CTCF Promotes Muscle Differentiation by Modulating the Activity of Myogenic Regulatory Factors

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CTCF nuclear factor regulates many aspects of gene expression, largely as a transcriptional repressor or via insulator function. Its roles in cellular differentiation are not clear. Here we show an unexpected role for CTCF in myogenesis. CTCF, whose DNA-binding elements have been mapped throughout the human genome (2, 3), is probably best known for its function as a genomic insulator (1, 4). Among many ascribed roles, CTCF participates in gene imprinting, X chromosome inactivation (5–7), interchromosomal interactions (8), subnuclear spatial organization (9), demarcation of lamina-associated domains (10), long range chromatin looping (11), chromosome pairing and counting (12), and regulation of cohesins-mediated gene expression (13, 14). The functional versatility of CTCF might be due to different interactions with multiple co-regulators (15). CTCF is essential for early embryo development (7), but whether CTCF functions in cell differentiation programs or specific signaling pathways to control morphogenesis remains poorly understood.

Myogenesis is regulated by the MyoD family of transcription factors, including Myf5, MyoD, MRF4, and myogenin, which activate muscle-specific gene expression (16, 17). These proteins, also known as myogenic regulatory factors (MRFs), have different activities on muscle genes controlling myogenic lineage establishment and differentiation (18, 19). The complexity of MRF function during myogenesis is exemplified by MyoD-mediated up- and down-regulation of specific gene clusters during myogenic differentiation (20). Thus, MyoD exerts modulated gene-specific responses. The modulated regulatory influence of MyoD over its target promoters is largely dependent, as for many other transcription factors, on co-activator or co-repressor interactions (21, 22). For example, heterodimerization of MyoD with the ubiquitous basic helix-loop-helix E proteins E12 or E47 confers high DNA recognition affinity and enhanced transcriptional potential (23–26). On the other hand, the interaction of MyoD with basic helix-loop-helix proteins Id and Twist results in transcriptionally incompetent complexes (27, 28). Similarly, MyoD-mediated recruitment of p300/CBP and PCAF to muscle promoters is required for maximal transcriptional activation (29, 30), but the presence of MyoD and HDAC1 at the Myogenin promoter correlates with transcriptionally inactive chromatin (31). These examples highlight the potential existence of unknown muscle-specific as well as ubiquitous MyoD partners that form part of regulatory

† This work was supported, in whole or in part, by National Institutes of Health Grant HL54737 (to D. Y. S.). This work was also supported by Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México Grants I2X230104, IN219043, and IN214407; Consejo Nacional de Ciencia y Tecnología Grants 42653-Q and 58767; and Third World Academy of Sciences Grant 01-055 RG/BO/IA (to F. R.-T.); and the J. David Gladstone Institutes (to B. G. B.).

‡ The on-line version of this article (available at http://www.jbc.org) contains supplemental references, Table S1, and Figs. S1–S7.

§ Supported by the California Institute of Regenerative Medicine.

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5 The abbreviations used are: MRF, myogenic regulatory factor; qPCR, quantitative PCR; E, embryonic day; hpf, h post-fertilization; MO, morpholino oligonucleotide.
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modules controlling muscle gene expression and affecting myogenesis and muscle development.

MRFs integrate signaling from pathways coordinating myogenic induction and differentiation during development (19). The Wnt signaling pathway participates in the activation of myogenesis (32, 33) by regulating MRF function (34–37). In particular, Wnt11 controls the fiber patterning of the limb muscle (33) and determines the oriented elongation of myocytes (38). In addition, WNT11 activates the canonical WNT/β-catenin-dependent pathway (39), which is required for the expression of Myf5 (37) and for MyoD-mediated transactivation (40). Thus, proteins regulating the expression of Wnts are likely important for myogenesis and muscle development.

In this study we demonstrate that CTCF promotes myogenesis by functionally interacting with MRFs. Mechanistically, CTCF stimulates muscle gene expression by favoring MyoD recruitment. In line with a critical role of CTCF in myogenesis, ctf loss of function in zebrafish results in defective muscle development. Furthermore, CTCF regulates Wnt signaling, linking CTCF to broad aspects of development.

EXPERIMENTAL PROCEDURES

In Situ Hybridization—RNA in situ hybridization in mouse and zebrafish embryos was performed as described (41, 42). Mouse Ctf mRNA was detected with a human CTCF cDNA-derived probe, which has over 90% identity with its mouse counterpart (supplemental Fig. S1). The zebrafish ctf antisense probe was synthesized from a 1054-bp DNA fragment corresponding to the 5′ end of the mRNA excluding the zinc fingers region (GenBank™ accession number BC097009).

Constructs and Plasmids—pCI-7.1 plasmid containing the full-length human CTCF was provided by Elena Klenova (University of Essex, Colchester, UK). pcDNA3-N-Myc, encoding a N-Myc tag, pcMyoD-N-Myc, and pcMyogenin-N-Myc, encoding a N-Myc-tagged MyoD and N-Myc-tagged Myogenin, respectively, were provided by Robin Meech (The Scripps Research Institute, La Jolla, CA). Constructs 1 and α-SGCP (43, 44), which include the α-SG full-length and core promoter (+4 to −76 relative to the transcription start site), respectively, were obtained from Ramón Coral-Vázquez (Instituto Mexicano del Seguro Social, Mexico City, Mexico). V5-tagged CTCF vectors were generated by PCR from constructs provided by Jeannie T. Lee (Harvard Medical School) (6). Probes for myod and myogenin were a gift from Eric S. Weinberg (University of Pennsylvania).

Cell Transfection—C2C12 and 10T1/2 cells were grown in DMEM supplemented with 10% fetal bovine serum. The α-SG promoter (1 µg) constructs were co-transfected with cDNAs encoding CTCF, MyoD, or Myogenin with Lipofectamine 2000 (Invitrogen). pRL/CMV vector, encoding Renilla reniformis luciferase, was co-transfected for normalization. 500,000 cells were seeded in six-well plates and transfected 24 h later. Luciferase activity was measured 48 h post-transfection. Luciferase activity was measured using the dual luciferase reporter assay system (Promega) in a TD-20/20 luminometer (Turner Designs). C2C12 cells stably expressing luciferase under the α-SG full-length promoter were as described (44). For overexpression of CTCF, 420,000 C2C12 cells were seeded in 6-well plates, and 24 h later, a CTCF encoding vector (5 µg) was transfected with FuGENE HD (Roche Applied Science), according to the manufacturer’s instructions. The cells were harvested 1.5, 3, and 4 days post-transfection and used to isolate RNA and prepare cell lysates for RT-PCR and Western blot analyses, respectively.

RT-PCR and qPCR—RNA was isolated with Trizol reagent (Invitrogen), according to the manufacturer’s instructions. RNA (2 µg) was used to synthesize cDNA with a first strand synthesis kit (Invitrogen). One µl of the resulting reaction was used for PCR. Primer sequences are available upon request. qPCR values were normalized against β-actin, used as endogenous control.

siRNA and shRNA-mediated CTCF Knockdown—200,000 C2C12 cells stably expressing luciferase under the α-SG full-length promoter were seeded in six-well plates. 24 h later, the cells were transfected with 0, 1, 2, 5, and 10 pm of CTCF siRNA (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen). After 24 h, the transfection was repeated. Luciferase assays and Western blot were performed 72 h post-transfection. Luciferase activity was normalized against protein concentration. The anti-CTCF shRNA vector was constructed with oligonucleotides targeting the sequence 5′-GGTGAGGCCGGTGAAAGCCA-3′ corresponding to the exon 1-intron 1 boundary. C2C12 cells were stably transfected with a vector expressing the shRNA under control of the H1 promoter or a control vector. Stable transfectants were induced for differentiation and seeded on coverslips for immunofluorescence or lysed to obtain RNA.

Myogenic Conversion Assays—190,000 10T1/2 cells were seeded on coverslips in 12-well plates. After 24 h, 2 µg of either phCTCF, encoding CTCF cDNA, pcDNA3-N-Myc, encoding a N-Myc tag, pcMyoD-N-Myc, or pcMyogenin-N-Myc encoding a N-Myc-tagged MyoD and N-Myc-tagged Myogenin, respectively, were co-transfected in different combinations with FuGENE HD reagent (Roche Applied Science). Transfection efficiency was assessed by immunofluorescence against the Myc tag. Immunofluorescence against MHC was performed 7 days later. The percentage of positive cells, with respect to the total number of cells, was compared between conditions.

Immunofluorescence—Whole mount immunofluorescence in zebrafish was performed as described (42).

Co-immunoprecipitation—In vitro synthesized proteins were produced with the TNT quick coupled transcription/translation system (Promega). The synthesis products were mixed in the presence of immunoprecipitation buffer (20 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100). Immunoprecipitation of TNT-produced proteins was done with Exacta-Cruz reagents (Santa Cruz Biotechnology). Antibodies for CTCF and MyoD were from Abcam and Santa Cruz Biotechnologies, respectively.

Chromatin Immunoprecipitation—C2C12 cells were transfected in 15-cm tissue culture dishes with 70 µg of either an empty vector or MyoD- or Myogenin-encoding cDNAs with FuGENE HD as recommended by the manufacturer. The cells were harvested 48 h after transfection and processed with the EZ-ChIP kit (Upstate). The analysis of immunoprecipitated chromatin was performed by qPCR using TaqMan (Applied
Biosystems) probes recognizing the \( \alpha \)-SG and serum response factor core promoters. Antibodies for CTCF and MyoD were from Abcam and Santa Cruz Biotechnologies, respectively. The anti-polymerase II antibody was from Upstate.

Morpholino Oligonucleotides Injection and Rescue Experiments—Morpholinos targeting the ATG (5’-CATTGAA-GGGGACCCTGAGCCG-3’) or the exon 1-intron 1 junction (5’-GGTTTCAGGTGTGATCTGTGTGT-3’) of the zebrafish \( ctf \) gene were purchased from Gene Tools. Human \( Ctf \) and zebrafish \( wnt11 \) mRNA was obtained using the MessageAmp II kit (Ambion). Zebrafish embryos at one cell stage were co-injected with 2 ng of \( ctf \) ATG morpholino and 50 pg of \( CTF \) mRNA. 4 ng of \( ctf \) ATG morpholino were co-injected with 100 ng of \( wnt11 \) mRNA.

Microarray Data Analysis—c2 morphants were pooled from five independent injections and used for RNA purification. RNA was hybridized on Affymetrix GeneChip® zebrafish genome arrays following the manufacturer’s recommendations. Linear models were fitted for each gene on morphant control embryos to derive morphant effect using the limma package in R/Bioconductor. Moderated t-statistics and the associated \( p \) values were calculated, adjusting for multiple testing by controlling for false discovery rate with the Benjamini-Hochberg method and for family-wise error rate using the Bonferroni correction (adj\( P \)). Genes with adj\( P < 0.05 \) were considered as differentially expressed.

RESULTS

CTCF Is Expressed in Somites in Developing Mouse and Zebrafish—Although CTCF is often considered as a widely expressed factor (45, 46), it is down-regulated in differentiating cells in vitro (47), raising questions about the regulation of its expression during specific biological processes. Because the expression pattern of \( Ctf \) in mammalian embryos is unknown, we observed its mRNA expression in the developing mouse by whole mount in situ hybridization. \( Ctf \) mRNA was widely detected at embryonic days (E) 10.5 and E12, with predominant expression in the forming jaw at E10.5 (Fig. 1A). \( Ctf \) expression in somites was observed at E10.5 and E12 (Fig. 1A). At E12, \( Ctf \) was detected in limbs, jaw, brain, and facial muscles (Fig. 1A). Expression of \( Ctf \) in the limbs is consistent with recent findings of a function of CTCF in mouse limb development (48). Later (E13), \( Ctf \) presented a more restricted expression pattern; it was predominantly detected in brain and facial muscles, whereas its presence was barely observed in intercostal musculature. \( Ctf \) decreased notably in E13 embryo forelimbs as compared with E12 embryos (Fig. 1A).

We extended our analysis to zebrafish embryos, in which \( ctf \) was strongly expressed and widely distributed during early development (data not shown) in agreement with a previous report (49). We focused our attention on \( ctf \) expression in somites, where myogenic precursors are determined. We used an anti-myod probe to identify somites in zebrafish (Fig. 1B). \( ctf \) mRNA was detected in somites beginning at 10–11 to 16–17 h post-fertilization (hpf) (Fig. 1, B and C), stages at which \( myod \) is strongly expressed. However, the presence of \( ctf \) was markedly reduced at 26 hpf (Fig. 1B), and by 36 hpf it was more diffused along the trunk and barely detected at the most distal somites (Fig. 1B). In 72-hpf embryos, \( ctf \) mRNA was absent from somites, whereas it was clearly detected in the gut, forebrain, midbrain, and hindbrain. As expected, at this stage MyoD was only detected in the jaw and eye muscles (50) (Fig. 1B). Sense probes for \( Ctf \) mRNA in mouse and zebrafish gave no detectable staining (supplemental Fig. S2 and data not shown).

To compare the expression patterns of \( Ctf \) and MRFs during myogenic cell differentiation, we used reverse transcription followed by qPCR in differentiating C2C12 cells. \( Ctf \) had a similar up-regulation pattern to that of MyoD, MRF4, and Myogenin (Fig. 1D). These results show that \( Ctf \) is expressed in developing muscle in mouse and zebrafish and suggest that CTCF expression is developmentally regulated.

CTCF Overexpression Increases C2C12 Cell Myogenic Differentiation—The observation that \( Ctf \) and the myogenic master regulator MyoD share expression domains during mouse and zebrafish development opened the possibility of CTCF involvement in myogenic differentiation. In a first attempt to address this issue, we overexpressed \( CTF \) in C2C12 cells and analyzed the enrichment of myogenic differentiation marker genes. RT-PCR showed enrichment of \( Myf5 \), MyoD, and Myogenin and premature expression of \( Myf4 \) and \( MHC \) (Fig. 2A) at day 1.5 post-transfection. Perinatal myosin heavy chain was not detected. \( \alpha \)-SG (\( \alpha \)-sarcoglycan, also known as \( Sgca \)), which is expressed in terminally differentiated muscle (51), and \( Myf5 \) were the only enriched markers at day 3 of differentiation (Fig. 2A). qPCR confirmed up-regulation of MyoD and Myogenin in CTCF-overexpressing cells at days 1.5 and 3 of differentiation (Fig. 2B). Accordingly, myogenic marker proteins were enriched in \( CTF \) transfected cells at day 1.5 post-transfection, and precocious \( \alpha \)-SG expression was detected at day 3 (Fig. 2A). Up-regulation of \( Myf5 \), whose expression normally decreases as differentiation proceeds (52), suggests that CTCF overexpression led to deregulation of the myogenic gene expression program. However, increased expression of terminal differentiation markers, such as MHC and \( \alpha \)-SG, suggests that CTCF overexpression led to increased myogenic differentiation. This raised the possibility of a functional interaction between CTCF and MRFs.

CTCF Enhances the Myogenic Potential of MRFs—Because CTCF overexpression affects muscle-specific gene expression (Fig. 2, A and B), which is orchestrated by MRFs, we determined whether CTCF affects the myogenic potential of MyoD and Myogenin. To this end, we performed myogenic conversion assays in 10T1/2 fibroblasts. Vectors encoding MyoD or myogenin fused to a Myc epitope tag were transiently transfected in absence or presence of a CTCF-encoding cDNA. Plasmid amounts were equaled for all conditions with empty vector. Myogenic converted cells were identified by the presence of MHC, which was revealed by immunofluorescence. The cell nuclei were stained with DAPI and counted, and the number of MHC-positive cells for each condition were compared (Fig. 2C). When \( CTF \) was co-transfected with an empty vector, no MHC-positive cells were observed (Fig. 2C), indicating that CTCF does not induce myogenic differentiation. Similarly, CTCF did not increase the myogenic potential of myogenin (supplemental Fig. S3). In contrast, \( CTF \) increased the myo-
genic potential of MyoD, because co-transfection of CTCF and MyoD resulted in more MHC-positive cells than did co-transfection of MyoD with an empty vector (Fig. 2). CTCF also increased the myogenic efficiency of MyoD and Myogenin together, although to a lesser extent than MyoD alone (supplemental Fig. S3). Thus, CTCF likely cooperates with MyoD in myogenic induction.

Ctcf Is Indispensable for Myogenic Cell Differentiation—To address the requirement of CTCF in myogenic differentiation, we stably knocked down Ctcf in C2C12 cells. We transfected a vector encoding an siRNA directed against Ctcf exon 1. Ctcf was effectively knocked down, whereas the levels of serum response factor were not altered, as shown by Western blot and qRT-PCR (Fig. 2D). The cells were induced to differentiation by culturing under serum starvation, and their differentiation capacity was compared with that of cells transfected with a control siRNA by means of MHC immunofluorescence and qPCR. Progressive MHC abundance and formation of multinucleated myofibers was observed in control cells during a 5-day differentiation time course (Fig. 2E). In contrast, Ctcf-deficient cells presented limited differentiation potential as shown by drastically decreased MHC and fewer myogenic fibers. Quantification of the MHC signal confirmed reduced MHC levels in siRNA-transfected cells (Fig. 2E). Furthermore, expression of MyoD and Myogenin decreased notably in CTCF-deficient cells during myogenic differentiation (Fig. 2D). The expression of MyoD and Myogenin was less affected by CTCF knockdown at day 5 of differentiation (Fig. 2D). A potential explanation for
this effect might be that factors stimulating expression of MyoD and Myogenin and not targeted by CTCF are up-regulated in differentiated myogenic cells. However, this possibility was not tested. Thus, Ctcf is indispensable for muscle-specific gene expression and myogenic cell differentiation.

CTCF Physically Interacts with MyoD—Our results suggest that CTCF stimulates myogenic cell differentiation potentially by cooperating with MRFs. To test this hypothesis, we assessed the capacity of CTCF to physically interact with MyoD. We used co-immunoprecipitations with in vitro synthesized Myc-tagged MyoD and V5-tagged full-length CTCF. CTCF was detected in Myc-MyoD immunoprecipitates by Western blot (Fig. 3 A, lane 2), indicating direct interaction between CTCF and MyoD. In a deletion series of Myc-tagged proteins, only the CTCF domain including the zinc fingers was co-immunoprecipitated with MyoD (Fig. 3A, lane 4), indicating that this domain mediates CTCF interaction with MyoD. In addition, endogenous CTCF and MyoD co-immunoprecipitated in C2C12 cells (Fig. 3B). Thus, CTCF might form part of a novel transcriptional regulatory module affecting muscle-specific gene expression.

Functional Interplay of CTCF and MRFs in Regulating Muscle Gene Expression—Because CTCF establishes physical and functional interactions with MRFs, we investigated whether CTCF cooperates in direct muscle-specific gene expression activation. First, we tested the activity of CTCF on the α-SG promoter in C2C12 myoblasts. We transiently transfected CTCF cDNA into C2C12 cells expressing luciferase under the full-length α-SG promoter (44), which contains multiple E-boxes and is activated by MyoD during myogenic differenti-
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In 10T1/2 cells in which we can control for the presence of specific MRFs. For these experiments, we used the α-SG core promoter, which lacks E-boxes and is targeted by MyoD in differentiated myogenic cells via interaction with basal transcription factors (44). We used this promoter to eliminate activities mediated by multiple E-boxes present in the full-length promoter (44). Similar to the α-SG full-length promoter (Fig. 4B), CTCF weakly enhanced the activity of MyoD on both the α-SG core promoter and the myogenin promoter in 10T1/2 cells (Fig. 4D, lanes 3 and 5), suggesting that additional myogenic factors might be required for transactivation. In support of this notion, CTCF enhanced the activity of MyoD and myogenin on both promoters (Fig. 4D, lanes 7 and 8). Thus, CTCF positively regulates muscle-specific gene expression through a MRF-dependent mechanism, perhaps as a modulator of MRF activity.

CTCF trans-activated muscle gene expression only in the presence of MyoD, raising the possibility that MyoD recruits CTCF to muscle genes. However, this possibility seemed unlikely because CTCF did not interact with a DNA probe containing two functional E-boxes (55) in the presence of MyoD, as revealed by EMSA (supplemental Fig. S6). Another possibility is that CTCF facilitates MRF recruitment to muscle promoters. To resolve this question, we examined the requirement of CTCF for MyoD recruitment to a muscle gene in the chromatin context (Fig. 4E). We performed chromatin immunoprecipitations on 10T1/2 cells transfected with CTCF alone or co-transfected with MyoD. We analyzed enrichment of the α-SG core promoter in chromatin immunoprecipitated with antibodies against MyoD, CTCF, or acetylated histone H3 (acH3) by qRT-PCR. CTCF was enriched in the α-SG promoter in the absence of MyoD (Fig. 4E, lane 2). However, this interaction is unlikely to be functional, because CTCF cannot stimulate α-SG promoter activity or promote myogenesis alone. In the absence of CTCF, MyoD interacted weakly with the α-SG core promoter in MyoD-transfected cells (Fig. 4E, lane 4), even though acH3 was enriched (Fig. 4E, lane 6), suggesting that the promoter was being remodeled for full gene expression activation. This finding agrees with robust interaction of MyoD with the α-SG core promoter only in C2C12 myotubes, as opposed to myoblasts, where the promoter is fully active (44). In contrast, in the presence of CTCF, the binding of MyoD to the α-SG core promoter increased, together with enrichment of CTCF and high acH3 (Fig. 4E, lanes 7–9), implying that CTCF promotes recruitment of MyoD to the α-SG core promoter. In agreement with this hypothesis, whereas MyoD was enriched on the α-SG core promoter in the presence of full-length CTCF (Fig. 4F, lane 3), MyoD did not bind the α-SG core promoter in the presence of a mutant CTCF lacking the zinc finger domain (Fig. 4F, lane 5), which mediates interaction with MyoD (Fig. 3A). Then we asked whether the endogenous CTCF is required for recruitment of MyoD to the α-SG promoter in the context of myogenic cells. We depleted CTCF in C2C12 cells by siRNA transfection and addressed the enrichment of MyoD and CTCF on the α-SG core promoter. qPCR on immunoprecipitated chromatin showed decreased MyoD and CTCF on the α-SG core promoter in cells transfected with CTCF siRNA, as compared with cells transfected with a control siRNA (Fig. 4G). Levels of

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**FIGURE 3.** CTCF physically interacts with MyoD. A, extracts with in vitro synthesized V5-tagged full-length CTCF (FL), the CTCF amino-terminal (N-ter), zinc finger-containing (ZF), or the carboxyl-terminal (C-ter) domains were mixed with Myc-MyoD for co-immunoprecipitations (IP). The tagged CTCF versions were identified by Western blot (WB) using an anti-V5 antibody. Inputs for MyoD and the CTCF proteins are shown in the middle and bottom panels, respectively. The numbers indicate kDa. In lane 1, Myc-MyoD was added as negative control. B, endogenous CTCF and MyoD co-immunoprecipitate in C2C12 cell extracts. CTCF and MyoD were revealed in immunoprecipitates obtained with anti-MyoD and anti-CTCF antibodies (α-Myc and α-CTCF, respectively) by Western blot (WB). The bottom panels show inputs. An anti-Myc (α-myc) antibody was used as a negative control.

CTCF trans-activated the α-SG promoter in a concentration-dependent manner (Fig. 4A, top panel). To test whether endogenous CTCF regulates the α-SG promoter, we depleted CTCF with anti-Ctcf siRNA in C2C12 cells. CTCF was efficiently depleted, as shown by Western blot (Fig. 4A, bottom panel). CTCF depletion caused decreased α-SG promoter activity (Fig. 4A, middle panel). A control siRNA did not affect promoter activity. Therefore, CTCF activates the α-SG promoter in the myogenic environment in the presence of MRFs. Then we addressed whether CTCF interacts with the α-SG promoter. We performed EMSA with in vitro synthesized CTCF and a DNA probe corresponding to the α-SG core promoter, in which a CTCF-binding site was predicted (supplemental Fig. S4). Results from EMSAs indicate that CTCF is capable of binding the core promoter of the muscle-specific gene α-SG (supplemental Fig. S5).

Because CTCF increased the myogenic potential of MyoD, we tested the requirement of MRFs for CTCF-mediated muscle gene activation. We analyzed the activity of CTCF over the full-length α-SG promoter in 10T1/2 fibroblasts, which do not express MRFs. MyoD (44), but not CTCF, trans-activated the α-SG promoter (Fig. 4B). However, CTCF and MyoD induced a modest but statistically significant increase in promoter activity (Fig. 4B), suggesting that CTCF requires MyoD to stimulate muscle gene expression. In agreement with this notion, two E-boxes known to mediate MyoD responsiveness (54) were necessary for CTCF-mediated trans-activation of the Myogenin promoter in C2C12 cells (Fig. 4C). To further understand the functional interaction between CTCF and MRFs, we assessed the combinatorial activity of MyoD, myogenin, and CTCF on the α-SG and Myogenin promoters, both MyoD targets (44, 54),
polymerase II on the serum response factor were comparable between conditions (Fig. 4G).

These results suggest that CTCF is necessary for recruitment of MyoD to at least some of its target promoters and for muscle-specific gene expression activation. In addition, CTCF seems to regulate myogenic induction and differentiation by co-activating muscle-specific gene expression through interaction with MRFs, predicting a function for CTCF in muscle development.

**ctcf Participates in Zebrafish Myogenesis**—Next, we tested the relevance of CTCF in myogenesis in vivo. ctcf was knocked down in zebrafish by injection of morpholino oligonucleotides (MOs) complementary to the ctcf mRNA translation start site (ATG) or the boundary between exon and intron 1 (SP) in one-cell stage embryos. Depletion of ctcf in embryos injected with either MO was shown by Western blot (Fig. 5A). Injection of either MO led to consistent phenotypes in zebrafish embryos (Fig. 5 and data not shown), suggesting that the observed phenotypes are due specifically to ctcf knockdown. This was further confirmed by rescue with ctcf mRNA injection (Fig. 5, F–H). Injection of a scrambled morpholino at the same concen-
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![Figure 5](link_to_figure_5)

**FIGURE 5.** Ctcf knockdown affects myogenic development in zebrafish embryos. A, Western blot analyses show the absence of Ctcf in zebrafish embryos injected with morpholino oligonucleotides complementary to ctcf translation start codon (ATG) or the exon 1/intron 1 boundary (SP). B, lateral views of zebrafish embryos injected with morpholino oligonucleotides complementary to the ctcf translation start codon (ATG). The embryos were grouped in class 1 or class 2. c1 presented mild somite disorganization at 24 and 48 hpf as compared with noninjected control embryos (ct). Severe somite disorganization was observed at 24 hpf in c2 embryos. The scale bars represent 200 μm. C, confocal micrographs showing MHC and actin signals in somites from c1 and c2 morphants at 24 and 48 hpf. Scale bars, 50 μm. D, transmission electron microscopy images show decreased myofibers (arrowheads) in c2 morphants (ATG), as compared with control (ct) embryos. E, in situ hybridization shows decreased myogenin in c1 and c2 morphants at 24 hpf. F, injection of CTCF mRNA partially rescued the abnormal phenotype of ctcf morphants. The percentage of embryos with normal somite shape and absence of curved tail obtained after injection of ATG alone or with CTCF mRNA is shown. G, lateral views of 32 hpf embryos injected with a control morpholino (CM), ATG morpholino (ATG) alone, or co-injected with CTCF mRNA (ATG + CTCF mRNA). H, in situ hybridization against myogenin on 48-hpf embryos injected with a control morpholino (CM), ATG morpholino alone (ATG), or ATG co-injected with CTCF mRNA (ATG + CTCF mRNA). The left and right panels show lateral and dorsal views, respectively. Superior oblique, lateral rectus, levator arcus palatini, transversus ventralis, lower jaw, pectoral fin, and hypaxial musculature are indicated.

trations as ATG and SP MO yielded no obvious phenotype (supplemental Fig. S7). Ctf morphants obtained by injection of MO-ATG were grouped into class 1 (c1) and class 2 (c2), characterized by somite disorganization with different levels of severity (Fig. 5, B and C). c1 embryos presented a mild phenotype including loss of the characteristic chevron-like form of the somites (Fig. 5, B and C) and a curved tail, evident at 24 and 48 hpf, respectively (Fig. 5B). Injection of a control morpholino at the same concentration did not cause obvious morphological abnormalities (Fig. 5G). c1 and c2 morphants had abnormal somite morphology, as shown by actin staining and a graded loss of slow MHC correlated with the phenotype severity of c1 and c2 morphants at 24 hpf (Fig. 5C). Furthermore, electron microscopy showed reduced muscle fibers in c2 morphants (Fig. 5D).

We examined the expression of MRFs in CTCF morphants. Myogenin expression decreased in c2 morphants at 24 hpf (Fig. 5E). Decreased myod and myogenin mRNA levels were also detected by qPCR in ctf morphants (Fig. 6B). In 48-hpf c1 morphants, myogenin was absent in specific muscles, including the superior oblique, lateral rectus, levator arcus palatini, transversus ventralis, lower jaw, pectoral fin, and the hypaxial musculature. Myogenin expression was lower also in pectoral fin muscles, but its expression in dorsal anterior myotomes was unaffected (Fig. 5E). Thus, Ctf functions in muscle differentiation in the zebrafish embryo.

Next, we determined whether counteracting ctcf knockdown rescues the abnormal phenotypes in ctf morphants. In vitro transcribed human CTCF mRNA was co-injected with MO-ATG (Fig. 5, F–H). Rescued embryos were considered as those co-injected embryos with normal somite shape as well as absence of curved tail (Fig. 5G). Only 2% of embryos injected with 1 ng of ATG-MO presented normal somite morphology. In contrast, injection of 2 ng of ATG-MO plus 25 pg of CTCF mRNA rendered 41% of embryos with normal somite morphology (Fig. 5F). Moreover, in situ hybridization revealed re-established myogenin expression in rescued embryos (Fig. 5H). Because a human CTCF mRNA rescued the ctcf deficiency in zebrafish, CTCF might have conserved functions in zebrafish and human, consistent with the high conservation of both orthologs (49). The fact that co-injection of CTCF mRNA mediated partial rescue of ATG-morphants suggests an essential function of ctcf in early zebrafish development, consistent with its abundant expression in early embryos (49). Thus, ctcf is indispensable for zebrafish muscle development.
Identification of Potential Ctf Target Genes—Early lethality from CTCF deficiency in the mouse has limited the study of the CTCF functions in vivo after preimplantation (55). To gain a broader view of the part of CTCF in developmental processes, we used microarrays to examine the global gene expression profile of 24-hpf Ctcf c2 morphants, which had the most severe phenotype. Of 187 genes that were differently expressed (Fig. 6A and supplemental Table S1), 100 were up-regulated, and 87 were down-regulated, indicating that ctcf-mediated transcriptional activation and repression function in zebrafish development. Expression of several genes involved in hematopoiesis decreased in ctcf morphants (Fig. 6A and C), as well as a putative Wnt receptor (pink) are shown. Color intensity reflects fold change in mRNA abundance as indicated in the reference bar. B, qPCR revealed down-regulation of myod and myogenin, but not mrf4, and up-regulation of myf5 in embryos injected with an ATG morpholino (ATG), as compared with control morpholino-injected (CM) embryos. C, number and percentage of genes enriched in the indicated gene ontologies. D, lateral views of whole embryos injected with CM and ATG alone or with ATG plus wnt11 mRNA. Injection of an ATG MO plus wnt11 mRNA re-established somite morphology and myogenin expression, as shown by in situ hybridization on 48-hpf embryos. E, the percentages of c1 and c2 embryos obtained after injection of a CM, an ATG, or the ATG morpholino plus wnt11 mRNA are shown.

**FIGURE 6. CTCF acts upstream of wnt11 during zebrafish muscle development.** A, heat map of the gene expression profiles of Ctcf morphants at 24 hpf. The numbers of down-regulated (green) and up-regulated (red) genes are shown on the left. Misregulated genes involved in hematopoiesis (black), muscle development and contraction (blue), as well as a putative Wnt receptor (pink) are shown. Color intensity reflects fold change in mRNA abundance as indicated in the reference bar. B, qPCR revealed down-regulation of myod and myogenin, but not mrf4, and up-regulation of myf5 in embryos injected with an ATG morpholino (ATG), as compared with control morpholino-injected (CM) embryos. C, number and percentage of genes enriched in the indicated gene ontologies. D, lateral views of whole embryos injected with CM and ATG alone or with ATG plus wnt11 mRNA. Injection of an ATG MO plus wnt11 mRNA re-established somite morphology and myogenin expression, as shown by in situ hybridization on 48-hpf embryos. E, the percentages of c1 and c2 embryos obtained after injection of a CM, an ATG, or the ATG morpholino plus wnt11 mRNA are shown.

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CTCF Regulates Myogenic Differentiation

**FIGURE 6. CTCF acts upstream of wnt11 during zebrafish muscle development.** A, heat map of the gene expression profiles of Ctcf morphants at 24 hpf. The numbers of down-regulated (green) and up-regulated (red) genes are shown on the left. Misregulated genes involved in hematopoiesis (black), muscle development and contraction (blue), as well as a putative Wnt receptor (pink) are shown. Color intensity reflects fold change in mRNA abundance as indicated in the reference bar. B, qPCR revealed down-regulation of myod and myogenin, but not mrf4, and up-regulation of myf5 in embryos injected with an ATG morpholino (ATG), as compared with control morpholino-injected (CM) embryos. C, number and percentage of genes enriched in the indicated gene ontologies. D, lateral views of whole embryos injected with CM and ATG alone or with ATG plus wnt11 mRNA. Injection of an ATG MO plus wnt11 mRNA re-established somite morphology and myogenin expression, as shown by in situ hybridization on 48-hpf embryos. E, the percentages of c1 and c2 embryos obtained after injection of a CM, an ATG, or the ATG morpholino plus wnt11 mRNA are shown.

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Ctf Acts Upstream of wnt11—Wnt11 participates in broad aspects of development and regulates myogenic differentiation (32–40). wnt11 was down-regulated in ctcf morphants, raising the possibility that besides acting as MRF co-activator in myogenic differentiation, CTCF might act upstream Wnt11 in the
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regulation of some aspects of muscle development. We approached this issue by addressing whether exogenously provided wnt11 mRNA rescues at least some of the phenotypes caused by ctcf deficiency in developing zebrafish. We co-injected embryos with ATG-MO and wnt11 mRNA and scored for c1 and c2 morphants (with mild and severe phenotype, respectively) at 24 hpf. The percentage of c1 morphants obtained was less after injecting ATG-MO plus wnt11 mRNA (0%) than after injecting a ctcf ATG-MO alone (64%) (Fig. 6E; p < 0.001), although the percentage of c2 morphants decreased from 36 to 17% (Fig. 6E; p < 0.02). Accordingly, co-injection of an ATG-MO and wnt11 mRNA led to increased numbers of embryos with normal somite morphology and myogenin expression (Fig. 6, D and E). Injection of 100 or 200 pg of wnt11 mRNA did not cause any visible abnormality when injected alone in zebrafish embryos (supplemental Fig. S7). However, injection of 300 pg did cause abnormal morphology, consistent with a previous report (57). Exogenously supplied wnt11 partially counteracted Ctcf deficiency; thus, Ctcf likely acts upstream of wnt11 to regulate some aspects of muscle development and potentially other developmental processes.

DISCUSSION

CTCF regulates broad aspects of transcription (1) as a gene expression activator and repressor (15) and mediates local and long range chromatin organization (8, 9), and its activity as an epigenetic modifier has deep implications in cancer (58). However, its role in developmental processes has only recently begun to be investigated (48) and remains poorly understood. In this work we show evidence of the involvement of CTCF in myogenic differentiation and development.

The physical and functional interactions between CTCF and MyoD implicate a mechanism in which CTCF co-activates muscle-specific gene expression. In in vitro experiments, CTCF could not activate the α-SG full-length and proximal promoter without MRFs. Myogenin alone did not activate the α-SG proximal promoter even with MyoD, but in the presence of MyoD and CTCF, myogenin contributed to promoter transactivation. This synergistic interaction also occurred on the Myogenin promoter. These results, in conjunction with the facts that CTCF promoted the interaction of MyoD with the α-SG core promoter and that it enhanced the myogenic conversion capacity of MyoD with myogenin but not myogenin alone, suggest that CTCF could act as mediator necessary for transactivation of common target genes (Fig. 7). MyoD and myogenin regulate an overlapping set of promoters during myogenic differentiation (59). MyoD induces the expression of early differentiation genes, and myogenin, which cannot activate such genes on its own, stimulates MyoD-activated genes later in differentiation (59). Thus, in at least a subset of promoters, myogenin activity depends on MyoD. Therefore, such dependence could lay in intermediary factors such as CTCF. Under this scenario, CTCF would be indispensable for the contribution of myogenin to muscle-specific gene expression in promoters previously contacted by MyoD (Fig. 7). We propose that CTCF functions in myogenic differentiation, at least in part, by stimulating MRFs and muscle structural gene expression by mediating functional interactions between MRFs (Fig. 7) and facilitating MRFs interaction with muscle-specific promoters. Although addressing the activity of CTCF in the presence of MRFs needs to be extended to other muscle-specific gene promoters, this hypothesis could explain the myogenic impairment caused by ctcf knockdown in zebrafish embryos, in which the expression of the myogenic regulator myogenin and the structural component slow-mhc was affected, highlighting the relevance of CTCF nuclear factor in muscle development.

CTCF is essential for development (7) and likely controls the expression of key genes regulating several developmental programs, including myogenesis. In this regard, our finding that Ctcf acts upstream of wnt11 provides a potential explanation for the essentiality of CTCF in wide aspects of development. For instance, wnt11 plays an important role in driving convergent extension movements during zebrafish gastrulation (60). The early requirement of wnt11 raises the possibility that the muscular deficiency in Ctcf morphants is secondary to early morphogenesis defects and not only due to disruption of the muscle-specific transcriptional circuitry. However, Wnt11 is involved in myogenesis in the chicken embryo (33, 38), and although Wnt11 deficiency in zebrafish causes only a mild defect in somite morphology (56), it is an important component of a planar cell polarity pathway that does play a critical role in somite morphology and muscle differentiation (38). Therefore, the partial rescue of Ctcf morphants by exogenously provided wnt11 suggests that Wnt11 is relevant downstream of Ctcf but is only a part of the potentially important targets of CTCF in the regulation of myogenesis and development. Although we did not rule out the nonmyogenic component of the phenotype caused by Ctcf deficiency, our results in in vitro models point to a specific function of CTCF as a direct co-regulator of MRFs in myogenic differentiation, and accordingly, the results in zebrafish support a function of CTCF in muscle development. With this in mind, and considering that Wnts are necessary for MRF function and myogenesis in birds and mammals (34–38), we propose that CTCF might function upstream and down-

![FIGURE 7. Model of CTCF involvement in myogenic differentiation.](image-url)
stream of a Wnt signaling pathway controlling at least some aspects of muscle development. Upstream, CTCF stimulates the expression of Wnt11 and thus MRF expression and activity, and downstream, CTCF modulates MRF functional interactions stimulating muscle-specific gene expression (Fig. 7). Our results reveal CTCF as a novel factor involved in myogenic regulation by modulating functional interactions between MRFs and link CTCF to broad aspects of development via Wnt signaling.

Acknowledgments—We thank Georgina Guerrero Avendaño for technical assistance, Inti A. De La Rosa-Velázquez for frequent discussions, J. A. Garcia-Sainz and the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México for support, Ru-Fang Yeh for microarray analysis, Jinny Wong from the Gladstone Electron Microscopy Core Facility, and Gary Howard for editorial assistance.

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