
Figures and figure supplements

Lack of *Tgfb β 1* and *Acvr1b* synergistically stimulates myofibre hypertrophy and accelerates muscle regeneration

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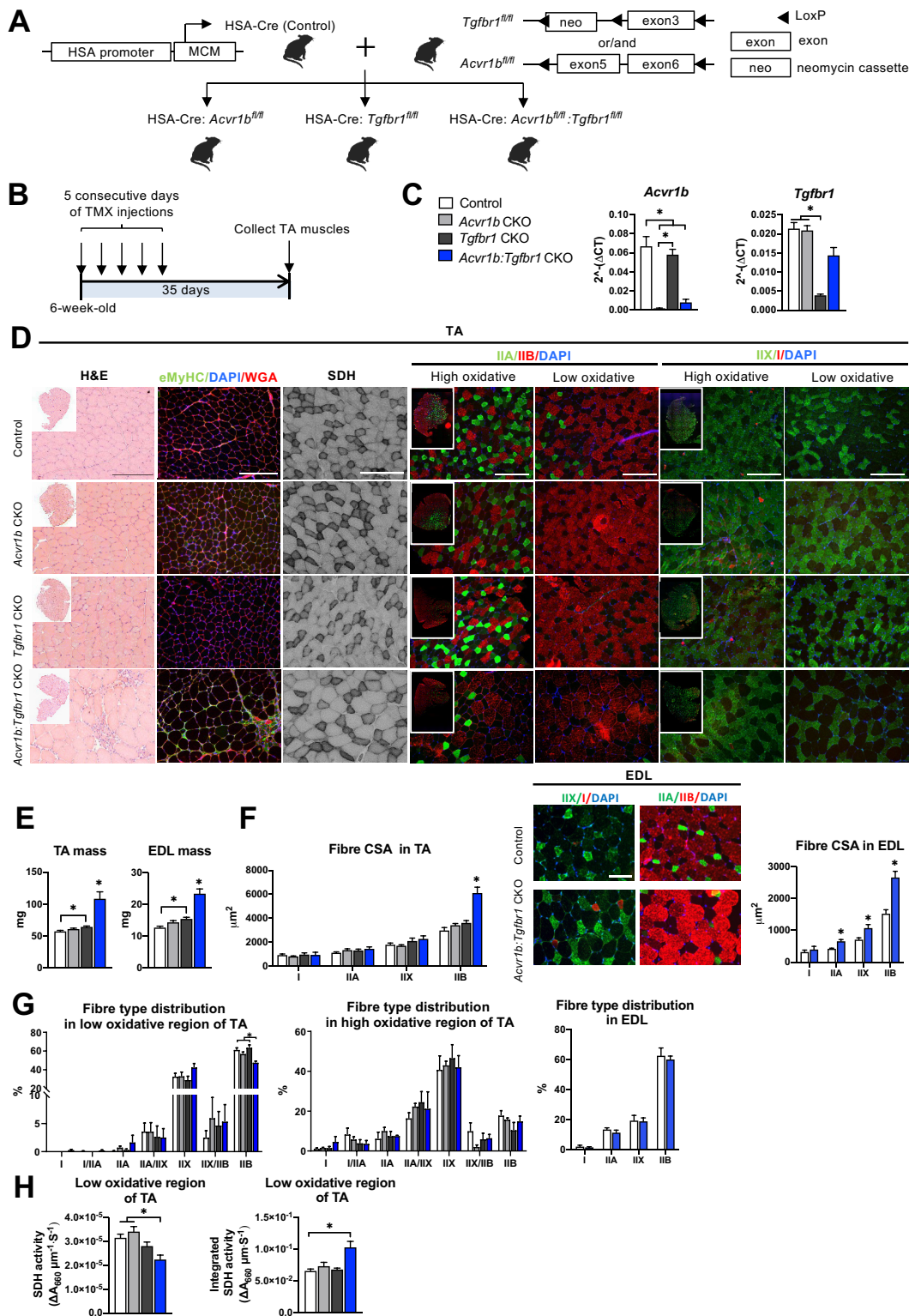


Figure 1. Simultaneous knockout of both *Acvr1b* and *Tgfb1* caused muscle hypertrophy. **(A)** Scheme showing cross-breeding of HSA-Cre mouse line with conditional knockout mouse lines *Acvr1b*^{fl/fl} and *Tgfb1*^{fl/fl}. LoxP sites are indicated by black arrows. A loxP-flanked neomycin (neo) cassette is inserted upstream of exon3 of *Acvr1b* genome. **(B)** Scheme demonstrating receptor knockout induced by tamoxifen (TMX) injection for consecutive 5 days. **(C)** Relative mRNA expression of *Acvr1b* and *Tgfb1* in TA muscles of experimental groups. **(D)** Histology stainings of TA muscles 35 days after

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first TMX injection. H&E staining and immunofluorescent staining of eMyHC (green) of TA showed regenerative regions containing eMyHC⁺ myofibres with central nuclei (DAPI, blue) in *Acvr1b:Tgfb1* CKO mice, wheat glucose agglutinin (WGA, red) was used to visualise cell membranes and ECM. *Acvr1b:Tgfb1* CKO mice showed lower staining intensity for SDH activity in low oxidative region of TA. MyHCs staining demonstrated type IIA (green), IIB (red), IIX (green) and I (red) myofibres in low and high oxidative regions of TA. Scale bars = 250 μm . **(E)** TA and EDL muscle mass and myofibre cross-sectional areas (CSAs) were increased in *Acvr1b:Tgfb1* CKO mice. **(F)** In TA, specifically CSA of type IIB myofibres was increased in *Acvr1b:Tgfb1* CKO animals, while in EDL CSA of all type II myofibres was increased. Myofibre types were stained in EDL. **(G)** Percentage of type IIB in low oxidative region of TA was reduced. No differences were observed in myofibre distribution in high oxidative region of TA or EDL. **(H)** SDH activity (absorbance units (ΔA_{660}) per micrometer section thickness per second of incubation time ($\Delta A_{660} \mu\text{m}^{-1} \text{s}^{-1}$)) was decreased, while the integrated SDH activity, SDH activity multiplied by CSA ($\Delta A_{660} \mu\text{m} \text{s}^{-1}$), increased in low oxidative region of TA of *Acvr1b:Tgfb1* CKO animals. N = 5–8 mice. Results are presented as mean + SEM. *: $p < 0.05$. Significant difference between individual groups is indicated by lines with a *. Single * indicates significant difference compared to all other groups.

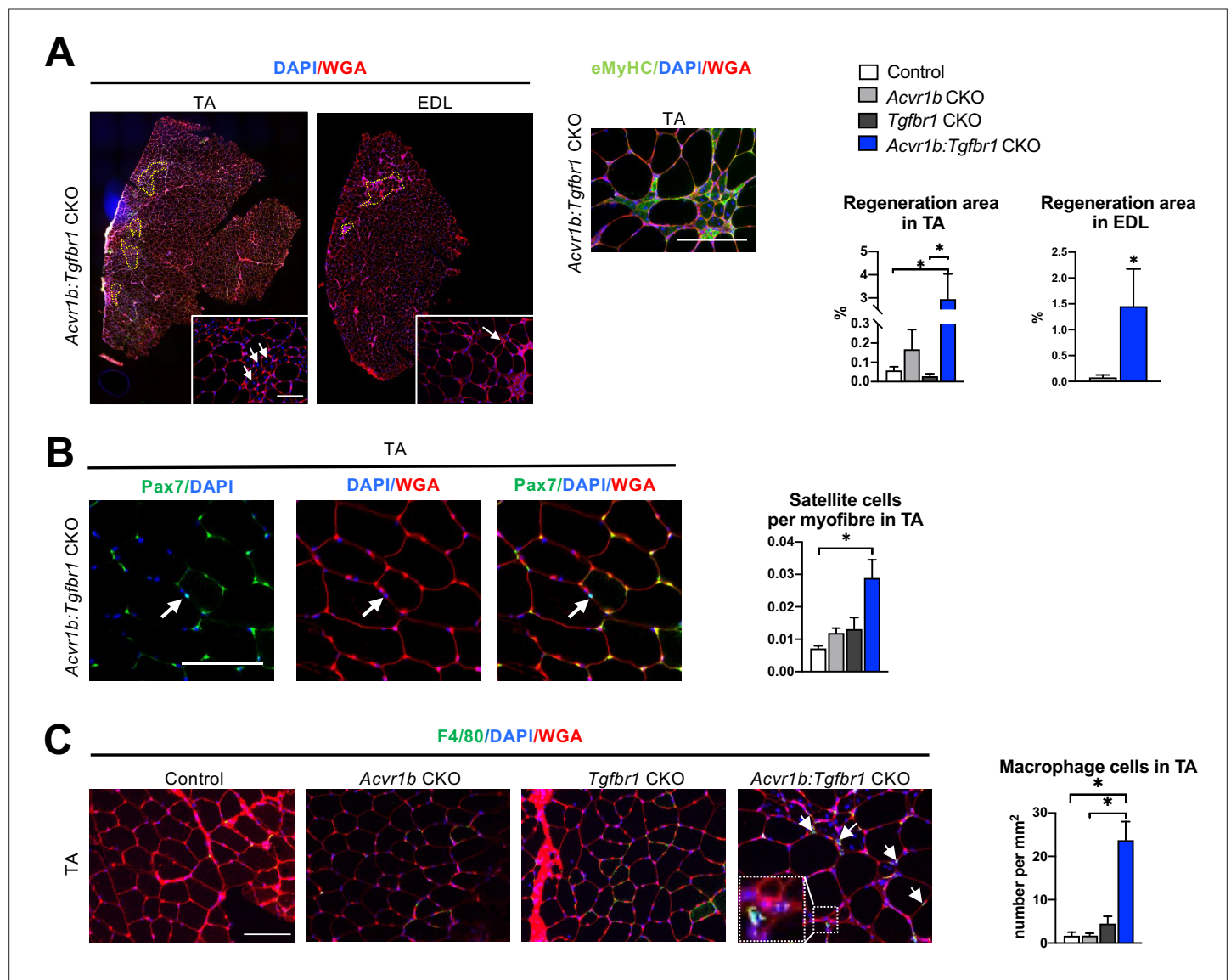


Figure 2. Increased heterogeneity of cell types was found in both TA and EDL of *Acvr1b:Tgfbf1* CKO animals. **(A)** Regions with spontaneously regenerating myofibres (circled by yellow dash lines) with central nuclei (indicated by arrows) were particularly present in low oxidative region of TA and EDL of *Acvr1b:Tgfbf1* CKO animals. **(B)** Increased number of Pax7⁺ cells per myofibre was found in TA of *Acvr1b:Tgfbf1* CKO mice. **(C)** IF staining of F4/80 (green) showed an increased number of macrophages (indicated by arrows) in TA muscle per mm² CSA of *Acvr1b:Tgfbf1* CKO mice compared to control. Macrophages (image with higher magnification on the left corner) were mainly located around myofibres with central nuclei. Scale bar = 100 μ m. N = 5–8 mice. Results are presented as mean + SEM. *. p < 0.05. Significant differences between individual groups are indicated by lines with a *. Single * indicates significant difference compared to all other groups at the same time point.

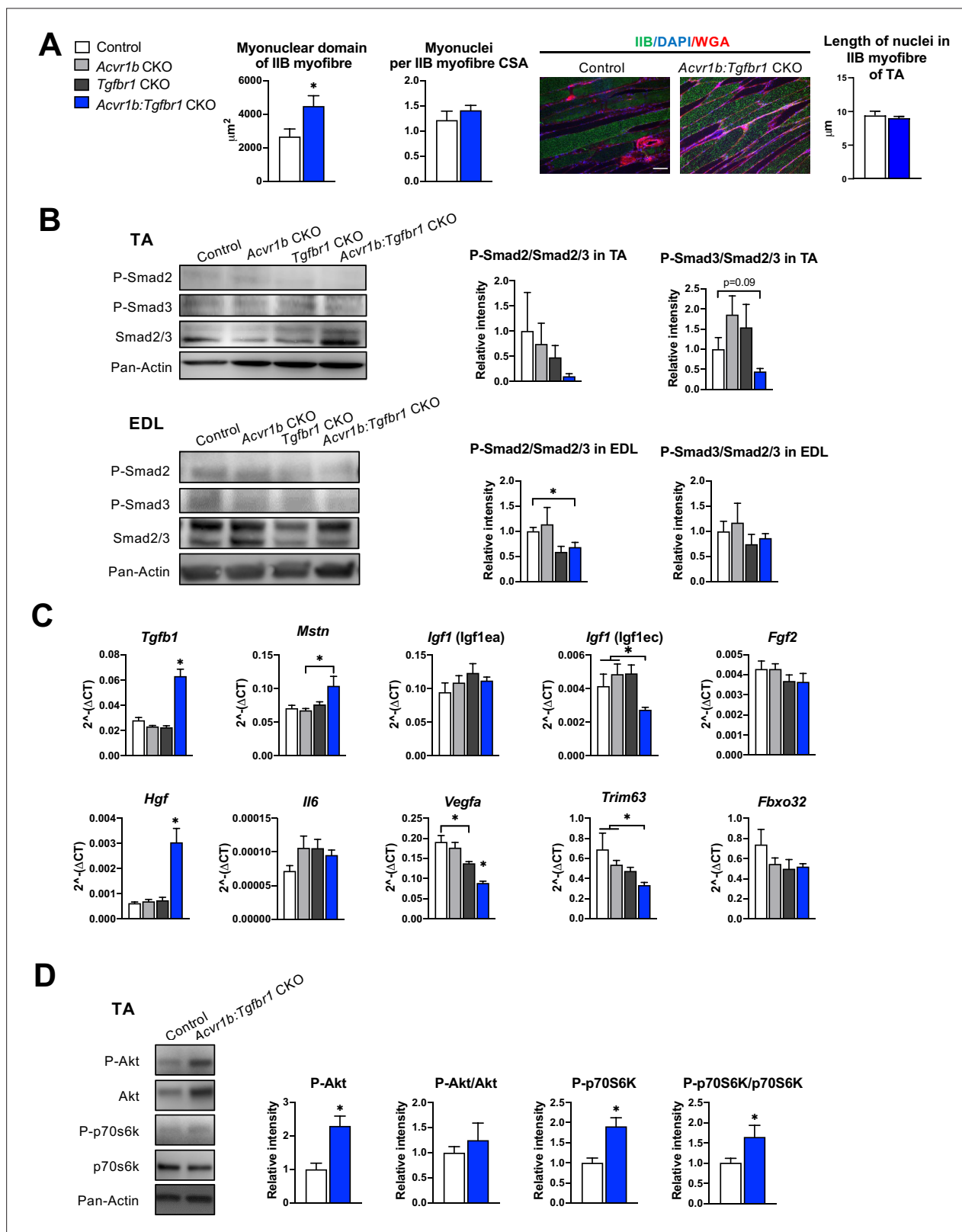


Figure 3. Effects of simultaneous knockout of both *Acvr1b* and *Tgfb1* on myonuclear number and signalling for protein synthesis as well as degradation. **(A)** No differences in myonuclear lengths were observed in longitudinal sections of TA type IIB myofibres of *Acvr1b:Tgfb1* CKO compared to control animals. This indicates that simultaneous knockout of *Acvr1b:Tgfb1* CKO did not affect the number of myonuclei per myofibre and that the myonuclear domain (i.e. cross-sectional area/ nuclei (μm^2)) was almost doubled. Scale bar = 100 μm . **(B)** Western blot analysis for

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Smad2/3 phosphorylation in TA and EDL muscle. **(C)** Relative gene expression of growth factors in non-injured muscle. **(D)** Western blot analysis of phosphorylated and total Akt and p70S6K in TA muscles. Results are presented as mean + SEM. N = 5–8 mice. *: $p < 0.05$. Significant differences between individual groups are indicated by lines with a *. Single * indicates significant difference compared to all other groups at the same time point.

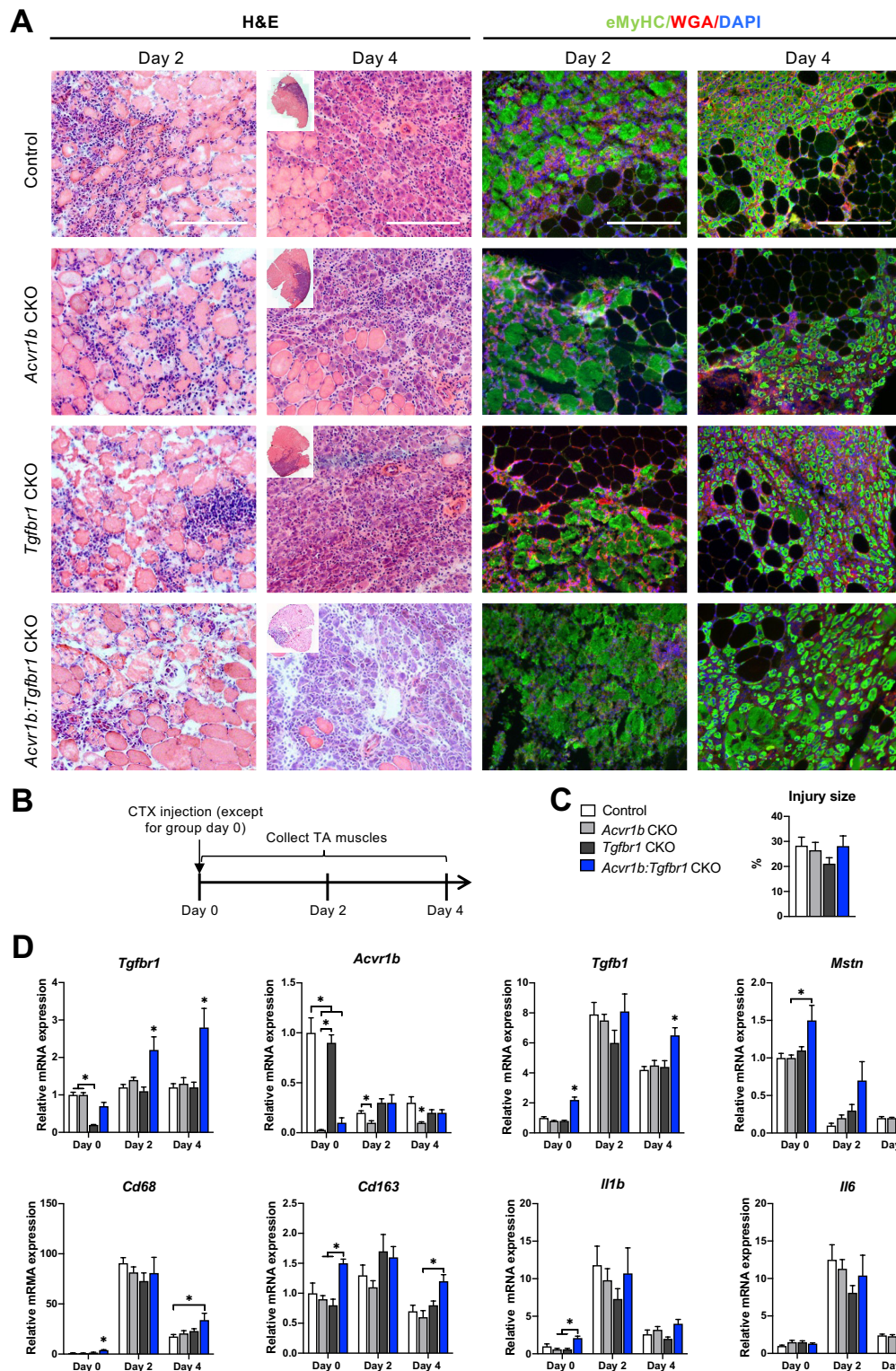


Figure 4. Immune response was slightly enhanced in muscle of *Acvr1b:Tgfb1* CKO mice. **(A)** Representative images of H&E and eMyHC staining of TA sections at 2 and 4 days after CTX injection. Scale bars = 250 μ m. **(B)** Scheme shows CTX injection in TA and sample collection. **(C)** Percentage of injury area was not significantly different between groups. **(D)** Relative gene expressions in TA in the absence (day 0) or presence of CTX injection after 2 and 4 days. Results are presented as mean + SEM. N = 5–8 mice, *; $p < 0.05$. Significant differences between individual groups are indicated by lines with a *. Single * indicates significant difference compared to all other groups at the same time point.

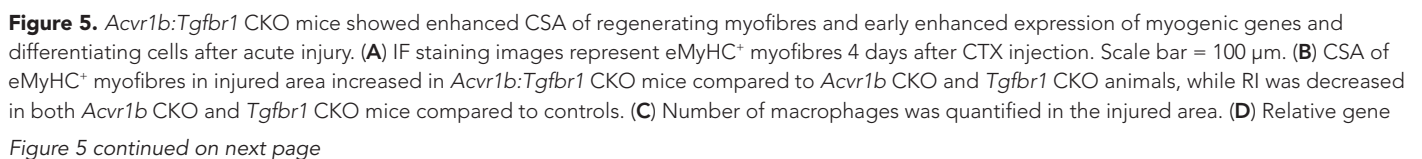


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expression in TA in absence (day 0) or presence of CTX injection after 2 and 4 days are presented. Increased number of Ki67⁺ cells (**E**) and Pax7⁺ (**F**) cells were found in TA of *Acvr1b:Tgfbf1* CKO mice in absence of injury as well as 4 days after CTX injection. (**G**) Four days post injury, number of Ki67⁺/Pax7⁺ cells was not different between control and *Acvr1b:Tgfbf1* CKO mice. (**H**) More Myogenin⁺ cells were found in injured area of *Acvr1b:Tgfbf1* CKO mice on day 4 post injury. Results are presented as mean + SEM. N = 5–8 mice, *: p < 0.05. Significant differences between individual groups are indicated by lines with a *. Single * indicates significant difference compared to all other groups at the same time point.

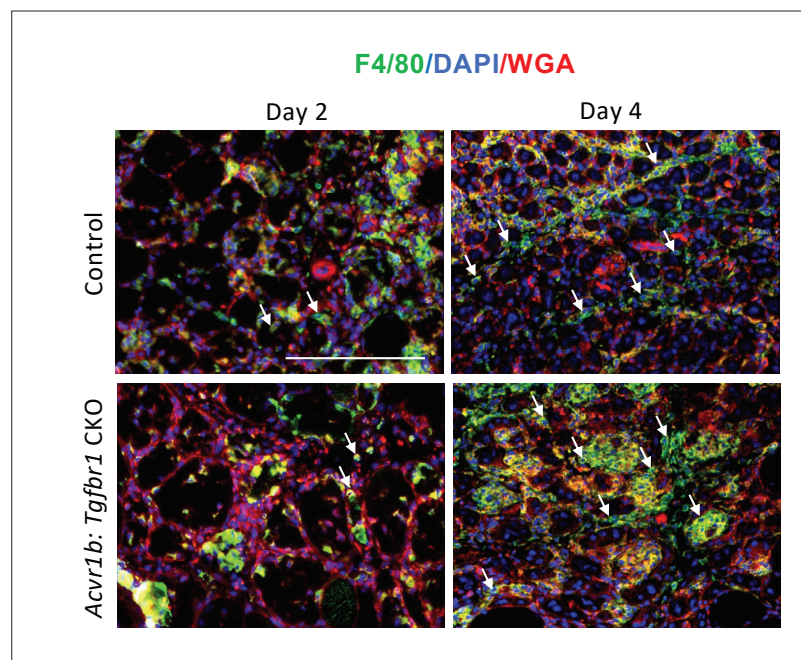


Figure 5—figure supplement 1. Number of macrophages in TA of *Acvr1b:Tgfbfr1* CKO animals was increased 4 days post injury. Number of macrophages (F4/80⁺, green) in control and *Acvr1b:Tgfbfr1* CKO animals on days 2 and 4 post injury. Nuclei were stained by DAPI (blue) and ECM of muscle were stained by WGA (red). Scale bar = 100 μ m. N = 5–7.

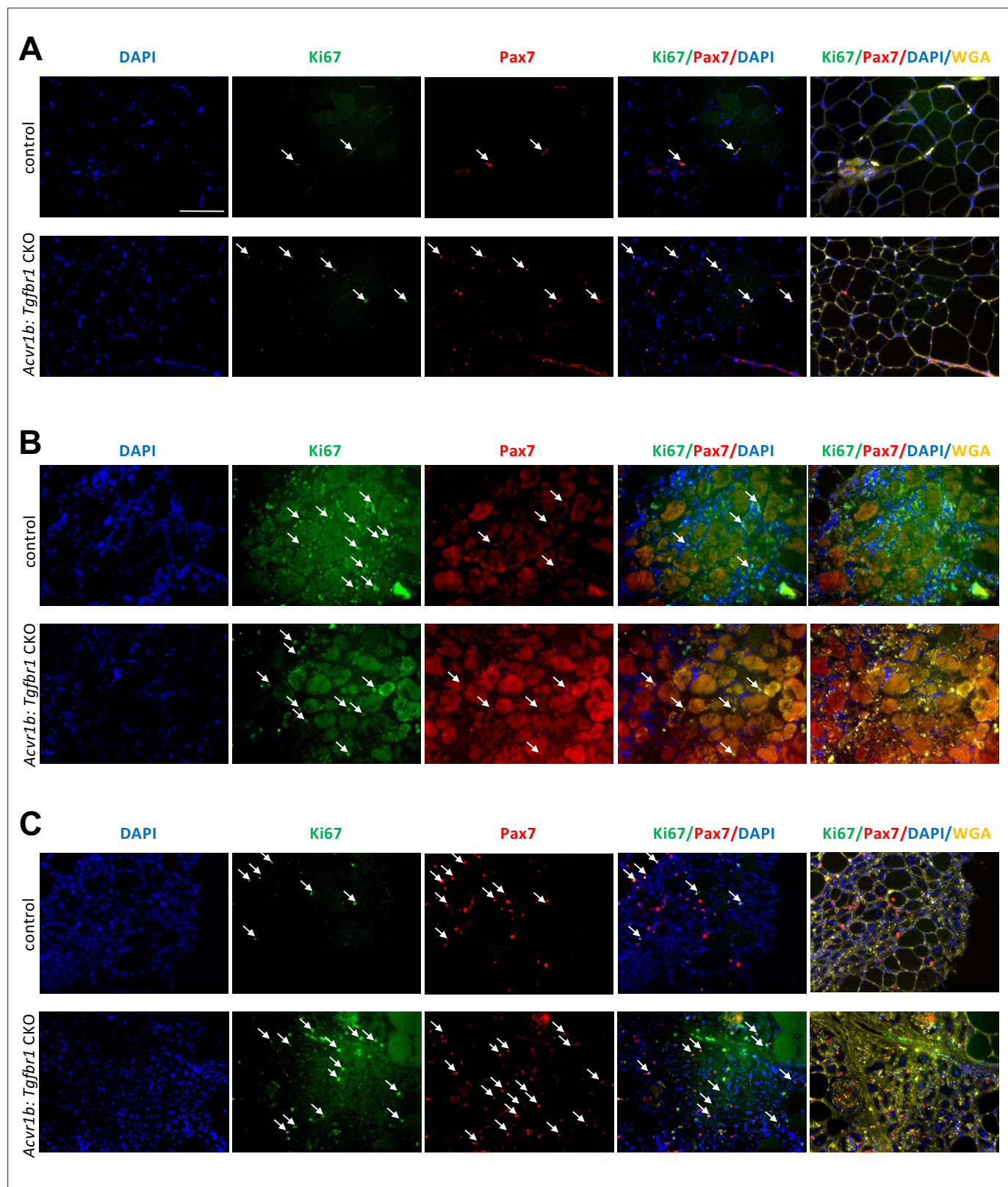


Figure 5—figure supplement 2. Increased number of proliferating cells and satellite cells in TA of *Acvr1b:Tgfbf1r1* CKO animals in absence of injury and 4 days post injury. **(A)** In absence of injury, proliferating cells (Ki67⁺, green, white arrows) and muscle satellite cells (Pax7⁺, red, white arrows) were shown by IF staining in low oxidative area of TA, where regeneration patches were found. Two **(B)** and 4 **(C)** days post injury, more Ki67⁺ and Pax7⁺ cells (white arrows) infiltrated in injured area. Nuclei were stained by DAPI (blue) and ECM of muscle were stained by WGA (yellow). Scale bar = 100 μ m. N = 5–7.

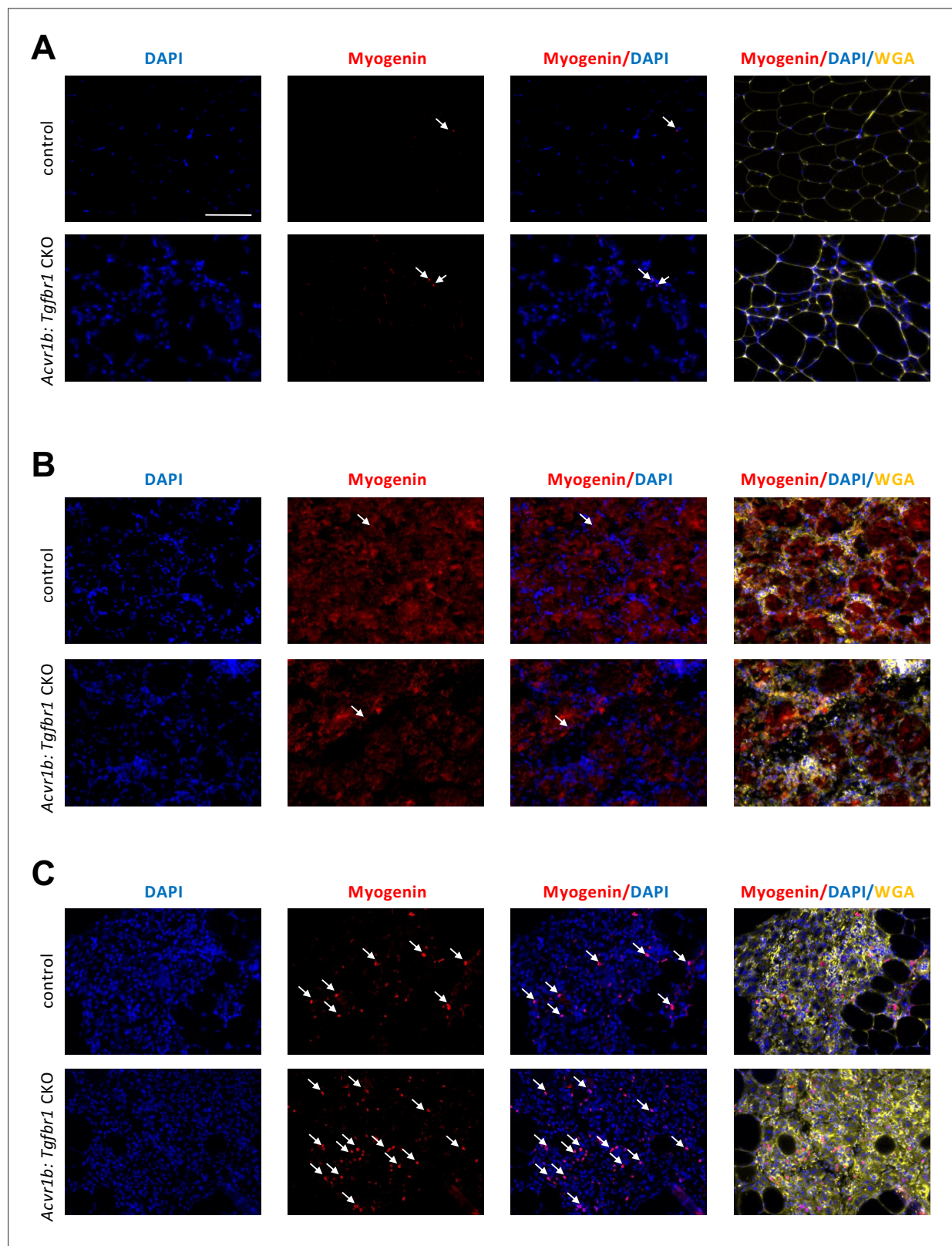


Figure 5—figure supplement 3. Increased number of differentiating muscle cells in TA of *Acvr1b:Tgfbbr1* CKO animals 4 days post injury. On day 0 (A) and 2 days (B) post injury, the number of differentiating myoblasts (myogenin⁺, red, white arrows) was of no difference between groups. Four days (C) post injury, more Myogenin⁺ cells were found in injured area of *Acvr1b:Tgfbbr1* CKO animals. Nuclei were stained by DAPI (blue) and ECM of muscle were stained by WGA (yellow). Scale bar = 100 μ m. N = 5–7.

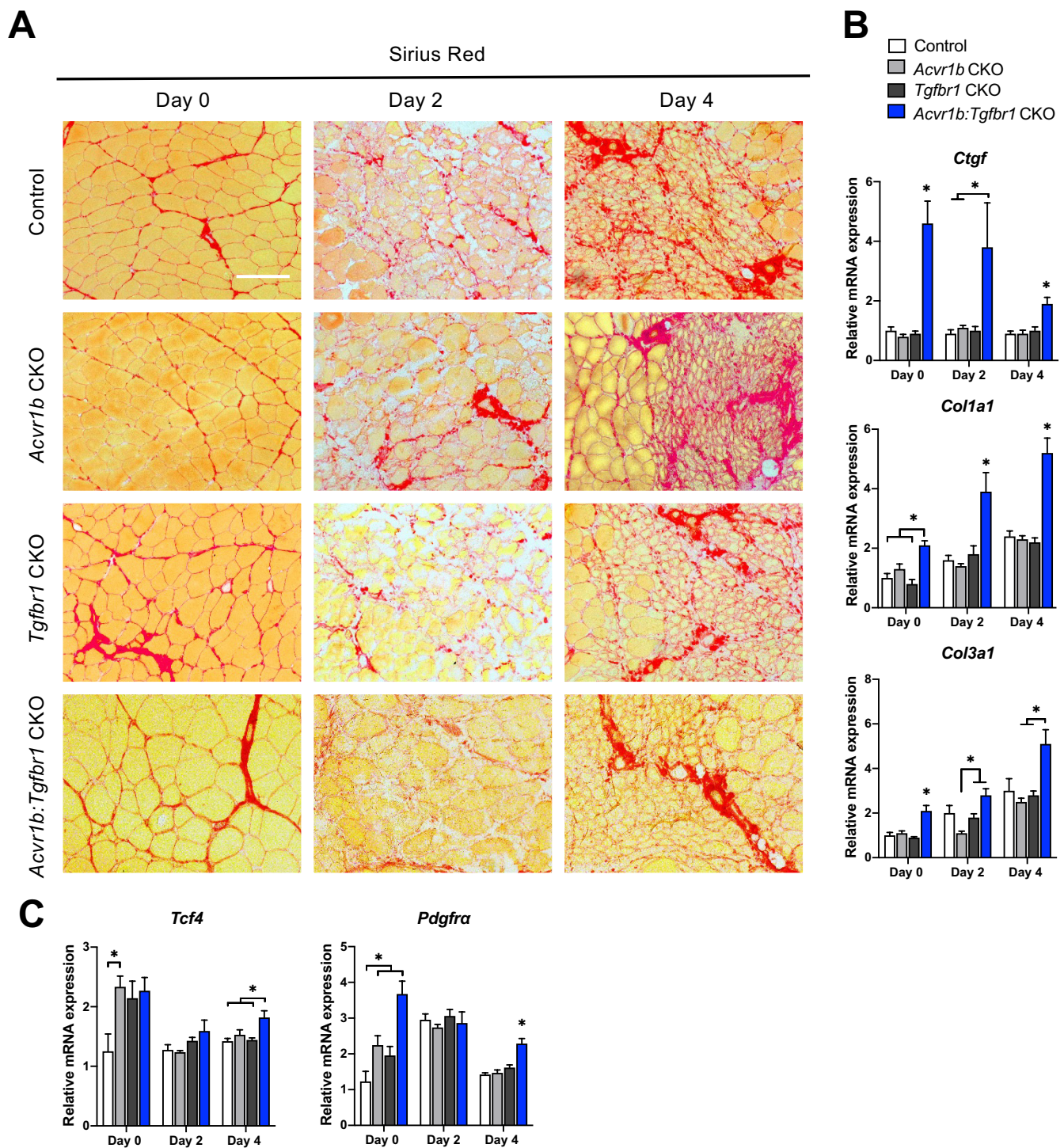


Figure 6. Relative mRNA expression levels of ECM components were enhanced in *Acvr1b:Tgfb1* CKO mice. **(A)** Sirius Red staining shows collagen deposition in absence (day 0) or presence of CTX injection after 2, and 4 days (scale bar = 100 μ m). **(B, C)** Relative gene expression in TA muscle in absence (day 0) or presence of CTX injection after 2 and 4 days. Results are presented as mean + SEM. N = 5–8 mice, *: $p < 0.05$. Significant differences between individual groups are indicated by lines with a *. Single * indicates significant difference compared to all other groups at the same time point.

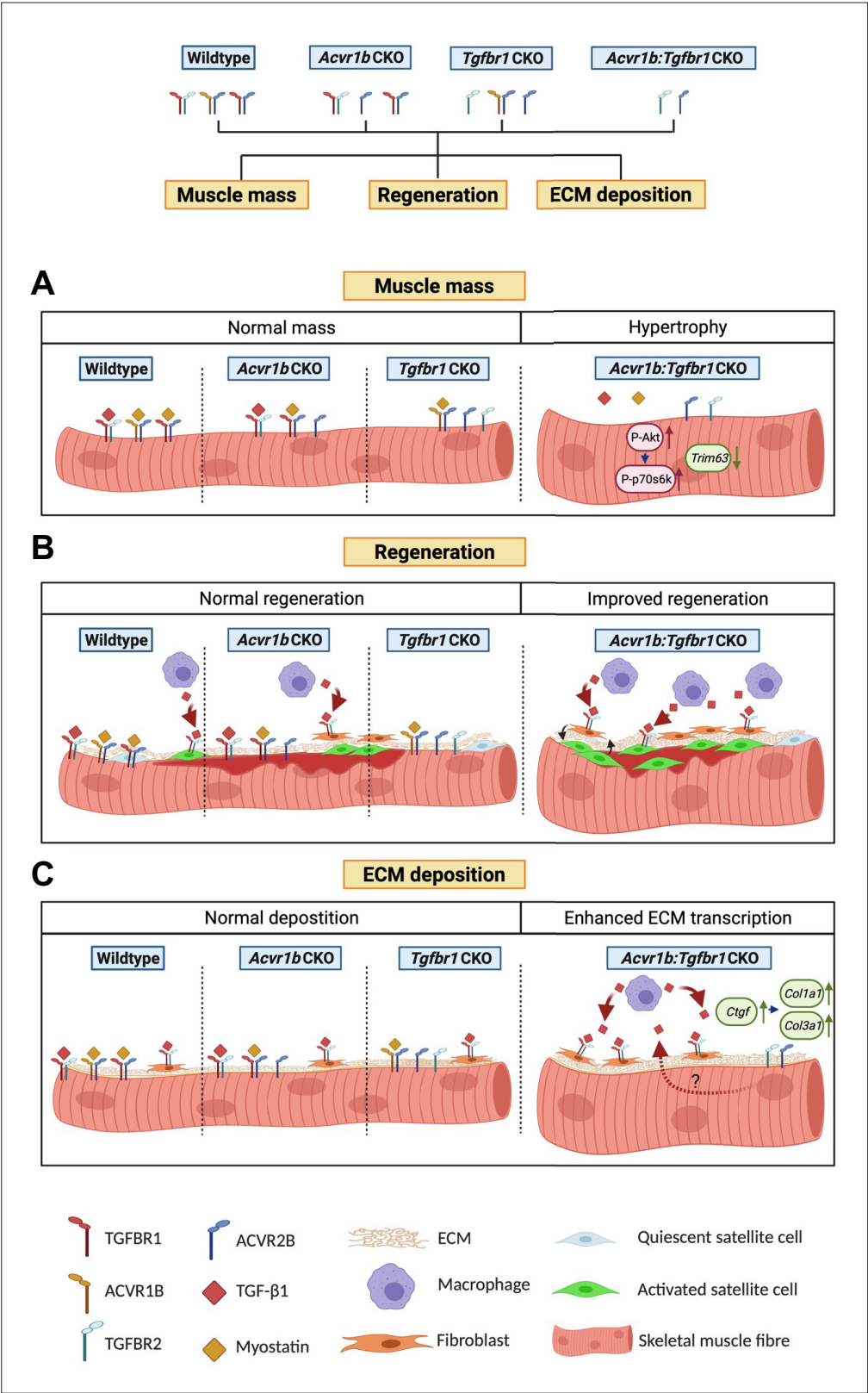


Figure 7. Schematic diagram of the effects of single or combined muscle-specific knockout of *Tgfb1* and/or *Acvr1b* receptors on muscle hypertrophy, regeneration, and expression of ECM components. **(A)** Myofibre size is not affected after individual knockout of *Acvr1b* or *Tgfb1*, which indicates that these receptors have redundant effects on muscle size and that myostatin signals via both receptors to control muscle mass. Simultaneous

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knockout of both *Acvr1b* and *Tgfb1* inhibits signaling of TGF- β , myostatin and activin A and stimulates protein synthesis via the Akt/mTOR/p70S6K pathway, while inhibiting protein breakdown through repression of *Trim63* levels, resulting in substantial muscle hypertrophy. **(B)** Upon acute injury, simultaneous knockout of combined *Acvr1b* and *Tgfb1* accelerates early muscle regeneration, as observed by increased myogenic gene expression as well as increased CSA of regenerating myofibres. An increased number of SCs likely contributes to these effects. **(C)** Simultaneous myofibre-specific knockout of *Acvr1b* and *Tgfb1* induces mRNA expression of ECM components. These effects are likely caused by enhanced TGF- β 1 signaling in fibroblasts. Schematic is created using BioRender.