MicroRNA regulation of the paired-box transcription factor Pax3 confers robustness to developmental timing of myogenesis

Katarzyna Goljanek-Whysall[a], Dylan Sweetman[a,1], Muhammad Abu-Elmagd[a], Elik Chapnik[c], Tamas Dalmay[b], Eran Hornstein[c], and Andrea Münsterberg[a,2]

Departments of [a]Cell and Developmental Biology and [b]Molecular Biology, School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, United Kingdom and [c]Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel

Edited by Clifford J. Tabin, Harvard Medical School, Boston, MA, and approved June 10, 2011 (received for review April 8, 2011)

Commitment of progenitors in the dermomyotome to myoblast fate is the first step in establishing the body musculature. Pax3 is a crucial transcription factor, important for skeletal muscle development and expressed in myogenic progenitors in the dermomyotome of developing somites and in migratory muscle progenitors that populate the limb buds. Down-regulation of Pax3 is essential to ignite the myogenic program, including up-regulation of myogenic regulators, Myf-5 and MyoD. MicroRNAs (miRNAs) confer robustness to developmental timing by posttranscriptional repression of genetic programs that are related to previous developmental stages or to alternative cell fates. Here we demonstrate that the muscle-specific miRNAs miR-1 and miR-206 directly target Pax3. Antagomir-mediated inhibition of miR-1/miR-206 led to delayed myogenic differentiation in developing somites, as shown by transient loss of myogenin expression. This correlated with increased Pax3 and was phenocopied using Pax3-specific target protectors. Loss of myogenin after antagomir injection was rescued by Pax3 knockdown using a splice morpholino, suggesting that miR-1/miR-206 control somite myogenesis primarily through interactions with Pax3. Our studies reveal an important role for miR-1/miR-206 in providing precision to the timing of somite myogenesis. We propose that posttranscriptional control of Pax3 downstream of miR-1/miR-206 is required to stabilize myoblast commitment and subsequent differentiation. Given that mutually exclusive expression of miRNAs and their targets is a prevailing theme in development, our findings suggest that miRNA may provide a general mechanism for the unequivocal commitment underlying stem cell differentiation.

chick and mouse embryo | progenitor-to-myoblast transition | locked nucleic acid in situ | onset of miR-1 and miR-206 expression

Posttranscriptional regulation of gene expression by microRNAs (miRNAs) is an important feature of development. miRNAs are short noncoding RNAs that bind to target sites typically found in the 3′-UTR of messenger RNAs, resulting in inhibition of translation and transcript degradation (1). The frequently exclusive temporal or spatial expression of miRNAs and their targets suggests that miRNAs confer accuracy to developmental gene expression programs, thereby ensuring tissue identity and supporting cell lineage decisions (2–4).

In skeletal muscle, miRNAs, including three highly related miRNAs of the same family—miR-1-1, miR-1-2, and miR-206—play important roles in proliferation, differentiation, and cell fate specification (5). The expression of these miRNAs is regulated by skeletal muscle–specific bHLH transcription factors of the myogenic regulatory factor (MRF) family (6, 7), and some of their targets have been investigated. In particular, miR-1 and miR-206 promote differentiation of C2C12 myoblasts by repression of HDAC4, connexin 43, utrophin, follistatin-like, and a subunit of DNA polymerase α (8–11). In addition, miR-1 and miR-206 facilitate differentiation of adult muscle stem cells through regulation of the paired box transcription factor Pax7 (12). However, potential roles of miR-1 and miR-206 during embryonic myogenesis have not yet been identified, possibly due to their overlapping functions. Deletion of miR-1-2 in mice leads to ventricular septal defects, although skeletal muscle in these mice was found to be grossly normal (13). Similarly, adult mice lacking miR-206 do not display an overt muscle phenotype (14).

In vertebrate embryos, miR-206 expression is restricted to skeletal myoblasts in somites, limb buds, and head muscles, whereas miR-1 is expressed in developing heart as well (13). Myogenic cells arising from embryonic somites give rise to skeletal muscles of the trunk and limbs, and skeletal myogenesis serves as a paradigm for cell fate commitment in response to extrinsic cues. Developmental signals that lead to activation of MRFs, and thus myogenic commitment, have been well characterized (15). Distinct regulatory networks underlie the formation of different muscle groups (16). The hierarchy of transcription factors controlling the myogenic program is also well understood. Myogenic progenitors express the paired-box transcription factor Pax3. In hypaxial muscle, Pax3 directly activates the muscle determination gene *Myf-5* (17), whereas in the epaxial dermomyotome, *Dmrt2* has been identified as a Pax3 target. *Dmrt2* in turn binds to the early epaxial enhancer of *Myf-5*, and this interaction is required for the onset of myogenesis (18). *MyoD*, another key regulator of myogenesis, has been identified as a direct target of FoxO3 and Pax3 in myoblasts (19). Once activated, the MRFs direct progenitor cells into the myogenic program, during which they express myogenin and differentiate. Pax3 activates ectopic myogenesis in the neural tube of chicken embryos and in somite explants (20), and the combined loss of Pax3 and Myf-5 abrogates muscle development in the trunk (21). Pax3 expression in the dermomyotome is down-regulated as cells move through the dorsomedial and ventrolateral lips into the myotome, where they express MRFs (22, 23). Some Pax3-positive cells translocate directly from the central dermomyotome into the myotome and contribute to satellite cells, stem cells in adult muscle (24, 25). Migratory muscle progenitors from the hypaxial dermomyotome require Pax3 in order to delaminate and migrate into limb buds (26, 27). In the myotome and in dorsal and ventral limb muscle masses, up-regulation of MRF expression correlates with down-regulation of Pax3, which is necessary for terminal differentiation, because high Pax3 levels interfere with this process (28). During activation of adult muscle stem cells, Pax3 is regulated by ubiquitination and proteasomal degradation (29). How Pax3 is regulated during somite and limb myogenesis in the embryo is not well understood, however.

Author contributions: K.G.-W., D.S., and A.M. designed research; K.G.-W., D.S., and M.A.-E. performed research; E.C. and E.H. contributed new reagents/analytic tools; K.G.-W., D.S., M.A.-E., T.D., and A.M. analyzed data; E.H. and A.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]Present address: Division of Animal Sciences, School of Biosciences, University of Nottingham, Sutton Bonington LE12 5RD, United Kingdom.

[2]To whom correspondence should be addressed. E-mail: a.munsterberg@uea.ac.uk.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1105362108/-/DCSupplemental.

Here we used complementary in vitro and in vivo assays to identify Pax3 as key target of miR-1/miR-206 in embryonic myoblasts. Injection of antagomirs (30, 31) leads to an increase of Pax3 and affects the timing of myoblast differentiation in developing somites. Pax3-specific target protector (TP) morpholinos mimic the phenotype induced by antagomirs. Myogenesis is restored in antagomir-injected somites by Pax3 knockdown, mediated by electroporation of splice morpholinos. We propose that after commitment, the complete posttranscriptional repression of Pax3 is the key event through which miR-1/miR-206 promote myogenic differentiation in the embryo.

Results

To confirm the requirement of miRNAs for early myogenesis, we used an available conditional allele to delete Dicer specifically in skeletal muscle progenitors (32). Dicer$^{fl/fl}$ females were crossed with Dicer$^{fl/+}$ males expressing cre-recombinase under the control of the Pax3 promoter (33), resulting in loss of Dicer in Pax3-expressing cells, including muscle progenitors in the dorsal somite. Embryos were examined at E10.5, E11.5, and E12.5. Homozygous Dicer$^{flox/flox/Cre}$ offspring had grossly normal morphology compared with their heterozygous littermates. Locked nucleic acid (LNA) in situ hybridization showed that conditional loss of Dicer led to loss of miR-206 in myoblasts (Fig. S1 *D–F*) compared with the heterozygous littermates (Fig. S1 *A–C*). This was first apparent in anterior somites of E10.5 and E11.5 mutant embryos (arrowheads in Fig. S1 *D* and *E*). At E12.5, miR-206 was absent in limb muscles, in migratory hypaxial muscle precursors, and in myoblasts of tail somites (Fig. S1*F*). Loss of miR-206 was accompanied by a delay in limb muscle differentiation, as shown by delayed myogenin expression (Fig. S1 *J–L*). Quantitative PCR (qPCR) on dissected limbs of E12.5 and E13.5 embryos demonstrated that loss of mature miR-206 and reduced myogenic differentiation in Dicer$^{flox/flox/Cre}$ mutant embryos was correlated with an increase in Pax3 transcripts (Fig. S1*M*). These results confirm that Dicer-mediated biogenesis of miRNA is required for the appropriate timing of myogenic differentiation, and that a delay in myogenesis is associated with elevated levels of Pax3, a marker for myogenic progenitor cells.

We previously used in situ hybridization to determine the onset of miR-1 and miR-206 expression during somite development in chicken embryos. LNA-containing probes discriminate between miR-1 and miR-206. Both miRNAs were expressed in committed myoblasts in the myotome, but miR-206 were expressed in almost all somites by HH16, ~24 h before miR-1 was detected at HH20 (7) (Fig. S2). This provided a window during which miR-206 was the only member of the family present in somites of the developing chicken embryo. This was not the case in mice, where miR-1 was weakly expressed in the first four somites and miR-206 was absent at E9.5, but both miR-1 and miR-206 were equally expressed in most somites by E10 (Fig. S2).

Expression of miRNAs and their targets is often mutually exclusive in development. Thus, to examine the relationships of miR-1 and miR-206 with Pax3 in vivo, we compared their spatiotemporal expression during skeletal myogenesis (Fig. 1). In somites, Pax3 transcripts were expressed in dorsomedial and ventrolateral lips of the dermomyotome; miR-1 and miR-206 were not detected in the dermomyotome, but were robustly expressed in committed myoblasts in the myotome (Fig. 1*A*). Interestingly, Pax3 and miR-1/miR-206 expression was overlapping in the central myotome, which contains precursors of satellite cells, stem cells in adult skeletal muscle (24); however, given that our in situ hybridization did not allow single-cell resolution, we could not determine whether Pax3 and miR-1/miR-206 are expressed in the same cells. In developing limb muscles, expression of miR-206 at HH28 was correlated with down-regulation of Pax3 in both forelimb and hindlimb muscles (Fig. 1*B*), whereas miR-1 was only faintly expressed in HH28 hindlimbs. The patterns suggested a possible role for miR-1/miR-206 in the complete down-regulation of Pax3 within the myotome and limb muscle masses, following myoblast commitment. Consistent with this, ectopic expression of miR-206 in the dorsomedial dermomyotome led to localized loss of Pax3 (Fig. 1*C*).

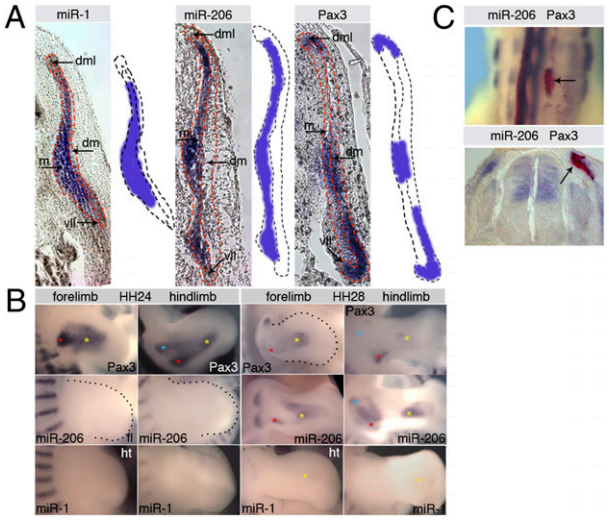

Fig. 1. Expression of miR-1 and miR-206 is inversely correlated with Pax3 in developing muscle. (*A*) Somite sections and schematics illustrating the distribution of miR-1, miR-206, and Pax3 in dermomyotome lips (dml, vll) and myotome (m); probes are indicated above each panel. (*B*) Whole-mount views of forelimbs (fl) and hindlimbs (hl) at HH stages 24 and 28, hybridized with Pax3, miR-1, or miR-206 probes as indicated. Pax3 transcripts detected at HH24 were significantly decreased by HH28, concomitant with increased miR-206 expression at that stage. miR-1 was detected faintly in the HH28 hindlimb but strongly in the heart (ht) and somites. Stippled lines indicate outline of limbs, and colored asterisks indicate equivalent regions. (*C*) Double in situ hybridization showing ectopic miR-206 expression (red, detecting GFP from miR-206 expression vector) leading to a loss of Pax3 transcripts (purple); a whole mount and a section are shown.

We used dual-luciferase reporter assays to examine whether miR-1/miR-206 might regulate embryo myogenesis by targeting Pax3. Two potential miR-1/miR-206 target sites in the Pax3 3′-UTR, termed TS1 and TS2, are conserved in human, mouse, rat, and chicken and have strong complementarity with the seed regions of both miRNAs (Fig. 2*A*). We generated sensors containing chicken Pax3 3′-UTR fragments downstream of firefly luciferase. Pax3 target sites were tested independently (TS1 or TS2) and in a sensor containing both sites (TS1 + TS2). Dual-luciferase assays showed that miR-1 and miR-206 directly target the Pax3 3′-UTR through both predicted target sites (Fig. 2*B*). TS1 was strongly down-regulated by miR-206, less so by miR-1, whereas TS2 was equally well regulated by both miR-1 and miR-206. Sensors containing both sites also were strongly down-regulated by both miRNAs, with miR-1 benefiting more from the presence of two sites. An unrelated miRNA not predicted to target Pax3, miR-140, had no effect on Pax3 sensors (Fig. 2*B*). Finally, mutant sensors containing point mutations within putative target sites (TS1^{m} and TS2^{m}) demonstrated that the sites were required for miRNA-mediated repression.

To investigate Pax3 regulation by miR-1/miR-206 in a cellular context, we screened several cell lines using RT-qPCR and Western and Northern blot analyses. We found that a glioma cell line, RuGli (34), expressed robust levels of Pax3, but no miR-1/miR-206. This allowed us to test the effects of miR-1/miR-206 on endogenous Pax3. Transfection with miR-1 or miR-206 led to reduced Pax3 transcript and protein expression, as shown by qPCR and Western blot analysis; this was restored by antimiR-206 cotransfection (Fig. 2 *C* and *D*). Northern blot analysis of C2C12 cells showed that antimiR-206 affects expression of both miR-1 and miR-206 (Fig. S3).

DEVELOPMENTAL BIOLOGY

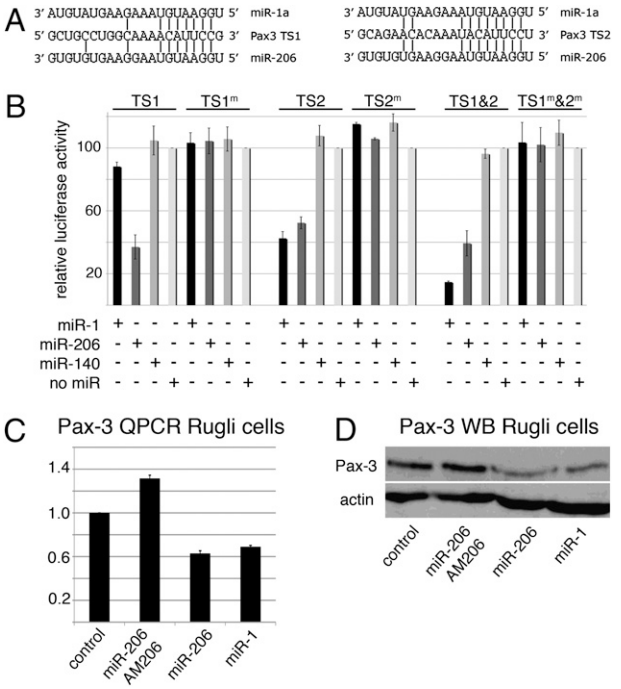

Fig. 2. The 3′-UTR of Pax3 is regulated by miR-1 and miR-206. (*A*) Alignment of target sites TS1 and TS2 with miR-1 and miR-206; *Materials and Methods* provides the genomic positions. (*B*) Normalized luciferase activity shown for Pax3–3′-UTR sensor constructs cotransfected with miR-1, miR-206, or miR-140 as indicated by (+). Activity was plotted relative to control (no miRNA). Experiments were repeated four times with triplicate samples and two independent DNA plasmid preparations. Error bars represent SE (n = 12). The constructs used are indicated above the graphs; TS^m represents constructs with mutant target sites. (*C*) qPCR of Pax3 expression in RuGli cells. Pax3 transcript levels in mock-transfected control cells (lane 1) were reduced after transfection of miR-206 (lane 3) and miR-1 (lane 4). Cotransfection of anti-miR-206 (AM) with miR-206 (lane 2) restored Pax3 expression. (*D*) Western blot analysis showing reduced Pax3 protein in RuGli cells transfected with miR-206 (lane 3) and miR-1 (lane 4). Protein levels were unaffected after cotransfection of antimiR-206 (AM) with miR-206 (lane 2).

We previously showed that MRFs activate miR-1/miR-206 expression during embryogenesis, with Myf-5 being of particular importance in both chick and mouse embryos (7). To examine the in vivo function of miRNAs in somite myogenesis, we injected 2′O-methyl modified antisense RNA (antagomir) into presegmented mesoderm of HH12–HH14 embryos. Presegmented mesoderm was allowed to form somites, and embryos were harvested after 24 h or 48 h of incubation (Fig. 3 *A*–*E*). The antagomirs were designed to inhibit miR-1 or miR-206; an identical antagomir-206 was previously shown to efficiently knock down miRNA levels in chicken embryos (35). A scrambled antagomir sequence was used as a negative control. Northern blot analyses confirmed the effects of antagomir-206 on miR-206 expression, which was no longer detected after antagomir-206 treatment. miR-1 expression was slightly affected by antagomir-206. Conversely, antagomir-1 had no effect on miR-206 expression, but led to loss of miR-1 (Fig. 3*L*). To examine whether miRNA inhibition affects myogenic differentiation in antagomir-injected embryos, we performed in situ hybridization for myogenin (Fig. 3 *A*–*E*). No myogenic phenotype was detected after 48 h of incubation; however, after 24 h, antagomir-206 or a combination of antagomir-206 and antagomir-1 led to complete or partial loss of myogenin expression in a significant number of embryos [n = 99/161 (61.5%) and n = 67/102 (65.6%); $P < 0.001$; Fig. 3 *B*, *D*, and *E*]. Microinjection of antagomir-1 affected myogenin expression in 12% of embryos (n = 13/109; Fig. 3 *C* and *E*). Injection of scrambled antagomir had no effect on somite myogenesis (n = 111; Fig. 3*A*). These findings suggest coordinated effects of miR-1 and miR-206 acting through the same seed sequence, which is important for the timely progression of myogenic progenitors into a differentiated state.

To corroborate the results from in situ hybridization, we used antibodies against Pax3 (red) and sarcomeric myosin (MF20, green), which label myogenic progenitors and differentiating myoblasts, respectively. This confirmed the effects of antagomir-206 on myogenic differentiation (Fig. 3 *F*, *F′*, and *G* and Fig. S4). Sectioned and stained embryos had fewer MF20-positive cells on the injected side (Fig. 3*F*), and Pax3-positive mesenchymal cells were detected in the myotome after antagomir-206 injection (red, Fig. 3*F′* and Fig. S4).

We used real-time qPCR and Western blot analysis to further examine the differences detected by in situ hybridization and immunohistochemistry. In dissected and pooled somites, we detected increased levels of Pax3 (Fig. 3*H*) and decreased levels of myogenin (Fig. 3*I*) after antagomir-206 injection compared with contralateral controls or scrambled antagomir-treated somites. In antagomir-1–injected somites, myogenin transcript levels were reduced by 20%, but there was no detectable change in Pax3, likely because only a small number of embryos injected with antagomir-1 showed any loss of myogenin, thus diluting any effect (Fig. 3*E*). Importantly, we did not expect to see any effect on Pax3 expression levels in the epithelial dermomyotome, given the lack of miR-1/miR-206 expression there (Fig. 1*A*); thus, the changes that we observed should be due to ectopic expression of Pax3 in myotome cells. Western blot analyses of dissected somites confirmed the effect of antagomir-206 on Pax3 protein, which was increased in injected somites (Fig. 3*K*). Conversely, Western blot analyses of antagomir-1-injected somites showed no effect on Pax3 (Fig. 3*J*), most likely for the reasons described above. Expression levels of Pax7 apparently remained unchanged (Fig. 3 *K* and *J*). These findings indicate that relatively small increases in Pax3 transcript and protein levels have a dramatic effect on the timing of myogenesis.

Because miRNAs have multiple targets, we investigated whether the interaction of miR-1/miR-206 specifically with Pax3 is the key event. For this, we made use of TPs, a powerful in vivo tool for investigating individual miR–target site interactions (36). Distinct TP morpholinos (TP1 and TP2, both FITC-labeled) were designed to protect miR-1/miR-206 target sites in the Pax3 3′-UTR (Fig. 4*A*). Cotransfection of TP1 and TP2 strongly inhibited the effect of miRNA on luciferase sensor constructs (Fig. S5). A TP1–TP2 mixture or a control morpholino was electroporated into presegmented mesoderm of HH14–HH16 chicken embryos. After 24 h, injected and noninjected contralateral somites were analyzed by Western blot (Fig. 4*B*) or qPCR (Fig. 4*C*). Targeted delivery of TP1/TP2 led to a detectable increase of Pax3 in injected somites compared with controls, consistent with the idea that TP1/TP2 interfere with the ability of miR-1/miR-206 to interact with the Pax3 3′-UTR. In addition, qPCR (Fig. 4*C*) and in situ hybridization (Fig. 4*D*) showed a dramatic reduction of myogenin expression in injected somites. Thus, antagomir injections (Fig. 3) and Pax3-specific TPs resulted in similar phenotypes, indicating that efficient repression of Pax3 by miR-1/miR-206 is crucial for timely progression of myogenic differentiation. This was confirmed by targeted misexpression of Pax3, which led to localized loss of myogenin expression in the myotome (Fig. 4*E*, *Upper*), demonstrating that persistent Pax3 expression in vivo is incompatible with myogenic differentiation. In contrast, morpholino-mediated knockdown of Pax3 at the same stage did not affect myogenesis (Fig. 4*E*, *Lower*).

Finally, we examined whether knockdown of Pax3 expression using morpholinos rescued the antagomir-206–induced loss of myogenin. We electroporated FITC-labeled Pax3 splice morpholino or control morpholino into somites previously injected with antagomir-206 (Fig. 4*F*, *Upper*). The morpholino is complementary to the exon 1–intron 1 splice junction (Fig. 4*G*). Western blot analysis of dissected and pooled somites showed a decrease of

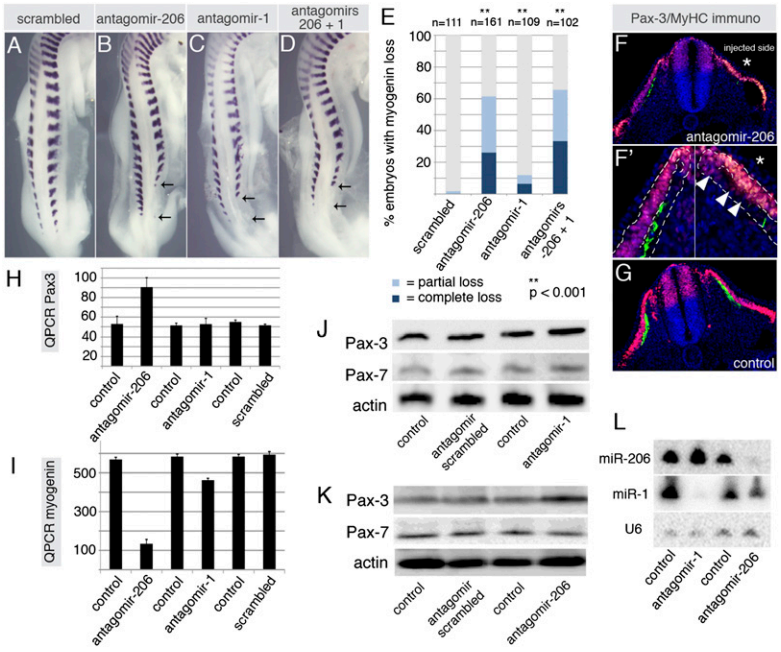

Fig. 3. Delayed myogenic differentiation after antagomir-mediated inhibition demonstrates a requirement for miR-1 and miR-206 activity. Antagomirs injected into presomitic mesoderm of HH12–14 embryos are indicated above each panel. Myogenic differentiation was assessed after 24 h by in situ hybridization with a myogenin probe. (*A–D*) Injection of scrambled antagomir had no effect (*A*); however, injection of antagomir-206 (*B*) or a 1:1 mixture of antagomir-206 and antagomir-1 (*D*) led to a loss of myogenin expression. Injection of antagomir-1 also affected myogenic differentiation (*C*). (*E*) Quantification of phenotypes observed. (*F*, *F′*, and *G*) Sections immunostained for Pax3 in the dermomyotome (red) and MF20 in the myotome (green) confirmed the inhibition of myogenic differentiation after antagomir-206 injection (*F*) compared with control embryos (*G*). *F′* is a higher-magnification view of *F* illustrating the increased number of Pax3-positive cells in the myotome (white arrowheads). Fewer myosin heavy chain (MF20)-positive cells were detected on the injected side, indicated by an asterisk, see also Fig. S4. (*H* and *I*) qPCR demonstrating the opposite effects on Pax3 and myogenin transcript levels in antagomiR-206 injected somites. (*J* and *K*) Western blots of dissected somites treated as indicated revealing increased Pax3 protein levels in antagomiR-206 injected somites with no change apparent on Pax7 protein levels. (*L*) Northern blot showing that antagomiR-1 specifically affects miR-1 and antagomiR-206 affects miR-206.

Pax3 protein after splice morpholino electroporation compared with controls (Fig. 4*H*). In situ hybridization showed a rescue of myogenin expression in regions of the somite that received the Pax3 splice morpholino (red), suggesting that miR-206 mediates its effect primarily via targeting of Pax3. In embryos injected with antagomir-206 followed by control morpholino electroporation, the loss of myogenin was not prevented (Fig. 4*F*, *Lower*).

Discussion

The miRNAs miR-1 and miR-206 have important functions in myogenesis. Their expression is positively regulated by MRFs (6, 7), and they have been shown to regulate a number of transcripts important for muscle biology (8–11). However, despite their striking, myotome-specific expression in developing somites, the role of miR-1/miR-206 in skeletal muscle development has not been resolved. Mice lacking either miR-1-2 or miR-206 have no overt phenotype in adult muscle (13, 14), most likely due to functional redundancy, given that these miRNAs have common targets. Here we made use of the accessibility of the chicken embryo for in vivo microinjection, which allowed us to inhibit miRNA function using antagomirs. We focused on a 24-h period in which miR-206 is the only family member expressed in somites to show that antagomirs inhibiting miR-1/miR-206 led to delayed myogenic differentiation (Fig. 3). We have provided evidence that this is due to the failure of complete down-regulation of Pax3. Inhibition of miRNA function in somites by antagomir-206 injection followed by quantitative analyses confirmed an increase in Pax3 expression (Fig. 3 *H* and *K*) accompanied by delayed muscle differentiation (Fig. 3 *B*, *E*, and *F*). The effects of miR-1 inhibition in vivo were similar, although less strong (Fig. 3 *C* and *E*). This could be due to the activity of miR-206 in these somites, which, based on Northern blot analysis, was not affected by antagomir-1 (Fig. 3*L*). We speculate that in chicken embryos, miR-206 is the primary regulator of skeletal myogenesis and miR-1 has a secondary role, with both miRNAs providing robustness to skeletal muscle differentiation, presumably through similar mechanisms. This is in agreement with the earlier expression of miR-206 in both somites and limbs (7) (Fig. 1*B* and Fig. S2 *A–D*). During mouse embryogenesis, within a few hours of detection of miR-1 in anteriormost somites at E9.5, both miRNAs were expressed in almost all somites along the axis by E10 (Fig. S2 *E–H*). This suggests that miR-1 and miR-206 likely have redundant functions, consistent with the observation that genetic deletion of either miR-1-2 or miR-206 in mice does not result in an overt skeletal muscle phenotype. This is in contrast to cardiogenesis, where deletion of miR-1-2 results in ventricular septal defects (13, 14).

After miR-1/miR-206 inhibition, the delay in muscle differentiation was transient, suggesting that additional mechanisms, potentially independent of miRNA, ensure the progression of myogenic differentiation. Functional interference experiments have indicated that miR-1 and miR-206 control the timing of the progenitor-to-myoblast transition during embryonic myogenesis, consistent with the view that miRNAs confer accuracy to developmental programs (2–4) (Fig. 5).

Pax3 sensors containing two conserved target sites were efficiently targeted by miR-1 and miR-206 (Fig. 2*B*), and endogenous Pax3 expression was regulated by miRNA at the level of both protein and RNA (Fig. 2 *C* and *D*), suggesting effects on both mRNA stability and repression of protein translation. The Pax3 TS1 site has greater complementarity to miR-206 compared with miR-1, and was more strongly affected by miR-206 (Fig. 2*A*). In addition, miR-206 seemed to be maximally effective in the presence of only one binding site, whereas miR-1 was much more effective in the presence of both TS1 and TS2. Interestingly, only a small number of targets have more than one putative target site for any given miRNA (2), and it is possible that effective down-regulation is enhanced by cooperation between sites.

Although miR-1 and miR-206 have been shown to target several genes involved in myogenesis in vitro and in adult muscles (8–12), our results show that interference with miRNA function or biogenesis at the time of myoblast commitment leads to changes in Pax3 expression in both somites (Fig. 3*H*) and limb buds (Fig. S1*M*). In somites, these changes are masked by continued expression of Pax3 in the dermomyotome, which is unaffected by antagomir-206 injection (Fig. 3*F*), because miR-206 expression is restricted to committed myoblasts in the myotome (Fig. 1). However, we detected a reproducible and significant increase in Pax3 levels using qPCR and Western blot analyses, and Pax3 increase was correlated with dramatic (albeit transient) changes in the muscle differentiation markers myogenin and myosin heavy chain (Fig. 3 *H–K*). In addition, we observed ectopic Pax3 staining in cells delaminating from the dermomyotomal sheet and moving into the myotome to become mesenchymal muscle cells (37) (Fig. 3 *F* and *F′* and Fig. S4); however, this was close to the limit of detection by immunohistochemistry, likely because the primary mechanism of Pax3 regulation is at the level

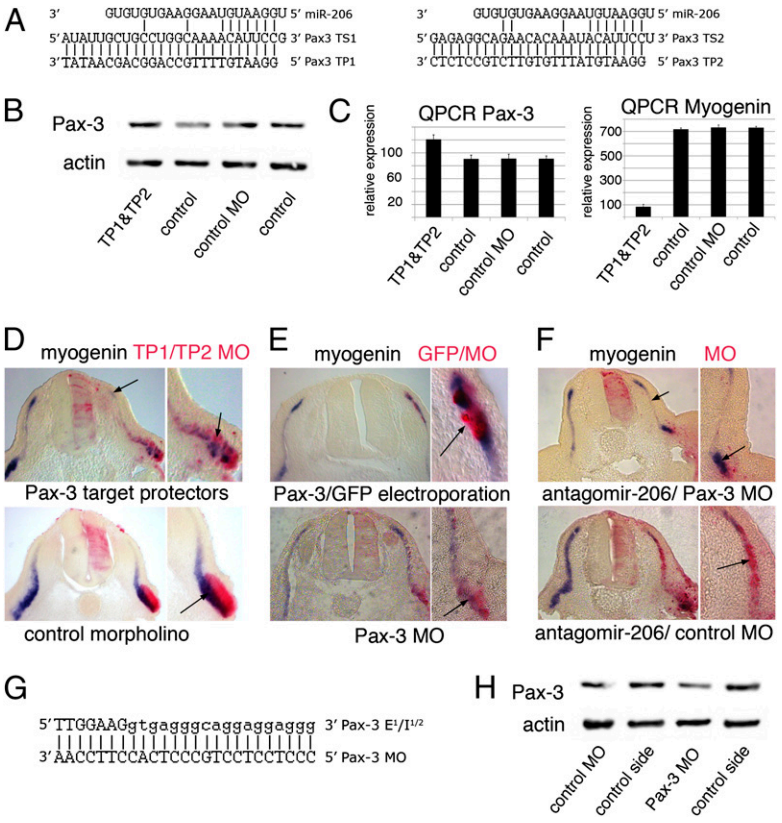

Fig. 4. miRNA affects somite myogenesis predominantly via Pax3. (*A*) FITC-labeled TP MO, designed to prevent miR-1/miR-206 interactions with both target sites in the Pax3 3′-UTR, were electroporated. After overnight incubation, somites were microdissected. (*B–D*) Western blot (*B*) and qPCR (*C*) demonstrated increases in Pax3 protein and transcripts after electroporation with TP1 plus TP2 compared with contralateral noninjected somites or somites electroporated with control morpholinos (MO). qPCR (*C*) and in situ hybridization (*D*) demonstrated a corresponding loss of myogenin expression (*Upper*); myogenin expression in control morpholino-injected somites was comparable to that in noninjected somites from the opposite side (*Lower*). (*E–H*) Electroporation of Pax3/GFP expression vectors (red) led to localized loss of myogenin (blue), consistent with the concept that elevated Pax3 levels are incompatible with differentiation (*Upper*). (*E* and *G*) Electroporation of Pax3 splice morpholinos (red) had no effect on myogenin expression (blue; *Lower*). (*H*) Western blot analysis confirmed reduced Pax3 expression in somites after Pax3 MO electroporation. (*F*) Cotransfection of Pax3 splice-MO (red) rescued antagomiR-206–induced loss of myogenin transcripts and restored normal expression of myogenin (blue; *Upper*), whereas control morpholino (red) did not rescue the antagomir-206 mediated inhibition of myogenin expression (blue; *Lower*).

of transcriptional repression (Figs. 1*A* and 5), which is not affected by antagomir. Down-regulation of Pax3 is also concomitant with up-regulation of MRFs, which themselves are involved in activation of miRNA expression (6, 7). Thus, miR-1 and miR-206 reinforce negative regulation of Pax3 expression and make a significant contribution to the complete silencing of Pax3, which leads to the timely expression of myogenin or sarcomeric myosin in committed myoblasts (Figs. 3 and 5).

We have shown that miR-1 and miR-206 act primarily through Pax3, as demonstrated by the fact that blocking this interaction with specific TPs also led to loss of myogenin in affected cells (Fig. 4 *C* and *D*). Furthermore, myogenin expression was rescued by Pax3 splice morpholinos in antagomir-206–injected somites (Fig. 4 *F* and *H*). The Pax3 morpholino by itself had no effect on myogenin expression (Fig. 4*E*, *Lower*), consistent with the lack of a somitic muscle phenotype in Pax3 mutant mice (22, 27). Thus, our data show that precise and robust developmental timing during myogenesis requires miR-1/miR-206, with Pax3 as a crucial target in embryonic myoblasts.

The importance of Pax3 in myogenesis is well established (20, 21, 38). It is expressed in proliferating muscle progenitors, before expression of MRFs, and down-regulated as myogenesis proceeds (22, 23). Interestingly, a population of cells in the central myotome, which contribute to adult muscle stem cells (24), retain Pax3 expression (Fig. 1*A*) to promote their survival and myotome growth (39). Whether or not these cells coexpress miR-1 and miR-206 remains to be determined; current in situ detection methods with LNA probes do not allow single-cell resolution. The behavior of adult muscle stem cells—particularly their proliferation, differentiation, and apoptosis—is regulated by Pax3 and the closely related Pax7. The protein levels of Pax3/7 were recently demonstrated to be tightly regulated by miRNAs, including miR-27b and miR-206 (12, 40, 41). Thus, embryonic myoblasts and adult muscle stem cells require mechanisms to tightly regulate Pax3/Pax7 activity. We suggest that miR-1 and miR-206 are responsible for regulation of Pax3 in newly committed myoblasts to control the timely progression of embryo myogenesis. This aspect of their function remains important in adult muscle stem cells.

Materials and Methods

DNA Constructs, Transfections, and Luciferase Assay. Sensors contained chick Pax3 3′-UTR fragments in a modified pGL3 vector (Promega; Pax3 TS1 sensor: nt 906–1,106; Pax3 TS2 sensor: nt 51–657; Pax3 TS1+2 sensor: nt 51–1,106). In mutant constructs, a BamHI site introduced point mutations within target sites. Chick dermal fibroblast cells (DF1 cells) were transfected with 200 ng of sensor with or without miR-206 or miR-1 (50 nM) (Sigma-Aldrich), using Lipofectamine 2000 (Invitrogen) in 96-well plates. miR-1 duplexes (5′-UGGAAUGUAAAGAAGUAUGUA-3′ and 5′-CAUACUUCUUUAUAUGCCCAUA-3′) and miR-206 duplexes (5′-UGGAAUGUAAGGAAGUGU GUGG-3′ and 5′-ACAUGCUUCUUUAUAUCCCCAUA-3′) were identical to endogenous miRNAs. pGL3 vector without 3′-UTR or with mutant 3′-UTRs, or transfection of unrelated miR-140, served as a negative control. Transfections had triplicate samples and were repeated four times using independent plasmid preparations. Firefly and renilla luciferase activities were measured after 48 h using a multilabel counter (Victor2; Perkin-Elmer), and relative activities were calculated. For overexpression of miR-206, we constructed

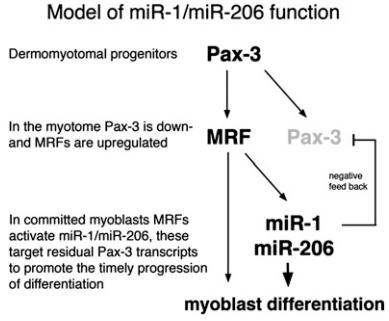

Fig. 5. Model illustrating that the complete silencing of Pax3 in committed myoblasts confers robustness to developmental timing of differentiation; see *Discussion* for details.

a vector encoding a short-hairpin precursor. Northern blot analysis demonstrated production of mature miR-206 was produced. A mouse Pax3 expression vector (MRC Geneservice) was used for overexpression.

Cell Culture, Western Blot Analysis, and Real-Time qPCR. Rat RuGli glioblastoma cells in DMEM, 10% FBS, and 1% pen/strep were transfected with miR-206 or miR-1 (50 nM; Sigma-Aldrich) with and without anti–miR-206 (50 nM; Ambion) using Lipofectamine 2000. Mock-transfected cells were used as negative controls. Protein was extracted at 48 h after transfection following standard protocols, and 20 μg was run on 8% polyacrylamide gels and blotted onto nitrocellulose using a semidry blotter (Biorad). Primary antibodies Pax3, Pax7 (1:500 dilution; Developmental Studies Hybridoma Bank), and actin (1:1,000; Abcam) and secondary antibodies (Jackson Laboratories) were applied for 1 h at room temperature.

RNA was extracted from cells, somites, or limbs using TRIzol (Invitrogen). cDNA synthesis was done using SuperScript II (Invitrogen). Predesigned TaqMan probes were used with the Applied Biosystems 7500 Fast Real-Time PCR system following the manufacturer's protocols. Data analysis was performed with Applied Biosystems SDS version 2.0.

In Situ Hybridization and Immunohistochemistry. Whole-mount in situ hybridization using double-labeled LNA oligos (mercury; Exiqon) or anti-sense RNA probes was carried out as described previously (7). Embryos were fixed in 4% PFA, embedded in paraffin, sectioned, and immunostained as described previously (42). The MF20 and PAX3 antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and maintained by the University of Iowa.

Injection of Antagomir, Target Protectors, and Antisense Morpholinos. Antagomirs were designed based on previously published methods (30, 31). All bases were replaced by 2′O-methyl bases; thiol bonds replaced phosphodiester bonds between bases 1 and 2, 2 and 3, 19 and 20, 20 and 21 and 21 and 22; and a 3′ cholesterol moiety (Dharmacon) was included. Scrambled sequences were used as controls. For injections, Fast Green (0.05%) was added to the antagomir, to a final concentration 100 μM. Embryos were injected into presegmented mesoderm as described previously (35), and the number of somites was recorded as 'n'. Embryos were harvested after 24 h, and somites n+1 to n+4 were dissected and lysed (42). Corresponding somites from the uninjected side were used as controls, Between 20 and 25 treated embryos were pooled for Western blot analysis using 20 μg of protein. Blots were repeated three times with somites from independent experiments.

Pax3 morpholino (CCCTCCTCCTGCCCTCACCTTCCAA) was designed to target both Pax3 splice variants. Control morpholino (CCTCTTACCTCAGTTACAATTTATA) was designed to not target any known gene. TPs complementary to miR-1/miR-206 target sites in the Pax3 3′-UTR were Pax3 TP1 (TATAACGACGGACCGTTTTGTAAGG) and Pax3 TP2 (CTCTCCGTCTTGTGTTTATGTAAGG). Morpholinos were 3′ fluorescein-labeled (Gene Tools), injected into somites at HH14-16, and electroporated using 6 × 20 ms pulses of 20V. Embryos were harvested after 24 h.

Dicer Conditional Null Mice. Dicer floxed ($Dicer^{fl/fl}$) mice and Pax3 Cre knock-in strains have been described previously (32, 33). Litters were genotyped by PCR of genomic DNA obtained from embryonic yolk sacs using the following primers: Dicer-F, AGTGTAGCCTTAGCCATTTGC; Dicer-R, CTGGTGGCTTGAGGACAAGAC; CreORF-F, ATCCGAAAAGAAAACGTTGA; and CreORF-R, ATCCAGGTTACGGATATAGT.

ACKNOWLEDGMENTS. We thank Uli Mayer and Grant Wheeler for discussions, Andrew Lassar for insightful comments on an early version of the manuscript, and Eddy McGlinn and Cliff Tabin for sharing of protocols. The work of K.G.-W., D.S., and M.A.E. was funded by a University of East Anglia Studentship and by Biotechnology and Biological Sciences Research Council and Medical Research Council project grants (BB/D016444/1 and G0600757, to A.M.).

1. Bartel DP (2009) MicroRNAs: Target recognition and regulatory functions. *Cell* 136: 215–233.
2. Stark A, Brennecke J, Bushati N, Russell RB, Cohen SM (2005) Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3′-UTR evolution. *Cell* 123:1133–1146.
3. Hornstein E, Shomron N (2006) Canalization of development by microRNAs. *Nat Genet* 38(Suppl):S20–S24.
4. Mann M, Barad O, Agami R, Geiger B, Hornstein E (2010) miRNA-based mechanism for the commitment of multipotent progenitors to a single cellular fate. *Proc Natl Acad Sci USA* 107:15804–15809.
5. van Rooij E, Liu N, Olson EN (2008) MicroRNAs flex their muscles. *Trends Genet* 24: 159–166.
6. Rao PK, Kumar RM, Farkhondeh M, Baskerville S, Lodish HF (2006) Myogenic factors that regulate expression of muscle-specific microRNAs. *Proc Natl Acad Sci USA* 103:8721–8726.
7. Sweetman D, et al. (2008) Specific requirements of MRFs for the expression of muscle-specific microRNAs, miR-1, miR-206 and miR-133. *Dev Biol* 321:491–499.
8. Anderson C, Catoe H, Werner R (2006) MIR-206 regulates connexin43 expression during skeletal muscle development. *Nucleic Acids Res* 34:5863–5871.
9. Kim HK, Lee YS, Sivaprasad U, Malhotra A, Dutta A (2006) Muscle-specific microRNA miR-206 promotes muscle differentiation. *J Cell Biol* 174:677–687.
10. Rosenberg MI, Georges SA, Asawachaicharn A, Analau E, Tapscott SJ (2006) MyoD inhibits Fstl1 and Utrn expression by inducing transcription of miR-206. *J Cell Biol* 175:77–85.
11. Chen JF, et al. (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat Genet* 38:228–233.
12. Chen JF, et al. (2010) microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. *J Cell Biol* 190:867–879.
13. Zhao Y, et al. (2007) Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2. *Cell* 129:303–317.
14. Williams AH, et al. (2009) MicroRNA-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice. *Science* 326:1549–1554.
15. Buckingham M (2001) Skeletal muscle formation in vertebrates. *Curr Opin Genet Dev* 11:440–448.
16. Buckingham M, Vincent SD (2009) Distinct and dynamic myogenic populations in the vertebrate embryo. *Curr Opin Genet Dev* 19:444–453.
17. Bajard L, et al. (2006) A novel genetic hierarchy functions during hypaxial myogenesis: Pax3 directly activates Myf5 in muscle progenitor cells in the limb. *Genes Dev* 20: 2450–2464.
18. Sato T, Rocancourt D, Marques L, Thorsteinsdóttir S, Buckingham M (2010) A Pax3/Dmrt2/Myf5 regulatory cascade functions at the onset of myogenesis. *PLoS Genet* 6:e1000897.
19. Hu P, Geles KG, Paik JH, DePinho RA, Tjian R (2008) Codependent activators direct myoblast-specific MyoD transcription. *Dev Cell* 15:534–546.
20. Maroto M, et al. (1997) Ectopic Pax-3 activates MyoD and Myf-5 expression in embryonic mesoderm and neural tissue. *Cell* 89:139–148.
21. Tajbakhsh S, Rocancourt D, Cossu G, Buckingham M (1997) Redefining the genetic hierarchies controlling skeletal myogenesis: Pax-3 and Myf-5 act upstream of MyoD. *Cell* 89:127–138.
22. Goulding M, Lumsden A, Paquette AJ (1994) Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. *Development* 120:957–971.
23. Williams BA, Ordahl CP (1994) Pax-3 expression in segmental mesoderm marks early stages in myogenic cell specification. *Development* 120:785–796.
24. Gros J, Manceau M, Thomé V, Marcelle C (2005) A common somitic origin for embryonic muscle progenitors and satellite cells. *Nature* 435:954–958.
25. Zammit PS, Partridge TA, Yablonka-Reuveni Z (2006) The skeletal muscle satellite cell: The stem cell that came in from the cold. *J Histochem Cytochem* 54:1177–1191.
26. Daston G, Lamar E, Olivier M, Goulding M (1996) Pax-3 is necessary for migration but not differentiation of limb muscle precursors in the mouse. *Development* 122:1017–1027.
27. Bober E, Franz T, Arnold HH, Gruss P, Tremblay P (1994) Pax-3 is required for the development of limb muscles: a possible role for the migration of dermomyotomal muscle progenitor cells. *Development* 120:603–612.
28. Epstein JA, Lam P, Jepeal L, Maas RL, Shapiro DN (1995) Pax3 inhibits myogenic differentiation of cultured myoblast cells. *J Biol Chem* 270:11719–11722.
29. Boutet SC, Disatnik MH, Chan LS, Iori K, Rando TA (2007) Regulation of Pax3 by proteasomal degradation of monoubiquitinated protein in skeletal muscle progenitors. *Cell* 130:349–362.
30. Krützfeldt J, et al. (2005) Silencing of microRNAs in vivo with "antagomirs." *Nature* 438:685–689.
31. Meister G, Landthaler M, Dorsett Y, Tuschl T (2004) Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *RNA* 10:544–550.
32. Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ (2005) The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proc Natl Acad Sci USA* 102:10898–10903.
33. Engleka KA, et al. (2005) Insertion of Cre into the Pax3 locus creates a new allele of *Splotch* and identifies unexpected Pax3 derivatives. *Dev Biol* 280:396–406.
34. Goodman SL, Deutzmann R, von der Mark K (1987) Two distinct cell-binding domains in laminin can independently promote nonneuronal cell adhesion and spreading. *J Cell Biol* 105:589–598.
35. McGlinn E, et al. (2009) In ovo application of antagomirs indicates a role for miR-196 in patterning the chick axial skeleton through *Hox* gene regulation. *Proc Natl Acad Sci USA* 106:18610–18615.
36. Choi WY, Giraldez AJ, Schier AF (2007) Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. *Science* 318:271–274.
37. Gros J, Scaal M, Marcelle C (2004) A two-step mechanism for myotome formation in chick. *Dev Cell* 6:875–882.
38. Lagha M, et al. (2008) Pax3 regulation of FGF signaling affects the progression of embryonic progenitor cells into the myogenic program. *Genes Dev* 22:1828–1837.
39. Relaix F, et al. (2006) Pax3 and Pax7 have distinct and overlapping functions in adult muscle progenitor cells. *J Cell Biol* 172:91–102.
40. Crist CG, et al. (2009) Muscle stem cell behavior is modified by microRNA-27 regulation of Pax3 expression. *Proc Natl Acad Sci USA* 106:13383–13387.
41. Hirai H, et al. (2010) MyoD regulates apoptosis of myoblasts through microRNA-mediated down-regulation of Pax3. *J Cell Biol* 191:347–365.
42. Smith TG, Sweetman D, Patterson M, Keyse SM, Münsterberg A (2005) Feedback interactions between MKP3 and ERK MAP kinase control scleraxis expression and the specification of rib progenitors in the developing chick somite. *Development* 132: 1305–1314.