Characterization of the DNA-binding Properties of the Mohawk Homeobox Transcription Factor

Received for publication, July 9, 2012. Published, JBC Papers in Press, August 24, 2012, DOI 10.1074/jbc.M112.399386

Douglas M. Anderson†‡, Rajani George§, Marcus B. Noyes†‖, Megan Rowton§*, Wenjin Liu**, Rulang Jiang**, Scot A. Wolfe†, Jeanne Wilson-Rawls‡, and Alan Rawls†‡‡

From the †School of Life Sciences, ‡Molecular and Cellular Biology Graduate Program, ‡‡Center for Evolutionary Medicine and Informatics, Biodesign Institute, Arizona State University, Tempe, Arizona 85287-4501, the †Program in Gene Function and Expression, †Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, and the **Department of Biomedical Genetics and Center for Oral Biology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Background: Mkx is a transcriptional repressor that regulates muscle and tendon differentiation.
Results: MKX binds to nnACA recognition sites as a homodimer. Mkx regulates transcription through recognition sites in the Mkx and Sox6 loci.
Conclusion: MKX has a novel DNA recognition mode and promotes slow muscle fiber type specification through Sox6.
Significance: We provide insight into Mkx regulation of musculoskeletal-specific transcription.

The homeobox transcription factor Mohawk (Mkx) is a potent transcriptional repressor expressed in the embryonic precursors of skeletal muscle, cartilage, and bone. Mkx has recently been shown to be a critical regulator of musculoskeletal tissue differentiation and gene expression; however, the genetic pathways through which Mkx functions and its DNA-binding properties are currently unknown. Using a modified bacterial one-hybrid site selection assay, we determined the core DNA-recognition motif of the mouse monomeric Mkx homeodomain to be A-C-A. Using cell-based assays, we have identified a minimal Mkx-responsive element (MRE) located within the Mkx promoter, which is composed of a highly conserved inverted repeat of the core Mkx recognition motif. Using the minimal MRE sequence, we have further identified conserved MREs within the locus of Sox6, a transcription factor that represses slow fiber gene expression during skeletal muscle differentiation. Real-time PCR and immunostaining of in vitro differentiated muscle satellite cells isolated from Mkx-null mice revealed an increase in the expression of Sox6 and down-regulation of slow fiber structural genes. Together, these data identify the unique DNA-recognition properties of Mkx and reveal a novel role for Mkx in promoting slow fiber type specification during skeletal muscle differentiation.

Mohawk (Mkx)† is a transcriptional repressor expressed in the embryonic progenitor cell populations of skeletal muscle,
idues) specifically within Helix III (1). Among the amino acids important for DNA recognition, mouse Mkx and Irx factors are identical at positions 8, 50, 51, 54, and 55, but differ at positions 2, 3, 5, 6, 7, and 47 (1). Because it has been shown previously that closely related homeobox genes recognize similar DNA recognition motifs, Mkx and Irx homeodomains may have similarity in their DNA-binding specificity (11, 17).

The minimal Iroquois-binding site (IBS) for Drosophila Iro-C family members has been identified using in vitro site selection assays (11, 18). The IBS is composed of an inverted repeat (ACAnnTGT) that consists of two monomeric recognition motifs for each Iro-C homeodomain (ACA). The arrangement of the two monomeric recognition motifs appears to be flexible, as Drosophila ara has been shown to recognize an opposite inverted repeat conformation (TGTnACA), but not a direct repeat ACAnnACA (17–19). The monomeric recognition motif of the Drosophila Mkx ortholog CG11617 has been characterized and is similar to Irx family members, except for a strong preference for a thymine (T) at position 1 of the binding motif (TnACA) (11).

The genetic networks through which Mkx functions in the normal differentiation and growth of the musculoskeletal system are currently unknown. To identify direct targets of MKX regulation, we characterized the DNA-binding specificity of mouse Mkx; its regulation of its own promoter sequence; and its ability to form a homodimer. Additionally, we have identified two Mkx-responsive elements within the locus of Sox6, a key repressor of slow fiber type specification during skeletal muscle differentiation. These characterized recognition elements provide a basis for identifying additional direct targets of MKX regulation and further our understanding of its role as a regulator of musculoskeletal development.

MATERIALS AND METHODS

Homeodomain-binding Site Selection—A fragment of mouse Mkx containing the homeodomain and adjacent conserved domain CD-A (amino acids 70–161) was amplified from the expression plasmid CS2MT-Mkx (2) (see supplemental Tables S1 and S2 for primers). Additionally, a stop codon, TAA (italics), was amplified from the expression plasmid CS2MT-Mkx (1–204)-VP16 was created by cloning the Mkx fragment into the Stul/XhoI sites in CS2MT and the activation domain of VP16 into the Xhol/XbaI sites (24). Primer sequences are located in supplemental Tables S1 and S2.

Transient Transfections and Luciferase Reporter Assays—Transcriptional activity of the Mkx and Sox6 genomic sequences was measured in NIH3T3-transfected mouse fibroblast cells as described in Anderson et al. (2). Luciferase activity was measured using an FLx800 microplate reader (BioTek Instruments, Inc., Winooski, VT). Experiments were performed in triplicate, and each experiment was repeated at least three times.

Electrophoretic Mobility Shift Assays (EMSA)—Recombinant Myc-epitope-tagged proteins used in EMSA were expressed in mouse NIH3T3 cells using transient transfection with Lipofectamine and Plus reagent (Invitrogen), as described in Ref. 2. Double-stranded EMSA probes were created by annealing complementary oligonucleotides in sodium chloride-Tris-EDTA buffer (50 mm NaCl, 10 mm Tris, pH 8.0, and 1 mm EDTA). Probe sequences are listed in supplemental Tables S1 and S2. Binding reactions were carried out at room temperature in 4× binding buffer and separated using polyacrylamide gel electrophoresis as described by Wilson-Rawls et al. (25).

Mkx Knock-out Mouse Genotyping—Targeted disruption of the Mkx locus has been reported previously (6). Mkx knock-out mice were genotyped using a duplex PCR with the primers (see supplemental Tables S1 and S2). Mice were maintained as heterozygotes for the Mkx-null allele and wild type, and null littersmates from heterozygous crosses were used for experimental analysis.

Primary Satellite Cell Isolation and Culture—Satellite cells were isolated from hindlimb muscle from six 4-month-old mice. Satellite cells were maintained in 20% fetal bovine serum (FBS) in Ham’s F-10 (supplemented with 10 ng/ml basic fibroblast growth factor (BD Biosciences) and Primocin (InvivoGen, San Diego, CA). To induce differentiation, satellite cells were plated in 60-mm dishes and changed to media containing DMEM containing 2% horse serum and Primocin. Differentiation medium was changed every day, and satellite cells were allowed to differentiate for 3 days. Expression of fast twitch myosin heavy chain isoforms (Myh1, 2, 4, and 8) and slow twitch myosin heavy chain (Myh7) was detected by immunohistochemistry using the MY32 and NOQ7.5.4D antibodies (Sigma-Aldrich), respectively. Immunohistochemistry was performed as described in Ref. 26.

Analysis of Satellite Cell Gene Expression Using Real-time PCR—Total RNA was isolated and purified from satellite cells cultured in 60-mm dishes using 1 ml of ice-cold TRIzol (Invitrogen) according to the manufacturer’s protocols. Reverse transcription of 2 μg of total RNA was performed using Super-Script III (Invitrogen). Real-time quantitative PCR analysis of the cDNA was performed using qPCR MasterMix Plus w/o UNG (Eurogentec, Fremont, CA) on an ABI 7900HT quantitative Real-Time PCR Machine (Applied Biosystems, Foster City, CA).
RESULTS

The Mouse Mkx Homeodomain Binds a Recognition Motif Common to the Irx Family—To characterize the DNA-binding specificity of the mouse Mkx homeodomain, we utilized a modified bacterial one-hybrid assay to select recognition sequences from a library of random decamer sequences. A fragment of mouse Mkx including the homeodomain and adjacent conserved domain CD-A (amino acids 70–161) was cloned into the pB1H2o2-12 vector, as a fusion with the ω subunit of RNA polymerase and the first and second zinc fingers of Zif268. This construct was co-transformed with a reporter plasmid that contains a region of random decamers adjacent to the binding site of the Zif268, and transformants were selected for their ability to grow under nutrient-deficient conditions (11, 19).

A total of 21 sequences were recovered and analyzed using the web-based program MotifSampler (Fig. 1A). The motif-detecting algorithm of this program uses Gibbs sampling to find the position probability matrix of the motif (20, 21). The preferred recognition sequence of mouse MKX was determined to be A-C-A (Fig. 1B). Thus, mouse MKX shares the same recognition motif as previously characterized Irx family members but diverges from the recently reported binding site (T-n-A-C-A) of the Drosophila MKX ortholog, which has a strong affinity for a T at position 1 (11).

Mkx Is Capable of Negative Autoregulation—DNA-binding transcription factors often participate in the regulation of their own transcription through autoregulatory feedback loops (27). Negative autoregulation is a well-established mechanism for both stabilizing transcription factor levels as well as modulating spatial and temporal expression patterns (23, 28, 29). To determine whether Mkx is capable of directly regulating its own transcription, we cloned a fragment of the mouse Mkx promoter (−3,576 to +127 bp) upstream of the luciferase reporter gene in pGL3Basic (3.5kbMkx-luc) (Fig. 2A). Full-length Mkx (MT-MKX) strongly repressed transcription (13.4-fold reduction) when compared with background (Fig. 2B). Deletion of the three Mkx repression domains (2) (MT-Mkx(AMRD1-3)) or Helix III of the homeodomain (MT-Mkx(ΔH3)) abrogated repression of 3.5kbMkx-luc (Fig. 2B). To confirm the responsiveness of 3.5kbMkx-luc to Mkx, the C-terminal region containing the three Mkx repressor domains (amino acids 205–352) was replaced by the potent transcriptional activation domain of VP16 (MT-Mkx-VP16) (30). The MT-Mkx-VP16 fusion resulted in 8.9-fold activation of 3.5kbMkx-luc. Additionally, neither a deletion mutant of MT-Mkx-VP16 that does not contain Helix III of the homeodomain (MT-Mkx(ΔH3)-VP16), nor VP16 alone, could activate the 3.5kbMkx-luc reporter to the same extent (Fig. 2B). This demonstrates that Mkx is capable of regulating its own promoter and that this regulation is dependent upon amino acids within the homeodomain.

Similar repression levels were observed with a smaller fragment of the Mkx promoter spanning –113 bp to +127 bp (113bpMkx-luc) (Fig. 2). This fragment was therefore further subdivided into three overlapping fragments (A, –113 to –17; B, –41 to +56; and C, +32 to +127) (Fig. 3A). Fragment A alone was responsive to MT-Mkx-VP16, which we will herein refer to as the MRE (Fig. 3B). A multiple sequence alignment revealed that the MRE is highly conserved in the promoters of vertebrate Mkx orthologs and is composed of a large inverted repeat (see supplemental Fig. S1).

MKX Interacts Directly with a Highly Conserved Sequence Within Its Promoter—The MRE includes a highly conserved inverted repeat, which contains the recognition motif we characterized, separated by 25 bases (AGTGT-N25-AACAT). To test if MKX can bind this sequence, we performed an EMSA using whole cell lysates from NIH3T3 cells expressing MT-MKX and a double-stranded DNA probe specific for the conserved inverted repeat sequence (Fig. 4). A band was consistently observed in the presence of MT-MKX and could be further supershifted by an anti-Myc antibody (Fig. 4A). A mutant fusion protein, MT-MKX with the Helix III of the homeodomain deleted (MT-Mkx(ΔH3)), was not able to shift the MRE probe (Fig. 4A). Further, MT-MKX-VP16 also formed a complex with the probe and was supershifted by the anti-Myc antibody (Fig. 4B). This demonstrated that MKX can form a complex with this inverted repeat, which we will refer to as the Mkx-binding sequence (MBS), and the homeodomain is necessary for this interaction.

The ability of MKX to regulate the MBS in cells was tested by cloning this sequence upstream in pGL3Promoter (MBS-luc). MT-Mkx-VP16 activated the MBS-luc vector 2.5-fold, when compared with pGL3Promoter alone (luc) (Fig. 4C). Reducing the number of flanking residues and randomizing the intervening 21 bp did not alter the level of activation (artificial MBS-aMBS-luc) (Fig. 4C). Multimerizing three copies of the aMBS in a head-to-tail fashion resulted in additive increases in luciferase activation (3X-aMBS-luc). Point mutation of nucleotides within both inverted repeats (mut-aMBS; ACAct N5 AGTTT) abolished activation by MT-Mkx-VP16 (Fig. 4C). Multimerized versions of the MBS in which one of the half-sites was mutated (Lmut-
MKx DNA-binding Properties

aMBS or Rmut-aMBS) abrogated activation by MT-Mkx-VP16 (Fig. 4C). This indicated that both half-sites of the MBS were required and given the inverted repeat nature of the MBS, suggested that MKX might bind the MBS as a homodimer.

MKX Can Homodimerize and Requires an Adjacent Conserved Domain to Recognize Its Binding Site—Homeodomain-containing proteins can function as monomers, homodimers, or heterodimers, which can lead to differential DNA-binding
specificity (31–33). To test whether MKX is capable of homodimerization, we used a modified mammalian two-hybrid approach using transient transfection in NIH3T3 cells. Gal4 DNA-binding domain (Gal4DBD)-fusion proteins with MKX-specific bait fragments were assayed for their ability to interact with the MT-Mkx-VP16. Dimerization of Mkx bait fragments

FIGURE 4. Identification of the MBS within the MRE. A and B, MT-Mkx (A) and MT-Mkx-VP16 (B) were able to bind the MBS sequence but not a mutated MBS sequence in an electrophoresis mobility shift assay. The Mkx-MBS complex could be supershifted with an anti-Myc antibody (MT-Ab). Deletion mutants of Mkx lacking Helix III of the homeodomain (MT-Mkx(ΔH3) and MT-Mkx(ΔH3)-VP16) were not sufficient to shift MBS. EMSA primers are provided in supplemental Table S1. C, cell-based luciferase transcription assays demonstrate that the MBS sequence is responsive to Mkx-VP16 activation. An artificial MBS (aMBS) that removes 5’-flanking sequence and randomizes the central 21 bp was equally responsive. Increasing the copy number of the MBS resulted in increased activation by Mkx-VP16. Mutation of either half-site demonstrated that both are required for Mkx-mediated regulation.
was scored by the level of activation of the Gal4DBD-responsive luciferase reporter, 5XUASpGL3Promoter, relative to Gal4DBD alone (2). Initially, full-length Mkx resulted in a reduction of reporter activity (Fig. 5A). Because the three Mkx repression domains (MRD1-3) may suppress luciferase transcription, a fusion construct that lacks these domains (Gal4DBD-Mkx(ΔMRD1-3)) was examined. This resulted in a 9-fold activation of the reporter, demonstrating that Mkx is capable of homodimerization. A fusion construct containing the Mkx homeodomain led to modest activation of the reporter, demonstrating that Mkx is capable of homodimerization dependent upon amino acids within the homeodomain and CD-A. Data are presented for each Gal4-Mkx bait as -fold activation relative to the level of luciferase activity obtained by co-transfection of the Gal4DBD alone with appropriate prey.

**The Sox6 Locus Contains Multiple Mkx-binding Sites**—To identify additional targets of MKX, a genome-scale DNA pattern search of the conserved MBS sequence was performed using the Regulatory Sequence Analysis Tools (22). The number of intervening nucleotides was allowed to vary from 0 to 25, and mismatches of 1 bp were allowed in either half-site (ATGTT N0–25 AACAT). A list of putative MRE sites identified by this approach is provided in supplemental Table S2. Within the intronic sequence in the Sox6 locus, we identified a well conserved MBS with an intervening space of 11 nucleotides (ACAnnTGT). This revealed an additional well conserved element within the Sox6 locus, we identified a well conserved MBS with an intervening space of 11 nucleotides (ACAnnTGT). This revealed an additional well conserved element within the Sox6 locus.

**Sox6 Is Up-regulated in Satellite Cells Isolated from Mkx−/− Mice**—To validate that Mkx was a repressor of Sox6 transcription in vivo, the levels of endogenous Sox6 mRNA were examined in primary muscle cells deficient for Mkx. Skeletal muscle satellite cells were isolated from the hindlimb muscles of 5-month-old Mkx−/− and wild type littermate mice. Quantitative RT-PCR using Sox6-specific primers revealed that Sox6 is expressed at a similar level in freshly isolated satellite cells from Mkx−/− and control muscle (Fig. 7A). However, satellite cells differentiating into myotubes demonstrated that Sox6 expression was 2.2-fold higher in the Mkx−/− mice (Fig. 7A). Sox6 has been shown to participate in fiber type specification in skeletal muscle by repressing the transcription of the slow twitch myosin heavy chain (MHC) isoform (Myh7) in fast twitch muscle fibers (34–36). Myh7 transcription was decreased in differentiating Mkx−/− myotubes but not the fast twitch myosin heavy chain isoforms (Myh1 and Myh2) (Fig. 7A). The differential reduction of the slow isoform was confirmed by immunostaining with antibodies specific to slow and fast MHC isoforms (Fig. 7B). This is consistent with Mkx regulating the transcription of Myh7 through repression of Sox6 and predicts a role in fiber type specificity.
DISCUSSION

The genetic pathways controlling the patterning and differentiation of the vertebrate musculoskeletal system require the combined actions of multiple regulatory factors. The Mx6 homeobox gene has been shown to be an important regulator of skeletal muscle and tendon differentiation (5, 6). We have characterized the DNA recognition motif of the mouse Mx6 homeodomain and identified an Mx6-responsive element within the proximal promoter of Mx6. Using the identified Mx6 regulatory element as a model, functional MREs within the Sox6 locus were identified, suggesting that MKX can directly repress the expression of Sox6. Combined with the observation of up-regulation of Sox6 in Mx6−/− satellite cells, this defines a potential pathway by which Mx6 promotes the expression of slow MHC during muscle development.

The DNA recognition motif for members of the homeodomain-containing transcription factor family is largely dependent on a core set of amino acids in Helix III and residues at the N terminus of the homeodomain (5, 6). A comprehensive analysis performed in Drosophila organizes this superfamily of genes into 11 distinct specificity groups (11). Consistent with sequence conservation in the homeodomain, the Drosophila Mx6 ortholog (CG11617) falls into the Iroquois group that is characterized by a monomeric nnACA motif. Interestingly, CG11617 diverges from Iroquois genes such as mirt through the preference for T at position 1 (TnACA) (11). Our data demonstrate that mouse Mx6 has lost specificity for a T and therefore can bind half-sites such as that found in the vertebrate MBS (GAACAT and AAACAT). Mouse and Drosophila Mx6 orthologs may recognize different recog-
Mkx DNA-binding Properties

ation motifs as the result of specific amino acid changes at critical residues of the homeodomain. At position 8 of the N-terminal arm of the homeodomain, mouse Mkx contains an alanine (Ala) and the Drosophila ortholog contains a phenylalanine (Phe) (7). Large hydrophobic residues in position 8 have been shown to influence binding specificity at position 1 of the recognition motif (11). The Iroquois factors, which share an affinity for the same monomeric site as mouse Mkx, similarly contain an Ala at position 8 of the N-terminal arm. Replacement of the Ala with the large hydrophobic Phe residue in Drosophila caup partially added a preference for T at binding position 1 (11). Whereas the amino acids critical for DNA recognition are identical among vertebrate Mkx orthologs, position 8 in the N-terminal arm is different among cephalochordates, hemichordates, and echinoderms. This predicts that the alteration in position 8, coupled with the adaptation of dimerization in vertebrates, expands the functionality of Mkx by increasing the complexity of binding sites and thereby target genes. Future studies will be required to determine whether changes in the amino acid identity at position 8 have altered Mkx DNA recognition among distantly related metazoans.

Whereas the mouse MKX homeodomain shares a common recognition motif with Irx family members, we have demonstrated that they differ in their ability to recognize half-site conformations. Our data show that Mnx can recognize and bind both MBS- and IBS-like conformations, whereas an IRX2-VP16 fusion protein is only capable of regulating the IBS conformation. MBS and IBS conformations are different in both the number of nucleotides separating the half-sites and the orientation of the half-sites. Inter-site spacing has been shown to be a critical parameter for homeobox gene regulation. The Drosophila bicoid homeobox transcription factor can bind strongly to sites that are spaced 25 bp apart but only weakly to sites separated by 11 bp (38). This reveals an additional layer of Mkx regulation by which the level repression could be mediated by specific MBS conformations that differ in spacer length between each half-site.

The inverted repeat arrangement of the MBS and IBS sequences is consistent with Mkx-binding DNA as dimers. The Iroquois family has been shown previously to bind DNA in both homo- and heterodimer arrangements, and these interactions are dependent upon sequences N-terminal to the homeodomain (18). Similarly, MKX was capable of homodimerization, Iroquois family has been shown previously to bind DNA in both homo- and heterodimer arrangements, and these interactions are dependent upon sequences N-terminal to the homeodomain (18). Similarly, MKX was capable of homodimerization, although this interaction was dependent upon a unique sequence immediately C-terminal to the MKX homeodomain. Deletion of this conserved domain also diminished the ability of MKX to regulate gene expression through the MBS, suggesting that MKX binds DNA as a requisite homodimer. It is interesting to speculate on whether members of the Irx family and MKX form functional heterodimers because their expression patterns overlap in the somites, limb buds, and muscle during development. Future studies will be required to determine the extent to which this interaction occurs in vivo and whether these closely related family members share functional redundancy during development.

Our studies predict that Mkx is able to repress its own transcription through the MBS found in its promoter. Such negative autoregulatory loops have been found at the core of several developmental processes, including the anterior-posterior colinear expression of the Hox genes, the oscillatory genetic networks associated with circadian rhythms, and the segmental clock of somitogenesis, stem cell potency, and cell differentiation (39–41). Further, it has been proposed that negative autoregulation can suppress the transcriptional noise intrinsic in genetic networks that reduces both the switching on and switching off time of gene expression (42). The Mkx-responsive element is highly conserved among vertebrate Mkx orthologs and clustered with other conserved inverted repeat sequences. This suggests that negative autoregulation is a conserved feature among Mkx orthologs and may be modulated in a larger regulatory complex. The significance of the negative autoregulation will become clearer as the Mkx genetic network is better understood.

Our initial characterization of the MKX-binding properties led to the identification of two well conserved MBS within the Sox6 locus. Sox6 has been shown to be an important regulator during musculoskeletal development and fiber type specification (35–37, 43). Sox6 possesses a redundant function with Sox5 during the differentiation of chondrocytes (44) and is required to block cells of the sclerotome from entering the tendon lineage (45). In skeletal muscle, Sox6 suppresses slow twitch fiber development through the direct repression of Myh7 transcription (34, 35). In primary satellite cells isolated from Mkx<sup>−/−</sup> mice, we observed that endogenous Sox6 transcription was elevated upon differentiation, suggesting it is a direct target of Mkx-mediated repression. Consistent with this result, Myh7 gene expression was reduced in these same cells, whereas fast MHC isomorph expression levels were unchanged. This provides the first evidence for a role for Mkx in the regulation of fiber type specificity during skeletal muscle differentiation. Further, it provides a possible mechanism by which Mkx is able to participate in the integration of the differentiation of multiple cell types in the musculoskeletal system.

Acknowledgments—We thank Dr. Ron Allen for advice on culturing primary satellite cells, Dr. Anthony Firulli for providing the CS2MT plasmid, Dr. Scott Bingham for assistance in DNA sequencing, and Dr. James Elser for the generous use of the FLx800 microplate reader.

REFERENCES

1. Anderson, D. M., Arredondo, J., Hahn, K., Valente, G., Martin, J. F., Wilson-Rawls, J., and Rawls, A. (2006) Mohawk is a novel homeobox gene expressed in the developing mouse embryo. Dev. Dyn. 235, 792–801
2. Anderson, D. M., Beres, B. J., Wilson-Rawls, J., and Rawls, A. (2009) The homeobox gene Mohawk represses transcription by recruiting the sin3A/HDAC co-repressor complex. Dev. Dyn. 238, 572–580
3. Liu, H., Liu, W., Malby, K. M., Lan, Y., and Jiang, R. (2006) Identification and developmental expression analysis of a novel homeobox gene closely linked to the mouse twirler mutation. Gene Expr. Patterns 6, 632–636
4. Takeuchi, J. K., and Bruneau, B. G. (2007) Irx1, a divergent Iroquois homeobox family transcription factor gene. Gene Expr. Patterns 7, 51–56
5. Ito, Y., Toriuchi, N., Yoshitaka, T., Ueno-Kudoh, H., Sato, T., Yokoyama, S., Nishida, K., Akimoto, T., Takahashi, M., Miyaki, S., and Asahara, H. (2010) The Mohawk homeobox gene is a critical regulator of tendon differentiation. Proc. Natl. Acad. Sci. U.S.A. 107, 10538–10542
6. Liu, W., Watson, S. S., Lan, Y., Keene, D. R., Ovitt, C. E., Liu, H., Schweitzer, R., and Jiang, R. (2010) The atypical homeodomain transcription factor Mohawk controls tendon morphogenesis. Mol. Cell. Biol. 30,
Mxx DNA-binding Properties

26. Wilson-Rawls, J., Hurt, C. R., Parsons, S. M., and Rawls, A. (1999) Differential regulation of epaxial and hypaxial muscle development by paraxis. Development 126, 5217–5229

27. Crews, S. T., and Pearson, J. C. (2009) Transcriptional autoregulation in development. Curr. Biol. 19, R241–246

28. Irvine, K. D., Botas, J., Jha, S., Mann, R. S., and Hogness, D. S. (1993) Negative autoregulation by Ultrabithorax controls the level and pattern of its expression. Development 117, 387–399

29. Semsey, S., Krishna, S., Erdossy, I., Horváth, P., Orosz, L., Sneppen, K., and Adhya, S. (2009) Dominant negative autoregulation limits steady-state repression levels in gene networks. J. Bacteriol. 191, 4487–4491

30. Sadowski, I., Ma, J., Triezenberg, S., and Ptashne, M. (1988) GAL4-VP16 is an unusually potent transcriptional activator. Nature 335, 563–564

31. Chang, C. P., Broccieri, L., Shen, W. F., Largman, C., and Cleary, M. L. (1996) Pbx modulation of Hox homedomain amino-terminal arms establishes different DNA-binding specificities across the Hox locus. Mol. Cell. Biol. 16, 1734–1745

32. Neuteboom, S. T., Petelenburg, L. T., van Dijk, M. A., and Murre, C. (1995) The hexapeptide LFPWMR in Hoxb-8 is required for cooperative DNA binding with Pbx1 and Pbx2 proteins. Proc. Natl. Acad. Sci. U.S.A. 92, 9166–9170

33. Furukawa, K., Iioka, T., Morishita, M., Yamaguchi, A., Shindo, H., Namba, H., Yamashita, S., and Tsukazaki, T. (2002) Functional domains of paired-like homeoprotein Cart1 and the relationship between dimerization and transcription activity. Genes Cells 7, 1135–1147

34. Hagiwara, N., Ma, B., and Ly, A. (2005) Slow and fast fiber isoform gene expression is systematically altered in skeletal muscle of the Sox6 mutant, p100H. Dev. Dyn. 234, 301–311

35. Hagiwara, N., Yeh, M., and Liu, A. (2007) Sox6 is required for normal fiber type differentiation of fetal skeletal muscle in mice. Dev. Dyn. 236, 2062–2076

36. von Hofsten, J., Elworthy, S., Gilchrist, M. J., Smith, J. C., Wardle, F. C., and Ingham, P. W. (2008) Pdn1- and Sox6-mediated transcriptional repression specifies muscle fiber type in the zebrafish embryo. EMBO Rep. 9, 683–689

37. Qiuat, D., Voelker, K. A., Pei, J., Grishin, N. V., Grange, R. W., Bassel-Duby, R., and Olson, E. N. (2011) Concerted regulation of myofiber-specific gene expression and muscle performance by the transcriptional repressor Sox6. Proc. Natl. Acad. Sci. U.S.A. 108, 10196–10201

38. Fu, D., Zhao, C., and Ma, J. (2003) Enhancer sequences influence the role of the amino-terminal domain of bicoid in transcription. Mol. Cell. Biol. 23, 4439–4448

39. Bessho, Y., and Kageyama, R. (2003) Oscillations, clocks, and segmentation. Curr. Opin. Genet. Dev. 13, 379–384

40. Pan, G., Li, J., Zhou, Y., Zheng, H., and Pei, D. (2006) A negative feedback loop of transcription factors that controls stem cell pluripotency and self-renewal. FASEB J. 20, 1730–1732

41. Wong, W. F., Kurokawa, M., Satake, M., and Kohu, K. (2011) Down-regulation of Runx1 expression by TCR signal involves an autoregulatory mechanism and contributes to IL-2 production. J. Biol. Chem. 286, 11110–11118

42. Zabet, N. R. (2011) Negative feedback and physical limits of genes. J. Theor. Biol. 284, 82–91

43. Dumitriu, B., Dy, P., Smits, P., and Lefebvre, V. (2006) Generation of mice with conditional null allele. Nature 444, 219–224

44. Lefebvre, V., Behring, R. R., and de Crombrugghe, B. (2001) L-Sox5, Sox6, and Sox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis Cartilage 9, Suppl. A, S69–75

45. Brent, A. E., Braun, T., and Tabin, C. J. (2005) Genetic analysis of interactions between the somitic muscle, cartilage, and tendon cell lineages during mouse development. Development 132, 515–528