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A Series of Cre-ER^{T2} Drivers for Manipulation of the Skeletal Muscle Lineage

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Summary

We report the generation of five mouse strains with the tamoxifen-inducible Cre (*Cre-ER^{T2}*; *CE*) gene cassette knocked into the endogenous loci of *Pax3*, *Myod1*, *Myog*, *Myf6*, and *Myl1*, collectively as a resource for the skeletal muscle research community. We characterized these *CE* strains using the Cre reporter mice, *R26R^{LacZ}*, during embryogenesis and show that they direct tightly controlled tamoxifen-inducible reporter expression within the expected cell lineage determined by each myogenic gene. We also examined a few selected adult skeletal muscle groups for tamoxifen-inducible reporter expression. None of these new *CE* alleles direct reporter expression in the cardiac muscle. All these alleles follow the same knock-in strategy by replacing the first exon of each gene with the *CE* cassette, rendering them null alleles of the endogenous gene. Advantages and disadvantages of this design are discussed. Although we describe potential immediate use of these strains, their utility likely extends beyond foreseeable questions in skeletal muscle biology.

Keywords

somite; limb; myogenesis; embryonic development; cell marking; lineage tracing; conditional knock-out

Introduction

Skeletal myogenesis is a fundamental process in biology, not only for embryonic development, but also for regeneration, homeostasis, and aging in normal and diseased states. Over the past few decades, the field has made many great leaps (reviewed in Bentzinger *et al.*, 2012; Bryson-Richardson and Currie, 2008; Buckingham and Rigby, 2014; Fan *et al.*, 2012; Kang and Krauss, 2011). We now know of many key transcriptional regulators of trunk skeletal muscle development. For example: the transcription factors Pax3

and Pax7 are regulators for myogenic progenitors; Myf5, Myod1, and Myf6 are regulators of myogenic commitment and differentiation; and Myog directs efficient myocyte fusion during embryogenesis. Analyses of various combinatorial mutations amongst these genes reveal a complex network of compensatory and overlapping roles. Not surprisingly, they also display complex temporal and spatial expression patterns. Historically, constitutive Cre recombinase driven by endogenous myogenic gene loci and by defined regulatory elements of these genes have been used for lineage tracing and gene manipulation (Engleka *et al.*, 2005; Gensch *et al.*, 2008; Haldar *et al.*, 2008; Kanisicak *et al.*, 2009; Keller *et al.*, 2004; Li *et al.*, 2005; Schuler *et al.*, 2005; Schienda *et al.*, 2006; Tallquist *et al.*, 2000). By contrast, Cre-ER offers tamoxifen (tmx)-inducible temporally controlled Cre activity (Feil *et al.*, 1997). Recent efforts by various laboratories have produced several myogenic *Cre-ER* alleles with different designs for temporal manipulation (Biressi *et al.*, 2013; Blum *et al.*, 2013; Lepper *et al.*, 2009; Murphy *et al.*, 2011). However, a systematically designed, coherent set of myo-genic *Cre-ERT²* (from here on referred to as *CE*) drivers in each gene locus to allow for gene manipulation at different steps of myogenesis is lacking. For this reason, we extended our previous effort of making the *Pax7^{CE}* mice (Lepper *et al.*, 2009; Lepper and Fan, 2010) openly available by creating additional *CE* knock-in (KI) alleles at the *Pax3*, *Myod1*, *Myogenin*, *Myf6*, and *Myf1* loci for public sharing. This “myogenicseries” of *CE* drivers should be of value to the community.

Results

The design for these *CE* KI alleles is similar to that of the *Pax7^{CE}* allele (Lepper *et al.*, 2009; Lepper and Fan, 2010) and diagrammed in Figure 1. We made two minor modifications. First, we eliminated the *IRES-DsRed* cassette since we were unable to visualize live fluorescence or detect DsRed by immunostaining. Second, we replaced the SV40 early polyA with the SV40 late polyA signal to enhance pre-mRNA processing. For each gene, an approximately 1.2–1.6 kb short arm and 8–12 kb long arm were used for homologous recombination: For *Pax3*, *Myod1*, and *Myf1* constructs, the 5′ arm is the short arm (Fig. 1A), while for *Myf6* and *Myog* constructs, the 3′ arm is the short one (Fig. 1B). The short arms were designed to aid in screening embryonic stem (ES) cell colonies by polymerase chain reaction (PCR). In each case, the coding region within the first exon, from the ATG to exon1/intron1 junction, was deleted. Additional PCR reactions using primer pairs across the 5′ and 3′ junction regions were performed to confirm homologous recombination. Germ line transmitted animals were crossed to *Actin-Flp* mice (Rodriguez *et al.*, 2000) to remove the *frt-neo-frt* cassette—confirmed by PCR using appropriate primers. Afterwards, new primers were used to distinguish each of these alleles (their generic positions are diagrammed in Fig. 1). Animals were then crossed to the Cre reporter *R26R^{LacZ}* mice (Soriano, 1999) for characterization. In all analyses, the *CE* driver alleles were transmitted through males, and the *R26R^{LacZ}* allele was kept homozygous to increase sensitivity. Because each *CE* driver marked cells with different efficiencies at various developmental stages, we standardized most of the stages and time of labeling for comparison, unless otherwise noted. For embryos, a single dose of tmx at 1 mg/40 g body weight of the pregnant female was administered, and embryos were harvested 36–38 h afterwards for analyses. We performed a series of analyses on embryos that received tmx at

time points from E8.75 to E14.75. For some *CE* drivers, we selected an embryonic stage for a longer term labeling as an example for “lineage tracing” to E16.25. Lastly, we surveyed for inducible cell marking in selected adult (4–5 months of age) hind limb muscle groups as a general guide for interested investigators. For adults, 3 mg/40 g body weight of tmx was administered for 5 consecutive days, and muscle groups were harvested 3–5 days later for analyses. In all cases, at least three animals or embryos were analyzed for each stage. Below, we report our characterization of these *CE* alleles.

For the *Pax3^{CE}* allele, we observed abundant LacZ activity (via X-gal reaction) derived from the *R26R^{LacZ}* reporter after tmx administration at the first time point examined (E8.75 to E10.25 labeling, 2 h X-gal reaction; Fig. 2A). The patterns observed for cell labeling at E9.75, E10.75, and E11.75 were consistent with *Pax3* expression in the paraxial mesoderm derivatives, dorsal neural tube, neural crest derivatives as well as craniofacial structures (Fig. 2B–D). As *Pax3* is expressed in progenitors of some of these lineages (e.g., myogenic and neural crest), their expansion over 36 h explains the labeled cell populations being broader than the reported *Pax3* expression by immunostaining (Horst *et al.*, 2006) or in situ hybridization (Relaix *et al.*, 2004). Of particular interest here is labeling of somites and limb muscles and/or myogenic progenitors. More ventrally extended lineage-labeling of body wall muscles was observed when comparing labeling of E11.75 to E13.25 (Fig. 2B) and E10.75 to E12.5 (Fig. 2C); labeling over the dorsal surface likely reflected subcutaneous muscles and/or melanocytes. Consistent with *Pax3* expression declining progressively in myogenic cells after E13.5 (Horst *et al.*, 2006), *Pax3^{CE}*-directed marking in this lineage started to diminish around this time (Fig. 2E). Even after 48 h of developing in the X-gal reaction, the staining intensity of the back muscles is low (Fig. 2E and F). Although removing the skin of E13.75 to E15.25 labeled embryos helped substrate penetration to detect labeled muscle groups (Fig. 2G), the same procedure performed on E14.75 to E16.25 embryos yielded only a few labeled muscle groups (Fig. 2H). By contrast, labeling of the spinal cord, snout, and melanocytes persisted to later time points (Fig. 2F,H–H’). We chose only E10.75 for a longer term tracing to E16.25. We observed labeling in the spinal cord, in the brown adipose tissue (spotty), and scattered cells beneath epidermis, presumably melanocytes (Fig. 2I). Importantly, axial, ventral wall, and limb muscles are all labeled (Fig. 2I,J). Consistent with previous data regarding minimal *Pax3* expression in adult tibialis anterior (TA) muscles (Kuang *et al.*, 2006; Lepper *et al.*, 2009; Relaix *et al.*, 2006), we found only a few labeled cells in the intrafusal muscle fibers (Fig. 2K), presumably those reported for the chick limb muscles (Kirkpatrick *et al.*, 2010). However, we were unable to detect *Pax3* in these cells by immunostaining (data not shown). Further in depth examination is underway to characterize the *Pax3^{CE}* allele in other adult muscle groups. Importantly, without exposure to tmx (insets at the upper right corner), *Pax3^{CE};R26R^{LacZ}* embryos displayed no observable X-gal staining signal above background.

For the *MyodI^{CE}* allele, we did not observe cell marking by tmx at E8.75, but found cell marking in the lateral edge of inter-limb somites when tmx was administered at E9.75 (Fig. 3A). Given the perdurance of tmx of ~ 12 h in vivo (Nakamura *et al.*, 2006), this period overlaps with the earliest *MyodI* expression timing and pattern (Sassoon *et al.*, 1989). Progressively more facial and distal limb muscles were labeled at later stages (Fig. 3B–F).

Eight to sixteen hours long X-gal reactions were needed to observe signals in these *Myod1^{CE}* embryos, likely reflecting that *Myod1^{CE}* marks mostly differentiating muscle cells that did not expand. Alternatively, lower levels of CE may be produced by *Myod1^{CE}* compared to *Pax3^{CE}*. The E13.75 to E15.25 treated embryos appeared to have less intense staining, likely due to lower substrate penetrance through the skin (Fig. 3E). Removal of skin from E14.75 to E16.25 treated embryos indeed increased staining signal (Fig. 3F). As E10.75 represents a time of robust labeling, we chose this time point for a longer term tracing to E16.25. These embryos showed staining of the trunk and body wall muscles as well as subcutaneous muscles (Fig. 3G). Although both fore and hind limb muscles were positive, the fore limb muscles showed a stronger staining, consistent with the more advanced developmental stage at E10.75 (the labeling time). The observation of strong staining after a 6-day chase suggests that minimally a fraction of myogenic progenitors at E10.75 were labeled by tamoxifen in the *Myod1^{CE}* embryos and subsequently amplified; thus, resulting in an expanded population of lineage-marked myogenic cells (in comparison to other *CE* alleles that did not display such a persistence, see below). This observation is consistent with the report that *Myod1^{Cre}* (constitutive *Cre* KI at the *Myod1* locus) directs lineage marking of muscle stem cells (Kanisicak *et al.*, 2009). For adult muscles, we selected the cardiac and various hind limb muscles for whole mount analyses. Although there was no staining of the heart (Fig. 3I), differential staining in different muscle groups was obvious, with the soleus (Fig. 3L) showing the lowest staining signal among gastrocnemius, plantaris, TA, and Extensor Digitorum Longus (EDL) muscles (Fig. 3J,K,M,N). Differential *Myod1* expression levels among adult limb muscles and fiber types have indeed been reported (Hughes *et al.*, 1993, 1997). Cross section of the TA muscles showed a mosaic pattern of labeling intensity (Fig. 3O), likely reflecting differential levels of CE and/or frequency of reporter recombination in different myofibers.

For the *Myf6^{CE}* allele, we observed sporadically labeled small clusters of cells in the somite as early as E8.75 (Fig. 4A), lasting through E10.75 (Fig. 4B,D). X-gal reactions had to be developed for 48 h to observe staining signals at these early stages. Reporter recombination by tmx at E11.75 gave weak labeling along the trunk muscles. By contrast, induced cell marking at E12.75, E13.75, and E14.75 (skinned) shows progressively more robust X-gal staining signals (E12.75, stained for 16 h; E13.75, 8 h; E14.75, 2 h), not only in the trunk but also in the limb muscles (Fig. 4E–G). The early weak and late strong cell marking is consistent with the reported temporal expression levels of *Myf6* (Hinterberger *et al.*, 1991). Because the reporter is driven by a ubiquitous promoter, the variation in X-gal signal presumably reflects the different number of cells having undergone recombination. We then chose E12.75 for labeling and tracing to E16.25. Very little staining was found in the intercostal muscles, but axial trunk muscles were labeled (Fig. 4H). We did not observe subcutaneous muscles labeled, although patches of fore and hind limb muscles were marked (Fig. 4I). As predicted by *Myf6*'s robust expression in adult skeletal muscles, we found gastrocnemius (not shown), plantaris, soleus, TA, and EDL muscles displaying strong X-gal staining signal (Fig. 4J–M; 1 h staining), but no staining in the heart (Fig. 4N). X-gal staining of sectioned TA muscles confirmed muscle-specific labeling (Fig. 4O).

For the *Myog*^{CE} allele, we observed little to no labeling at E8.75, but weak staining in the inter-limb somites of E9.75 and E10.75 tmx-treated embryos (Fig. 5A,B), consistent with the reported *Myog* expression at these stages (Sassoon *et al.*, 1989). From E11.75 onward (Fig. 5C–F), *Myog*^{CE}-directed cell marking showed increased labeling in the trunk as well as proximal and distal limb muscles. E13.75 to E15.25 labeling showed staining covering the dorsal trunk surface, likely the subcutaneous muscles, in addition to the limb and craniofacial muscles (Fig. 5D). Embryos labeled by tmx at E14.75 were skinned for substrate penetrance (Fig. 5F). For longer term tracing from E11.75 to E16.5, we observed few limb and body wall muscles labeled after prolonged staining on thick sections (Fig. 5G). The difficulty to observe a broader pattern of persistent labeling may be explained by dilution of labeled cells (or nuclei) by fusion with un-labeled cells derived from the expanding pool of myogenic progenitors which did not express *Myog* at the time of labeling by tamoxifen. By contrast, in E12.75 to E16.5 lineage-traced embryos, X-gal staining was seen throughout the limb, body wall, and subcutaneous muscles (Fig. 5H), albeit 24 h of reaction time was needed. Presumably at E12.75, substantial myocytes and myonuclei express *Myog*^{CE}, leading to sufficient number of cells (or nuclei) producing LacZ that could withstand dilution by fusion with unlabeled cells and therefore allow for detection. Tmx treatment of adults did not induce cell labeling in the heart (Fig. 5I), but induced weak and mosaic labeling of various hind limb muscle groups (Fig. 5J–N), with the EDL showing the least signal (Fig. 5N). Indeed, differential expression of *Myog* in hind limb muscle groups has been documented (Hughes *et al.*, 1993). Analysis of sections of the TA muscle confirmed the mosaic labeling amongst myofibers (Fig. 5O).

Lastly, for the *Myf1*^{CE} allele, we found very little labeling prior to E11.75, and a gradual increase of labeling from E12.75 onward (Fig. 6A–D), consistent with the reported developmental expression of *Myf1* (Lyons *et al.*, 1990). Different embryonic stages represented here required different lengths of time of X-gal reactions (see Fig. 6 legends). For E14.75 and E15.75 labeling, we were able to observe various muscles groups differentially labeled only after skinning. We do note a sudden robust labeling of TA muscles in E15.75 to E17.25 embryos (4 h of reaction time). After various trials of E12.75 to E16.5 longer term labeling, we detected very few loosely clustered myofibers retained weak signals (Fig. 6E,F). We presume that continuous fusion with unlabeled progenitors diluted reporter signal during the chase period, rendering most E12.75 labeled fibers without a sufficient level of LacZ for detection. As would be predicted, the adult heart was not labeled after tmx treatment (Fig. 6G). The gastrocnemius, plantaris, TA, and EDL were strongly labeled, but the soleus was poorly labeled (Fig. 6H–M). The differential labeling in different muscles reflects *Myf1* being preferentially expressed in the fast fibers (Barton *et al.*, 1989); the soleus is mainly comprised of slow fibers. Staining of TA muscle sections confirmed *Myf1*^{CE}-directed labeling only in the myofibers. As the TA muscle is of mixed fiber types, we did observe differential labeling between fibers (Fig. 6N).

Discussion

We are providing basic information for this new collection of myogenic *CE* drivers using the *R26R*^{LacZ} reporter. Because each laboratory has different questions to address, we encourage interested investigators to perform more focused characterization for each of

these *CE* drivers prior to their own studies. Along with our descriptions, we made various assumptions to explain the observed cell labeling patterns. Additional studies are needed to prove or refute these assumptions. We envision a few immediate uses of these alleles.

First, one can use these lines to decipher the time window for non-myogenic lineages derived from cells expressing these genes as has been performed using the *Pax7^{CE}* allele (Lepper and Fan, 2010). Constitutive Cre drivers have been routinely used for collective lineage documentation, but they lack temporal control to define the developmental timing of lineage restriction. For *Pax3*, this kind of lineage tracing has been performed (Engleka *et al.*, 2005; Hutcheson *et al.*, 2009; Schienda *et al.*, 2006). We are currently extending our study to determine temporal lineage restriction of *Pax3*-derivatives, that is, neural crest, dermal, endothelial, and brown adipose lineages. Similarly, it would be interesting to define the time window when *Myod1* becomes expressed in the *Pax7⁺* muscle stem/progenitor cells (Kanisicak *et al.*, 2009), or whether/when *Myod1⁺* non-myogenic lineages may arise. Along this line of thoughts, investigators in non-myogenic fields may find one of these genes selectively expressed in their favorite non-muscle cell types. For example, many of our *CE* alleles display craniofacial muscle labeling and may be used in this less explored system both in development and after birth.

Second, they can be used to define the time window for each of these genes' *CE* drivers that is still capable of co-labeling muscle progenitor or stem cells during embryogenesis and/or perinatal period using the recently developed reporter at the *Pax7* locus (Sambasivan *et al.*, 2013). We envision that they can also be used to define whether, during the muscle injury-regeneration process, differentiating myogenic cells (based on *CE* transcribed from each endogenous locus) can reverse course and regain stem cell status in vivo. Third, they can be used to test whether different subtypes of rhabdomyosarcomas may arise from either activating oncogenes and/or inactivating recessive onco-genes at distinctive steps of myogenic differentiation (Blum *et al.*, 2013; Clark *et al.*, 1991; Rubin *et al.*, 2011) at time of choosing. Although the tmx used here lasts about 12 h in vivo (Nakamura *et al.*, 2006), one may consider using 4OH-tmx with a much shorter in vivo half-life for a more precise time window of cell marking.

The *CE* drivers being null alleles have a preconceived disadvantage of lineage analysis or manipulation of gene activity in a heterozygous background. However, constitutive *Cre* KI/KO alleles of *Pax3*, *Myod1*, *Myf5*, and *Myog* had been used with no un-anticipated issues (Engleka *et al.*, 2005; Gensch *et al.*, 2008; Kanisicak *et al.*, 2009; Kuang *et al.*, 2006; Tallquist *et al.*, 2000). By contrast, the use of *IRES* or 2A peptide to direct *CE* expression from endogenous loci is more likely to preserve the wild type background. However, these approaches are not suitable for temporal conditional inactivation of the same gene. The advantages of making these as null alleles thus include: (1) time-controlled cell marking/lineage tracing of mutant cells of the same gene—gene expressing cells can be identified by *CE* expression, while identification of descendant cells accompanied by a Cre reporter; (2) time-controlled generation of compound conditional mutants between these genes and other genes using corresponding floxed alleles to determine synthetic defects (e.g., between *Myog* and another myogenic gene) in *CE* expressing cells. We do note that related myogenic *CE* alleles with different designs have been reported, including the transgenic *HSA-CE* under the

control of human skeletal muscle actin enhancer/promoter (Schuler *et al.*, 2005), a *Myod1-CE* KI allele using the IRES (Blum *et al.*, 2013), and a *Myf5-CE* KI allele using the IRES (Biressi *et al.*, 2013). Although the transgenic *HSA-CE* allele is generally used for gene manipulation in skeletal muscles, the *Myf1^{CE}* allele will provide selectivity in fast muscle fibers and also the advantage for mosaic gene manipulation in muscles with mixed fiber types, if so desired. In addition, the IRES-directed *Myod1-CE* and *Myf5-CE* alleles are not suitable for conditional inactivation of their corresponding genes. These myogenic *CE* alleles together—existing and reported here—should greatly facilitate the advancement of the skeletal muscle field.

Materials and Methods

For embryos, noon of the vaginal plug date is designated as E0.5 of development. Tamoxifen (Sigma, T5648) was prepared as 20 mg/ml stock in corn oil (Sigma), and used either by oral gavage (for embryos) or by intraperitoneal injection (adults). All animal protocols are approved by Carnegie IACUC.

For generating recombination constructs, BAC clones (C57BL/6 genomic DNA) containing each gene were purchased from CHORI. Genomic segments according to design were looped out and the *CE-frt-neo-frt* cassette was inserted into desired location of each gene locus by the recombineering method described in Liu *et al.* (2003) and Wu *et al.* (2008). Nucleotide sequences for these constructions are available upon request. The final constructs were in the pWS-tk6 vector with two flanking tk-negative selection cassettes. C57BL/6;129sv hybrid ES cells (gift from Dr. Yixian Zheng) were used for electroporation and selection to obtain homologously recombined clones. Blastocyst injection of ES cell clones was used to generate chimeras. Actin-Flp mice were gifts from Dr. Susan Dymecki. *R26^{LacZ}* reporter mice were made by Dr. Philip Soriano, and purchased from the Jackson Lab. X-gal reactions followed Hogan *et al.* (1994), either performed with whole mount embryos or muscles. Cryo-sectioning at 10 μ m thickness was typically used, unless otherwise indicated. PCR primers for genotyping and respective product sizes for each *CE* alleles are as follows:

Pax3^{CE}: GGAGCCTGTGGACTTGGATCTATTAG; GGT TGAGAAGTACCACGG GAAGAG; CTCACCCTGAAGTTC TCAGGATC: wt = 391 bp, mutant = 220 bp.

Myod1^{CE}: GTAGAGGACAGCCGGTGTGCATTC; CAT GCTCATCCTCACGAGCGC CT; CTCACCCTGAAGTTCT CAGGATC: wt = 368 bp, mutant = 190 bp.

Myf6^{CE}: TCGCTGTAATCACTTTGGGAGACTG; AGAA CGTGTTCTCTCCACTG CTG; CTCACCCTGAAGTTCT CAGGATC: wt = 375 bp, mutant = 225 bp.

Myog^{CE}: GGGAATCACATGTAATCCACTGGA; CCTTA CATGCCCACGGCAGGC ACT; CTCACCCTGAAGTTCT CAGGATC: wt = 410 bp, mutant = 245 bp.

Myf1^{CE}: TCCAATCCAGCCGCGTGTGTCAAG; TCCTC ACTGGGATTGTATGCT CCAG; CTCACCCTGAAGTTCT CAGGATC: wt = 500 bp, mutant = 220 bp.

The following PCR program is suitable for all above genotyping primer sets, 94°C initial denaturation for 2 min, 94°C denaturation 30 s, 63°C annealing 30 s, 72°C extension 45 s for 35 cycles, followed by 2 min 72°C extension.

Importantly, all lines will be made available to the community through Jackson Laboratory upon publication of the manuscript.

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Per original proposal and in compliance with NIH regulations, these mouse strains will be deposited to the Jackson laboratory for public sharing after publication. The authors also thank Ms. Eugenia Dikovsky for generating the chimeras, rodent facility team for animal husbandry, and Ms. Samantha Satchell and Stephanie Kuo for genotyping animals.

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Literature Cited

- Barton PJR, Harris AJ, Buckingham M. Myosin light chain gene expression in developing and denervated fetal muscle in the mouse. *Development*. 1989; 107:819–824. [PubMed: 2534378]
- Bentzinger CF, Wang YX, Rudnicki MA. Building muscle: Molecular regulation of myogenesis. *Cold Spring Harb Perspect Biol*. 2012; 4:a008342. [PubMed: 22300977]
- Biresi S, Bjornson CR, Carlig PM, Nishijo K, Keller C, Rando TA. Myf5 expression during fetal myogenesis defines the developmental progenitors of adult satellite cells. *Dev Biol*. 2013; 379:195–207. [PubMed: 23639729]
- Blum JM, Añó L, Li Z, Van Mater D, Bennett BD, Sachdeva M, Lagutina I, Zhang M, Mito JK, Dodd LG, Cardona DM, Dodd RD, Williams N, Ma Y, Lepper C, Linardic CM, Mukherjee S, Grosveld GC, Fan CM, Kirsch DG. Distinct and overlapping sarcoma subtypes initiated from muscle stem and progenitor cells. *Cell Rep*. 2013; 5:933–940. [PubMed: 24239359]
- Bryson-Richardson RJ, Currie PD. The genetics of vertebrate myogenesis. *Nat Rev Genet*. 2008; 9:632–646. [PubMed: 18636072]
- Buckingham M, Rigby P. Gene regulatory networks and transcriptional mechanisms that control myogenesis. *Dev Cell*. 2014; 28:225–238. [PubMed: 24525185]
- Clark J, Rocques PJ, Braun T, Bober E, Arnold HH, Fisher C, Fletcher C, Brown K, Gusterson BA, Carter RL. Expression of members of the myf gene family in human rhabdomyosarcomas. *Br J Cancer*. 1991; 64:1039–1042. [PubMed: 1764365]
- Engleka KA, Gitler AD, Zhang M, Zhou DD, High FA, Epstein JA. Insertion of Cre into the Pax3 locus creates a new allele of Splotch and identifies unexpected Pax3 derivatives. *Dev Biol*. 2005; 280:396–406. [PubMed: 15882581]
- Fan CM, Li L, Roza ME, Lepper C. Making skeletal muscle from progenitor and stem cells: Development versus regeneration. *WIREs Dev Biol*. 2012; 1:315–327.
- Feil R, Wagner J, Metzger D, Chambon P. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem Biophys Res Commun*. 1997; 237:752–757. [PubMed: 9299439]
- Gensch N, Borchardt T, Schneider A, Riethmacher D, Braun T. Different autonomous myogenic cell populations revealed by ablation of Myf5-expressing cells during mouse embryogenesis. *Development*. 2008; 135:1597–1604. [PubMed: 18367555]
- Haldar M, Karan G, Tvrdik P, Capecchi MR. Two cell lineages, myf5 and myf5-independent, participate in mouse skeletal myogenesis. *Dev Cell*. 2008; 14:437–445. [PubMed: 18331721]
- Hinterberger TJ, Sassoon DA, Rhodes SJ, Konieczny SF. Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. *Dev Biol*. 1991; 147:144–156. [PubMed: 1715299]
- Hogan, B.; Beddington, R.; Constantini, F.; Lacey, E. Manipulating mouse embryos: A laboratory manual. 2nd. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1994.

- Horst D, Ustanina S, Sergi C, Mikuz G, Juergens H, Braun T, Vorobyov E. Comparative expression analysis of Pax3 and Pax7 during mouse myogenesis. *Int J Dev Biol*. 2006; 50:47–54. [PubMed: 16323077]
- Hughes SM, Koishib K, Rudnicki M, Maggs AM. MyoD protein is differentially accumulated in fast and slow skeletal muscle fibres and required for normal fibre type balance in rodents. *Mech Dev*. 1997; 61:151–163. [PubMed: 9076685]
- Hughes SM, Taylor JM, Tapscott SJ, Gurley CM, Carter WJ, Peterson CA. Selective accumulation of MyoD and Myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. *Development*. 1993; 118:1137–1147. [PubMed: 8269844]
- Hutcheson DA, Zhao J, Merrell A, Haldar M, Kardon G. Embryonic and fetal limb myogenic cells are derived from developmentally distinct progenitors and have different requirements for beta-catenin. *Genes Dev*. 2009; 15:997–1013. [PubMed: 19346403]
- Kang JS, Krauss RS. Muscle stem cells in developmental and regenerative myogenesis. *Curr Opin Clin Nutr Metab Care*. 2011; 13:243–248. [PubMed: 20098319]
- Kanisicak O, Mendez JJ, Yamamoto S, Yamamoto M, Goldhamer DJ. Progenitors of skeletal muscle satellite cells express the muscle determination gene, MyoD. *Dev Biol*. 2009; 332:131–141. [PubMed: 19464281]
- Keller C, Hansen MS, Coffin CM, Capecchi MR. Pax3:Fkhr interferes with embryonic Pax3 and Pax7 function: Implications for alveolar rhabdomyosarcoma cell of origin. *Genes Dev*. 2004; 18:2608–2613. [PubMed: 15520281]
- Kirkpatrick LJ, Yablonka-Reuveni Z, Rosser BW. Retention of Pax3 expression in satellite cells of muscle spindles. *J HistochemCytochem*. 2010; 58:317–327.
- Kuang S, Charge SB, Seale P, Rudnicki M. Distinct Roles for Pax7 and Pax3 in adult regenerative myogenesis. *J Cell Biol*. 2006; 172:103–113. [PubMed: 16391000]
- Lepper C, Conway SJ, Fan CM. Adult satellite cells and embryonic muscle progenitors have distinct genetic requirements. *Nature*. 2009; 460:627–631. [PubMed: 19554048]
- Lepper C, Fan CM. Inducible lineage tracing of Pax7-descendant cells reveals embryonic origin of adult satellite cells. *Genesis*. 2010; 48:424–436. [PubMed: 20641127]
- Li S, Czubyrt MP, McAnally J, Bassel-Duby R, Richardson JA, Wiebel FF, Nordheim A, Olson EN. Requirement for serum response factor for skeletal muscle growth and maturation revealed by tissue-specific gene deletion in mice. *Proc Natl Acad Sci USA*. 2005; 102:1082–1087. [PubMed: 15647354]
- Liu P, Jenkins NA, Copeland NG. A highly efficient recombineering-based method for generating conditional knockout mutations. *Genome Res*. 2003; 13:476–484. [PubMed: 12618378]
- Lyons GE, Ontell M, Cox R, Sassoon D, Buckingham M. The expression of myosin genes in developing skeletal muscle in the mouse embryo. *J Cell Biol*. 1990; 111:1465–1476. [PubMed: 2211821]
- Murphy MM, Lawson JA, Mathew SJ, Hutcheson DA, Kardon G. Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. *Development*. 2011; 138:3625–3637. [PubMed: 21828091]
- Nakamura E, Nguyen MT, Mackem S. Kinetics of tamoxifen-regulated Cre activity in mice using a cartilage-specific CreER(T) to assay temporal activity windows along the proximodistal limb skeleton. *Dev Dyn*. 2006; 235:2603–2612. [PubMed: 16894608]
- Relaix F, Montarras D, Zaffran S, Gayraud-Morel B, Rocancourt D, Tajbakhsh S, Mansouri A, Cumano A, Buckingham M. Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J Cell Biol*. 2006; 172:91–102. [PubMed: 16380438]
- Relaix F, Rocancourt D, Mansouri A, Buckingham M. Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes Dev*. 2004; 18:1088–1105. [PubMed: 15132998]
- Rodriguez CI, Buchholz F, Galloway J, Sequerra R, Kasper J, Ayala R, Stewart AF, Dymecki SM. High-efficiency deleter mice show that FLPe is an alternative to Cre-loxP. *Nat Genet*. 2000; 25:139–140. [PubMed: 10835623]
- Rubin BP, Nishijo K, Chen HI, Yi X, Schuetze DP, Pal R, Prajapati SI, Abraham J, Arenkiel BR, Chen QR, Davis S, McCleish AT, Capecchi MR, Michalek JE, Zarzabal LA, Khan J, Yu Z, Parham DM, Barr FG, Meltzer PS, Chen Y, Keller C. Evidence for an unanticipated relationship between

- undifferentiated pleomorphic sarcoma and embryonal rhabdomyosarcoma. *Cancer Cell*. 2011; 19:177–191. [PubMed: 21316601]
- Sambasivan R, Comai G, Le Roux I, Gomès D, Konge J, Dumas G, Cimper C, Tajbakhsh S. Embryonic founders of adult muscle stem cells are primed by the determination gene *Mrf4*. *Dev Biol*. 2013; 381:241–255. [PubMed: 23623977]
- Sassoon D, Lyons G, Wright WE, Lin V, Lassar A, Weintraub H, Buckingham M. Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. *Nature*. 1989; 341:303–307. [PubMed: 2552320]
- Schienda J, Engleka KA, Jun S, Hansen MS, Epstein JA, Tabin CJ, Kunkel LM, Kardon G. Somitic origin of limb muscle satellite and side population cells. *Proc Natl Acad Sci USA*. 2006; 103:945–950. [PubMed: 16418263]
- Schuler M, Ali F, Metzger E, Chambon P, Metzger D. Temporally controlled targeted somatic mutagenesis in skeletal muscles of the mouse. *Genesis*. 2005; 41:165–170. [PubMed: 15789425]
- Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet*. 1999; 21:70–71. [PubMed: 9916792]
- Tallquist MD, Weismann KE, Hellstrom M, Soriano P. Early myotome specification regulates PDGFA expression and axial skeleton development. *Development*. 2000; 127:5059–5070. [PubMed: 11060232]
- Wu S, Ying G, Wu Q, Capecchi MR. A protocol for constructing gene targeting vectors: Generating knockout mice for the cadherin family and beyond. *Nat Protoc*. 2008; 3:1056–1076. [PubMed: 18546598]

FIG. 1.

Construction of homologous recombination plasmids. Generic diagrams for constructs used to obtain homologously recombined (A) *Pax3, Myod1, Myl1* CE, (B) *Myf6* and *Myog* CE alleles. Genomic structures are depicted with a generic exon1 (e1) at the top, while recombination constructs are at the bottom. Thick lines indicate genomic sequence not included in recombination constructs, while thin lines indicate homologous regions (5' and 3' arms). Plasmid backbone (pWS-tk6) is not included. Abbreviations: i, rabbit β -globin intron; CE, Cre-ER^{T2} cassette; pA, SV40 late polyA signal sequence; frt-neo-frt, frt-flanked neo cassette; atg, translation initiation codon. The arrows indicate the positions of redesigned primers (sequences in Materials and Methods section) used to genotype and distinguish each CE allele.

FIG. 2.

Pax3^{CE} characterization. Time of tmx administration is the first number at the bottom of each figure, and time of embryo harvest, the second number. (A–E) Embryos were subjected to whole mount X-gal reaction for 2 h, (F) for 24 h, and (G, H) 48 h in sagittal view; insets are control embryos without tmx but with the same X-gal reaction time. (G, H) The skinned side of these embryos is shown; (H') dorsal surface view of un-skinned side of the embryo in (H); (H'') dorsal view (over skinned area) of the spinal cord (sc). (I, J) Cross sections of E10.75 to E16.25 long-term tracing at the brachial level (I), as well as at the fore limb (FL) and hind limb (HL) levels (J); BAT, brown adipose tissue; h, humerus; u, ulnar; t, tibia. Adult TA muscle section stained for X-gal (48 h) and Nuclear Fast Red. Asterisks, the snout; arrows, limb muscles, white arrows, trunk and tail muscles; white arrowheads, melanocytes; open arrowheads, staining signal in the intrafusal fibers. Scale bars = 500 μ m, except for in (I), 100 μ m.

FIG. 3. *Myod1*^{CE} characterization. Time of tmx administration and embryo harvest is as in Figure 2. (A–F) Embryos (sagittal view) were subjected to X-gal reactions (8–16 h); insets are control embryos; embryo in (F) was skinned. (G) Transverse section of the embryo at the brachial level, and (H) fore limb (FL, left) and hind limb (HL, right) levels (24 h reaction); sc, spinal cord; s, scapula; h, humerus; u, ulna; f, femur. (I–N) Adult muscles subjected to whole mount X-gal reactions (overnight); muscle names at bottom. Corresponding muscles from animals without tmx treatment (inset in J, and left muscles in K–M) were photographed side by side. (O) Cross section of TA muscles stained for X-gal (overnight) and Nuclear Fast Red. Open stars, facial muscles; arrows, limb muscles, white arrows, trunk muscles. Scale bars = 500 μm, except for in (O), 100 μm.

FIG. 4. *Myf6*^{CE} characterization. Time of tmx administration and embryo harvest are as in Figure 2. (A–G) Embryos were subjected to X-gal reaction for 48 h in (A–C), 16 h in (D), 8 h in (E), and 2 h in (F, G); upper insets, embryos without tmx; lower insets, magnified view of the boxed area. (H) Cross sections at the brachial level. (I) Sections at the fore limb (FL, left) and hind limb (HL, right) levels (24 h reaction). Abbreviations: sc, spinal cord; BAT, brown adipose tissue; arrows, limb muscles; white arrows, trunk and neck muscles. (J–N) Adult muscles subjected to whole mount X-gal staining (1 h); muscle names at bottom. Equivalent muscles without tmx treatment (left in [J–M]) were photographed side by side. (O) Cross section of adult TA muscles stained for X-gal and Nuclear Fast Red. Scale bars= 500 μm, except for in (O), 100 μm.

FIG. 5. *Myog^{CE}* characterization. Time of tmx administration and embryo harvest are as in Figure 2. (A–F) Embryos were subjected to X-gal reaction for 24 h in (A–C), and 6 h in (D–F); (F) skinned embryo; upper right insets, control embryos without tmx treatment. (G, H) Cross sections at fore limb level (50 mm thickness in G; 48 h reaction) and hind limb level (H, 24 h reaction); h, humerus; p, pelvis; f, femur. Open asterisks, facial muscles; arrows, limb muscles, white arrows, trunk and neck muscles. (I–N) Adult muscles subjected to whole mount X-gal reactions (overnight); muscle names at bottom. (O) Cross section of adult TA muscles stained for X-gal (overnight) and Nuclear Fast Red. Scale bars = 500 μ m, except for in (O), 100 μ m.

FIG. 6. *Myl1^{CE}* characterization. Time of tmx administration and embryo harvest is labeled as in Figure 2. (A–D) Embryos were subjected to X-gal reactions for (A, B) 24 h, (C, skinned) for 16 h, and (D, skinned) 4 h; insets, control embryos without tmx treatment. (E, F) Cross sections at the fore limb (FL in E) and hind limb (HL in F) levels (48 h reaction). (G–M) Adult muscles (name at the bottom) subjected to whole mount X-gal reactions: 24 h for (G), and 2 h for (H–M). (N) Cross section of adult TA muscles stained for X-gal (2 h) and Nuclear Fast Red. White asterisks, facial muscles; arrows, limb muscles, white arrows, trunk and neck muscles; open triangles, unstained fibers; r, rib; s, scapula; f, femur. Scale bars = 500 μm, except for in (N), 100 μm.