerably elevated in the *cIAP1*$^{-/-}$ myoblasts. To confirm the results, I examined processing of p100 into the active p52 fragment, which is the critical step in alternative pathway activation, by Western blotting. p52 levels decreased over the course of differentiation in both wildtype and *cIAP1*$^{-/-}$ myoblasts. Nevertheless, they remained consistently higher in differentiating *cIAP1*$^{-/-}$ myoblasts than in wildtypes (Fig. 2.13). Collectively, these data suggest that NF-κB activity along both classical and alternative pathways is sustained in the absence of cIAP1.

Loss of cIAP1 increases myogenesis by activating alternative NF-κB signalling

The evidence so far suggests that down-regulation of cIAP1 activates the classical NF-κB pathway in primary myoblasts, resulting in a considerable delay in expression of biochemical markers of differentiation. The role played in myogenesis by the alternative pathway, whose activation is also elevated in the absence of cIAP1, is unclear. I thus asked whether increased myoblast fusion in the absence of cIAP1 is a direct consequence of

Fig. 2.12: Loss of cIAP1 leads to sustained activation of classical and alternative NF-κB pathways.

(A, B) To determine the effect of cIAP1 loss on NF-κB activity, wildtype and *cIAP1*$^{-/-}$ myoblasts were differentiated for the indicated durations, and were harvested for NF-κB activity analysis by ELISA. This assay quantified the amount of p65-containing (A) or p52-containing (B) NF-κB complexes in 10 μg of each sample that could bind to an immobilized NF-κB consensus sequence oligomer. Light units are expressed as relative to undifferentiated (time 0) wildtype myoblasts. These results indicate that both classical (p65-containing) and alternative (p52-containing) NF-κB pathways remain active over the course of differentiation, in the absence of cIAP1. * indicates significantly different, with $p < 0.05$. ** indicates $p < 0.01$.

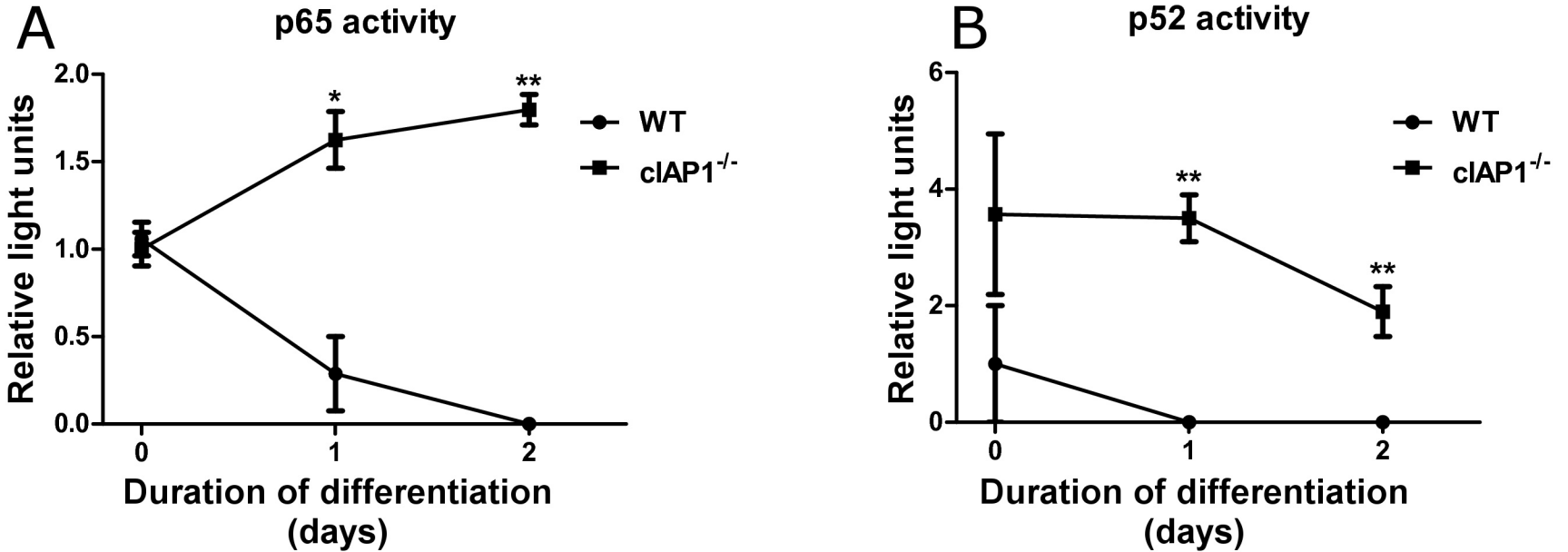
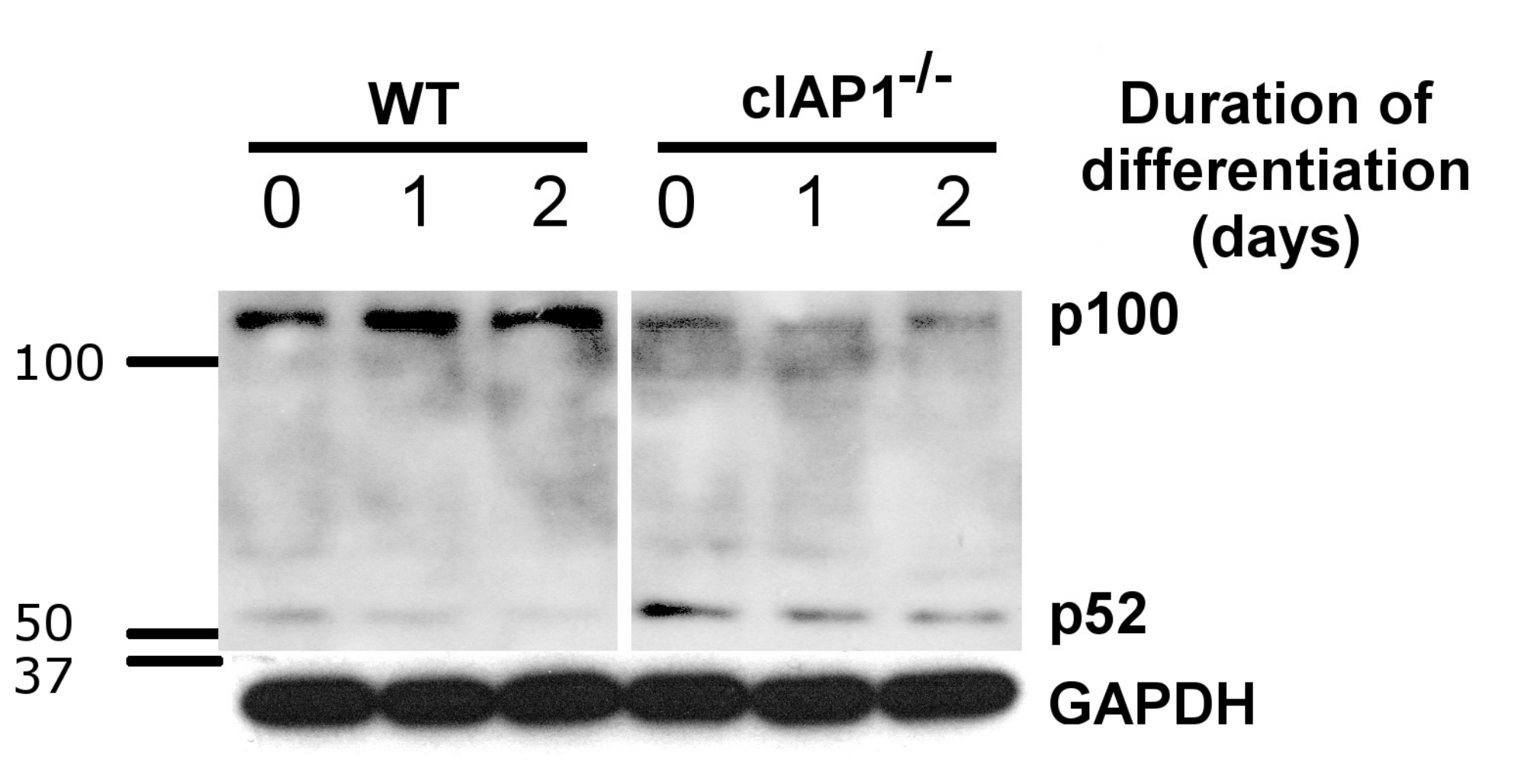

A
p65 activity
Relative light units
2.0
1.5
1.0
0.5
0.0
*
**
WT
clAP1-/-
0
1
2
Duration of differentiation
(days)
B
p52 activity
Relative light units
6
4
2
0
**
**
WT
clAP1-/-
0
1
2
Duration of differentiation
(days)

Fig. 2.13: Loss of cIAP1 leads to sustained activation of the alternative NF-κB pathway. To confirm the results of the NF-κB ELISA, wildtype and *cIAP1*$^{-/-}$ myoblasts were differentiated for the indicated number of days, and samples were analyzed by Western blotting for p100 processing. The extent to which p100 is processed into p52 is an indication of pathway activity; there is considerably more processing in the absence of cIAP1.


WT
cIAP1-/-
Duration of differentiation (days)
0
1
2
0
1
2
100
50
37
p100
p52
GAPDH

alternative pathway activation. To test this, I used siRNA to knock down p100 in *cIAP1*$^{-/-}$ myoblasts (Fig. 2.14A-C). This resulted in a 50% reduction in myotube diameter when compared to *cIAP1*$^{-/-}$ myoblasts transfected with non-targeting siRNA controls (Fig. 2.15A), as well as an almost complete loss of hypernucleated myotubes (Fig. 2.15B). I repeated the experiment using siRNAs targeting IKKα and RelB, both of which are also essential components of the alternative pathway (Bonizzi et al., 2004). In each case, knockdown of these gene products in *cIAP1*$^{-/-}$ myoblasts resulted, upon differentiation, in reduced myotube diameter and a loss of hypernucleated myotubes (Fig. 2.16, 2.18A). Similar results were seen when IKKα and RelB were down-regulated (Fig. 2.17) in wildtype myoblasts, and in wildtype myoblasts treated with SMC (Fig. 2.16, 2.18B). Next, I took a more direct approach and asked if activation of the alternative NF-κB pathway was sufficient to produce hypernucleated myotubes, even in the presence of cIAP1. Wildtype myoblasts were transiently co-transfected with p52 and HA-tagged RelB, or with GFP as a control, and differentiated for 48 hours. Overexpression of p52 and RelB increased the percentage of hypernucleated myotubes and the average myotube diameter as compared to GFP controls (Fig. 2.19, 2.20). The myotubes produced upon overexpression of p52 and RelB were visually indistinguishable from those formed from *cIAP1*$^{-/-}$ myoblasts. Taken together, these results suggest that cIAP1 controls myotube size by negatively regulating the alternative NF-κB pathway.

Fig. 2.14: The alternative NF-κB pathway is required for increased myoblast fusion in the absence of cIAP1.

(A, B) To determine if the alternative NF-κB pathway is required for increased myoblast fusion, *cIAP1*$^{-/-}$ myoblasts were transfected for 24 hours with 120 nM of siRNA targeting p100, or with non-targeting siRNA controls. The myoblasts were subsequently differentiated for 48 hours. Myotubes were immunolabeled for MHC (red). Knockdown of p100 abolished the fusion phenotype of the *cIAP1*$^{-/-}$ myotubes. Scale bar represents 50 μm.

(C) Efficacy of siRNA knockdown of p100/p52 was assessed by Western blotting after 48 hours of differentiation. GAPDH serves as loading control.

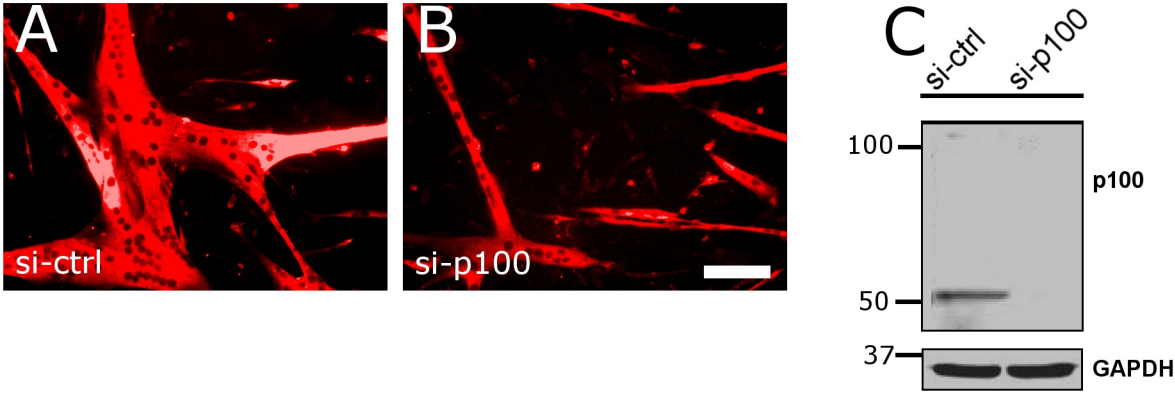

A
si-ctrl
B
si-p100
C
si-ctrl
si-p100
100
50
37
p100
GAPDH

Fig. 2.15: Attenuating the alternative pathway reduces myoblast fusion in the absence of cIAP1.

(A, B) To quantify the effect of p100 knockdown on fusion of *cIAP1*$^{-/-}$ myoblasts, the average myotube diameter and percentage of hypernucleated myotubes were measured from photomicrographs of the siRNA-transfected *cIAP1*$^{-/-}$ myotubes, as described in Fig. 2.14. * indicates significantly different, with $p < 0.05$.

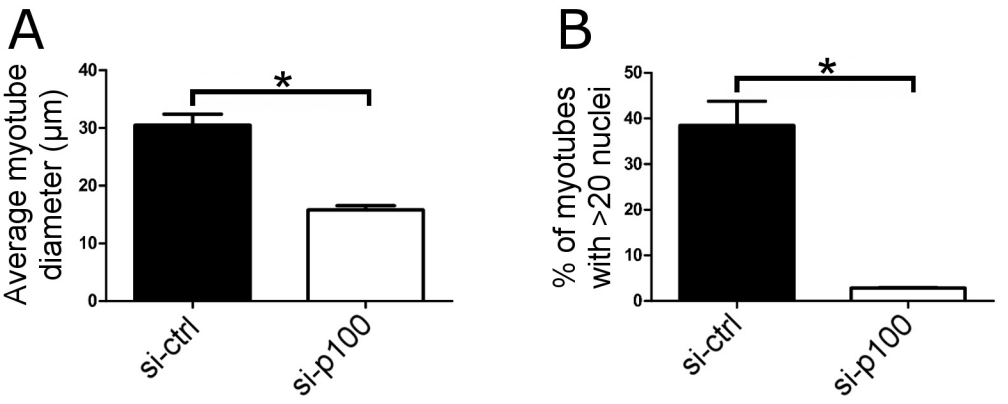

A
Average myotube diameter (µm)
40
30
20
10
0
*
si-ctrl
si-p100
B
% of myotubes with >20 nuclei
50
40
30
20
10
0
*
si-ctrl
si-p100

Fig. 2.16: The alternative NF-κB pathway is required for increased myoblast fusion in the absence of cIAP1.

To confirm that the alternative NF-κB pathway is essential for myoblast fusion, I targeted different components of the pathway (IKKα and RelB) and examined the effect on the myotubes. Wildtype, SMC-treated wildtype, and *cIAP1*$^{-/-}$ myoblasts were transfected with the indicated siRNAs. Twenty-four hours after transfection, they were seeded at equal densities, differentiated for two days, and immunolabeled for MHC (red). The upper panel shows the expected phenotype resulting from wildtype, wildtype + SMC, and *cIAP1*$^{-/-}$ myoblasts. The middle panel shows the considerable attenuation of myoblast fusion that results from IKKα knockdown. The lower panel is similar to the middle panel in the attenuation of fusion, this time from down-regulation of RelB. Scale bar represents 50 μm.

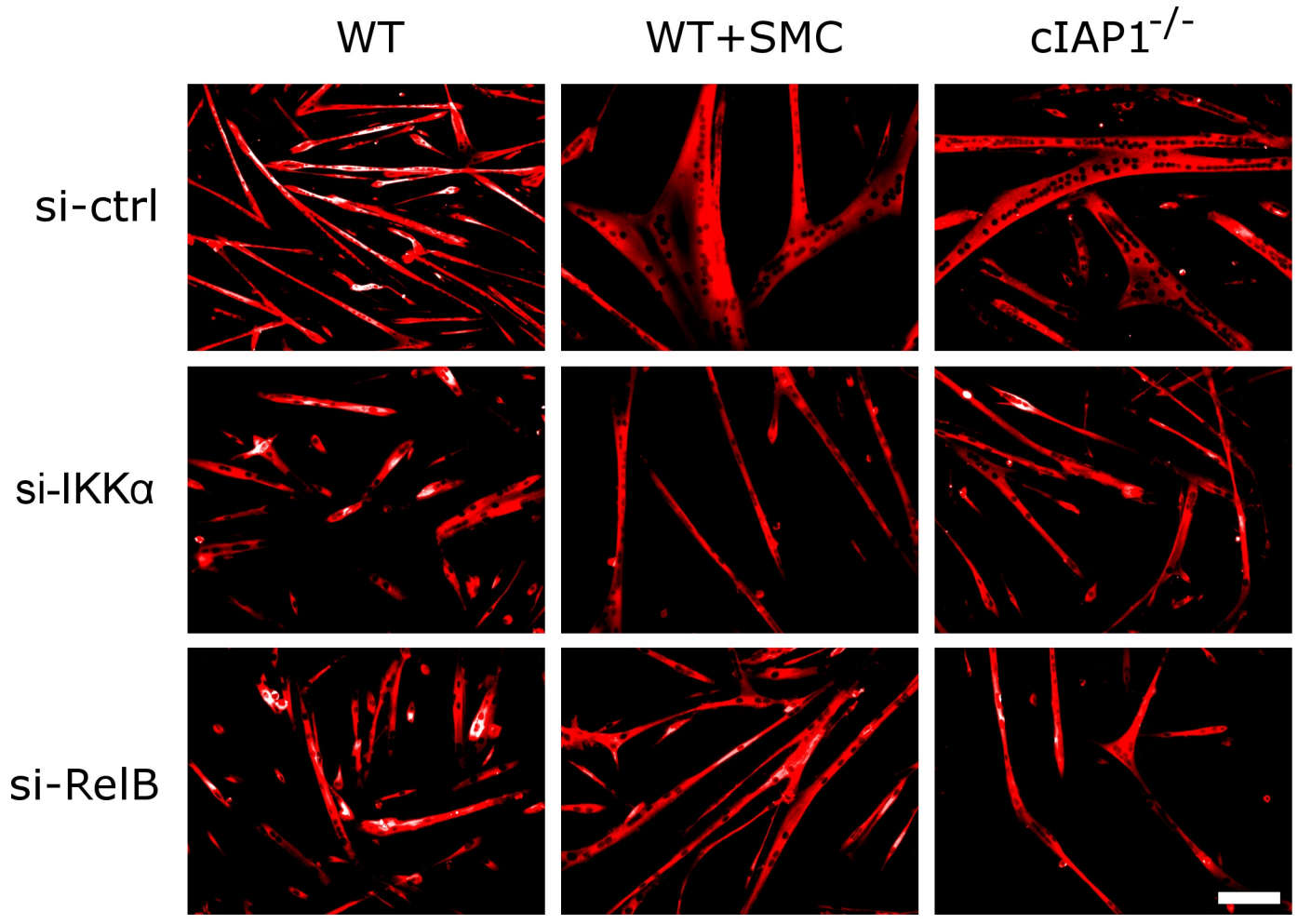

WT
WT+SMC
cIAP1-/-
si-ctrl
si-IKKα
si-RelB

Fig. 2.17: Western blot analyses of myoblasts transfected with specific or non-targeting siRNAs.

(A, B) *cIAP1*$^{-/-}$ myoblasts were transfected with the indicated siRNAs to inactivate the alternative NF-κB pathway. To verify the efficacy of knockdown, samples were harvested for Western blotting three days after transfection to ensure that the knockdown remained effective throughout the course of differentiation. GAPDH is used as a loading control.

A
si-ctrl
si-IKKα
75
37
IKKα
GAPDH
B
si-ctrl
si-RelB
75
37
RelB
GAPDH

Fig. 2.18: Inactivation of the alternative NF-κB pathway reduces myoblast fusion in the absence of cIAP1.

(A, B) To determine if the alternative NF-κB pathway was required for myoblast hyperfusion, *cIAP1*$^{-/-}$ myoblasts (A) and wildtype myoblasts (B) were transfected with siRNAs targeting critical components (RelB and IKKα) of the alternative pathway. These were differentiated for two days and immunolabeled for MHC. The average myotube diameter and percentage of hypernucleated myotubes were quantified from photomicrographs of the siRNA-transfected myotubes. * indicates significantly different, with $p < 0.05$.

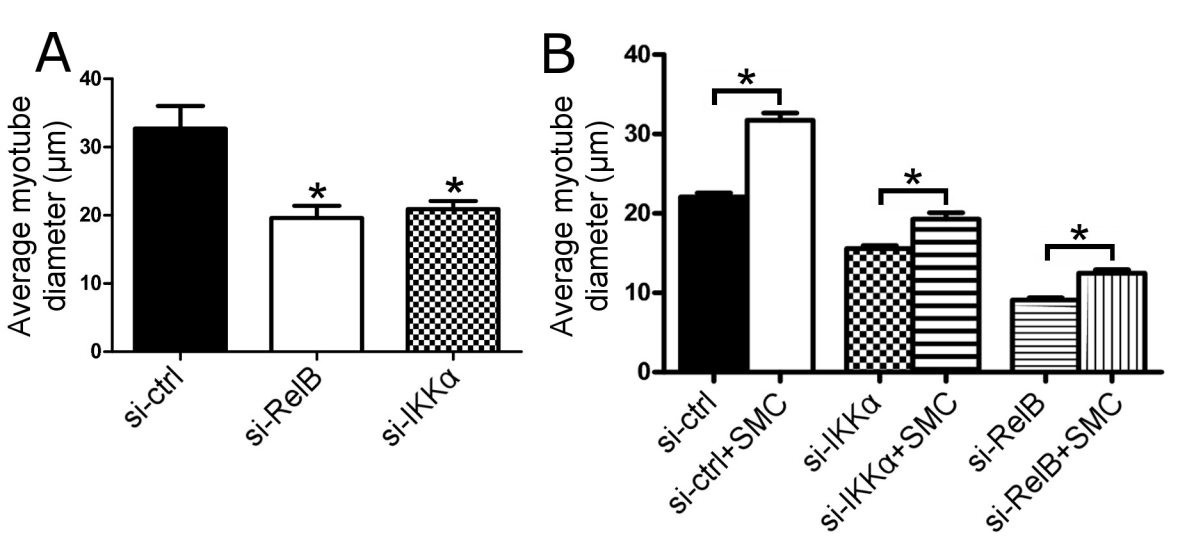

A
Average myotube diameter (μm)
40
30
20
10
0
*
*
si-ctrl
si-RelB
si-IKKα
B
Average myotube diameter (μm)
40
30
20
10
0
*
*
*
si-ctrl
si-ctrl+SMC
si-IKKα
si-IKKα+SMC
si-RelB
si-RelB+SMC

Fig. 2.19: Activation of the alternative NF-κB pathway is sufficient to increase myoblast fusion.

(A) Wildtype myoblasts were co-transfected with expression vectors coding for p52 and HA-tagged RelB, or with a vector coding for GFP as a control. Twenty-four hours after transfection, the myoblasts were differentiated for two days, and subsequently immunolabeled for MHC (red). Overexpression of p52:RelB was sufficient to reproduce the increased fusion phenotype seen with *cIAP1*$^{-/-}$ myotubes. Scale bar represents 50 μm.

(B) Overexpression of p52 and HA-RelB after 48 hours of differentiation was verified by Western blotting.

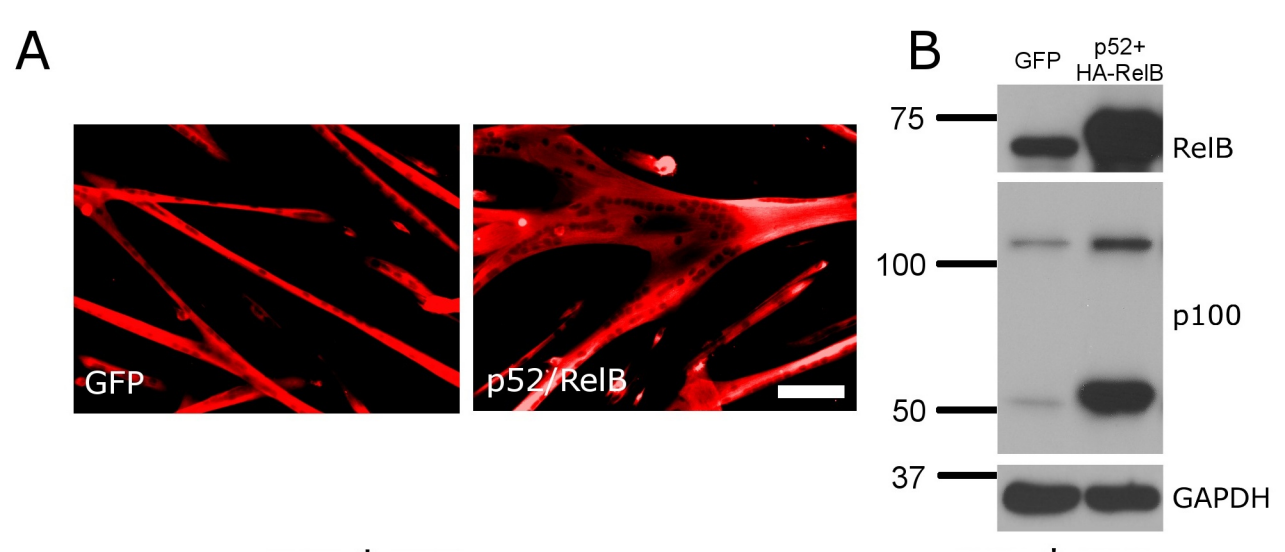
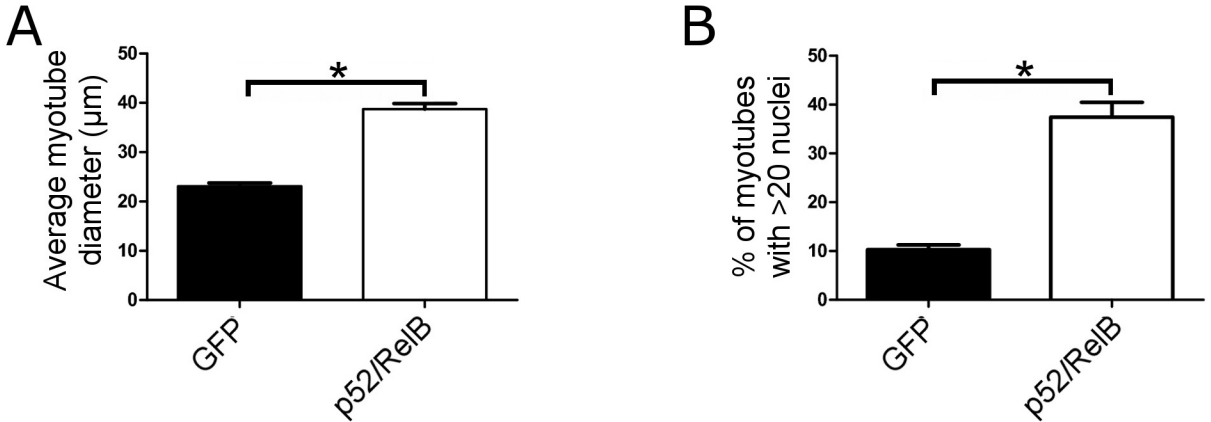

A
GFP
p52/RelB
B
GFP
p52+
HA-RelB
75
RelB
100
p100
50
37
GAPDH

Fig. 2.20: Activation of the alternative NF-κB pathway increases myoblast fusion.

(A, B) The effect of overexpression of p52 and RelB on myoblast fusion was measured by quantifying the average myotube diameter (A) and percentage of hypernucleated myotubes (B), as described in Fig. 2.18. * indicates significantly different, with $p < 0.05$.


A
Average myotube diameter (µm)
50
40
30
20
10
0
*
GFP
p52/RelB
B
% of myotubes with >20 nuclei
50
40
30
20
10
0
*
GFP
p52/RelB

Loss of cIAP1 increases muscle size

I next asked whether the aforementioned effects of the absence of cIAP1 on myotube formation produced a similar effect *in vivo*. Previous studies of *cIAP1*$^{-/-}$ mice revealed no overt phenotype, other than the corresponding upregulation of cIAP2 in various tissues (Conze et al., 2005; Mahoney et al., 2008). None of the previous analyses, however, included histological, physiological or functional assessments of skeletal or cardiac muscle. Here, I found that the hind limb muscles of *cIAP1*$^{-/-}$ mice, particularly the gastrocnemius, quadriceps and tibialis anterior (TA) muscles, were more massive than in their wildtype counterparts (Fig. 2.21A). Surprisingly, this was not associated with a significant increase in overall body mass (Fig. 2.21B). To examine the basis of the increased musculature, I took cross-sections through the TA and gastrocnemius muscles of wildtype and *cIAP1*$^{-/-}$ mice, and performed morphometry of fibre and muscle cross-sectional areas. In *cIAP1*$^{-/-}$ gastrocnemii, the muscle fibre and whole-muscle cross-sectional areas were greater than in wildtype controls, whereas the *cIAP1*$^{-/-}$ TA contained more fibres per muscle than controls (Fig. 2.22A-C). To determine whether satellite cell numbers were affected by the loss of cIAP1, I counted the numbers of Pax7-positive satellite cells in the muscle cross-sections. The *cIAP1*$^{-/-}$ TA and gastrocnemii contained 80% to 100% more satellite cells per unit area than the controls, respectively (Fig. 2.23). These results suggest that cIAP1 acts *in vivo* to control skeletal muscle mass and to expand the stem cell niche.

Fig. 2.21: Loss of cIAP1 increases muscle mass.

(A) To determine if the absence of cIAP1 plays a role in maintaining muscle mass, the indicated muscle groups were isolated and weighed from *cIAP1*$^{-/-}$ and wildtype mice, matched for age and sex. Each measured muscle group was heavier in the *cIAP1*$^{-/-}$ mice. Gastroc, gastrocnemius; Quad, quadriceps; TA, tibialis anterior.

(B) Wildtype and *cIAP1*$^{-/-}$ mice used in (A) were weighed before being euthanized for histology. n = 3-4 per group. Data are mean ± standard error. * indicates significantly different, with $p < 0.05$.

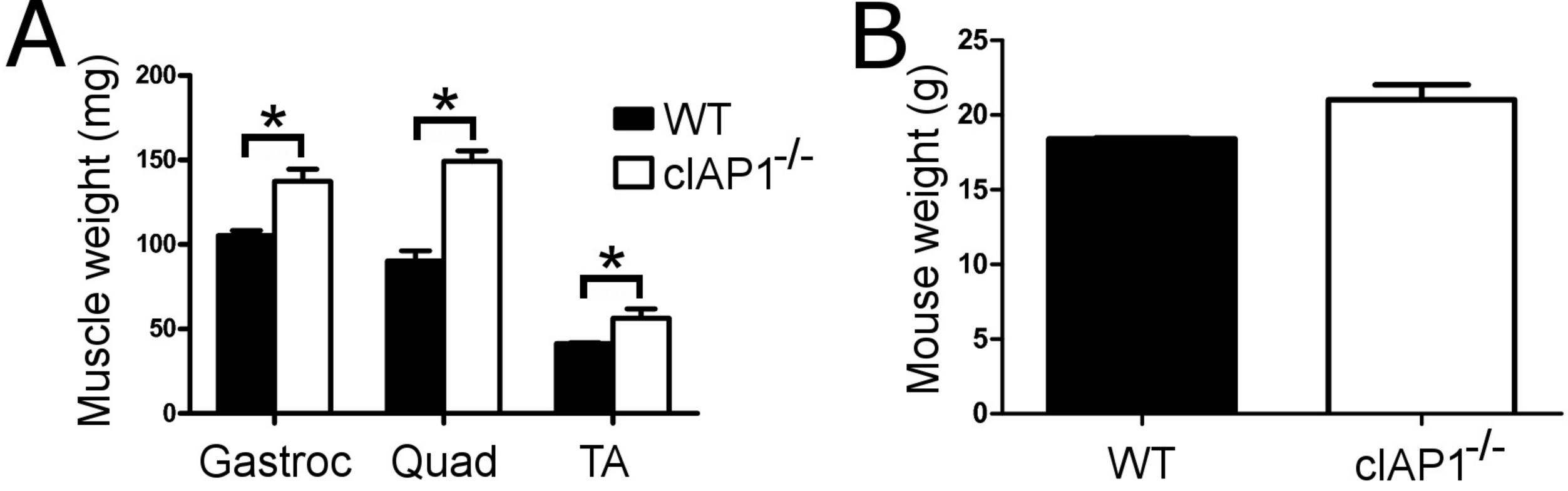
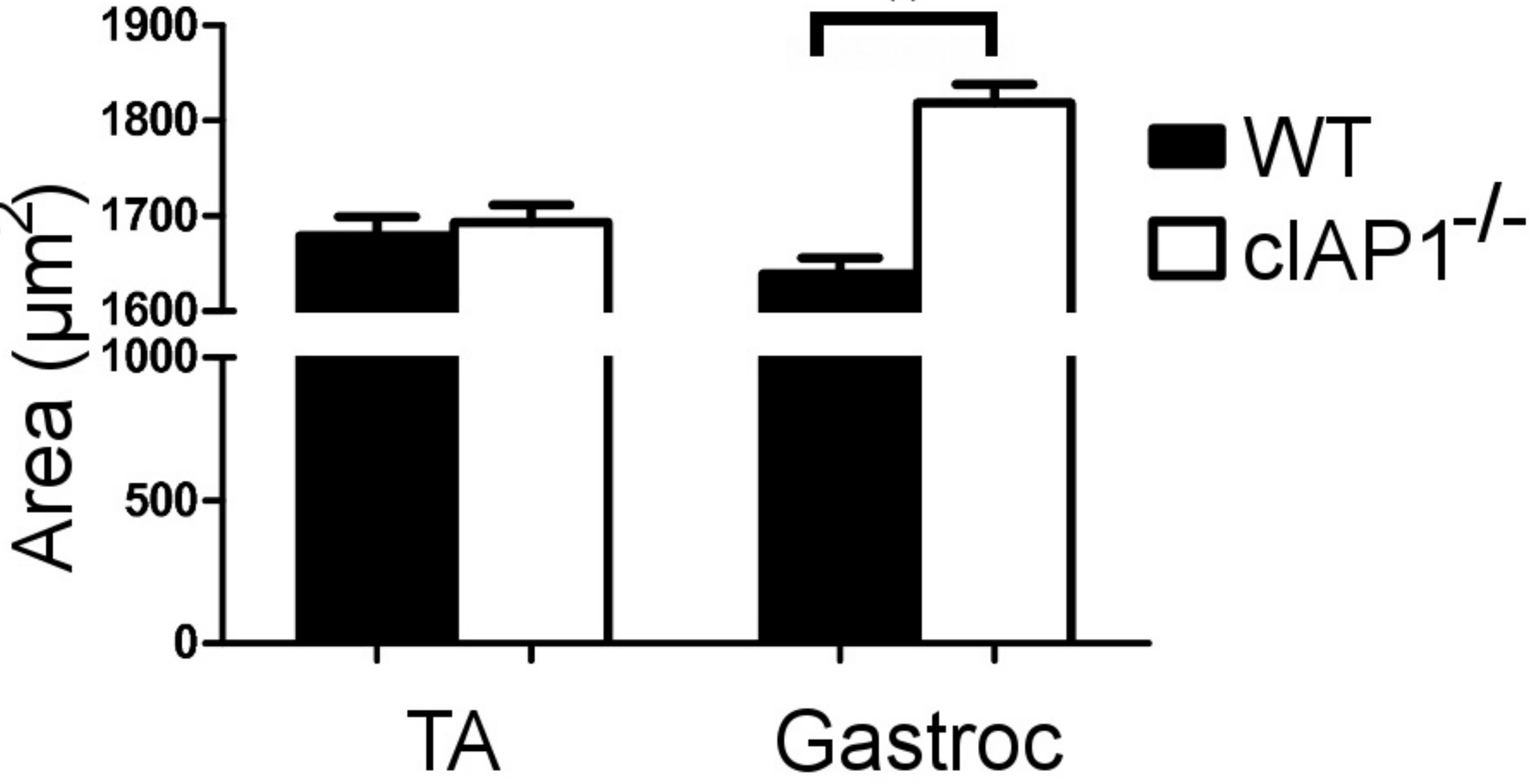

A
Muscle weight (mg)
200
150
100
50
0
*
*
*
WT
cIAP1-/-
Gastroc
Quad
TA
B
Mouse weight (g)
25
20
15
10
5
0
WT
cIAP1-/-

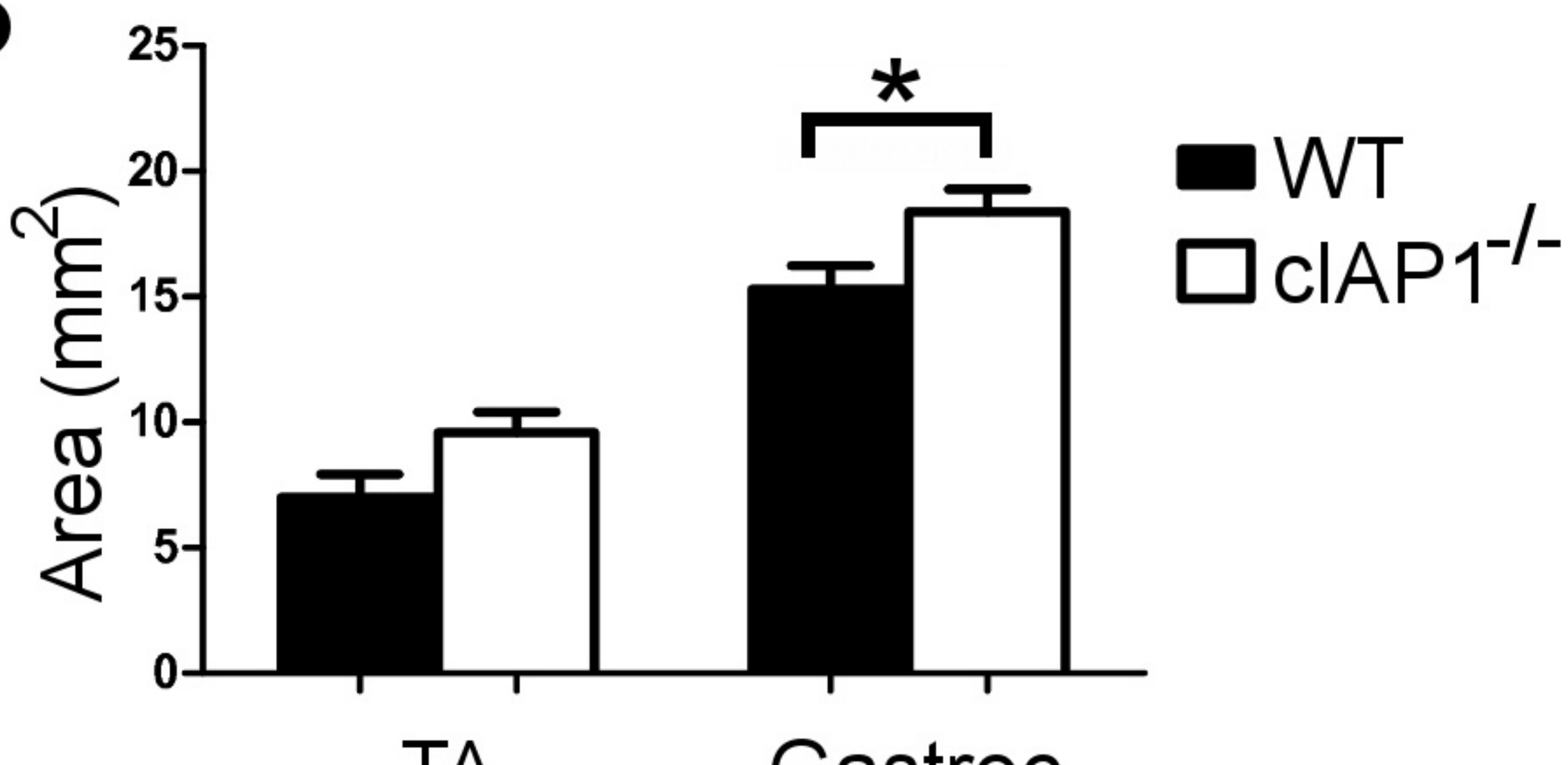
Fig. 2.22: Loss of cIAP1 increases muscle size.

(A, B, C) Ten-micrometer cross-sections through the TA and gastrocnemius muscles were stained with haematoxylin and eosin. For each genotype, I quantified the average fibre cross-sectional area (A), the average cross-sectional areas of the individual muscles (B), and the total number of fibres per muscle (C), using Northern Eclipse software. n = 3-4 per group. Data are mean ± standard error. * indicates significantly different, with $p < 0.05$.

A


1900
1800
1700
1600
1000
500
0
Area (μm2)
*
WT
clAP1-/-
TA
Gastroc

B


25
20
15
10
5
0
Area (mm2)
*
WT
clAP1-/-
TA
Gastroc

C

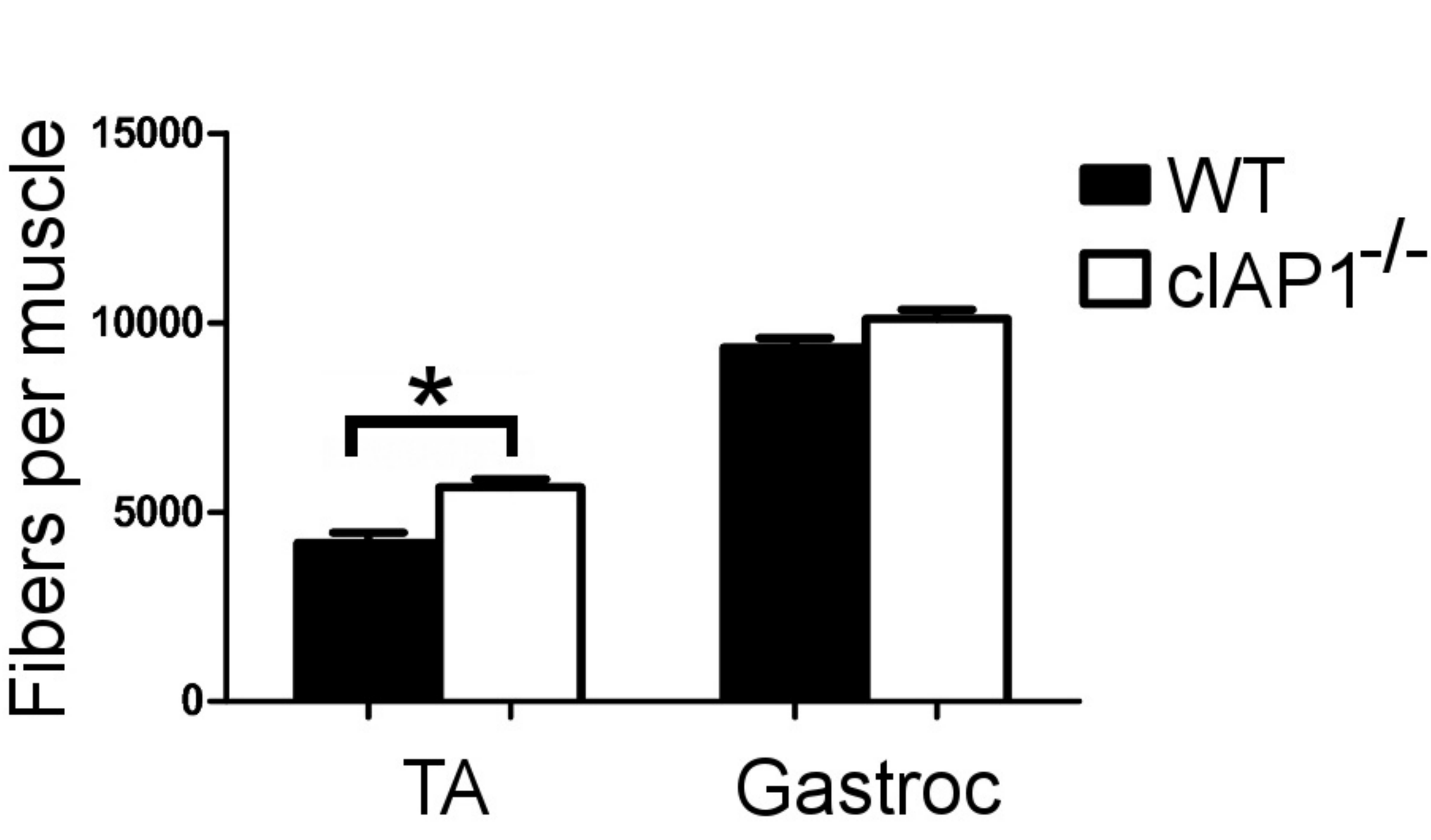
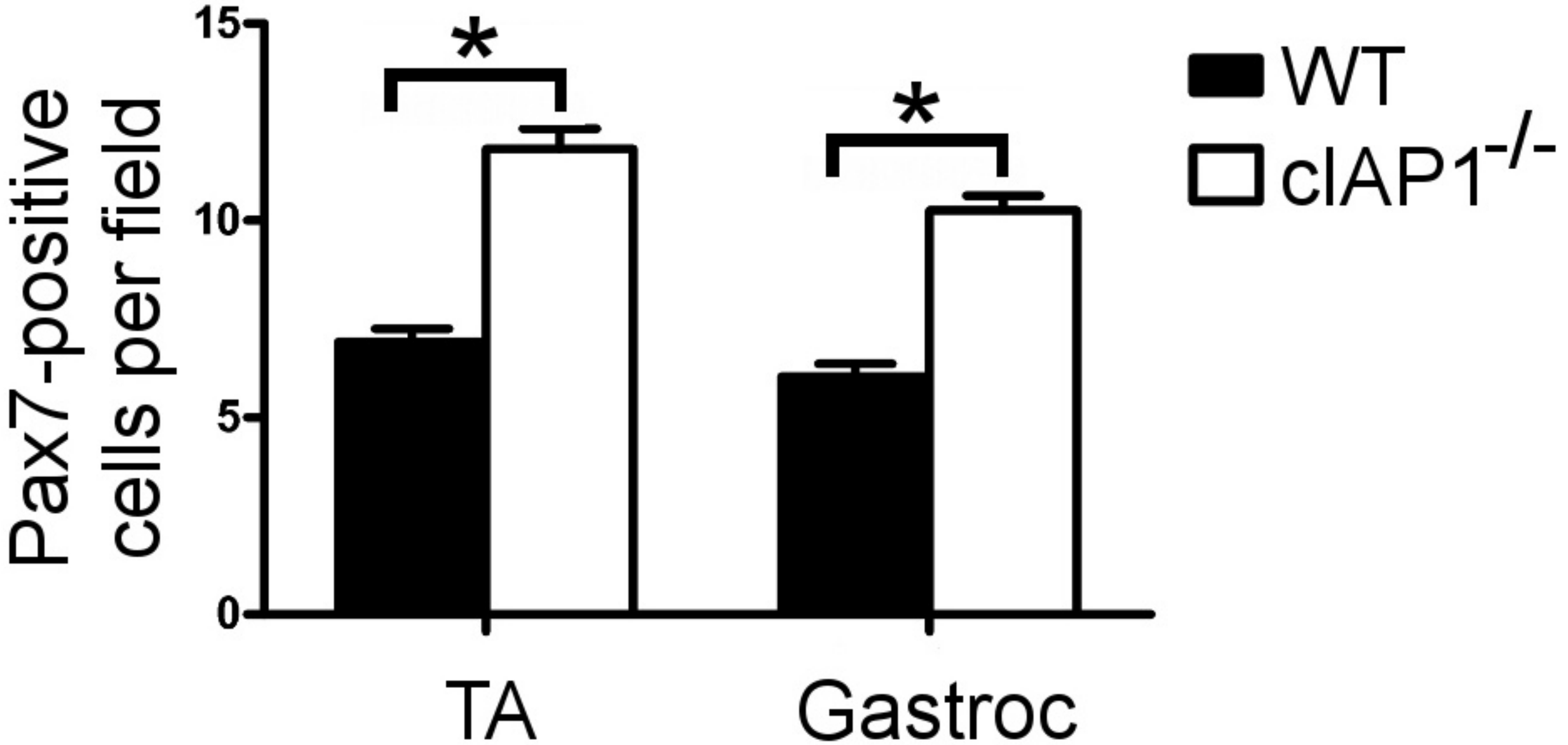

15000
10000
5000
0
Fibers per muscle
*
WT
clAP1-/-
TA
Gastroc

Fig. 2.23: Loss of cIAP1 increases the number of muscle satellite cells.

To determine if the loss of cIAP1 influenced muscle satellite cell numbers, the satellite cell density was quantified from cross-sections immunolabeled for the satellite cell marker Pax7. There was an increase in number of satellite cells in both TA and gastrocnemius muscles. *n* = 3-4 per group. Data are mean ± standard error. * indicates significantly different, with $p < 0.05$.

Figure produced in collaboration with Radoslav Zinoviev in the lab of Dr. Michael Rudnicki.


Pax7-positive cells per field
15
10
5
0
*
*
TA
Gastroc
WT
clAP1-/-

Discussion

Skeletal muscle as a tissue is highly adaptive and responsive to environmental and functional requirements, and employs a wide range of regulatory processes to maintain the organism's metabolic health. These processes are thus often implicated when degenerative and disease states disrupt muscle homeostasis. Evidence to date places the NF-κB pathways as key molecular sensors in many muscle regulatory processes, including hypertrophy (Glass, 2005), atrophy (Hunter et al., 2002; Cai et al., 2004; Hunter and Kandarian, 2004), development (Dahlman et al., 2010) and regeneration (Munz et al., 2002; Mourkioti et al., 2006; Acharyya and Guttridge, 2007). The data presented here indicate that cIAP1 regulates skeletal muscle mass, producing a 5-10 fold increase in myonuclear number *in vitro*, by controlling both NF-κB signaling arms, so that with the loss of cIAP1, sustained activity of the alternative pathway increases myogenesis. This positions cIAP1 as a potential master regulator with functions that potentially extend through the full range of roles already ascribed to NF-κB in muscle biology.

Many studies that involve cIAP1 also address similar roles played by its cIAP2 paralogue. cIAP1 is expressed ubiquitously, and in most situations functions interchangeably with cIAP2 to regulate NF-κB signalling (Vince et al., 2007; Mahoney et al., 2008; Zarnegar et al., 2008). The effects on myogenesis in this study are ascribed to cIAP1 alone, because cIAP2 is not expressed in muscle (Mahoney et al., 2008). Consequently, cIAP2$^{-/-}$ myoblasts differentiate to produce myotubes identical in size to wildtypes (E.K.E. and R.G.K., unpublished observations). Nevertheless, this functional redundancy may explain the general lack of phenotype previously ascribed to the cIAP1-knockout (Conze et al., 2005). The

cIAP2$^{-/-}$ mouse also has no overt phenotype, but is highly resistant to the toxic effects of LPS-induced sepsis (Conte et al., 2006). The difference in functionality here occurs largely because cIAP2, unlike cIAP1, is a transcriptional target of NF-κB (Chu et al., 1997; Schoemaker et al., 2002). This permits macrophages, which upregulate cIAP2 upon activation, to survive the high levels of reactive oxygen species and inflammatory cytokines released upon sepsis. As macrophages invade damaged muscle and function critically in the regenerative process (Acharyya et al., 2007), this opens the possibility that cIAP2 is involved, albeit indirectly, in muscle physiology.

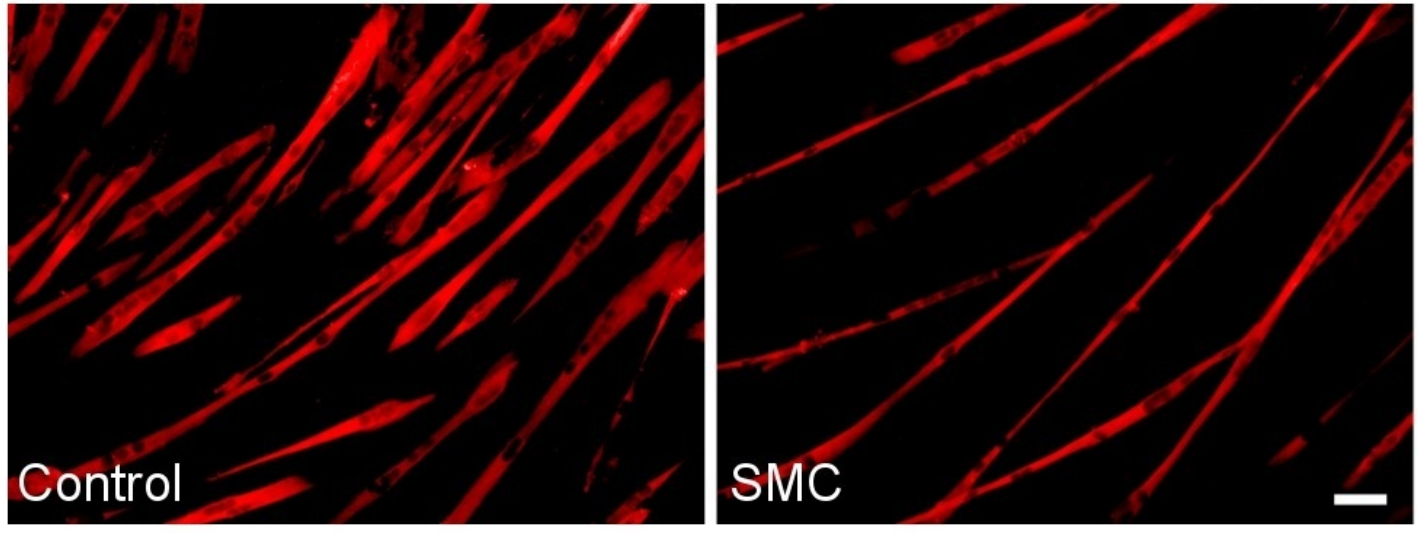
While the literature on the classical NF-κB pathway in myogenesis is extensive, relatively few studies have addressed roles played by the alternative pathway. Canicio and colleagues (Canicio et al., 2001) showed that IKKα, the upstream kinase of p100/p52, is essential for differentiation of L6 rat myoblasts. More recently, Bakkar and colleagues (2008) reported that in C2C12 myoblasts and MyoD-transformed fibroblasts, alternative signalling via p52:RelB acts to promote myotube maintenance, but plays no role in myotube formation. A key difference between that study and the present one is in our use of primary myoblasts. The consequences of NF-κB activation by SMC treatment differ considerably between immortalized C2C12 cells and primary myoblasts under similar differentiation conditions. SMC inhibits differentiation in both types of cells, but increases myogenesis only in primary myoblasts (Fig. 2.24). Nevertheless, I believe that in this context the cultured primary cells produce a more accurate representation of how these cells respond in a physiological context, as demonstrated by our findings that *cIAP1*$^{-/-}$ mice display increased muscle mass. While it is possible that alternative NF-κB signalling would improve primary myotube maintenance, the size and spontaneous contractility of the *cIAP1*$^{-/-}$ or p52:RelB-

Fig. 2.24. SMC does not increase myoblast fusion in C2C12 cells.

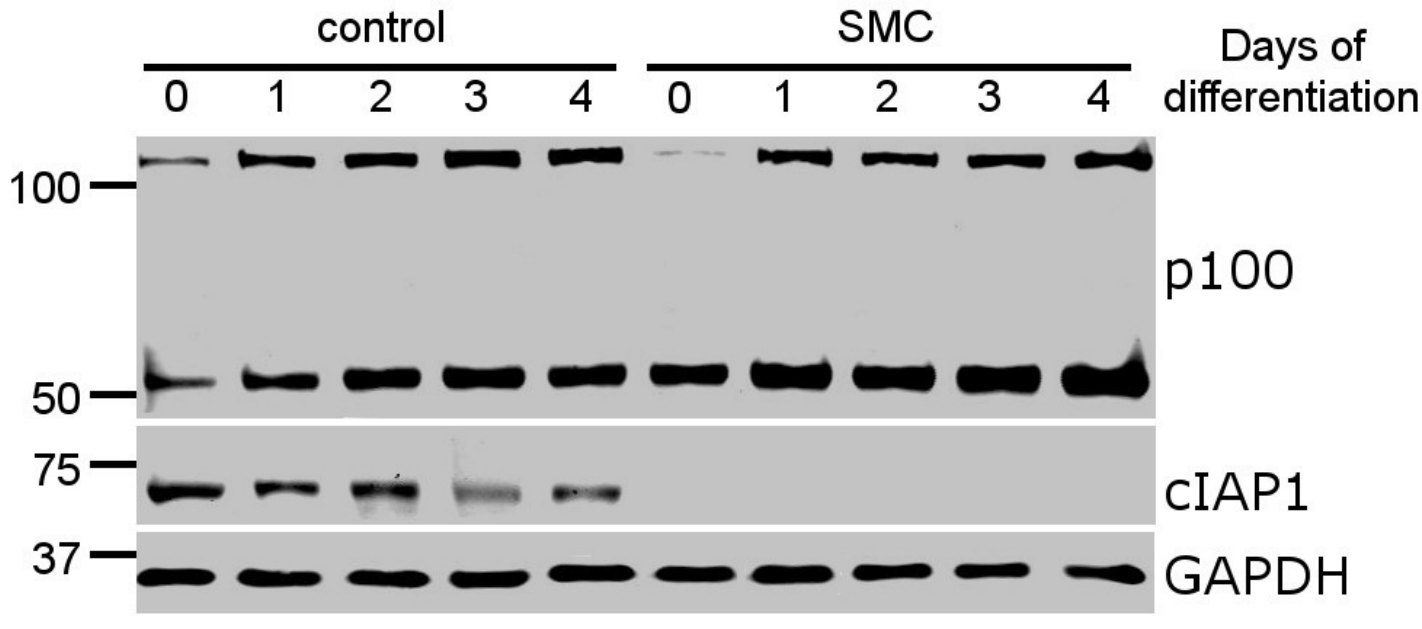
(A) To determine if loss of cIAP1 increases fusion in C2C12s, equal numbers of cells were differentiated for four days in the presence or absence of 500 μM SMC. The resulting myotubes were immunolabeled for MHC (red). Differentiation in the presence of SMC produced fewer and smaller myotubes, a result quite different from that seen in primary myoblasts. Scale bar represents 50 μm.

(B) Lysates from C2C12s differentiated for up to four days were analyzed for expression of p100 and cIAP1 by Western blotting. Samples at time point 0 were pre-treated with SMC or DMSO control for 24 hours prior to harvesting. Processing of p100 to p52 was increased in C2C12s, in the absence of cIAP1. GAPDH was used as a loading control.

A


Control
SMC

B


control
SMC
Days of differentiation
0 1 2 3 4 0 1 2 3 4
100
50
75
37
p100
cIAP1
GAPDH

overexpressing myotubes was such that they detach very readily from tissue culture plates, making long-term examination technically challenging.

The genetic and chemical ablation of cIAP1 expression in muscle cells has clearly revealed an important role for the alternative NF-κB pathway in regulating myoblast fusion, as well as muscle fibre size and mass. Classical and alternative NF-κB pathways have both shared and discrete transcriptional targets which mediate the diverse functions of the separate pathways. There are no known targets of the alternative pathway in muscle (Bakkar and Guttridge, 2010), and thus the next step in the mechanism employed by cIAP1 to promote myogenesis remains unknown. One possibility involves NF-κB activation by the cytokine TWEAK, through its receptor Fn14. TWEAK, like TNFα, causes robust skeletal muscle atrophy (Dogra et al., 2007b). The TWEAK-Fn14 axis was recently shown to be the key effector pathway in disuse atrophy (Mittal et al., 2010). As TWEAK triggers degradation of cIAP1 (Vince et al., 2008), and Fn14 is essential for myogenesis (Dogra et al., 2007a), it is tempting to speculate whether TWEAK and similar physiological ligands modulate alternative NF-κB signalling in muscle to control fusion processes under normal and disease circumstances. The NFAT proteins NFATc1, NFATc2 and NFATc3 have been implicated in promoting myoblast fusion and increasing myotube size (Horsley et al., 2003; Cowling et al., 2008; Giordano et al., 2009). NFATc2 and NFATc3 have κB sites within their promoters, leading to the possibility that these could be p52:RelB target genes. In either case, the availability of soluble, bioavailable Smac-mimetic compounds to systemically down-regulate cIAP1/2 makes it possible to address further mechanistic and functional questions about the roles played by the cIAPs in muscle homeostasis, development and regeneration.

In conclusion, the data presented here on *cIAP1*$^{-/-}$ myoblasts *in vitro* and skeletal muscle *in vivo* clearly establish roles for cIAP1 and NF-κB signalling in regulation of myoblast fusion, satellite cell homeostasis, and muscle mass. The expansion of the satellite cell niche observed in the *cIAP1*$^{-/-}$ animals warrants further investigation as to its mechanism of action, and its implications for muscle development and repair.

Chapter 3: Loss of cIAP1 attenuates soleus muscle pathology and improves diaphragm function in mdx mice

Abstract

The cellular inhibitor of apoptosis protein 1 (cIAP1) is a regulator of NF-κB signaling pathways involved in myogenesis and myoblast fusion. Here I asked whether the loss of cIAP1 would influence the pathology of skeletal muscle in the *mdx* mouse model of Duchenne muscular dystrophy. In double-mutant mice lacking both cIAP1 and dystrophin (*cIAP1*$^{-/-}$*;mdx*), the slow-twitch soleus muscles exhibited reduced proportions of centronucleated fibres, and reduced muscle damage compared to single-mutant (*cIAP1*$^{+/+}$*;mdx*) controls. No such improvements were observed in the double-mutant extensor digitorum longus (EDL) muscle. The reduction in damage of the double-mutant solei was associated with reduced infiltration of CD68-expressing macrophages. *In vitro*, the force produced at stimulation frequencies between 10 and 60 Hz was impaired in single-mutant solei, but was normal in double-mutant solei as compared to C57BL/6 controls. Finally, the double-mutant mice exhibited improved treadmill running endurance, which was associated with reduced exercise-induced damage to the diaphragm. These results suggest that loss of cIAP1 can ameliorate the pathology resulting from systemic loss of dystrophin.

Introduction

Duchenne muscular dystrophy (DMD) is a severe and progressive X-linked neuromuscular disorder resulting from mutations causing the loss of dystrophin (Hoffman et al., 1987). Dystrophin functions as a major structural protein, and its loss renders skeletal muscle extremely susceptible to damage. In humans, continuing cycles of degeneration and regeneration eventually result in the exhaustion and senescence of the satellite cells, and eventual replacement of muscle with fat and connective tissue. Affected individuals present with muscle weakness as early as two years of age, and usually die in their late teens or early twenties from pulmonary or cardiac failure. To date, there are no durable treatments or cures for this disorder; it is thus crucial to develop strategies that either improve the regenerative potential or decrease the susceptibility to dmamage of affected muscle.

Most approaches to DMD therapy fall into two broad categories. The first category includes gene replacement therapies, which employ a variety of approaches to introduce functional dystrophin constructs into affected muscle. These include transplantation of myoblasts from dystrophin-competent individuals, in the hopes that the myoblasts will fuse with and introduce dystrophin to the affected muscle. This approach suffers from low viability of the transplanted cells, and, more importantly, their inability to repopulate the satellite cell niche and thus expand the therapeutic potential away from the transplant site. Another aspect of gene replacement involves direct delivery of dystrophin constructs to the affected muscle. The sheer size of the complete open reading frame (14 kilobases, from a 2.1 megabase template) makes this a challenging task: most approaches make use of truncated but functional “micro-dystrophin” constructs that are suitable for packaging into an

adenoviral or plasmid vector. This method often requires chronic immunosuppression, but clinical trials have progressed to various stages based on this approach, particularly using so-called third-generation adeno-associated viral vectors. A routine downside of this approach is the difficulty of balancing suitable viral tropism with acceptable safety limits for use in humans, both of which encourage development of alternative approaches to treatment.

A second emerging category of treatment for DMD involves mobilizing endogenous resources to either produce dystrophin or increase muscle resistance to damage. For instance, the most common therapy for DMD is the use of corticosteroids, such as prednisone and deflazacort (Angelini, 2007). Multiple clinical trials over the past 30 years have reaffirmed their positive effects on degenerative pathology. These corticosteroids serve to increase muscle strength and resilience, and can extend the lives of sufferers for years beyond the normal disease course. The exact method by which such corticosteroids work is unclear, but progress into the cellular biology of the disease provides insight into possible mechanisms. Corticosteroids are potent immunosuppressants, and considerably modulate a number of signaling pathways in both skeletal muscle and infiltrating immune cells. These observations offer starting points to many approaches towards a better understanding of the biology of muscle regeneration.

A point of convergence for corticosteroid therapy, immune system biology, and skeletal muscle regeneration is the NF-κB pathway. In recent years, a considerable number of studies have addressed the role of NF-κB signaling in DMD, using the *mdx* mouse model in which a nonsense mutation in the *Dys* gene causes a loss of functional dystrophin (Ryder-Cook et al., 1988; Sicinski et al., 1989). Classical NF-κB activity is chronically elevated in dystrophic muscle, and most reports suggests this elevation contributes to the resulting

pathology (Durham et al., 2006; Messina et al., 2006; Acharyya et al., 2007; Siegel et al., 2009; Acharyya et al., 2010; Graham et al., 2010). No studies have examined the roles played by the alternative pathway, or of the cIAPs, in this context. While the loss of cIAP1 may trigger ligand-independent NF-κB activity, it may also have the opposite effect in the presence of sufficient exogenous TNFα. Furthermore, the effects of the alternative pathway on myoblast fusion and myotube maintenance (Bakkar et al., 2008) may prove positive on dystrophic muscle, leading to a possible therapeutic advantage to SMC use in this context.

Here, I examined the effect of the absence of cIAP1 on the pathology of skeletal muscle in the *mdx* mouse. I show that the loss of cIAP1 is associated with decreased damage and improved contractile responsiveness of the soleus muscle. I also show that in the absence of cIAP1, *mdx* mice exhibit reduced diaphragm permeability and improved exercise endurance. These results suggest that depletion of cIAP1 from muscle may decrease pathology and offer potential clinical application in the treatment of DMD.

Methods and Materials

Experimental mice

All experiments were approved by the Animal Care Committee of the University of Ottawa. Mice were fed ad libitum, and housed according to the guidelines of the Canadian Council for Animal Care. Female *mdx* mice homozygous for the point mutation in the dystrophin gene were mated with male *cIAP1*$^{-/-}$ mice described elsewhere (Conze et al., 2005). They were further backcrossed to obtain double-mutant *cIAP1*$^{-/-}$*;mdx* mice, as well as *cIAP1*$^{+/+}$*;mdx* controls. The *mdx* mice were derived from a C57BL/10 background and the *cIAP1*$^{-/-}$ mice on a C57BL/6 background. Male mice of age 10 – 12 weeks were used for the experiments described here. Age-matched C57BL/6 males were used as dystrophin-containing controls where indicated. Genotypes of the mice were determined by PCR screening of tail snip or ear notch DNA for the cIAP1 deletion, and by sequencing the locus in Exon 23 of the dystrophin gene containing the nonsense mutation.

Treadmill running

Separate groups of mice were used for both treadmill-running tasks. For determination of time-to-exhaustion, 12 *cIAP1*$^{+/+}$*;mdx* and eight *cIAP1*$^{-/-}$*;mdx* mice were run for two minutes at 10 m/min on a 15° incline, followed by a 15° decline until they could no longer maintain the speed even with gentle stimulation on the rump. For the assessment of diaphragm resilience, one group of mice was run for 10 minutes at 10 m/min on a 0° incline. A second group remained unexercised; both groups were subsequently injected intraperitoneally with

10 mg/kg of Evans Blue (Sigma-Aldrich, Oakville, Ontario, Canada) in sterile phosphate-buffered saline. Diaphragms were excised 24 hours after injection.

Quantitative RT-PCR

Total RNA was extracted from soleus and EDL samples using the Qiagen RNeasy Mini kit (Qiagen, Valencia, California) following manufacturer's instructions. One-step real-time RT-PCR reactions were carried out with each sample in triplicate, using 10 nanograms of RNA per reaction. RT-PCR reactions were performed in a 50 µl reaction volume using the QuantiTect SYBR Green RT-PCR (Qiagen, Valencia, California). *GAPDH* was used as the housekeeping gene. Primers used were as follows: sense CD68: 5'-TTC CAA GAG AAG GCA AAG-3'; antisense CD68: 5'-GCT GGT GTG AAC TGT GAC-3'; sense GAPDH: 5'-AGG TCG GTG TGA ACG GAT TTG-3'; antisense GAPDH: 5'- TGT AGA CCA TGT AGT TGA GGT CA-3'.

Histology and immunohistochemistry

Ten-micron sections were cut on a cryostat from fresh frozen samples embedded in OCT (Tissue Tek II), and frozen in isopentane precooled in liquid nitrogen. Hematoxylin and eosin staining were used to identify centronucleated fibres and regions of degeneration. For immunohistochemistry, antibodies used were: rabbit anti-laminin (catalog #L9393, Sigma), mouse anti-dystrophin (MANDYS1, Developmental Studies Hybridoma Bank, Iowa) and mouse anti-myosin type I (A4.840, Developmental Studies Hybridoma Bank). Slide-mounted cross-sections were incubated overnight with primary antibodies diluted in PBS containing 0.3% Triton X-100 (Sigma) and 0.5% bovine serum albumin (Invitrogen). After

washing, cover slips were incubated with the appropriate secondary antibody, diluted in PBS with Hoechst 33258 (Sigma) as a nuclear counterstain, and incubated for one hour at room temperature. This was followed by washing, mounting of cover slips, and visualization. Sections were photographed using a Zeiss Axiophot microscope equipped with a digital camera and Northern Eclipse image analysis software, version 6.0 (Empix Imaging, Mississauga, Ontario). Areas of degeneration were measured in Northern Eclipse from the entire area of each analyzed muscle section. For the soleus and EDL muscles, data were collected from all fibres in a cross-section; for the diaphragm, randomly selected 400 – 500 fibres were counted for the indicated analyses.

Creatine kinase analysis

Creatine kinase activity was measured from mouse sera with the EnzyChrom Creatine Kinase Assay kit (BioAssay Systems, Hayward, CA, USA), in accordance with manufacturer's specifications.

Muscle force measurements

Determination of force-frequency responses from EDL and soleus muscles were done essentially as previously described (Liu et al., 2005). Briefly, following terminal deep anaesthesia, the muscles were dissected and mounted in a bath containing oxygenated Ringer's solution at 25°C. The maximum twitch force at optimum muscle length (L_o) was determined following in-bath stimulation with electrodes. After a five-minute rest, the muscles were stimulated at two-minute intervals for 500 milliseconds at 20, 40, 60, 80, 100, 120 and 140 Hz.

Statistical analyses

All quantitative data are represented as mean ± SEM. Statistical analyses were performed in GraphPad Prism 5 (GraphPad Software, La Jolla, CA) using a t-test with significance set at $p < 0.05$.

Results

cIAP1$^{-/-}$*;mdx* double-mutant mice have decreased pathology in the soleus muscle

To determine the role played by cIAP1 in the pathology of *mdx* muscle, I generated *cIAP1*$^{-/-}$*;mdx* double-mutant mice, as well as single-mutant *cIAP1*$^{+/+}$*;mdx* as controls. Sequencing, immunoblotting for cIAP1, and immunohistochemistry for dystrophin expression (Fig. 3.1) confirmed genotypes. The presence of nuclei at the center of a fibre is an indicator of recent regeneration, as mature healthy fibres have nuclei at the periphery (Cheng et al., 2006; Stupka et al., 2006). Cross-sections of 12-week-old male soleus and EDL muscles were stained with H&E for analysis. The *cIAP1*$^{-/-}$*;mdx* solei had about 50% fewer centronucleated fibres than their *cIAP1*$^{+/+}$*;mdx* counterparts (28 ± 9.7% versus 57 ± 1.2% respectively, Fig. 3.2A,B), suggesting that in the absence of cIAP1, fewer fibres required regeneration. There was no difference in the proportion of centronucleated fibres between single- and double-mutant EDLs (Fig. 3.2C). I also measured the cross-sectional areas of necrotic regions (from H&E-stained sections), marked by macrophage infiltrations and fibre loss. There was a considerable reduction in size of necrotic regions in *cIAP1*$^{-/-}$*;mdx* solei, but not in the EDL (Fig. 3.3A,B). These results suggest that loss of cIAP1 decreases damage in the soleus associated with the *mdx* pathology.

A secondary indicator of regeneration is inflammation and influx of macrophages into the muscle (Acharyya et al., 2007). I reasoned that a reduction in muscle fibre damage would be associated with a reduction in macrophage infiltration into the dystrophic muscle. To test this possibility, I immunolabeled soleus and EDL cross-sections with an antibody to

Fig. 3.1: Generation of *cIAP1*$^{-/-}$*;mdx* double-mutant mice.

The mice were generated from crossings of *cIAP1*$^{-/-}$ males with *mdx* females. Genotype was confirmed using multiple approaches.

(A) Representative chromatogram of a sequencing reaction spanning the region of Exon 23 containing the *mdx* mutation. The T nucleotide (asterisk) turns the Glu codon into a stop codon (underlined).

(B) Immunohistochemistry for dystrophin and laminin identifies dystrophin-deficient muscle fibres in gastrocnemius sections. Gastrocnemii from C57BL/6 mice were used as controls.

(C) Immunoblotting for cIAP1 in EDL muscle samples confirms the absence of cIAP1 in *cIAP1*$^{-/-}$*;mdx* double-mutants. The RIAP1 antibody detects cIAP2 which is upregulated in macrophages in the absence of cIAP1. Scale bar represents 100 μm.

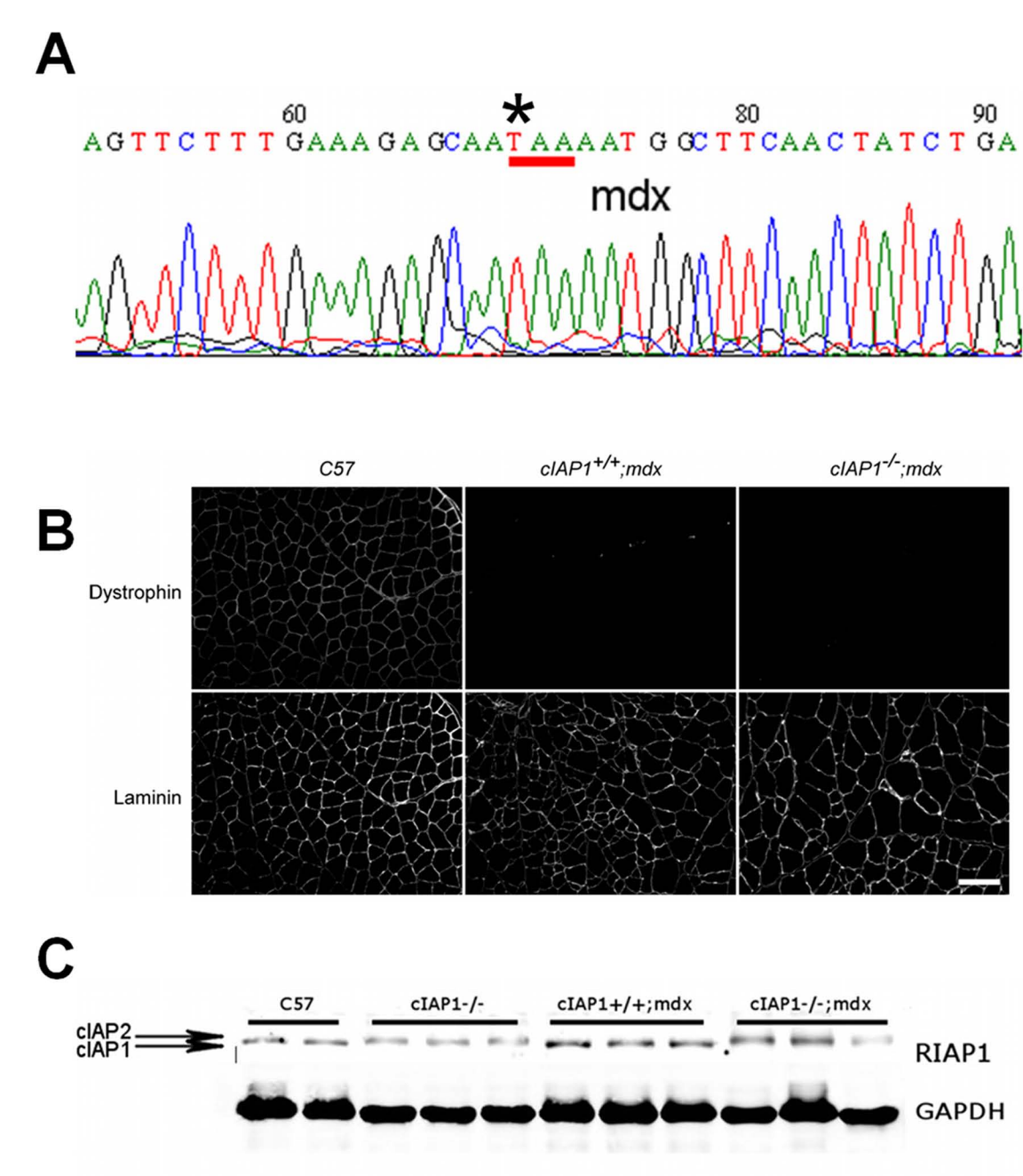

A
60
*
80
90
AGTTCTTTGAAAGAGCAATAAAATGGCTTCAACTATCTGA
mdx
B
C57
cIAP1+/+;mdx
cIAP1-/-;mdx
Dystrophin
Laminin
C
C57
cIAP1-/-
cIAP1+/+;mdx
cIAP1-/-;mdx
cIAP2
cIAP1
RIAP1
GAPDH

Fig. 3.2: Loss of cIAP1 reduces damage in *mdx* solei.

(A) Cross-sections of single- and double-mutant solei were stained with H&E, highlighting centronucleated fibres and regions infiltrated with macrophages (arrow). Centronucleation is an indication that the fibre has recently regenerated; thus, a reduction in centronucleation indicates increased muscle fibre resistance to damage.

(B, C) The percentage of fibres containing centrally located nuclei in soleus (B) and EDL (C) muscles was measured from the H&E-stained cross-sections using Northern Eclipse software. Double-mutant solei exhibited a considerable reduction in centronucleation; there was no difference between groups in centronucleation in the EDL. Data are means ± SEM, $n = 5$. *$p < 0.05$ versus $cIAP1^{+/+}$;*mdx* controls. Scale bar represents 100 μm.

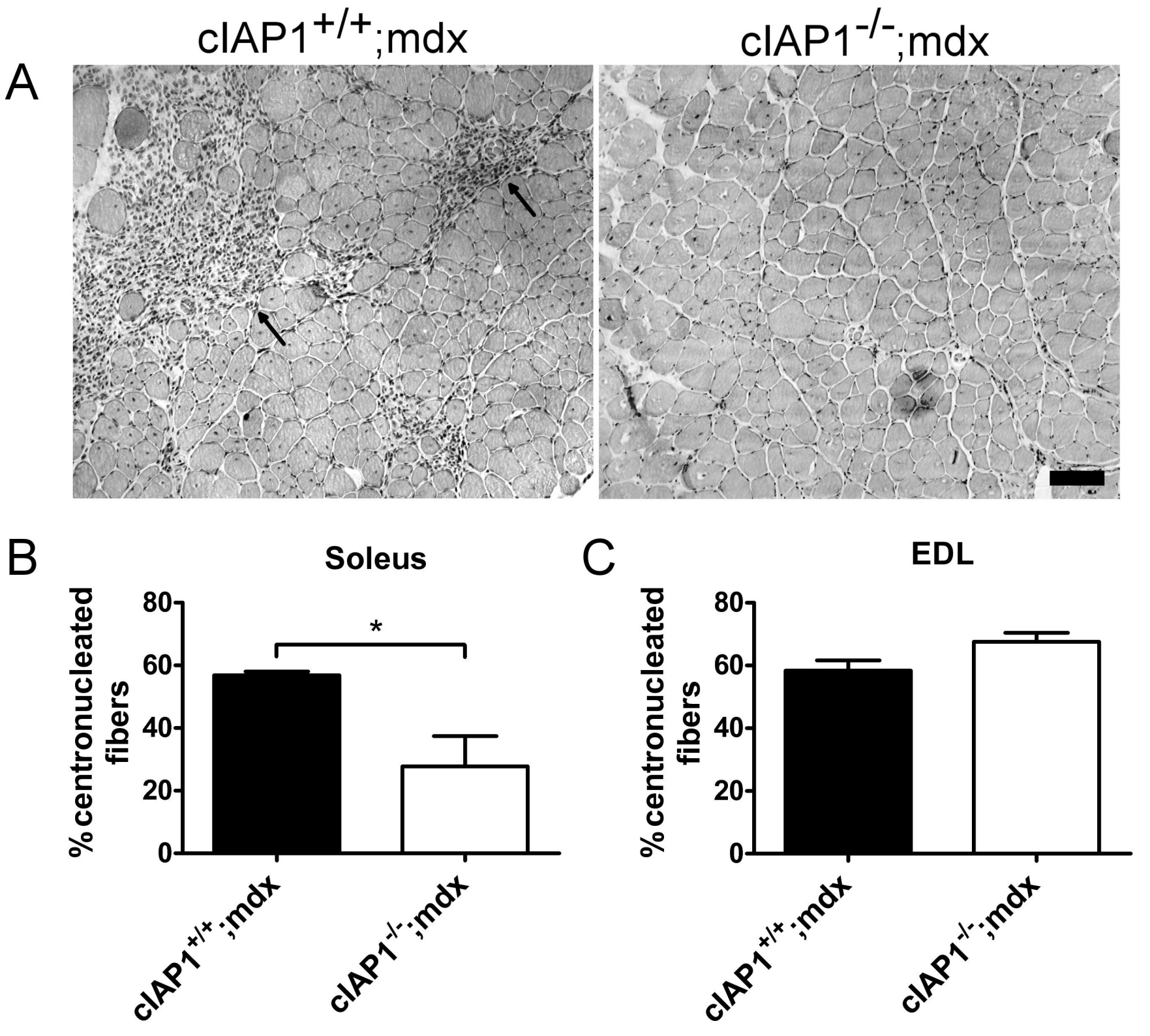

cIAP1+/+;mdx
cIAP1-/-;mdx
A
B
Soleus
% centronucleated fibers
80
60
40
20
0
*
cIAP1+/+;mdx
cIAP1-/-;mdx
C
EDL
% centronucleated fibers
80
60
40
20
0
cIAP1+/+;mdx
cIAP1-/-;mdx

Fig. 3.3: Loss of cIAP1 reduces macrophage infiltration into *mdx* solei.

(A, B) Macrophages infiltrate regenerating muscle, and their presence is another indicator of damage. Cross-sections of soleus and EDL muscles were stained with H&E, and the total area of muscle occupied by macrophage infiltrations was measured from the H&E-stained cross-sections using Northern Eclipse software. There was a reduction in areas of infiltration in the *cIAP1*$^{-/-}$*;mdx* solei compared to the *cIAP1*$^{+/+}$*;mdx* controls, suggesting a decrease in damage and associated macrophage influx. Data are means ± SEM, $n = 5$. *$p < 0.05$ versus *cIAP1*$^{+/+}$*;mdx* controls. Scale bar represents 100 μm.

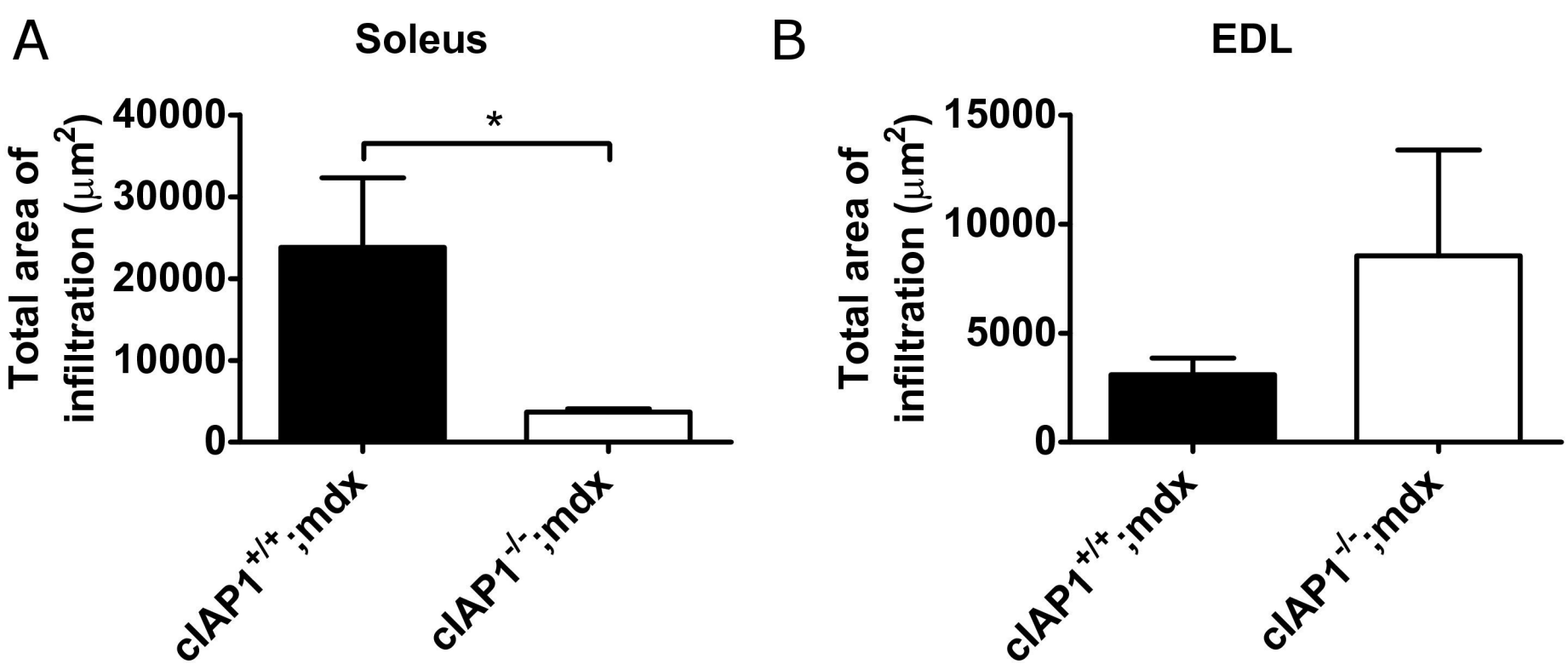
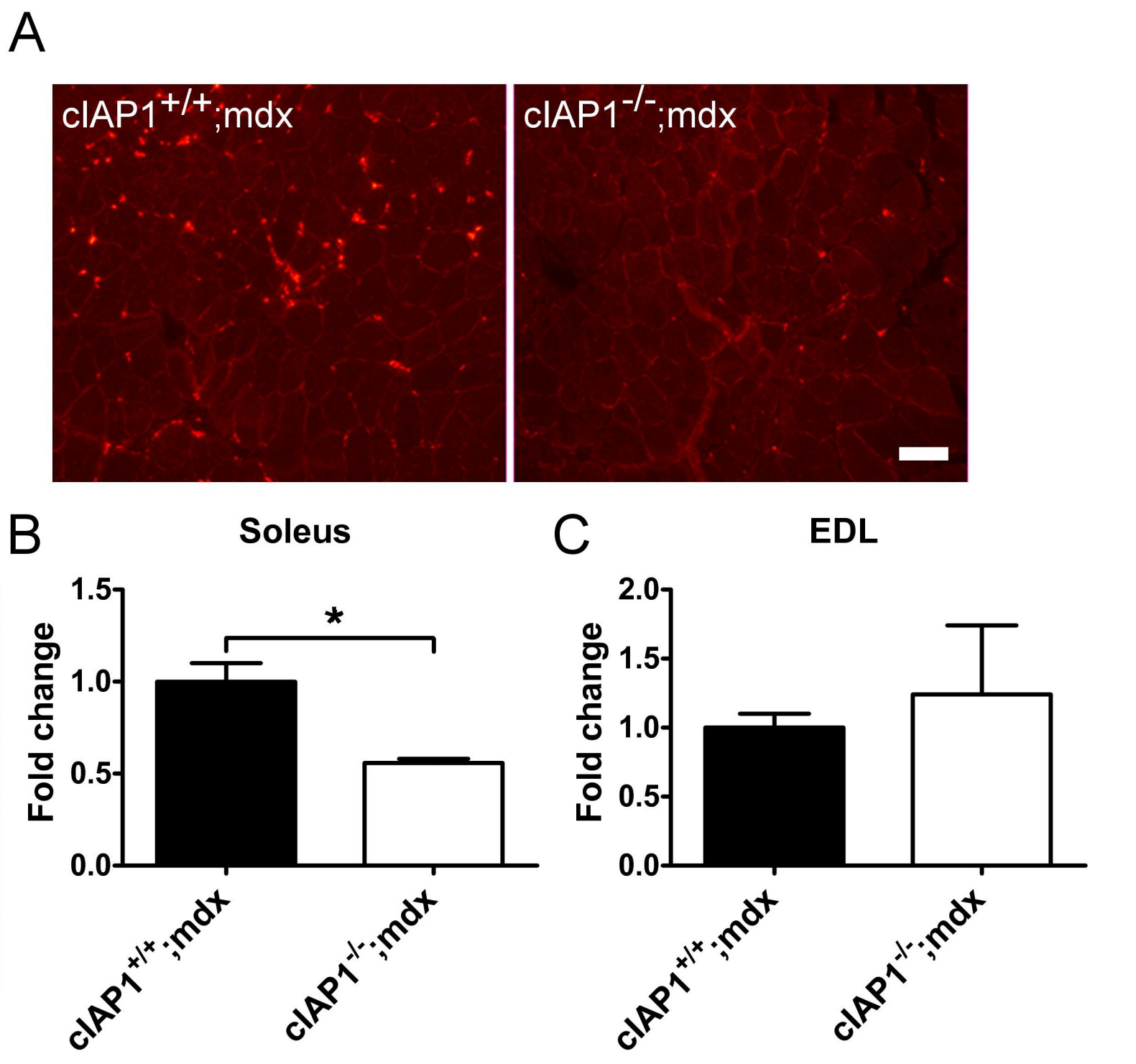

A
Soleus
Total area of infiltration (μm²)
40000
30000
20000
10000
0
*
cIAP1+/+;mdx
cIAP1-/-;mdx
B
EDL
Total area of infiltration (μm²)
15000
10000
5000
0
cIAP1+/+;mdx
cIAP1-/-;mdx

Fig. 3.4: Loss of cIAP1 leads to a reduction in macrophage infiltration of soleus muscle.

(A) Macrophages infiltrate regenerating muscle, and their presence is another indicator of damage. To determine their presence, cross-sections of soleus muscles from *cIAP1*$^{+/+}$*;mdx* and *cIAP1*$^{-/-}$*;mdx* mice were immunolabeled for CD68 (red) to detect macrophages. Fewer macrophages are evident in the photomicrographs taken from the double-mutant solei.

(B, C) To quantify the macrophage presence in muscle, total RNA was isolated for quantitative RT-PCR detection of *CD68* mRNA in soleus (B) and EDL (C) samples. Values were expressed relative to *cIAP1*$^{+/+}$*;mdx* data. Data are means ± SEM, $n = 3$. *$p < 0.05$ versus *cIAP1*$^{+/+}$*;mdx* controls. Scale bar represents 100 μm.


A
cIAP1+/+;mdx
cIAP1-/-;mdx
B
Soleus
Fold change
1.5
1.0
0.5
0.0
*
cIAP1+/+;mdx
cIAP1-/-;mdx
C
EDL
Fold change
2.0
1.5
1.0
0.5
0.0
cIAP1+/+;mdx
cIAP1-/-;mdx

the macrophage marker CD68 (Travaglione et al., 2002). *cIAP1*$^{-/-}$*;mdx* solei demonstrated decreased macrophage staining as compared to the *cIAP1*$^{+/+}$*;mdx* (Fig. 3.4A). To quantify this difference, I performed quantitative RT-PCR on RNA extracted from solei and EDL samples, to detect *CD68* mRNA. Double-mutant solei contained about half the *CD68* as single-mutant counterparts (Fig. 3.4B). *CD68* expression was unchanged between the genotypes in the EDL (Fig. 3.4C). These data suggest that a decreased susceptibility to damage in the double-mutant soleus reduces the requirement for regeneration.

cIAP1$^{-/-}$*;mdx* solei demonstrate improved contractile properties

Changes in the functional properties of *mdx* muscle are well-documented effects of the disease pathology (Sacco et al., 1992; Chan et al., 2007). Specifically, differences emerge in the amount of specific force produced by a muscle at a particular stimulation frequency. I asked if the loss of cIAP1 would restore normal force-frequency characteristics to *mdx* muscle. To test this, I determined the maximum isometric force produced by isolated solei and EDLs, and then measured the percentage of that force produced at different stimulating frequencies. Forces produced at different frequencies were expressed as percentages of maximum force per muscle, and produced a force-frequency curve for each muscle (Fig. 3.5A). While the *cIAP1*$^{+/+}$*;mdx* solei produced distinctively different curves from the C57BL/6 controls, the *cIAP1*$^{-/-}$*;mdx* solei were essentially identical in force-frequency characteristics to C57 solei (Fig. 3.5B). In contrast, *cIAP1*$^{-/-}$*;mdx* EDLs demonstrated a slight but distinct shift away from both C57 and *cIAP1*$^{+/+}$*;mdx* EDLs by producing a greater percentage of maximum force at lower frequencies (Fig. 3.5C). To determine whether the preservation of force in the *cIAP1*$^{-/-}$*;mdx* mice represented an increase in proportion of slow

fibres, I immunolabeled cross-sections of *cIAP1*$^{+/+}$*;mdx* and *cIAP1*$^{-/-}$*;mdx* solei with antibodies to Type I (slow) MHC, and quantified the percentage of fibres expressing this MHC isoform. Unexpectedly, the double-mutant solei had about 25% fewer slow fibres than their single-mutant counterparts (Fig. 3.6). These data suggest that the loss of cIAP1 preserves *mdx* soleus function in spite of a reduction in available slow muscle fibres.

Loss of cIAP1 increases exercise endurance of *mdx* muscle

I next asked if the improvements seen with muscle pathology and responsiveness in the absence of cIAP1 led to functional recovery. Muscle fibre "leakiness" is a hallmark of DMD, and results in permeation of muscle creatine kinase into the serum (Quinlan et al., 1990; Rooney et al., 2009; Spurney et al., 2009). I assessed the amount of creatine kinase in *cIAP1*$^{+/+}$*;mdx* and *cIAP1*$^{-/-}$*;mdx* sera. The *cIAP1*$^{-/-}$*;mdx* mice demonstrated a 14% reduction in serum creatine kinase as compared to *cIAP1*$^{+/+}$*;mdx* mice (Fig. 3.7). This relatively modest decline was consistent with improvements seen predominantly in slow muscle. I asked if this was sufficient to produce a detectable outcome in the animals' behavior. I tested mice on a two-part treadmill-running task: first, they were run at a 15° incline at 10 m/min for two minutes. Subsequently, they were switched to a decline at 10 m/min and their time-to-exhaustion was recorded. The *cIAP1*$^{-/-}$*;mdx* mice ran about three times longer than the *cIAP1*$^{+/+}$*;mdx* controls (Fig. 3.8A).

Fig. 3.5: Force frequency curves from soleus (SOL) and EDL muscles of C57 and mutant mice.

(A) To determine the physiological functionality of the single- and double-mutant muscle, baseline values for force-frequency curves were established from soleus and EDL muscles of C57 mice. These curves demonstrate a clear difference in responsiveness between soleus and EDL muscles, and establish baselines for normal functioning. The baseline data were compared with data from single- and double-mutant solei (B) and EDLs (C). The force-frequency curve derived from double-mutant solei is indistinguishable from that of the baseline established with the C57 control mice (B), whereas the single-mutant curve remains shifted away from the baseline. This is in contrast to the EDL, where both single- and double-mutant curves are shifted away from baseline. Data are means ± SEM, $n = 5$.

Figure produced in collaboration with Louise Boudreault in the lab of Dr. Jean-Marc Renaud

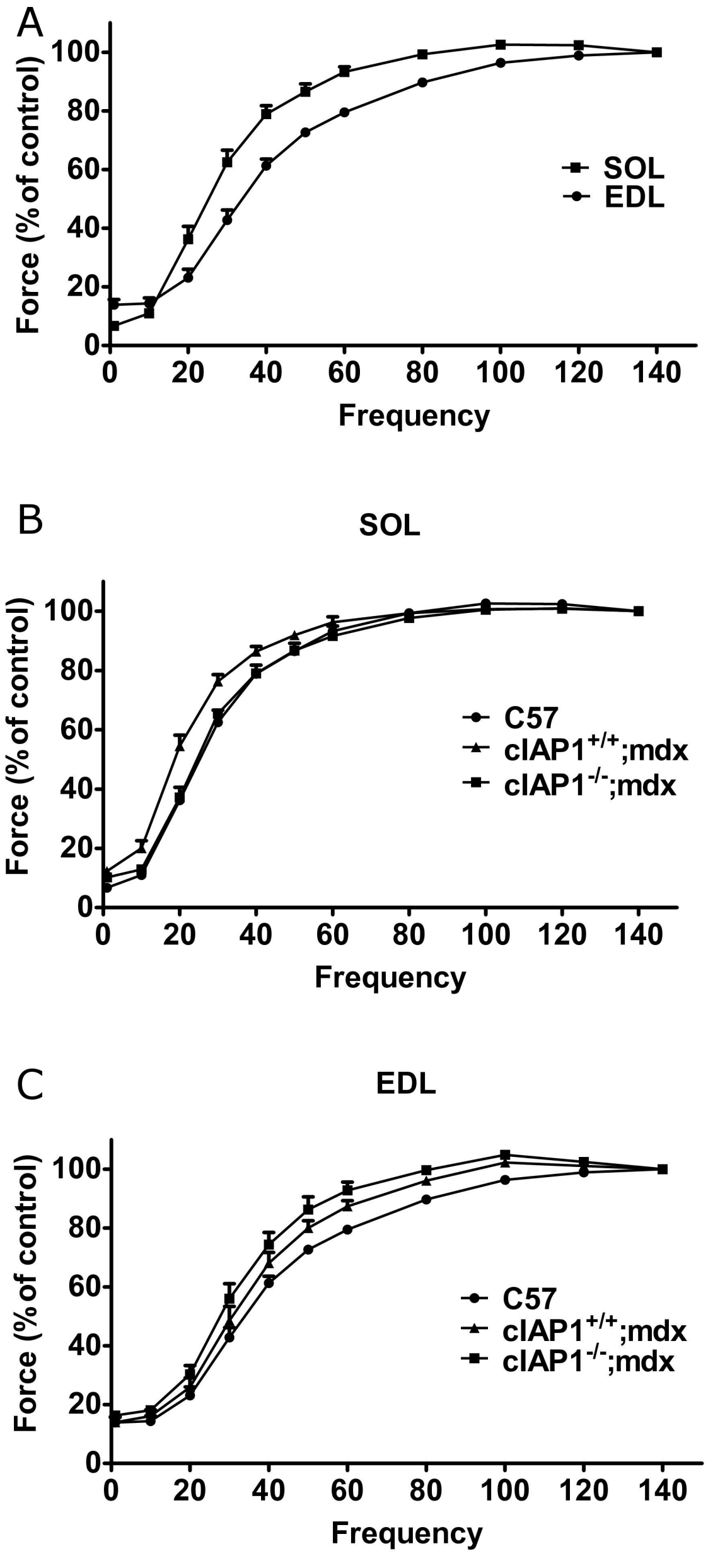

A
Force (% of control)
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Frequency
SOL
EDL
B
SOL
Force (% of control)
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Frequency
C57
cIAP1+/+;mdx
cIAP1-/-;mdx
C
EDL
Force (% of control)
100
80
60
40
20
0
0
20
40
60
80
100
120
140
Frequency
C57
cIAP1+/+;mdx
cIAP1-/-;mdx

Fig. 3.6: Loss of cIAP1 reduces proportion of Type I fibres in *cIAP1$^{-/-}$;mdx* solei.

(A) To determine the abundance of slow-twitch fibres in the soleus, cryostat sections of single- and double-mutant solei were immunolabeled for type I myosin (red). The number of fibres expressing type I myosin was expressed as a percentage of all (laminin-labeled) fibres (B). Evident is a reduction in the proportion of fibres expressing type I myosin in the *cIAP1$^{-/-}$;mdx* solei. Data are means ± SEM, $n = 3$. *$p < 0.05$ versus *cIAP1$^{+/+}$;mdx* controls.

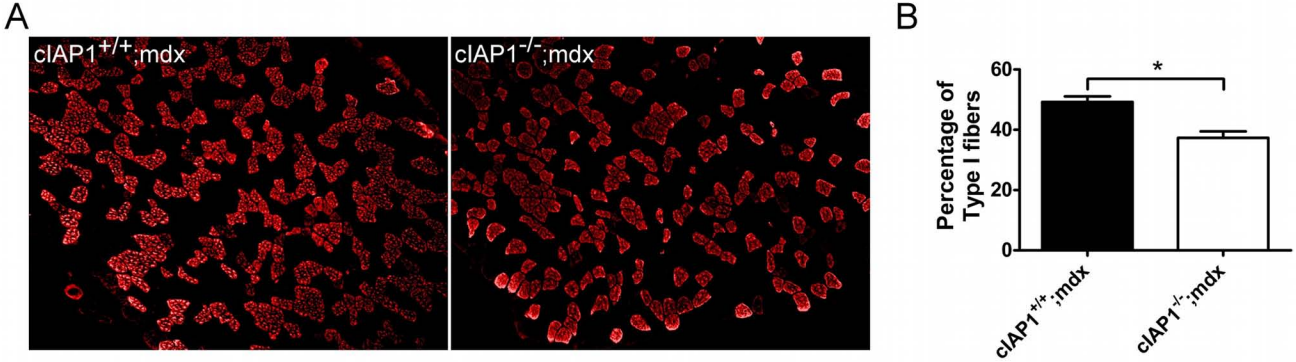

A
cIAP1+/+;mdx
cIAP1-/-;mdx
B
Percentage of Type I fibers
60
40
20
0
*
cIAP1+/+;mdx
cIAP1-/-;mdx

Fig. 3.7: Loss of cIAP1 decreases muscle permeability.

A hallmark of damage resulting from loss of dystrophin is the release of creatine kinase from skeletal muscle into the blood. Consequently the amount of creatine kinase present in the blood is an indication of the extent of muscle damage systemically. To determine whether the loss of cIAP1 affected general muscle permeability, sera were collected from *cIAP1*$^{+/+}$*;mdx* and *cIAP1*$^{-/-}$*;mdx* mice and used in a creatine kinase assay. Data are means ± SEM, $n = 3$. *$p < 0.05$ versus *cIAP1*$^{+/+}$*;mdx* controls.

Creatine Kinase activity (units/L)
8000
6000
4000
2000
0
*
cIAP1+/+;mdx
cIAP1-/-;mdx

Fig. 3.8: Loss of cIAP1 increases muscle endurance.

(A) To determine if the loss of cIAP1 improved muscle endurance, mice were run on a treadmill, at a 15° incline at 10 m/min for two minutes. They were then switched to a 15° decline and the time-to-exhaustion was recorded. Data plotted are for individual animals tested. Horizontal bar indicates the mean time-to-exhaustion for each group. (B) To evaluate muscle damage in response to exercise, single- and double-mutant mice were run at 10 m/min for 10 minutes, and injected with 10 mg/kg Evans Blue. Another group of mice were injected with Evans Blue without treadmill exercise. Twenty-four hours later, the mice were euthanized and the diaphragms were sectioned and the percentage of fibres labeled with Evans Blue was counted. The double-mutant diaphragms contained considerably fewer Evans Blue-labeled fibres than the single-mutant *mdx* mice. Furthermore, the percentage of Evans Blue-labeled fibres was the same in the double-mutant mice with or without treadmill exercise, further indicating the improved damage resistance of the diaphragm muscles in these mice. Values were expressed relative to $cIAP1^{+/+}$*;mdx* data. Data are means ± SEM, $n = 4$. *$p < 0.05$ versus $cIAP1^{+/+}$*;mdx* controls.

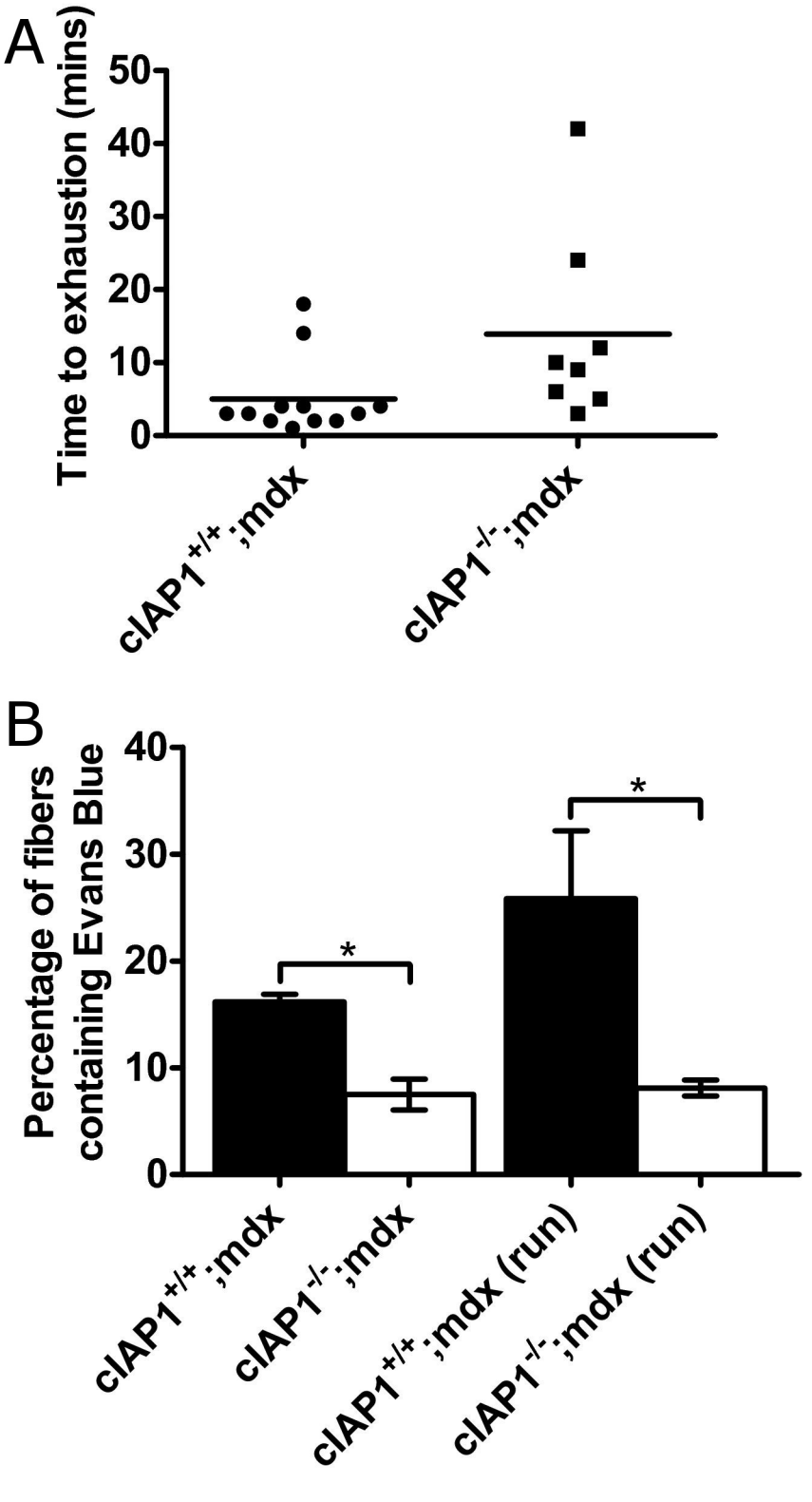

A
Time to exhaustion (mins)
50
40
30
20
10
0
cIAP1+/+;mdx
cIAP1-/-;mdx
B
Percentage of fibers containing Evans Blue
40
30
20
10
0
*
*
cIAP1+/+;mdx
cIAP1-/-;mdx
cIAP1+/+;mdx (run)
cIAP1-/-;mdx (run)

Fig. 3.9: Loss of cIAP1 decreases diaphragm permeability and increases endurance.

I asked if loss of cIAP1 protected the diaphragm from exercise-induced damage. Diaphragms from mice engaged in the 10-minute treadmill task in Fig. 3.8 were immunolabeled for laminin to identify muscle fibres (green). Evans Blue-labeled fibres were identified by red fluorescence. The *cIAP1*$^{-/-}$*;mdx* mouse diaphragms contained less baseline and exercise-induced damage than the *cIAP1*$^{+/+}$*;mdx* counterparts. Scale bar represents 250 μm.

Laminin

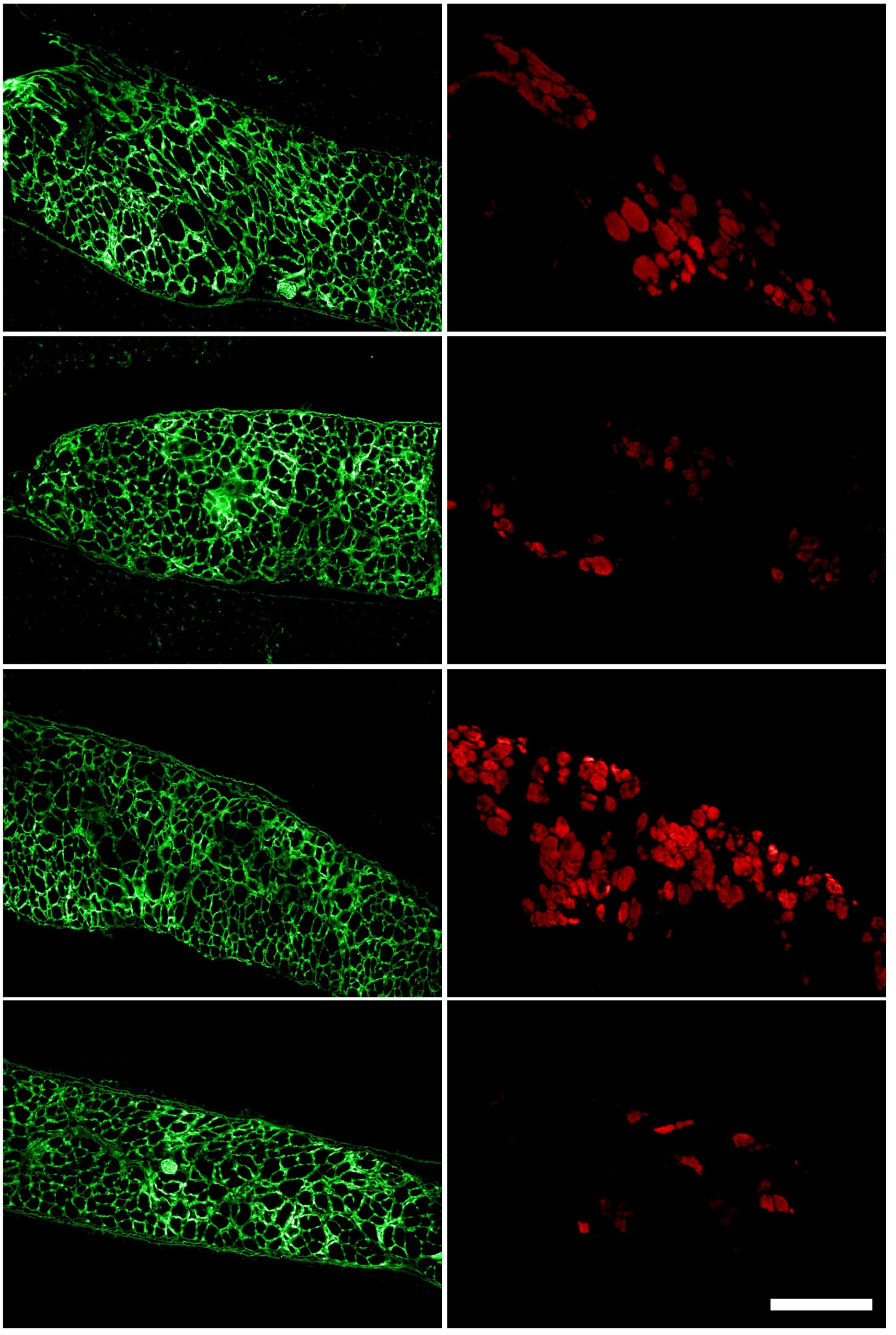
Evans Blue

cIAP1$^{+/+}$;mdx (no exercise)

cIAP1$^{-/-}$;mdx (no exercise)

cIAP1$^{+/+}$;mdx (exercised)

cIAP1$^{-/-}$;mdx (exercised)



A number of factors, including skeletal and cardiovascular endurance, could have accounted for this improvement. The diaphragm is one of the most susceptible muscles to eccentric damage in the *mdx* mouse, and is also the muscle that most closely mimics the degeneration seen in DMD (Stedman et al., 1991; Matecki et al., 2004; Hnia et al., 2006). An improved resistance of the diaphragm to damage may explain the increased runtime of the double-mutant mice. To test this, single- and double-mutant mice were treadmill-exercised at 10 m/min for 10 minutes, and injected with Evans Blue dye, a membrane-impermeable dye that labels damaged fibres. Another cohort of animals were injected with Evans Blue, but were not exercised. Twenty-four hours later, the diaphragms were isolated, sectioned, and immunolabeled for laminin to identify fibres that contained Evans Blue (which fluoresces red). *cIAP1*$^{-/-}$*;mdx* diaphragms had 50% fewer Evans Blue-labeled fibres than the *cIAP1*$^{+/+}$*;mdx* controls without exercise, and 75% fewer labeled fibres after exercise (Fig. 3.8B, 3.9). Notably, there was no increase in the proportion of Evans Blue-labeled fibres in the double-mutant diaphragms with exercise. Taken together, these data suggest that loss of cIAP1 improves exercise performance at least in part by decreasing associated damage of the diaphragm.

Discussion

These results demonstrate that the loss of cIAP1 can ameliorate the pathology of *mdx* soleus muscle. This occurs through a reduction of fibre damage and inflammation, improved contractile properties, and increased exercise endurance. Histologically, the improvements were predominant in the slow-twitch soleus versus the fast-twitch EDL. Nevertheless, they were sufficient to produce a significant reduction in serum creatine kinase and reduced permeability of the diaphragm.

The data indicate that *mdx* solei are more resistant to damage in the concurrent absence of cIAP1. The double-mutant mice had about half the percentage of centronucleated fibres as the SM controls, as well as a considerable reduction in size of necrotic foci. One could hypothesize that the corresponding reduction in macrophage infiltration of double-mutant solei reflects the decreased damage of the muscle rather than a defect in the inflammatory response. There are two lines of evidence to support this position. First, while loss of the cIAP1 paralogue cIAP2 renders peritoneal macrophages prone to lipopolysaccharide-induced apoptosis (Conte et al., 2006), no such response has been reported in *cIAP1*$^{-/-}$ mice. Second, while loss of cIAP1 can activate the classical NF-κB signaling pathway in macrophages and increase the inflammatory response in muscle, macrophages also express cIAP2, which is upregulated in macrophages in the absence of cIAP1 (Conze et al., 2005; Conte et al., 2006). cIAP1 and cIAP2 act redundantly to regulate the NF-κB signaling cascade, and consequently the loss of cIAP1 would have no effect in the context of macrophage infiltration of dystrophic muscle (Acharyya et al., 2007; Mahoney et al., 2008).

A further outcome of this study was that loss of cIAP1 led to extended treadmill time-to-exhaustion in *mdx* mice. The time-to-exhaustion task was designed predominantly to test function of slow-twitch muscle by exhausting the fast-twitch muscle early in the trials. The results suggest that the increased resistance of slow-twitch muscle to damage may confer endurance benefits to the animal. The diaphragm is subject to continuous eccentric contractions and is consequently the most reliable indicator of damage or recovery in the *mdx* mouse (Stedman et al., 1991; Dupont-Versteegden and McCarter, 1992; Louboutin et al., 1993). On equivalent tasks, the *cIAP1*$^{-/-}$*;mdx* diaphragms displayed less exercise-induced damage than in the *cIAP1*$^{+/+}$*;mdx* counterparts, suggesting that improved cardiovascular resilience partially explained the differences in performance between the groups.

A notable aspect of this study is the disparity in effect of cIAP1 on slow-twitch versus fast-twitch muscle. The relatively modest reduction in serum creatine kinase further suggests that loss of cIAP1 is protective only in a subset of muscle. The reasons for this disparity are currently unknown. Previous reports indicate that slow-twitch muscle is more resistant than fast-twitch muscle to the usual pathology associated with DMD (Webster et al., 1988). The data indicate that the loss of cIAP1 offers a significant improvement in the slow-twitch-specific protection against dystrophic damage. In Chapter 2 I reported that NFATc3 was upregulated in differentiating *cIAP1*$^{-/-}$ myoblasts. NFATc3 is a Ca^{2+}-calcineurin-regulated transcription factor associated with myoblast fusion, but also regulates expression of Type I MHC (Delling et al., 2000; Pandorf et al., 2009). It is thus possible that in the soleus, loss of cIAP1 confers a hitherto uncharacterized protection by upregulating NFATc3. This outcome would be consistent with previous reports that inhibition of Ca^{2+} signaling exacerbates the pathology of *mdx* muscle (Chakkalakal et al., 2006). Another

possibility emerges from the recent reports that activation of the alternative NF-κB pathway increases muscle mitochondrial content (Bakkar et al., 2008). This would be most effective in the mitochondria-dense, oxidative slow fibres, suggesting that improved energy utilization may provide unanticipated structural benefits to the associated muscle groups.

In summary, the loss of cIAP1 is associated with decreased soleus damage and increased exercise endurance and performance of *mdx* mice. This opens the way for therapeutic approaches using pharmacological inhibitors of cIAP1. The Smac mimetic compounds mentioned earlier can be administered orally and are well tolerated (Weisberg et al., 2007). Their use in *mdx* animals without a genetic deletion of cIAP1 would provide a clearer understanding of the role of this protein in the pathology of muscular dystrophy.

Chapter 4: General Discussion

The data from the second and third chapters of this thesis focused on the roles played by cIAP1 in a regenerative and functional context. I began with a protocol for the rapid and efficient isolation of myoblasts from skeletal muscle. Using this tool, I examined the function of cIAP1 in differentiating myoblasts using genetic and pharmacological tools. This approach identified cIAP1 as a repressor of myoblast fusion, particularly the early stage in which myoblasts fuse with other myoblasts. Paradoxically, while the loss of cIAP1 led to a considerable increase in myoblast fusion, it also delayed differentiation of the myoblasts. I hypothesized that these disparate effects may result from an effect of cIAP1 on separate aspects of the same pathway. ELISA and Western blotting analyses indicated that loss of cIAP1 leads to sustained activation of classical and alternative NF-κB pathways. The classical pathway is a well-established inhibitor of myoblast differentiation. The alternative pathway, however, is not as clearly understood. A combination of siRNA and over-expression experiments revealed that loss of cIAP1 promotes myoblast fusion by activating the alternative NF-κB pathway. *In vivo*, the loss of cIAP1 led to increased muscle mass and cross-sectional area, as well as an increase in the number of resident satellite cells. I concluded that in the absence of cIAP1, increased NF-κB activity along both arms of the pathway combined to improve myogenesis by increasing myoblast fusion and potentially increasing the number of satellite cells present in adult muscle.

The observations of the first part of this thesis led me to ask whether the combined effects of cIAP1 on myogenesis may lead to pathological and functional improvements in a model of degenerative muscle disease. Surprisingly, the loss of cIAP1 decreased pathology and macrophage infiltration predominantly in the slow-twitch soleus muscle, with no significant effect on the fast-twitch EDL muscle. The loss of cIAP1 also improved

physiological functionality of the soleus muscle as determined by *ex vivo* experiments performed in collaboration with the lab of Dr. Jean-Marc Renaud. Overall, these improvements in physiology and pathology increased the exercise endurance of *cIAP1*$^{-/-}$*;mdx* double-mutant mice as compared to their *cIAP1*$^{+/+}$*;mdx* counterparts. This increased endurance was at least in part due to decreased exercise-induced damage of the diaphragm, as assessed by fibre permeability to Evans Blue dye. I concluded that loss of cIAP1 accorded multifactorial improvements to the pathology and functioning of skeletal muscle in the *mdx* mouse.

The involvement of cIAP1 in the classical and alternative NF-κB pathways driving both myoblast fusion and satellite cell maintenance form one aspect of the work reported here. The increased damage resistance seen in the *mdx* model may or may not rely on the NF-κB aspect. In this chapter, I will discuss how these findings affect the state of knowledge of the satellite cell, and speculate on the unexplained functions of this IAP in skeletal muscle biology.

1. cIAP1 function in myogenesis: Aims revisited

In the introductory chapter, I laid out a number of possibilities for the roles cIAP1 might play in skeletal myogenesis. I suggested that cIAP1 might a) inhibit apoptosis and promote differentiation b) promote apoptosis and inhibit differentiation c) impair myotube survival and biogenesis, or d) carry out some unknown other function unrelated to the above. At the conclusion of this dissertation, as often happens in research, the actual functions of cIAP1 could not be readily predicted. Loss of cIAP1 inhibits differentiation of primary myoblasts and C2C12 cells, due to ligand-independent activation of the classical NF-κB pathway; there

was no apparent effect on myoblast apoptosis. Furthermore, I discovered a hitherto uncharacterized effect of cIAP1 on myoblast fusion, not evident in C2C12 cells, and resulting from the concordant activation of the alternative pathway with loss of cIAP1. Finally, *cIAP1*$^{-/-}$*;mdx* double-mutant mice exhibited decreased pathology that was specific to slow versus fast-twitch muscle, resulting in improvements in behaviour and function in the diaphragm and on appropriate treadmill tasks.

An aspect of myogenesis that is still poorly understood is the extent to which paracrine and autocrine factors affect the course of myoblast differentiation. This information is essential to resolve the evident changes in many cellular signalling pathways seen with differentiation, and to determine the effects of these changes on muscle formation. For instance, the activity states of both classical and alternative NF-κB pathways change during differentiation, with the classical pathway turned off and the alternative pathway turned on. Is this in response to autocrine signalling of unknown ligands? Some evidence of this exists in the literature on myoblast fusion. The process of differentiation promotes Ca^{2+} signalling both by intake from the external environment (Bijlenga et al., 2000; Konig et al., 2006) and from intracellular stores in the endoplasmic reticulum (Nakanishi et al., 2007). Ca^{2+} influx activates the calcium phosphatase calcineurin, which dephosphorylates the NFAT family proteins and permits their nuclear translocation and signalling. Downstream of NFAT, autocrine release and signalling of interleukin-4 drives myoblast fusion (Horsley et al., 2003). The placement of alternative NF-κB signalling into the myoblast fusion scheme suggests that Ca^{2+} may also affect that pathway during differentiation. Recent studies indicate this chain of events is possible. For example, in response to DNA damage, the

kinase ataxia telangiectasia mutated (ATM) recruits TRAF6 and cIAP1 to drive IKK ubiquitination in a Ca^{2+}-dependent manner (Culver et al., 2010; Hinz et al., 2010).

The data reported here raise the question of whether cIAP1 functions through one (NF-κB) or more pathways to produce the indicated effects on muscle fusion, formation and regeneration. Several signalling pathways play roles in maintaining the balance between self-renewal and differentiation of satellite cells, both during and after the prenatal development period. The Notch signalling pathway represses differentiation in a number of systems, such as the brain, pancreas, and muscle (Nakhai et al., 2008; Shimojo et al., 2008; Pisconti et al., 2010). The Notch pathway is of particular interest in the context of adult myogenesis. Activation of Notch1 promotes proliferation of myoblasts, whereas cells expressing the Notch inhibitor Numb preferentially express commitment markers such as Myf5 and desmin (Conboy and Rando, 2002). More recently, Notch-3-deficient mice were shown to respond to repeated muscle injuries with muscle hypertrophy (Kitamoto and Hanaoka, 2010). Furthermore, these mice contained considerably more satellite cells than did their wildtype counterparts. Both phenotypes are similar to our findings in the *cIAP1*$^{-/-}$ mice, raising the possibility of functional crosstalk among cIAP1, NF-κB, and Notch pathways.

There is precedent for the interaction between NF-κB and Notch signalling. Several studies have shown that the cytokine TNFα activates classical NF-κB signalling to inhibit myoblast differentiation (Langen et al., 2001; Li and Schwartz, 2001; Langen et al., 2004). A recent study by Denis Guttridge's group suggested that TNFα secreted by regenerating muscle fibres inhibits regeneration by repressing Notch1 (Acharyya et al., 2010). This would limit the proliferation of myoblasts and reduce their regenerative capacity. Since cIAP1 in muscle is required for TNFα-dependent NF-κB activation, the absence of cIAP1 would

attenuate this activation and improve muscle regeneration. A subsequent microarray study confirmed the repression of Notch expression by TNFα in muscle cells (Bhatnagar et al., 2010). The method proposed in the paper by Guttridge's group involved histone and DNA methylation of the Notch1 promoter, but several direct interactions between the two pathways have been reported. These include both Notch regulation of the NF-κB pathway (Song et al., 2008; Monsalve et al., 2009) and cooperation between elements of both pathways (Fukushima et al., 2008; Morga et al., 2009). These studies complement the evidence indicating that chronic NF-κB activation due to DMD has adverse effects on muscle regeneration (Monici et al., 2003; Acharyya et al., 2007; Tang et al., 2010), and that these effects are ameliorated by reducing pathway activity through genetic or pharmacological means (Carlson et al., 2005; Acharyya et al., 2007; Siegel et al., 2009).

Before drawing conclusions about the role cIAP1 plays in the pathology of DMD, we must take into account the divergent effects of this IAP on NF-κB signalling. The loss of muscular cIAP1 is sufficient to activate the classical pathway in a ligand-independent manner. On the other hand, with pathway activation by TNFα, the downregulation of cIAP1/2 blunts NF-κB signalling. A hypothesis that the loss of cIAP1 is beneficial for dystrophic muscle only holds for as long as we can show that the absence of cIAP1 attenuates this pathway to improve regeneration and decrease pathology. There are several lines of evidence suggesting that the latter possibility is correct. First, immune system cells such as helper and killer T cells, macrophages, lymphocytes, and eosinophils are abundant in dystrophic muscle, and all produce inflammatory cytokines including TNFα (Spencer et al., 2001). Second, a reduction in numbers of these cytokine-producing cells has a dramatic effect on muscle pathology (Spencer et al., 2001; Acharyya et al., 2007). In the end, we still

have a chicken-and-egg cycle that mocks attempts to rationalize the cIAP1 phenotype in dystrophic muscle: is the muscle more resistant to damage due to a reduction in macrophages, or is there a reduction in macrophages because the muscle is more resistant to damage? From a clinical perspective, the answer is irrelevant so long as the effect is sound, but for a hardheaded biologist the lack of concrete mechanism remains unsatisfying.

Skeletal muscle regeneration is also regulated by factors that control the transition of myoblasts from proliferation to differentiation. The classical NF-κB pathway impairs regeneration by maintaining myoblasts in a proliferative state, thus limiting their availability to form new muscle fibres. Notch acts in a similar fashion, and cIAP1 could consequently promote regeneration by blunting NF-κB inhibition of Notch signalling. Conversely, Wnt/β-catenin signalling is activated in both regenerating and differentiating muscle, and is essential for myoblast differentiation (Brack et al., 2008; Le Grand et al., 2009). Wnt and NF-κB pathways are linked and exhibit crosstalk at multiple levels and in several systems. Briefly, in canonical Wnt signalling, Wnt binds to the receptor Frizzled, which complexes with the low-density lipoprotein receptor-related protein (LRP). This interaction triggers degradation of a cytosolic complex containing the kinase GSK3β, leading to nuclear translocation and signalling of β-catenin/T cell factor (TCF). GSK3β is often a focal point of multiple pathways, particularly with respect to the IAPs (Sun et al., 2009).

2. The NF-κB contradiction

While the effects of cIAP1 depletion on myoblast fusion were placed within a general mechanistic framework, the same claim does not apply to the outcome seen in the *mdx* double-mutant mice. One reason for this exclusion is that the data linking the loss of cIAP1

to a phenotype in the *mdx* mice are purely correlational. While association by correlation is not necessarily a flaw, it limits the strength of the conclusions drawn for a number of reasons.

a) We cannot say that the increased muscle resilience is due to the loss of cIAP1 in *muscle* rather than in, say, macrophages or neutrophils.

b) There is no direct evidence that the NF-κB pathway plays any role whatsoever in this context; and

c) The NF-κB hypothesis appears contradictory when the loss of cIAP1 activates the pathway in one instance and blunts the pathway in another.

Nevertheless, it is possible to argue that the tentative conclusions drawn here are the most likely explanations for the observed results. To this end, I will address each of the above points in turn.

a) In most tissues, the loss of cIAP1 leads to the corresponding upregulation of cIAP2, which compensates for the absence of cIAP1 in most tissues (Conze et al., 2005; Zarnegar et al., 2008). Consequently, the non-muscle cells should function normally; accordingly, the *cIAP1*$^{-/-}$ mice have no overt phenotypes, other than the reported effect on skeletal muscle. This leaves the muscle tissue itself, which in the absence of cIAP1 has no compensating cIAP2 expression.

b) While the NF-κB pathway is currently the best-studied signalling pathway in the context of cIAP biology, it is certainly not necessarily the only one. XIAP, for instance, acts as an adaptor protein in the BMP/TGFβ axis by binding to TAK1 (Yamaguchi et al., 1999; Sanna et al., 2002; Kaur et al., 2005). Nevertheless, the little available literature to this point suggests that the alternative NF-κB pathway may improve mitochondrial biogenesis and

muscle fibre endurance under stress (Bakkar et al., 2008). This possibility would explain both the increased resilience of *cIAP1*$^{-/-}$*;mdx* muscle and the predominance of the improvement in the mitochondria-rich slow-twitch solei. It does not preclude, however, the contribution, in major or minor part, of other hitherto uncharacterized pathways in the protective mechanism.

c) The attenuation of NF-κB activity following treatment with TNFα (ligand-dependent activation) in the absence of cIAP1, and the corresponding stimulation of activity following SMC treatment (ligand-independent activation), are well-established phenomena (Petersen et al., 2007; Vince et al., 2007; Mahoney et al., 2008). These apply to the classical pathway, however: the alternative pathway is consistent in not responding to TNFα treatment, but responding with activation to the depletion of cIAP1. If both the fusion phenotype in the *cIAP1*$^{-/-}$ and the increased resilience in the *cIAP1*$^{-/-}$*;mdx* mice are results of increased alternative NF-κB signalling, then the results would indicate synergy, rather than dichotomy, of the proposed mechanisms.

3. The developmental context

An aspect of the findings reported here that I addressed only peripherally, and which lies largely outside the scope of this thesis, is the extent to which cIAP1 affects skeletal myogenesis during development. Fortunately (for me), the adult *cIAP1*$^{-/-}$ mice demonstrated a muscle phenotype; nevertheless, the findings reported are the summary outcomes of all events that occurred between the earliest stages of myogenesis and the age of the mice at examination (six weeks). It would be intriguing to ask if cIAP1, and by extension the classical and alternative NF-κB pathways, plays different roles at different stages of muscle

development. As described in the introductory chapter, myogenesis occurs in three waves, all regulated by independent cellular pathways. Embryonic myogenesis spans the production and migration of muscle progenitors from the somites to the back, trunk and limbs, as well as the differentiation of said progenitors into primary muscle fibres. Fetal myogenesis, which runs from E16.5 until birth, involves fusion of a secondary population of myoblasts with the embryonic muscle fibres, as well as fusion between embryonic and fetal fibres. Postnatally, myogenesis continues for the first two weeks of life, followed by continuous muscle hypertrophy as the animal grows to adulthood. Growth factors such as BMP (Wang et al., 2010) and transcription factors such as NFix (Messina et al., 2010) regulate the extent of satellite cell proliferation and muscle fibre formation in the embryonic versus fetal myogenic context. While Pax3 and Pax7 play significant roles in myogenesis developmentally, satellite cells remain fully functional when these genes are conditionally ablated from adult muscle (Lepper et al., 2009). The possibilities of temporally restricted gene function offer ample research opportunities into muscle form and function in the future.

4. Conclusions and future questions

In conclusion, the loss of cIAP1 can have diverse context-dependent effects on skeletal muscle regeneration and development. While it is tempting to view the possibilities solely under the auspices of the NF-κB pathway, the unknown diversity of binding partners and ubiquitination targets leave ample room for further investigations. What other pathways are involved? What are the transcriptional targets of p52/RelB that promote the fusion phenotype? Would treatment with a SMC recapitulate the observed effects of a genetic ablation of cIAP1? I look forward to future studies in a similar vein down the road, and

though many of these questions will likely be solved, the only guaranteed outcome is that still more questions will emerge from the answers.

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