Telomere Length Regulation of Muscle Stem Cells in Chronic Injuries

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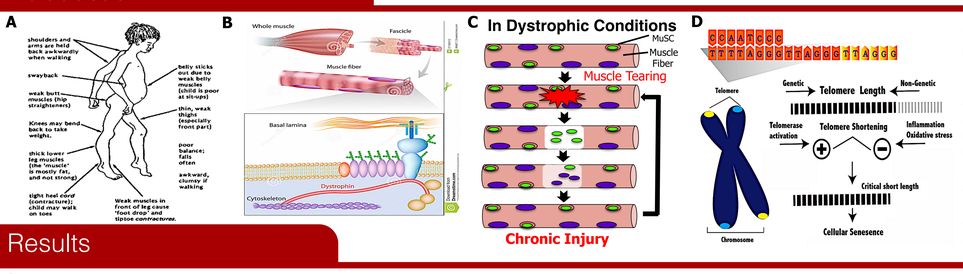
Abstract

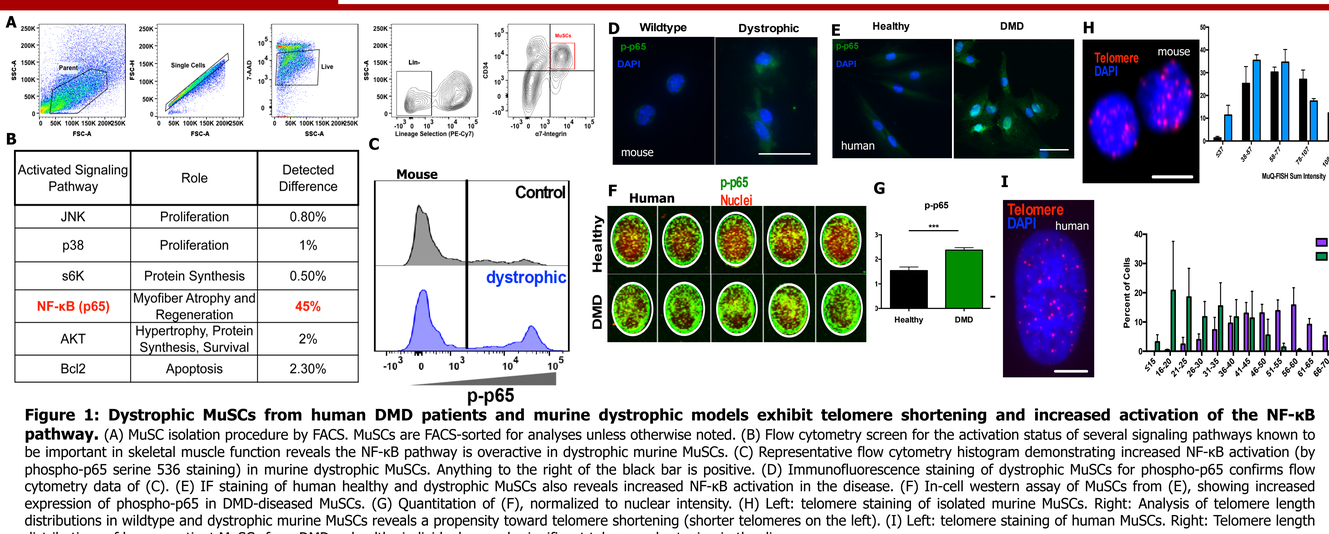
Duchenne muscular dystrophy (DMD) is a muscle disease characterized by multiple rounds of skeletal muscle injury and repair. Skeletal muscle regeneration relies on the proper functioning of muscle stem cells (MuSCs), which upon injury, become activated, proliferate, differentiate, and fuse to form a repaired muscle fiber. Previous studies by the lab have shown that telomeres play a pivotal role in the severity and progression of dystrophy in mouse models, particularly in MuSCs. Recently, we have shown that telomeres are also shortened in DMD-diseased MuSCs from young patients, compared with those from healthy donors. To identify dysregulated signaling pathways in DMD that may contribute to telomere length changes, we screened dystrophic murine MuSCs, and we found that the NF-κB signaling pathway was aberrantly upregulated in the dystrophic MuSCs, as compared with controls. Using an inducible, MuSC-specific mouse model, where NF-κB signaling is upregulated in the absence of stimuli, we queried the effects of enforced NF-κB signaling in MuSCs during in both normal and chronic muscle injury conditions. We found no ill effects resulting from NF-κB activation in either MuSCs or skeletal muscle tissue, when muscles were not injured. However, upon chronic injury, we observed rapid telomere shortening of NF-κB MuSCs compared to controls, where more repetitive injuries resulted in more significant telomere shortening. This finding correlated well with increasingly poor muscle histology. We next asked whether increased activation of NF-κB signaling in mice experiencing dystrophy enhanced the phenotype of the disease. We observed a much more severe pathology, increased muscle damage, reduced muscle strength, and telomere shortening in dystrophic mice and MuSCs, when NF-κB signaling was further enhanced. To identify the mechanism by which NF-κB promotes telomere attrition in chronic injury conditions, we investigated several different aspects. We found that NF-κB MuSCs do not exhibit increased proliferation, but they do show a downregulation of Ku80 RNA. Ku80 can reside at the telomere, but its function at this location is still being elucidated. However, Ku80 is also involved in DNA damage repair by nonhomologous end joining. To assess whether more DNA damage is present at the telomere, MuSCs from injured NF-κB mice were stained with a DNA damage marker, a MuSC marker, and a telomere probe. We found increased DNA damage at the telomere, which is known mechanism for induction of telomere shortening. We observed the same phenomenon in MuSCs derived from DMD patients, as compared to healthy human MuSCs. Finally, we asked whether the NF-κB-dependent reduction of telomere length correlated with stem cell exhaustion, similar to what is observed in the DMD disease process. Using two-photon microscopy and GFP-labeled MuSCs, we observed a significant reduction in MuSC numbers in chronically injured NF-κB mice, compared to control mice. These findings are the first to identify a role for NF-κB signaling in telomere length loss and stem cell exhaustion in chronic muscle injuries, and may lead to alternative avenues for the treatment of patients with DMD.

Introduction



DMD is a lethal disease that affects 1/3500 males and disturbs muscle strength and function (A). The cause of DMD is a nonsense mutation in dystrophin (B), which links the interior of the muscle fiber with the exterior of the fiber. Loss of this connector results in muscle tearing/damage with movement. Muscle stem cells (MuSCs) are responsible for the repair of muscle damage. However, DMD causes repetitive, chronic muscle injuries, and over time, muscle tissue is replaced by fat/fibrotic tissue, leading to loss of strength (C). Since MuSCs are constantly utilized to repair dystrophic muscle damage, they are likely to exhibit shortened telomeres, which could explain why regeneration at later stages of the disease is impaired (D).

Results



Activated Signaling Pathway	Role	Detected Difference
JNK	Proliferation	0.80%
p38	Proliferation	1%
s6K	Protein Synthesis	0.50%
NF-κB (p65)	Myofiber Atrophy and Regeneration	45%
AKT	Hypertrophy, Protein Synthesis, Survival	2%
Bcl2	Apoptosis	2.30%

Figure 1: Dystrophic MuSCs from human DMD patients and murine dystrophic models exhibit telomere shortening and increased activation of the NF-κB pathway. (A) MuSC isolation procedure by FACS. MuSCs are FACS-sorted for analyses unless otherwise noted. (B) Flow cytometry screen for the activation status of several signaling pathways known to be important in skeletal muscle function reveals the NF-κB pathway is overactive in dystrophic murine MuSCs. (C) Representative flow cytometry histogram demonstrating increased NF-κB activation (by phospho-p65 serine 536 staining) in murine dystrophic MuSCs. Anything to the right of the black bar is positive. (D) Immunofluorescence staining of dystrophic MuSCs for phospho-p65 confirms flow cytometry data of (C). (E) IF staining of human healthy and dystrophic MuSCs also reveals increased NF-κB activation in the disease. (F) In-cell western assay of MuSCs from (E), showing increased expression of phospho-p65 in DMD-diseased MuSCs. (G) Quantitation of (F), normalized to nuclear intensity. (H) Left: telomere staining of isolated murine MuSCs. Right: Analysis of telomere length distributions in wildtype and dystrophic murine MuSCs reveals a propensity toward telomere shortening (shorter telomeres on the left). (I) Left: telomere staining of human MuSCs. Right: Telomere length distributions of human patient MuSCs from DMD or healthy individuals reveals significant telomere shortening in the disease.

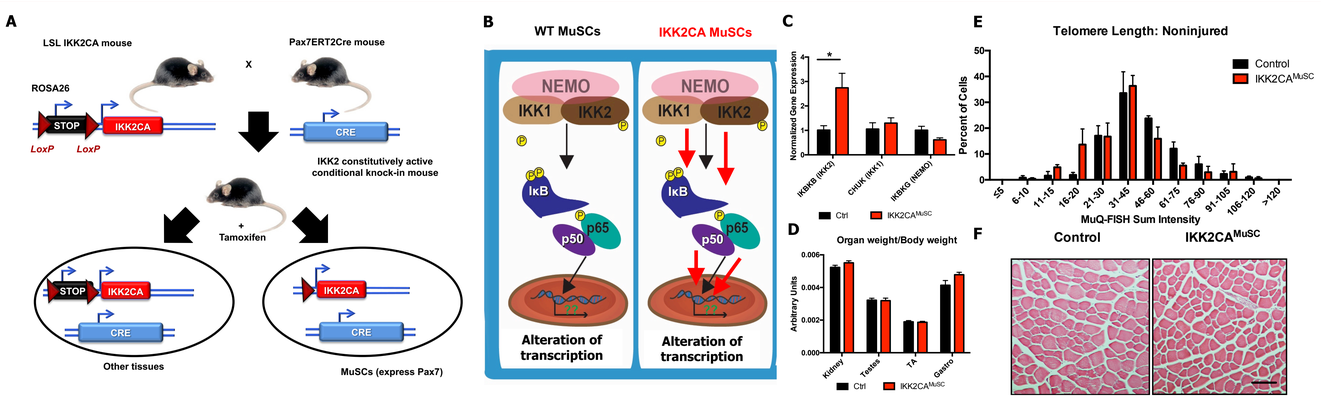


Figure 2: Enforced activation of NF-κB signaling in MuSCs has no effect on muscle histology, weight, or telomere length. (A) Design of a mouse model where NF-κB is induced in MuSCs in adults. (B) NF-κB signaling pathway in wildtype and IKK2CA MuSCs. (C) Assessment of IKK2 expression following tamoxifen administration. (D) Skeletal muscle weight comparisions reveal no overt differences between genotypes. (E) Telomere length distributions of MuSCs from wildtype and IKK2CA MuSCs demonstrates no significant differences in telomere length in noninjured animals. (F) H&E staining of tibialis anterior muscles from noninjured wildtype and IKK2CA mice.

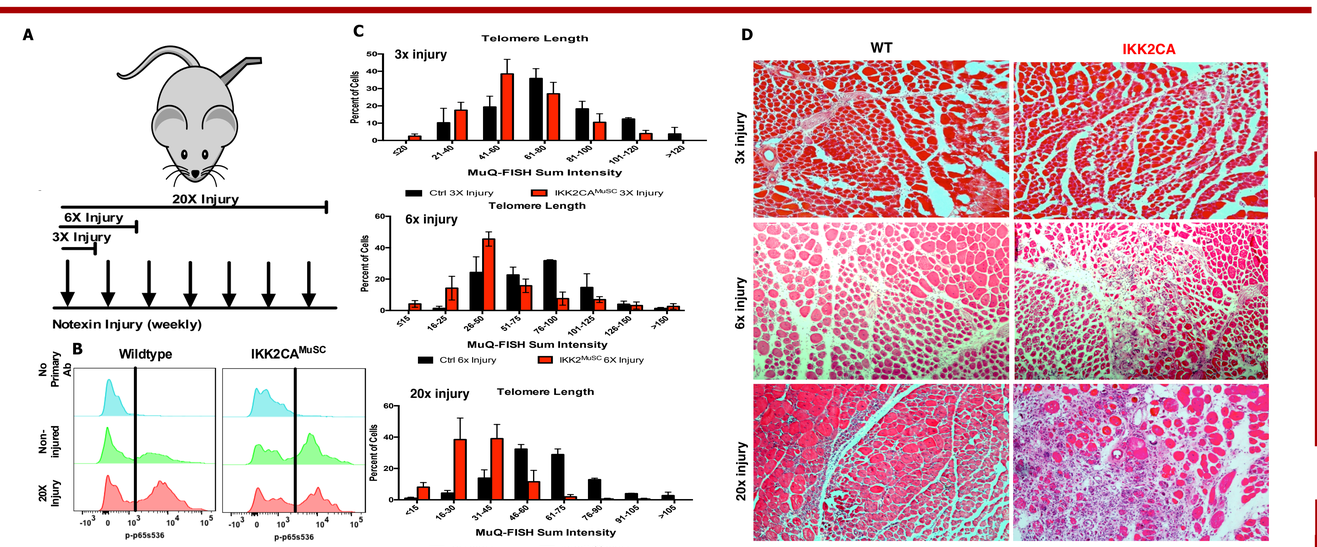
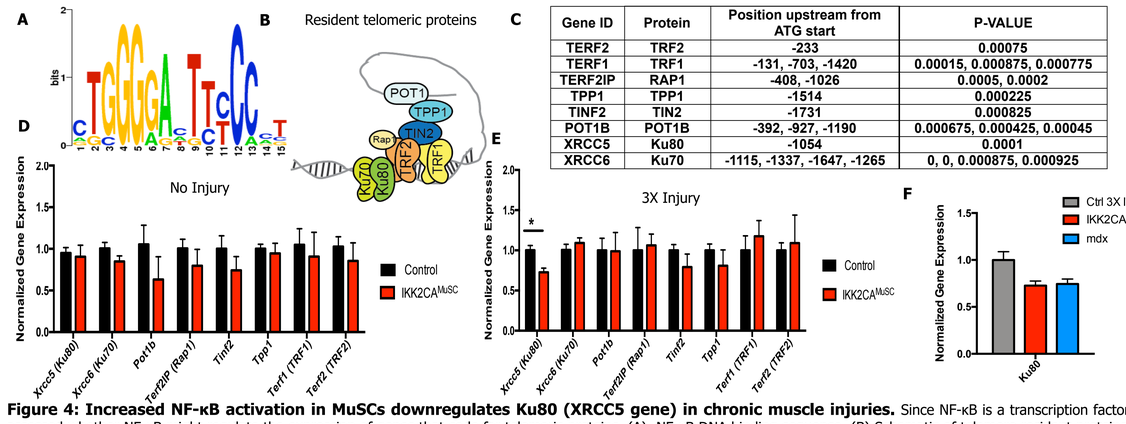
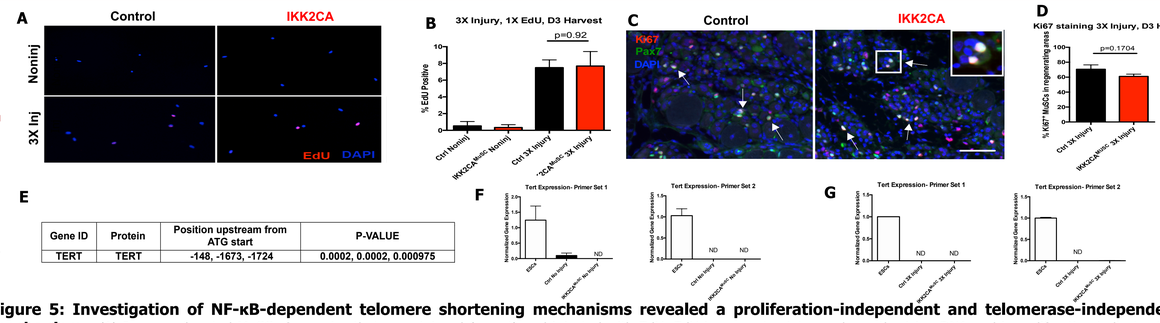


Figure 3: Overactivated NF-κB signaling promotes telomere shortening in MuSCs and poor muscle regeneration during chronic injury. (A) Scheme of chronic injury induction. Mouse muscles were injured with notexin, a myotoxic agent, weekly, as shown. (B) Flow cytometry staining for activated NF-κB (by phospho-p65 serine 536 quantitation) in control and IKK2CA MuSCs, derived from either noninjured or 20X injured mice. Note that NF-κB activation (right of black line) is low in noninjured control mice, but gets induced following repetitive (chronic) injury. However, IKK2CA mice already exhibit activated NF-κB in the absence of injury, which remains unchanged following injury. (C) Telomere length distribution in MuSCs from control (black) or IKK2CA (red) MuSCs following 3 injuries (top), 6 injuries (middle), or 20 injuries (bottom). Note the dramatic shifts to the left (shorter telomeres), in IKK2CA MuSCs, relative to control MuSCs. (D) H&E staining of muscles from mice shown in (C).



Gene ID	Protein	Position upstream from ATG start	P-VALUE
TERF2	TRF2	-233	0.00075
TERF1	TRF1	-131, -703, -1420	0.00015, 0.000875, 0.000775
TERF2IP	RAP1	-408, -1026	0.0005, 0.0002
TPP1	TPP1	-1514	0.000225
TINF2	TIN2	-1731	0.000825
POT1B	POT1B	-392, -927, -1190	0.000675, 0.000425, 0.00045
XRCC5	Ku80	-1054	0.0001
XRCC6	Ku70	-1115, -1337, -1647, -1265	0, 0, 0.000875, 0.000925

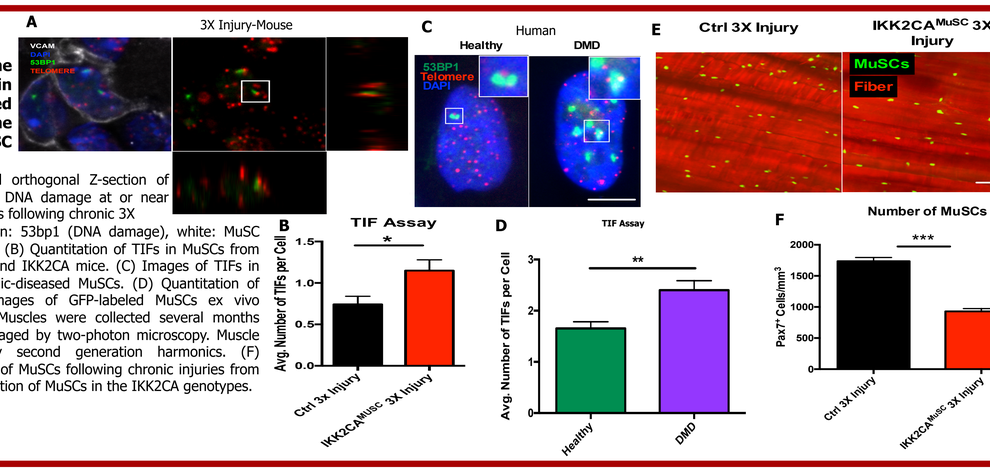
Figure 4: Increased NF-κB activation in MuSCs downregulates Ku80 (XRCC5 gene) in chronic muscle injuries. Since NF-κB is a transcription factor, we assessed whether NF-κB might regulate the expression of genes that code for telomeric proteins. (A) NF-κB DNA binding sequence. (B) Schematic of telomere resident proteins. (C) In-silico analysis of putative NF-κB binding sites in promoters of telomeric proteins using the Lasagna 2.0 algorithm. (D). QRT-PCR of telomeric gene expression in noninjured control and IKK2CA MuSCs. No significant differences were observed. (E) Gene expression analysis of control and IKK2CA MuSCs following chronic injury (3 times). Ku80, the XRCC5 gene product, was downregulated in IKK2CA MuSCs. (F) QRT-PCR of 3X injured control and IKK2CA MuSCs, compared with murine dystrophic (mdx) MuSCs.



Gene ID	Protein	Position upstream from ATG start	P-VALUE
TERT	TERT	-148, -1673, -1724	0.0002, 0.0002, 0.000975

Figure 5: Investigation of NF-κB-dependent telomere shortening mechanisms revealed a proliferation-independent and telomerase-independent mechanism. (A) Images of sorted MuSCs from mice that were injured (or not) and injected with EdU. EdU incorporates into newly synthesizing DNA and is proliferation indicator that can by stained for by IF. (B) Quantitation of (A). (C) Assessment of proliferation in muscle cryosections following 3X chronic injury in control and IKK2CA mice. Red: Ki67 (proliferation marker), Green: Pax7 (MuSC marker), Blue (DAPI). (D) Quantitation of (C). (E) In silico analysis of NF-κB binding sites in the Tert promoter. (F) QRT-PCR of Tert (telomerase) expression in noninjured IKK2CA and control MuSCs. Murine embryonic stem cells served as a positive control. (G) Assessment of Tert expression in chronically injured control and IKK2CA mouse MuSCs.

Figure 6: NF-κB-dependent downregulation of the DNA repair protein Ku80 leads to increased DNA damage at the telomere and to MuSC exhaustion.



(A) Representative confocal orthogonal Z-section of telomere-induced foci (TIF; DNA damage at or near telomeres) in muscle sections following chronic 3X injury. Red: telomere, Green: 53bp1 (DNA damage), white: MuSC marker (Vcam), blue: DAPI. (B) Quantitation of TIFs in MuSCs from chronically injured control and IKK2CA mice. (C) Images of TIFs in healthy human or dystrophic-diseased MuSCs. (D) Quantitation of (C). (E) Representative images of GFP-labeled MuSCs ex vivo following chronic injuries. Muscles were collected several months after the last injury and imaged by two-photon microscopy. Muscle fibers were identified by second generation harmonics. (F) Quantitation of the number of MuSCs following chronic injuries from (E). Note the dramatic reduction of MuSCs in the IKK2CA genotypes.

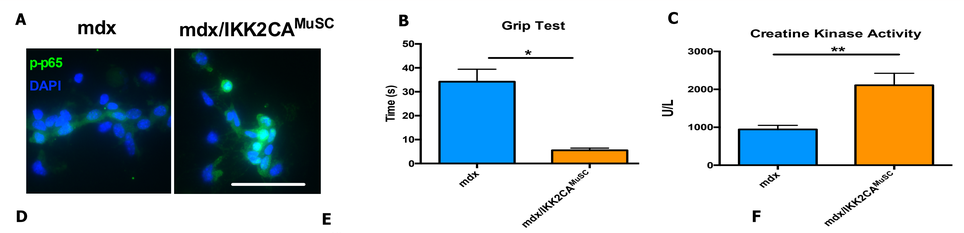
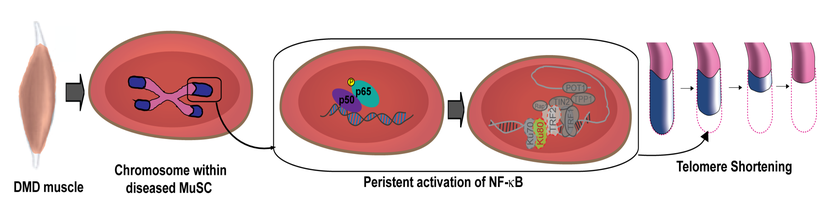


Figure 7: The combination of the endogenous injuries from dystrophy with the MuSC-specific IKK2CA mice results in a severe muscle phenotype. (A) Staining for phospho-p65 serine 536 in isolated MuSCs shows increased staining in the combination mouse model. (B) Dystrophic mice overexpressing overactivated IKK2 have reduced muscle strength with age. (C) mdx/IKK2CA mice exhibit more muscle damage compared to dystrophic mice only. (D) The combination mouse exhibits severe kyphosis with age, similar to dystrophic patients. (E) Telomere length distributions reveal the combination mouse has shorter telomeres compared with dystrophic mice. (F) MuSCs from combination mice have more DNA damage at telomeres, compared with dystrophic mice only.

Conclusions



Current Direction

- Rescue experiment- restoration of Ku80 in IKK2CA myogenic cells and assessment of telomere length