The Role of Effector caspases in DNA Damage Response and Chromatin Remodeling during Myogenic Differentiation

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Abstract

Effector caspase activation is a critical regulatory step in apoptosis, as well as an essential inductive cut in numerous non-death related processes that occur within complex cell systems. Here we report two novel studies detailing mechanisms by which effector caspase activation advances muscle cell differentiation. In the first study, we demonstrate that caspase 3 triggered DNA damage leads to rapid formation of XRCC1 repair foci within differentiating myonuclei, which dissipates as the maturation program proceeds. Skeletal myoblast deletion of XRCC1 does not impact cell growth, yet leads to perinatal lethality, with sustained DNA damage and impaired myofiber development. These observations demonstrate that the temporal deployment of the XRCC1-related DNA repair mechanisms are effector caspase mediated, and essential for muscle cell differentiation. In the second study, we sought to investigate whether effector caspase enzymes altered chromatin structure to promote the early differentiation of muscle progenitor cells. Past research has shown that Matrix Attachment region proteins known as Special AT-rich binding proteins are expressed abundantly in stem and progenitor cells, showing rapid decrease in expression as the cell advances into its mature phenotype. Here we demonstrate that effector caspase-7 is responsible for cleavage of Satb2, rather than caspase 3. Satb2 degradation alters the expressed genetic profile leading to acceleration of the muscle differentiation program. Our cumulative work adds novel roles in which effector caspases are vital in the development of cells.

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APAF1	Apoptotic Protease Activating Factor 1
APE	Apurinic Endonuclease
BCL2	B-Cell CLL/Lymphoma 2
BER	Base Excision Repair
Bfgf	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
CAD	caspase Activated Dnase
CASBAH	caspase Substrate Database Homepage
CASPASE	Cysteinyl-Aspartate Specific Protease
Caspdb	caspase Data Base
Chip	Chromatin Immunoprecipitation
CK2	Casein Kinase 2
CMA	Chaperone Mediated Autophagy
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAPI	4’,6-Diamidino-2-Phenylindole
DDR	DNA Damage Response
DISC	Death Inducing Signalling Complex
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
Dnapol-Beta	DNA Polymerase Beta
Dnase	DNA Nuclease
Drice	Drosophila Interleukin-1-Converting Enzyme
DSHB	Developmental Studies Hybridoma Bank

dUTP	2'-Deoxyuridine 5'-Triphosphate
ESC	Embryonic Stem Cell
EZH2	Enhancer Of Zeste Homolog 2
FAS	Fas Cell Surface Death Receptor
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GO	Gene Ontology
HMG	High Mobility Group
HR	Homologous Recombination
ICAD	Inhibitor Of caspase Activated Dnase
ISNT	In Situ Nick-Translation
MAR	Matrix Attachment Region
MCK	Muscle Creatine Kinase
MEF2C	Myocyte Enhancer Factor 2c
MHC	Myosin Heavy Chain Protein
MMR	Mis-Match Repair
Mrna	Messenger RNA
MS	Mass Spectrometry
MST1	Mammalian Sterile Twenty-Like Kinase
Myod	Myoblast Determination Protein
Myod	Myogenic Differentiation Gene
NHEJ	Non-Homologous End Joining
OGG1	8-Oxoguanine Glycosylase
PAGE	Polyacrylamide Gel Electrophoresis

PARP	Poly (ADP-Ribose) Polymerase
PAX7	Paired Box Protein 7
PBS	Phosphate-Buffered Saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PNK	Polynucleotide Kinase
PRC2	Polycomb Repressive Complex 2
PTM	Post Translational Modification
RNA	Ribonucleic Acid
Rnase	RNA Nuclease
SASP	Senescence-Associated Senescence Phenotype
SATB	Special AT-Rich Binding Protein
SDS	Sodium Dodecyl Sulfate
Shrna	Short Hairpin RNA
SOX2	SRY-Related HMG-Box Gene 2
SSB	Single Strand Break
STS	Staurosporine
SWI/SNF	Switch/Sucrose Nonfermentable
TBST	Tris Buffered Saline With Tween 20
TNF	Tumor Necrosis Factor
TopoIIb	Topoisomerase II Beta
TRAIL	TNF-Related Apoptosis-Inducing Ligand
UV	Ultra Violet
XRCC	X-Ray Cross Complementing Protein

Yca1	Yeast Metacaspase 1
Z.DEVD.Fmk	N-Benzyloxycarbonyl-Asp-Glu-Val-Asp-Fluomethylketone

Chapter 1.0: General Introduction

1.1: General Proteolytic Regulatory Mechanisms

At its simplest form of categorization, most cellular components are made up of amino acid building blocks for proteins, and nucleic acid building block making up DNA or RNA [Ingber, 2000, Trevors, 2003]. Biochemical synthesis of complex structures from these basic building blocks is the genesis of life as it is understood today [Ingber, 2000, Trevors, 2003]. And while synthesis of complex structures is essential and conceptually easy to understand, it is just as important to understand the role that breakdown of complex structures plays in the maintenance and advancement of biological systems, specifically in the cellular context driving replication as well as the cell specialization that occurs in biological systems [Neurath, 1984].

Protein breakdown within cellular systems is an evolutionarily conserved process that extends from the simplest unicellular organisms, up to the most complex multicellular life forms. Also known as proteolysis, this process is essential in disposal of damaged or expired proteins in the cellular system, as well as allowing for the recycling of the basic amino acid building blocks to produce future proteins beneficial to the cell [Mizushima and Komatsu, 2011, Bell et al., 2016]. The most studied pathways in general proteolysis are: the lysosomal degradation pathway and the ubiquitin/proteasome degradation pathway [Ciechanover, 2005]. The lysosome was initially discovered by Dr. C du Duve [du Duve et al., 1953] and its proteolytic function is conducted on engulfed proteins that are subject to targeted recruitment by the organelle [Dice et al., 1986, Kornfeld and Mellman, 1989, Bohley and Seglen, 1992]. The lysosome houses more than 60 known enzymes capable of degrading cellular content [Xu and Ren, 2015]. This enzyme localization depends on a sophisticated sorting mechanism that includes the golgi apparatus and various membrane receptors that trigger shuttling of these proteins into the lysosome [Braulke and Bonifacino, 2008]. Intracellular protein transport to the lysosome is a function known as

autophagy [Mizushima et al., 2008], which in itself is sub-organized into microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy [Settembre et al., 2013], each having a distinct method of protein targeting. This generalized method of proteolysis is activated in response to multiple cellular needs such as content recycling, secretion, repair, signaling and energy metabolism [Mizushima et al., 2008].

The other well studied mechanism of proteolysis is the ubiquitin/proteasome pathway [Hershko, 1991, Smalle and Vierstra, 2004]. This system works on the basis of post-translational modification of proteins (adding an ubiquitin moiety), which then allows for motif-based regulated disposal and recycling. Ubiquitin tagged proteins are then transported to a large disassembly complex (proteasome) that catalyzes the breakdown of protein into its essential amino acid building blocks, which are then free to be recycled and used in the making of new proteins [Hershko, 1991, Hellmann and Estelle, 2002, Smalle and Vierstra, 2004]. Substrate proteins enter the proteasome and are degraded, generating products that are between 3 and 30 amino acids in length, with an average of 7 to 8 residues [Zwickl et al. 200]. The methods utilized by biological systems to tag proteins with ubiquitin (also known as ubiquitination) is subject to varied forms of regulation, including other regulatory post-translational modifications [Karin and Ben-Neriah, 2000], as well as alterations of protein structure. [Pickart, 2001].

An additional method of altering protein structure in the cell is by fragmenting the protein via directed cleavage of bonds between the amino acid chains. Fragmentation of the three-dimensional structure of proteins can be an efficient method to promote ubiquitination, as well as facilitate the transport of smaller protein fragments into the proteasome for complete degradation [Denault and Salvesen, 2002, Paquette et al., 2010]. As previously noted, the two major categories of generalized proteolysis are lysosomal degradation and the ubiquitin/proteasome

pathway [Ciechanover, 2005]. Eukaryotic cells also employ other directed forms of proteolysis that rely on specific subclasses of protease enzymes. One of the most widely studied forms of protease directed cleavage is the cellular function known as apoptosis, which is simply defined as programmed cell death [Budihardjo et al., 1999]. From an evolutionary perspective, apoptosis provides a mechanism whereby multicellular organisms can eliminate compromised or supernumerary cells, allowing neighboring tissues to uptake (and the organism in general) and recycle the cellular constituents [Hershko, 1991, Smalle and Vierstra, 2004]. Indeed, programmed cell death is an evolutionarily conserved molecular mechanism that is found from the simplest unicellular organisms such as phytoplankton [Bidle and Falkowski, 2004] to nematodes [Ellis and Horvitz, 1986], through to the highest order mammalian species [Shalini et al., 2015].

1.2: caspase Proteases and Apoptosis

The operative proteases in apoptosis derive from a large protein family known as caspases (cysteine-dependent aspartate-directed proteases), with 18 identified to date in mammalian species [Shalini et al., 2015]. These are highly specific endopeptidases that recognize substrates through an aspartic acid residue at the P1 position within a generally conserved 4 amino acid domain [Budihardjo et al., 1999, Chang and Yang, 2000, Denault and Salvesen, 2002]. The enzymatic activity of caspase proteases are dependent on a conserved cysteine residue found in the active site and within the general protease fold region. caspase activation is a 2 step biochemical process: first is the deprotonation of a thiol functional group followed by the nucleophilic attack by the deprotonated cysteine anionic sulfur on the substrate carbonyl carbon. This deprotonation results in a fragment of the substrate released with an amine group and a thioester intermediate linking the new carboxy-terminus of the substrate to the cysteine thiol of

the enzyme. Finally, the thioester bond is hydrolyzed to generate a carboxylic acid moiety on the remaining substrate fragment, while regenerating the free enzyme [Thornberry and Lazebnik, 1998, Budihardjo et al., 1999, Chang and Yang, 2000].

While all caspases retain this core biochemical activation (caspase 9 being the exception), a plethora of studies have established a clear hierarchy within the caspase family during engagement of apoptosis. For example, caspases are broadly categorized as either initiator caspases (enzymes that target and cleave downstream caspases) or effector caspases (the primary substrate targets of initiator caspases), which are widely known to be the final executioner enzymes that catalyze the cleavage of target proteins and initiate cell death [Thornberry and Lazebnik, 1998, Denault and Salvesen, 2002]. In the mammalian system, initiator caspases are activated by either intrinsic or extrinsic stimuli that signal an activation of caspase-directed proteolysis [Boatright and Salvesen, 2003]. Initiator caspases natively reside in the cell as monomers, and are activated by dimerization to produce the cleaving site [Renatus et al., 2001, Pop and Salvesen, 2009]. Effector caspases are synthesized as dimerized inactive zymogens, requiring the cleavage of the procaspase form by the active dimerized initiator caspases to become enzymatically functional [Budihardjo et al., 1999, Reidl and Shi, 2004]. This level of control is extremely important, as accelerated activation of effector caspases can rapidly compromise cell integrity.

Intrinsic pathway activation, originates from the mitochondria causing release of proteins, such as cytochrome c, from the inner-mitochondrial membrane space [MacFarlane and Williams, 2004]. This process regulated in part by Bcl2 family members, with both anti-apoptotic and pro-apoptotic members inhibiting or promoting the release, respectively. Once released, cytochrome c binds to apoptotic protease-activating factor 1 (Apaf1), which results in formation of the larger

Apaf1–caspase 9 apoptosome complex and activation of the initiator caspase 9. Activation of the extrinsic cell death pathway is initiated via ligand based receptor signaling events. Here, cell surface death receptors of the tumour necrosis factor (TNF) receptor superfamily, including CD95 and TNF-related apoptosis-inducing ligand (TRAIL)R1/R2, initiate the death signal. Following ligand stimulation, these receptor complexes recruit the initiator caspase 8/10 to a larger protein complex referred to as the death receptor ligand complex (DISC) through adaptor molecules such Fas-associated death domain protein (FADD) [MacFarlane and Williams, 2004]. Subsequently, both the caspase-9/apoptosome complex and the caspase-8/DISC complex recruit and cleavage activate one of three known members of the effector caspase family (caspases-3, -6, -7) to complete the death centric proteolysis signal [Pop and Salvesen, 2009].

Effector caspases have different structural and functional qualities [Slee et al., 2001, Brentnall et al., 2013]. The most well-studied functions of effector caspases are found in research related to apoptosis [Budihardjo et al., 1999, Boatright and Salvesen, 2003,], and inflammation within the context of immune system response [Shalini et al., 2015, Fernández DJ, Lamkanfi, 2015] including caspase-1 in pyroptosis [Fernandes-Alnemri et al., 2007, Miao et al., 2010]. These models provide a clear and useful illustration of the proteolytic role that caspases play in cellular systems and has led many researchers to investigate this proteases as targets for disease therapies, i.e. limit caspase activation as a means to limit human pathology associated with excess cell death [Degterev et al., 2005, Adams and Cory, 2007, Jana and Paliwal, 2007]. Currently, there are over 500 identified human proteins that are catalogued as targets of caspase cleavage (these cleavage events have been annotated in databases such as CASBAH and CaspDB, [Luthi and Martin, 2007; Kumar et al. 2014, respectively]. These databases are critical to understand caspase biology and allow researchers to predict physiologically relevant

substrates without conducting broad based proteolytic screens. In addition to promoting cell death, caspase proteases have been shown to perform vital cell functions, from processing and activating cytokines [Gurung et al., 2014, Lamkanfi and Dixit, 2014] to remodeling cellular structures and organelles [Hetz and Glimcher, 2008, Feinstein-Rotkopf and Arama 2009].

1.3: Non Death Role of caspases

Over the past decade, a new body of research has emerged demonstrating vital physiologic roles for caspase activity, independent of cell death [Shrestha and Megeney, 2012, Dick and Megeney, 2013, Connolly et al., 2014, Unsain and Barker, 2015, Vesela et al., 2015]. Entrenched death and non-death functions for caspase proteases may have been a critical feature to ensure that this enzyme class would be retained over an evolutionary timeframe, as a death only protein function would be the focus of intense negative selection pressure [Fernando and Megeney 2007]. Although apoptosis is clearly beneficial in the context of multicellular organisms, programmed cell death provides little advantage to unicellular life forms, although there has been recent work showing potential analogous pathways existing in phytoplankton, reviewed in [Bidle et al., 2004], and in certain types of bacteria [Gautam et al., 2002].

In other unicellular organisms researchers have discovered families of genes related to caspases, termed as paracaspases and metacaspases [Uren et al 2000], which led to the identification of a metacaspase present in *S. cerevisiae* yeast organisms [Madeo et al., 2002]. Apoptotic function in these unicellular organisms is hypothesized to be a form of community altruism [Rice and Bayles, 2008], self-sacrificing cells ensure the survival of neighboring cells by not competing for limited resources or spreading infections, however this concept is based on a bias that suggests single cell organisms have a quorum sensing capacity. As such, it is reasonable to suggest that

primordial cells may have selected for non-death roles of caspases over evolutionary timescales. In support of this, research into non-cannonical function of caspase activity has yielded multiple examples of non-death roles for these enzymes. Seminal work done using the *S. cer* yeast model [Lee et al., 2008, Lee et al., 2010, Hill et al., 2014] has shown non-death roles of metacaspase Yca1.

In the first study, Lee et al. show using both genetic deletion of Yca1 and expression of a catalytically inactive mutant strain of Yca1 results in a significant reduction in cell growth/cell survival [Lee et al., 2008]. Both Yca1 deleted and enzymatically inactive strains displayed longer G1/S phase transition leading to slower growth in fermentation conditions, and a failure in response to reagents that induce the G2/M checkpoint. In the follow up study, Lee et al. characterized a novel molecular function for Yca1. Using a series of complimentary proteomic screens, Lee et al reported that Yca1 formed physical interactions with proteins that controlled proteostasis functions, including protein folding and aggregate formation [Lee et al., 2010]. The loss of Yca1 led to accumulation of vacuolar structures and protein aggregates during both cell cycle progression, and heat stress conditions. This new function of Yca1, limiting protein aggregation within the cell, provided a non-overlapping role for the metacaspase, separate from the traditional promotion of PCD. Further research in Yca1 has elucidated several specific cellular pathways affected by this novel non death role of the metacaspase. In Shrestha et al., the authors used wildtype and mutant knockouts of Yca1, both under normal and heat stressed conditions, and showed that the loss of Yca1 leads to a significant decrease of proteins that control ribosome biogenesis and protein [Shrestha et al., 2013]. In a study done by Hill et al., Yca1 overexpression showed a decrease in accumulation of unfolded proteins and an increase in the life span of the mutant cells [Hill et al., 2014]. The research in non-death roles of caspase-

like proteases in unicellular organisms continues to provide valuable understanding of the alternative functions of these proteins that can justify their positive evolutionary selection.

Early studies on the caspase protease family in mammalian organisms revealed that these enzymes could be arranged within two broad categories that reflect the respective function: the pro-inflammatory caspases and the pro-apoptotic caspases [Wang and Lenardo, 2000]. For example: mouse caspase-1 and -11 are pro-inflammatory caspases. caspase-$1^{-/-}$ mice have no mature IL-1β, IL-18 [Kuida et al., 1995; Li et al., 1995] or IL-33 [Ogura et al., 2006], demonstrating that caspase-1 is an essential regulator of inflammatory responses through its capacity to process and activate the pro form of these cytokines. caspase-$11^{-/-}$ mice show a similar phenotype to caspase-$1^{-/-}$ mice and have defects in caspase-1 activation [Wang et al., 1998], as well, Wang et al. showed that caspase-11 can physically interact with caspase-1 to promote its activation. Moreover, the canonically categorized pro-apoptotic caspases, have also been implicated in non death roles. For example, studies have shown that caspase-8 is implicated in the proliferation of hemopoietic progenitor cells, proliferation and activation of T lymphocytes, B lymphocytes and natural killer cells [Kang et al., 2004].

1.4: caspase Function in Cellular Differentiation

The most widely studied role for caspase signaling (independent of cell death) is in the management of cell differentiation. Briefly, differentiation is a term describing the cumulative events at the genetic and protein level that result in cells specializing in form and function [Chen and Dent., 2014, Alvarado and Yamanaka, 2014]. Morphologic transformation at this scale requires a dramatic alteration in gene expression, as well as extensive structural alterations that finalize cellular architecture [Kilian et al., 2010, Guo et al., 2011]. Not surprisingly, enzymes

capable of peptide bond cleavage at a common amino acid residue provide a potent mechanism to alter the function of a large number of proteins, which in itself may spur alterations in cell fate.

One of the earliest studies examining the non-death role of caspases reported the involvement of caspase-3 in mediating differentiation of lens fiber epithelial cells [Ishizaki et al., 1998]. At the time of publication, this study described the activity of caspase-3 as being "at least part of the machinery of Programmed Cell Death", arguing that differentiation of this cell lineage was an attenuated form of apoptosis. Similarly, another early publication [Zermati et al., 2001], noted that transient activation of caspases was required for promoting differentiation of erythroid cells. Again, as with the Ishizaki et al. study, the authors suggested that the consequence of caspase action in this cell type was synonymous with a partial or aborted apoptotic process, as evidenced by the apoptotic like changes that are coincident with erythroid differentiation such as chromatin condensation, loss of organelles, and enucleation [Zermati et al., 2001].

In addition to these early studies, a separate group of pioneering observations suggested that caspase activity engaged cell differentiation without apoptosis-like outcomes. These studies included demonstrations of caspase control of sperm differentiation [Arama et al., 2003], monocyte differentiation [Sordet et al., 2002], and muscle cell differentiation [Fernando et al. 2002]. The Arama et al. study focused on postmeiotic sperm cell differentiation, which requires a sequence of conserved changes in the morphology and structural organization. The authors investigated the role of apoptotic effector caspase drICE during *Drosophila* spermatogenesis [Arama et al., 2003]. The study found that terminal differentiation of elongated spermatids involved an apoptosis-like process, carried out by drICE activity. However, unlike drICE activation during true apoptotic events, drICE activation during spermatid maturation was

restricted to the cytoplasmic compartment of sperm cells, where removal of cytoplasm is necessary for generation of functional sperm. Next, in a study by Sordet et al, the authors followed up on early research demonstrating the non-death role of the pro-inflammatory caspases, specifically the role of caspase-1 in activating several cytokines [Kuida et al., 1995; Li et al., 1995], by seeking to investigate the role of caspase-3, previously categorized as solely pro-apoptotic in function. This study identified that an activation of caspase-3 and caspase-9 in human peripheral blood monocytes induced them to differentiate into macrophages in response to a stimulating factor [Sordet et al., 2002].

In parallel to these studies, work from the Megeney group demonstrated that either chemical inhibition or direct deletion of caspase-3 expression resulted in reduced myotube formation and decreased expression of muscle specific proteins in vitro, and severe attenuation of myofiber maturation in vivo [Fernando et al., 2002]. The authors noted an increase in caspase-3 activity that spiked at early differentiation time points then dissipated toward the late stages of differentiation. The authors reported that one of the targets of caspase-3 cleavage during this early differentiation phase was the Mammalian Sterile Twenty-like kinase (MST1). Prior observations had shown that caspase cleavage of MST1 leads to its activation and translocation into the nucleus where it is known to target histones for phosphorylation [Creasy et al., 1996]. Fernando et al demonstrated that restoring the cleaved form of MST1 in caspase-3 null myoblasts allowed for restoration of the differentiation phenotype, establishing a novel role for effector caspases in regulating proteins that drive myogenic differentiation. Also of importance in this context is that downstream phosphorylation by MST1 has been shown to lead to chromatin remodeling events that promoted differentiation [Larsen et al., 2010, Li et al., 2013, Du et al., 2014]. The study of Fernando et al was one of the earliest demonstrations that pro-

apoptotic caspases function in differentiation that is not categorized as aborted or incomplete apoptosis (in contrast to lens fiber epithelial or erythrocyte differentiation). This landmark study laid the groundwork for an expanding research effort into non-death roles of caspases. For example, a study by Murray et al demonstrated that in the muscle cell differentiation model, initiator caspase-9 which is implicated in the intrinsic (mitochondrial/cytochrome-C) pathway of activating the caspases, is required for activating caspase-3 during this process [Murray et al., 2008]. This work clarified the upstream molecular pathway involved in the early transient caspase-3 activity during myoblast differentiation described first in [Fernando et al., 2002].

More recently, caspase-3 has also been implicated in regulating very early steps in cell differentiation programs. A study by Fujita et al has demonstrated this requirement by investigating the regulation of a number of the early identified pluripotency factors, namely OCT4, SOX2, and NANOG [Fujita et al., 2008]. The authors hypothesized that negative regulation of these factors would come in the form of a post-translational modification (PTM), possibly by directed protease cleavage. Using mouse embryonic stem cell (ESC) cultures, it was elucidated that 1) differentiating ESCs show increased caspase activity that is not associated with programmed cell death, 2) caspase-$3^{-/-}$ ESCs showed delay in differentiation compared with wild type and heterozygous cells, and 3) human Nanog contained a caspase-3 cleavage site at D69. Taken together, this work demonstrated that transient caspase-3 activation was an essential step in differentiation, through targeted degradation of the pluripotency factor Nanog.

Similar to the role of caspase 3 in ESC self-renewal, a recent study by the Megeney group have demonstrated that caspase-3 is also implicated in muscle progenitor cell fate decisions (commonly referred to as satellite cells) by targeting of Pax7 protein [Dick et al., 2015]. Briefly, the study uncovers novel caspase-3 cleavage sites on the Pax7 protein, initially described in

[Seale et al., 2000] to be essential in the specification of lineage in myogenic satellite cells, with further research elucidating its role in allowing activated satellite cells to reacquire a quiescent, undifferentiated state [Olguin and Olwin, 2004]. Dick et al show post-translational modifications of Pax7, either by caspase-mediated cleavage, or by phosphorylation on CK2 kinase (to prevent the cleavage event), resulted in satellite cell differentiation or self-renewal, respectively. Prior studies by the Litchfield group had demonstrated that casein kinase 2 (CK2) and caspase 3 have overlapping consensus motifs, that allow the modulation of caspase activity by inhibiting its access to the preferred cleavage site following phosphorylation of an adjacent serine residue [Duncan et al., 2010, Turowec et al., 2011].

1.5: caspase Mediated DNA Damage. An Inductive Cue for Muscle Cell Differentiation

Effector caspases, principally caspase-3, are involved in initiating genomic fragmentation associated with apoptotic cell death [Fernandes-Alnemri et al., 1994]. One of the primary enzymes implicated in DNA fragmentation within the context of apoptosis is caspase Activated DNase (CAD) [Liu et al., 1997]. This enzyme is activated by the removal of ICAD, an inhibitory chaperone protein that could be cleaved and inactivated by caspase 3 [Enari et al, 1998, Sakahira et al, 1998]. Inactive pro-caspase-3 is localized in the cytoplasm, however once activated it can localize to the nucleus and inactivate ICAD via proteolytic cleavage [Lechardeur et al., 2000]. The active CAD homodimerizes and forms a scissors-like structure [Woo et al., 2004] that lacks sequence specificity, and is able to indiscriminately target both naked DNA, as well as inter-nucleosomal DNA within chromatin structure [Widlak et al., 2000]. While DNA fragmentation in apoptosis is a biologically programmed event, DNA damage can result from various non-intentional assaults on the genome such as microbiological infection [Baik et al., 1996, Cuevas-Ramos et al., 2010], genotoxic chemicals [Poirier, 2004], external environmental ingredients like

ultraviolet rays from the sun [Rastogi et al., 2010], all can trigger DNA damage causing events within cells.

The conservation of caspase mediated signaling in metazoan cell differentiation suggests that the terminal DNA damage event may also be a key feature of the differentiation program. For example, DNA damage by Neocarzinostatin was shown to promote myogenic differentiation of cultured Drosophila Schneider cells [Hossain et al, 2003]. Similarly, DNA damage in melanocyte stem cells prompted cell cycle exit and differentiation to mature melanocytes in the absence of apoptosis [Inomata et al, 2009]. A more striking example of DNA damage altering gene expression independent of cell death was noted in the study of Ju et al. in 2006. Here, the authors reported that topoisomerase II beta (TopoIIB) was recruited to and induced DNA strand breaks within the promoter elements of glucocorticoid responsive genes in MCF-7 cells [Ju et al, 2006]. Inhibitors to TopoIIB prevented the nucleosome specific exchange of H1 for HMGB1/2 that impedes the expression of the stimulated gene. This study demonstrated a form of site-specific DNA strand breaks that was essential for gene expression, yet was independent of cell death or apoptosis.

These studies provided a rationale to explore whether the caspase mediated DNA damage that occurs during apoptosis, would be similarly conserved in cell differentiation. In the seminal study of Larsen et al. (2010), the authors demonstrated that the caspase-3/CAD signal nexus was critical for muscle cell differentiation. Briefly, the authors demonstrated that DNA strand breaks were present early in myoblast differentiation, as evidenced by in situ nick translation incorporation of dUTP, COMET assay, and through increased nuclear expression and foci formation of phospohorylated-H2A.X, all of which dissipated later in the differentiation program. The authors then demonstrated that of these DNA damage metrics were caspase 3

sensitive, and use of pharmacologic or biologic caspase inhibitors resulted in a complete absence of DNA damage concurrent to a blockade in differentiation. Subsequently, the authors demonstrated that the source of these transient DNA strand breaks originated from the specific activity of caspase-3 cleaving ICAD to allow for CAD to target and induce the formation of DNA strand breaks (figure 1). Finally, the study reported that a primary target of CAD strand break formation occurred within the promoter of the cell cycle arrest protein p21. Also known as WAF1/CIP1, this gene was first discovered as an inhibitor of a number of cyclin dependent kinases (CDKs) [Harper et al., 1993], inducing cell cycle arrest at the G1/S checkpoint. Cell cycle exit is a prerequisite to terminal differentiation, and thus p21 increased expression is known to be essential to the initiation of cellular differentiation [Gartel and Tyner, 1999]. Loss of CAD expression or inhibition of caspase 3 activity resulted in a complete loss of p21 expression, suggesting that the CAD targeted strand break within the p21 promoter was an essential cue to initiate p21 transcription and propel the differentiation program.

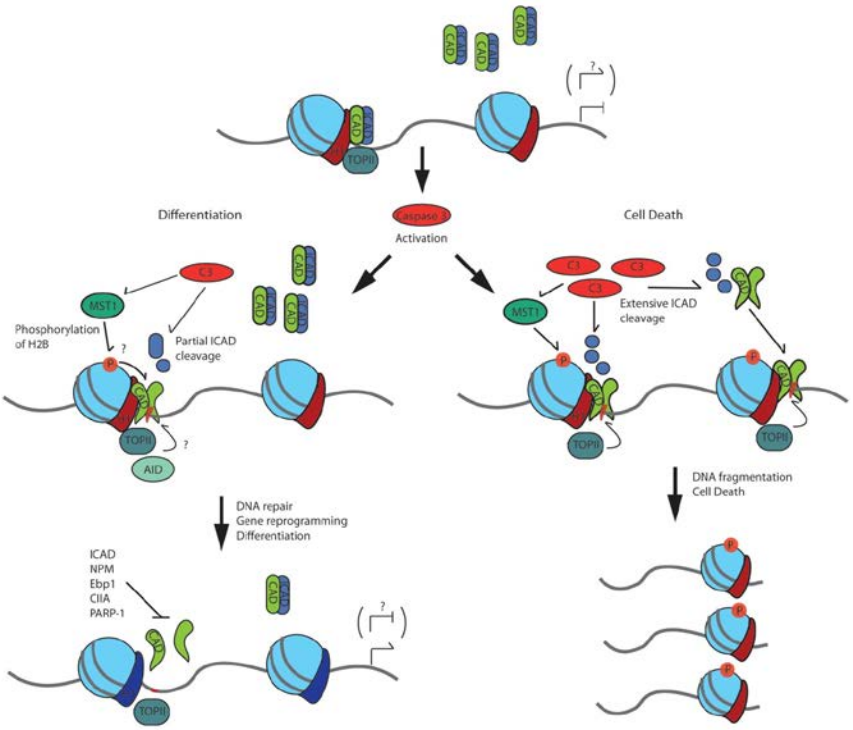

Figure 1 caspase 3 activation balances the dissemination of CAD induced DNA strand breaks for gene reprogramming or fragmentation. This model depicts several factors that regulate CAD induced DNA strand breaks in physiologic and apoptotic conditions. [Larsen and Megeney, 2010; used with permission]

1.6: Base Excision Repair. An Essential DDR Mechanism for Genome Stability

The transient/temporal formation of CAD induced DNA strand breaks implies that the differentiating muscle cell must utilize a mechanism to repair the damage. Indeed, the experimental evidence provided by Larsen et al. strongly suggests that CAD mediated strand breaks are extensive and genome wide. As such, long term cell viability would be compromised without an efficient mechanism(s) to repair DNA damage and avoid the induction of DNA surveillance mechanisms that themselves are pro-apoptotic by design, i.e. p53. Depending on the type of damage, repair mechanisms specific for that have evolved. Mispaired DNA bases are replaced with correct bases by mismatch repair (MMR); single strand breaks and small alterations of DNA bases are repaired by base excision repair (BER); double strand breaks are processed either by nonhomologous end joining (NHEJ) or homologous recombination (HR). NHEJ promotes the potentially inaccurate re-ligation of double strand breaks [Weterings and Chen, 2008]. HR precisely restores the genomic sequence of the broken DNA ends by utilizing sister chromatids as template for repair [Li and Heyer, 2008].

DNA repair is carried out by a number of proteins that lead to repairing the damage [Harper and Elledge, 2012]. The list includes a continually expanding number of nucleases, helicases, polymerases, topoisomerases, recombinases, ligases, glycosylases, demethylases, kinases, phosphatases, and various non-enzymatically active proteins that scaffold and direct the interaction of these various enzymes [Harper and Elledge, 2012]. These proteins are precisely

regulated, therefore cells have developed strategies to recruit and activate the right factors in the right place at the right time [Harper and Elledge, 2012]. In general, repair is initiated after sensor proteins localize and bind to the site of the damage, with different types of damage recruiting different types of sensor proteins [Zhou and Elledge, 2000]. This is followed by a cascade of DDR-related repair factors to target and mend the damage site, leading to the correction and disassembly and dispersal of the repair machinery to be utilized at other lesion site. To illustrate this repair mechanism, we can focus on the case of base excision repair pathway (BER) as an example. The justification for focusing on BER stems from the fact that it is considered to be the principle method of providing genome stability [Dianov and Hübscher, 2013], with deficiencies in this mechanism showing links to premature aging, cancer, neurodegenerative diseases and general development [Freitas and de Magalhaes, 2011, Poirier, 2004, Madabhushi et al., 2014]. This type of DDR is used to fix single strand breaks that occur on the DNA double helix.

BER is initiated by a damage-specific DNA glycosylase that recognizes a damaged DNA base, cleaving the N-glycosylic bond that links that DNA base to the sugar phosphate backbone [Lindahl, 1979]. The now baseless site is then processed by an AP endonuclease (APE) that cleaves the phosphodiester bond 5′- to the baseless site, generating a single strand break (SSB), sometimes called a nick, containing a hydroxyl residue at the 3′-end and deoxyribose phosphate at the 5′-end [Dianov and Parsons, 2007]. Polynucleotide kinase (PNK) is the principal enzyme involved in the restoration of 5′-phosphate and 3′-hydroxyl ends in SSBs. It has been characterised as having DNA 5′-kinase and 3′-phosphatase functions, to phosphorylate the 5′-end of SSBs and removing the blocking phosphate lesions from the 3′-end [Jilani et al., 1999]. This allows a DNA polymerase to incorporate a new nucleotide, and a DNA ligase to seal the DNA ends. The DNA polymerase involved in this process is DNApol-beta, and the ligase is DNA

Ligase III. DNApol-beta adds a nucleotide to the 3′-end (while simultaneously removing the phosphate group at the 5′-end) [Matsumoto and Kim, 1995], and DNA Ligase III (complexed with XRCC1, to be functional) seals the ends together [Caldecott et al., 1994]. Coordinating this whole series of events is the specific to base excision repair protein known as X-ray cross complementing protein 1 (XRCC1) [Caldecott et al., 1996, Whitehouse et al., 2001, Caldecott, 2003]. This protein is not known for having an enzymatic function, the main role of XRCC1 is to act as a scaffold protein, recruiting and providing docking platform for the required enzymes to interact and mend the DNA nick [Caldecott, 2003]. XRCC1 participates in the first step of BER by interacting either with poly (ADP-ribose) polymerase-1 (PARP-1) which acts as a sensor identifying the single strand break sites [El-Khamisy et al., 2003], or with DNA glycosylases, stimulating the activities of these enzymes [Campalans et al., 2005]. The recruitment of XRCC1 specifically at SSBs was clearly first demonstrated by experiments employing UV radiation in nucleotide excision repair-defective cells [Okano et al., 2003].

1.7: The role of Chromatin Remodeling in Myogenic Differentiation

Changes in DNA structure are hallmarks of the maturation process that convert a stem cell into an adult differentiated cell type [Ju et al., 2006, Sherman et al., 2011, Puc et al., 2015]. Cells progress from the proliferative state into a mature, post-mitotic state concurrent to a major reorganization of the genome structure [Kimmins and Sassone-Corsi., 2004, Fisher and Fisher, 2011]. These chromatin remodeling events arrest transcription of cell division genes and activate expression of differentiation specific genes [Muller and Leutz, 2001]. During myogenesis, satellite cells are converted into differentiating myocytes, concurrent to dramatic alterations in genetic expression. These differentiating cells require a great deal of architectural modifications

within the nuclear space, some of which are caused by DNA damage inducing agents such as caspase-Activated DNase, CAD [Larsen et al., 2010].

Early research in the myogenesis field established that chromatin remodeling was essential for the activation myogenic genes. Specifically, research demonstrated that chromatin remodeling proteins interacted with muscle determination genes (transcription factors from the myogenic regulatory family, MRFs) to modify the local chromatin environment and enhance differentiation specific gene expression. One of the first co-activators discovered in this regard was p300/CBP [Sartorelli et al., 1997] and its associated histone acetyltransferase enzyme PCAF [Puri et al., 1997]. Active gene expression is associated with histone acetylation and loss of histone/DNA interaction, and it was determined that recruitment of histone acetyltransferase P300/CBP-associated factor (PCAF) by MyoD, through p300/CBP, is crucial for activation of the myogenic program [Puri et al., 1997]. Further research in this pathway has shown the direct role that MyoD plays in controlling chromatin structure. In a study by Gerber et al, the authors reported that MyoD can remodel chromatin at binding sites in muscle gene enhancers and activate transcription at previously silent loci [Gerber et al., 1997]. These results established a mechanism for chromatin modeling in skeletal muscle and define domains of MyoD that participate in chromatin reorganization.

Following these early investigations, considerable research efforts have helped to elucidate the mechanisms that govern chromatin modification and gene expression during myogenesis. Within this context, post translational modifications (PTM) have emerged as significant regulatory event. PTM types include methylation of arginine residues, acetylation, ubiquitination, ADP-ribosylation, sumolation of lysine residues; and phosphorylation of serine and threonine residues [Li et al., 2007]. Modifications that lead to active transcription, such as

acetylation of H3 and H4 histones, di- or tri-methylation (me) of H3K4, are referred to as euchromatin modifications, while modifications that are localized to repressed genes, such as H3 K9me and H3 K27me, are often referred to as heterochromatin modifications [Li et al., 2007]. Research has elucidated multiple PTM mechanisms that specifically target and promote muscle specific gene expression. For example, the work of [Rampalli et al., 2007], examined the role of a core histone methyltransferase protein called Ash2L, which was recruited to muscle-specific genes like myogenin, in a Mef2d dependent manner. A different method of affecting chromatin structure has also been demonstrated by protein complexes named chromatin remodelers. These multi-protein complexes are characterized by the highly conserved SWItch/Sucrose NonFermentable (SWI/SNF) ATPase like proteins (either BRG1 or BRM) [Workman and Kingston, 1998], as well as multiple additional subunits that function to read and interpret epigenetic marks. This SWI/SNF complexes then assemble, reposition, and disassemble nucleosomes in an ATP-dependent fashion [Zhang et al., 2006, Tang et al., 2010]. The biological roles of the SWI/SNF complexes are varied, and may have positive and negative roles in gene expression, depending on the composition of the various subunits [Nagl et al., 2007].

The SWI/SNF chromatin remodeler complex has been associated with regulating many essential components in the myogenic differentiation pathway. In a study by Ivana et al, it was found that SWI/SNF interacts with MyoD in initiation of muscle differentiation [Ivana et al., 2001]. In a study by Simone et al, the authors established a link between the differentiation-activated p38 pathway and the recruitment of SWI/SNF complexes to transcriptionally active loci during skeletal myogenesis [Simone et al., 2004]. It was also discovered that myogenin and Mef2D direct the chromatin remodeler Brg1-based SWI/SNF complex to late differentiation specific gene promoters, such as MCK and desmin [Ohkawa et al., 2006]. These studies provided early

evidence for an essential interaction between chromatin remodeling proteins and myogenic transcription factors implicated in the various stages of muscle differentiation. More recent work has suggested that SWI/SNF protein interactions are a two step process , where preassembly of the BAF60c subunit with MyoD then leads to direct recruitment of SWI/SNF to muscle gene loci in response to differentiation cues [Forcales et al., 2012].

1.8: Proteolytic Regulation of Chromatin Remodeling Proteins during Cell Differentiation

The function of chromatin remodeling proteins during cell differentiation has also been linked to directed proteolysis events. An example in this regard is the protein named Enhancer of Zeste Homolog 2 (EZH2). This methyltransferase enzyme is a critical enzymatic subunit of the polycomb repressive complex 2 (PRC2), which trimethylates histone H3 (H3K27) to mediate gene repression, with its overexpression or hyperactivation of EZH2 being implicated in the pathological proliferation and decrease in differentiation, hallmarks of cancer development [Sahasrabuddhe et al., 2015]. Recent work has identified a specific E3 ubiquitin ligase responsible for the polyubiquitination and proteasome-mediated degradation of EZH2, which is required for neuronal differentiation [Yu et al., 2013]. Most recently, controlled EZH2 proteolysis has been documented during myogenesis, where a MyoD induced E3 ligase, Praja1 targets EZH2 for ubiquitin mediated destruction, a step that appears to be essential for the differentiation program to proceed [Consalvi et al. 2017].

In addition to ubiquitin mediated control of chromatin remodeling proteins, caspase targeted cleavage of chromatin remodeling proteins may also represent a key regulatory event in the muscle differentiation program. For example, as previously discussed in [Fernando et al., 2002], caspase-3 targets and cleavage activates the ste20 like kinase MST1, which is both necessary and

sufficient for myoblast differentiation. The exact target of the MST1 kinase in myoblasts was not elucidated; however there is significant data in the literature to suggest that the protein substrate may be a chromatin regulatory factor. MST1 has been linked to the regulation of chromatin compaction and decompaction during apoptosis [Ura et al., 2001; Ura et al., 2007]. In the yeast system this nuclear remodeling event has been linked to the direct phosphorylation of histone H2B [Cheung et al., 2003] and in mammalian cells MST1 has also been shown to directly phosphorylate and to inhibit the DNA repair histone protein, histone H2AX [Wen et al. 2010]. This later study is interesting in light of Larsen et al. and suggests that MST1 may be activated to ensure that H2AX mediated repair events do not preclude or limit the initial DNA damage event, which is essential for early stages of the differentiation program. This body of evidence indicates the direct relationship between proteolytic mechanisms and chromatin remodeling involved in cell fate decisions. The architectural remodeling occurring in the nucleus of progenitor cells during the transformative stage involves not only an alteration of structure at the DNA level in the form of DNA damage/DNA repair, but also an alteration of proteins that directly remodel the chromatin landscape [Ju et al., 2006, Singh et al., 2015, Puc et al., 2015].

caspase-3 targeted upregulation of the nuclease CAD and the concomitant DNA damage suggests that effector caspase enzymes may target additional proteins to alter gene expression and that this may occur through chromatin remodeling events. For example, there is a large number of observations in the literature demonstrating that caspase mediated apoptosis triggers chromatin structural changes, with alterations in both matrix components as well as genome wide Chromatin condensation [Robertson et al., 2000, Norbury and Zhivotovsky, 2004]. Whether a similar caspase dependent structural modification occurs during differentiation remains unknown. The nuclear matrix was originally described as the structure that maintained the

spherical shape of the nucleus [Berezney and Coffey, 1974]. Further investigations established that the nuclear matrix was essential in organizing higher-order chromatin loops, and that specific DNA sequences bind to the nuclear matrix in loop formation [Mirovitch et al., 1984]. These DNA sequences are referred to as matrix attachment regions (MARs) or scaffold-associated regions. MARs are approximately 200 bp long, AT-rich, contain topoisomerase II consensus sequences and other AT-rich sequence motifs [Wang et al., 2010]. Many proteins interact with MARs, and the function of these domains may involve many cellular pathways, from regulating gene transcription and expression, to packaging of chromosomes, and affecting cell fate decisions like development and apoptosis [Wang et al., 2010, Barboro et al., 2012]. MARs binding proteins are of great interest and a number of studies have implicated these factors as critically regulatory proteins that alter stem cell differentiation kinetics [Savrese et al., 2009, Wang et al., 2014].

One class of MARs factors that have been linked to regulating both apoptosis and cell differentiation are the Special AT-rich binding proteins (Satb1 and Satb2), which are expressed abundantly in stem cells and progenitor cells and generally decline as cells mature and differentiate [Galande et al., 2001, Britanova et al., 2005, Han et al., 2008, Dong et al., 2015]. Depending on the lineage, cells may express either Satb1, Satb2, or both [Savarese et al., 2009, Zhao et al., 2014]. Satb1 has been extensively studied in a number of model systems, for its chromatin modifying capacity and as a potential transcription factor [Galande et al., 2003, Wen et al., 2005, Balamotis et al., 2009, Han et al., 2008, Will et al., 2013, Wang et al., 2014]. Most interestingly, caspase 6 has been demonstrated to target and cleave Satb1, which then abolishes its DNA binding activity [Galande et al., 2001]. Nevertheless, Satb1 is not expressed at an appreciable level in skeletal muscle. However, recent gene expression profiling of murine

satellite cells (fetal, adolescent, adult and aged satellite cells) has shown that Satb2 expression is dynamic, with a notable decrement in expression from fetal to adult satellite cell populations (Log Fold Change = -0.47). Satb2 has been broadly implicated as a regulator of stemness in both normal stem cell populations [Dong et al., 2014], as well as a wide variety of cancer subtypes including as colorectal cancer [Mansour et al., 2015, Brocato and Costa, 2015], pancreatic cancer [Yu et al., 2016], and bone [Soeng et al., 2015]. Given these observations, it is reasonable to suggest that a decline in Satb2 expression is essential for the cell to acquire differentiation competence. How Satb2 is down regulated in progenitor cell populations remains unclear, yet a caspase mediated degradation event may be reasonable supposition (given the upregulation of caspase activity during cell differentiation, as well as the fact that the highly related protein Satb1 has been shown to be targeted by an effector caspase).

1.9: Thesis Aims and Hypothesis

We sought to expand the research on non-death roles mediated by caspase cleavage to investigate not only the immediate target of caspase cleavage, which could be an event that triggers either apoptosis or differentiation, but on the subsequent chain reaction occurring as a result of the cleavage. Parallel to the cleavage activation role of caspases is the role of altering function of protein via cleavage that is unrelated to increased enzymatic activity, what can be referred to as caspase mediated protein remodeling. This mode of operation is more centered on the concept that physical alteration of a protein caused by effector caspase cleavage is sufficient to provide a new role, or terminate a physical function that the protein performed prior to cleavage. This type of view of caspase cleavage as an initiator step for cell fate decisions, either pro-death or non-death pathways, is the underlying scientific concept we explore in the projects making up this thesis.

For our first project we sought to further investigate the role of caspase cleavage as an initiator of enzymatic activity leading to promotion of differentiation; building on the published work of [Larsen et al., 2010]. Our hypothesis was that caspase-3 cleavage of ICAD leading to the activation of CAD is in fact an initiator event that could lead to cellular differentiation if the proper DNA damage repair mechanisms are triggered with the cell. We focus on the role of base excision repair mediated by XRCC1 as the essential DNA damage response occurring in early myogenic differentiation**.** In our second project towards this thesis, we sought to illustrate a role for caspase cleavage as a driver of differentiation that utilizes its protein structure alteration. For this aim, we specifically sought to look into the chromatin remodeling events that occur at the transition of cells from a proliferative state to a differentiation state, considering this type of transformation required an enormous alteration to nuclear architecture, theoretically providing an environment ripe for the utilization of caspase cleavage potential. Here, using the well-studied muscle progenitor cell (C2C12) differentiation model, we investigate the expression and role that Satb2 plays in cellular differentiation, and whether effector caspases modulate chromatin structure by targeting Satb2 for proteolytic cleavage.

Chapter 2.0: Temporal Activation of XRCC1 mediated DNA repair is essential for muscle differentiation

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Running Title: XRCC1 mediated repair is essential for differentiation

AUTHOR CONTRIBUTIONS:

M.H.A-K, L.E.B, B.D.L, and R.A.B. performed all experiments. S.B. provided technical support. R.J.P., M.A.R., and P.J.M. contributed essential experimental resources. F.J.D. provided scientific advice and helpful comments on the project. **M.H.A-K** and L.E.B analyzed all data. **M.H.A-K**, L.E.B and L.A.M. wrote the paper. **M.H.A-K**, L.A.M. planned and L.A.M. supervised the project.

2.1 Abstract:

Transient DNA strand break formation has been identified as an effective means to enhance gene expression in living cells. In the muscle lineage, cell differentiation is contingent upon the induction of caspase mediated DNA strand breaks, which act to establish the terminal gene expression program. This coordinated DNA nicking is rapidly resolved, suggesting that myoblasts may deploy DNA repair machinery to stabilize the genome and entrench the differentiated phenotype. Here, we identify the base excision repair pathway component XRCC1 as an indispensable mediator of muscle differentiation. caspase triggered XRCC1 repair foci form rapidly within differentiating myonuclei, then dissipate as the maturation program proceeds. Skeletal myoblast deletion of *Xrcc1* does not impact cell growth, yet leads to perinatal lethality, with sustained DNA damage and impaired myofiber development. Together, these results demonstrate that XRCC1 manages a temporally responsive DNA repair process to advance the muscle differentiation program.

Keywords: Base Excision Repair, XRCC1, muscle differentiation, DNA strand breaks

2.2: INTRODUCTION

Genome stability is of utmost importance for the survival and development of all organisms. Accordingly, eukaryotic cells have evolved a variety of mechanisms to maintain and repair DNA, of which these mechanisms are collectively referred to as the DNA damage response (DDR) [Insinga et al., 2014, Iyama and Wilson, 2013]. The DDR is further delineated by the type of DNA damage that is targeted and repaired, which may involve single [Gassman and Wilson, 2015] or double strand breaks [O'Driscoll and Jeggo, 2006] and formation of toxic DNA adducts [Roos and Kaina, 2013]. Although persistent DNA damage is generally regarded as detrimental for maintaining cell viability, transient DNA strand break formation has been identified as an effective means to enhance gene expression in living cells [Ju et al., 2006, Puc et al, 2015, Szekvolgyi et al., 2007, Sherman et al., 2011]. The best studied example in this regard is V(D)J recombination, a purposeful DNA damage and strand rearrangement event that underlies the genetic diversity of antibody and T cell receptor generation in the adaptive immune system [Schatz and Swanson, 2011].

More recently, DNA strand breakage has been demonstrated to exert a profound effect on cell fate, acting to limit stem cell self-renewal and stimulate differentiation, without a concomitant increase in cell death [Larsen et al., 2010, Behrens et al., 2014, Narciso et al., 2007, Caldecott, 2003, Larsen and Megeney, 2010, Inomata et al., 2009, Santos et al., 2014, Sinha et al., 2014, Houlihan and Feng, 2014]. In the skeletal muscle lineage, cell differentiation is dependent upon a temporal activation of the caspase 3 protease and its cognate DNase CAD (caspase activated DNase), which act to enhance muscle gene expression through targeted DNA strand breaks [Larsen et al., 2010]. These observations imply that a differentiating cell must utilize/recruit an equally potent DNA repair mechanism to stabilize the genome and secure the

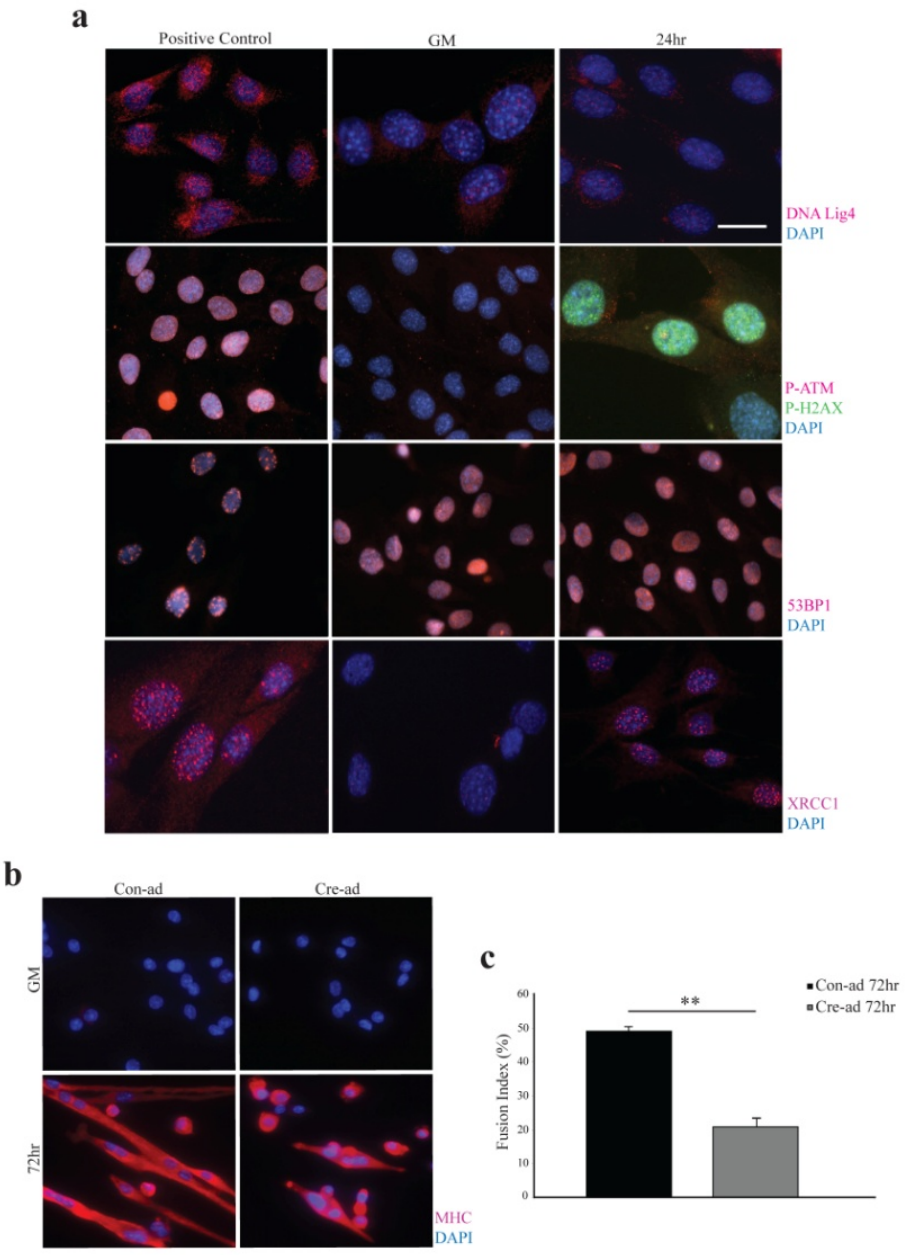
cell fate selection. The corollary to this hypothesis predicts that loss or inhibition of such a repair mechanism would result in failure to establish the differentiated state. Here, we sought to identify the repair machinery/proteins that target differentiation induced DNA strand breaks and whether this operant mechanism was similar to or divergent from existing DDRs. Our observations indicate that base excision mediated repair, as exemplified by the scaffold protein XRCC-1, is essential for resolving differentiation associated DNA damage and for securing this cell fate choice.

2.3: RESULTS

Base Excision Repair pathway is involved in early myoblast differentiation

Skeletal muscle cells cultured in low serum conditions recapitulate the differentiation program and are characterized by transient strand break formation [Larsen et al., 2010]. Using this model system, we noted that the standard components of the nonhomologous end joining (NHEJ) pathway were not active during differentiation, as muscle cells did not display increased foci formation for the ATM kinase, 53BP1, Ligase IV (**Supplementary Figure S1a**). Differentiating muscle cells give rise to long lived cell types, therefore we reasoned that the DNA damage may be more reflective of single strand breaks/nicks vs. catastrophic double strand breaks. In support of this supposition we noted that XRCC1, a key scaffold protein in the base excision repair (BER) pathway [Caldecott, 2003, London, 2015, Marsin et al., 2003], forms discrete foci during differentiation (**Figure 1a, Supplementary Figure S1a**). The XRCC1 foci are transient and match the temporal DNA strand breaks that form during muscle cell differentiation (**Figure 1a-c**; as measured by DNA polymerase guided in situ nick translation). Next, we ascertained whether these XRCC1 foci resulted from the caspase 3/CAD mediated

strand break activity that characterizes muscle cell differentiation [Larsen et al., 2010, Tebbs et al., 2003]. Using shRNA CAD muscle cell lines (previously described by our group [Larsen et al., 2010]), we noted that loss of CAD expression resulted in a complete absence of XRCC1 foci formation (**Figure 1d,e**). Moreover, peptide inhibition of caspase 3 activity during muscle cell differentiation results in a complete loss of XRCC1 foci formation (**Figure 1f**). These results confirm that XRCC1 clustering in differentiating cells is a direct response to the caspase 3/CAD induced DNA damage. These results also establish that this DNA break/replication repair cycle is a differentiation specific event rather than a terminal mitosis response.



Supplementary Figure S1 NHEJ pathway is not active during early differentiation. (**a**) Proliferating C2C12 muscle cells were induced to differentiate using low serum media for the indicated time period and were then fixed and stained for DNA Damage repair proteins DNA Ligase4, p-ATM, p-H2AX, 53BP1 and XRCC1. Scale bar, 20µm. (**b**) *Xrcc1* knockout myoblasts fail to fuse into myotubes. *Xrcc1*$^{flox/flox}$ primary myoblast induced to differentiate for 72hr then stained for Myosin Heavy Chain (red) and counterstained with DAPI (blue). (**c**) *Xrcc1* knockout cells have significantly reduced fusion potential, resulting in the inability to form myotubes, as determined by two-tailed student t-test analysis with ***P*-value<0.025

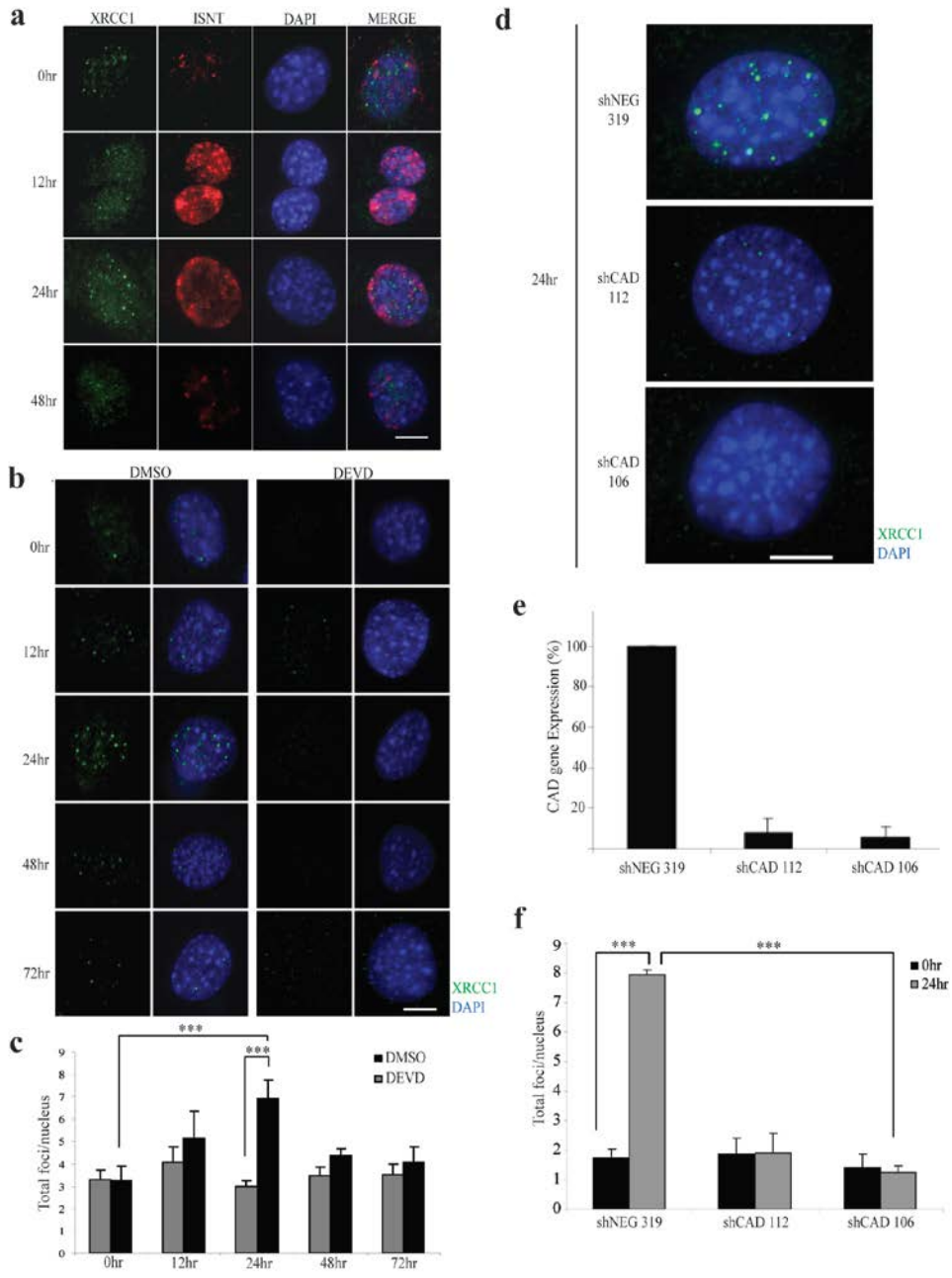


Figure 1 DNA repair during early myoblast differentiation is associated with XRCC1 foci. (**a**) Co-staining for XRCC1 foci formation and *in situ* nick translation, to measure DNA polymerase activity, in C2C12 myoblast cells over differentiation time course. Scale bar, 10µm. (**b**) Immunofluorescent staining for XRCC1 in caspase 3 inhibited (DEVD) differentiating C2C12 cells and immunofluorescently stained for XRCC1 and counter stained using DAPI. Scale bar, 10µm. Images are representative from n=3 experimental replicates. (**c**) Data were quantified by counting the total number of foci per nucleus as represented in the histogram. (**d**) Targeted shRNA mediated knock down of CAD in differentiating C2C12 cells. The cells were

induced to differentiate for 24hr then were immunofluorescently stained for XRCC1. (**e**) Quantitative real-time PCR reveals an 80% reduction in CAD expression in the KD lines compared to the control. (**f**) Quantification of total XRCC1 foci per nucleus in proliferating cells (GM) and after 24hr differentiation (24hr) is represented by histogram (n=3). Asterisks (***) indicate that the changes in foci formation between the two conditions indicated are statistically significant as determined by student t two-tailed analysis with *P*-value<0.01.

XRCC1 deletion halts C2C12 myoblasts fusion and myotube formation

To assess the impact of XRCC1 expression in differentiating muscle cells, we initially performed shRNA targeted *Xrcc1* (shXRCC1) gene repression. C2C12 muscle cells were co-transfected with shXRCC1/dsRED plasmid or negative control shRNA (shNEG) and dsRED immediately prior to low serum induction of differentiation (**Figure 2a, Supplemental Figure S2a**). At 96hr post low serum exposure, shXRCC1 transfected muscle cells displayed significant impairment in the ability to form multi-nucleate myotubes with a concurrent reduction in the expression of differentiation specific proteins (myosin heavy chain) (**Figure 2c, Supplemental Figure S2c**). At all timepoints of equal transfection with shNEG or shXRCC1 (**Supplemental Figure S2b**), we noted a significant reduction in *Xrcc1* gene expression (**Figure 2b**), as well as XRCC1 protein foci (**Figure 2a**).

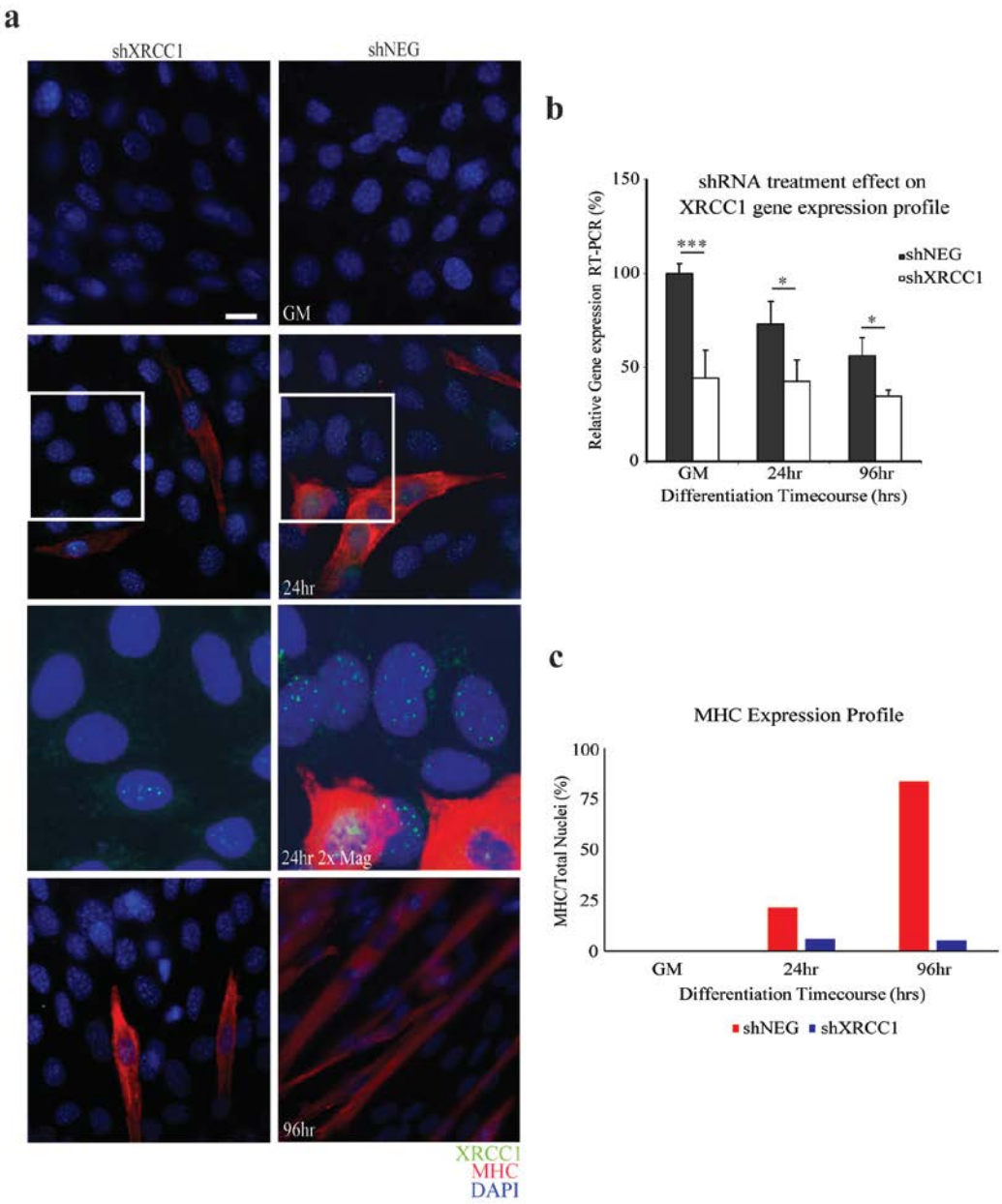
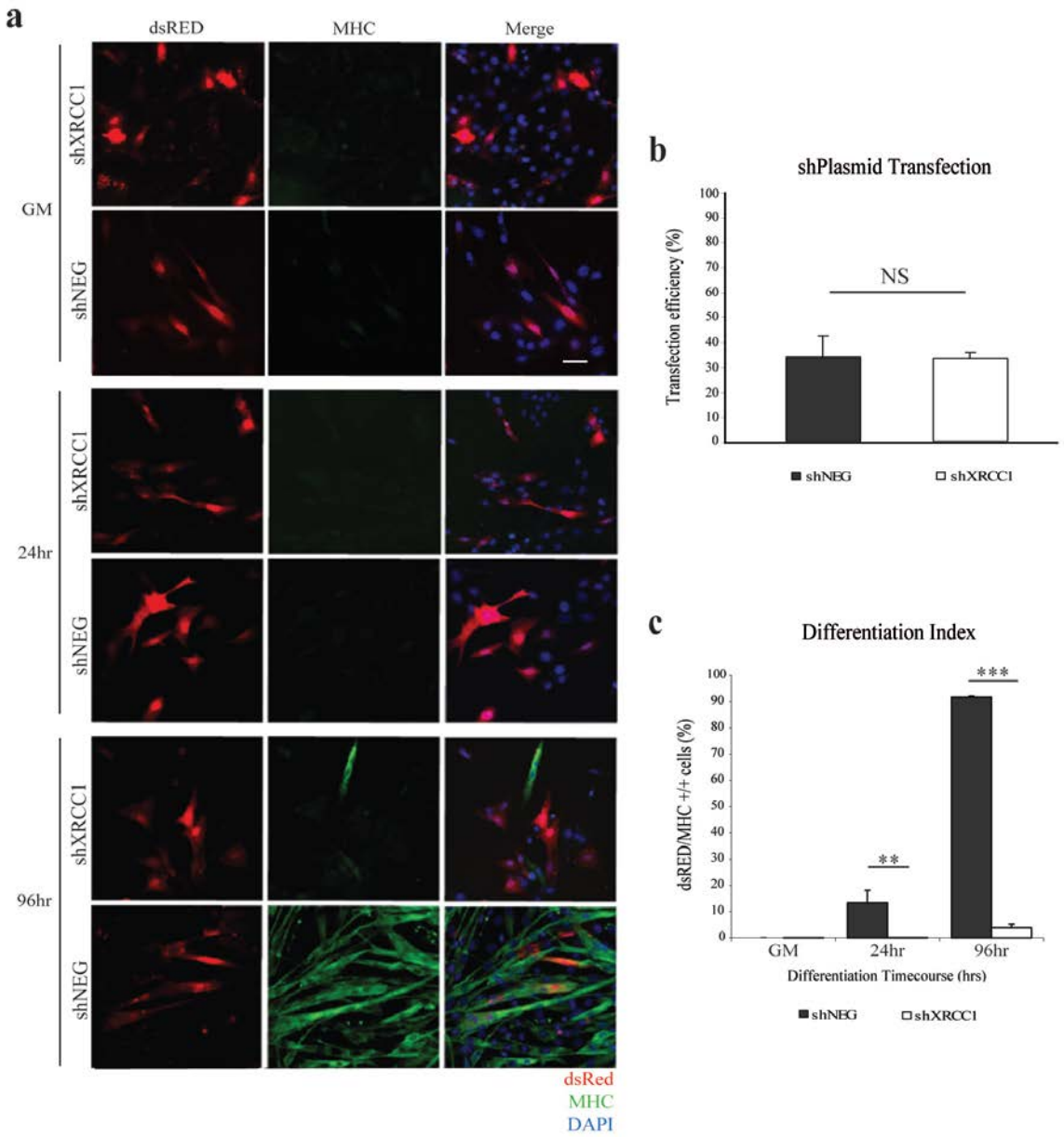


Figure 2 shRNA mediated loss of *Xrcc1* impedes myoblast differentiation. (**a**) Differentiation time course of shRNA treated C2C12 myoblasts. Magnified panels included for 24hr differentiated conditions 2x performed using Photoshop C3 software. (**b**) Real time-PCR demonstrates a reduction in *Xrcc1* gene expression by 50% in shRNA transfected cells at growth. (n=3, one-tailed student t-test analysis with ****P*-value<0.01 *p<0.05). (**c**) Scoring the percentage of differentiated C2C12 cells (MHC cells/total nuclei) shows a decrease in differentiation after loss of XRCC1. Images are representative from n=3 experimental replicates. Scale bar, 50μm.

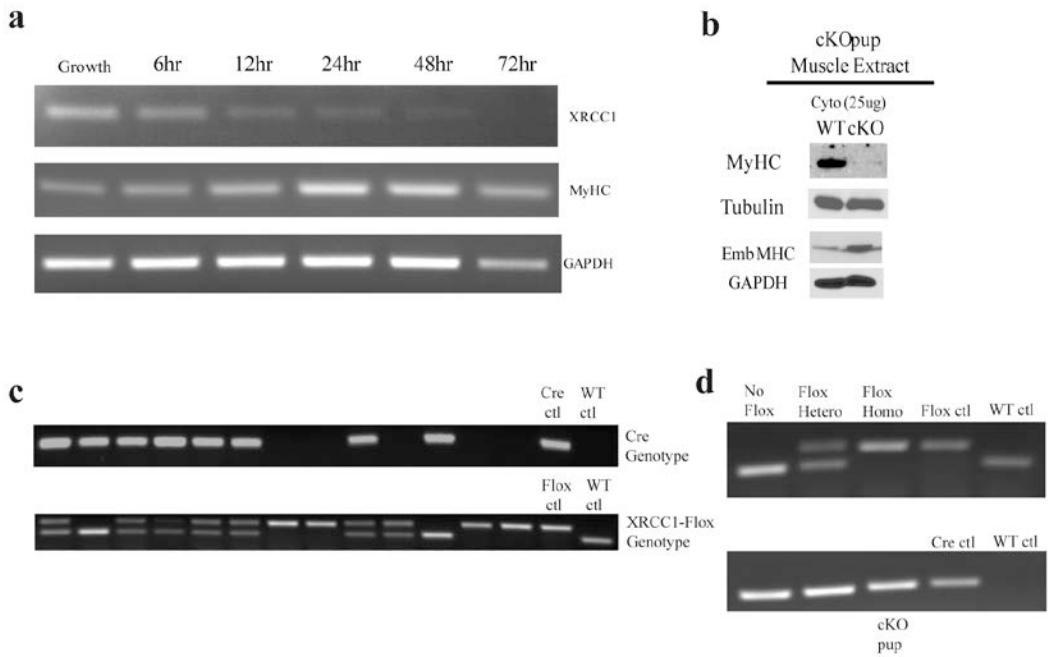


Supplementary Figure S2 shRNA mediated loss of *Xrcc1* impedes myoblast differentiation. (**a**) shRNA mediated knock down of *Xrcc1* in differentiating C2C12 muscle cells impairs the formation of myotubes. Proliferating C2C12 myoblast cells were co-transfected with dsRED plasmid and shRNA directed against *Xrcc1* or a scrambled shRNA negative control plasmid 24hr prior to being induced to differentiate using low serum media. (**b**) Transfection efficiency is similar between control scrambled shRNA and *Xrcc1* targeted shRNA. (**c**) The number of Myosin Heavy Chain positive cells is significantly reduced in shRNA mediated *Xrcc1* knockdown cells, as measured by immunofluorescence. Statistical significance determined by two-tailed student t-test analysis with ****P*-value<0.01 ***P*-value<0.025

in vivo deletion of Xrcc1 gene during early differentiation inhibits muscle tissue development

Next, we sought to assess the consequences of *in vivo* disruption of XRCC1 expression on skeletal muscle cell differentiation, and muscle fiber maturation. *Xrcc1* null mice are early embryonic lethal [Karo et al., 2014], as such we generated a skeletal muscle conditional *Xrcc1* knockout model by cross breeding *Xrcc1*$^{flox/flox}$ mice [Lee et al., 2009] with the *Myf5-Cre* mouse strain [Tallquist et al., 2000, Huh et al., 2004]. *Myf5-Cre*/ *Xrcc1*$^{flox/flox}$ mice were born in normal Mendelian ratios (**Supplementary Figure S3c,d**), yet these animals all suffered early perinatal lethality (**Figure 3a**, **Supplementary Figure S4a**). Nuclear protein extracted from pooled hind leg muscle showed a marked decrease in XRCC1 expression in the *Myf5-Cre*/ *Xrcc1*$^{flox/flox}$ mice relative to non-*Cre Xrcc1* controls **(Figure 3a, Supplementary Figure S3a,b**). This was specific to muscle, as tissue collected from non-*Cre* targeted organs, such as liver, did not show any marked change in XRCC1 protein expression (**Figure 3a**). *Myf5-Cre*/ *Xrcc1*$^{flox/flox}$ mice (body weight =1.10g ±0.1g, n=5) were significantly smaller by 35% than wildtype (body weight =1.70g ±0.2g, n=5) littermates and displayed a severe lack of skeletal muscle development. Histologic examination of *Myf5-Cre*/ *Xrcc1*$^{flox/flox}$ skeletal muscle revealed blunted myofiber formation, increased prevalence of mononucleated muscle cells and increased interstitial space (**Figure 3b, Supplementary Figure S4b**), changes that are consistent with a severe perturbation in the muscle cell maturation program. To confirm that the *Myf5-Cre*/ *Xrcc1*$^{flox/flox}$ skeletal muscle phenotype arose from a differentiation deficit, we examined differentiation kinetics in primary myoblasts isolated from *Xrcc1*$^{flox/flox}$ mice. *Cre*-adenovirus (Cre-ad) infected *Xrcc1*$^{flox/flox}$ myoblasts displayed a thorough inhibition of the differentiation program, characterized by a loss in expression of myosin heavy chain and a complete inability to form multi-nucleate myotubes

compared to control-Adenovirus (Ctl-ad) infected cells (**Figure 3c,d and Supplementary Figure S1b,c).**



Supplementary Figure S3 XRCC1 PCR, Genotyping, and Myosin heavy chain (MHC) protein expression. (**a**) Primary myoblast differentiation time course PCR for *Xrcc1*, *MHC-IIb* and *Gapdh* for control. (**b**) Adult and Embryonic MHC protein expression from WT and cKO hind leg proteins extraction from PN<1day pups, n=2 per genotype. (**c**) Genotyping PCR for *Xrcc1*$^{flox/flox}$ transgene and (**d**) *Cre* transgene from genomic DNA isolated from pup ear clips.

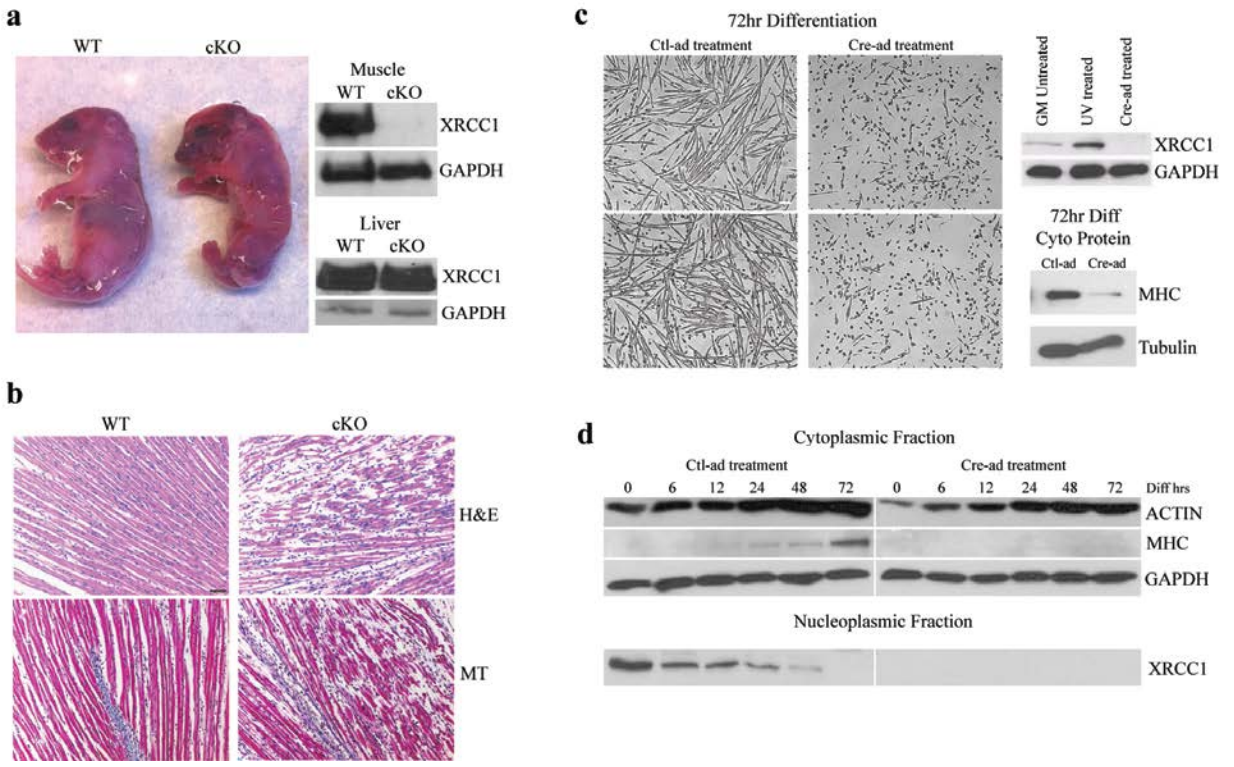
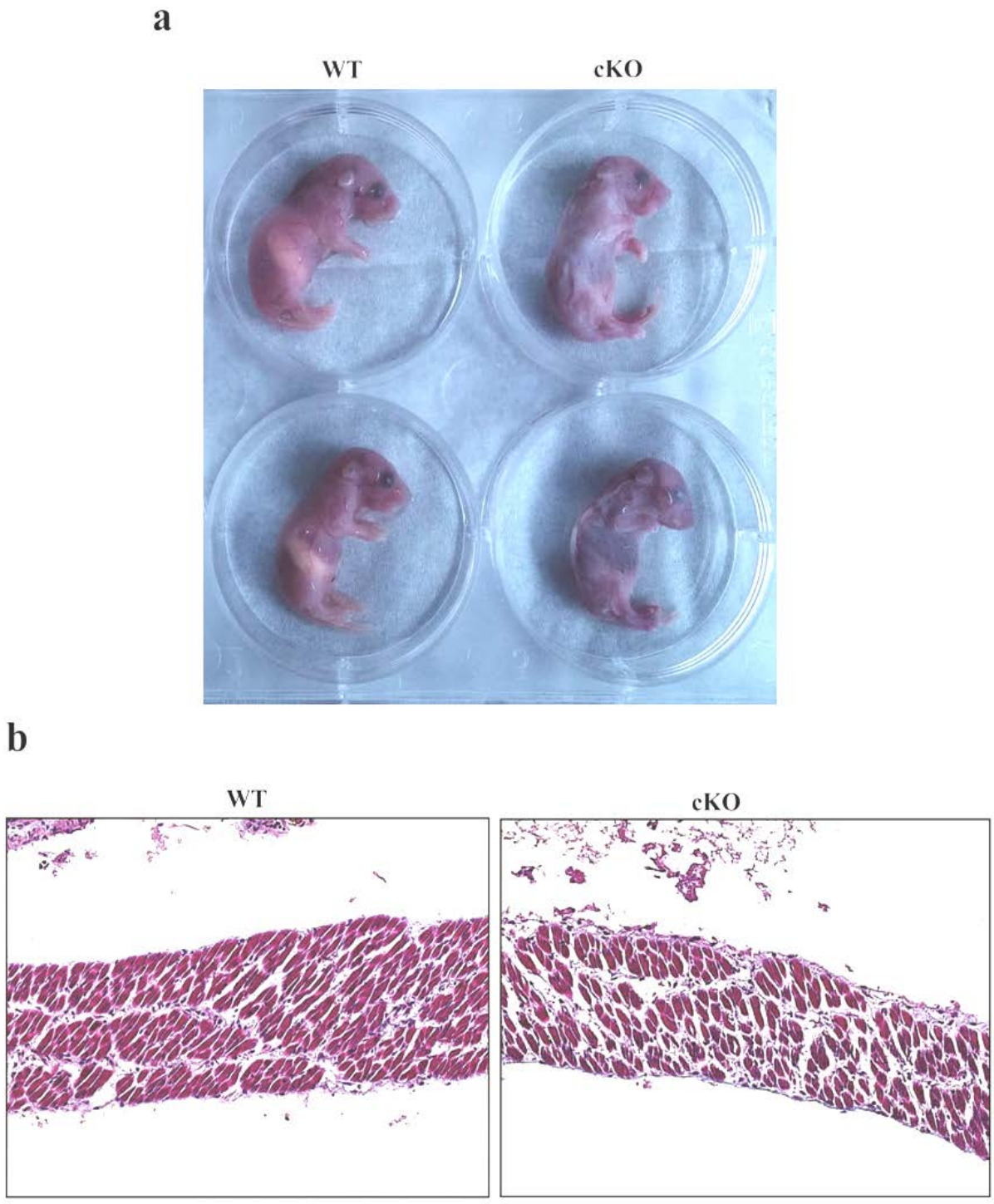


Figure 3 Gene targeted loss of XRCC1 leads to attenuation of myofiber development and perinatal lethality. (**a**) Wildtype (WT) and *Myf5-Cre*/ *Xrcc1*$^{flox/flox}$ conditional knockout of *Xrcc1* (cKO) pups images taken immediately post birth. Nuclear protein extraction from pooled hind-leg muscles, and from liver as control, is used for western blot and probed for XRCC1 and GAPDH. Images representative from n=5 for each genotype. Western blot is representative from n=3 per genotype. (**b**) Longitudinal skeletal muscle sections stained for H&E or Masson's Trichrome. Images representative from n=3. Scale bar, 200µm. (**c**) Primary myoblasts from *Xrcc1*$^{flox/flox}$ mice are treated with Cre-ad or Negative control-adenovirus (Ctl-ad) then induced to differentiate for 72hr. Images representative from n=5. Scale bar, 200µm. Western blot probed for XRCC1 is from nuclear protein fraction isolated from *Xrcc1*$^{flox/flox}$ primary myoblasts, either untreated, UV-treated to induce DNA damage, or Cre-ad treated to delete the *Xrcc1* gene. Western blot is representative from n=3. Western blot for Myosin Heavy Chain (MF20) in cytoplasmic protein lysates from *Xrcc1*$^{flox/flox}$ primary myoblasts that were treated with either Cre-ad or Ctl-ad, and induced to differentiate for 72hr. Western blot is representative from n=3. (**d**) Western blot analysis from a time course treatment of *Xrcc1*$^{flox/flox}$ primary myoblasts induced to differentiate by low serum exposure. Blots representative from n=3.



Supplementary Figure S4 Wild type and *Xrcc1* conditional gene targeted phenotypes. (**a**) WT and *Xrcc1* conditional deletion (*Myf5*-Cre/*Xrcc1*$^{flox/flox}$ genotype) pups PN<1day. (**b**) Diaphragm cross sections from WT and cKO pups stained with Masson's Trichrome solutions.

Loss of XRCC1 affects late muscle differentiation specific and cell cycle specific genes

The shRNA depletion and the *Cre*-mediated excision of *Xrcc1* gene strongly suggest that muscle cell differentiation is dependent on engaging a temporally sensitive XRCC1 mediated DNA repair event. Real-time PCR for canonical skeletal muscle differentiation markers was performed on control and *Xrcc1* deleted primary myoblasts, and the results show that while early differentiation markers *myoD* and *myogenin* were not significantly altered by the loss of XRCC1, late differentiation markers *Mef2c* and *MCK* were significantly reduced compared to control conditions (**Figure 4a**). In addition to muscle specific genes, prior observations from our laboratory have established that caspase/CAD induced DNA strand breaks act as a priming event to engage gene expression of non-lineage specific regulatory factors. For example, a CAD induced strand break in the promoter region of the cyclin-dependent kinase inhibitor 1 (p21) leads to induction of *p21* expression [Larsen et al., 2010], a gene coding for a protein critical for induction of differentiation across a broad spectrum of cell types [Parker et al., 1995]. Here using ChIP-PCR, we show that XRCC1 binds directly on the same *p21* promoter region upon induction of differentiation to repair the SSB induced by CAD (**Figure 4c**). When XRCC1 is absent, the p21 gene is not transcribed, as we are able to show using Cre-ad infection of primary *Xrcc1* null myoblast cultures, leading to a complete failure to induce *p21* expression during differentiation (**Figure 4b**), suggesting that the post-strand break repair event is critical for gene induction at this loci.

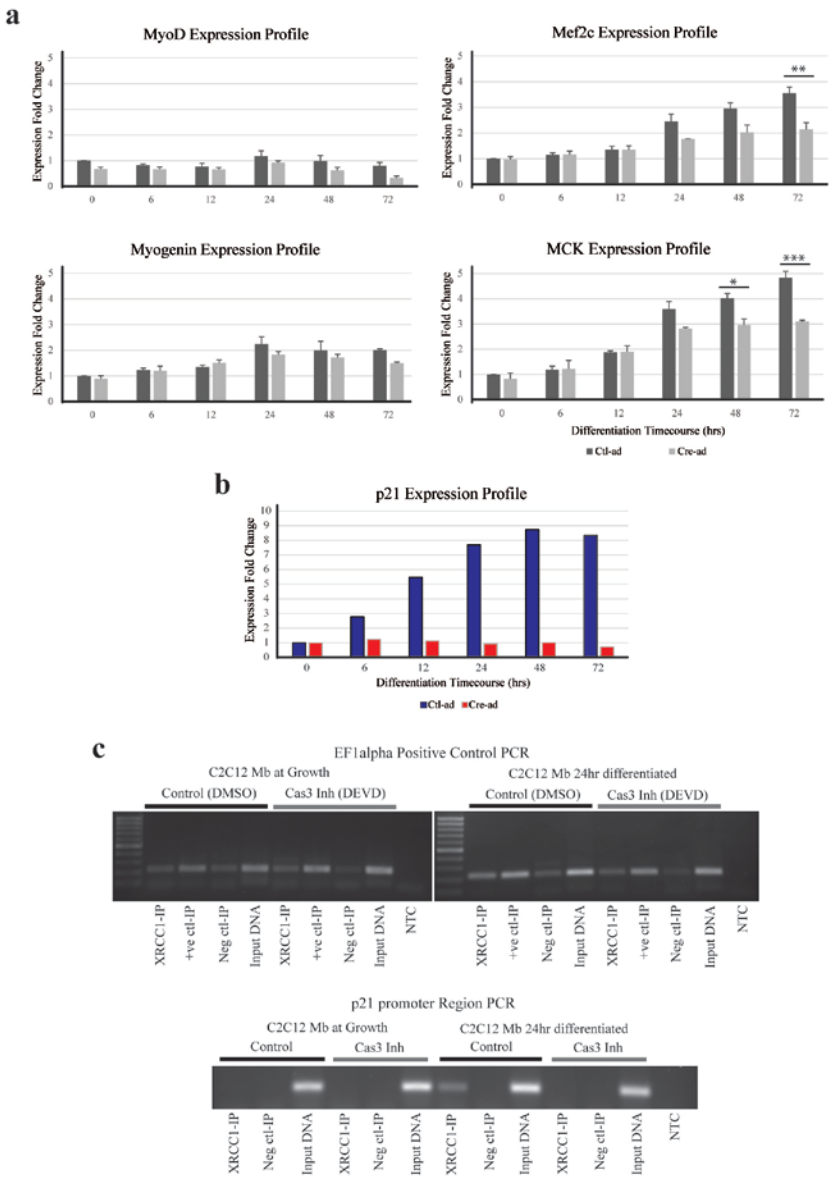
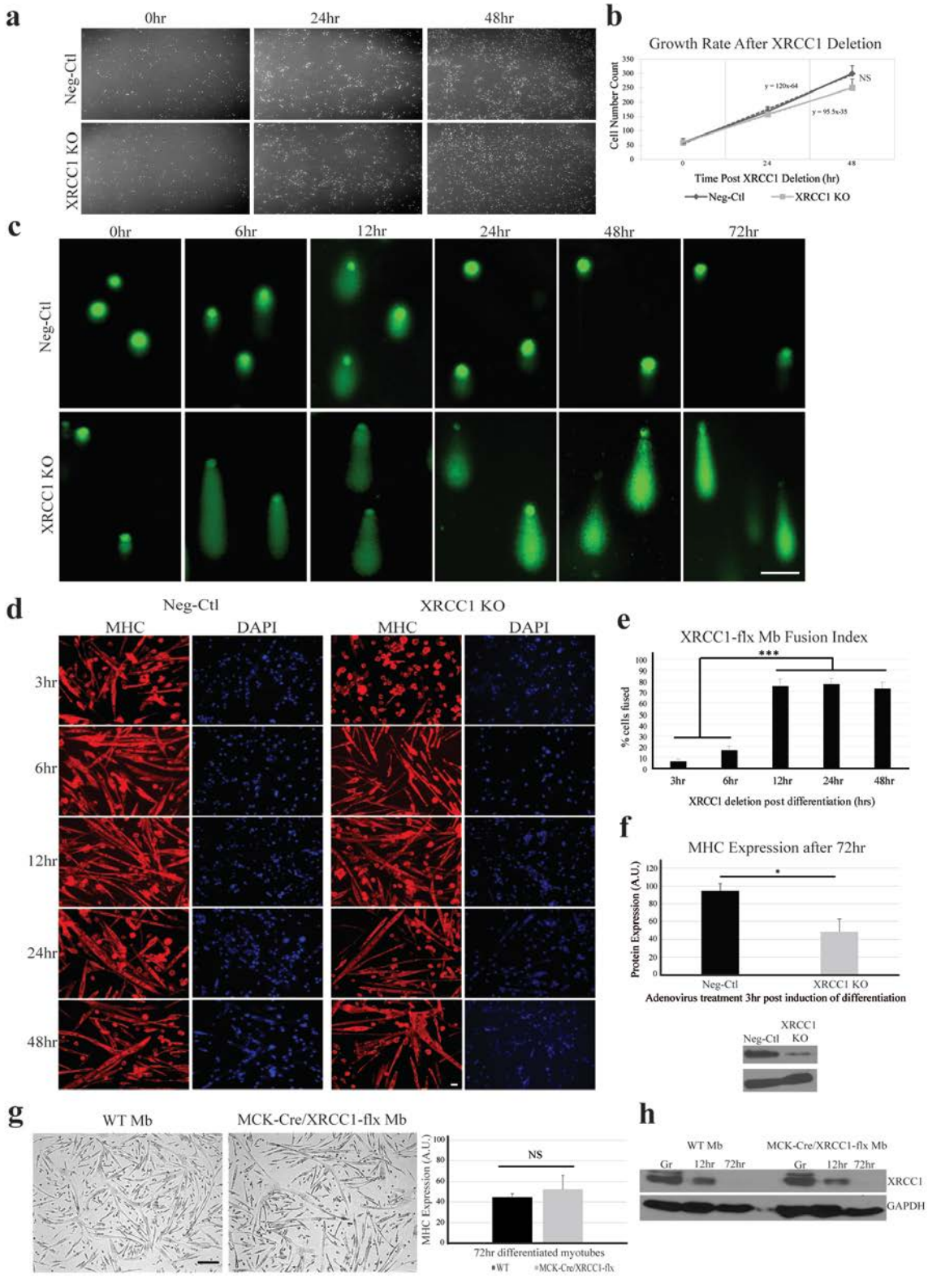


Figure 4 Skeletal muscle gene expression profiles are altered with *XRCC1* deletion. (a) Muscle specific gene markers *myoD*, *myogenin* are unaffected by XRCC1 expression, while *Mef2c* and *MCK* induction are significantly reduced in XRCC1 deleted cells**.** (n=3, two-tailed student t-test analysis with ****P*-value<0.01 ***P*-value<0.025 **P*-value<0.05). (**b**) *p21* gene expression profile during early differentiation. *Xrcc1*$^{flox/flox}$ primary myoblasts infected with Ctl-ad (blue) or Cre-ad (red) and induced to differentiate up to 72hr displayed reduced p21 induction (**c**) ChIP-Endpoint PCR of the p21-promoter region shows enhancement of XRCC1 binding during early differentiation of C2C12 cells compared to growth conditions (n=3). caspase 3 inhibition (DEVD) leads to loss of XRCC1-p21 promoter binding. EF1alpha is used as non-target genomic control for the ChIP experiment.

Temporal activation of Xrcc1 gene is responsible for progression of myoblast differentiation

In addition to acting as a gene expression regulatory event, the loss of XRCC1 in cycling myoblasts may also lead to the accumulation of DNA strand breaks, a genotoxic phenotype that could preclude myoblasts from engaging the differentiation program. However, the doubling time of *Xrcc1* deleted myoblast cultures was similar to wildtype cells, suggesting that loss of XRCC1 prior to the onset of differentiation was not a significant impediment for myoblast growth and survival (**Figure 5a,b**). Cycling *Xrcc1* deleted myoblasts display a moderate increase in the frequency of DNA strand breakage (as measured by comet assay), yet the transient DNA damage that characterizes normal differentiation is dramatically enhanced in XRCC1 deleted myoblasts. Here, >90% of all *Xrcc1* deleted myoblasts accumulate extensive DNA damage compared to <40% wildtype myoblasts, which resolve the transient breaks as the differentiation program proceeds (**Figure 5c, Supplementary Figure S5a,b**). To confirm the differentiation specific function of XRCC1 (and the origin of the muscle phenotype in the gene-targeted mice) we performed temporal excision experiments in *Xrcc1*$^{flox/flox}$ myoblasts, where *Xrcc1* was deleted following the induction of differentiation. Cre-ad infection of *Xrcc1*$^{flox/flox}$ myoblasts at 3 and 6 hours post low serum exposure led to a complete block in differentiation, similar to the results obtained for shRNA targeting of *Xrcc1*gene and *Cre*-mediated deletion of *Xrcc1* in cycling myoblasts (**Figure 5d**). This experiment confirmed the temporally sensitive role of XRCC1 in stabilizing the differentiation phenotype. Cre-ad addition at 12-48 hours post low serum induction did not impair formation of myosin heavy chain positive myotubes in *Xrcc1*$^{flox/flox}$ myoblast cultures, suggesting that loss of XRCC1 at later stages was inconsequential for completion of the differentiation program (**Figure 5e**), while early deletion of XRCC1 shows significant loss of fusion, as well as overall MHC protein expression (**Figure 5d,f**). In addition,

flox-flox myocyte/post-myoblast specific deletion of *Xrcc1* via the generation of *MCK-Cre*/ *Xrcc1*$^{flox/flox}$ mice had no observable impact on muscle development (these animals were born with normal Mendelian ratios), while primary myoblasts derived from the *MCK-Cre*/ *Xrcc1*$^{flox/flox}$ strain displayed the conventional differentiation response as with wildtype myoblasts (**Figure 5g**). These myoblasts possess the same XRCC1 protein expression profile as wildtype primary myoblasts differentiated up to 72hrs (**Figure 5h**).


a
0hr
24hr
48hr
Neg-Ctl
XRCC1 KO
b
Growth Rate After XRCC1 Deletion
Cell Number Count
y = 120x-64
y = 95.5x-35
NS
Time Post XRCC1 Deletion (hr)
Neg-Ctl
XRCC1 KO
c
0hr
6hr
12hr
24hr
48hr
72hr
Neg-Ctl
XRCC1 KO
d
Neg-Ctl
XRCC1 KO
MHC
DAPI
3hr
6hr
12hr
24hr
48hr
e
XRCC1-flx Mb Fusion Index
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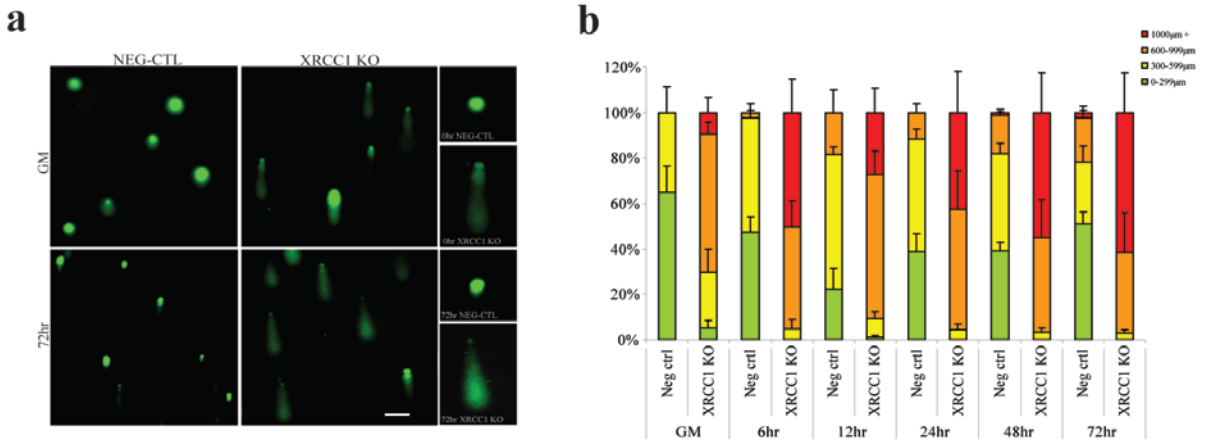
% cells fused
XRCC1 deletion post differentiation (hrs)
f
MHC Expression after 72hr
*
Protein Expression (A.U.)
Neg-Ctl
XRCC1 KO
Adenovirus treatment 3hr post induction of differentiation
XRCC1
Neg-Ctl
KO
g
WT Mb
MCK-Cre/XRCC1-flx Mb
MHC Expression (A.U.)
NS
72hr differentiated myotubes
WT
MCK-Cre/XRCC1-flx
h
WT Mb
MCK-Cre/XRCC1-flx Mb
Gr
12hr
72hr
XRCC1
GAPDH

Figure 5 XRCC1 has a temporally sensitive requirement to mitigate DNA damage in differentiating myoblasts. (**a**) Knockout of *Xrcc1* gene expression does not significantly affect myoblast proliferation potential. (**b**) Cell number counts quantified in graph. Images are representative from n=6. (**c**) Single cell gel electrophoresis (Comet assay) was performed on differentiating *Xrcc1*$^{flox/flox}$ primary myoblast cells treated with either Cre-ad or Ctl-ad. Following electrophoresis, cells were stained with SYBR green and visualized to assess the length of migration of DNA from the nucleus. Results show increased and persistent DNA damage in *Xrcc1* knockdown cells while control cells exhibit damage early in differentiation that is resolved over the differentiation time course. Images are representative from n=4 for each experimental condition, minimum number of cells counted =50 for each condition. Scale bar, 15µm. (**d**) Cre-ad mediated knockdown of *Xrcc1* gene expression post induction of differentiation. Immunofluorescence staining for Myosin Heavy Chain (MHC) was used to assess myotube formation. (**e**) Fusion Index graph quantifies myotube formation. (n=3, student t-test two-tailed analysis with ****P*-value<0.01). Scale bar, 20µm. (**f**) Quantification of MHC protein at 3hr post induction of differentiation following adenovirus treatment. Endpoint is 72hrs post induction of differentiation. (n=3, two-tailed student t-test analysis with **P*-value<0.05). (**g**) Primary myoblasts isolated from *MCK-Cre*/*Xrcc1*$^{flox/flox}$ transgenic mice show equivalent differentiation capacity to WT myoblasts. Scale bar, 200µm. Graph shows MHC expression is equivalent between WT and *MCK-Cre*/*Xrcc1*$^{flox/flox}$ myoblasts differentiated for 72hrs. (**h**) XRCC1 protein expression profile is similar between WT and *MCK-Cre*/*Xrcc1*$^{flox/flox}$ myoblasts differentiated for 72hrs.



Supplementary Figure S5 *Xrcc1* gene deletion causes accumulation of damage. (**a**) Single cell gel electrophoresis (Comet assay) was performed on differentiating *Xrcc1*$^{flox/flox}$ primary myoblast cells treated with either *Cre*-adenovirus or control-adenovirus. Following electrophoresis, cells were stained with SYBR green and visualized to assess the length of

migration of DNA from the nucleus. (**b**) The comet tails were quantified based on tail length (in μm) using ImageJ software and bin sorted into short (green), medium-short (yellow), long (orange) or extra-long (red) tail lengths. Results show increased and persistent DNA damage in *Xrcc1* knockdown cells while control cells exhibit damage early in differentiation that is resolved over the differentiation time course (a statistically significant decrease in the number of short length tails was observed between the 0hr and 12hr time points). Images are representative from n=3 for each experimental condition, minimum number of cells counted = 50 for each condition. Scale bar, 50μm.

2.4: DISCUSSION

Taken together, these results demonstrate that the temporal deployment of the BER related DNA repair mechanisms (as exemplified by XRCC1) are essential for muscle cell differentiation. Clearly, p21 expression is XRCC1 dependent, an observation that suggests XRCC1 manages a gene induction program that may be applicable to a wide range of cell lineages. Whether XRCC1 directly promotes a muscle specific differentiation program remains undefined. Prior observations from our laboratory, along with the current study, suggest that certain elements of the skeletal muscle gene expression program are not influenced by caspase mediated signaling events, such as the induction of myogenin expression [Fernando et al., 2002]. Nevertheless, other muscle specific genes appear to be responsive to the caspase/CAD/XRCC1 circuit, including Mef2c, MCK and myosin heavy chain. Identifying the range of XRCC1 responsive genes in a differentiating myoblast will clarify to what extent XRCC1 is a general vs lineage specific differentiation cue.

XRCC1 is also a component of the backup NHEJ (b-NHEJ) DNA repair pathway, which shares many common features with BER including the recruitment of PARP and DNA ligase III to the strand break site [Iliakis, 2009]. We have not directly ascertained if the repair machinery is a BER or b-NHEJ mechanism, however we favor the hypothesis that XRCC-1 is scaffolding a

BER repair factory. This supposition is based on the nature of the strand breaks that predominant during the differentiation program. For example, we have observed that differentiating myoblasts are readily labeled via ISNT using a DNA polymerase, a process that will preferentially label a strand break/nick rather than a double strand break. In addition, our COMET analysis on differentiating myoblasts display a qualitatively different electrophoresis profile compared to myoblasts engaging true apoptosis, a cell fate that is characterized by double strand breaks. While these measures are not conclusive, they strongly suggest that XRCC1 is participating in a BER mechanism rather than a b-NHEJ repair pathway.

The prevalence of DNA strand breaks and XRCC1 repair foci in early differentiating muscle cells suggest that this regulated form of damage may target a large number of gene induction events, what remains unknown is whether this targeted break/repair mechanism may also repress gene expression at discrete loci. Given that caspase mediated signaling is a broadly conserved inductive cue for differentiation, a reasonable supposition is that caspase driven DNA damage/XRCC1-mediated repair may act as an essential genomic reprogramming event in many cell lineages [Karo et al., 2014, Dick and Megeney, 2013, Cattoglio et al., 2015], including gene induction and gene repression. Our observations establish an XRCC1-BER mediated repair mechanism during differentiation, whether other unrelated or unknown factors assist this process will require future investigations.

ACKNOWLEDGMENTS:

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Regulome Consortium (IRC). L.A.M. is a former Chair in Cardiac Research supported by the Mach-Gaensslen Foundation. The authors declare no competing or conflicts of interest for the current work.

2.5: MATERIALS AND METHODS

Mice and *in vivo* procedures. All transgenic mice were housed and treated at the University of Ottawa Animal Care and Veterinary Services. Mice used in our studies were housed and cared for according to Canadian Council on Animal Care (CCAC) guidelines and University of Ottawa Animal Care Committee protocols. *Xrcc1*$^{flox/flox}$ mouse strain was obtained from Dr. McKinnon [21]. *Myf5-Cre* and *MCK-Cre* mouse strains were obtained from Dr. Rudnicki [23].

Transgenic pups and their wild type litter mates were taken shortly after birth, Post Natal<1 day. Hind limb muscle is extracted and frozen in microfuge tubes at -20°C until further experiments are performed. Whole pup bodies were placed in OCT filled embedding molds (Polysciences Inc) then flash frozen in liquid nitrogen to preserve for sectioning and staining. All sectioning and H&E and Masson's Trichrome staining was performed by the University of Ottawa department of Pathology and Laboratory Medicine, Morphology Unit Lab.

Cell Culture Growth and Differentiation Protocols. For primary myoblast isolations, surgeries were performed as described previously [11]. Briefly, skeletal muscle from hind limbs is extracted, and finely cut with scissors until sufficiently liquefied isolate is achieved. The liquefied muscle chunks are then cultured in a dispase/collagenase solution (dispase II, Roche and 1% collagenase (reconstituted in sterile PBS) with 2.5mM CaCl2). Several rounds of trituration and low speed centrifugation followed by resuspension in Hams's F-10 Enriched growth media (Ham's F-10 medium from Gibco Life Technologies, 20% FBS, 2% Pen-Strep, 0.6μg/mL Fungizone and 2.5 μg/mL βFGF) will yield a population of primary myoblasts

sufficiently free of any cross contaminating tissue remnants. The cells were maintained by replacing the media every 48hr. The cells were allowed to grow on collagen coated plastic plates (2mL of rat-tail collagen, Roche, NY containing 0.2% acetic acid was coated onto tissue culture plates). Ham's F-10 media is replenished every 48 hours to maintain high nutrient content in the solution to maximize the growing potential of primary myoblasts and prevent unaccounted for differentiation. Primary myoblasts are passaged before reaching 70% confluence in the growth dish to further eliminate the chance of spontaneous differentiation. Differentiation was induced in primary myoblast cells by replacing the complete growth media with primary differentiation media (DMEM containing 5% horse serum and 2% Pen-Strep). Cells were collected by scrapping off the plates and centrifugation to pellet cells at the predetermined time points, or differentiation media was changed every 24hr until time point is reached.

Cell Culture shRNA, ISNT, Adenovirus, and caspase Inhibition protocols. Target sequences of shRNA were cloned into the appropriate vector (TRC mouse shRNA individual clone lentiviral pLKO.1 targeted to *Xrcc1*, Open Biosystems/Thermo Scientific, AL, pCMV-dsRed-Express Vector, Clontech, CA or nonsilencing pGIPZ (shNEG), Open Biosystems/Thermo Scientific, CA) and the plasmid DNA was amplified by culturing the E. coli engineered to express each plasmid in antibiotic media containing either Ampicillin or Kanamycin (40μg/mL in LB broth). After colony expansion the bacterial plasmid DNA was purified using HiSpeed Plasmid Midi Kit from Qiagen, ML. The purified plasmid DNA was verified for size by performing a restriction digest using the restriction enzymes Bam HI and ClaI. The digests were separated on 1% agarose gels (plus 0.1μg/mL EtBr) to check that the DNA fragments were of the appropriate size as predicted from the restriction map for the plasmid of interest. One day before transfection C2C12 myoblast cells were cultured in 35mm tissue culture plates in PenStrep free

growth media to a confluence of 50-60%. The cells were rinsed with 1X OptiMEM I reduced serum media, Gibco prior to transfection. The shRNA was prepared by diluting 16µg/mL DNA per condition in OptiMEM. Similarly, the Lipofectamine 2000 was diluted to 4% in Opti-Mem. The solutions were allowed to incubate for 5min at room temperature. The Lipofectamine 2000 solution was then mixed into the shRNA solution and gently mixed. The Lipofectamine-shRNA solution was allowed to incubate for 20min at RT. Next 500µL of the transfection solution was added to each plate and incubated at 37°C for 3hr. After incubation 1mL of antibiotic free growth media was added to each plate and the cells were incubated for a further 12-18hr. At this point cell cultures were either induced to differentiate using low serum media or collected for growth condition.

To perform in situ Nick Translation (ISNT), C2C12 mouse myoblasts were plated onto UV irradiated glass cover slips in 35mm tissue culture dishes at a concentration of 0.5x106 cells/plate. One plate was prepared for each experimental condition, plus one DNaseI positive control plate for each experimental parameter. The cells were allowed to grow to confluence in a 37°C incubator. Prior to initiating the in situ nick translation assay the 10X ISNT reaction buffer (0.5M TRIS-HCl pH 7.9, 50mM MgCl2, 100mM β mercaptoethanol) and ISNT reaction mix (1X ISNT reaction buffer, 10µM dNTP (1mM dCTP, dATP, and dGTP), 1µM dTTP, 1µM digoxigenin (DIG)-II-dUTP, 0.1% DNA Polymerase I, New England Biolabs) were prepared. Positive control plates were washed in 1X PBS. Next the cover slips were treated with 200µL of DNaseI treatment mix (1% DNaseI, 1X DNase Buffer) and incubated for 10min at room temperature. After incubation the cover slips were washed 2 times in 1X PBS. The DNaseI and experimental condition cover slips were all rinsed with 1X ISNT reaction buffer (made by diluting the 10X reaction buffer 1:10). After rinsing each cover slip was incubated with 200µL of

ISNT reaction mix and incubated at 37°C for 45min with gentle agitation. After 45min the cover slips were washed 2 times in 1X PBS then immuno-stained using a mouse anti-DIG primary antibody at a concentration of 1:500 in 3% BSA for 1hr at RT.

Adenovirus treatments protocols were used as previously described [23]. Briefly, primary myoblasts isolated from *Xrcc1*$^{flox/flox}$ mice are cultured on collagen coated plates until 50% confluence is reached. Media is removed and cells are washed with PBS. Cre-ad at MOI 10 or Ctl-ad are added to warm reduced serum OptiMEM media. Cre-ad or Ctl-ad media is then added to myoblast plates and allowed to incubate for 1hr at 37°C, followed by addition of regular Ham's F-10 enriched growth media and left overnight for virus to infect close to 100% cells on plate. Next day we collect samples for growth, or initiate normal differentiation time course protocol described above. For caspase 3 inhibition, cultured myoblasts were pre-treated with either 15μM z.DEVD-FMK (DEVD) from BioVision or 15μM Dimethyl Sulphoxide (DMSO) from Sigma, MO for 2hr at 37°C. After pre-treatment the cells were induced to differentiate using low serum media or continued in growth media both containing 15μM DEVD or DMSO as a vehicle only control. The inhibition or control media was changed every 48hr until the end of the time course. The cells were collected at the predetermined time points and analyzed as described.

Single Cell Gel Electrophoresis Assay (COMET assay). Primary myoblast cells from *Xrcc1*$^{flox/flox}$ mice were grown on a 10cm tissue culture plate, treated with either Ctl-ad or Cre-ad as described above, and collected in freezing media and frozen at -80°C. For the comet assay the cells were thawed in 37°C water bath and transferred to growth media. The thawed cells were then centrifuged at 720xg for 5min. The media was then removed and the cells were resuspended in ice cold 1X PBS. At this point the cells were ready to proceed using the Comet Assay Reagent

kit for Single Cell Gel Electrophoresis from Trevigen, MD. In preparation for the comet assay the Lysis Solution (included in kit) was chilled on ice for at least 20min prior to use. Next the low melting-point agarose (LMA) was melted by submerging in a beaker of boiling water for 5min with the cap loosened. The agarose was maintained in a liquid state by transferring to a 37°C water bath. The LMA was allowed to cool at 37°C for at least 20min prior to use. Cell samples, at a concentration of 1x105 cells/mL, were combined with LMA at a ratio of 1:10 cells:LMA and immediately pipetted onto labeled Gelbond film strip (agarose gel support medium) Lonza, ME in 75µL aliquots. Cells were applied to the hydrophilic side of the film to ensure that the sample spread evenly into a circle of approximately 25mm in diameter. Each film was placed flat at 4°C in the dark for 10min or until the gel solidified. The slide was then immersed in pre-chilled Lysis Solution and incubated at 4°C for 45min. After incubation the lysis solution was removed and the slide was immersed in freshly prepared Alkaline Solution pH>13 (6g NaOH, 250µL 200mM EDTA, dH2O) for 45min in the dark. The slide was removed from the alkaline solution and transferred to a horizontal electrophoresis apparatus where it was placed equidistant from each electrode in alkaline electrophoresis solution (12g/L NaOH, 1mM EDTA pH8). Temperature fluctuations were minimized in the non-buffered system by running the electrophoresis at 4°C in walk in refrigerator. The voltage was set to 30V for 30min at constant amperage of 300mA. Following electrophoresis the slide was rinsed several times in dH2O then immersed in 70% ethanol to fix. Next the slide was dried in an air tight container containing desiccant overnight at room temperature. Once dry, the slide was stained by submerging in SYBR Green stain at 1:10000 in TE buffer pH 7.5 for 5min. The slide was then removed and allowed to air dry. The dry slides were mounted with coverslips and the cells were visualized by fluorescence microscopy using the GFP channel (494nm) at 20x magnification.

Tail length (in µm) for each nucleus is measured using ImageJ software and is bin sorted into short (green), medium-short (yellow), long (orange) or extra-long (red) bar graphs.

Chromatin immunoprecipitation. 100 million C2C12 cells were collected per timepoint and fixed in 1% formaldehyde-DMEM solution for 5 minutes. Arrest of fixation and PBS washing is then performed and cells are scrapped, pelleted and stored at -80°C until ready for lysis and shearing. We used the Enzymatic shearing kit from Active Motif (ChIP-IT Express Enzymatic Cat# 53009) with minor modifications to the procedure. Briefly, we follow instructions in cell lysis using hand held dounce homogenizer. We enzymatically shear the samples at 37°C for 10 minutes with periodic full speed vortex as instructed. We perform the immunoprecipitation using the amounts indicated for higher volume reactions in the instruction manual, with one major alteration, we use 5µg of antibody per sample instead of the indicated 1-3µg. All volumes are adjusted to 200µl and left on rotators at 4°C overnight. The following day we proceed to washing the beads, eluting the chromatin and reverse-crosslinking as described in the manual with one major alteration, we perform the reverse-crosslink for 4 hours at 65°C and always continue through the proteinase K treatment afterwards without storing the samples between steps. The resulting samples (XRCC1-IP, RNApol II-IP, IgG-IP and input DNA samples for each time point) can be used to perform end point PCR at this stage, or the samples can be purified using gDNA purification kits to yield qPCR or Chip-sequencing ready samples. We perform p21-promoter region PCR using primers previously published [11]. EF1alpha primers used for positive control of ChIP is provided in the Active Motif Kit. For caspase 3 inhibition, we perform DEVD-fmk and DMSO treatments of cells as described above.

Immunofluorescence Staining and Microscopy. Cells were cultured on 25mm coverslips in 35mm plates and exposed to the described experimental conditions. At appropriate time points

the cell plates were place on ice and the media was aspirated and replaced with ice cold 1XPBS. The plates were washed a further 2 times with PBS. The cells were fixed in 90% methanol for 8min on ice. The fixative was removed and the cells were washed with PBS 2 times. Next the cells were covered with PBS and the plates were wrapped with Parafilm and stored at 4°C until such time that all time points had been fixed. The plates were removed to RT and blocked for 24hr in 3% FBS. After blocking the cells were incubated in primary antibody (DNA ligase IV (H-300) rabbit polyclonal IgG (200μg/mL), Santa Cruz Biotechnology, CA 1:00, Anti-XRCC1 antibody produced in rabbit (1mg/mL), Sigma-Aldrich, MO 1:100, Anti-Myosin Heavy Chain (MF20) mouse IgG, Developmental Studies Hybridoma Bank, IO 1:50) made up in blocking solution for 24hr at 4°C. After primary antibody incubation the cells were washed 3 times in PBS and incubated in secondary antibody (2mg/mL Alexa Fluor 488 goat anti-rabbit IgG (H+L), Invitrogen, CA 1:500, 2mg/mL Alexa Fluor 594 goat anti-rabbit IgG (H+L), Invitrogen, CA 1:500, 2mg/mL Alexa Fluor 594 goat anti-mouse IgG (H+L), Invitrogen, CA 1:500, AP129F Donkey anti-mouse IgG FLUOR, Chemicon International, CA 1:500) diluted in PBS for 1hr (RT) to 24hrs (4°C). After incubation the cells were washed 2X in PBS and 1X in dH20 then counterstained with DNA specific 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), Sigma Aldrich, MO made up in dH2O (1:10 000) for 10min at RT. After incubation the cells were washed 2 times in PBS and 1 time in dH2O. The coverslips were mounted on microscope slides using Dako Fluorescent Mounting Medium and visualized using a Zeiss Observer Z1 inverted fluorescence microscope. All images are developed using AxioVision 4.8 software. The quantification of XRCC1 foci was done by manually visualizing and counting the highest intensity foci within each individual nucleus and recording the number of foci/nucleus for a minimum of 50 nuclei per experimental condition.

RNA extraction, PCR. Cells from C2C12 and primary myoblast cultures were washed with ice-cold PBS and lysed using TRIzol reagent. Appropriate volume of chloroform is added to the lysates, followed by the samples being vortexed then allowed to incubate for 3min at RT after which time they were centrifuged at 10 000xg for 10min at 4°C to separate the phases. The top phase was removed to a fresh, labeled, RNase free tube. Appropriate amount of isopropanol was added to each sample and the tubes were gently inverted to mix. The tubes were rocked on a nunator for 20min at RT after which time the RNA was pelleted by centrifugation at 10 000xg for 15min at 4°C. The supernatant was discarded and the pellet was washed in 500µL 70% ethanol-DEPC (Ethanol in Dimethyl Pyrocarbonate (DEPC) containing sterile water) and re-centrifuged for 5min at 7500xg at 4°C. The supernatant was discarded and the RNA pellet was allowed to air dry in the fume hood. Once dry the RNA pellet was resuspended in 80µL nuclease free water and allowed to dissolve at 4°C for 1hr before being stored at -20°C. First strand cDNA synthesis was performed using iScript RT Supermix from Bio-Rad according to manufacturer instructions. All primers used are appended in Supplementary Information section.

Protein Extraction and Western Blotting. Cells from C2C12 and primary myoblast cultures were washed with ice-cold PBS and lysed on ice for 45 min in modified cytoplasmic/nuclear fractionation NE-PER extraction kit from Thermo Scientific. Immunoblot analyses were performed as described previously[4] by using the following antibodies: mouse monoclonal XRCC1 antibody from Abcam (ab1838, 1:1000), myosin heavy chain and tubulin from Development Studies Hybridoma Bank, Iowa City (MF20, Tub 1:100), and GAPDH from Cell Signaling (#2118, 1:2000). Probe analysis and quantification was performed using ImageJ software.

Chapter 3.0: caspase 7 cleavage of chromatin structure protein Satb2 alters the genetic profile to accelerate cellular differentiation

3.1: Abstract

Chromatin remodeling is essential in the control of the global positioning and accessibility of genes related to activation of differentiation pathways. Research has explored the post-translational modifications that control the epigenetic mechanisms of activation/repression of genes; however additional higher-order chromatin structural changes may also play a vital role. In addition, the capacity of caspase activity to modify fundamental aspects of DNA damage and repair, suggests that these proteases may also target chromatin modification proteins to manage cell differentiation. The Special AT-rich binding protein 2 (Satb2) is a matrix attachment region protein that is implicated in the regulation of gene activation and repression, and related proteins have been demonstrated to be targeted by effector caspases during apoptotic related chromatin modification events. Here, we sought to investigate whether Satb2 was an effector caspase substrate and whether this chromatin structural protein influenced the early differentiation of muscle progenitor cells. In this study we show that effector caspase 7 targets and cleaves Satb2 during early myoblast differentiation, leading to accelerated myotube formation. ChIP and RNA sequencing experiments revealed that Satb2 targets and binds to the promoters, and alters a variety of genes that modify the differentiation program. Taken together, effector caspases target and cleave chromatin remodeling proteins, causing a robust change in the gene expression profile directed toward cellular differentiation.

3.2: Introduction

Caspase cleavage and degradation of nuclear proteins during apoptosis leads to the condensation of the nucleus and eventual collapse of the whole structure [Fernandes-Alnemri et al., 1994, Widlak et al., 2000]. This well defined cellular process is performed by multiple effector caspases targeting different proteins within the nucleus, such as caspase 3 targeting of ICAD/CAD [Lechardeur et al., 2000], caspase 6 targeting of nuclear lamin [Zhao et al., 2016], as well as Satb1 [Galande et al., 2001], caspase 7 cleavage of HDAC3 [Xia et al., 2007], and other nuclear residing proteins. The targeting of nuclear proteins by effector caspases also occurs during cell differentiation, which is coincident with DNA damage and DNA repair events [Larsen et al., 2010, Al-Khalaf et al., 2016]. For example, caspase 3 cleaves the paired box transcription factor Pax7 to limit satellite cell self renewal and initiate myoblast maturation [Dick et al., 2015]. Caspase 3 has also been shown to cleavage activate the histone modifying kinase MST1 in this system, which propels the differentiation program [Fernando et al., 2002].

These results suggest that caspase activity may also exert a direct impact on chromatin structure itself, which in turn can modify the activation or repression of gene expression [Muller and Leutz, 2001, Kimmins and Sassone-Corsi., 2004, Fisher and Fisher, 2011]. Indeed, chromatin conformation is clearly divergent in replicating versus differentiating cells, where alterations occur that repress or open access to previously opened or repressed gene loci respectively [Muller and Leutz, 2001, Forcales et al., 2012]. As such, identifying caspase substrates that alter chromatin structure is of great interest to the field.

The special AT-rich binding protein 2 (Satb2) may represent a bona fide chromatin remodeling protein that is subject to caspase mediated targeting. Satb2 is a nuclear matrix attachment protein

[Wang et al., 2010, Barboro et al., 2012], and has been shown to maintain patterns of gene expression specific to stem and cycling progenitor cells [Savrese et al., 2009, Wang et al., 2014]. Consistent with a growth specific function, Satb2 gene expression and protein content is also rapidly downregulated during early stages of differentiation Galande et al., 2001, Britanova et al., 2005, Han et al., 2008, Dong et al., 2015]. These observations coupled with prior studies showing caspase 3 targeting of the related Satb1 protein, imply that Satb2 may also be subject to a similar mechanism of protein degradation. Here, we explored whether caspase 3 targets Satb2 during early stages of myoblast differentiation, and whether this proteolytic event was essential to establish the appropriate chromatin environment to engage cell differentiation.

3.3: Materials and Methods

Mice and *in vivo* procedures. All mice were housed and treated at the University of Ottawa Animal Care and Veterinary Services. Mice used in our studies were housed and cared for according to Canadian Council on Animal Care (CCAC) guidelines and University of Ottawa Animal Care Committee protocols.

Cell Culture Growth and Differentiation Protocols. For primary myoblast isolation, surgeries were performed as described previously [Al-Khalaf et al., 2016]. Briefly, skeletal muscle from hind limbs were extracted, and finely minced to generate a tissue isolate. The liquefied muscle chunks were then cultured in a dispase/collagenase solution (dispase II, Roche and 1% collagenase (reconstituted in sterile PBS) with 2.5mM CaCl2). Several rounds of trituration and low speed centrifugation followed by resuspension in Hams's F-10 Enriched growth media (Ham's F-10 medium from Gibco Life Technologies, 20% FBS, 2% Pen-Strep, 0.6μg/mL Fungizone and 2.5 μg/mL βFGF) yielded a population of primary myoblasts sufficiently free of any cross contaminating tissue remnants. The cells were maintained by replacing the media

every 48hr. The cells were allowed to grow on collagen coated plastic plates (2mL of rat-tail collagen, Roche, NY containing 0.2% acetic acid was coated onto tissue culture plates). Ham's F-10 media is replenished every 48 hours to maintain high nutrient content in the solution to maximize the growth potential of primary myoblasts and to limit contact mediated differentiation. Primary myoblasts were passaged before reaching 70% confluence to further eliminate the chance of spontaneous differentiation. Differentiation was induced in primary myoblast cells by replacing the complete growth media with primary differentiation media (DMEM containing 2% horse serum and 2% Pen-Strep). Cells were collected by scraping off the plates and centrifugation to pellet cells at the predetermined time points, or differentiation media was changed every 24hr until time point is reached. For C2C12 cells, similar procedures were followed with the following notable differences: Tissue culture plates did not require collagen coating prior to cell seeding, as C2C12 cells were more adherent to plastic than primary myoblast cells. Growth media was composed of 10% FBS-DMEM with 2% Pen-Strep added to final solution, and differentiation media was 2% HS-DMEM with 2% Pen-Strep added.

caspase Cleavage Assays. Recombinant Satb2 protein (250-500ng; Abnova) and recombinant active-caspase 3 (0.5µg; Chemicon) or recombinant active-caspase 7 (0.5µg; Biovision) were incubated for 3hr in cleavage assay buffer [50 mM Hepes, pH 7.5; 0.1 M NaCl; 10% (vol/vol) glycerol; 0.1% Chaps; 10mM DTT] containing either DMSO or z.DEVD.fmk (20µM; BioVision) as indicated. Reactions were stopped by addition of Laemmli sample buffer, and subjected to SDS/PAGE. Mass spectral analysis was performed at the OHRI Proteomics Core Facility (Ottawa, Canada). MASCOT 2.3.01 software (Matrix Science) was used to infer peptide and protein identities from the mass spectra.

caspase Inhibition Assays. For caspase 3/7 inhibition, cultured myoblasts were pre-treated with either 15μM Z-DEVD-FMK (DEVD) from BioVision or 15μM Dimethyl Sulphoxide (DMSO) from Sigma, MO for 2hr at 37°C. After pre-treatment the cells were induced to differentiate using low serum media or continued in growth media both containing 15μM DEVD or DMSO as a vehicle only control. The inhibition or control media was changed every 48hr until the end of the time course. Cells were collected at the predetermined time points and analyzed as described.

siRNA Knockdown of Satb2 Gene Expression. 27mer siRNA duplexes were used to suppress Satb2 gene expression (10nmol trial kit (Origene Technologies) contains: 3 unique siRNAs for Satb2, 1 universal scrambled siRNA control, and siRNA resuspension buffer). We tested the 3 siRNAs designated A, B, C and determined the highest efficiency of knockdown for came from SR419652B, which became our exclusively utilized siRNA for experimentation. 500nM of siRNA was transfected using Lepofectamine RNAiMAX reagent as a carrier into myoblasts plated in 10% FBS-DMEM media at 25% confluence. After 24hr incubation, fresh siRNA-Lepofectamine was replaced with the 10% FBS-DMEM media. Incubation for another 24hr period allowed cells to reach 100% confluence on plates, and growth time point si-CTL/si-Satb2 samples were collected. For other time points, plates were washed with PBS and siRNA-Lipofectamine mixed in 2% HS-DMEM media is added on the plates and allowed to reach the endpoint desired before collection (24hr, 48hr, 72hr Diff time points mostly required).

Chromatin immunoprecipitation. Cells were grown on 15cm plates and allowed to reach 100% confluence before either collecting or switching to differentiation media and allowed to incubate until the desired time point was achieved. 20 million cells per time point were used for ChIP sample. Cells were fixed for 10mins using 1% Formaldehyde in blank DMEM. Fixation was quenched by removing fixation solution, rinsing plates with PBS solution, then pouring

0.125M Glycine in PBS solution and incubating for 5mins. Plates were washed 2x with PBS solution, then cells were scraped from plates and pellets were stored at -80°C until used. Considering Satb2 is a nuclear matrix attachment protein, it required an alternative protocol for chromatin shearing than standard preparations. First we performed cellular lysis using commercial hypotonic solution (Active Motif) to burst cellular membrane but retained intact nuclear membranes. We performed additional lysis using hand held glass Dounce homogenizer. After centrifugation and discarding the supernatant, we treated the pelleted nuclei with commercial pro-enzymatic digestion nuclear extraction solution (Active Motif). After incubation at 37°C for 5mins, we supplemented the reaction with 1 unit Micrococcal nuclease (New England Biolabs) and incubated for 10mins, with occasional stripping of reaction vial. This enzymatic shearing step helps further breakdown of chromatin to allow access to Satb2. We then supplemented the reaction with SDS to a final volume 2%SDS-chromatin solution. We proceeded to the second stage of shearing using sonication via Covaris M220 Focused-Ultrasonicator instrument, setting the parameters to produce sheared DNA of 200bp. After sonication, we performed final centrifugation at 16000xg at 4°C for 20mins and collected supernatant containing sheared chromatin. We used 20% of the final volume to perform standard shearing efficiency checks using agarose gel electrophoresis. After determining shearing efficiency and concentration of chromatin samples, we performed immunoprecipitation with 5µg 1°Ab (Target: Satb2, Abcam, positive control: RNA pol II, Active Motif, negative control: mouse IgG, Santa Cruz) using magnetic beads (Active Motif) diluted in commercial ChIP-buffer (Active Motif) overnight rotating at 4°C. Captured chromatin was separated from solution using magnetic stands to pulldown the beads. Standard elution, reverse cross-linking, protein digestion, and finally RNA digestion was all performed using commercial solutions (Active Motif) or lab

made ingredients. Resulting samples of DNA are further purified using classic phenol:chloroform extraction procedures. Final DNA was quantified using NanoDrop spectrophotometer, with typical 25µl final volume having concentrations of 75-100ng/µl.

ChIP-sequencing and Bioinformatics. For genome-wide analysis, IP DNA was amplified, and 75bp single read sequencing was performed on a Illumina HiSeq 2500 at the Next-Generation Sequencing Facility at The Centre for Applied Genomics in The Hospital for Sick Children (Toronto, Ontario, Canada). Analysis was performed with the participation of OHRI Bioinformatics research technician Alphonse Chu. Briefly, data was summarized and basic comparisons performed using the Excel spreadsheet program (Microsoft). Reads were aligned to the NCBI build 38 (UCSC mm10, Dec/2011) genome from UCSC genome browser using default options. Data was summarized and basic comparisons performed using MACS version 2.1.0.20140616, Gene Ontology (GO) term annotation was performed using GREAT v3.0.0 available online from the Bejerano lab at Stanford University [McLean et al, 2010].

RNA-sequencing and Bioinformatics. Total RNA was isolated using RNesay kit (QIAGEN) using an on-column DNase digestion (RNase-Free DNase Set, QIAGEN) to avoid genomic DNA contamination. Library preparation and 126-bp paired-end RNA-seq were performed by the Next-Generation Sequencing Facility at The Centre for Applied Genomics in The Hospital for Sick Children (Toronto, Ontario, Canada). RNA integrity was assessed using the Bioanalyzer platform (Agilent Technologies, Inc.). Sequencing was performed using standard procedures for the Illumina HiSeq 2500 platform. Analysis was performed with the participation of OHRI Bioinformatics research technician Alphonse Chu. Gene expression quantification was performed using CuffDiff 2 software [Trapnell et al 2013]. Data was summarized and basic comparisons were performed using the Excel spreadsheet program (Microsoft).

Immunofluorescence Staining and Microscopy. Cells were cultured on 25mm coverslips in 35mm plates and exposed to the described experimental conditions. At designated time points the cell plates were placed on ice and the media was aspirated and replaced with ice cold 1X PBS. The plates were washed a further 2 times with PBS. The cells were fixed in 4% Para-Formaldehyde in PBS for 10min on ice. The fixative was removed and the cells were washed with PBS 2 times. Next the cells were covered with PBS and the plates were wrapped with Parafilm and stored at 4°C until such time that all time points had been fixed. The plates were removed to RT and incubated in 0.5%Triton-100X in PBS for 10mins to allow for membrane permeabilization. After washing off the Triton-PBS solution twice with PBS, the cells were incubated in block solution for 1hr in 5% Horse Serum in PBS. After blocking, the cells were incubated in primary antibody (Satb2 mouse 1°Ab 1:50 (ab51502, Abcam), HP1alpha rabbit 1°Ab 1:200 (#2616, Cell Signaling), GAPDH rabbit 1°Ab 1:400 (#2118, Cell Signaling), Desmin rabbit 1°Ab 1:400 (ab15200, Abcam)) reconstituted in blocking solution for 2hr with rocking at room temperature. After primary antibody incubation, the cells were washed 3 times in PBS and incubated in secondary antibody (2mg/mL Alexa Fluor 488 goat anti-rabbit IgG (H+L), Invitrogen, CA 1:1500, 2mg/mL Alexa Fluor 594 goat anti-mouse IgG (H+L), Invitrogen, CA 1:1500) diluted in PBS for 1.5hr rocking at room temperature. After incubation the cells were washed 2X in PBS and 1X in dH20 then counterstained with DNA specific 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), Sigma Aldrich, MO made up in dH2O (1:10000) for 10min at RT. After incubation the cells were washed 2 times in PBS and 1 time in dH2O. The coverslips were mounted on microscope slides using Dako Fluorescent Mounting Medium and visualized using a Zeiss Observer Z1 inverted fluorescence microscope. All images

were developed using AxioVision 4.8 software. The quantification of nuclear Satb2 was performed by densitometry analysis using ImageJ/Photoshop C3 software.

RNA extraction, PCR. Cells from C2C12 and primary myoblast cultures were washed with ice-cold PBS and lysed using TRIzol reagent. The appropriate volume of chloroform was added to the lysates, followed by the samples being vortexed, and then allowed to incubate for 3min at RT after which time they were centrifuged at 10000xg for 10min at 4°C to separate the phases. The top phase was removed to a fresh, labeled, RNase free tube. Isopropanol was added to each sample and the tubes were gently inverted to mix. The tubes were rocked on a nunator for 20min at RT after which time the RNA was pelleted by centrifugation at 10000xg for 15min at 4°C. The supernatant was discarded and the pellet was washed in 500µL 70% ethanol-DEPC (Ethanol in Dimethyl Pyrocarbonate (DEPC) containing sterile water) and re-centrifuged for 5min at 7500xg at 4°C. The supernatant was discarded and the RNA pellet was allowed to air dry in the fume hood. Once dry the RNA pellet was resuspended in 80µL nuclease free water and allowed to dissolve at 4°C for 1hr before being stored at -20°C. First strand cDNA synthesis was performed using iScript RT Supermix from Bio-Rad according to manufacturer instructions.

Protein Extraction and Western Blotting. Cells from C2C12 and primary myoblast cultures were washed with ice-cold PBS and lysed on ice for 45min in modified cytoplasmic/nuclear fractionation NE-PER extraction kit from Thermo Scientific. Immunoblot analyses were performed as follows: Proteins fractioned via SDS-PAGE were transferred to 0.45 µM PVDF membrane (Millipore) on a TRANS-BLOT SD apparatus (Bio-Rad). Membranes were blocked with TBST containing 5% skim milk for minimum of 1hr at room temperature; after which they were supplemented with primary antibody and further incubated at 4ºC overnight on a rotation station. List of primary antibodies used and dilutions were as follows: Satb2 mouse 1°Ab 1:1000

(ab51502, Abcam), MF20 mouse 1°Ab 1:250 (DSHB), GAPDH mouse 1°Ab 1:4000 (#2118, Cell Signaling).

Statistical Analysis. Statistical analysis was performed using one factorial ANOVA when comparing 3 or more sample sets. For comparison between 2 sample sets, an unpaired, 2-tailed t test was performed. $p< 0.05$ was considered statistically significant.

3.4: Results

To establish the normal biological expression profile of Satb2, we measured Satb2 protein content in proliferative muscle cells, through early differentiation of myocytes, to late stage fully differentiated myotubes. As figure 1 demonstrates, the expression profile of full-length Satb2 protein was as expected and replicated a similar pattern to other published observations [Asanoma et al., 2012]. For example, proliferative cells expressed the highest level of Satb2 protein, localized in the nucleus. As differentiation progressed, the FL-Satb2 levels decreased and we could detect prospective fragments of Satb2 protein (Mass-Spec analysis confirmed these bends to be Satb2 proteolytic fragments, see figure 5). We performed the Satb2 expression profile analysis using both the C2C12 muscle cell model (figure 1a), as well as primary mouse myoblast cells (figure 1b). In both cell types, the expression of Satb2 followed the same profile, highest in proliferative cells, gradually decreasing in all subsequent differentiation time points, until completely disappearing by late differentiation stages.

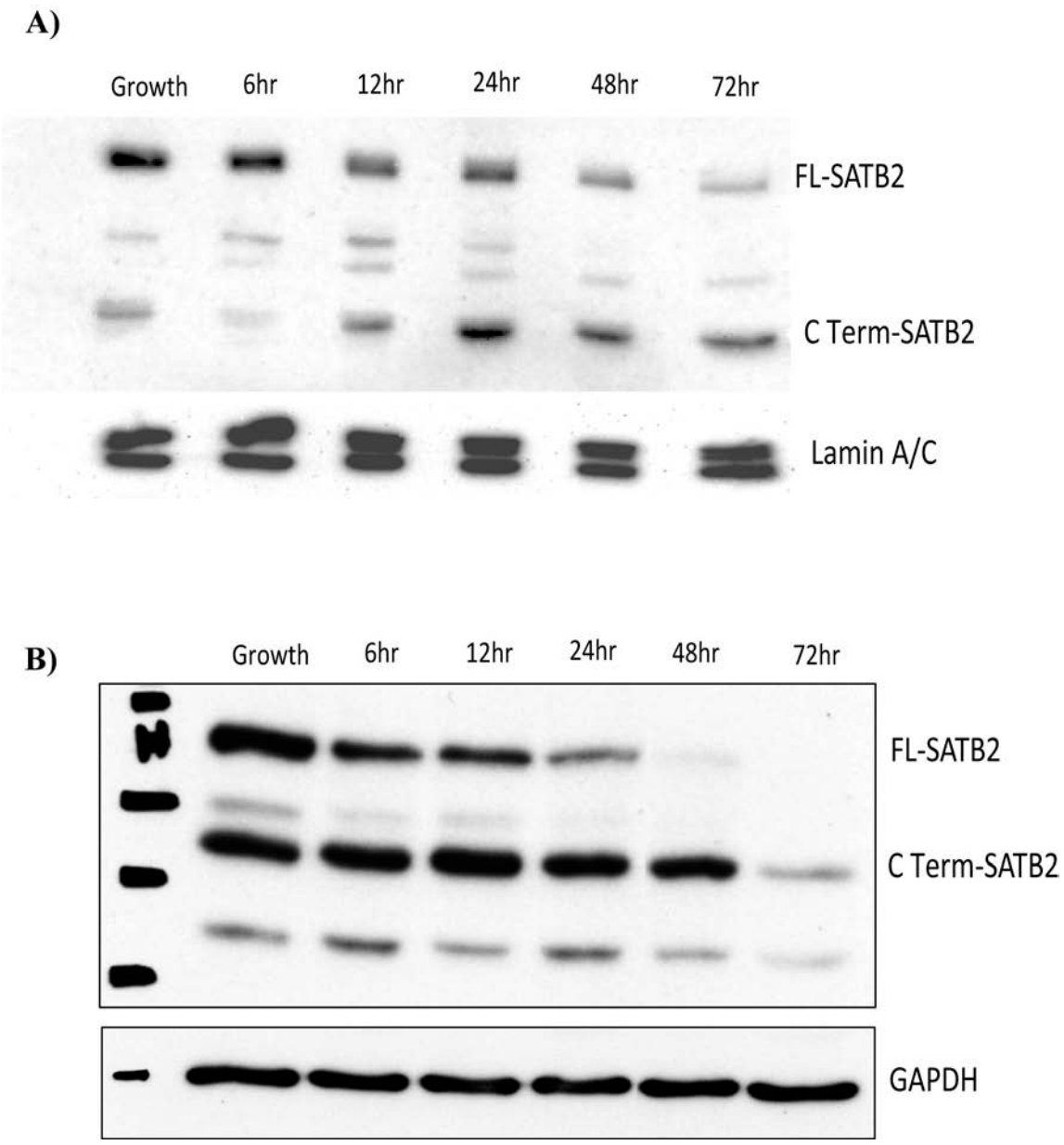


Figure 1 Satb2 protein expression during growth and early myoblast differentiation timecourse. (a) Western blot of Satb2 nuclear fraction from C2C12 myoblasts, 25µg of protein loaded per sample, N=6 independent samples. (b) Western blot of Satb2 whole cell fraction from primary mouse myoblasts, 50µg of protein loaded per sample, N=3 independent samples.

To accurately visualize, and characterize Satb2 in the nucleus of proliferating and differentiating C2C12 cells, we performed immunofluorescence staining (figure 2). The time course consisted of growth (proliferating myoblasts), intermediate differentiation (myocyte formation, 24hr post

induction), and late stage differentiation (fusion and myotube formation, 48hr post induction). Staining patterns of Satb2 protein and DAPI matched the results we observed in the western blotting data of figure 1. The highest nuclear localization of Satb2 protein was found in the growth (proliferation) time point, decreasing as the differentiation program progressed. We performed immunofluorescence staining with Satb2 and HP1-alpha antibodies to answer whether the localization was heterochromatic or euchromatic (i.e. HP1-alpha is typically used to verify heterochromatic localization [Sdek et al., 2013]). As figure 3 demonstrates, Satb2 does not co-localize exclusively with HP1-alpha, instead Satb2 is more generally localized to euchromatic space, dispersed and found outside the ultra dense heterochromatin ultrastructure. This type of staining indicates that Satb2 may participate in regulating gene transcription in physically more pliable regions, rather than the tightly controlled and repressed gene loci (at any particular stage of the gene).

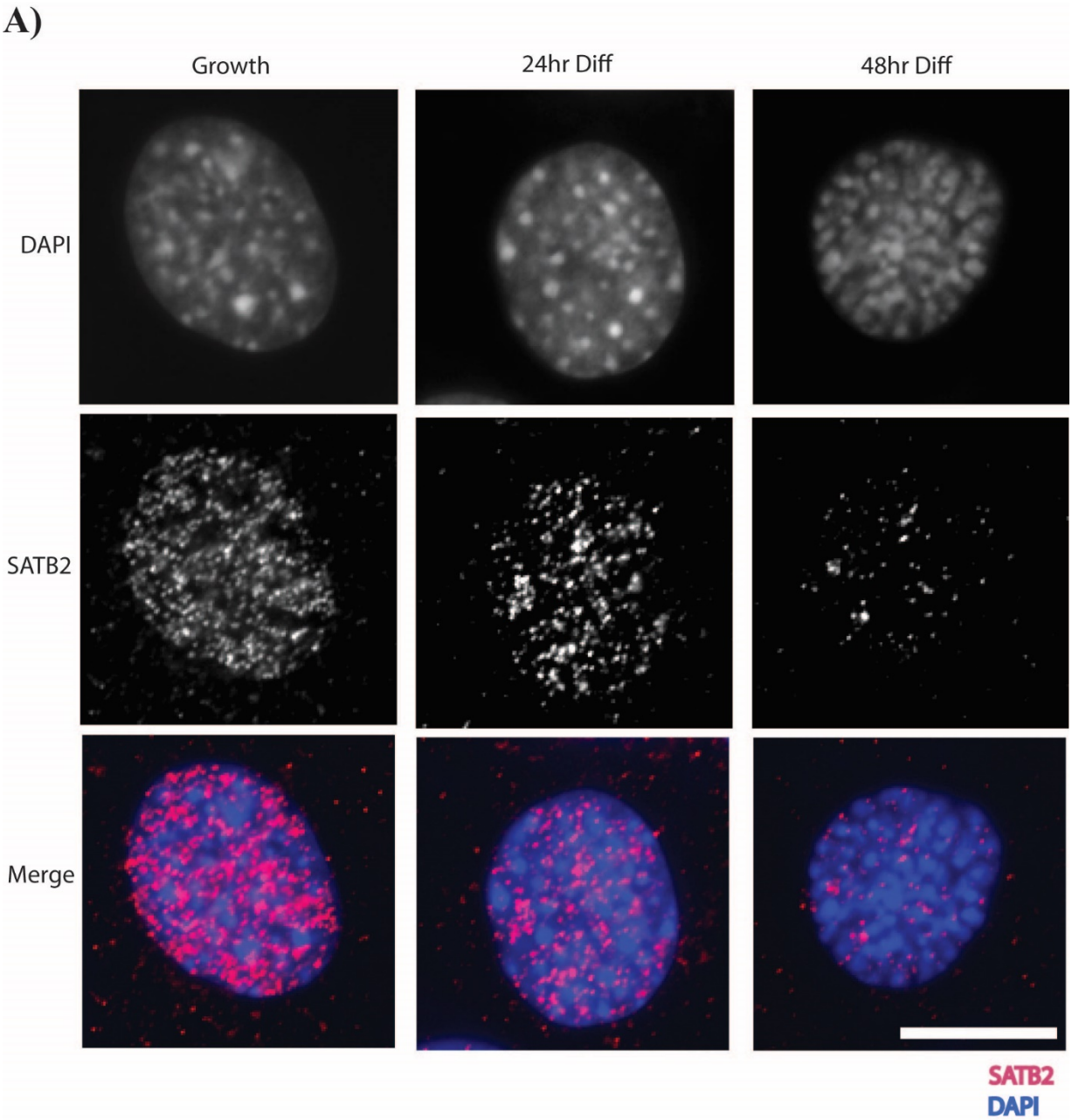


Figure 2 Satb2 nuclear localization profile during growth and early myoblast differentiation timecourse. (a) High magnification to isolate single nucleus per panel. Satb2 stained with Alexa-Flour 594, indicated in Red color, DAPI indicated in Blue color. Scale bar is 10µm. N=5 independent samples, 50-100 cells per time point isolated for staining analysis.

The appearance of distinct fragments of consistent size on the western blots, as well as previous published works showing that during apoptosis Satb1 is targeted for degradation by caspase 6 [Galande et al., 2001], led us to examine if Satb2 was subject to similar caspase dependent

cleavage during early differentiation stage of development. caspase cleavage of protein targets is a well described mechanism that is critical for the initiation and promotion of cell differentiation in addition to the role for this protease family in apoptosis induction. For example, previous work has shown an absolute requirement for active-caspase 3 during early muscle cell differentiation [Fernando et al., 2002, Dick et al., 2015]. Here, we also demonstrate that expression of active-caspase 7 and its nuclear localization is increased during early differentiation of our progenitor muscle cell model C2C12 cell line (figure 4). These sets of data provide us with two potential active caspases that could cleave Satb2 during the early stage of myotube formation that we observe in figure 1.

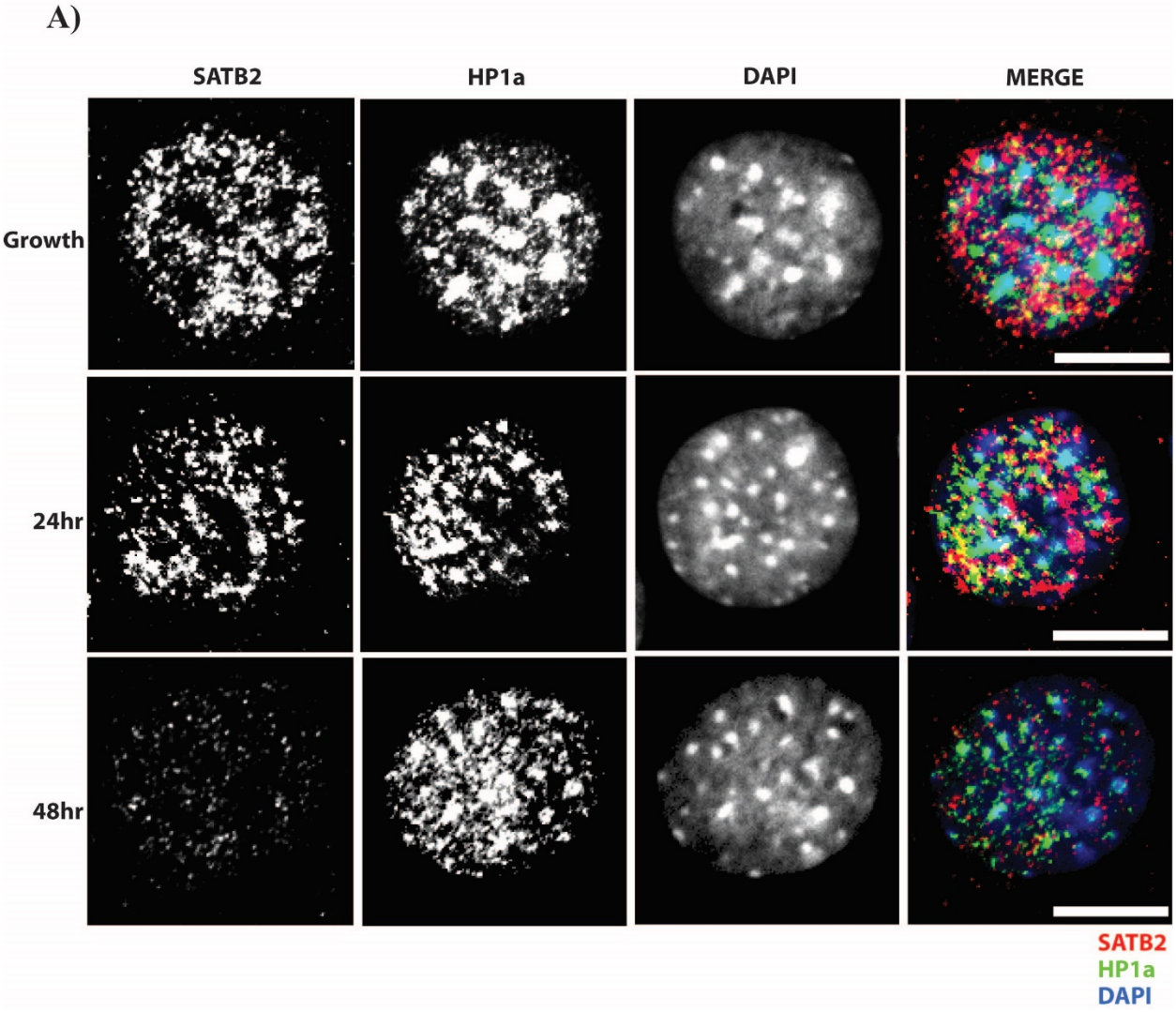


Figure 3 Satb2 localization is staining mostly in euchromatic nuclear space. (a) High magnification to isolate single nucleus per panel. Satb2 stained with Alexa-Flour 594 indicated in Red color, Heterochromatin Protein 1-alpha (HP1a) stained with Alexa-Flour 488 indicated in Green color DAPI indicated in Blue color. Scale bar is 10µm. N=3 independent samples, 50-100 cells per time point isolated for staining analysis.

As figure 5a demonstrates, our in-vitro caspase cleavage assay indicates a robust cleavage of Satb2 by effector caspases. However, unlike other well characterized caspase substrate relationships in cell differentiation, we noted that caspase 7 was far more robust at targeting and cleaving Satb2 protein. caspase 7 cleavage of Satb2 protein, provides another example for a

prospective substrate that is differentially sensitive between caspase 3 and 7 [Walsh et al., 2008, Lamkanfi and Kanneganti, 2010]. Mass-spec analysis of the most dominant cleavage fragments of Satb2 (that resulted from the caspase 7 cleavage assay) demonstrated an optimal cleavage site at D477, as noted in figure 5b. However, we have observed that in the *in vitro* cleavage assay, as well as the biological expression profile of Satb2, there appears to be multiple cleavage fragments, suggesting that further proteolysis of Satb2 occurs. Whether these cleavage events occur concurrent to or following cleavage of D477 is not clear. These results taken together confirm that a) active-caspase 7 is upregulated in the nucleus of early differentiating progenitor muscle cells, and b) Satb2 is targeted for cleavage in early differentiation by active-caspase 7.

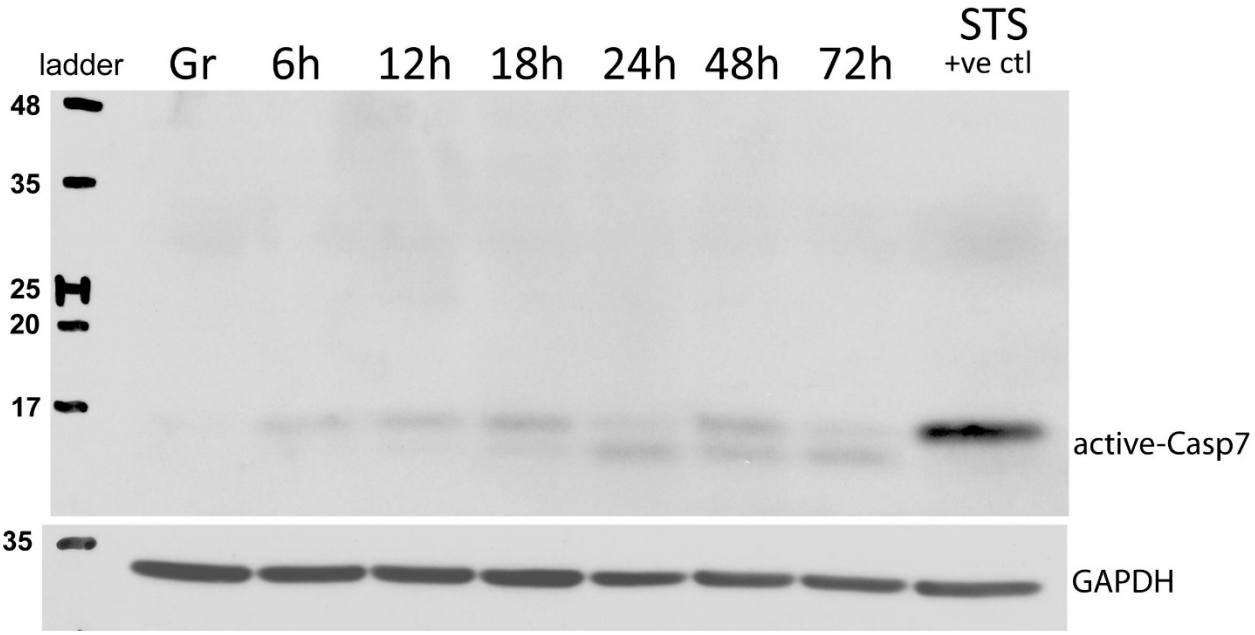
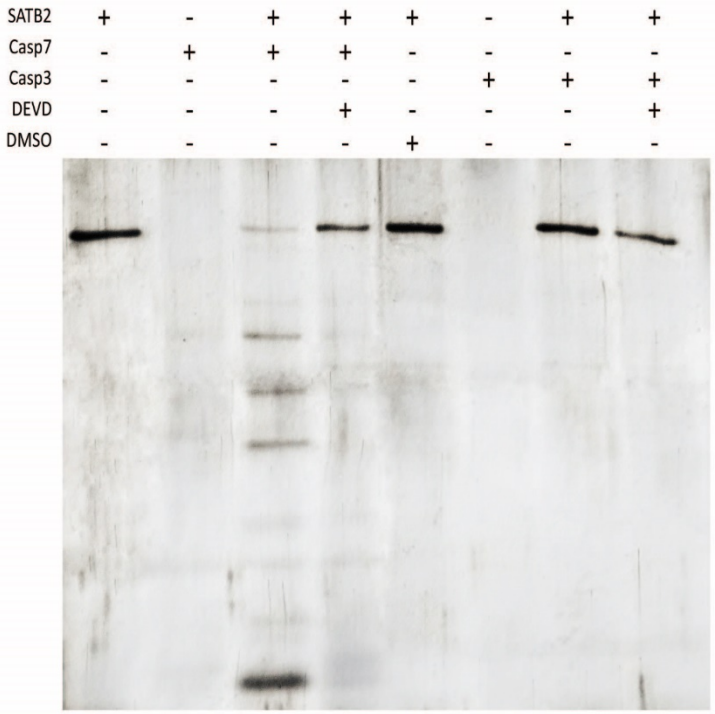


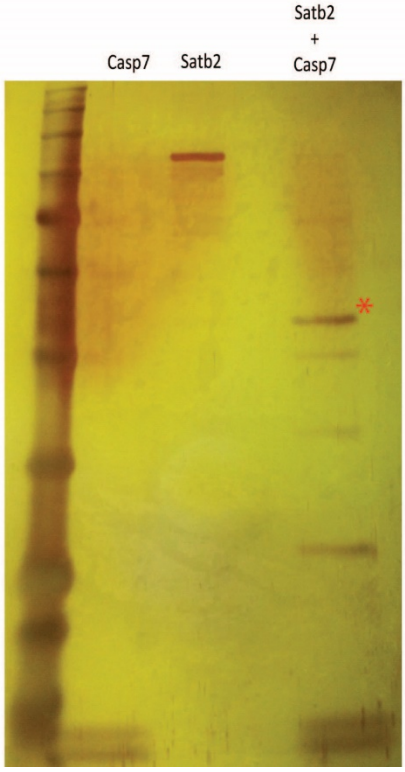
Figure 4 caspase 7 nuclear expression profile during growth and early myoblast differentiation. Western blot of cleaved active fragment of caspase-7 exclusively found in the nuclear fraction of C2C12 myoblasts at the proliferative phase (Gr-growth media) and during early and late differentiation phases marked by number of hours in timecourse, post induction of differentiation culturing media. 50µg loaded per sample. N=3 independent samples.

Effector caspase inhibition by designer small molecules is a valuable and widely used method to verify the cleavage effects of caspases in cellular system. We have performed caspase inhibition experiments previously to verify the role of caspase in initiating DNA damage events that occur early post induction of differentiation [Larsen et al., 2010] and we have shown the importance of DNA damage repair pathways [Al-Khalaf et al., 2016] as well as discovering the role of caspase 3 cleavage of Pax 7 protein [Dick et al., 2015] and as Mst1 [Fernando et al., 2002], amongst others. In an effort to validate the caspase dependent cleavage and essential removal of Satb2 during early myoblast differentiation, we performed caspase inhibition using the modified peptide z.DEVD.fmk which is designed to specifically inhibit caspases 3 and 7 and not any other caspase proteases in the cell. As figure 6 demonstrates, we performed a time course comparison between control and caspase 3/7 inhibited cells, and used immunofluorescence staining of Satb2 to monitor its expression and nuclear localization during our target of interest window of early differentiation (figure 6a). Our results show that caspase 7 inhibition using DEVD led to significant accumulation of Satb2 in the nucleus compared to lysates derived from control cells (figure 6b).

A)



B)



Start - End	Observed	Mr(expt)	Mr(calc)	ppm	M	Score	Expect	Rank	U	Peptide
478 - 496	1069.5300	2137.0455	2137.0361	4.41	0	77	1.8e-06	1	U	D.GANINITAAIYDEIQQEMK.R
478 - 496	1069.5302	2137.0458	2137.0361	4.52	0	41	0.037	1	U	D.GANINITAAIYDEIQQEMK.R
478 - 497	760.0575	2277.1507	2277.1423	3.68	1	51	0.0027	1	U	D.GANINITAAIYDEIQQEMKR.A
478 - 497	1139.5853	2277.1561	2277.1423	6.07	1	48	0.00016	1	U	D.GANINITAAIYDEIQQEMKR.A
478 - 497	765.3890	2293.1451	2293.1372	3.45	1	18	0.58	1	U	D.GANINITAAIYDEIQQEMKR.A
478 - 497	765.3898	2293.1475	2293.1372	4.49	1	15	0.59	1	U	D.GANINITAAIYDEIQQEMKR.A
482 - 497	647.6520	1939.9341	1939.9196	7.45	1	7	1.6	3	U	I.NITAAIYDEIQQEMKR.A + 2
498 - 507	531.8209	1061.6273	1061.6233	3.76	1	7	0.23	1	U	R.AKVSQALFAK.V

Figure 5 in vitro Satb2 Cleavage Assay by effector caspases 3 and 7 and fragment identification via MASS-SPEC. (a) Silver Stain gel of Satb2 recombinant protein, active-caspase-3 and -7 recombinant proteins, with caspase chemical inhibitor Z-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)- fluoromethylketone (Z-DEVD-FMK, marked as DEVD on WB) or control chemical Dimethyl sulfoxide (DMSO) **B)** Silver stain of Casp-7 with Satb2, * identifies major cleavage fragment analyzed by MASS-SPEC to include D477 cleavage site.

Next, we employed silencing RNA targeting of *Satb2* gene and examined the effects of the knockdown at the proliferative phase of cell state, then at the early differentiation phase of cells. We hypothesized that caspase-7 targeting of Satb2 may be an essential step in either initiating muscle cell differentiation, or can positively regulate the rate of muscle differentiation. The results showed that si-Satb2 knockdown during muscle cell growth had no effect on the morphology (figure 7) or on the proliferative capacity of the seeded myoblasts (figure 8). We performed a cell counting assay over a period of 96h post siRNA knockdown of Satb2 gene, and as we show in figure 8, the number of cells increased in both control and si-Satb2 in equal amounts, with no significant difference between the two groups. These results indicate that Satb2 may not be essential for cell proliferation and expansion of myoblasts. These results also suggest that Satb2 does not lead to spontaneous or precocious differentiation of these cells.

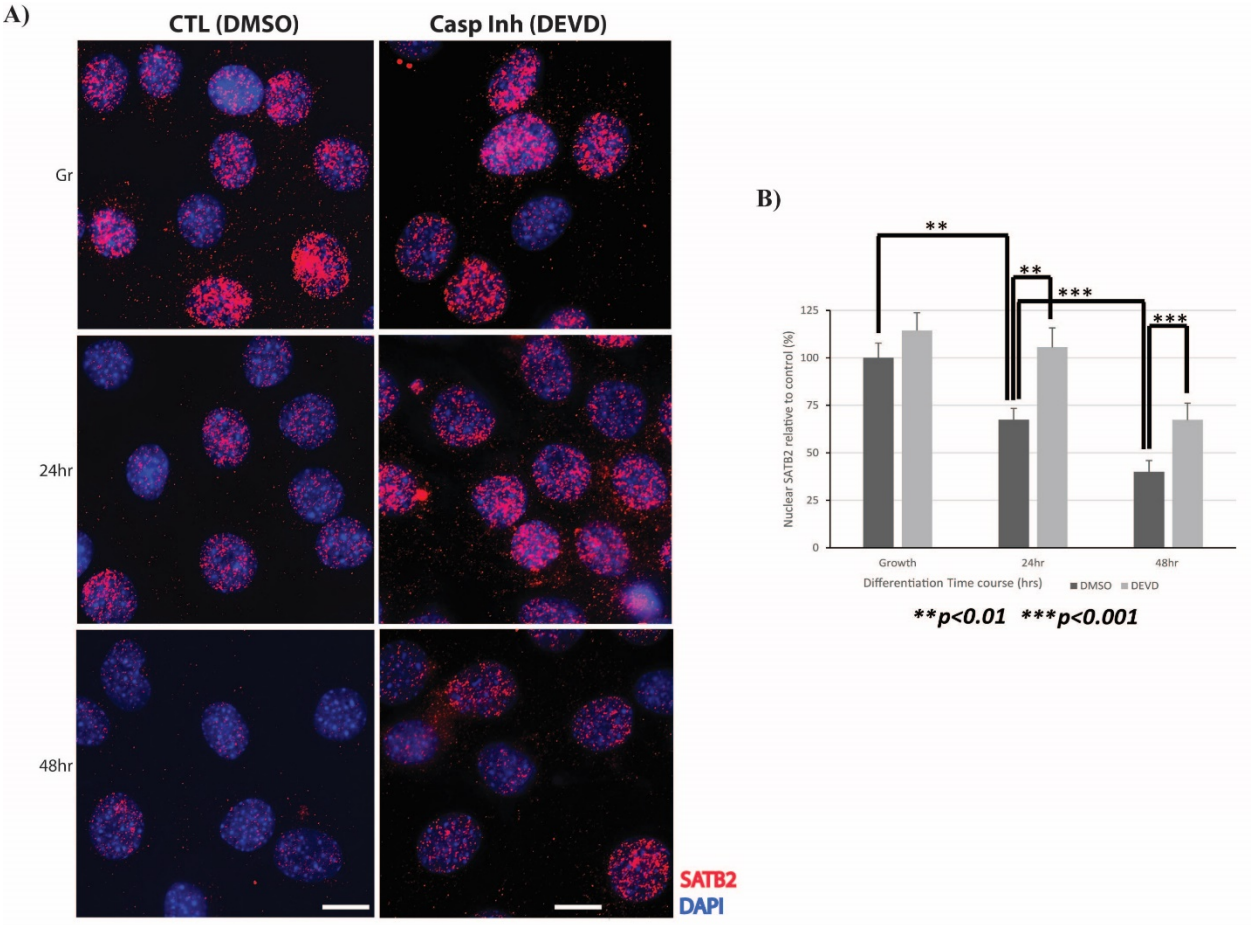


Figure 6 Inhibition of caspase 3/7 leads to an accumulation of nuclear Satb2. (a) Nuclear Satb2 during growth (Gr), 24hr and 48hr post induction of differentiation is stained with Alexa-Flour 594, indicated in Red color, DAPI indicated in Blue color. Scale bar is 10µm (b) Satb2 nuclear expression in relation to control growth conditions quantification bar graph. Dark bars indicate DMSO control samples, Light bars indicate DEVD caspase inhibitor treated samples. N=3 independent samples, 75-100 cells per condition/time point isolated for staining analysis. ** indicates $p<0.01$ *** indicates $p<0.001$

Next, we examined whether Satb2 knockdown had any impact on the myoblast differentiation process. We performed siRNA knockdown of Satb2, then allowed the seeded cells to reach 100% confluence on culture dishes, at which point we initiated differentiation by exposure to low serum culture conditions. In figure 9a, western blot analysis of si-CTL and si-Satb2 treated cells revealed a robust reduction in Satb2, as well as cleaved Satb2 protein fragments.

Interestingly, there is a marked increase in the expression of differentiation markers such as myosin heavy chain (pan-myosin heavy chain antibody MF20) seen at the late differentiation time point of 72hr, which is an indicator of increased myotube formation. In figure 10, we demonstrate the resulting phenotype of Satb2 knockdown cells. As differentiation progresses, we noted an acceleration in myocyte fusion and myotube formation, which corroborated the western blot results for MF20. Taken together, this data indicates that loss of Satb2 protein leads to a more robust differentiation of muscle progenitor cells.

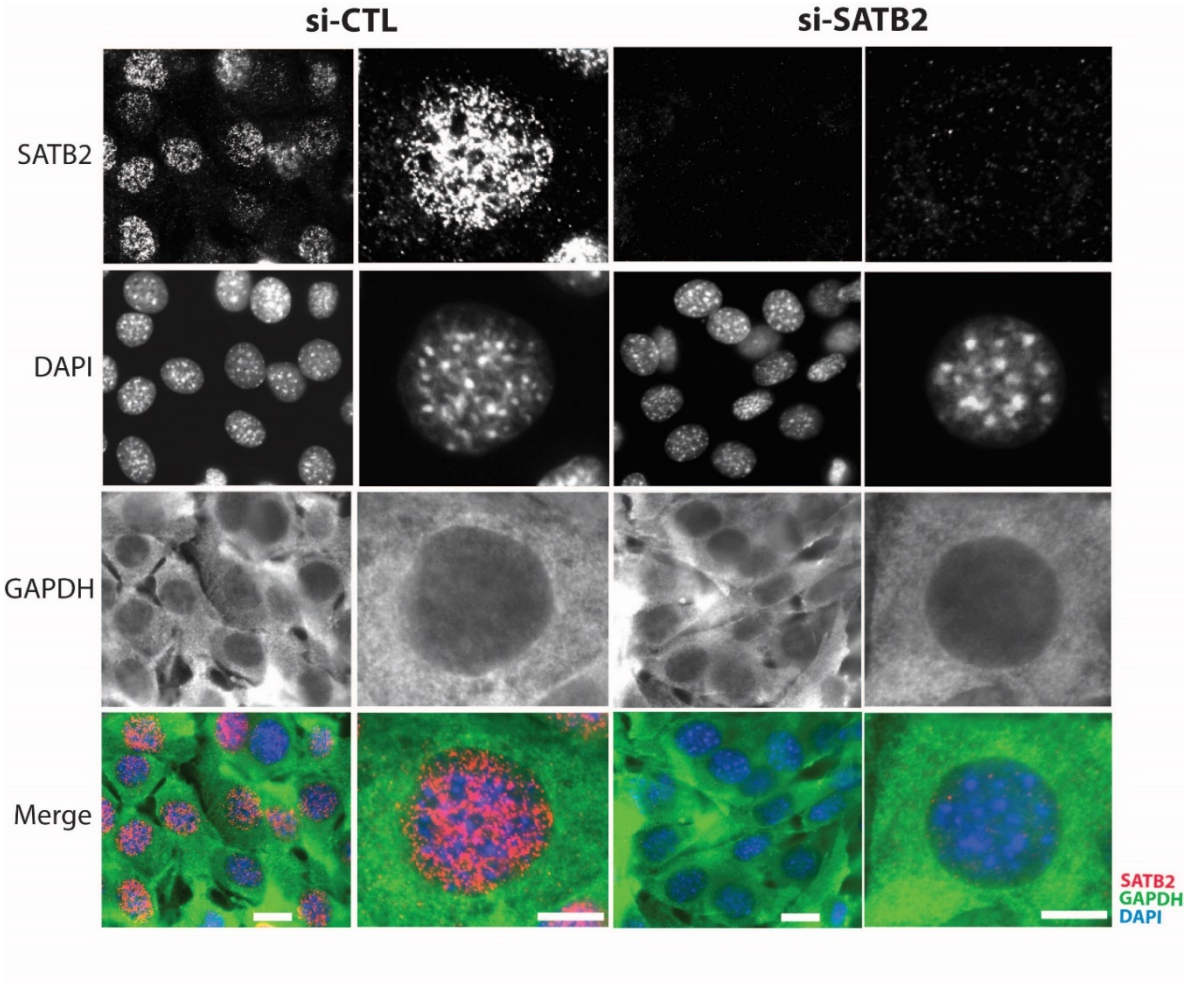


Figure 7 *Satb2* knockdown does not affect proliferating progenitor muscle cells.
si-CTL are control samples treated with sham siRNA; si-Satb2 are knockdown samples treated with siRNA targeting *Satb2* gene. Lower magnification columns scale bar is 10µm; Higher magnification columns scale bar is 5µm. Satb2 nuclear stain is Alexa-Flour 594 indicated in Red

color, GAPDH whole cell stain is Alexa-Flour 488 indicated in Green color; DAPI nuclear stain is indicated in Blue color. N=3 independent samples, 50-100 cells per condition isolated for staining analysis.

Next, we sought to understand the precise role of Satb2, and how this MAR protein affects the growth and differentiation of myoblasts. Specifically, we were interested in identifying genome wide targets of Satb2 and how this MAR may modify myoblast gene expression. To this end, we performed Chromatin immunoprecipitation (ChIP) followed by next generation sequencing on proliferation phase C2C12 myoblasts using Satb2 antibody from Abcam (ab51502). First, as figure 11a shows, Chip-seq data analysis followed by standard Gene Ontology categorization using GREAT software [McLean et al., 2010] of Satb2 binding peaks identified approximately 92% of the reads mapped to the genome. The data analysis revealed a wide distribution of Satb2 binding across the mouse genome, covering 38% of known classified genes (figure 11b). The table in figure 11c lists the top 25 GO Biological Process categorizations for Satb2 binding, as determined by statistical analysis using the FDR method. As expected the association of Satb2 with genes categorized as Chromatin modification (GO:0016568) is the top category recognized in our dataset. Satb2 binds more frequently to genes that are categorized as either chromatin or chromosome organization role players (figure 11c). This data also demonstrates that Satb2 is implicated in epigenetic control of gene expression, acting as a higher order regulator of modifiers to chromatin structure and function. Interestingly, Satb2 binding was also enriched for genes categorized as Cell division (GO:0051301), and Mitosis (GO:0007067), suggesting that Satb2 may influence cell proliferation mechanisms (despite the observation that siSatb2 does not result in any alteration in myoblast growth rates).

si-CTL	Sample 1	Sample 2	Sample 3	AVG	ST DEV	SE
24h post siRNA	29	32	31	30.67	1.53	0.88
48h post siRNA	45	51	49	48.33	3.06	1.76
72h post siRNA	81	126	112	106.33	23.03	13.30
96h post siRNA	237	255	229	240.33	13.32	7.69

si-SATB2	Sample 1	Sample 2	Sample 3	AVG	ST DEV	SE
24h post siRNA	28	41	37	35.33	6.66	3.84
48h post siRNA	46	61	56	54.33	7.64	4.41
72h post siRNA	125	87	106	106.00	19.00	10.97
96h post siRNA	254	231	263	249.33	16.50	9.53

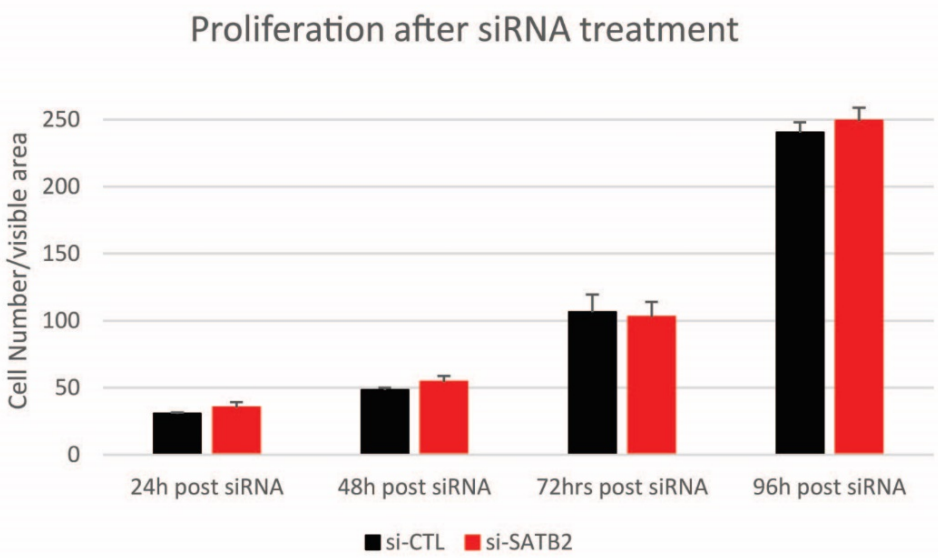


Figure 8 *Satb2* knockdown cell counting assay quantification tables and graph. C2C12 myoblasts seeded in 35mm well plates were counted by acquiring bright field microscope images at low magnification setting and each individual cell was marked and counted using ImageJ cell counter software. N=3 sample wells/treatment, counted 48h post initial siRNA treatment, and recounted at the specified time points indicated. Statistical analysis showed no significant difference between si-CTL and si-Satb2.

Our observations and previous studies have shown that Satb2 expression is typically highest in stem cells and cells that retain their proliferative capacity, demonstrating a marked downregulation in expression as cells initiate differentiation, leading to complete lack of expression in late mature cell stages. Additionally, we observed no discernible effect on the morphology or proliferative capacity of myoblasts lacking Satb2 expression while in growth promoting condition. This suggests that the role of Satb2 is crucial during the early window of transformation out of the proliferative state and into the differentiation state. Considering Satb2 is a structural protein that directly binds and interacts with a large number of genes, we sought to investigate the role Satb2 plays in the early differentiation window by identifying and monitoring the expression profile of genes that bind to Satb2, and genes that are most affected by the lack of Satb2 expression induced by siRNA knockdown experiments. Our DNA analysis and RNA-seq data analysis was performed in order to shed light on this question.

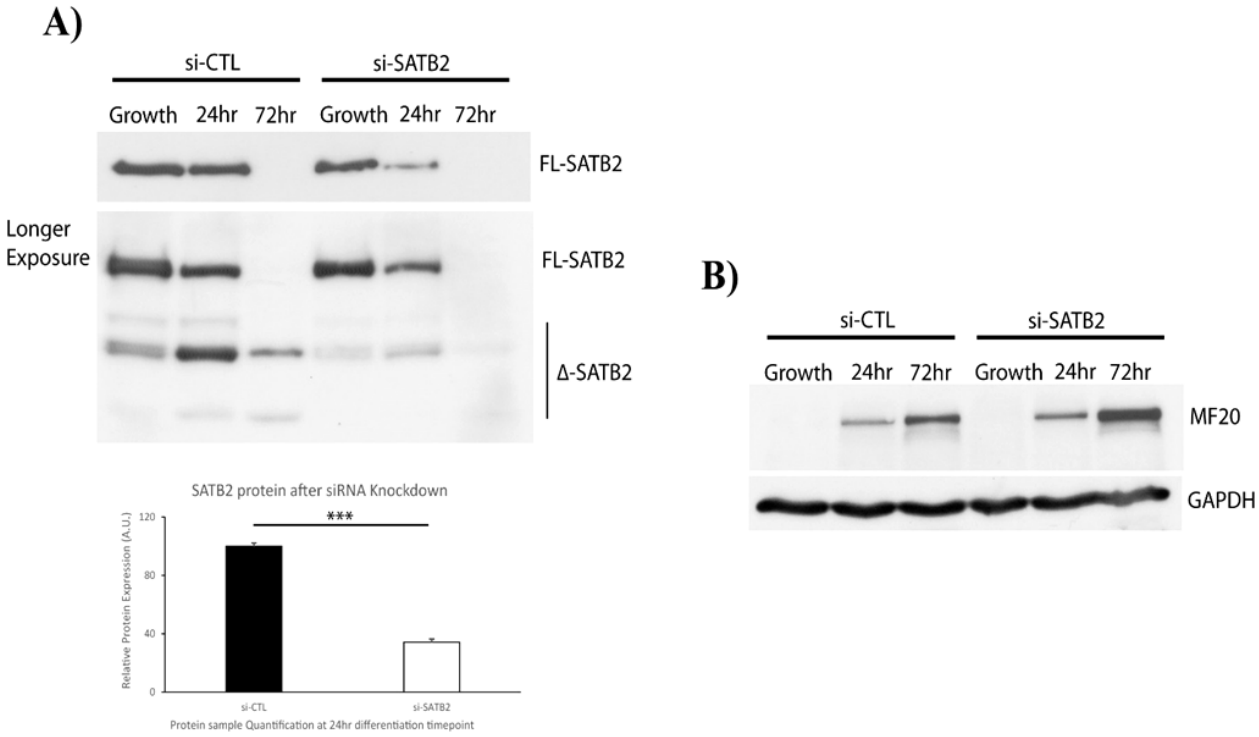


Figure 9 *Satb2* knockdown leads to accelerated myotube formation post initiation of differentiation. (a) Growth, early differentiation and late differentiation time points showing expression of full length Satb2 (FL-Satb2) protein in normal conditions (si-CTL) vs *Satb2* knockdown conditions (si-Satb2); longer exposure western blot used to show Satb2 cleavage fragments (Δ-Satb2) (b) Growth, early differentiation and late differentiation time points showing expression of pan adult myosin heavy chain proteins (MF20) in normal conditions (si-CTL) vs *Satb2* knockdown conditions (si-Satb2); GAPDH used as blot loading control. N=3 independent experiments. *** indicates $p<0.001$

We performed RNA-seq analysis comparing control 24hr-differentiated C2C12 cells to Satb2 knockdown (si-Satb2) 24hr-differentiated cells, in order to assess what gene expression changes occur due to lack of Satb2 in the nucleus in cells progressing through early differentiation. We decided on 24hr-differentiated time point based on the observed expression profile of Satb2, seeing how the decrease in its expression is not particularly rapid (about 50% decrease by 24hr, 75% decrease by 48hr). It is important here to point out that Satb2 sensitive gene expression may be variable across a differentiation time course, yet the phenotypic response noted with siRNA depletion of Satb2, suggests that monitoring gene expression at 24 hours was a reasonable supposition.

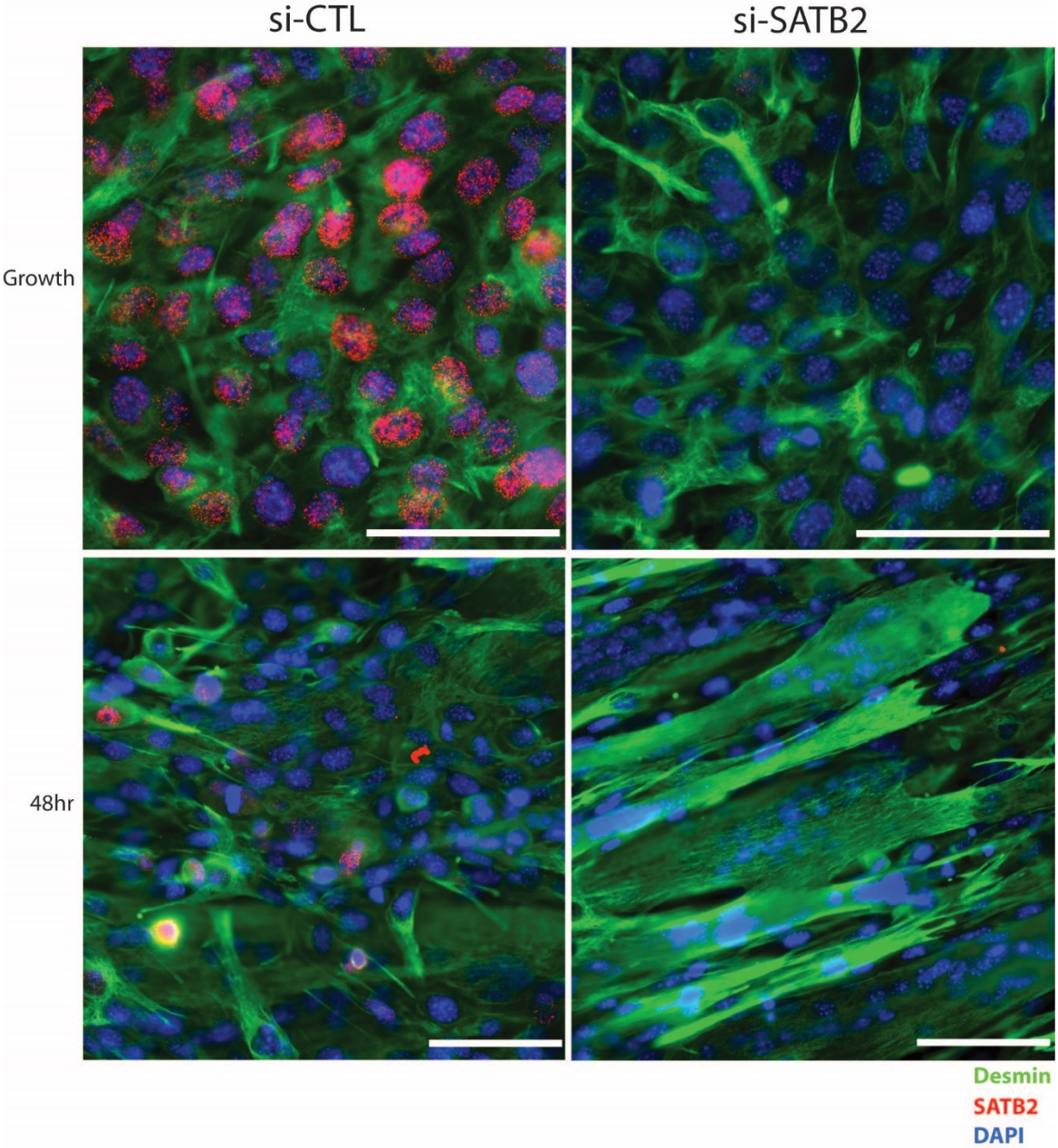
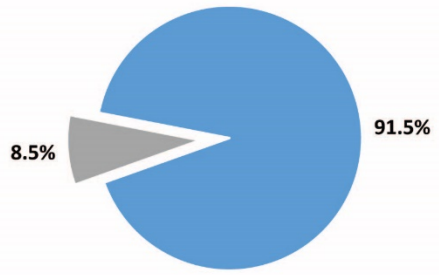


Figure 10 Immunofluorescence staining shows *Satb2* knockdown leading to early development of myotubes phenotype. Satb2 stained with Alexa-Flour 594, indicated in Red color, to visualize expression in control (si-CTL) and knockdown (si-Satb2) cells; Desmin stained with Alexa-Flour 488, indicated in Green color, DAPI indicated in Blue color. Scale bar is 50µm. N=3 independent samples.

First, our RNA-seq data showed that at 24hr post induction of differentiation, the expression of Satb2 was reduced by approximately 2-fold (figure 12a), showing an effective knockdown, and allowing us to validate and progress in comparing the whole set. We performed our RNA-seq

computational analysis using Cuffdiff 2 [Trapnell et al., 2012]. In figure 12b, we show that 2021 genes were identified to have significantly been altered from control expression levels in the Satb2 knockdown samples, with 942 genes being significantly upregulated and 1085 genes being significantly downregulated upon knockdown of Satb2 using siRNA. We cross-analyzed the gene list from the RNA-seq with the gene list of our ChIP-seq in order to identify specifically the genes that were physically binding to Satb2 that showed significantly altered gene expression at the intermediate differentiation time point we performed the RNA-seq on. In figure 12c, we found that there were 312 genes that are identified in the ChIP-seq data that were also significantly altered in the RNA-seq data. Of those genes, we found that roughly half showed significant upregulation (148 genes) and the other half showed significant downregulation (164 genes). In figure 12d, we highlight some of the genes found in the ChIP & RNA sequencing subset (38 of 312) in order to shed light on the direct role Satb2 plays on proliferation and differentiation pathways, especially through chromatin remodeling agents.

A)



B)

■ Identified peaks ■ Unidentified peaks ■ SATB2 associated ■ SATB2 unassociated

Name	Raw P-Value	FDR Q-Val	Fold Enrichment	Observed Region Hits	Region Set Coverage	FDR Q-Val	Fold Enrichment	Observed Gene Hits	Total Genes	Gene Set Coverage
chromatin modification	1.60E-16	3.75E-14	1.5418	397	4.41%	9.41E-06	1.3246	202	397	2.40%
mitosis	3.46E-15	6.71E-13	1.6686	268	2.97%	5.42E-04	1.3154	144	285	1.71%
cell division	4.58E-15	8.38E-13	1.5102	392	4.35%	2.97E-08	1.3872	219	411	2.60%
organelle fission	4.74E-14	7.23E-12	1.6069	282	3.13%	5.81E-04	1.3017	154	308	1.83%
RNA splicing	8.40E-11	6.87E-09	1.6012	212	2.35%	3.86E-03	1.2867	129	261	1.53%
chromatin remodeling	2.08E-09	1.40E-07	1.8943	104	1.15%	6.87E-05	1.7118	48	73	0.57%
negative regulation of chromosome organization	2.41E-09	1.57E-07	2.4968	55	0.61%	3.82E-02	1.6021	24	39	0.29%
regulation of chromatin organization	5.28E-08	2.66E-06	1.7836	102	1.13%	4.59E-02	1.3732	48	91	0.57%
regulation of chromosome organization	5.91E-08	2.96E-06	1.6378	135	1.50%	3.76E-03	1.4341	65	118	0.77%
maintenance of location	2.60E-07	1.18E-05	1.722	102	1.13%	9.61E-03	1.4293	56	102	0.67%
maintenance of location in cell	4.07E-07	1.80E-05	1.8656	77	0.85%	8.47E-03	1.5072	44	76	0.52%
negative regulation of gene expression, epigenetic	5.14E-07	2.23E-05	2.6368	35	0.39%	1.08E-02	1.9242	17	23	0.20%
maintenance of protein location in cell	5.50E-07	2.36E-05	1.9241	69	0.77%	1.35E-02	1.5154	39	67	0.46%
blastocyst formation	9.09E-07	3.71E-05	2.1548	50	0.55%	3.82E-02	1.6567	21	33	0.25%
chromatin silencing	9.82E-07	3.97E-05	2.8006	30	0.33%	2.84E-02	1.9183	14	19	0.17%
ATP-dependent chromatin remodeling	1.08E-06	4.34E-05	2.682	32	0.36%	2.46E-02	1.9908	13	17	0.15%
maintenance of protein location	1.16E-06	4.62E-05	1.7497	86	0.95%	7.38E-03	1.4876	48	84	0.57%
maintenance of protein localization in organelle	1.95E-06	7.40E-05	3.5622	20	0.22%	2.46E-02	1.9908	13	17	0.15%
response to interleukin-1	6.38E-06	2.19E-04	1.6823	84	0.93%	1.04E-02	1.562	36	60	0.43%
regulation of proteasomal protein catabolic process	8.52E-06	2.82E-04	1.6322	91	1.01%	3.82E-02	1.42	42	77	0.50%
trophectodermal cell differentiation	1.27E-05	4.03E-04	2.1595	39	0.43%	1.08E-02	1.9242	17	23	0.20%
CDP-choline pathway	1.40E-05	4.41E-04	4.7449	12	0.13%	4.11E-02	2.6034	6	6	0.07%
RNA splicing, via transesterification reactions	2.87E-05	8.64E-04	1.7009	69	0.77%	2.00E-03	1.6172	41	66	0.49%
mRNA splicing, via spliceosome	3.53E-05	1.04E-03	1.6964	68	0.75%	1.99E-03	1.6271	40	64	0.48%
labyrinthine layer development	7.38E-05	1.99E-03	1.6604	67	0.74%	4.62E-03	1.6521	33	52	0.39%

C)

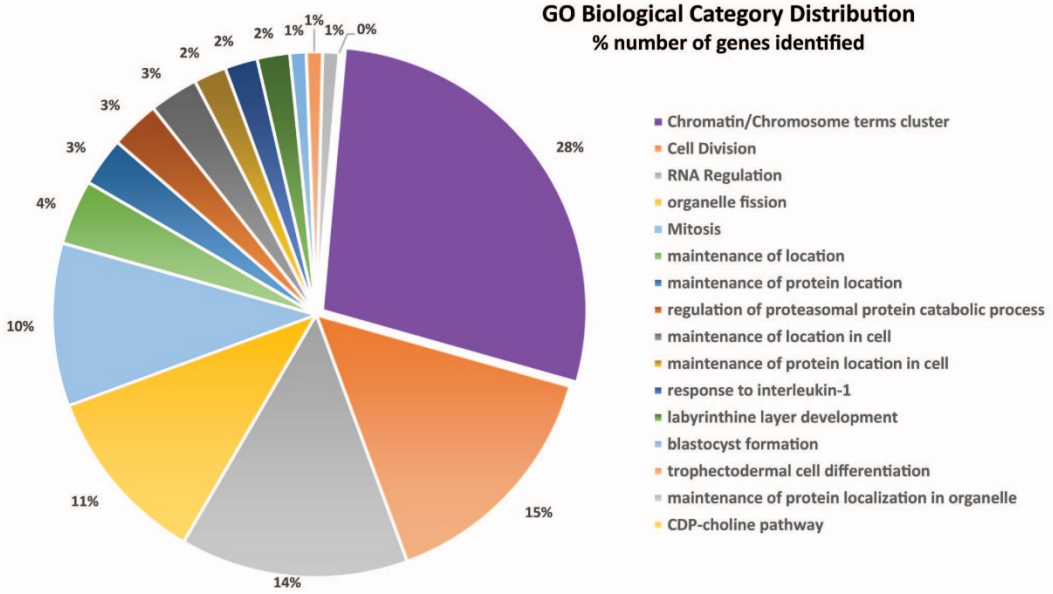


Figure 11 Satb2 ChIP-seq analysis from proliferating myoblasts. (a) GREAT-input identified 8413 (91.5%) of the 9010 peaks generated from from Satb2 ChIP-sequencing; identified peaks corresponded to 38% of the 21902 mus musculus genes classified in the UCSC mm10 NCBI build 38 species assembly; (b) Table shows the top 25 Gene Ontology (GO) Biological Process categories identified within the Satb2 ChIP-seq dataset **C)** Pie chart shows the distribution of the number of genes identified as it relates to GO category clusters.

We tabulated our selected subsets of downregulated genes in Table 1 and upregulated genes in Table 2. Gene Ontology analysis of the downregulated genes subset shows that Satb2 knockdown affected a number of genes categorized as: positive cell proliferation genes (GO 0008283: Hdgfrp3, Slc29a2); general transcription control (GO 0006351: Zfp808, Hdac9, Erbb2; GO 0006357: Nfyc, Hmgn1; GO 0000122: Nedd4l, Rara), as well as a number of other genes implicated in pathways identified to be active during growth/proliferative cell stages. As for Gene Ontology analysis of the upregulated genes subset, Satb2 knockdown affected as number of genes in categorizes such as: chromatin modification (GO 0016568: Taf9, Smarca4, Kat2b); general transcription (GO 0006351: Gtf3c3, Zfp521) as well as a number of differentiation specializing genes. Lastly, the focus of this thesis work is on the gene profile switch from proliferative cells into differentiated myotubes, to that extent we show the effect of Satb2 on a selection of genes that are known to be involved either directly in differentiation, or as upstream chromatin modifiers leading into the differentiation pathway (figure 11b,c and figure 12d). Our ChIP and RNA-sequencing analysis indicates a large number of genes are associated with Satb2, and others are altered by its knockdown, that have no direct link to the proliferation or differentiation pathways studied in this thesis. Here we will state that Satb2 is a higher order chromatin structure protein that regulates genes in a context dependent manner, and that its effects extend beyond the control of proliferation/differentiation gene profile switch.

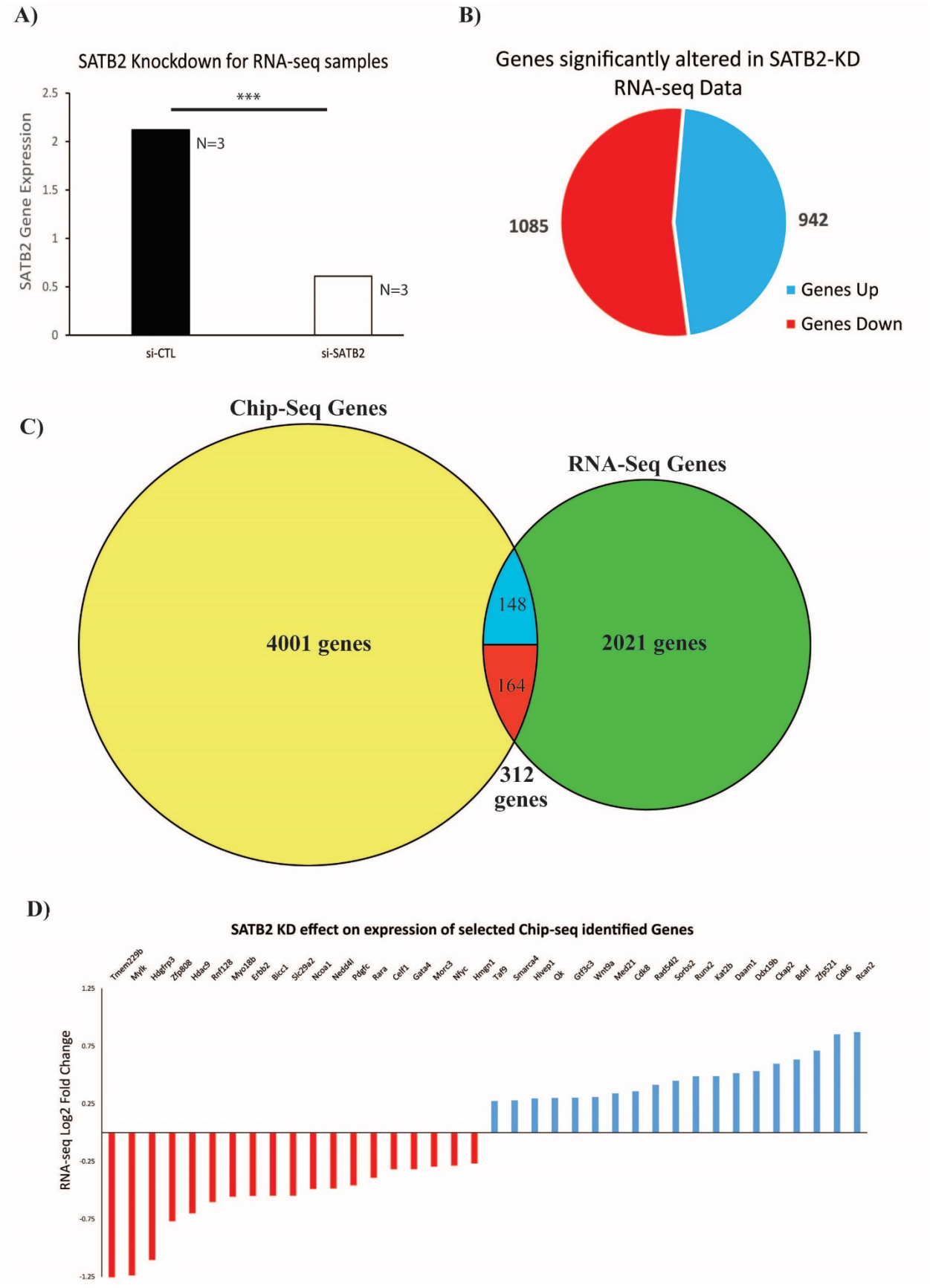

A)
SATB2 Knockdown for RNA-seq samples
***
N=3
N=3
SATB2 Gene Expression
2.5
2
1.5
1
0.5
0
si-CTL
si-SATB2
B)
Genes significantly altered in SATB2-KD RNA-seq Data
1085
942
Genes Up
Genes Down
C)
Chip-Seq Genes
RNA-Seq Genes
4001 genes
148
164
2021 genes
312 genes
D)
SATB2 KD effect on expression of selected Chip-seq identified Genes
Tmem229b
Mylk
Hdgfrp3
Zfp808
Hdac9
Rnf128
Myo18b
Erbb2
Bcc1
Slc29a2
Ncoa1
Nedd4l
Pdgfc
Rara
Celf1
Gata4
Morc3
Nfyc
Hmgn1
Taf9
Smarca4
Hivep1
Ok
Gtf3c3
Wnt9a
Med21
Cdk8
Rad54l2
Sorbs2
Runx2
Kat2b
Daam1
Ddx19b
Ckap2
Bdnf
Zfp521
Cdk6
Rcan2
RNA-seq Log2 Fold Change
1.25
0.75
0.25
-0.25
-0.75
-1.25

Figure 12 *Satb2* knockdown RNA-seq analysis from 24hr differentiated cells. (a) Validation of *Satb2* knockdown in tested samples, Black bar shows control condition, white bar shows knockdown samples; (b) Cuffdiff analysis of dataset show that 2027 genes were identified to have significantly altered expression between control and knockdown condition, with 1085 genes being significantly downregulated in expression and 942 genes being significantly upregulated in expression; (c) Venn diagram comparing MACS-identified Satb2 ChIP-seq genes (4001) at growth condition with Cuffdiff-identified Satb2 knockdown RNA-seq genes (2021) at 24hr differentiated cells. 312 genes were found to be bound to Satb2 in proliferating cells that became significantly altered in expression upon knockdown and *Satb2* gene, of those 312 genes 164 genes were significantly downregulated and 148 were significantly upregulated; (d) Graph highlights a selection from the 312 genes identified in common between the ChIP-seq and RNA-seq datasets. These selected genes (19 upregulated, 19 downregulated, see table I and II for expanded characterizations) are all known to be involved in the genetic switch from a proliferation to a myoblast differentiation molecular program. RNA-seq samples N=3 per condition, ***p<0.001

Gene ID	Log2 Fold	Gene Name	GO Term ID	GO Biological Process Category
Tmem229b	-1.262	Transmembrane Protein 229B	GO:0016021	integral component of membrane
Mylk	-1.239	Myosin Light Chain Kinase	GO:0006939	smooth muscle contraction
Hdgfrp3	-1.105	Hepatoma-Derived Growth Factor, Related Protein 3	GO:0008283	cell proliferation
Zfp808	-0.770	Zinc Finger Protein 594	GO:0006351	transcription, DNA-templated
Hdac9	-0.701	Histone Deacetylase 9	GO:0006351	transcription, DNA-templated
Rnf128	-0.602	Ring Finger Protein 128	GO:0031647	regulation of protein stability
Myo18b	-0.556	Myosin 18B	GO:0001701	in utero embryonic development
Erbb2	-0.549	Erb-B2 Receptor Tyrosine Kinase 2	GO:0006351	transcription, DNA-templated
Bicc1	-0.547	BicC Family RNA Binding Protein 1	GO:0090090	negative regulation of canonical Wnt signaling pathway
Slc29a2	-0.547	Solute Carrier Family 29 Member 2	GO:0008283	cell proliferation
Ncoa1	-0.489	Nuclear Receptor Coactivator 1	GO:0016568	chromatin modification
Nedd4l	-0.485	Neural pre. Expressed, Developmentally Downregulated 4-Like	GO:0000122	negative regulation of transcription from RNA pol II promoter
Pdgfc	-0.458	Platelet Derived Growth Factor C	GO:0009790	embryo development
Rara	-0.392	Retinoic Acid Receptor Alpha	GO:0000122	negative regulation of transcription from RNA pol II promoter
Celf1	-0.319	RNA-Binding Protein BRUNOL-2	GO:0009790	embryo development
Gata4	-0.318	GATA Binding Protein 4	GO:0003208	cardiac ventricle morphogenesis
Morc3	-0.296	Nuclear Matrix Protein 2	GO:0009791	post-embryonic development
Nfyc	-0.287	Nuclear Transcription Factor Y Subunit C	GO:0006357	regulation of transcription from RNA pol II promoter
Hmgn1	-0.268	High Mobility Group Nucleosome Binding Domain 1	GO:0006357	regulation of transcription from RNA pol II promoter

Table 1 Satb2 ChIP-seq identified RNA-seq Downregulated Genes.

Gene ID	Log2 Fold	Gene Name	GO Term ID	GO Biological Process Category
Taf9	0.274	TATA-Box Binding Protein Associated Factor 9	GO:0016568	chromatin modification
Smarca4	0.279	SWI/SNF rel. Matrix Assoc. Actin-dep. Regulator Chromatin A4	GO:0016568	chromatin modification
Hivep1	0.295	Positive Regulatory Domain II Binding Factor 1	GO:0006366	transcription from RNA pol II promoter
Qk	0.300	Quaking Homolog, KH Domain RNA Binding	GO:0008380	RNA splicing
Gtf3c3	0.302	General Transcription Factor IIIC Subunit 3	GO:0006351	transcription, DNA-templated
Wnt9a	0.309	Wnt Family Member 9A	GO:0008285	negative regulation of cell proliferation
Med21	0.339	Mediator Of RNA Polymerase II Transcription Subunit 21	GO:0006357	regulation of transcription from RNA pol II promoter
Cdk8	0.357	Cyclin Dependent Kinase 8	GO:0051726	regulation of cell cycle
Rad54l2	0.413	Helicase ARIP4	GO:0005634	nucleus
Sorbs2	0.449	Arg Binding Protein 2	GO:0007015	actin filament organization
Runx2	0.487	Runt Related Transcription Factor 2	GO:0001501	skeletal system development
Kat2b	0.489	Histone Acetyltransferase KAT2B	GO:0016568	chromatin modification
Daam1	0.515	Dishevelled Associated Activator Morphogenesis 1	GO:0030036	actin cytoskeleton organization
Ddx19b	0.533	DEAD-Box Helicase 19B	GO:0006406	mRNA export from nucleus
Ckap2	0.597	Cytoskeleton Associated Protein 2	GO:0045944	positive regulation of transcription from RNA pol II promoter
Bdnf	0.633	Brain Derived Neurotrophic Factor	GO:0031547	brain-derived neurotrophic factor receptor signaling pathway
Zfp521	0.711	Zinc Finger Protein 521	GO:0006351	transcription, DNA-templated
Cdk6	0.852	Cyclin Dependent Kinase 6	GO:0000082	G1/S transition of mitotic cell cycle
Rcan2	0.870	Regulator Of Calcineurin 2	GO:0070884	regulation of calcineurin-NFAT signaling cascade

Table 2 Satb2 ChIP-seq identified RNA-seq Upregulated Genes

3.5: Discussion

Remodeling chromatin structure is a hallmark of cellular differentiation, a process that is concurrent to, and influences global alterations in gene expression [Muller and Leutz, 2001, Kimmins and Sassone-Corsi., 2004, Fisher and Fisher, 2011]. Chromatin higher-order structure proteins, such as matrix attachment region proteins (MARs), play a key role in remodeling and access of genetic material depending on what transition cells are progressing to [Wang et al., 2010, Barboro et al., 2012, Zhao et al., 2014]. Here our presented results suggest that MARs proteins such as Satb2 strongly influence global gene expression patterns, modulating the progress of muscle cell differentiation. Specifically, we show that Satb2 protein is targeted and cleaved by effector caspases (caspase 7), which leads to a robust drive of the myogenic differentiation program.

The role of effector caspases during cellular differentiation has been intensely studied over the past decade [Bell et al., 2016]. As such it was reasonable to investigate whether MARs proteins could be subject to caspase targeting, and whether this enzymatic event would influence similar cell fate outcomes. Indeed, studies showing a link between nuclear matrix attachment region proteins and cleavage by effector caspases during apoptosis [Galande et al., 2001, reviewed in Wang et al., 2010] provide an interesting basis for exploring a wider set of interactions between chromatin structure proteins and effector caspases active during early differentiation. We focused on Special AT-rich binding protein 2 because of the original discovery of SATB1 sensitivity to caspase 6 cleavage, as well as the genetic profile comparing SATB1 and Satb2 in muscle progenitor cells [Zhao et al., 2014, Price et al., 2015] showing a significant change in the expression of Satb2 in this model system compared to the constant expression and non-alteration of SATB1 profile.

We initially established the Satb2 protein expression profile from growth up to 72hrs post induction of differentiation in both C2C12 cells, as well as mouse primary myoblasts. The highest expression of full length Satb2 was noted during the proliferative growth phase, followed by a gradual decrease as the differentiation program progressed. The protein fragments we observed in the western blot data led us to test whether Satb2 was subject to proteolytic cleavage, specifically the two active effector caspases that are highly expressed in skeletal muscle myoblasts [Fernando et al., 2002]. The in vitro cleavage assays in figure 5a demonstrated that Satb2 was a bona fide caspase 7 substrate, but not targeted by caspase 3. Although similar in structure and classically determined to have the same cleavage site targets, caspase 3 and 7 have been shown to have unique protein targets in numerous cell types [Walsh et al., 2008, Lamkanfi and Kanneganti, 2010]. Our data provide another example of this differential sensitivity to cleavage that some proteins exhibit for caspase 3 vs. 7.

We analyzed the main cleavage fragments obtained from caspase 7-Satb2 assays in figure 5b, and we have identified a previously unknown cleavage site on Satb2 located at aspartic acid residue D477. Typically, caspase proteases target aspartic acid (D) residues in substrate proteins, provided the D amino acid falls in a structurally accessible region of the protein, usually the region for caspase 3 and caspase 7 is a stretch D-E-V-D of amino acids, with the cleavage coming after the D following the V amino acid. In the case of Satb2, the prospective caspase 7 cleavage site comes after a stretch of I-K-V-D amino acids, which provides a local structure sufficiently similar to the classic DEVD site, yet somewhat variable from the prototypical caspase 3 site DEVD/DXXD. Nevertheless, more in depth biochemical and structural examination of Satb2 is required to provide a definitive understanding why this protein is preferentially targeted by caspase 7. of this novel site identified in the present study. As an

initial proof of effector caspase/Satb2 sensitivity, we performed caspase 3/7 inhibition on growing cells using the chemical molecule z.DEVD.fmk and initiated differentiation 24hrs post treatment with the inhibitor.

Having established a functional relationship between Casp7-Satb2, we proceeded to elucidate the specific roles that Satb2 played in cell differentiation, and whether Satb2 regulated gene expression that controlled the transition from growth to differentiation. To address this, we performed a series of Satb2 knockdown experiments in our muscle progenitor cell model, looking separately at the effects of the loss of Satb2 during proliferative cells, then at the effects during actively differentiating cells. In figures 7 and 8 we show that in proliferation phase, the loss of Satb2 by silencing RNA knockdown did not affect the progenitor cell ability to expand, which was an unexpected outcome. We originally hypothesized that Satb2 knockdown may lead cells to directly abort proliferation and initiate cellular differentiation, however our results negate that theory and present a new, more nuanced role that Satb2 played in this mechanism, which required further elucidation. We investigated the effect of Satb2 knockdown in differentiation phase next. In figures 9 and 10, our results showed cells lacking in Satb2 were able to accelerate differentiation by exhibiting the ability to fuse and form myotubes at an earlier stage compared to normal cells. Expressions of mature muscle specific proteins were observed, as well as an overall phenotype of myotube formation was evident.

These data led us to conclude that Satb2 may repress the genetic programming that promotes differentiation/myogenesis. Therefore, we sought to investigate on a global level, what part of the genome is targeted by Satb2, and whether a loss of Satb2 would engage a global gene expression alteration that was consistent with activation of a pro-differentiation gene expression program. To establish the starting point of Satb2 genetic relationships, we chose to perform

chromatin immunoprecipitation on proliferating C2C12 myoblasts, considering cells at this stage express the highest levels of Satb2. Our results, in figure 11, show that in the mouse genome, Satb2 is found to bind to approximately 40% of known genes during cell proliferation phase, which matches the broad foci immunolocalization profile in proliferating myoblast nuclei. ChIP-sequencing data analysis has given us an understanding that Satb2 is most likely a protein that acts as a general chromatin remodeler because of the high proportion of binding to genes identified as chromatin modification agents, as figure 10c,d shows. Satb2 has been described previously as acting in a transcription factor manner [Dobreva et al., 2003, Britanova et al., 2005, Chung et al., 2011], and while our ChIP-seq results do not contradict these results, we propose that Satb2 binding to many chromatin modifiers during proliferation is indicative of a chromatin structural modification role, rather than a classic transcriptional factor function.

To further characterize the functional activity that Satb2 exerts on myoblasts we performed RNA-sequencing analysis, comparing normal control cells with Satb2 knockdown cells at an early point in the differentiation program (24 hours post low serum induction). In addition, we conducted a comparative analysis between the RNA-seq and ChIP-seq datasets to identify whether genes bound by Satb2 displayed significant alteration in gene expression. Accordingly, we were able to identify 312 genes that were present in both sets of genomic sequencing, with approximately half the genes showing a significant upregulation in expression after Satb2 knockdown and the other half showing a significant downregulation in expression after Satb2 knockdown and differentiation for 24hrs. This data suggested that Satb2 is not strictly an inductive or repressive regulator of gene expression, with equivalent degrees of both gene expression trends. This data leaves space for more exploration and need to understand the type

of structural interaction Satb2 shares with genes it binds to, in order to characterize more accurately the effect of Satb2 binding to genes both during proliferation and early differentiation.

Here we have provided sufficient evidence that Satb2 knockdown does in fact affect genes known to be involved in chromatin modification pathways, as well we see a downregulation of proliferation promoting genes and an upregulation of differentiation promoting genes. All together, the genomics data analyzed does provide a basis for the phenotype observed of accelerated differentiation with the loss of Satb2. Further analysis will be required to fully understand the extent of control Satb2 has on the full genomic field that Satb2 plays a critical role in. Chromatin remodeling is essential in the control of the global positioning and accessibility of genes related to activation of differentiation pathways. While a lot of research explores the post-translational modifications that control the epigenetic mechanisms of activation/repression of genes, there are other higher-order chromatin structural changes that also play a vital role in this type of control. Special AT-rich binding protein 2 (Satb2) is a nuclear matrix attachment region protein that is implicated in the regulation of gene activation and repression. We sought to investigate the mechanism responsible for controlling Satb2-related chromatin restructure within the early differentiation of progenitor muscle cells. In this study we show that effector caspase 7 is responsible for cleavage of Satb2 altering the genetic profile expressed leading to acceleration of the differentiation program. Our study adds yet another role in which effector caspases are vital in the development of cells, and are not strictly confined to their well-studied role in apoptosis.

Chapter 4.0: General Discussion

4.1: Summary of Thesis:

Muscle cell differentiation has been shown in part to be regulated by the cleavage activity of effector caspase-3 [Fernando et al., 2002, Larsen et al., 2010, Dick et al., 2015]. DNA damage has also been shown to be a positive regulator of gene expression [Ju et al., 2006, Larsen et al., 2010, Puc et al., 2015]. In this thesis, we demonstrate for the first time that DNA damage events lead to the recruitment of base excision repair protein XRCC1, and that this scaffold protein is essential for DNA repair and the ensuing progression of progenitor muscle cell differentiation [Al-Khalaf et al., 2016].

Additionally in this thesis, we provide evidence of the activation and nuclear localization of effector caspase-7 during early myoblast differentiation. This is a new and parallel pathway of caspase regulation to be investigated for possible roles that caspases play in the chromatin remodeling events occurring in the transformation of progenitor cells into mature myotubes. Here, we present evidence that the matrix attachment region protein Satb2 is a target of caspase-7 cleavage, facilitating its removal from the nucleus and altering gene expression, which promotes the differentiation of skeletal muscle cells.

Taken together, these observations suggest that caspase signaling promotes muscle cell differentiation through a coordinated targeting of substrates. In turn, cleavage of these substrates leads to genome wide alterations that are permissive for establishing a differentiation specific gene expression program (see summary figure of novel proposed model figure 1).

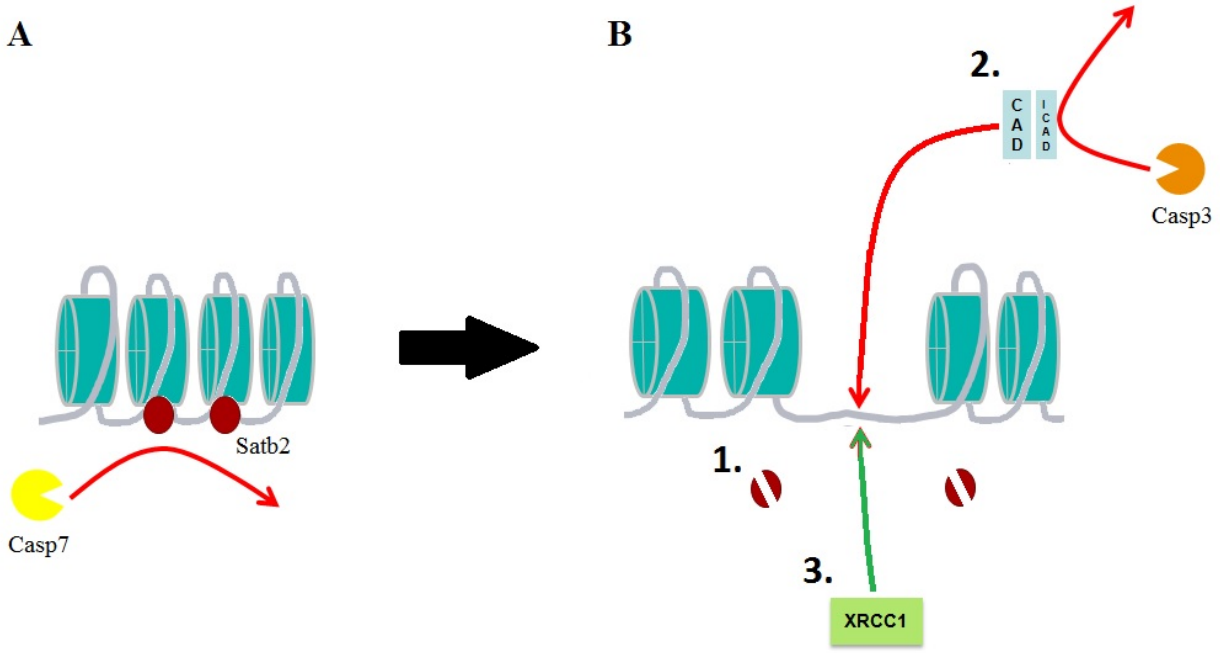


Figure 1: Coordinated effector caspases cleavage of multiple targets is essential for muscle cell differentiation. A) Muscle progenitor cells in active proliferation. Satb2 is part of the higher order chromatin structure preferred by multiplying cells. **B)** caspase 7 can target and cleave Satb2 (1.), initiating chromatin conformational changes that promote exit from the cell cycle, including caspase 3 cleavage of ICAD, leading to the activation of CAD (2.) followed by the recruitment of XRCC1 (3.) to specific genomic loci, and initiation of myogenic differentiation.

4.2: DNA Damage and repair mechanisms a novel control mechanism to control gene expression

CAD is a caspase sensitive endonuclease that induces DNA damage in the cell, and as mentioned earlier, CAD has been shown to trigger p21 gene expression by inflicting a strand break within the p21 promoter region [Larsen et al., 2010]. XRCC1 is a base excision repair protein and the present thesis work has demonstrated that this scaffold protein is responsible for repairing the strand break on the p21 promoter produced by CAD [Al-Khalaf et al., 2016]. We provide evidence in vivo that loss of XRCC1 (via conditional knockout of the Xrcc1 gene) leads to a

severe disruption of skeletal muscle differentiation, confirming the importance of DNA repair mechanisms in the maturation of this cell lineage.

If DNA strand breaks are not resolved, the accumulation of damage initiates a host of deleterious consequences within a cell [Roos and Kaina, 2013, Insinga et al., 2014]. One of the most studied phenomenon associated with strand break accumulation/lack of proper repair process are the transformation events associated with the development of cancer [Poirier, 2004, Santos et al., 2014]. Research has shown that some genes become overexpressed due to increased DNA damage assault, such as cell cycle arrest proteins $p16^{INK4a}$ and $p21^{CIP1}$ [Alcorta et al., 1996, Brown et al., 1997], and the senescence-associated senescence phenotype (SASP) proteins [Rodier et al., 2009, Tchkonia et al., 2013, Wiley et al., 2016]. These observations provide evidence that gene expression profiles are governed by the DNA damage/repair mechanisms. The data in this thesis and from previous work by [Larsen et al., 2010] provide a concrete example of endogenous targeting of a specific gene promoter (p21) by a DNA damaging protein, and the subsequent essential repair process required to activate the expression of the gene and promote the muscle differentiation pathway. Unlike the cancer example, our observations suggest that DNA damage and DNA repair can be utilized to alter cell fate during normal developmental processes, without inducing a cancer like phenotype.

DNA strand breaks/damage have been assumed to be pathologic for a cell [Zhao and Elledge, 2000, Rastogi et al., 2010, Freitas and de Magalhaes, 2011], yet there is growing evidence to indicate that this modification can be used to alter gene expression without impairing cell viability. This is similar to the classical view of caspase proteases as being agents of cell death due to their cleavage action on their target proteins. Presently caspases are realized to have a multitude of non-death roles due to their ability to modify the structure of proteins [Shrestha and

Megeney, 2012, Dick and Megeney, 2013]. There remains much to be investigated in this novel discovery relating to caspase initiating DNA damage (through activation of CAD) and gene expression control. Specifically the need to fully understand the ways in which targeted DNA damage induction and selective repair can be used by cells as a reliable gene expression altering mechanism [Saada et al., 2017]. One reasonable hypothesis is that CAD induced strand breaks on gene promoters may serve as a mechanism to co-recruit the DNA repair factors and transcriptional machinery, allowing for activation of the gene. This type of interplay between DNA damage/ and repair proteins with transcriptional machinery proteins has been demonstrated by [Ju et al., 2006], specifically elucidating the role of topoisomerase IIb in inducting double strand breaks at the promoter region of certain genes. Additionally, work by [Perillo et al., 2008] has shown that estrogen, OGG1 and TopoIIB are all involved in initiating strand breaks (first by generating single strand breaks to act as entry points for TopoIIB to induce double strand breaks) to modify local chromatin architecture. DNA damage could also be used within gene exons or other regulatory areas to cause arrest of gene expression or temporary downregulation of its expression. A study by [Williamson and Lees-Miller, 2010] presents data that shows DNA double strand breaks caused by TopoIIB at estrogen inducible genes persist for a prolonged period of time and are thought to be a physical barrier to DNA replication. Most recently, a review of the emerging science behind the DNA damage and repair link to regulation of gene activation has been published by [Puc et al., 2017], coining the term “programmed DNA nicks and breaks” as a mechanism of gene regulation. Investigating how cells can physically and temporally target specific gene loci will greatly add to the understanding of how cells can utilize DNA damage and repair mechanisms to regulate gene expression within a healthy biological context.

4.3: caspase mediated cleavage activity as a mechanism to induce chromatin remodeling

Caspase regulation of chromatin structure has been extensively studied in apoptotic cells, leading to the identification of a number of targeted proteins such as MST1 [Graves et al., 2001, Du et al., 2014], CBP/p300 [Rouaux et al., 2003], Helicard [Kovacsovics et al., 2002] and SATB1 [Galande et al., 2001]. However, it is likely that similar proteolytic targeting events modify chromatin structure during cell differentiation. Indeed caspase targeting of chromatin structural proteins could provide a rapid mechanism to alter gene expression on a genome wide level without additional alterations [Kimmins and Sassone-Corsi, 2005, Li et al., 2007, Fisher and Fisher, 2011, Chen and Dent, 2014]. The ultrastructure of the genome is responsible for both repression and activation of a broad array of genes. Organisms that have tens of thousands of genes have evolved complex packaging components to house and manipulate their large genomes. Chromatin proteins serve two main purposes within the nucleus: bind, condense, and pack DNA to protect it from physical damage, as well as control the transcriptional access [Li et al., 2007, Chen and Dent, 2014]. The primary structure of chromatin is defined as a 10nm fibre, frequently described as the “beads on a string” structure describing nucleosomes with the DNA double helix loop connecting individual nucleosomes together [Felsenfeld and Groudine, 2003]. The secondary structure is named the 30nm fibre, which describes a more packed set of nucleosomes folded closely together, and finally the higher order structures describe various levels of compaction of the chromosome components [Felsenfeld and Groudine, 2003]. Chromatin modification occurring on the primary structure can influence the expression of single genes, while chromatin modifications occurring in higher order structures can influence the expressions of hundreds or thousands of genes [Müller and Leutz, 2001, Fisher and Fisher, 2011, Chen and Dent, 2014]. Higher order chromatin modifications are necessary in cell-fate decisions

that occur when stem cells are triggered to differentiate, since such a transformation requires a great number of genes to change their expression profiles.

Histone modifiers such as methyltransferases, acetyltransferases (sometimes called “readers”), and histone demethylases, and deacetylases (called “erasers”) are all part of the dynamic epigenetic regulation mechanisms that utilize chromatin modifications to control gene expression [Falkenberg and Johnstone, 2014]. Chromatin remodeling proteins such as the SWI/SNF complex can destabilize the association between DNA and the histone octamer causing an effect named nucleosome sliding [Zhang et al., 2006, Tang et al., 2010]. This allows for easier access to gene promoter regions and activation of gene transcription [Ivana et al., 2001, Ohkawa et al., 2006, Forcales et al., 2012]. Other chromatin proteins such as the high mobility group proteins (HMG) play a role in associating with DNA independently from histones, causing structural changes like bending or circling of the DNA ribbon [Bustin and Reeves, 1996, Reeves, 2015], providing further control on transcription. More research needs to be performed to elucidate the roles in which caspase directed cleavage of chromatin and chromatin-associated proteins is a mechanism of gene regulation, specifically in the context of cellular differentiation.

As work from this thesis has demonstrated, effector caspase cleavage activity can be an effective method in modifying chromatin higher order structure. It can cause remodeling events that alter the expression profile of a large number of genes, culminating in major cell-fate decisions, such as the transformation from proliferation to differentiation gene profile. The activation and nuclear localization of caspase-7 during early myoblast differentiation was shown to target the matrix attachment region binding protein Satb2, leading to its cleavage and removal from the nucleus. This event was followed by the alteration of a large number of genes, ultimately leading to a robust differentiation phenotype observed in the model cell line studied. Effector caspase-7

cleavage of Satb2 is presented here as a novel mechanism in the regulation of chromatin structure within the context of normal biological events leading to a mature cell formation, not in a classical pro-apoptotic context. We hypothesize that this maybe a core biological mechanism used in cells undergoing major gene expression profile changes such as in differentiation pathways.

4.4: Conclusions and Future Directions

Taken together, our work supports a model in which effector caspases act as essential modifiers of gene expression during myoblast differentiation. We show that caspase-3 initiated DNA damage events are specifically recruiting XRCC1 mediated base excision repair, leading directly to the activation of cell cycle arrest gene p21, as well as affecting the expression levels of known muscle differentiation markers MEF2c and MCK. We also show a novel role for effector caspase-7 cleavage of matrix attachment region binding protein Satb2, initiating the removal of this higher-order chromatin structure protein from the nucleus, leading to the alteration of the gene expression profile of muscle progenitor cells towards a more robust differentiation phenotype.

Questions remain regarding the exact nature in which cells can utilize DNA damage agents such as CAD to target specific genes for activation or repression. The demonstration of the specific CAD induced strand break within the p21 promoter region [Larsen et al., 2010], and the resulting necessary repair process by the XRCC1 [Al-Khalaf et al., 2016] shed light on the molecular mechanism occurring on the gene locus prior to the initiation of transcription of this specific gene. Presently, knowledge as to how CAD is directed to induce a strand break within the p21 promoter region is unknown. It has been reported that the p21 promoter region resides in a

DNase I hypersensitivity site [Braastad et al., 2003], which could be a lead towards identifying the targeting potential of CAD. Furthermore, there remains to be established if there is a direct interaction between the DNA damage repair components necessary for activation of gene, as demonstrated with XRCC1 [Al-Khalaf et al., 2016] and the transcriptional machinery involved in activation of the gene. Presently there is little known evidence linking the two well studied molecular pathways. Biochemical experiments such as co-immunoprecipitation and verification using immunofluorescent staining colocalization can be utilized to investigate whether components of the DDR pathway, such as XRCC1, directly interact with the transcriptional machinery components, such as RNA polymerase II. Identifying whether there is a direct interaction between DDR proteins and RNA polII or whether mediator proteins bridge this interaction to drive gene expression should be a high priority for the field [Puc et al., 2017]. The investigation of this pathway could potentially complete the link between the original active caspase-3 triggering of CAD to induce strand breaks, followed by the recruitment of DDR leading to transcriptional activation of targeted genes. This could be a major discovery connecting the DNA damage and response field to the well studied activation of gene expression pathway.

Satb2 plays a critical role in regulating the differentiation pathway of muscle progenitor cells; however the exact nature of Satb2 control of differentiation requires further elucidation. Analysis of the full effect of caspase cleavage of Satb2 would necessitate the study of the Satb2 caspase-uncleavable mutant in both *in vitro* and *in vivo* models. We hypothesize that using either a D477A overexpression plasmid transfection approach, or a more elaborate CRISPR-Cas9 genetic mutant of Satb2, would result in compromised muscle differentiation ability. This would implicate Satb2 as a novel master regulator of muscle cell differentiation and the essential role

effector caspase-7 plays in activation of this pathway. A complete investigation of how effector caspases modify chromatin structure may require more extensive proteomic analysis, where systematic identification of all relevant proteins and respective cleavage sites are categorized in differentiating myoblasts. Caspase cleavage screening assays would provide valuable data towards understanding the full extent in which caspases are participating in the relevant pathways of interest. Photometric analysis can be performed of the theoretically identified potential cleavage targets incubated with effector caspase-3/7, with further follow-up performed on the most efficiently cleaved proteins. This method will produce a higher than desired rate of false positives, as proteins *in vitro* are of greater sensitivity to cleavage by effector caspases than in normal functional cellular biology context. Identifying potential candidates, followed by investigating their caspase sensitivity within the cellular context will yield novel targets of interest in the chromatin remodeling targeted by caspases during differentiation. There remains a lot to uncover in the search of non-death roles of caspases, with potential of high impact biological outcomes in understanding basic cell science and utilization in therapeutic purposes.

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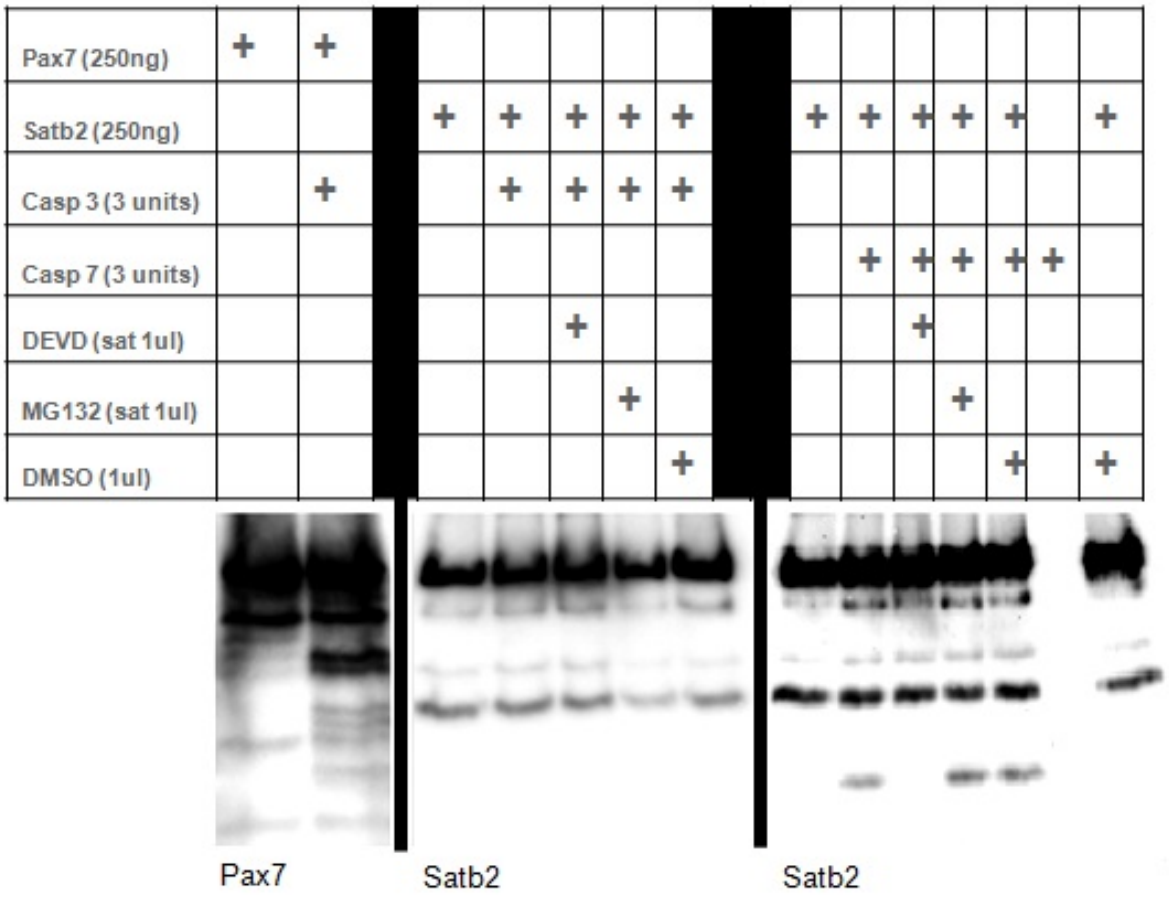
Appendix I



Comprehensive caspase Cleavage Assay Western blots. Pax7 protein is used as a positive control of caspase 3 cleavage potential. Equal amounts of Pax7 and Satb2 were used for the assays. Equal amounts of caspase 3 (Casp3) and caspase 7 (Casp7) were used to perform the cleavage assays. DEVD is the small molecule inhibitor of both Casp3/7. MG132 is the small molecule inhibitor of proteasomes. DMSO is used as control for DEVD treatments.

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Temporal activation of XRCC1-mediated DNA repair is essential for muscle differentiation

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Transient DNA strand break formation has been identified as an effective means to enhance gene expression in living cells. In the muscle lineage, cell differentiation is contingent upon the induction of caspase-mediated DNA strand breaks, which act to establish the terminal gene expression program. This coordinated DNA nicking is rapidly resolved, suggesting that myoblasts may deploy DNA repair machinery to stabilize the genome and entrench the differentiated phenotype. Here, we identify the base excision repair pathway component XRCC1 as an indispensable mediator of muscle differentiation. Caspase-triggered XRCC1 repair foci form rapidly within differentiating myonuclei, and then dissipate as the maturation program proceeds. Skeletal myoblast deletion of *Xrcc1* does not have an impact on cell growth, yet leads to perinatal lethality, with sustained DNA damage and impaired myofiber development. Together, these results demonstrate that XRCC1 manages a temporally responsive DNA repair process to advance the muscle differentiation program.

***Keywords*:** base excision repair; XRCC1; muscle differentiation; DNA strand breaks

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Introduction

Genome stability is of utmost importance for the survival and development of all organisms. Accordingly, eukaryotic cells have evolved a variety of mechanisms to maintain and repair DNA, of which these mechanisms are collectively referred to as the DNA damage response (DDR) [1, 2]. The DDR is further delineated by the type of DNA damage that is targeted and repaired, which may involve single [3] or double-strand breaks [4] and formation of toxic DNA adducts [5]. Although persistent DNA damage is generally regarded as detrimental for maintaining cell viability, transient DNA strand break formation has been identified as an effective means to enhance gene expression in living cells [6–9]. The best studied example in this regard is V(D)J recombination, a purposeful DNA damage and strand rearrangement event that underlies the genetic diversity of antibody and T-cell receptor generation in the adaptive immune system [10].

More recently, DNA strand breakage has been demonstrated to exert a profound effect on cell fate, acting to limit stem cell self-renewal and stimulate differentiation, without a concomitant increase in cell death [11–19]. In the skeletal muscle lineage, cell differentiation is dependent upon a temporal activation of the caspase 3 protease and its cognate DNase CAD (caspase-activated DNase), which act to enhance muscle gene expression through targeted DNA strand breaks [11]. These observations imply that a differentiating cell must utilize/recruit an equally potent DNA repair mechanism to stabilize the genome and secure the cell fate selection. The corollary to this hypothesis predicts that loss or inhibition of such a repair mechanism would result in failure to establish the differentiated state. Here, we sought to identify the repair machinery/proteins that target differentiation-induced DNA strand breaks and whether this operant mechanism was similar to or divergent from existing DNA damage responses (DDRs). Our observations

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indicate that base excision-mediated repair, as exemplified by the scaffold protein XRCC1, is essential for resolving differentiation-associated DNA damage and for securing this cell fate choice.

Results

The base excision repair pathway is involved in early myoblast differentiation

Skeletal muscle cells cultured under low-serum conditions recapitulate the differentiation program and are characterized by transient strand break formation [11]. Using this model system, we noted that the standard components of the nonhomologous end joining (NHEJ) pathway were not active during differentiation, as muscle cells did not display increased foci formation for the ATM kinase, 53BP1 and Ligase IV (Supplementary Figure S1a). Differentiating muscle cells give rise to long-lived cell types; therefore, we reasoned that the DNA damage may be more reflective of single-strand breaks/nicks versus catastrophic double-strand breaks. In support of this supposition, we noted that XRCC1, a key scaffold protein in the base excision repair (BER) pathway [14, 20, 21], forms discrete foci during differentiation (Figure 1a and Supplementary Figure S1a). The XRCC1 foci are transient and match the temporal DNA strand breaks that form during muscle cell differentiation (Figure 1a,b and c; as measured by DNA polymerase-guided *in situ* nick translation (INST)). Next, we ascertained whether these XRCC1 foci resulted from the caspase 3/CAD-mediated strand break activity that characterizes muscle cell differentiation [11, 22]. Using short hairpin RNA (shRNA) CAD muscle cell lines (previously described by our group [11]), we noted that loss of CAD expression resulted in a complete absence of XRCC1 foci formation (Figure 1d and e). Moreover, peptide inhibition of caspase 3 activity during muscle cell differentiation results in a complete loss of XRCC1 foci formation (Figure 1f). These results confirm that XRCC1 clustering in differentiating cells is a direct response to the caspase 3/CAD-induced DNA damage. These results also establish that this DNA break/replication repair cycle is a differentiation-specific event rather than a terminal mitosis response.

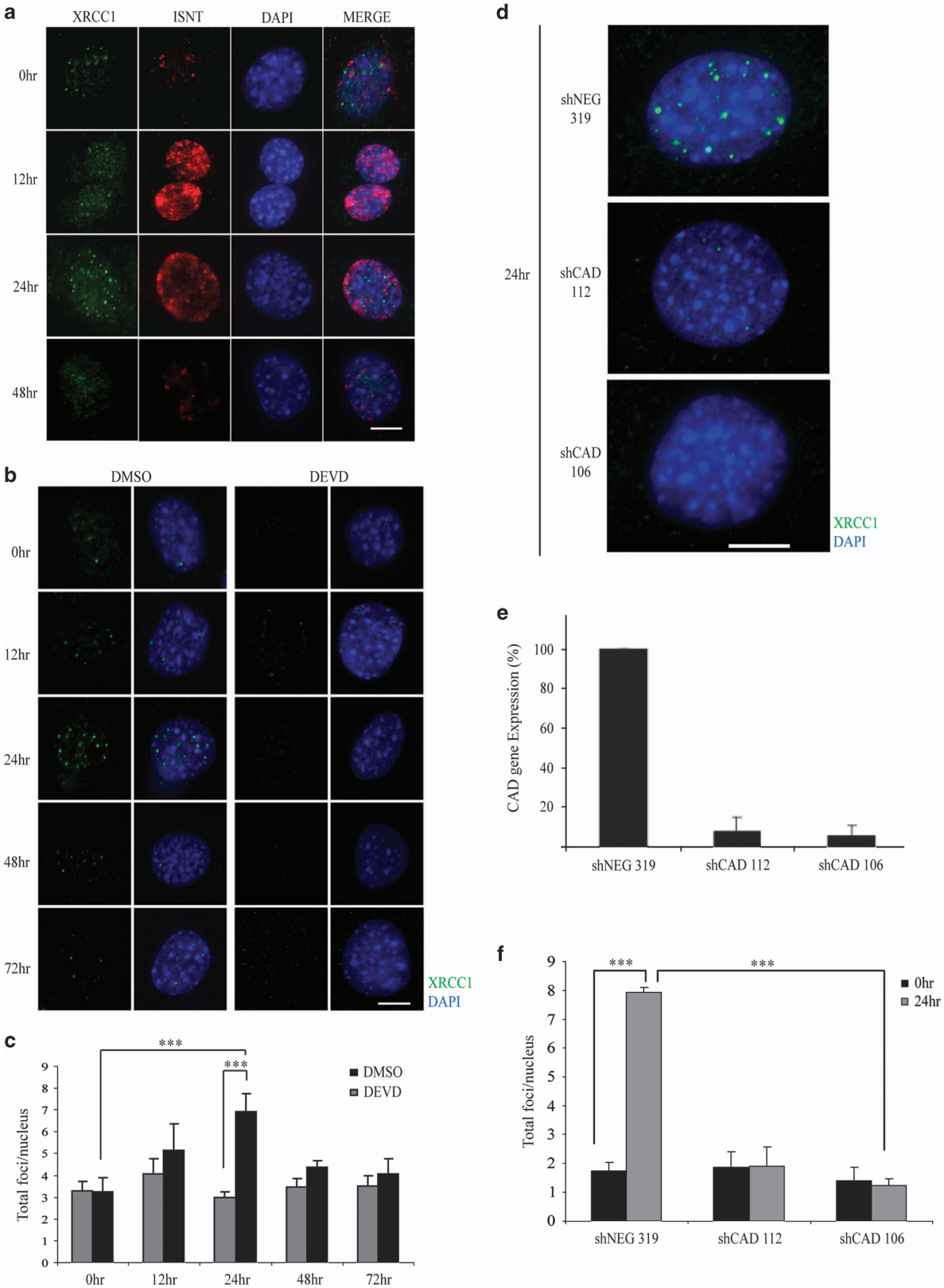
XRCC1 deletion halts C2C12 myoblast fusion and myotube formation

To assess the impact of XRCC1 expression in differentiating muscle cells, we initially performed shRNA-targeted *Xrcc1* (shXRCC1) gene repression. C2C12 muscle cells were co-transfected with shXRCC1/dsRED plasmid or negative control shRNA (shNEG) and dsRED immediately before low-serum induction of differentiation (Figure 2a and Supplementary Figure S2a). At 96 h post-low-serum exposure, shXRCC1-transfected muscle cells displayed significant impairment in the ability to form multinucleate myotubes with a concurrent reduction in the expression of differentiation-specific proteins (myosin heavy chain; Figure 2c and Supplementary Figure S2c). At all time points of equal transfection with shNEG or shXRCC1 (Supplementary Figure S2b), we noted a significant reduction in *Xrcc1* gene expression (Figure 2b), as well as XRCC1 protein foci (Figure 2a).

In vivo deletion of the Xrcc1 gene during early differentiation inhibits muscle tissue development

Next, we sought to assess the consequences of *in vivo* disruption of XRCC1 expression on skeletal muscle cell differentiation and muscle fiber maturation. *Xrcc1* null mice are early embryonic lethal [23]; as such, we generated a skeletal muscle conditional *Xrcc1* knockout model by cross-breeding $Xrcc1^{flox/flox}$ mice [24] with the *Myf5-Cre* mouse strain [25, 26]. *Myf5-Cre/* $Xrcc1^{flox/flox}$ mice were born in normal Mendelian ratios (Supplementary Figure S3c and d), yet all these animals suffered early perinatal lethality (Figure 3a and Supplementary Figure S4a). Nuclear protein

Figure 1 DNA repair during early myoblast differentiation is associated with XRCC1 foci. (**a**) Co-staining for XRCC1 foci formation and *in situ* nick translation, to measure DNA polymerase activity, in C2C12 myoblast cells over differentiation time course. Scale bar, 10 μm. (**b**) Immunofluorescent staining for XRCC1 in caspase 3 inhibited (DEVD) differentiating C2C12 cells and counterstained using 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI). Scale bar, 10 μm. Images are representative from $n = 3$ experimental replicates. (**c**) Data were quantified by counting the total number of foci per nucleus as represented in the histogram. (**d**) Targeted shRNA-mediated knockdown of CAD in differentiating C2C12 cells. The cells were induced to differentiate for 24 h and then were immunofluorescently stained for XRCC1. (**e**) Quantitative real-time PCR reveals an 80% reduction in CAD expression in the knockdown lines compared with the control. (**f**) Quantification of total XRCC1 foci per nucleus in proliferating cells (growth media) and after 24-h differentiation (24 h) is represented by histogram ($n = 3$). Asterisks (***) indicate that the changes in foci formation between the two conditions indicated are statistically significant as determined by two-tailed Student's *t*-test analysis with P-value < 0.01.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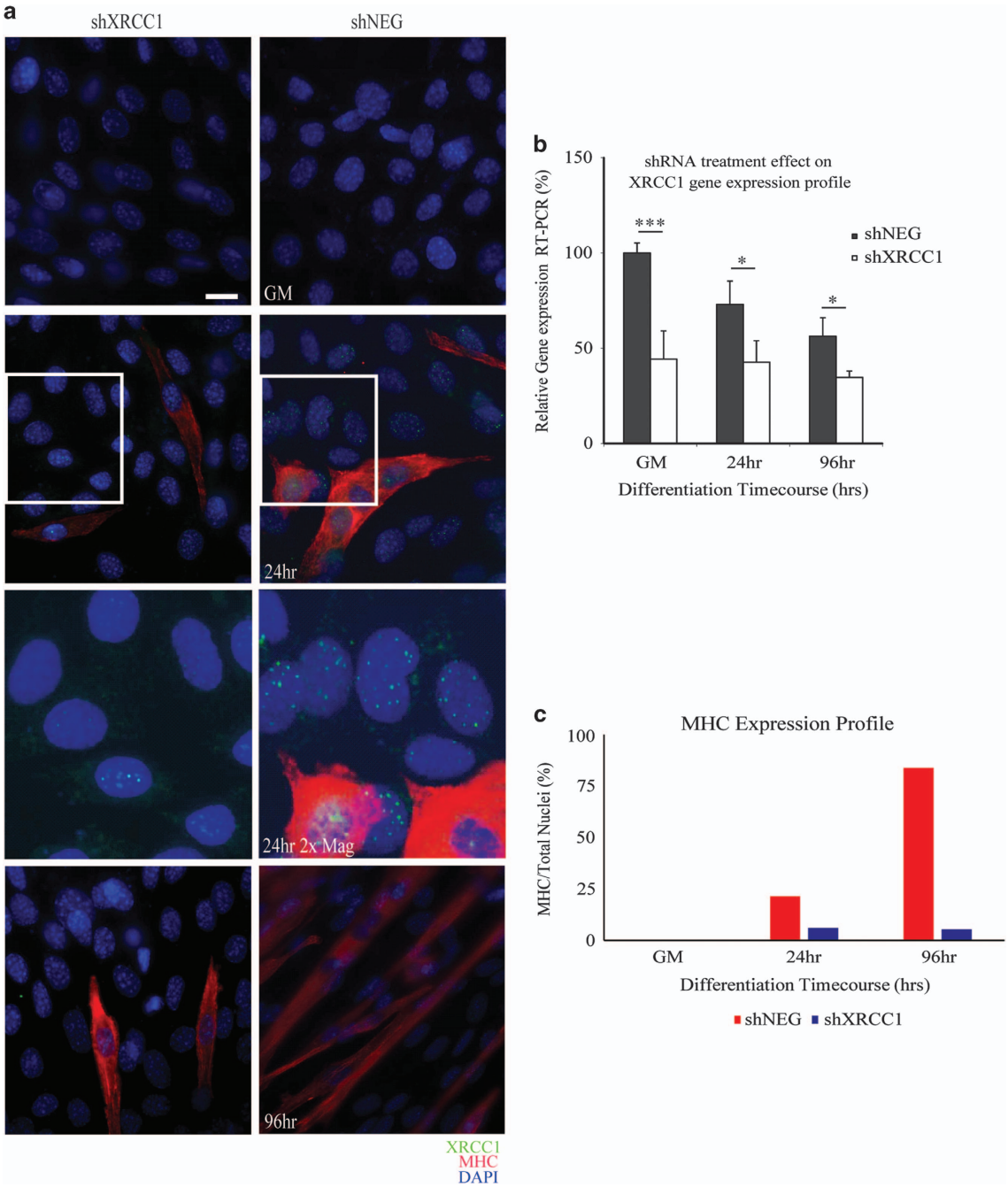


Figure 2 shRNA-mediated loss of *Xrcc1* impedes myoblast differentiation. (**a**) Differentiation time course of shRNA-treated C2C12 myoblasts. Magnified inset panels included for 24-h differentiated conditions performed using the Photoshop CS3 software (Adobe Systems Inc., San Jose, CA, USA). (**b**) Real-time PCR demonstrates a reduction in *Xrcc1* gene expression by 50% in shRNA-transfected cells at growth ($n = 3$, one-tailed Student's *t*-test analysis with ***P-value < 0.01 and *$P < 0.05$). (**c**) Scoring the percentage of differentiated C2C12 cells (myosin heavy chain (MHC) cells/total nuclei) shows a decrease in differentiation after loss of XRCC1. Images are representative from $n = 3$ experimental replicates. Scale bar, 50 μm.

extracted from pooled hindleg muscle showed a marked decrease in XRCC1 expression in the *Myf5-Cre/ Xrcc1*$^{flox/flox}$ mice relative to non-*Cre Xrcc1* controls (Figure 3a and Supplementary Figure S3a and b). This was specific to muscle, as tissue collected from non-*Cre*-targeted organs, such as the liver, did not show any marked change in XRCC1 protein expression (Figure 3a). *Myf5-Cre/ Xrcc1*$^{flox/flox}$ mice (body weight $= 1.10 \pm 0.1$ g, $n = 5$) were significantly smaller by 35% than wild-type littermates (body

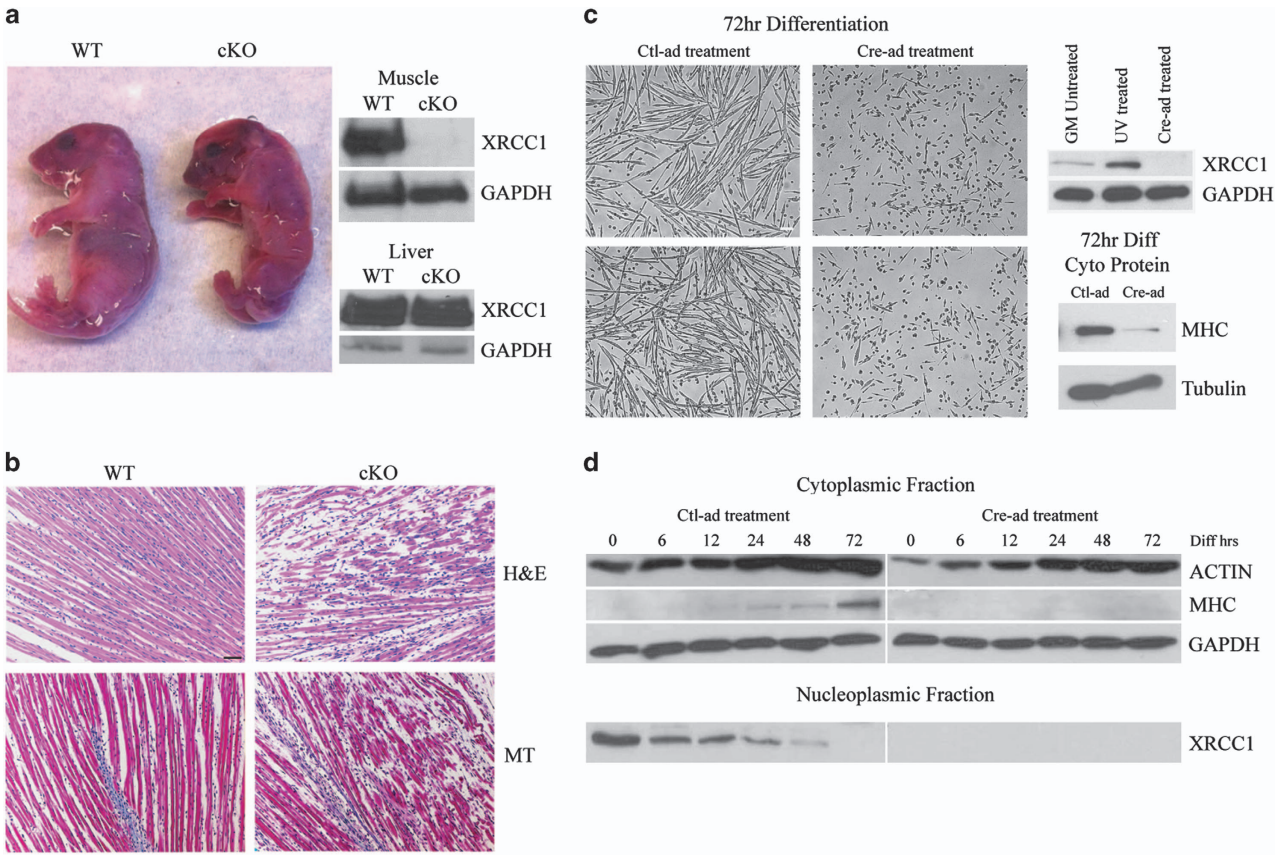


Figure 3 Gene-targeted loss of XRCC1 leads to attenuation of myofiber development and perinatal lethality. (**a**) Wild-type (WT) and *Myf5-Cre*/ *Xrcc1*$^{flox/flox}$ conditional knockout (cKO) of *Xrcc1* pup images taken immediately post birth. Nuclear protein extraction from pooled hindleg muscles, and from liver as control, is used for western blot and probed for XRCC1 and GAPDH. Images representative from $n = 5$ for each genotype. Western blot is representative from $n = 3$ per genotype. (**b**) Longitudinal skeletal muscle sections stained for hematoxylin and eosin (H&E) or Masson's Trichrome. Images representative from $n = 3$. Scale bar, 200 μm. (**c**) Primary myoblasts from *Xrcc1*$^{flox/flox}$ mice are treated with Cre-ad or Negative Ctl-ad and then induced to differentiate for 72 h. Images representative from $n = 5$. Scale bar, 200 μm. Western blot probed for XRCC1 is from nuclear protein fraction isolated from *Xrcc1*$^{flox/flox}$ primary myoblasts, either untreated, ultraviolet-treated to induce DNA damage or Cre-ad-treated to delete the *Xrcc1* gene. Western blot is representative from $n = 3$. Western blot for myosin heavy chain (MF20) in cytoplasmic protein lysates from *Xrcc1*$^{flox/flox}$ primary myoblasts that were treated with either Cre-ad or Ctl-ad, and induced to differentiate for 72 h. Western blot is representative from $n = 3$. (**d**) Western blot analysis from a time course treatment of *Xrcc1*$^{flox/flox}$ primary myoblasts induced to differentiate by low-serum exposure. Blots representative from $n = 3$.

weight $= 1.70 \pm 0.2$ g, $n = 5$) and displayed a severe lack of skeletal muscle development. Histologic examination of *Myf5-Cre*/ *Xrcc1*$^{flox/flox}$ skeletal muscle revealed blunted myofiber formation, increased prevalence of mononucleated muscle cells and increased interstitial space (Figure 3b and Supplementary Figure S4b)—changes that are consistent with a severe perturbation in the muscle cell maturation program. To confirm that the *Myf5-Cre*/ *Xrcc1*$^{flox/flox}$ skeletal muscle phenotype arose from a differentiation deficit, we examined differentiation kinetics in primary myoblasts isolated from *Xrcc1*$^{flox/flox}$ mice. *Cre*-adenovirus (Cre-ad)-infected *Xrcc1*$^{flox/flox}$ myoblasts displayed a thorough inhibition of the differentiation program, characterized by a loss in expression of myosin heavy chain and a complete inability to form multinucleate myotubes compared with control-Adenovirus (Ctl-ad)-infected cells (Figure 3c and d and Supplementary Figure S1b and c).

Loss of XRCC1 affects late muscle differentiation-specific and cell cycle-specific genes

The shRNA depletion and the *Cre*-mediated excision of the *Xrcc1* gene strongly suggest that muscle cell differentiation is dependent on engaging a temporally sensitive XRCC1-mediated DNA repair

event. Real-time PCR for canonical skeletal muscle differentiation markers was performed on control and *Xrcc1*-deleted primary myoblasts, and the results show that, while early-differentiation markers *myoD* and *myogenin* were not significantly altered by the loss of XRCC1, late-differentiation markers *Mef2c* and *MCK* were significantly reduced compared with control conditions (Figure 4a). In addition to muscle-specific genes, prior observations from our laboratory have established that caspase/CAD-induced DNA strand breaks act as a priming event to engage gene expression of nonlineage-specific regulatory factors. For example, a CAD-induced strand break in the promoter region of the cyclin-dependent kinase inhibitor 1 (p21) leads to induction of *p21* expression [11], a gene coding a protein critical for induction of differentiation across a broad spectrum of cell types [27]. Here, using chromatin immunoprecipitation-polymerase chain reaction (ChIP-PCR), we show that XRCC1 binds directly on the same *p21* promoter region upon induction of differentiation to repair the single-strand break (SSB)-induced by CAD (Figure 4c). When XRCC1 is absent, the *p21* gene is not transcribed, as we are able to show using Cre-ad infection of primary *Xrcc1* null myoblast cultures, leading to a complete failure to induce *p21* expression during differentiation (Figure 4b), suggesting that the post-strand break repair event is critical for gene induction at this loci.

Temporal activation of the Xrcc1 gene is responsible for progression of myoblast differentiation

In addition to acting as a gene expression regulatory event, the loss of XRCC1 in cycling myoblasts may also lead to the accumulation of DNA strand breaks, a genotoxic phenotype that could preclude myoblasts from engaging the differentiation program. However, the doubling time of *Xrcc1*-deleted myoblast cultures was similar to wild-type cells, suggesting that loss of XRCC1 before the onset of differentiation was not a significant impediment for myoblast growth and survival (Figure 5a and b). Cycling *Xrcc1*-deleted myoblasts display a moderate increase in the frequency of DNA strand breakage (as measured with comet assay); yet the transient DNA damage that characterizes normal differentiation is dramatically enhanced in *Xrcc1*-deleted myoblasts. Here, $>90\%$ of all *Xrcc1*-deleted myoblasts accumulate extensive DNA damage compared with $<40\%$ wild-type myoblasts, which resolve the transient breaks as the differentiation program proceeds (Figure 5c and Supplementary Figure S5a and b). To confirm the differentiation-specific function of XRCC1 (and the origin of the muscle phenotype in the gene-targeted mice), we performed temporal excision experiments in $Xrcc1^{flox/flox}$ myoblasts, where *Xrcc1* was deleted following the induction of differentiation. Cre-ad infection of $Xrcc1^{flox/flox}$ myoblasts at 3 and 6 h post-low-serum exposure led to a complete block in differentiation, similar to the results obtained for shRNA targeting of the *Xrcc1* gene and *Cre*-mediated deletion of *Xrcc1* in cycling myoblasts (Figure 5d). This experiment confirmed the temporally sensitive role of XRCC1 in stabilizing the differentiation phenotype. Cre-ad addition at 12–48 h post-low-serum induction did not impair formation of myosin heavy chain-positive myotubes in $Xrcc1^{flox/flox}$ myoblast cultures, suggesting that loss of XRCC1 at later stages was inconsequential for completion of the differentiation program (Figure 5e), whereas early deletion of XRCC1 shows significant loss of fusion, as well as overall myosin heavy chain protein expression (Figure 5d and f). In addition, flox-flox myocyte/post-myoblast-specific deletion of *Xrcc1* via the generation of *MCK-Cre*/ $Xrcc1^{flox/flox}$ mice had no observable impact on muscle development (these animals were born with normal Mendelian ratios), whereas primary myoblasts derived from the *MCK-Cre*/ $Xrcc1^{flox/flox}$ strain displayed the conventional differentiation response as with wild-type myoblasts (Figure 5g). These myoblasts possess the same XRCC1 protein expression profile as wild-type primary myoblasts differentiated up to 72 h (Figure 5h).

Discussion

Taken together, these results demonstrate that the temporal deployment of the BER-related DNA repair mechanisms (as exemplified by XRCC1) is essential for muscle cell differentiation. Clearly, p21 expression is XRCC1-dependent, an observation suggesting that XRCC1 manages a gene induction program that may be applicable to a wide range of cell lineages. Whether XRCC1 directly promotes a muscle-specific differentiation program remains undefined. Prior observations from our laboratory, along with the current study, suggest that certain elements of the skeletal muscle gene expression program are not influenced by caspase-mediated signaling events, such as the induction of myogenin expression [28]. Nevertheless, other muscle-specific genes appear to be responsive to the caspase/CAD/XRCC1 circuit, including Mef2c, MCK and myosin heavy chain. Identifying the range of XRCC1-responsive genes in a differentiating myoblast

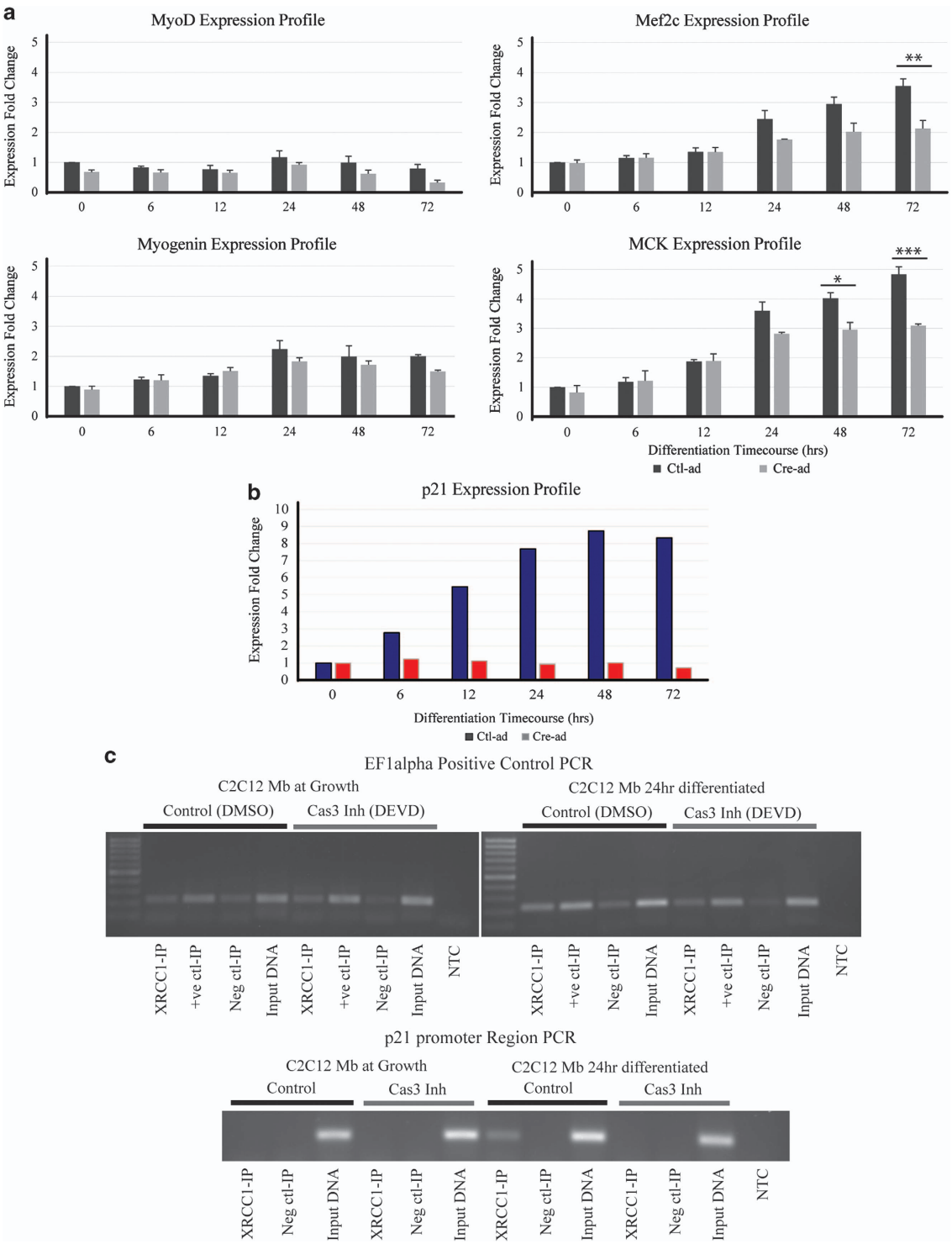


Figure 4 Skeletal muscle gene expression profiles are altered with *XRCC1* deletion. (**a**) Muscle-specific gene markers *myoD*, *myogenin* are unaffected by XRCC1 expression, whereas *Mef2c* and *MCK* inductions are significantly reduced in XRCC1-deleted cells ($n = 3$, two-tailed Student's *t*-test analysis with ***P-value < 0.01, **P-value < 0.025, *P-value < 0.05). (**b**) *p21* gene expression profile during early differentiation. *Xrcc1*$^{flox/flox}$ primary myoblasts infected with Ctl-ad (blue) or Cre-ad (red) and induced to differentiate up to 72 h displayed reduced p21 induction (**c**) ChIP-end point PCR of the p21 promoter region shows enhancement of XRCC1 binding during early differentiation of C2C12 cells compared with growth conditions ($n = 3$). Caspase 3 inhibition (DEVD) leads to loss of XRCC1-p21 promoter binding. EF1alpha is used as a non-target genomic control for the ChIP experiment.

will clarify to what extent XRCC1 is a general versus lineage-specific differentiation cue.

XRCC1 is also a component of the backup NHEJ (b-NHEJ) DNA repair pathway, which shares many common features with BER including the recruitment of PARP and DNA ligase III to the strand break site [29]. We have not directly ascertained whether the repair machinery is a BER or b-NHEJ mechanism; however, we favor the hypothesis that XRCC1 is scaffolding a BER repair factory. This supposition is based on the nature of the strand breaks that is predominant during the differentiation program. For example, we have observed that differentiating myoblasts are readily labeled via ISNT using a DNA polymerase, a process that will preferentially label a strand break/nick rather than a double-strand break. In addition, our COMET analysis on differentiating myoblasts displays a qualitatively different electrophoresis profile compared with myoblasts engaging true apoptosis, a cell fate that is characterized by double-strand breaks. Although these measures are not conclusive, they strongly suggest that XRCC1 is participating in a BER mechanism rather than a b-NHEJ repair pathway.

The prevalence of DNA strand breaks and XRCC1 repair foci in early-differentiating muscle cells suggests that this regulated form of damage may target a large number of gene induction events; what remains unknown is whether this targeted break/repair mechanism may also repress gene expression at discrete loci. Given that caspase-mediated signaling is a broadly conserved inductive cue for differentiation, a reasonable supposition is that caspase-driven DNA damage/XRCC1-mediated repair may act as an essential genomic reprogramming event in many cell lineages [23, 30, 31], including gene induction and gene repression. Our observations establish an XRCC1-BER-mediated repair mechanism during differentiation; whether other unrelated or unknown factors assist this process will require future investigations.

Materials and Methods

Mice and in vivo procedures

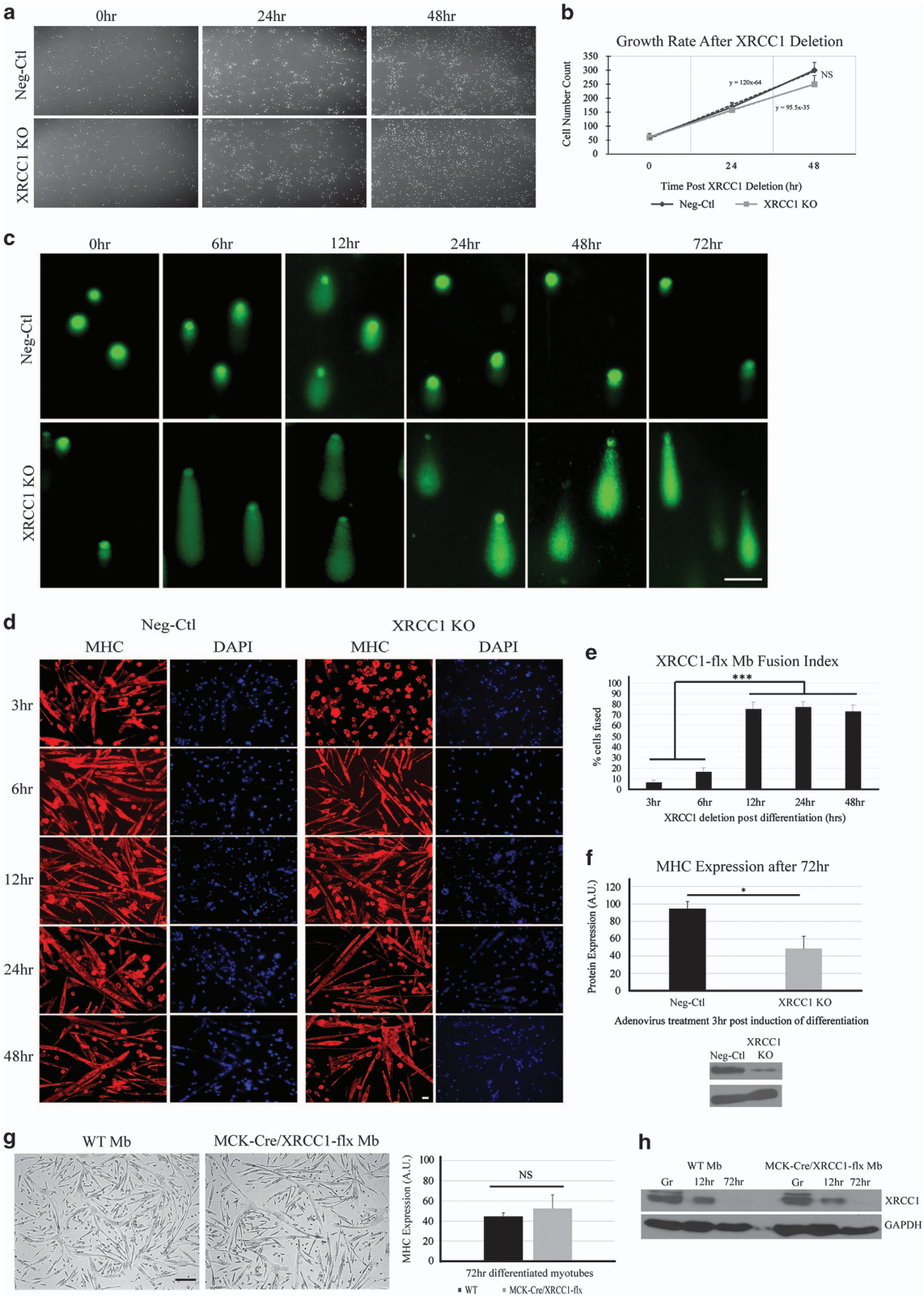
All transgenic mice were housed and treated at the University of Ottawa Animal Care and Veterinary Services. Mice used in our studies were housed and cared for according to the Canadian Council on Animal Care guidelines and University of Ottawa Animal Care Committee protocols. *Xrcc1*$^{flox/flox}$ mouse strain was obtained from Dr McKinnon [21]. *Myf5-Cre* and *MCK-Cre* mouse strains were obtained from Dr Rudnicki [23].

Transgenic pups and their wild-type littermates were taken shortly after birth, post natal < 1 day. Hindlimb muscle is extracted and frozen in microfuge tubes at -20 °C until further experiments are performed. Whole-pup bodies were placed in optimum cutting temperature gel-filled embedding molds (Polysciences Inc.) and then flash-frozen in liquid nitrogen to preserve for sectioning and staining. All sectioning and hematoxylin and eosin and Masson's Trichrome staining were performed by the University of Ottawa, Department of Pathology and Laboratory Medicine, Morphology Unit Lab.

Cell culture growth and differentiation protocols

For primary myoblast isolations, surgeries were performed as described previously [11]. Briefly, skeletal muscle from hindlimbs is extracted and finely cut with scissors until sufficiently liquefied isolate is achieved. The liquefied muscle chunks are then cultured in a dispase/collagenase solution (dispase II, Roche, New York City, NY, USA, and 1% collagenase (reconstituted in sterile phosphate-buffered saline (PBS)) with 2.5 mM $CaCl_2$). Several rounds of trituration and low-speed centrifugation followed by resuspension in Hams's F-10 Enriched growth media (Ham's F-10 medium from Gibco Life Technologies, Waltham, MA, USA, 20% fetal bovine serum, 2% penicillin-streptomycin (Pen-Strep), 0.6 μg/ml Fungizone and 2.5 μg/ml βFGF) will yield a population of primary myoblasts sufficiently free of any cross-contaminating tissue

Figure 5 XRCC1 has a temporally sensitive requirement to mitigate DNA damage in differentiating myoblasts. (**a**) Knockout of *Xrcc1* gene expression does not significantly affect myoblast proliferation potential. (**b**) Cell number counts quantified in graph. Images are representative from $n = 6$. (**c**) Single-cell gel electrophoresis (Comet assay) was performed on differentiating *Xrcc1*$^{flox/flox}$ primary myoblast cells treated with either Cre-ad or Ctl-ad. Following electrophoresis, cells were stained with SYBR green and visualized to assess the length of migration of DNA from the nucleus. Results show increased and persistent DNA damage in *Xrcc1* knockdown cells, whereas control cells exhibit damage early in differentiation that is resolved over the differentiation time course. Images are representative from $n = 4$ for each experimental condition; minimum number of cells counted $= 50$ for each condition. Scale bar, 15 μm. (**d**) Cre-ad-mediated knockdown of *Xrcc1* gene expression post induction of differentiation. Immunofluorescence staining for MHC was used to assess myotube formation. (**e**) The fusion Index graph quantifies myotube formation ($n = 3$, two-tailed Student's *t*-test analysis with ***P-value < 0.01). Scale bar, 20 μm. (**f**) Quantification of MHC protein at 3 h post induction of differentiation following adenovirus treatment. End point is 72 h post induction of differentiation ($n = 3$, two-tailed Student's *t*-test analysis with *P-value < 0.05). (**g**) Primary myoblasts isolated from *MCK-Cre*/*Xrcc1*$^{flox/flox}$ transgenic mice show equivalent differentiation capacity to WT myoblasts. Scale bar, 200 μm. Graph shows that MHC expression is equivalent between WT and *MCK-Cre*/*Xrcc1*$^{flox/flox}$ myoblasts differentiated for 72 h. (**h**) The XRCC1 protein expression profile is similar between WT and *MCK-Cre*/*Xrcc1*$^{flox/flox}$ myoblasts differentiated for 72 h.


a
0hr
24hr
48hr
Neg-Ctl
XRCC1 KO
b
Growth Rate After XRCC1 Deletion
Cell Number Count
y = 120x-64
y = 95.5x-35
NS
Time Post XRCC1 Deletion (hr)
Neg-Ctl
XRCC1 KO
c
0hr
6hr
12hr
24hr
48hr
72hr
Neg-Ctl
XRCC1 KO
d
Neg-Ctl
XRCC1 KO
MHC
DAPI
3hr
6hr
12hr
24hr
48hr
e
XRCC1-flx Mb Fusion Index
% cells fused
***
XRCC1 deletion post differentiation (hrs)
f
MHC Expression after 72hr
Protein Expression (A.U.)
*
Neg-Ctl
XRCC1 KO
Adenovirus treatment 3hr post induction of differentiation
XRCC1
g
WT Mb
MCK-Cre/XRCC1-flx Mb
MHC Expression (A.U.)
NS
72hr differentiated myotubes
WT
MCK-Cre/XRCC1-flx
h
WT Mb
MCK-Cre/XRCC1-flx Mb
Gr
12hr
72hr
XRCC1
GAPDH

remnants. The cells were maintained by replacing the media every 48 h. The cells were allowed to grow on collagen-coated plastic plates (2 ml of rat-tail collagen, Roche, containing 0.2% acetic acid was coated onto tissue culture plates). Ham's F-10 media is replenished every 48 h to maintain high nutrient content in the solution to maximize the growing potential of primary myoblasts and prevent unaccounted for differentiation. Primary myoblasts are passaged before reaching 70% confluence in the growth dish to further eliminate the chance of spontaneous differentiation. Differentiation was induced in primary myoblast cells by replacing the complete growth media with primary differentiation media (DMEM containing 5% horse serum and 2% Pen-Strep). Cells were collected by scrapping off the plates and centrifugation to pellet cells at the predetermined time points, or differentiation media was changed every 24 h until time point is reached.

Cell culture shRNA, ISNT, adenovirus and caspase inhibition protocols

Target sequences of shRNA were cloned into the appropriate vector (TRC mouse shRNA individual clone lentiviral pLKO.1 targeted to *Xrcc1*, Open Biosystems/Thermo Scientific, Waltham, MA, USA, pCMV-dsRed-Express Vector, Clontech-Mountain View, CA, USA, or nonsilencing pGIPZ (shNEG), Open Biosystems/Thermo Scientific) and the plasmid DNA was amplified by culturing the *Escherichia coli* engineered to express each plasmid in antibiotic media containing either Ampicillin or Kanamycin (40 μg/ml in LB broth). After colony expansion the bacterial plasmid DNA was purified using HiSpeed Plasmid Midi Kit from Qiagen, Valencia, CA, USA. The purified plasmid DNA was verified for size by performing a restriction digestion using the restriction enzymes *Bam*HI and *Cla*I. The digests were separated on 1% agarose gels (plus 0.1 μg/ml ethydium bromide) to check that the DNA fragments were of the appropriate size as predicted from the restriction map for the plasmid of interest. One day before transfection, C2C12 myoblast cells were cultured in 35-mm tissue culture plates in Pen-Strep-free growth media to a confluence of 50–60%. The cells were rinsed with 1 × OptiMEM I reduced serum media (Gibco Life Technologies) before transfection. The shRNA was prepared by diluting 16 μg/ml DNA per condition in OptiMEM. Similarly, the Lipofectamine 2000 was diluted to 4% in Opti-Mem. The solutions were allowed to incubate for 5 min at room temperature. The Lipofectamine 2000 solution was then gently mixed into the shRNA solution. The Lipofectamine–shRNA solution was allowed to incubate for 20 min at room temperature (RT). Next, 500 μl of the transfection solution was added to each plate and incubated at 37 °C for 3 h. After incubation, 1 ml of antibiotic-free growth media was added to each plate and the cells were incubated for a further 12–18 h. At this point, cell cultures were either induced to differentiate using low-serum media or collected for growth condition.

To perform ISNT, C2C12 mouse myoblasts were plated onto ultraviolet-irradiated glass coverslips in 35-mm tissue culture dishes at a concentration of 0.5×10^6 cells/plate. One plate was prepared for each experimental condition, plus one DNaseI-positive control plate for each experimental parameter. The cells were allowed to grow to confluence in a 37 °C incubator. Before initiating the INST assay, the 10 × ISNT reaction buffer (0.5 M TRIS-HCl, pH 7.9, 50 mM $MgCl_2$ and 100 mM β-mercaptoethanol) and ISNT reaction mix (1 × ISNT reaction buffer, 10 μM dNTP (1 mM dCTP, dATP and dGTP), 1 μM dTTP, 1 μM digoxigenin (DIG)-II-dUTP, 0.1% DNA Polymerase I, New England Biolabs, Whitby, ON, Canada) were prepared. Positive control plates were washed in 1 × PBS. Next, the coverslips were treated with 200 μl of DNaseI treatment mix (1% DNaseI, 1 × DNase buffer) and incubated for 10 min at room temperature. After incubation, the coverslips were washed two times in 1 × PBS. The DNaseI control coverslip and experimental condition coverslips were all rinsed with 1 × ISNT reaction buffer (made by diluting the 10 × reaction buffer 1:10). After rinsing, each coverslip was incubated with 200 μl of ISNT reaction mix and incubated at 37 °C for 45 min with gentle agitation. After 45 min, the coverslips were washed two times in 1 × PBS and then immunostained using a mouse anti-digoxigenin (DIG) primary antibody at a concentration of 1:500 in 3% BSA for 1 h at RT.

Adenovirus treatment protocols were used as previously described [23]. Briefly, primary myoblasts isolated from *Xrcc1*$^{flox/flox}$ mice are cultured on collagen-coated plates until 50% confluence is reached. Media is removed and cells are washed with PBS. Cre-ad at multiplicity of infection 10 or Ctl-ad are added to warm reduced serum OptiMEM media. Cre-ad or Ctl-ad media is then added to myoblast plates and allowed to incubate for 1 h at 37 °C, followed by addition of regular Ham's F-10-enriched growth media, and was left overnight for virus to infect close to 100% cells on plate. Next day, we collect samples for growth or initiate normal differentiation time course protocol described above.

For Caspase 3 inhibition, cultured myoblasts were pre-treated with either 15 μM z.DEVD-FMK (DEVD) from BioVision or 15 μM dimethyl sulphoxide (DMSO) from Sigma-Aldrich (Oakville ON, Canada) for 2 h at 37 °C. After pre-treatment, the cells were induced to differentiate using low-serum media or continued in growth media both containing 15 μM DEVD or DMSO as a vehicle only control. The inhibition or control media was changed every 48 h until the end of the time course. The cells were collected at the predetermined time points and analyzed as described.

Single-cell gel electrophoresis assay (COMET assay)

Primary myoblast cells from *Xrcc1*$^{flox/flox}$ mice were grown on a 10-cm tissue culture plate, treated with either Ctl-ad or Cre-ad as described above, collected in freezing media and frozen at − 80 °C. For the comet assay, the cells were thawed in 37 °C water bath and transferred to growth media. The thawed cells were then centrifuged at 720 *g* for 5 min. The media was then removed and the cells were resuspended in ice-cold 1 × PBS. At this point, the cells were ready to proceed using the Comet Assay Reagent kit for Single Cell Gel Electrophoresis from Trevigen, Gaithersburg, MD, USA. During preparation for the comet assay, the Lysis Solution (included in kit) was chilled on ice for at least 20 min before use. Next, the low-melting-point agarose (LMA) was melted by submerging in a beaker of boiling water for 5 min with the cap loosened. The agarose was maintained in a liquid state by transferring it to a 37 °C water bath. The LMA

was allowed to cool at 37 °C for at least 20 min before use. Cell samples, at a concentration of 1×10^5 cells per ml, were combined with LMA at a ratio of 1:10 cells:LMA and immediately pipetted onto labeled Gelbond film strip (agarose gel support medium) Lonza, ME, USA, in 75-µl aliquots. Cells were applied to the hydrophilic side of the film to ensure that the sample spreads evenly into a circle of ~25 mm in diameter. Each film was placed flat at 4 °C in the dark for 10 min or until the gel solidified. The slide was then immersed in pre-chilled lysis solution and incubated at 4 °C for 45 min. After incubation, the lysis solution was removed and the slide was immersed in freshly prepared alkaline solution pH > 13 (6 g NaOH, 250 µl 200 mM EDTA, dH_2O) for 45 min in the dark. The slide was removed from the alkaline solution and transferred to a horizontal electrophoresis apparatus where it was placed equidistant from each electrode in alkaline electrophoresis solution (12 g l^{-1} NaOH, 1 mM EDTA pH 8). Temperature fluctuations were minimized in the non-buffered system by running the electrophoresis at 4 °C in walk in refrigerator. The voltage was set to 30 V for 30 min at constant amperage of 300 mA. Following electrophoresis, the slide was rinsed several times in dH_2O and then immersed in 70% ethanol to fix. Next, the slide was dried in an air-tight container containing desiccant overnight at room temperature. Once dry, the slide was stained by submerging in SYBR Green stain at 1:10 000 in TE buffer pH 7.5 for 5 min. The slide was then removed and allowed to air-dry. The dry slides were mounted with coverslips and the cells were visualized using fluorescence microscopy using the green fluorescent protein channel (494 nm) at × 20 magnification. Tail length (in µm) for each nucleus is measured using the ImageJ software and is bin-sorted into short (green), medium-short (yellow), long (orange) or extra-long (red) bar graphs.

Chromatin immunoprecipitation

Hundred million C2C12 cells were collected per time point and fixed in 1% formaldehyde-DMEM solution for 5 min. Arrest of fixation and PBS washing are then performed, and cells are scrapped, pelleted and stored at − 80 °C until ready for lysis and shearing. We used the Enzymatic shearing kit from Active Motif (Carlsbad, CA, USA, ChIP-IT Express Enzymatic Cat# 53009) with minor modifications to the procedure. Briefly, we follow instructions in cell lysis using hand-held dounce homogenizer. We enzymatically shear the samples at 37 °C for 10 min with periodic full-speed vortex as instructed. We perform the immunoprecipitation using the amounts indicated for higher volume reactions in the instruction manual, with one major alteration: we use 5 µg of antibody per sample instead of the indicated 1–3 µg. All volumes are adjusted to 200 µl and left on rotators at 4 °C overnight. The following day we proceed to washing the beads, eluting the chromatin and reverse-cross-linking, as described in the manual with one major alteration: we perform the reverse-crosslinking for 4 h at 65 °C and always continue through the proteinase K treatment afterward without storing the samples between steps. The resulting samples (XRCC1-IP, RNApol II-IP, IgG-IP and input DNA samples for each time point) can be used to perform end point PCR at this stage, or the samples can be purified using gDNA purification kits to yield qPCR or Chip-sequencing ready samples. We perform p21 promoter region PCR using primers previously published [11]. EF1alpha primers used for positive control of ChIP is provided in the Active Motif Kit. For Caspase 3 inhibition, we perform DEVD-fmk and DMSO treatments of cells as described above.

Immunofluorescence staining and microscopy

Cells were cultured on 25-mm coverslips in 35-mm plates and exposed to the described experimental conditions. At appropriate time points, the cell plates were place on ice and the media was aspirated and replaced with ice-cold 1 × PBS. The plates were washed a further two times with PBS. The cells were fixed in 90% methanol for 8 min on ice. The fixative was removed and the cells were washed with PBS for two times. Next, the cells were covered with PBS and the plates were wrapped with Parafilm and stored at 4 °C until a time when all time points had been fixed. The plates were tranferred to RT and blocked for 24 h in 3% fetal bovine serum. After blocking, the cells were incubated in primary antibody (DNA ligase IV (H-300) rabbit polyclonal IgG (200 µg ml^{-1}), Santa Cruz Biotechnology, Dallas, TX, USA, 1:00, Anti-XRCC1 antibody produced in rabbit (1 mg ml^{-1}), Sigma-Aldrich, 1:100, Anti-Myosin Heavy Chain (MF20) mouse IgG, Developmental Studies Hybridoma Bank, Iowa City, IA, USA, 1:50) made up in blocking solution for 24 h at 4 °C. After primary antibody incubation, the cells were washed three times in PBS and incubated in secondary antibody (2 mg ml^{-1} Alexa Fluor 488 goat anti-rabbit IgG (H+L), Invitrogen, CA, USA, 1:500, 2 mg ml^{-1} Alexa Fluor 594 goat anti-rabbit IgG (H+L), Invitrogen, 1:500, 2 mg ml^{-1} Alexa Fluor 594 goat anti-mouse IgG (H+L), Invitrogen, 1:500, AP129F Donkey anti-mouse IgG FLUOR, Chemicon International, CA, USA, 1:500) diluted in PBS for 1 h (RT) to 24 h (4 °C). After incubation, the cells were washed 2 × in PBS and 1 × in dH_2O and then counterstained with DNA-specific DAPI, Sigma-Aldrich, made up in dH_2O (1:10 000) for 10 min at RT. After incubation, the cells were washed two times in PBS and one time in dH_2O. The coverslips were mounted on microscope slides using Dako Fluorescent Mounting Medium (Dako, Burlington, ON, Canada) and visualized using a Zeiss Observer Z1 inverted fluorescence microscope (Carl Zeiss, North York, ON, Canada). All images are developed using the AxioVision 4.8 software. The quantification of XRCC1 foci was performed by manually visualizing and counting the highest-intensity foci within each individual nucleus and recording the number of foci/nucleus for a minimum of 50 nuclei per experimental condition.

RNA extraction and PCR

Cells from C2C12 and primary myoblast cultures were washed with ice-cold PBS and lysed using TRIzol reagent. Appropriate volume of chloroform is added to the lysates, followed by the samples being vortexed, and was then allowed to incubate for 3 min at RT after which time they were centrifuged at 10 000 *g* for 10 min at 4 °C to separate the phases. The top phase was removed to a fresh, labeled, RNase-free tube. Appropriate amount of isopropanol was added to each sample and the tubes were gently inverted to mix. The tubes were rocked on a nunator for 20 min at RT, after which time the RNA was

pelleted by centrifugation at 10 000 *g* for 15 min at 4 °C. The supernatant was discarded and the pellet was washed in 500 µl 70% ethanol in dimethyl pyrocarbonate containing sterile water) and re-centrifuged for 5 min at 7 500 *g* at 4 °C. The supernatant was discarded and the RNA pellet was allowed to air-dry in the fume hood. Once dry, the RNA pellet was resuspended in 80-µl nuclease-free water and allowed to dissolve at 4 °C for 1 h before being stored at − 20 °C. First, strand cDNA synthesis was performed using iScript RT Supermix from Bio-Rad (Saint-Laurent, QC, Canada) according to the manufacturer's instructions. All primers used are appended in Supplementary Information section.

Protein extraction and western blot analysis

Cells from C2C12 and primary myoblast cultures were washed with ice-cold PBS and lysed on ice for 45 min in modified cytoplasmic/nuclear fractionation NE-PER extraction kit from Thermo Scientific. Immunoblot analyses were performed as described previously by using the following antibodies: mouse monoclonal XRCC1 antibody from Abcam (Cambridge, UK, ab1838, 1:1 000), myosin heavy chain and tubulin from Development Studies Hybridoma Bank (MF20, Tub 1:100) and GAPDH from Cell Signaling (Danvers, MA, USA, #2118, 1:2 000). Probe analysis and quantification was performed using the ImageJ software.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

MHA-K, LEB, BDL and RAB performed all experiments. SB provided technical support. RJP, MAR and PJM contributed essential experimental resources. FJD provided scientific advice and helpful comments on the project. MHA-K and LEB analyzed all data. MHA-K, LEB and LAM wrote the paper. MHA-K and LAM planned, and LAM supervised the project.

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Skeletal Muscle

REVIEW Open Access

The beneficial role of proteolysis in skeletal muscle growth and stress adaptation

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Abstract

Muscle atrophy derived from excessive proteolysis is a hallmark of numerous disease conditions. Accordingly, the negative consequences of skeletal muscle protein breakdown often overshadow the critical nature of proteolytic systems in maintaining normal cellular function. Here, we discuss the major cellular proteolysis machinery—the ubiquitin/proteosome system, the autophagy/lysosomal system, and caspase-mediated protein cleavage—and the critical role of these protein machines in establishing and preserving muscle health. We examine how ordered degradation modifies (1) the spatiotemporal expression of myogenic regulatory factors during myoblast differentiation, (2) membrane fusion during myotube formation, (3) sarcomere remodeling and muscle growth following physical stress, and (4) energy homeostasis during nutrient deprivation. Finally, we review the origin and etiology of a number of myopathies and how these devastating conditions arise from inborn errors in proteolysis.

Keywords: Proteolysis, Proteasome, Autophagy, Caspase, Muscle growth, Muscle cell differentiation

Background

Maintaining protein homeostasis (proteostasis) is a critical factor in preventing cellular dysfunction and the propagation of many disease states. Proteostasis requires both protein synthesis and degradation, although the latter is more often associated with pathological states than with normal cellular functioning. The negative connotation of protein degradation is particularly evident for skeletal muscle, where excessive protein destruction is the principle mechanism behind muscle atrophy and weakness. This view is propagated by the fact that activation of proteolysis is a feature of a number of pathologies, including cancer, sepsis, uremia, acquired immune deficiency syndrome, and diabetes mellitus (reviewed in [72]). While the concern regarding the aberrant activation of proteolysis is justified in these disease states, a strictly negative association ignores the fact that the long-term viability and maintenance of any organ system will require regular protein turnover.

The necessity for properly functioning proteolytic systems in skeletal muscle is especially acute when one considers the unique requirements for this tissue. Skeletal muscles are the force-generating structures of the body and are constantly challenged by mechanical, heat, and oxidative stress. These events increase protein damage and require efficient protein turnover to maintain optimal functioning. Furthermore, skeletal muscle repair and regeneration is dependent upon the differentiation of satellite cell-derived myoblasts [13], which requires extensive remodeling and the spatiotemporal expression of myogenic factors. This, of course, requires the temporal destruction and synthesis of the appropriate proteins. These examples illustrate the necessity of proteolysis in skeletal muscle, and this review will focus on the critical nature of proteolytic systems—namely proteasome-, autophagy-, and caspase-mediated proteolysis—to sustain skeletal muscle health and development.

Proteasome-mediated proteolysis

Perhaps the most well-known cellular proteolytic system is the ubiquitin/proteasome pathway (UPP), which is responsible for degrading the majority of cellular proteins [93]. This is a system whereby proteins meant for destruction are enzymatically tagged with the polypeptide ubiquitin via E3 ubiquitin ligases. These tagged

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proteins are recognized by the 26S proteasome, which is a large barrel protein complex that consists of a 20S core particle associated with two 19S regulatory subunits. The latter subunits recognize and bind the ubiquitinated proteins and begin their adenosine triphosphate (ATP)-dependent destruction within the catalytic core [119]. In the skeletal muscle, protein degradation via the proteasome is often associated with muscle atrophy. Indeed, muscle wasting is characterized by increased proteolysis via the UPP, increased ubiquitin conjugation to muscle proteins, and an up-regulation of ubiquitin-protein ligases (reviewed by [58]). However, this clear role in muscle atrophy minimizes the necessity of the UPP in maintaining normal muscle functioning. The following sections highlight the essential roles of the UPP in the skeletal muscle.

Proteasome and muscle cell differentiation

Myogenesis is a complex process that is dependent on the spatiotemporal expression of a variety of myogenic regulatory factors. As myoblast differentiation proceeds, there is a need for timely synthesis and degradation of the appropriate myogenic proteins, which suggests a significant role for adaptive proteolysis in the myogenic process. Indeed, early studies indicated that proteasome inhibition decreased myoblast fusion and differentiation [36, 75] and prevented the degradation of key myogenic proteins, such as MyoD. MyoD degradation via the UPP was further confirmed by a series of studies [1, 48, 104, 105] and was accompanied by the revelation that many of the other proteins critical to the progression of the myogenic program were degraded in a similar manner (Fig. 1). This extends to the myogenic regulatory factors Myf5 [61], myogenin [47], Id1 (negative regulator of MyoD; [105]), E2A proteins [106], and filamin B [8]. Interestingly, both Pax3 and Pax7 are also subject to ubiquitin-mediated degradation, implying that the acquisition of differentiation competence requires proteolytic cleavage [10, 11]. In addition, recent evidence has suggested that the immunoproteasome—so called due to the interferon-γ-induced expression of three alternative proteasome β subunits—also plays a role in myogenesis, with its suppression leading to decreased myoblast differentiation [16].

The necessity of proper proteasome functioning during myoblast differentiation may extend beyond the effects on the spatiotemporal expression of transcription factors. For instance, myogenesis is a period of intense restructuring that relies on increased mitochondrial energy production. As a by-product, differentiating myoblasts produce increased levels of reactive oxygen species (ROS) and an elevated level of oxidized proteins, which must be degraded [67]. Without proper proteasome functioning, the accumulated oxidized proteins

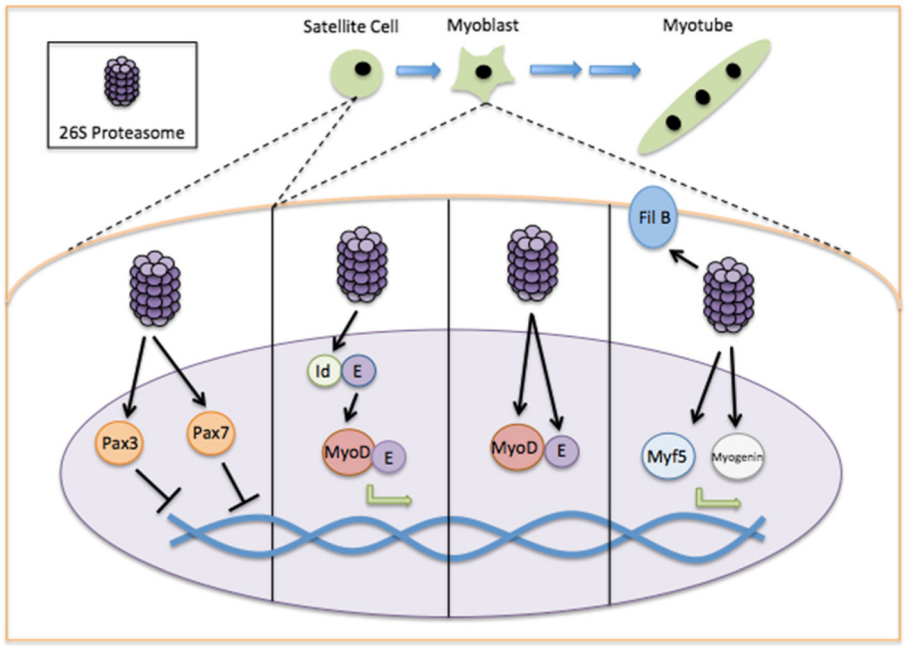

Fig. 1 The role of the UPP in skeletal muscle cell differentiation. The fidelity of muscle cell differentiation is dependent upon the spatiotemporal expression of particular myogenic proteins. Indeed, UPP involvement in satellite cell differentiation begins with its role in the removal of Pax3 and Pax7, which maintain satellite cells in their stem cell niche. Further, the 26S proteasome appears critical for the early activation of a key myogenic factor, MyoD, through the removal of an endogenous MyoD inhibitor, Id. The continuation of the myogenic program relies on UPP-dependent degradation of MyoD and its binding partner E2A (E), as well as Myf5, myogenin, and filamin B (Fil B) during later stages of differentiation

may halt the differentiation process [100] and possibly initiate apoptosis [27]. Taken together, myogenesis appears to integrate proteasome-mediated proteolysis to achieve effective myoblast differentiation.

Proteasome and muscle growth

Recently, researchers have demonstrated that the muscle-specific knockout of an essential 26S proteasome protein, Rpt3 (also known as Psmc4), leads to a significant deficit in muscle growth and force generation in mice [52]. This sentiment is echoed in earlier publications on proteasome function in *Drosophila* muscle, where the conditional expression of a mutant proteasome β subunit (within the 20S core particle) led to the deterioration of muscle architecture [40].

The apparent role of the UPP in muscle growth and integrity suggests that proteasome-mediated protein degradation may be important during exercise. Indeed, acute bouts of resistance exercise have been shown to increase both protein synthesis and breakdown in skeletal muscle [92]. Moreover, numerous studies have indicated that the expression of two muscle-specific ubiquitin ligase genes, muscle really interesting novel gene (RING) finger-1 (*MuRF1*) and muscle atrophy F-box (*MAFbx*; also called *atrogin-1*), increased following acute resistance exercise (reviewed by [78]), suggesting increased proteasome-mediated proteolysis post-exercise. Similarly, both acute and chronic bouts of endurance exercise appear to increase proteasome-mediated proteolysis. In the former case, a single bout of endurance running led to increased expression of MuRF1 and atrogin-1 immediately after the run (0–4 h post-exercise), suggesting increased UPP flux [64, 85]. Interestingly, chronic endurance exercise (i.e., 8-week running regimen) in mice also elicited a sustained increase in *MuRF1* expression and proteasome activity [18]. The reason for the sustained activation of the UPP as compared to untrained animals can only be speculated; however, it may stem from the increased oxidative capacity (and therefore ROS-derived protein damage) that is a characteristic of trained skeletal muscle. More recently, Baehr et al. [7] found that chronic loading of mice skeletal muscle using the functional overload model led to skeletal muscle hypertrophy that was characterized by increased protein synthesis and degradation via the UPP. However, in contrast to the study by Cunha et al. [18], this increased proteasome activity was independent of MuRF1 (and MAFbx) expression. Interestingly, recent studies have indicated that several other ubiquitin ligases may have important roles in determining skeletal muscle-associated phenotypes, including TRIM32 [54, 80], MUSA1 [98], MG53 [121], and Nedd4-1 [79]. In any case, the surge in protein breakdown following resistance and endurance exercise has been hypothesized to be adaptive, as it rids muscles of damaged proteins and facilitates myofilament restructuring and muscle growth (Fig. 2). Collectively, these studies offer an alternative function for the proteasome, for what otherwise has been largely considered to be a conveyor of muscle wasting and pathology.

Autophagy/lysosome-mediated proteolysis

Autophagy is one of the major protein degradative pathways within virtually all cells of the body. It involves the sequestration of dysfunctional proteins or organelles in membrane bound vesicles (termed autophagosomes) and the subsequent fusion of these vesicles with lysosomes, where the encapsulated cytoplasmic material is degraded and essential biomolecules recycled [59]. Autophagy was originally identified as a form of programmed cell death and is often thought of as one of the principle mechanisms that spur muscle wasting [96]. Nevertheless, autophagy is important in maintaining healthy muscle and is critical in muscle adaptation to sublethal cellular stress. The following sections will explore the various roles of autophagy in maintaining skeletal muscle functioning, as well as the role of this process in skeletal muscle relevant stress responses.

Autophagy and muscle mass maintenance

Several studies over the past decade have indicated that excessive autophagy aggravates muscle wasting and contributes to muscle weakness [25, 68, 111, 118, 122]. Indeed, autophagosome accumulation has been observed in nearly all myopathies [66]. However, recent evidence has indicated that basal autophagy is necessary to maintain muscle mass and prevent atrophy. Much of this evidence is derived from studies of autophagy-deficient mice, where critical autophagy-related genes have been knocked out (i.e., *Atg5* and *Atg7*). In the latter case, muscle-specific knockout of *Atg7* causes muscle cells to adopt myopathic characteristics such as misalignment of the Z-line, abnormal enlargement of mitochondria, distended sarcoplasmic reticulum (SR), and the formation of aberrant membranous structures [70]. Moreover, $Atg7^{-/-}$ mice showed a 20–40 % age-dependent reduction in muscle fiber cross-sectional area with a corresponding decrease in force generation. A similar decrease in muscle cross-sectional area was observed in $Atg5^{-/-}$ mice; however, this decrease did not appear to translate into decreased muscle performance [90]. Moreover, $Atg5^{-/-}$ mice displayed further similarities to the *Atg7* knockout mice including the accumulation of membranous structures and the formation of protein aggregates. Taken together, these initial studies highlight the necessity for basal autophagy in muscle mass maintenance.

One of the key factors in muscle mass maintenance is the regenerative capacity of the muscle satellite cells. Recent evidence has now placed autophagy at the heart of muscle regeneration, with this process being

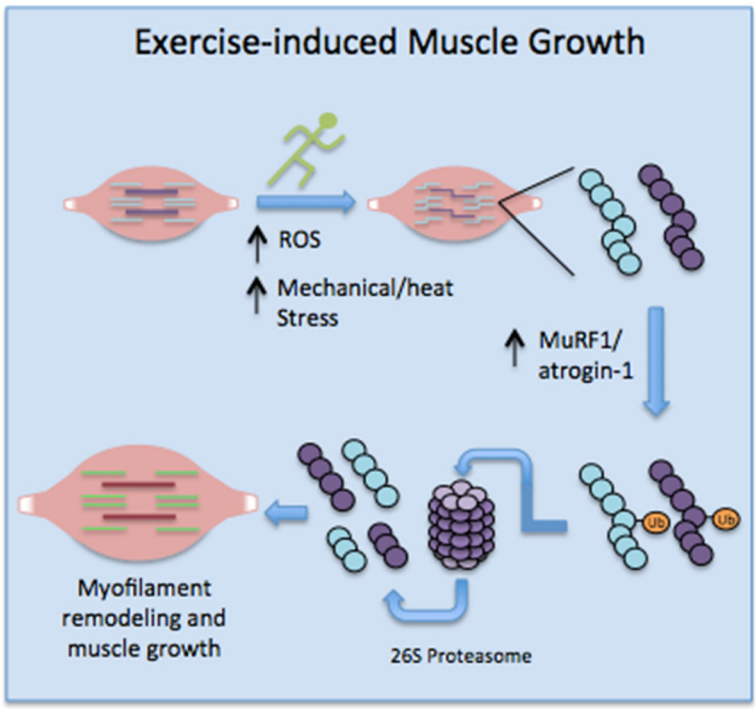

Fig. 2 The role of the UPP in skeletal muscle growth. Exercise-induced protein damage via increased ROS/mechanical and heat stress necessitates an increase in proteasome-mediated proteolysis to rid the cells of non-functional myofibrillar proteins. This is typically dependent on a prerequisite increase in key muscle-specific ubiquitin ligases, MuRF1 and atrogin-1 (MAFbx), which ubiquitinate and target damaged proteins for degradation by the 26S proteasome. Efficient removal of damaged proteins is critical to skeletal muscle growth and remodeling following exercise

responsible for preventing quiescent muscle stem cells from taking on a senescent state. Stem cell senescence appears to be the main culprit limiting muscle regeneration in aging mammalian muscle, which thereby suggests that efficient autophagic signaling is necessary in preventing sarcopenia [35]. Moreover, muscle stem cell activation also appears reliant on autophagy, as it is thought to provide the necessary nutrients to meet the bioenergetics demands of satellite cells transitioning from quiescence to activation [109].

In addition to the basal requirement of autophagic flux to maintain muscle mass and muscle regeneration capacity, there is a growing evidence for an essential role for autophagy in exercise-induced muscle growth. Most recently, *Redd1*$^{-/-}$ (regulated in development and DNA damage responses 1; an inhibitor of mTORC1) mice display decreased autophagic flux and a significant decline in exercise capacity [87]. Indeed, the mechanical stress and the simultaneous production of ROS during physical exercise [75] increase the necessity for autophagic removal of damaged cellular components. Moreover, autophagy may be necessary during exercise-induced energy stress to provide muscle cells with an alternative energy source.

Some of the more convincing evidence for the importance of autophagy during physical exercise stem from studies of ultra-endurance runners. For instance, a recent study indicated that these runners display a 50 % decrease in FoxO3 phosphorylation (consistent with its activation and translocation into the nucleus), a fivefold increase in the phosphatidylethanolamine-conjugated microtubule-associated protein 1A/1B-light chain 3 (LC3-II) expression (indicative of increased autophagy), and a significant increase in Atg5-Atg12 complex formation (important for autophagosome formation) [45]. A study by the same group also indicated that a bout of ultra-endurance running increased skeletal muscle expression of key autophagy genes such as *Atg4b*, *Atg12*, *Gabarap1*, *LC3*, *Bnip3*, and *Bnip3l* [46]. It is likely that these studies on endurance runners represent an extreme example of skeletal muscle stress, which requires autophagy to meet energetic demands and allow for the removal of dysfunctional proteins/organelles that will inevitably accumulate with extreme contractile demands.

In juxtaposition to the studies on ultra-endurance running, the effects of acute and chronic exercise on the autophagic response in skeletal muscle cells appear to be inconclusive. With respect to acute bouts of exercise, one investigation demonstrated that murine skeletal muscle autophagosome formation increased after ~30 min of treadmill running. Furthermore, the transgenic mice used

in this study, which lack the ability to activate exercise-induced autophagy through the release of beclin-1 from the B cell lymphoma 2 (BCL2)-beclin-1 complex (Fig. 3), display a marked reduction in endurance during acute treadmill running [41]. Thus, this study indicates not only that autophagy is induced during short-term exercise but that the lack of autophagic flux hinders muscle performance. Grumati and colleagues [39] also found that a 60-min bout of treadmill running was capable of increasing autophagic flux in murine skeletal muscles, as evidenced by increased LC3 lipidation (i.e., conversion of LC3-I to LC3-II via phosphatidylethanolamine conjugation). Most recently, an acute bout of exercise (60–90 min in duration) caused a significant increase in autophagic signaling in both mouse and human skeletal muscle [74, 109]. One potential aspect of increased autophagy following acute exercise appears to be in mediating mitochondrial turnover (termed mitophagy), which was recently shown to be coordinated, in part, by the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) [116]. In contrast to these studies, Kim et al. [50] demonstrated that 50 min of treadmill running decreased the expression of autophagy-related genes (i.e., *LC3*, *beclin-1*, and *Atg7*), a few hours post-exercise. It is important to note that the exercise regimens between these studies were not identical and varied in aspects such as speed, treadmill inclination, and time. These are likely significant factors given the recent research by Schwalm et al. [99] that indicates that exercise intensity may be the crucial factor in determining the induction of autophagy.

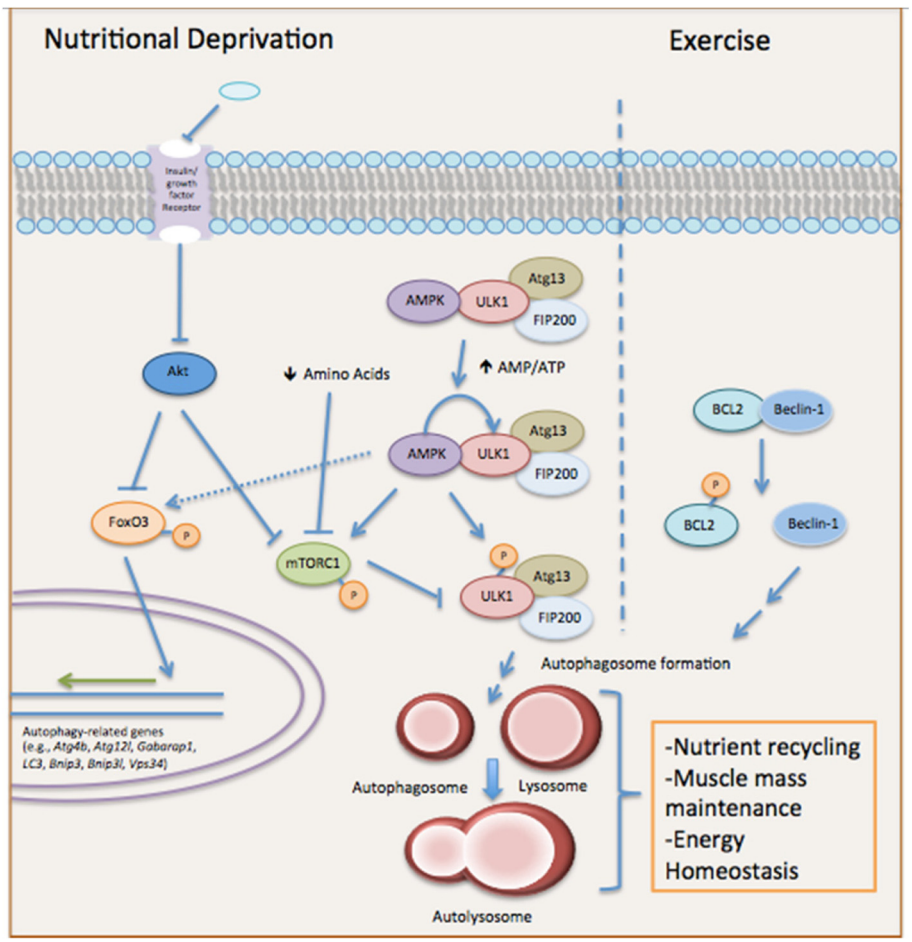

Fig. 3 Exercise- and starvation-induced autophagy pathways and their beneficial role in muscle stress adaptation. Nutrient deprivation decreases signaling through insulin/growth factor receptors, which decreases Akt activation and allows for the AMPK-dependent phosphorylation of FoxO3. FoxO3 is then able to translocate into the nucleus and initiate the transcription of autophagy-related genes. Activated AMPK also phosphorylates mTORC1 (preventing its action on ULK1, a key autophagy-related kinase) and ULK1 to allow for efficient autophagosome formation and clearance of encapsulated material. Moreover, the lack of intake of essential amino acids further prevents mTORC1 activation and promotes autophagy induction. Taken together, these processes recycle nutrients for muscle cells and the body as a whole during lean periods. While starvation-induced autophagy is undoubtedly a part of muscle biochemistry during exercise, physical activity also activates beclin-1 through its phosphorylation-dependent release from the BCL2-beclin-1 complex. Beclin-1 is critical to autophagosome formation and the efficient clearance of damaged organelles and proteins that arise from physical stress

Moreover, the differences in the level of autophagic induction may stem from intrinsic differences in muscle glycogen reserves and pre-exercise energy status (i.e., AMP to ATP ratio and the corresponding activation state of AMP-dependent protein kinase (AMPK)). Indeed, it is reasonable to assume that a threshold may exist during aerobic exercise where muscle cells engage in autophagy-mediated breakdown of damaged organelles/proteins to ensure normal functioning and energy homeostasis.

Similar to the above situation, skeletal muscle autophagic flux following chronic exercise appears to be quite variable. For instance, analysis of the tibialis anterior (TA) muscle in mice that were allowed to spontaneously exercise on a running wheel for 3 months did not show any evidence of LC3 lipidation [39]. Conversely, investigation of the plantaris muscle in mice subjected to 4 weeks of voluntary running displayed increased LC3 lipidation, decreased p62 protein content (indicating greater autophagic flux), and increased expression of a number of autophagy-related proteins (i.e., *Atg6*, *LC3*, and *Bnip3*) [62]. A recent study confirmed these findings with basal autophagy in mice plantaris muscle being induced after a 20-week period of voluntary exercise [108]. One major difference between these two studies and the previous study on the TA muscle is the difference in muscle types analyzed. Plantaris is composed of mixed muscle fiber types (glycolytic/oxidative) while the TA is a mainly composed of glycolytic (type II) fibers [3, 110]. Muscles with mixed muscle fiber types often become more oxidative with chronic aerobic exercise [108], and thus, an induction of autophagy may be necessary as an adaptive mechanism to deal with increased oxidative stress and mitochondrial turnover. Recently, a study of the rectus femoris muscle (consisting of mixed fiber types) following 2 months of exercise training displayed increased LC3 expression, decreased p62 expression, and an overall increase in autophagic flux [44]. Similarly, analysis of rat soleus muscle (composed of mainly oxidative muscle fibers) after chronic exercise (1 h/day, 6 days/week, for 8 weeks) showed increases in a variety of autophagy-related genes such as *Atg7*, *beclin-1*, and *LC3* [28]. These rats also displayed mitochondrial dysfunction and the induction of an oxidative stress response, which may explain the increases in autophagy markers.

Autophagy and nutrient stress response

Nutrient deprivation is one of the most potent activators of autophagy, and while this generally promotes muscle wasting, the process appears necessary in times of energy stress to supply the body with catabolic substrates to allow continued functioning [91]. Indeed, in vivo analysis has indicated that there is a significant increase in autophagosome formation in skeletal muscle following a 24-h period of starvation in mice [73]. Interestingly, autophagosome formation differed between muscle fiber types, with fast-twitch muscle fibers showing a significantly greater autophagic response as compared to slow-twitch muscle fibers [73]. Previous studies on the overall protein degradation have indicated that slow-twitch muscle fibers are more resilient than fast-twitch fibers during starvation [31, 60], which may be adaptive given the importance of slow-twitch fibers in maintaining posture.

In recent years, significant strides have been made in determining the mechanism by which nutrient deprivation triggers skeletal muscle autophagy (Fig. 3). Early evidence indicated that starvation-induced autophagy was mediated via the Akt/FoxO3 axis [68, 122]. Specifically, the lack of growth factor- and insulin-dependent signaling during nutrient deprivation suppresses Akt activation and subsequent FoxO3 phosphorylation (at Akt specific sites). This then allows for the nuclear translocation of FoxO3 and the transcriptional initiation of autophagy-related genes, such as those essential for autophagosome formation (*Atg12l*, *Atg4b*, *Gabarapl1*, and *LC3*) and the regulation of autophagy (*Bnip3*, *Bnip3L*, and *Vps34*) [68, 122]. Further research indicated that transcriptional activation of FoxO3 was promoted by AMP-dependent protein kinase (AMPK)-dependent phosphorylation. AMPK also appears to bind and regulate Unc-51 like autophagy activating kinase 1 (ULK1), which is important in the induction of autophagy and the formation of autophagosomes. Sanchez et al. [95] found that AMPK and ULK1 are interacting partners during periods of nutrient abundance, with nutrient stress causing their dissociation and an increase in ULK1 activity (through AMPK-dependent phosphorylation), thereby aiding in autophagosome biogenesis.

Surprisingly, the aforementioned studies generally found that the nutrient-responsive kinase, mammalian target of rapamycin (mTOR), contributed little to the induction of autophagy in skeletal muscle during starvation. Zhao et al. [122] found that inhibition of mTOR complex 1 (mTORC1) in C2C12 myotubes by rapamycin only resulted in a slight increase in autophagy, while inhibition of the upstream kinase, Akt, via API-2, induced an autophagic response that was fivefold greater than that with rapamycin treatment. Similarly, Mammucari and colleagues [68] found that rapamycin inhibition and knockdown of mTOR had little effect on the induction of lysosomal proteolysis. This same study also indicated that the RNAi-mediated knockdown of the rapamycin-insensitive mTOR complex (mTORC2) did induce FoxO3 translocation into the nucleus. In contrast to these findings, a recent study indicated that constitutive activation of mTORC1 in the skeletal muscle of tuberous sclerosis 1 (TSC1)-deficient mice inhibited autophagy despite AMPK and FoxO3 activation [12]. In these

mice, active skeletal muscle mTORC1 was shown to phosphorylate ULK1 and thereby prevent the AMPK-dependent phosphorylation of ULK1 that is necessary for autophagosome formation [49]. Indeed, rapamycin administration or institution of a ULK1 mutant that was insusceptible to mTORC1 phosphorylation was sufficient to restore autophagic flux. While this study does not discount the importance of AMPK/FoxO3 signaling, it does indicate that inhibition of mTORC1 may be necessary for efficient autophagy induction. Interestingly, these two signaling pathways have been linked, with AMPK being capable of phosphorylating mTORC1 and disrupting any interaction with ULK1 [95]. Thus, the discrepancies in the aforementioned studies may suggest that an initial decrease in ATP to AMP ratio during nutrient deprivation may activate AMPK, which will subsequently initiate FoxO3 signaling and, concurrently, inhibit mTORC1 to allow ULK1 function and autophagy to be initiated.

An additional aspect of nutrient deprivation that directly affects mTOR and thereby autophagic signaling is the lack of intake of essential amino acids (e.g., leucine). This deficiency prevents amino acid-induced mTORC1 translocation to lysosomal membranes where mTORC1 would be activated through an interaction with Rag GTPases and the ragulator protein complex [94]. Given that growth factor and amino acid-dependent signaling converge at the same point (i.e., mTOR), it is likely that these two signaling pathways act in concert during nutrient deprivation to activate autophagy and recycle essential building blocks.

Autophagy and metabolism

The link between autophagy and metabolism has been well established based on its function in recycling damaged proteins and organelles during energy stress. However, recently, autophagy has been suggested to play a role in carbohydrate and lipid metabolism. In the latter case, evidence has emerged that autophagic activity is inversely correlated with intramyocellular triglyceride levels in morbidly obese patients following bariatric surgery [55]. These researchers also found that L6 rat myocytes cultured with free fatty acids showed increased lipid accumulation and cell death when autophagy was inhibited and decreased lipid accumulation when autophagy was activated. These results clearly imply a role for autophagy in lipid metabolism; however, further experiments will be needed to elucidate its exact role.

Skeletal muscle carbohydrate metabolism also appears to be affected by autophagic flux, with the strongest evidence emerging from the study of various muscle pathologies. For instance, in patients with Danon disease, glycogen granules accumulate in autophagosomes that are unable to fuse with lysosomes for proper clearance, which leads to muscle weakness [82]. Interestingly, patients with Pompe disease exhibit a defect in glycogen breakdown within lysosomes, and muscle-specific inhibition of autophagic transport of glycogen to lysosomes confers some therapeutic benefits [33, 90]. Autophagy may also be linked to glucose homeostasis. Mice deficient in exercise-induced autophagy (i.e., inhibited release of beclin-1 from the BCL2-beclin-1 complex) also show decreased insulin sensitivity and impaired redistribution of glucose transporter 4 (GLUT4) in skeletal muscle cells in response to acute exercise [41]. Moreover, this study demonstrated that impaired exercise-induced autophagy negated the exercise-mediated benefits to glucose tolerance in obese mice. In direct opposition to this study, muscle-specific knockout of *Atg7* (suppressing autophagy) led to increased lipid oxidation and decreased high-fat diet-induced insulin resistance [51]. While in not identical studies, the discrepancies do indicate the need for further investigation regarding the role (if any) of autophagy in insulin sensitivity and glucose homeostasis. These studies aside, it appears likely that autophagy plays an important role in lipid and glycogen metabolism within skeletal muscle cells; however, the underlying mechanisms remain unclear.

Autophagy dysfunction and disease

The necessity for proper autophagic flux in maintaining skeletal muscle functioning is most evident when one considers the pathologies that accompany a dysfunctional autophagic process. The aforementioned Danon disease is a prime example of myopathy caused by disturbed autophagic flux, with impaired autophagosome/lysosome fusion leading to muscle weakness and various extra-muscular effects. Similarly, there are a variety of other related diseases that likely develop, at least in part, due to defects in autophagosome/lysosome fusion. These include Vici syndrome [17], X-linked myopathy with excessive autophagy, adult-onset vacuolar myopathy with multiorgan involvement, and infantile vacuolar autophagic myopathy [81]. Moreover, muscle disorders can arise from the defects in the autophagic clearance of disease-causing molecules. For instance, in sporadic inclusion body myositis, one of the principle mechanisms for its initiation is the accumulation of amyloid precursor protein and its fragment, β-amyloid in muscle cells [5]. These two proteins associate with LC3 in cultured muscle cells and biopsied degenerating muscle fibers, which suggests that they may be cleared through autophagy [65]. Similarly, limb girdle muscular dystrophy type 2B and Miyoshi myopathy arise due to the aggregation of mutant dysferlin (typically a sarcolemmal protein) in the endoplasmic reticulum. Activation of the stress-induced autophagic pathway increases mutant dysferlin degradation, while a blockade in autophagy

(i.e., through Atg5 depletion) promotes aggregate formation [32]. Accumulation of mutant filamin C, an actin-binding protein that functions at the Z-disk, also triggers protein aggregation and the development of myofibrillar myopathy. This accumulation is thought to be due in part to a disruption in chaperone-assisted selective autophagy (CASA) [53]. Additionally, skeletal muscles of one of the more detrimental myopathies, Duchenne muscular dystrophy, were recently found to display impaired autophagy. This was evidenced by decreased LC3 lipidation, the accumulation of damaged organelles, and decreased expression of autophagy-related genes [20]. Lastly, disrupted autophagy is thought to play an important role in the progression of myopathies derived from mutated collagen VI genes, such as Ullrich congenital muscular dystrophy and Bethlem disease (reviewed in [97]). These genes encode for a key skeletal muscle extracellular matrix (ECM) protein [56], and studies of skeletal muscle *Col6a1*$^{-/-}$ mice and humans have indicated that this defect leads to the formation of abnormal mitochondria and SR, as well as the initiation of apoptosis [4, 43]. Subsequent experiments revealed that these aberrant organelles were the result of a malfunction in the autophagic process, specifically impairment in autophagosome formation [38]. Indeed, nutritional or pharmacological reactivation of autophagy attenuated the dystrophic phenotype in *Col6a1*$^{-/-}$ mice. Taken together, these myopathies further illustrate the importance of skeletal muscle autophagy in maintaining normal muscle function and identifying the constellation of relevant autophagy/vacuolar proteases that manage this process will be a high priority.

Caspase-mediated proteolysis

Caspases (cysteine-aspartic proteases) are a family of proteolytic enzymes that are most commonly known for their role in initiating apoptosis. Caspases are typically classified as initiator caspases (caspase-2, caspase-8, caspase-9, caspase-10) or effector caspases (caspase-3, caspase-6, caspase-7), with the former being responsible for activating the effector caspases. The role of caspases in apoptosis and the association of these proteases with various forms of muscle atrophy have reinforced the negative stereotype of this protein family, as conduits of cell destruction [26]. Despite the prevailing death-centric view, caspase-mediated signaling events have been linked to a diverse array of vital cell tasks, which are independent of inducing apoptosis [24].

Caspases and satellite cell commitment

Satellite cell commitment to the muscle cell lineage is an essential step in muscle growth and regeneration. Caspase activity appears to be intimately involved in this process as recent studies have indicated that caspase 3 activity directly limits satellite cell self renewal, by cleaving and inactivating the paired box transcription factor Pax7 [23, 83]. Pax7 is essential to maintain the satellite cell niche and must be removed for satellite cells to acquire differentiation competence [84]. Given that Pax7 is subject to both caspase and ubiquitin targeted degradation, a reasonable conjecture may be that these processes work in tandem to ensure the acquisition of a differentiation competent state. One could envision the initial caspase-dependent cleavage of Pax7, followed by ubiquitination and removal of Pax7 fragments. How and whether these proteolytic systems engage in crosstalk will require further experimentation.

Caspases and skeletal myoblast differentiation

Skeletal myoblast differentiation and the early steps in apoptosis possess a remarkable number of similarities. For instance, actin fiber disassembly/reorganization and phospholipid reorientation are features of both apoptosis [15] and differentiation [34, 88, 117]. Moreover, these seemingly conflicting cell fates both require increased activity of select matrix metalloproteinases [69, 120]. These similarities spurred a controversial hypothesis that suggested muscle cell differentiation and apoptosis may utilize overlapping signaling cascades, a supposition that was initially addressed in 2002. Here, Fernando et al. [29] demonstrated that transient caspase-3 activity is required for myoblast differentiation and that this non-death activity is mediated in part through the cleavage activation of the mammalian sterile 20-like kinase-1, MST1. Subsequent studies have established that the key elements of the intrinsic mediated cell death pathway are fully conserved to engage caspase 3 during myoblast differentiation [37, 77]. Once activated, caspase 3 targets multiple substrates to engage the differentiation program (Fig. 4). These substrates include promyogenic kinases such as MST1, HIPK2, NEK5 [19, 29, 101], the post-transcriptional regulatory protein ELAV-like protein (HuR) [6], and caspase-activated DNase (CAD), where the latter is activated by cleaving and removing its nascent inhibitor inhibitor of caspase-activated DNase (ICAD) [57]. CAD promotes myoblast differentiation by inflicting transient DNA strand breaks at the promoters of critical regulatory factors such as the cell cycle inhibitor p21, an event that leads to *p21* induction [2, 57]. Interestingly, CAD-sensitive strand breaks are detectable throughout differentiating myonuclei, suggesting that the DNase may engage a genome wide reprogramming event to alter the expression of a large number of gene targets. It is important to note that these DNA strand breaks need to be resolved quickly for proper muscle cell differentiation, and recent evidence has indicated that the base excision repair protein, XRCC1, is a key player in mending CAD-dependent breakage events [2].

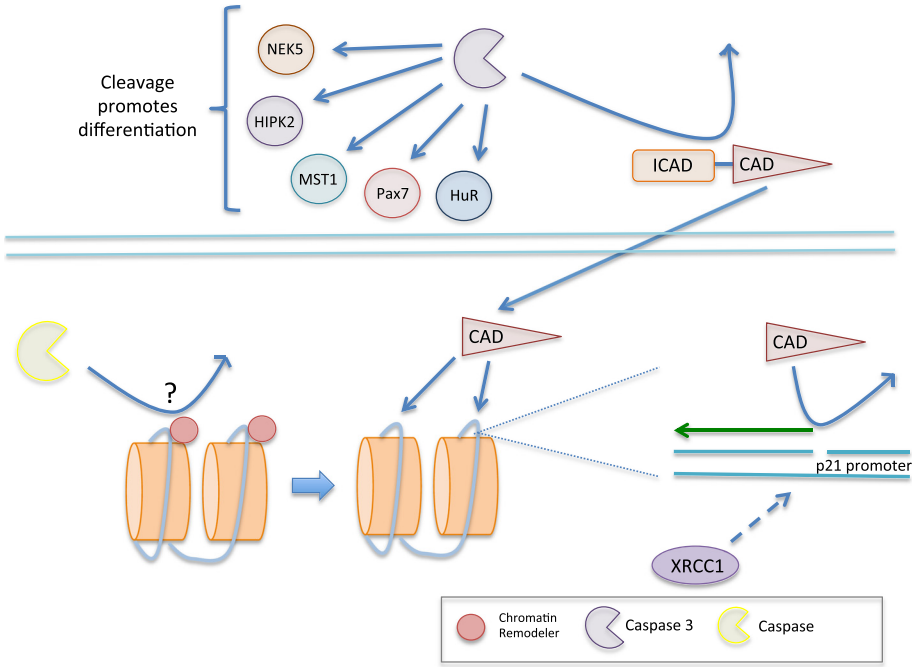

Fig. 4 The role of caspases in skeletal myoblast differentiation. Caspase 3 has a multifaceted role in regulating myogenesis. It is responsible for the proteolytic cleavage of the transcription factor Pax7, which maintains satellite cells in their stem cell niche and prevents myoblast differentiation. Moreover, caspase 3 cleaves the promyogenic kinases MST1, HIPK2, and NEK5 to promote myogenesis. The posttranscriptional regulator, HuR, is also cleaved by caspase 3 and is necessary for muscle fiber formation. Additionally, preliminary evidence (unpublished) suggests that the myogenic differentiation program appears to rely on the caspase-mediated cleavage of chromatin remodeling proteins to increase DNA accessibility for CAD (activated by caspase 3 cleavage of ICAD), which produces DNA strand breaks that are critical to regulating myogenic gene expression. For instance, CAD cleavage of the p21 promoter stimulates p21 expression, which is essential for cell cycle arrest and terminal differentiation. The CAD-derived DNA strand breaks require rapid resolution, which is mediated by the base excision repair protein XRCC1. This mending of DNA strand breaks is necessary to stabilize the genome and ensure the fidelity of the myogenic differentiation program

In addition to augmenting myogenic gene expression, caspase 3 regulates other key features of myogenesis. For instance, phosphatidylserine receptor-mediated caspase 3 activation has been reported to enhance myoblast fusion to existing myofibers. The relevant caspase substrates have not been characterized in this process, yet the protease activity appears to be a critical step in effecting muscle maturation and regeneration via cell fusion [42].

These observations confirm that caspase 3 acts at multiple points to secure the differentiation program, yet the mechanism that spurs caspase activity at these specific temporal junctures remains unknown. Satellite cell activation has been reported to trigger engagement of the extrinsic mediated cell death pathway such as the Fas-associated protein with death domain (FADD) receptor [14], yet the requisite initiator caspase for this pathway, caspase 8, does not appear to be appreciably activated at this stage [37]. Given the central role of caspase 3 signaling in the differentiation process, there is a significant need to identify (1) the pathways that engage the activation of this protease, (2) the full range of substrates that facilitate its capacity to induce differentiation, and (3) the mechanisms that restrain protease activity and direct it to a non-death cell function.

Caspases and skeletal muscle adaptation

The central role of caspase 3 in directing myoblast differentiation suggests that this protease and its cognate signaling pathways may retain non-death functions within fully formed myofibers. Notably, Wang et al. [113] have reported that caspase 3 targets and cleaves proteins that lead to acetylcholine receptor dispersal in postsynaptic membranes, a key step in the development of the neuromuscular junction. Skeletal muscle fibers express the repertoire of caspase regulatory proteins, and caspase 3 has been linked to a wide array of remodeling activities in various cell lineages, including postsynaptic remodeling in neurons and cardiomyocyte hypertrophy [24, 86]. The link between caspase 3 and cardiomyocyte hypertrophy is of particular interest as this form of cell growth is an adaptive stress response. As cardiac and skeletal muscle cells share a remarkable overlap in regulatory gene expression, sarcomere assembly/content etc., it is not unreasonable to suggest that caspase 3 activity may also manage the

hypertrophy of skeletal muscle fibers through discrete proteolytic targeting steps.

How caspase 3 activity translates to a beneficial stress adaptation in skeletal muscle fibers is currently unknown. One probable mechanism may involve direct communication between caspase 3 and other proteostatic control mechanisms within the muscle fiber (i.e., caspase signaling may serve to control both proteasome and autophagy-related signaling during muscle adaptation). To date, over 500 physiologic substrates have been identified for caspase 3/7, as such the ability of an effector caspase to target and integrate regulatory control over disparate proteolytic mechanisms is an entirely probable event. In *Drosophila* oogenesis, the effector caspase equivalent, death caspase-1 (DCP-1), has been shown to promote autophagy flux by cleaving and inhibiting the key autophagy suppressing protein SesB [22]. Whereas autophagy has been generally associated with muscle atrophy/wasting, a number of studies have shown that resistance trained skeletal muscle is associated with enhanced autophagic flux [62, 114]. The observations in *Drosophila* have established the existence of a caspase-directed autophagy signal, whether skeletal muscle utilizes a similar beneficial regulatory cascade will require further investigation. Simultaneous caspase activation and proteosome signaling in skeletal muscle are understood to have generally negative outcomes, associated with wasting and atrophy in a variety of disease settings [26, 71, 102]. Nevertheless, caspase 3 has been demonstrated to target and cleave subunits of the 19S proteasome (Rpt2 and Rpt6), leading to an obligatory increase in proteasome activity during myoblast differentiation [112]. Mutation of the respective caspase 3 cleavage sites in Rpt2 and Rpt6 resulted in failure to up-regulate the 19S proteasome, with a profound block in the differentiation program. Clearly, the uncontrolled engagement of this signaling interaction would have dire consequences for myofiber viability, yet one can easily envision that a transient activation of these proteases may act to remodel the ultrastructure of the myofiber in response to physiologic demands.

What remains unknown are the biochemical controls that allow for the activation of effector caspases, while avoiding induction of cell death/apoptosis. As noted above, caspase-mediated cell differentiation is a broadly conserved mechanism that spans all cell lineages and is extant from worms to humans [24]. The key distinction between death and non-death caspase function is the duration of the signaling cascade, where cell death is characterized by sustained caspase 3/7 activity, non-death responses by a transient activity pattern [23, 37, 77]. A probable loci of caspase control may reside with the inhibitor of apoptosis (IAPs), a class of RING Finger domain proteins that target and inhibit caspase activity by both structural blockade and through self directed ubiquitination of the IAP/caspase complex [21, 63, 107]. One IAP, X-linked inhibitor of apoptosis (XIAP), has been investigated for its capacity to modify caspase activity during myoblast differentiation, with studies supporting a role for XIAP in this regard [77, 103] and a study that concludes otherwise [9]. The additional IAPs such as cIAP1 and cIAP2 may provide similar levels of control yet they have not been investigated in this context. Finally, it is relevant to note, that while protein interactions with the effector caspases may direct the activation of these proteases for non-death outcomes, the ultimate arbiter of control may originate with the initiation signal per se. Activation of intrinsic and/or extrinsic cell death signal cascades during non-apoptotic cell adaptation(s) are themselves uniformly transient [30, 115], suggesting that external physiologic inputs may be the deciding factor in managing effector caspase-mediated outcomes in any tissue, including skeletal muscle.

Conclusions

In addition to its role in simply maintaining proteostasis, proteolysis is an essential part of the production of new skeletal muscle fibers and adapting muscle fibers to cellular stress. Current research clearly indicates a critical role of protein degradation in the regulation of the myogenic differentiation program, ensuring timely protein expression for myoblast differentiation while also mediating myoblast fusion and myotube formation. The mature muscle fiber then relies on appropriate protein degradation to rid the cell of damaged proteins from the mechanical and oxidative stress that accompanies the force-bearing/force-generating function of skeletal muscle. Additionally, during nutrient deprivation the organism depends on skeletal muscle proteolysis to maintain whole-body energy homeostasis. Not surprisingly, defects within these proteolytic systems often result in the development of myopathic conditions. Thus, future work in this field should focus on delineating the mechanistic details of protease function in healthy skeletal muscle. In our opinion, this information is essential, as indiscriminate targeting of proteolytic pathways as a means to treat muscle atrophy may engender more harm than benefit.

Abbreviations

AMP: adenosine monophosphate; AMPK: AMP-dependent protein kinase; ATP: adenosine triphosphate; BCL2: B cell lymphoma 2; CASA: chaperone-assisted selective autophagy; DCP-1: death caspase-1; ECM: extracellular matrix; FADD: Fas-associated protein with death domain; GLUT4: glucose transporter 4; HuR: ELAV-like protein; HIPK2: homeodomain-interacting protein kinase 2; IAP: inhibitor of apoptosis; LC3: phosphatidylethanolamine-conjugated microtubule-associated protein 1A/1B-light chain 3; MAFbx: muscle atrophy F-box; MST1: macrophage stimulating 1; mTOR: mammalian target of rapamycin; MuRF1: muscle really interesting novel gene (RING) finger-1; NEK5: NIMA-related kinase 5; PGC-1α: peroxisome proliferator-activated receptor-γ coactivator-1α;

ROS: reactive oxygen species; SR: sarcoplasmic reticulum; TA: tibialis anterior; TSC1: tuberous sclerosis 1; ULK1: Unc-51 like autophagy activating kinase 1; XIAP: X-linked inhibitor of apoptosis.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
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