# Thyroid hormone regulates muscle fiber type conversion via miR-133a1

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It is known that thyroid hormone (TH) is a major determinant of muscle fiber composition, but the molecular mechanism by which it does so remains unclear. Here, we demonstrated that *miR-133a1* is a direct target gene of TH in muscle. Intriguingly, miR-133a, which is enriched in fast-twitch muscle, regulates slow-to-fast muscle fiber type conversion by targeting TEA domain family member 1 (TEAD1), a key regulator of slow muscle gene expression. Inhibition of miR-133a in vivo abrogated TH action on muscle fiber type conversion. Moreover, TEAD1 overexpression antagonized the effect of miR-133a as well as TH on muscle fiber type switch. Additionally, we demonstrate that TH negatively regulates the transcription of *myosin heavy chain I* indirectly via miR-133a/ TEAD1. Collectively, we propose that TH inhibits the slow muscle phenotype through a novel epigenetic mechanism involving repression of TEAD1 expression via targeting by miR-133a1. This identification of a TH-regulated microRNA therefore sheds new light on how TH achieves its diverse biological activities.

## Introduction

Skeletal muscles are composed of a mixture of different muscle fiber types with different contractile and metabolic properties that are optimally suited for their tasks. The performance and function of skeletal muscle are mainly dependent on the intrinsic contractile properties of myocytes. Myosin heavy chain (MyHC) is the major contractile protein of skeletal muscle cells and the primary determinant of the efficiency of muscle contraction (Baldwin and Haddad, 2001). The major isoforms of MyHC are MyHC I, IIa, IIx/d, and IIb in mammals, ranked in order of increasing catalytic turnover rate. Slow-twitch (type I) fibers express MyHC I, whereas fast-twitch (type II) fibers express MyHC IIa, IIx/d, and IIb. To meet various physiological demands, the expression of myosin genes is modulated by a complex regulatory network (Bassel-Duby and Olson, 2006).

The type of an adult skeletal muscle fiber is determined to a great extent by the type of innervation and use, and the level of several hormonal factors, particularly that of thyroid hormone (TH; Pette and Staron, 1997; McLennan, 1994). In general, the development and maintenance of the slow phenotype is dependent on innervation by a slow motor neuron (Pette and Staron, 1997; Pette and Staron, 2001). Development of fast characteristics depends on TH rather than innervation (Pette and Staron, 1997; Pette and Staron, 2001; Salvatore et al., 2014). TH induces a consecutive shift from MyHC I to IIa, to IIx/d, and to IIb (Izumo et al., 1986; Mahdavi et al., 1987). In hyperthyroidism, most type I fibers are converted to a mixed oxidative/ glycolytic type IIa or IIx/d phenotype (Müntener et al., 1987; van der Linden et al., 1992). In contrast, neonatal hypothyroidism results in a delay in fast MyHC isoforms expression (Butler-Browne et al., 1984; Mahdavi et al., 1987).

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The molecular mechanisms underlying the regulation of muscle fiber type and MyHC isoforms by TH remain unclear. TH is one of the most potent regulators of many muscle genes, whose promoters might contain TH response elements (TREs; Izumo and Mahdavi, 1988; Salvatore et al., 2014). All members of the *MyHC* multigene family respond to TH (Gambke et al., 1983; Diffee et al., 1991; Swoap et al., 1994; Schiaffino et al., 1998) in a muscle- or muscle fiber–specific manner (Izumo et al., 1986; d'Albis et al., 1990; Caiozzo and Haddad, 1996; Li et al., 1996; Yu et al., 1998). However, only a few of TREs have been identified and confirmed in all TH-regulated genes, indicating that some of the regulations by TH are suspected to be indirect.

It is widely accepted that the action of TH is mainly mediated through TH receptors (TRs), which regulate transcription in a ligand-dependent manner (Yen, 2001). There are two major TR isoforms, TRα1 and TRβ1, both of which are expressed in skeletal muscle. The muscle-specific difference in response to hypothyroidism in rodent fast- and slow-twitch muscles (Caiozzo and Haddad, 1996) was mimicked in *TRα1⁻/⁻TRβ⁻/⁻* mice, which suggests that the effects of TH on MyHC isoforms expression are mediated by TRs. The study using TR isoform–specific knockout mouse models indicated that both receptors mediate the action of TH (Yu et al., 2000). However, some other studies suggested that TRα1 is the major player that mediates the effect of TH on gene expression in slow-twitch muscle fibers (Yu et al., 2000; Johansson et al., 2003; Miyabara et al., 2005). In addition to different TR isoforms, TH action could be modulated by multiple nuclear cofactors or microRNA (miRNA) through epigenetic mechanisms. The fiber-specific variation of these mediators might contribute to the muscle- and muscle fiber–specific regulation by TH. miR-208 probably was the first miRNA identified and characterized in response to TH signaling (van Rooij et al., 2007). However, how miRNAs mediate TH action has not been extensively explored.

Here, by using hypo- and hyperthyroid mouse models and TR isoform–specific knockout mice, as well as an in vitro approach, we discovered that TH directly regulates *miR-133a1* transcription in a TR-dependent manner. Interestingly, we discovered that miR-133a is enriched in fast-twitch muscle fibers and has the ability to promote slow-to-fast muscle switch. Importantly, inhibition of miR-133a is able to attenuate the effect of TH on muscle fiber determination both in vitro and in vivo. Mechanically, we demonstrated that miR-133a regulates slow-to-fast muscle fiber type conversion by targeting TEA domain family member 1 (TEAD1), a key modulator of slow muscle gene (Tsika et al., 2008). Thus, we proposed that TH promotes slow-to-fast muscle fiber conversion by repressing TEAD1 via targeting miR-133a1.

## Result

### TH regulates miR-133a expression in skeletal muscle

Because it has been reported that slow fibers exhibit a greater sensitivity to TH than fast fibers (Simonides and van Hardeveld, 2008), to identify miRNAs regulated by TH, we checked the expression of muscle-specific miRNAs (MyomiRs) in the soleus (SOL) muscles under different TH status. As shown by either qRT-PCR or Northern blot analysis, the levels of miR-133a were increased by 3,3′,5-triiodo-L-thyronine (T3) injection in methimazole (MMI)-treated hypothyroid mice, indicating that miR-133a expression is positively regulated by TH (Figs. 1 A and S1 A). This effect could be detected as early as 2 h after T3 injection (Fig. 1 B). Importantly, this response was diminished in SOL muscle of mice deficient in both *TRα1* and *TRβ*, which suggests that the regulation of miR-133a by TH is mediated by TRs (Fig. 1 C). Note that we did not observe any significant change in miR-1 levels after T3 treatment (Figs. 1 A and S1 A).

To see whether the regulation of miR-133a by TR has isoform specificity, we first checked the expression pattern of different TR isoforms in the liver, slow-twitch SOL, and fast-twitch gastrocnemius (GAS) muscle of mice. In agreement with the current concept (Yu et al., 2000), TRβ1 is the major isoform in liver (Fig. 1 D). In contrast, we found that TRα1 expression was higher than that of TRβ1 in both SOL and GAS muscles, which suggests that TRα1 is the major isoform in muscle (Fig. 1 D). In addition, both TRα1 and TRβ1 exhibited higher expression in slow-twitch SOL muscle compared with that in fast-twitch GAS muscle (Fig. 1 D), which suggests that the higher expression of TRs in SOL muscle might contribute to its greater sensitivity to TH. Consistent with the finding that TRα1 was the major TR isoform in muscle, miR-133a expression was lower in SOL muscle of mice deficient in *TRα1* (Fig. 1 E). In contrast, loss of *TRβ* did not cause a detectable change in miR-133a levels in SOL muscles (Fig. 1 F). The decrease of miR-133a expression was more evident in SOL muscle of mice lacking both *TRα1* and *TRβ* (Fig. 1 G).

Consistent with the in vivo data, T3 treatment increased the expression of miR-133a but not miR-1 in primary myotubes (Fig. S1 B). Interestingly, the induction of miR-133a expression in C2C12 myotubes by T3 was time dependent and could be detected as early as 2 h after T3 addition, which suggests that the regulation might be direct (Fig. 1 H). The effect of T3 on miR-133a expression also could be observed in cultured C2C12 myoblasts (Fig. 1 I) as well as in C2C12 cells during differentiation (Fig. 1 J). We also confirmed the regulation of miR-133a expression by T3 in isolated SOL muscles (Fig. 1 K).

To rule out the possibility that T3 controls *miR-133a* transcription indirectly, we pretreated C2C12 myoblasts with the translational inhibitor cycloheximide (CHX) to see whether new protein synthesis is required for the up-regulation of miR-133a by T3. As shown in Fig. 1 L, CHX treatment did not affect the levels of miR-133a in the presence of T3, which indicates that T3 regulates *miR-133a* transcription directly.

### miR-133a1 is a direct target gene of TH

It has been reported that *miR-1-1/133a2* and *miR-1-2/133a1* are transcribed as bicistronic transcripts on different chromosomes (Fig. 2 A; Liu et al., 2007). To further study the regulation of miR-133a by TH, *miR-1-2/133a1* and *miR-1-1/133a2* enhancers were cloned. We found that *miR-1-2/133a1* enhancer but not *miR-1-1/133a2* enhancer was positively regulated by TRα1 in a ligand-dependent manner in 293T cells (Fig. 2, B and C). The
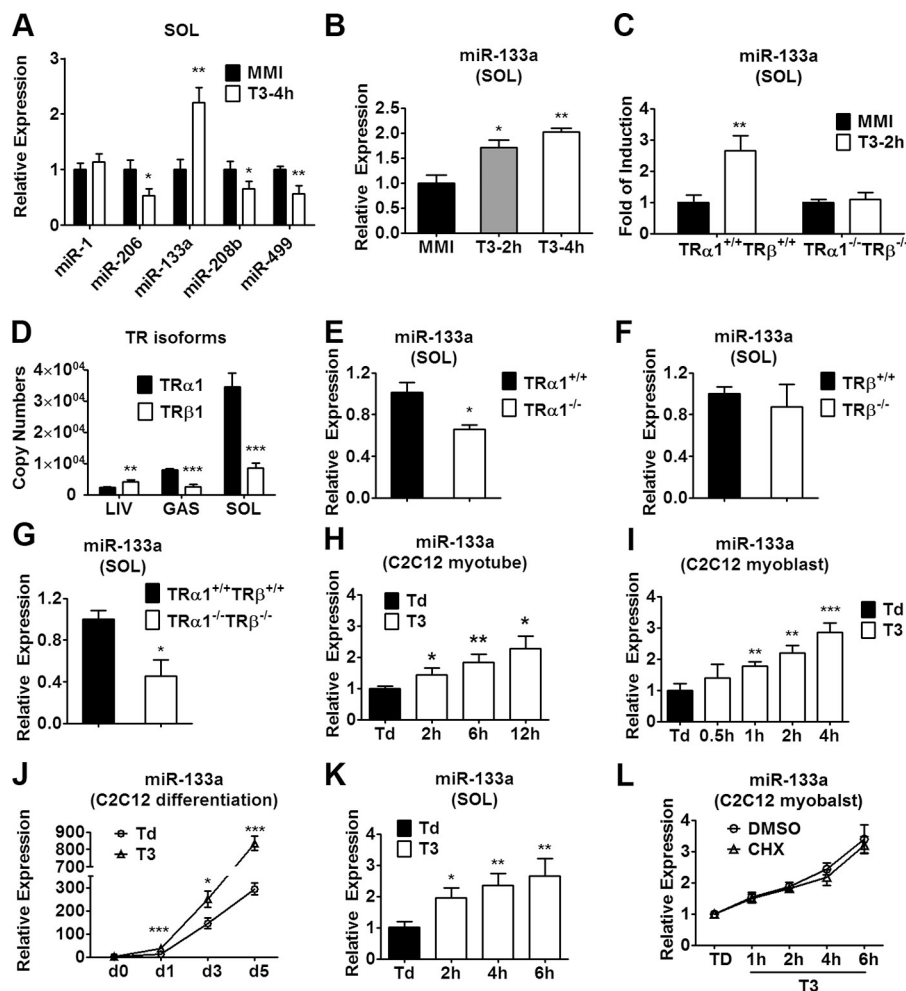
Figure 1. **TH regulates miR-133a expression in skeletal muscle.** (A) qRT-PCR analysis of miR-1, miR-206, miR-133α, miR-208b, and miR-499 in SOL muscle of MMI-treated mice injected with T3 for 4 h ($n = 3$). (B) qRT-PCR analysis of the time-course expression of miR-133α in SOL muscle of MMI-treated mice after T3 injection. Expression levels for miR-133α were normalized to U6 small nucleolar RNA (snRNA) expression ($n = 3$). (C) MMI-treated $TR\alpha 1^{+/+}TR\beta^{+/+}$ mice and $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice were injected with T3 for 2 h. Expression levels of miR-133α in SOL muscle were determined by qRT-PCR ($n = 4$). (D) Quantification of TRα1 and TRβ1 copy number in liver (LIV), GAS, and SOL by absolute quantification RT-PCR using a standard curve ($n = 3$). (E–G) qRT-PCR analysis of miR-133α in SOL muscle of $TR\alpha 1^{-/-}$ mice (E), $TR\beta^{-/-}$ mice (F), and $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice (G; $n = 4$). (H–J) qRT-PCR analysis of miR-133a in C2C12 cells under different culture conditions: C2C12 myotubes induced by Td differentiation medium for 3 d after T3 treatment (H), C2C12 myoblasts cultured in Td growth medium for 24 h before T3 treatment (I), and C2C12 myogenesis induced by Td differentiation medium or Td differentiation medium with T3 added (J). (K) SOL muscles isolated from mice were cultured in Td growth medium for 4 h before T3 treatment. The time-course expression of miR-133α in SOL muscles was analyzed with qRT-PCR ($n = 3$). (L) CHX pretreatment did not affect the regulation of miR-133α by T3 in C2C12 myoblasts. Means ± SD (error bars) are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

same results were obtained in C2C12 myoblasts expressing endogenous TR (Fig. 2, D and E). These data clearly indicated that TH could activate *miR-133a1* transcription through the upstream enhancer. Subsequently, we found three putative TREs (TRE1, TRE2, and TRE3) in this *miR-1-2/133a1* enhancer (Fig. 2 F). To determine which putative TRE is regulated by TH, reporters containing truncated *miR-1-2/133a1* enhancer-F1, -F2, and -F3 were constructed (Fig. 2 F). As shown in Fig. 2 (G–I), only the reporter containing enhancer-F2 could be activated by TRα1 in the presence of T3, which suggests that a TRE is located in the F2 region. To further confirm this finding, we performed a luciferase assay using a reporter with a deletion of TRE2 from enhancer-F2 (enhancer-F4) and a reporter with a mutation in TRE2 (enhancer-F2-mutation; Fig. 2 F). As expected, the reporter with either a deletion or a mutation in TRE2 did not respond to TRα1 in the presence of T3 (Fig. 2, J and K).

We also performed a chromatin immunoprecipitation (ChIP) assay to see whether TR would be recruited to *miR-1-2/133a1* enhancer (Fig. 2, L–O). As shown in Fig. 2 (L and N), we detected the recruitment of exogenous TRα1 to the *miR-1-2/133a1* enhancer in a 293T cell. More importantly, we observed the recruitment of endogenous TR to the enhancer in both the presence and absence of T3 in C2C12 myotubes (Fig. 2, M and O). Moreover, the recruitment of steroid receptor coactivator (SRC-1), a coactivator for TR (Cheng et al., 2010), was

increased by T3 treatment, which suggests that the enhancer would be activated in the presence of T3 (Fig. 2, M and O).

We also determined the effect of T3 on the levels of primary transcripts encoding miR-1-2, miR-133a1, miR-1-1, or miR-133a2 (pri-miR-1-2, pri-miR-133a1, pri-miR-1-1, or pri-miR-133a2). Consistent with the data from enhancer analysis, we only observed the effect of T3 on the levels of pri-miR-133a1 and pri-miR-1-2 but not on the levels of pri-miR-133a2 and pri-miR-1-1 in SOL muscle as well as in C2C12 myoblasts, indicating that the transcription of *miR-1-2/133a1* but not *miR-1-1/miR-133a2* is controlled by TH (Fig. S2, A and B). These data also suggested that the increase of pri-miR-133a1 levels might contribute to the elevated levels of matured miR-133a in SOL muscle of mice treated with T3 (Fig. S2 vs. Figs. 1 A and S1 A). In contrast, the elevated levels of pri-miR-1-2 did not result in an increase of matured miR-1 in SOL muscle of mice receiving T3 injection (Fig. S2 vs. Figs. 1 A and S1 A), which indicates that posttranscriptional regulation might be involved to affect the maturation process or stability of miR-1-2.

### miR-133a is enriched in fast-twitch muscle

Because TH has the capacity to alter myofiber type composition, we hypothesized that TH-regulated miR-133a might be able to affect muscle fiber specification. To test our hypothesis, we first checked whether miR-133a is differentially expressed
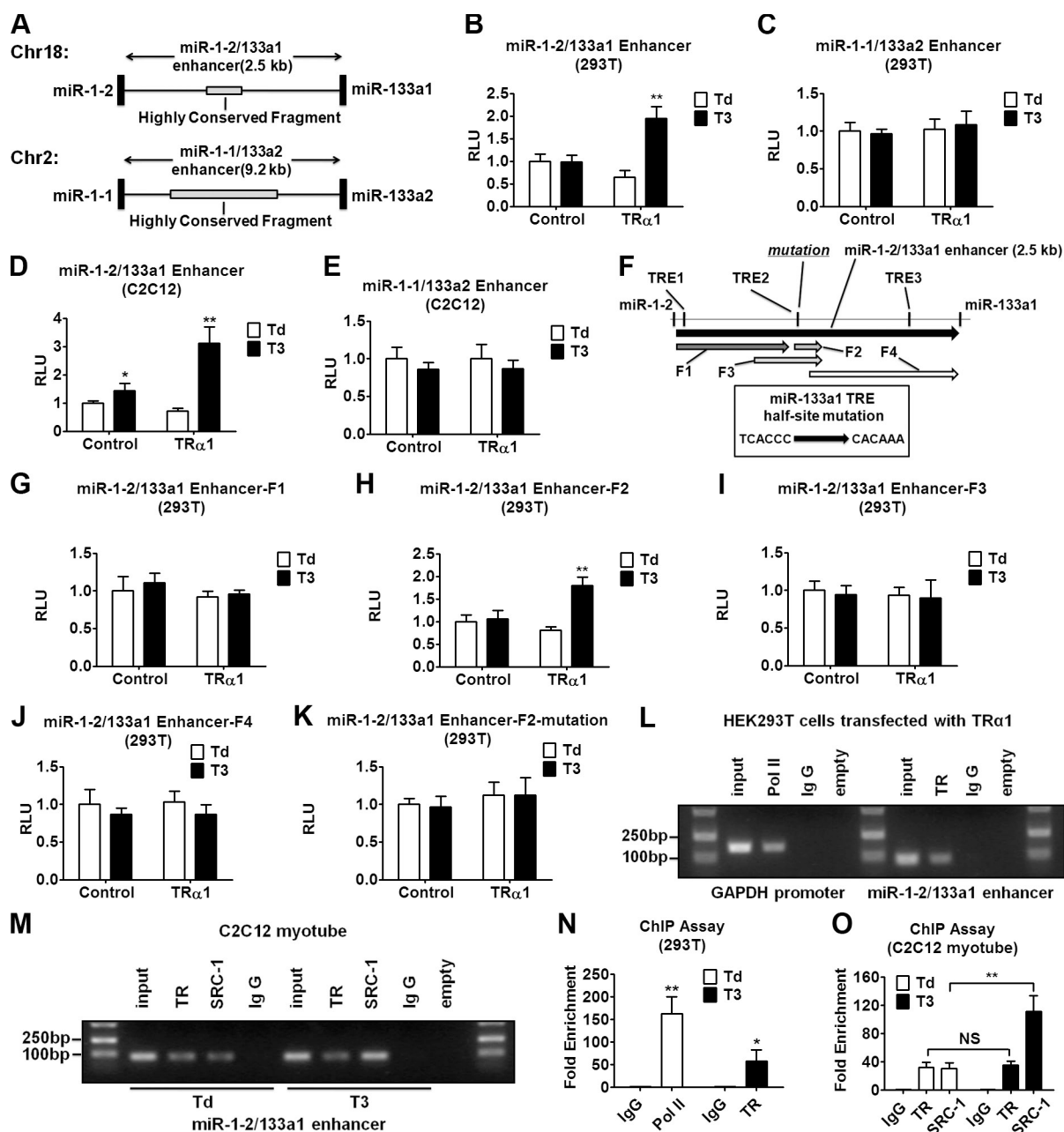
Figure 2. **miR-133a1 is a direct target gene of TH.** (A) The genomic location of mouse *miR-1* and *miR-133a*. Evolutionarily conserved fragments are shown. (B–E) The activities of *miR-1-2/miR-133a1* enhancer (B and D) or *miR-1-1/miR-133a2* enhancer (C and E) in HEK293T cells or C2C12 myoblasts were measured with a luciferase assay. Cells were transfected with TRα1 and/or treated with T3 as indicated. (F) Schematic representation of the *miR-1-2/miR-133a1* enhancer and truncated reporters. The sequence of TRE mutation is shown. (G–K) The activities of reporters containing truncated *miR-1-2/miR-133a1* enhancer-F1 (G), -F2 (H), -F3 (I), -F4 (J), and -F2 with TRE mutation (K) were measured with a luciferase assay in HEK293T cells. (L–O) A ChIP assay was performed using chromatin from TRα1-transfected HEK293T cells (L) or C2C12 myotubes (M). Anti-RNA Polymerase II (Pol II), normal mouse IgG, anti-TR (C4), and anti-SRC-1 antibodies were used for immunoprecipitation. Purified DNA was then analyzed by PCR using control primers for human GAPDH promoter or the TRE region in *miR-1-2/miR-133a1* enhancer. Water was used as a negative control for PCR (empty). Purified DNA was also analyzed by qPCR, and fold enrichment is expressed as the ratio of positive signal to IgG signal calculated by extrapolation from a standard curve of input DNA dilutions (N and O). Means ± SD (error bars) are shown. *, P < 0.05; **, P < 0.01.

in different muscle fibers by performing qRT-PCR. Consistent with previous reports, we found that miR-499 and miR-208b were enriched in SOL muscles (van Rooij et al., 2009) and miR-206 expression was significantly higher in SOL muscles (Williams et al., 2009; Liu et al., 2012), whereas miR-486 showed much lower expression in SOL muscles (Fig. S3 A). Interestingly, we observed that the expression of miR-133a and

miR-1 was higher in fast-twitch GAS muscles compared with slow-twitch SOL muscles (Fig. S3 A). The differential expression pattern of miR-1, miR-133a, and miR-206 was also confirmed by Northern blot analysis (Fig. S3 B).

We also compared the miR-133a levels in medial GAS (GAS-M), SOL, and tibialis anterior (TA) muscles from either mice or rats. As shown by metachromatic ATPase staining

(Fig. 3, A and B), GAS-M, SOL, and TA muscles of C57BL/6 mice were composed of 7%, 42%, and 1% type I fibers, and 93%, 58%, and 99% type II fibers, respectively (Fig. 3, C and E). In Sprague-Dawley rats, GAS-M, SOL, and TA muscles contained 28%, 88%, and 3% type I fibers, and 72%, 12%, and 97% type II fibers, respectively (Fig. 3, D and F). The expression of miR-133a was lower in slow-twitch SOL muscle and relatively higher in fast-twitch GAS-M and TA muscles in both mice and rats (Fig. 3, G and H). Moreover, consistent with the fact that the SOL muscles in rats contain more type I fibers than those in mice, the miR-133a level was lower in rats than in mice (Fig. S3 C). These results strongly indicated that miR-133a is enriched in fast-twitch muscle.

### miR-133a controls muscle fiber composition

Based on our findings, we speculated that miR-133a might be involved in the maintenance of muscle phenotype. To test the miR-133a action on muscle fiber type specification, we evaluated the expression of transcripts encoding individual MyHC isoforms (type I, MyHC-I; type II, MyHC-IIa, MyHC-IIx/d, and MyHC-IIb) as well as myoglobin and Tnni1 (markers of type I fibers) in SOL muscles overexpressing miR-133a. Overexpression of miR-133a either by miR-133a expression plasmid or synthetic miR-133a mimics in SOL muscles led to decreases in type I MyHC as well as myoglobin and Tnni1, and increases in type II MyHCs (Fig. 3, I and J). Consistently, inhibition of miR-133a either by miR-133a sponges or synthetic miR-133a inhibitor led to an increase in type I MyHC as well as myoglobin and Tnni1, and decreases in type II MyHCs in SOL muscles (Fig. 3, K and L). The effect of miR-133a on myofiber phenotype was also investigated in C2C12 myotubes. miR-133a mimic transfection reduced the expression of type I MyHC, myoglobin, and Tnni1, and increased the expression of type II MyHCs (Fig. 3 M). In contrast, miR-133a inhibitor transfection enhanced the expression of type I MyHC and myoglobin and suppressed the expression of type II MyHCs (Fig. 3 N). These data collectively suggested that miR-133a regulates the expression of MyHC isoforms as well as other myofiber markers.

To see whether the effect of miR-133a on the mRNA expression of these muscle fiber markers would affect the muscle fiber specification, we performed immunofluorescent staining and ATPase staining. Because it has been reported that miR-133 plays a regulatory role in C2C12 cells differentiation (Chen et al., 2006), differentiated C2C12 myotubes were used for transfection to rule out these effects. In addition, the cell numbers and differentiation status were visualized by DAPI staining and MHC staining. Overexpression of miR-133a in C2C12 myotubes increased the percentage of myosin-fast–positive myotubes and decreased the percentage of myosin-slow–positive myotubes (Fig. 3, O–Q; and Table S1). In contrast, miR-133a inhibitors decreased the percentage of myosin-fast–positive myotubes and increased the percentage of myosin-slow–positive myotubes (Fig. 3, O–Q; and Table S1). Note that overexpressing miR-133a in C2C12 myotubes did not change the amount of MHC-positive cells, which indicates that miR-133a did not affect differentiation under these experimental conditions (Table S1). Importantly, overexpression of miR-133a was able to reduce

the percentage of type I fibers in rat SOL muscles (Fig. 3, R–T). Collectively, these in vivo and in vitro experiments provided solid evidence that miR-133a is able to regulate the specification of muscle fiber identity.

### miR-133a is required for the TH action on myofiber type conversion

Because our data suggested that miR-133a is able to promote slow-to-fast muscle fiber type conversion, we hypothesized that miR-133a mediates the TH action in myofiber specification. To test whether miR-133a is essential for the regulatory role of TH on myofiber type conversion, we evaluated the TH effect after knockdown of miR-133a by miR-133a antagomir (ant-133a) in vivo. As shown in Fig. S4, ant-133a significantly reduced the miR-133a level in SOL muscle of mice. Interestingly, under our experimental condition, ant-133a neutralized the T3 effect on miR-133a levels in SOL muscle of hypothyroid mice. In agreement with our hypothesis, ant-133a injection abolished the T3 effect on SOL myofiber composition in hypothyroid mice (Fig. 4, A and B). Consistently, ant-133a diminished the T3 effect on the mRNA expression of type I MyHC and type II MyHCs in SOL muscle of hypothyroid mice (Fig. S4). This in vivo result strongly indicated that the effect of T3 on miR-133a is required for the TH action on myofiber type conversion. Similar results were obtained in C2C12 myotubes. As shown in Fig. 4 (C–F), in the absence of T3, miR-133a mimics promoted the fast phenotype and suppressed the slow phenotype in C2C12 myotubes. In the presence of T3, C2C12 myotubes underwent a slow-to-fast transition. miR-133a inhibitors antagonized the effect of T3 on the percentage of myosin-fast–positive and myosin-slow–positive myotubes. These in vitro results further supported the notion that miR-133a is essential for the regulatory role of TH on muscle fiber type composition.

### TEAD1 is a direct target gene of miR-133a

To identify which target gene might be responsible for miR-133a–mediated muscle fiber type specification, we used TargetScan to predict the miRNA targets, and found TEAD1 (Fig. 5 A). Subsequently, we found that miR-133a mimics significantly reduced the protein levels of TEAD1 in both C2C12 and L6 myoblasts at different time points, as indicated (Fig. 5 B). The repressive effect of miR-133a on TEAD1 protein levels was also observed in C2C12 and L6 myotubes (Fig. 5, C and D). In contrast, miR-133a inhibitor was able to elevate the protein levels of TEAD1 in C2C12 and L6 myotubes (Fig. 5, C and D). To further confirm that TEAD1 is a direct target of miR-133a, the 3′ UTR of TEAD1 containing the miRNA response element (MRE)-1/2 (Fig. 5 A) for miR-133a was cloned and constructed into a reporter. A luciferase assay demonstrated that miR-133a mimics were able to repress the 3′ UTR of TEAD1 in a dose-dependent manner (Fig. 5 E). The inhibitory effect of miR-133a was reduced when one of the two MREs was mutated (Fig. 5, F and G). The reporter with two mutations in both MREs did not respond to miR-133a (Fig. 5, F and G). These results clearly indicated that TEAD1 is a direct target of miR-133a in muscle cells.

The TEAD family consists of four members including TEAD1, TEAD2, TEAD3, and TEAD4. We found that the
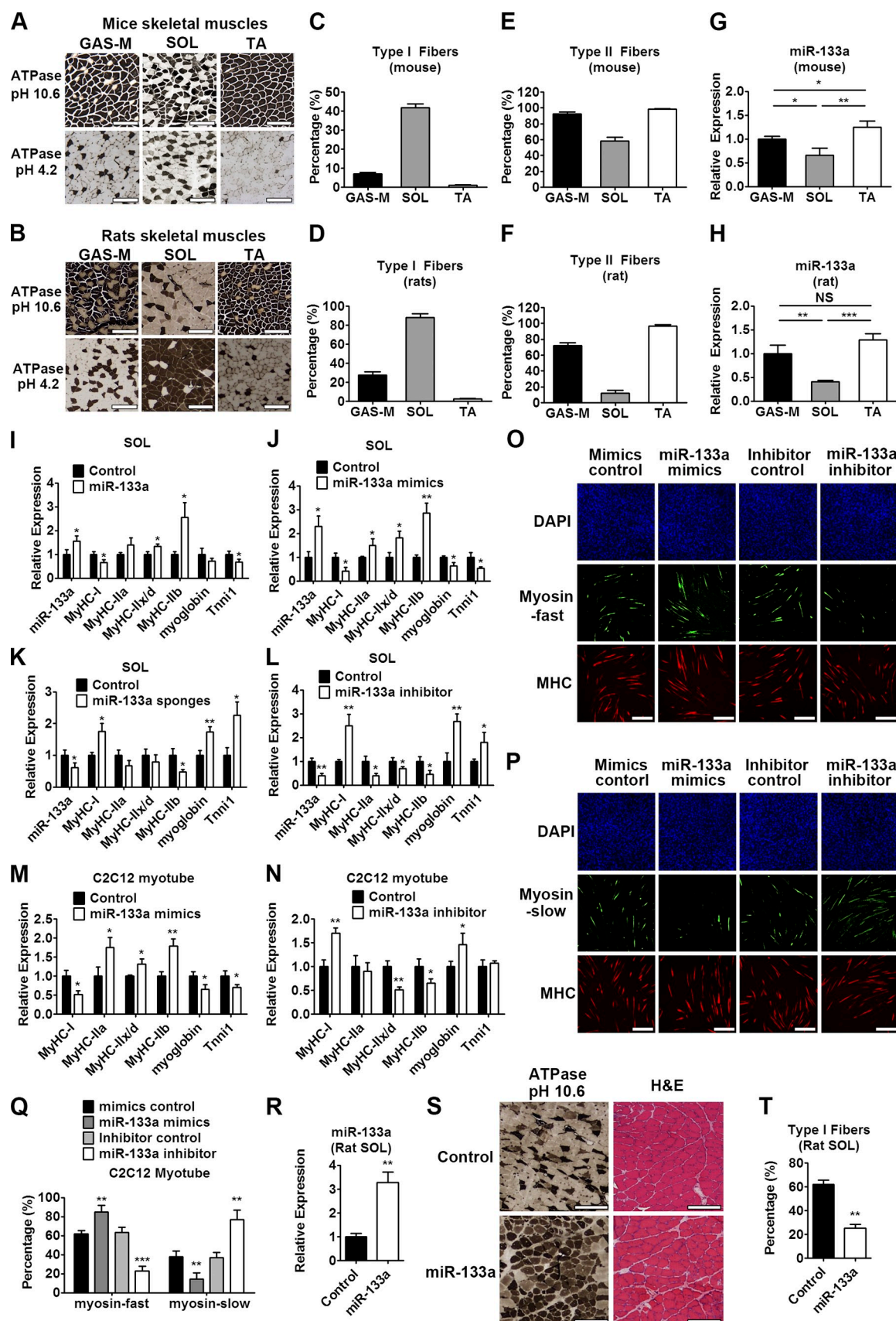
Figure 3.   **miR-133a is abundant in fast twitch muscle and controls the muscle fiber type phenotype.** (A and B) Metachromatic ATPase staining of GAS-M, SOL, and TA muscle from adult mice (A) and rats (B). (C–F) Percentage of type I (C and D) and type II (E and F) fibers in various muscles of mice (C and E) and rats (D and F) according to the ATPase staining are shown. (G and H) Expression of miR-133a in mouse (G) or rat (H) GAS-M, SOL, and TA muscle
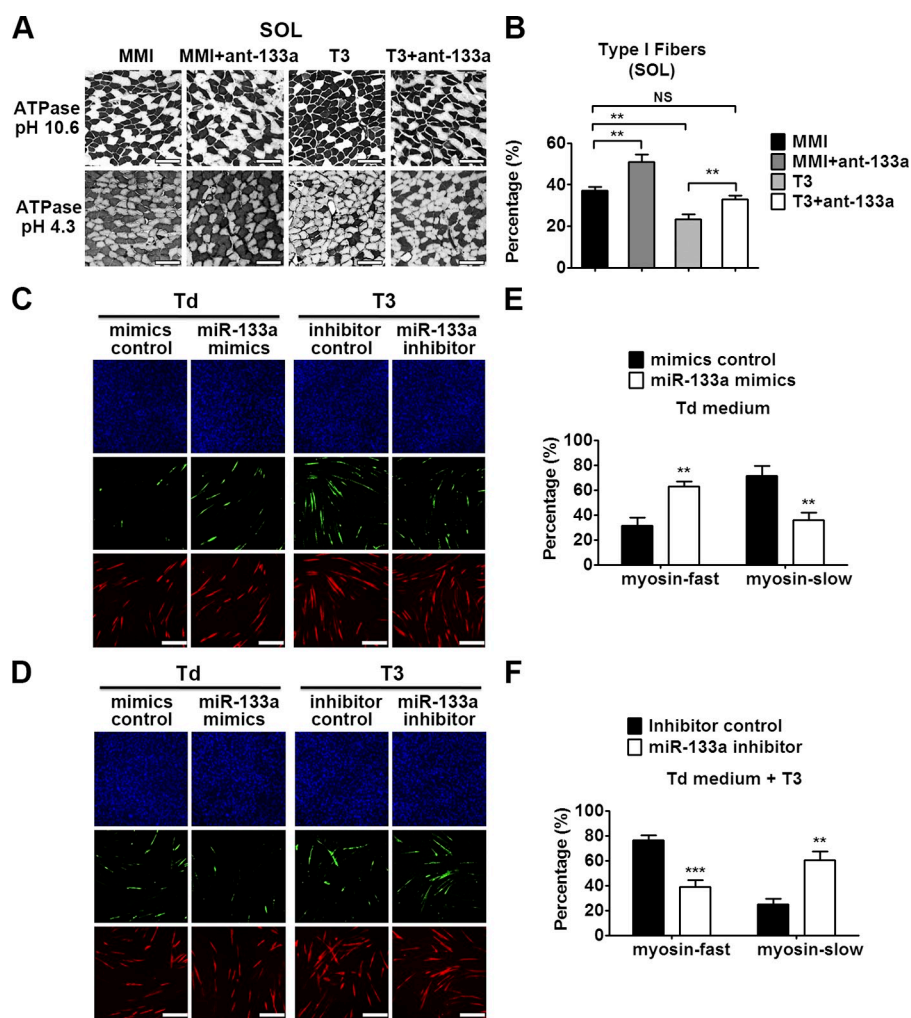
**Figure 4. miR-133a is required for the TH action on myofiber type conversion.** (A) ATPase staining of mouse SOL muscle from hypothyroid mice (MMI group), hypothyroid mice injected with ant-133a (MMI+ant-133a group), T3-treated hypothyroid mice (T3 group), and T3-treated hypothyroid mice injected with ant-133a (T3+ant-133a group). The treatment of T3 and/or ant-133a lasted for 14 d. (B) The percentage of type I fibers was quantified in SOL muscle of these mice. (C and D) C2C12 myotubes were transfected with miR-133a mimics, mimics control, miR-133a inhibitor, or inhibitor control in the absence or presence of T3. Immunostaining of C2C12 myotubes was performed using antibody against MHC and myosin-fast (C) or myosin-slow (D) 48 h after transfection. Representative results were shown. (E and F) Quantitative values were determined in four random fields for each group. Means ± SD (error bars) are shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Bars: (A) 150 µm; (C and D) 200 µm.

mRNA level of TEAD1 is the most abundant in both GAS and SOL muscles, which suggests that TEAD1 might be the major family member in muscles (Fig. S5 A). We also tested whether miR-133a has an effect on other TEADs. As shown in Fig. S5 B, miR-133a mimic transfection did not affect the 3′ UTRs of TEAD2, TEAD3, and TEAD4. Additionally, the protein levels of TEAD3 and TEAD4 did not respond to miR-133a mimic or inhibitor transfection (Fig. S5, C and D). We could not detect TEAD2 in adult muscles using Western blot analysis.

Because miR-133a is enriched in fast-twitch muscle, we speculated that the expression of TEAD1 would be correspondingly lower in fast-twitch muscle and higher in slow-twitch muscle. As we expected, the highest expression of TEAD1 was

observed in mouse SOL muscles, whereas the relative lower expression of TEAD1 was seen in GAS-M and TA muscles (Fig. 5 H). A similar result was obtained in muscles from rats (Fig. 5 I). In addition, because miR-133a is positively regulated by T3, we hypothesized that TEAD1 protein levels would be altered by T3 treatment in mice. In agreement with the accepted concept, we found that T3 treatment reduced the mRNA expression of type I MyHC and increased the mRNA expression of type II MyHCs in hypothyroid mice (Fig. S5 E). TEAD1 protein expression was down-regulated in SOL muscle of hypothyroid mice with T3 treatment for either 2 h or for 5 d (Fig. 5 J), which was associated with the elevated miR-133a levels (Fig. S5 F). The repressive effect of T3 on TEAD1 protein

was determined by qRT-PCR (n = 3). (I–L) In vivo gene transfer into adult mice SOL muscle using plasmid MDH1-miR-133a (I) or miR-133a mimics (J), MDH1-miR-133a sponge (K), or miR-133a inhibitor (L) for 7 d as indicated. Expression levels of MHC isoforms and oxidative fiber markers were determined by qRT-PCR (n = 4). (M and N) C2C12 myoblasts were transfected with miR-133a mimics (M) or miR-133a inhibitor (N) as indicated. 24 h after transfection, C2C12 myogenesis was induced. qRT-PCR was performed to quantify the relative levels of MHC isoforms and oxidative fiber markers in C2C12 myotubes at day 3 of differentiation. (O–Q) C2C12 myoblasts were induced into myotubes by changing differentiation medium. 24 h after induction, C2C12 cells were transfected with miR-133a mimics or miR-133a inhibitor as indicated. Immunostaining of C2C12 myotubes was performed using antibody against MHC and myosin-fast (O) or myosin-slow (P) 48 h after transfection. MHC was stained for normalization. Representative images of cells were taken with a fluorescence microscope. Quantitative values were determined in four random fields for each group (Q). (R) Expression levels of miR-133a were determined 14 d after electroporation in rat SOL muscle by qRT-PCR (n = 3). (S) ATPase and hematoxylin and eosin (H&E) staining of rat SOL muscle 14 d after electroporation using plasmid MDH1-miR-133a. (T) Percentage of type I fibers in rat SOL muscle 14 d after electroporation according to the ATPase staining (n = 3). Means ± SD (error bars) are shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Bars: (A and B) 150 µm; (O and P) 200 µm; (S) 150 µm.
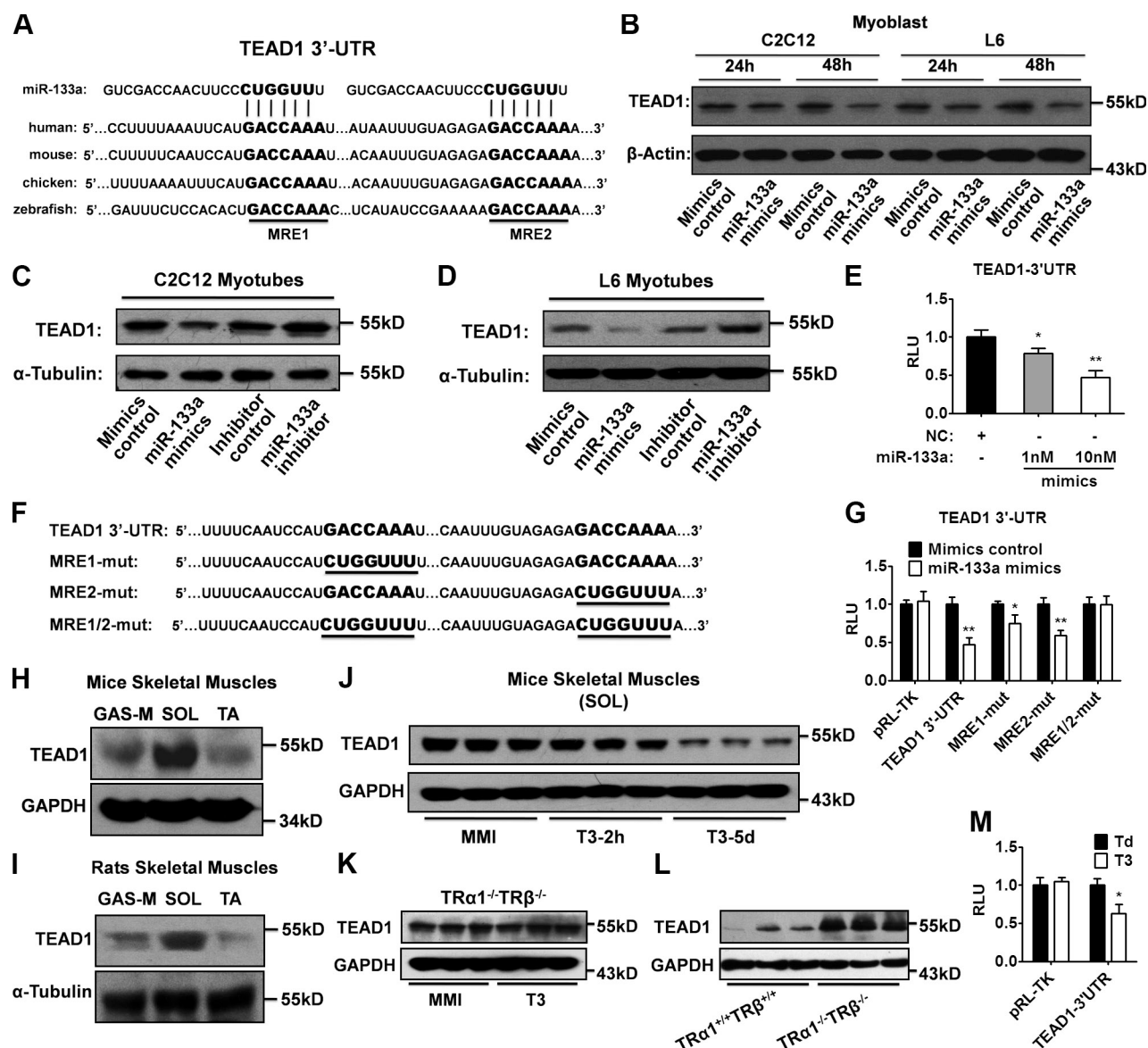
Figure 5. **TEAD1 is a direct target gene of miR-133a.** (A) Position of miR-133a regulatory element in TEAD1 3′ UTR and sequence alignment of miR-133a and the TEAD1 3′ UTR from various species are shown. (B) Western blot analysis of TEAD1 in C2C12 and L6 myoblasts transfected with miR-133a mimics. (C and D) Western blot analysis of TEAD1 in C2C12 (C) and L6 (D) myotubes transfected with miR-133a mimics or inhibitor. C2C12 and L6 myoblasts were transfected with miR-133a mimics or inhibitor. 24 h after transfection, myoblasts were induced into myotubes for 3 d. (E) Luciferase reporter containing TEAD1 3′ UTR sequences was cotransfected into HEK293T cells with miR-133a mimics. 48 h after transfection, Renilla luciferase activity was measured and normalized to firefly luciferase activity. (F) Mutations (underlined) were introduced into TEAD1 3′ UTR to disrupt base-pairing with miR-133a seed sequence. MREs for miR-133a are shown in bold. (G) Luciferase reporter containing wild type and mutant TEAD1 3′ UTR was cotransfected into HEK293T cells with miR-133a mimics. 48 h after transfection, luciferase activity was measured and normalized to firefly luciferase activity. (H and I) TEAD1 expression was measured in GAS-M, SOL, and TA muscle from mice (H) and rats (I) by Western blot analysis. (J) Western blot analysis of TEAD1 was performed on protein lysates from SOL muscle of MMI-treated mice with or without T3 treatment for 2 h or 5 d. (K) Western blot analysis of TEAD1 in SOL muscle of MMI-treated $TR\alpha1^{-/-}TR\beta^{-/-}$ mice with or without 5 d of T3 treatment. (L) Western blot analysis of TEAD1 in SOL muscle of $TR\alpha1^{-/-}TR\beta^{-/-}$ mice. (M) Luciferase activity of the reporter containing the 3′ UTR of TEAD1 was analyzed in C2C12 cells in the presence or absence of T3. Means ± SD (error bars) are shown. *, P < 0.05; **, P < 0.01.

expression was attenuated in SOL muscle of $TR\alpha1^{-/-}TR\beta^{-/-}$ mice and $TR\alpha1^{-/-}$ mice but not in SOL muscle of $TR\beta^{-/-}$ mice, which is consistent with the data of miR-133a expression (Figs. 5 K and S5, G–K). All these results again supported the notion that TRα1 is the major isoform in muscle. We also observed that the protein levels of TEAD1 were elevated in SOL muscle of $TR\alpha1^{-/-}TR\beta^{-/-}$ mice with reduced levels of miR-133a (Figs. 5 L and 1 G). Additionally, we also found that T3

treatment could repress the 3′ UTR of TEAD1 in a reporter assay (Fig. 5 M), further supporting the conclusion that *TEAD1* is a target gene of miR-133a.

## TEAD1 mediates miR-133a and TH action in myofiber specification

It has been shown that TEAD1 plays a regulatory role in muscle fiber determination (Karasseva et al., 2003; Tsika et al., 2008);
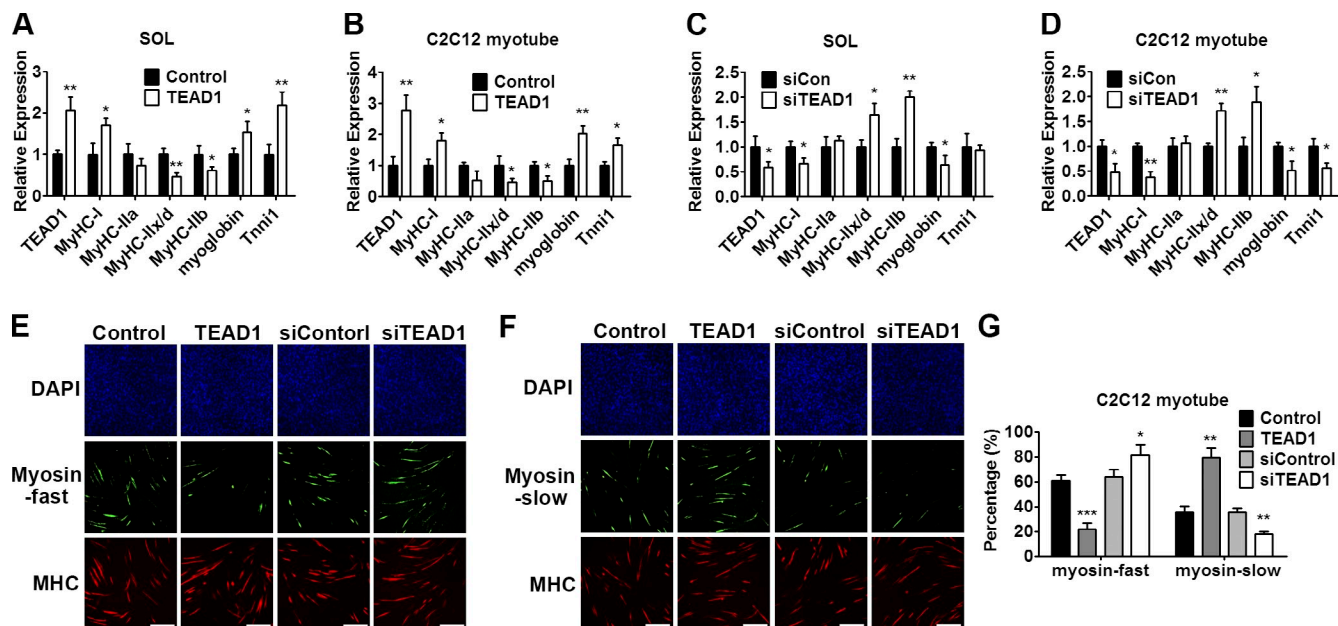
Figure 6. **TEAD1 promotes fast-to-slow myofiber type conversion.** (A and B) qRT-PCR analysis of the levels of MHC isoforms and oxidative fiber markers in SOL muscles (A) after TEAD1 plasmid electrotransfer and C2C12 myotubes (B) transfected with TEAD1 ($n = 3$). (C and D) qRT-PCR analysis of the levels of MHC isoforms and oxidative fiber markers in SOL muscle (C) after siTEAD1 electrotransfer and C2C12 myotubes (D) transfected with siTEAD1 ($n = 3$). (E and F) Immunofluorescent staining of C2C12 myotubes transfected with TEAD1 or siTEAD1 using antibodies against MHC and myosin-fast (E) or -slow (F). Bars, 200 μm. (G) Quantitative values were determined in four random fields for each group. Means ± SD (error bars) are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

however, the molecular mechanism is not very clear. Here we tested the effect of TEAD1 on the expression of individual MyHC isoforms as well as myoglobin and Tnni1 both in vivo and in vitro. As shown in Fig. 6 (A and B), TEAD1 increased the expression of type I MyHC, myoglobin, and Tnni1, and reduced the expression of type II MyHC isoforms in SOL muscles and C2C12 myotubes, which suggests that TEAD1 promotes fast-to-slow conversion. Consistently, knockdown of TEAD1 decreased the expression of type I MyHC and increased the expression of type II MyHC isoforms in SOL muscles and C2C12 myotubes (Fig. 6, C and D). The effect of TEAD1 on muscle fiber type determination was further confirmed by immunofluorescent staining in C2C12 myotubes transfected with either TEAD1 or siTEAD1 (Fig. 6, E–G).

To determine whether TEAD1 mediates miR-133a function in muscle fiber type transition, we designed a rescue experiment. As expected, overexpressing TEAD1 protein using *TEAD1* coding sequence without 3′ UTR significantly antagonized the effect of miR-133a mimics (Fig. 7, A–C). In contrast, TEAD1 with 3′ UTR showed an attenuated effect (Fig. 7, A–C). All these results indicated that TEAD1 mediates the effect of miR-133a as a miR-133a direct target in the regulation of muscle fiber type specification.

We also tested whether overexpression of TEAD1 could attenuate TH function in fiber type specification. As expected, TEAD1 overexpression promoted MyHC isoform transition from fast to slow and antagonized the effect of TH in either C2C12 myotubes (Fig. 7 D) or SOL muscle of mice (Fig. 7 E). Collectively, our results suggest a novel mechanism of TH-mediated muscle fiber specification, which involves TH-regulated miR-133a1 and its direct target TEAD1 (Fig. 7 F).

## TH regulates MyHC-I promoter indirectly through miR-133a/TEAD1

It has been proposed that TH negatively regulates *MyHC-I* transcription through nTREs. However, many basic issues including consensus sequence and dynamic recruitment of cofactors remain far from understood regarding nTRE (Chiamolera and Wondisford, 2009; Santos et al., 2011; Costa-e-Sousa and Hollenberg, 2012). Here we cloned *MyHC-I* promoter containing putative nTREs and an MCAT element (Fig. 8 A) to study the transcription of *MyHC-I* in C2C12 myoblasts. Surprisingly, T3 repressed the activities of the reporter containing the TRE region as well as the parental reporter pGL3-Basic (Fig. 8 B). Because there was no difference in the degree of repression between these two reporters, we concluded that TH is not able to affect *MyHC-I* transcription through nTREs as previously proposed. The finding that T3 repressed pGL3 reporter activity supported the known finding that the activity of the firefly luciferase reporter gene is suppressed by T3 and TR (Tillman et al., 1993). Consistent with previous reports, we showed that TEAD1 positively regulated *MyHC-I* promoter (Fig. 8 C). The regulation of *MyHC-I* transcription by TEAD1 was further confirmed with a ChIP assay using two sets of primers designed for MCAT region. As shown in Fig. 8 D, TEAD1 could be recruited to the MCAT region in the *MyHC-I* promoter. In agreement with our finding in Fig. 8 D, we found that *miR-133a1*, as a downstream target gene of TH and an upstream regulator of TEAD1, was able to inhibit *MyHC-I* promoter activity (Fig. 8 E). Moreover, the activity of the reporter containing MCAT element was inhibited by miR-133a, further suggesting that miR-133a suppresses *MyHC-I* transcription
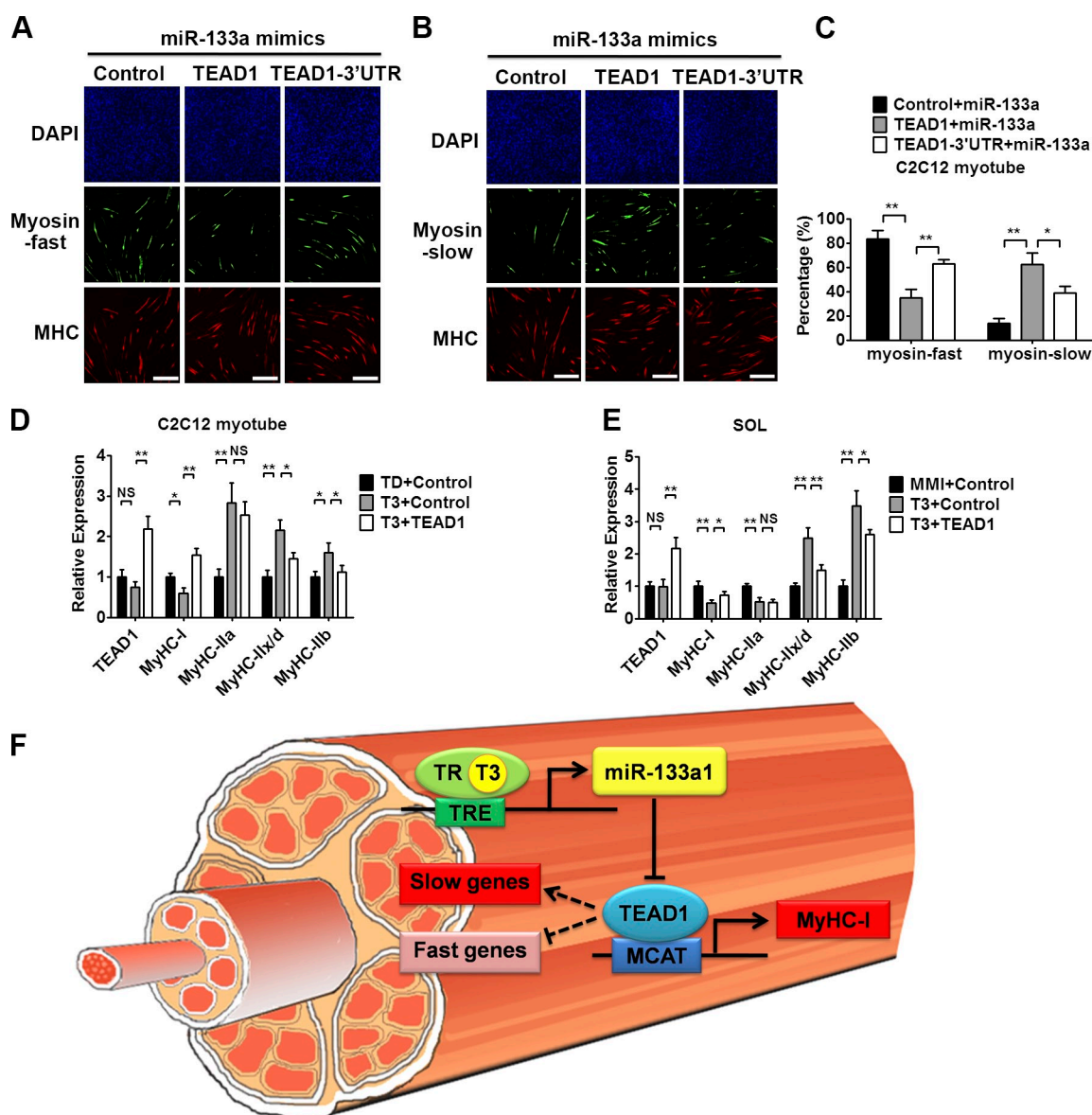
Figure 7. **TEAD1 mediates miR-133a and TH action in myofiber specification.** (A and B) C2C12 myotubes were cotransfected with miR-133a mimics and/or TEAD1 with or without 3′ UTR. 48 h after transfection, immunostaining was performed. Bars, 200 µm. (C) Quantitative values were determined in four random fields for each group. (D) qRT-PCR analysis of the expression of TEAD1 and MyHC isoforms in C2C12 myotubes transfected with TEAD1 in the presence of T3. (E) qRT-PCR analysis of the expression of TEAD1 and MyHC isoforms in SOL muscles of MMI-treated mice, MMI-treated mice with T3 treatment for 5 d, and MMI-treated mice with electrotransfer of TEAD1 and T3 treatment for 5 d ($n = 3$). (F) Schematic representation of miR-133a–mediated TH function in muscle fiber type determination. Means ± SD (error bars) are shown. *, $P < 0.05$; **, $P < 0.01$.

through targeting TEAD1 (Fig. 8 E). These results indicated that TH might negatively regulate *MyHC-I* transcription through an miRNA-mediated mechanism.

## Discussion

The remodeling of slow-twitch oxidative or fast-twitch glycolytic muscle fibers is mechanistically unclear. Extensive literature to date highlights the fact that TH is one of the major determinants of the muscle phenotype. However, the molecular mechanisms of TH action on skeletal muscle and MyHC isoform composition are unclear. Growing evidence has demonstrated that formation and maintenance, as well as physiological and

pathophysiological responses of skeletal muscles, are subject to the regulation by miRNAs. Here, our study demonstrated that TH modulates muscle fiber type specification through its direct target gene *miR-133a1*. We also proposed that the negative regulation by TH could be mediated by TH-regulated miRNA. Our study provided a novel mechanism of TH-mediated gene regulatory network involving miRNA.

In this study, we found that miR-133a plays a critical role in muscle remodeling under different TH status. Most importantly, our in vivo experiment demonstrated that inhibition of endogenous miR-133a by ant-133a diminished the effect of T3 on muscle fiber type conversion, indicating that miR-133a is required for the TH action on myofiber type transition. Together with in vitro data, we proposed that the regulation of miR133a
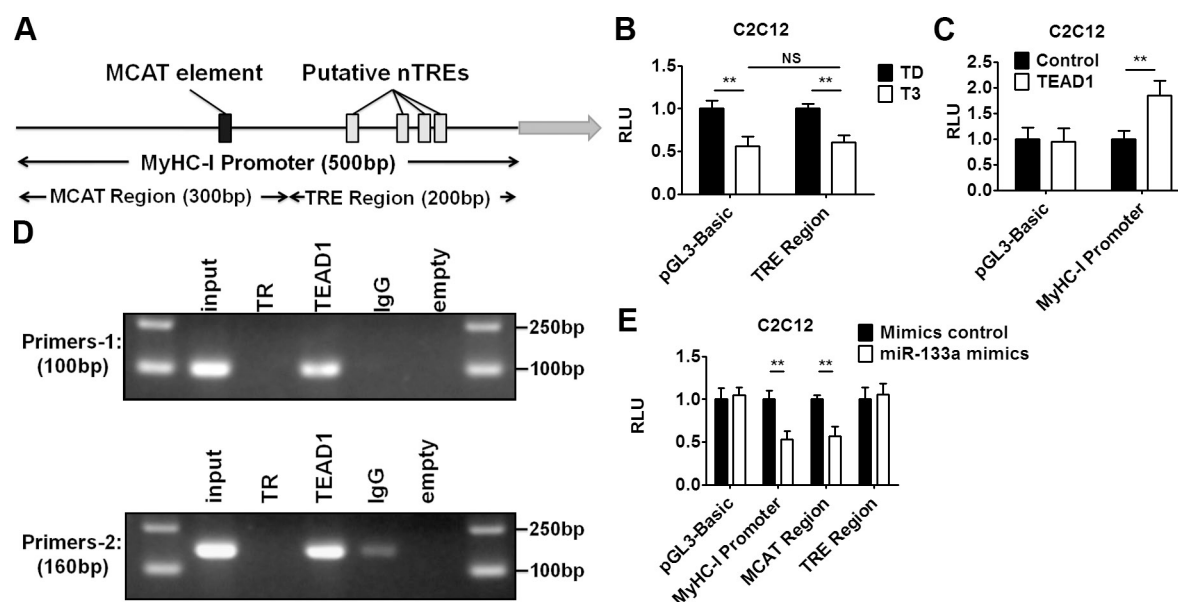
Figure 8. **The effect of T3, TEAD1, miR-133a on the promoter activity of MyHC-1.** (A) Mouse *MyHC-I* gene promoter containing an MCAT element and putative TREs. (B) C2C12 myoblasts cultured in Td medium transfected with pGL3-Basic or a reporter containing TRE region. 24 h after transfection, T3 was added for 1 d. Promoter activities were evaluated with a luciferase assay. (C) C2C12 myoblasts were cotransfected with a reporter containing *MyHC-I* promoter and TEAD1 expression vector. Promoter activities were determined with a luciferase assay. (D) A ChIP assay was performed using chromatin from C2C12 myotubes. Anti-TEAD1, normal mouse IgG, and anti-TR (C4) antibodies were used for immunoprecipitation. Purified DNA was then analyzed by PCR using two sets of primers specific for the MCAT region. Water was used as a negative control for PCR (empty). (E) C2C12 myoblasts were cotransfected with miR-133a mimics or mimics control, and reporters containing *MyHC-I* promoter, MCAT element, or TRE region as indicated. Promoter activities were examined with a luciferase assay. Means ± SD (error bars) are shown. **, P < 0.01.

by TH plays a major role in TH-mediated muscle fiber type conversion. Moreover, we show that restoration of TEAD1 was able to antagonize the T3 effect on the mRNA expression of slow and fast muscle genes both in vitro and in vivo, which suggests that TEAD1 is involved in T3-stimulated slow-to-fast conversion. Our study provided a new perspective on the understanding of TH-related pathophysiologic mechanisms and opened the door to a whole new class of therapeutics with a wide range of applications. Moreover, miR-133a and TEAD1 could serve as novel targets for drug development.

Recently, it was reported that mice with genetic deletions of *miR-133a1* and *miR-133a2* developed adult-onset centronuclear myopathy in fast-twitch myofibers, accompanied by impaired mitochondrial function and fast-to-slow myofiber conversion (Liu et al., 2011). The finding of the regulatory role of miR-133a in myofiber type transition is consistent with the results of our present study. The fact that there is no difference in the number of slow fibers in neonatal muscles of *miR-133a* KO mice, whereas the percentage of slow-twitch myofibers in SOL muscles is increased in *miR-133a* KO mice at the age of 2 and 4 wk, is also consistent with the known increase in TH after birth (Liu et al., 2011). However, why lacking *miR-133a* in muscle led to a fast-to-slow conversion of myofibers was not further explored in that study. Here we provide evidence suggesting that miR-133a promotes slow-to-fast myofiber conversion by targeting the transcription factor TEAD1, which was previously shown to promote the slow muscle phenotype (Tsika et al., 2008).

TR-mediated transcription could be regulated at multiple levels or by various factors, including the circulating TH level, the intracellular TH level, TH metabolites, the expression pattern of TR isoforms, and posttranslational modification of TR, as well as TR coregulators (Shi, 2009; Brent, 2012; Fondell, 2013; Salvatore et al., 2014). Compared with positive regulation by TH, negative regulation by TH remains under debate (Ortiga-Carvalho et al., 2005; Chiamolera and Wondisford, 2009; Santos et al., 2011; Costa-e-Sousa and Hollenberg, 2012). Here, we proposed that TH and its receptors might suppress gene expression indirectly through an miRNA-mediated network. The novel mechanism identified in the current study added a new intricate layer to the regulation by TH.

The inhibitory effect of TH on endogenous expression of the *MyHC-I* gene has been reported (Baldwin and Haddad, 2001). The proposed nTREs are located within the basal promoter, where binding of the transcription machinery is necessary to initiate transcription (Wright et al., 1999; Baldwin and Haddad, 2001). However, our data here showed that the negative regulation of *MyHC-I* transcription by TH is indirect. This discrepancy prompted us to check previous reports in which nTREs of *MyHC-I* were identified. Interestingly, we found that previous conclusions were all based on a luciferase assay using reporters derived from the region of the basal promoter. Deletion of any sequences in this region resulted in very low reporter activity, which may only reflect the basal activity of the parental reporter. Consequently, the effect of TH stimulation could not be detected, which led to a misleading conclusion that the deleted region contained a TRE. Recently, it has been proposed that TR might repress the *MyHC-I* transcription through a tethering mechanism via binding to TEAD1 (Iwaki et al., 2014). This model also indicated that TH does not regulate *MyHC-I* transcription through nTREs. However, this study did

not provide data showing what will happen after the binding of TR to TEAD1. It is still not clear whether the interaction between TR and TEAD1 will affect the binding of TEAD1 to the promoter, or will cause the recruitment of TR. Moreover, because the GST pull-down assay in that paper showed that the binding of TR to TEAD1 is T3 independent, how T3 achieves transcriptional repression on *MyHC-I* promoter is still not clear and requires further investigation. In our study, we did not detect the recruitment of TR to the MCAT region in a ChIP assay (Fig. 8 D), which supported our proposed model in which T3 controls the *MyHC-I* promoter activity indirectly through an miR-133a1–mediated mechanism. Just based on our current data, we believe that T3 and TR might not use the tethering mechanism to control the *MyHC-I* transcription in skeletal muscle.

Collectively, our studies revealed a novel role of TH-regulated miR-133a1 in controlling muscle type specification. The identification of an miRNA-mediated mechanism of TH-regulated muscle type specification undoubtedly increased our understanding of TH-controlled normal muscle physiology and adaptive responses.

## Materials and methods

### Animal study
Our study was reviewed and approved by the Institutional Review Board of the Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Animals were maintained and experiments were performed according to protocols approved by the Animal Care and Use Committees of Institute for Nutritional Sciences (permit No. 2011-AN-14). $TR\alpha 1^{-/-}$ mice and $TR\beta^{-/-}$ mice were obtained from B. Vennstrom (Karolinska Institute, Stockholm, Sweden) and D. Forrest (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD; Forrest et al., 1996; Wikström et al., 1998). Regarding the generation of $TR\beta^{-/-}$ mice, a targeted mutation was designed by inserting a neomycin cassette into exon 3, which disrupts both $TR\beta 1$ and $TR\beta 2$ isoforms. $TR\alpha 1$ is encoded by exons 1–9, whereas $TR\alpha 2$ results from alternative splicing from a donor site in exon 9 (exon 9a) to an acceptor site for exon 10. Deletion of nucleotides in exon 9, which are after the splice site (exon 9b), would selectively disrupt $TR\alpha 1$. However, the orphan hormone receptor *rev-erbA$\alpha$* is encoded by the opposite strand of the $TR\alpha$ locus, and its last coding exon overlaps exon 10 of the $TR\alpha$ locus. Therefore, as for the development of $TR\alpha 1^{-/-}$ mice, to ablate only TR$\alpha$1 expression, in order to leave that of TR$\alpha$2 intact and keep the region of the $TR\alpha$ exon 10, which overlaps the *rev-erbA$\alpha$* locus undisturbed, a targeting construct with the neomycin transcriptional unit was designed to change exon 9a+b to an exon encoding 9a + exon 10. Both mouse strains with hybrid genetic background had been backcrossed to C57BL/6 for at least 10 generations. Mice were rendered hypothyroid by the addition of MMI and $NaClO_4$ for 2 wk. Hypothyroid mice were injected intraperitoneally with 5 µg T3/20 g body weight per day for 5 d or 2 h or as indicated (Song et al., 2012).

Regarding in vivo muscle transfection by electroporation (Donà et al., 2003), the plasmids for transfection were prepared by using ethanol precipitation. The pellets of plasmids were resuspended in PBS solution containing 25% sucrose. Plasmids were stored at 4°C for at least 12 h before transfection. The mice were anesthetized through intraperitoneal injection (1% pentobarbital sodium, 50 mg/kg). Sterile scissors were used to make a small incision on the lateral aspect of the rear leg before intact SOL muscles were isolated. The plasmids (2 mg/ml) were injected throughout the longitudinal axis of the SOL muscle by using a syringe. Five electric pulses (20-ms duration pulses at an interval of 200 ms, voltage-to-distance ratio 50 V/cm) were given by using an electric pulse generator (Electro Square porator ECM-830; BTX). After insertion of the needle electrode (two-needle array; BTX) into SOL muscle belly, electric pulses were then applied. The incision on the leg was sutured using a silk thread before the mice were transferred back to their home cages. 7 or 14 d after surgery, animals were anesthetized, and SOL muscles were extracted and frozen in liquid nitrogen.

Chemically modified antisense oligonucleotides (antagomir) have been used to inhibit miR-133a expression. The sequence of ant-133a was as follows: 5′-CAGCTGGTTGAAGGGGACCAAA-3′. The 3′ end of the antagomir was conjugated to cholesterol and all the bases were modified with 2′-OMe (Shanghai GenePharma). C57BL/6 mice (6 wk old) received antagomir every other day at doses of 20 mg/kg body weight through tail injection.

### Cell culture, transfection, and luciferase assay
The mouse C2C12 myoblast cell line and rat L6 myoblast cell line were maintained in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco). For induction of myoblasts into myotubes, DMEM containing 2% horse serum (Gibco) and 1% penicillin/streptomycin was used. Trimmed and minced muscle tissue was incubated with 0.1% Pronase in DMEM at 37°C on a rocker for 1 h. After removal of supernatant by centrifugation, the pellet was resuspended and filtered with a 40-mm nylon mesh cell strainer (2350; Falcon). To enrich the myoblasts, cells were collected by centrifugation, resuspended, and preplated in a noncoated dish for 1 h to remove fibroblasts. The floating cells with enriched myoblasts were then transferred to Matrigel-coated plates (BD; Zhang et al., 2012). To make TH-deficient medium (Td medium), fetal bovine serum and horse serum were stripped with 10% resin. For T3 treatment, 100 nM T3 was added to the medium. Plasmid transfection into C2C12 myoblasts was performed using Lipofectamine LTX (Invitrogen) according to the standard protocol. MicroRNA-133a mimics and inhibitors were transfected into myoblasts using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 48 h after transfection, a luciferase assay was performed with a Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Firefly and Renilla luciferase activities were measured on a luminometer (Berthold Technologies). Cycloheximide (C4859) was purchased from Sigma-Aldrich and added to the medium of C2C12 myoblasts (final concentration 10 µM) before T3 treatment.

### Plasmid and RNA oligonucleotide
The miR-133a1 precursor was amplified by PCR using mouse genomic DNA as a template, and the PCR product was cloned into MDH1-IRES-GFP vector (*H1* promoter; Addgene) to generate miRNA expression plasmids (MDH1-miR-133a). Oligonucleotides of the miR-133a binding site (5′-CAGCTGGTTGTCCGGACCAAA-3′) with 10-bp linker sequences containing XhoI and EcoRI sites were chemically synthesized. To obtain MDH1-miR-133a sponge plasmid with six tandemly arrayed miRNA-133a binding sites, the oligonucleotides were annealed, ligated, and inserted into MDH1-IRES-GFP vector (Zhang et al., 2012). Mouse *TEAD1* cDNA amplified by RT-PCR was subcloned into the BamHI and EcoRI sites of pcDNA3.1 vector (*CMV* promoter; Invitrogen) to obtain pcDNA-TEAD1, and the primer sequences used for cloning are provided in Table S2. The fragment of the TEAD1, TEAD2, TEAD3, and TEAD4 3′ UTRs were amplified from mouse cDNA by PCR and inserted into pRL-TK vector containing a cDNA encoding Renilla luciferase and HSV-TK promoter (Promega). To construct pcDNA-TEAD1-3′ UTR plasmid, the 3′ UTR fragment was inserted into the pcDNA-TEAD1 plasmid downstream of the *TEAD1* cDNA sequence. Enhancers of *miR-1-2/miR-133a1* and *miR-1-1/miR-133a2* were amplified by using mouse genomic DNA as a template and cloned into pTK109-luc vector (containing a firefly luciferase gene and minimal thymidine kinase promoter). To find the TRE location in *miR-133a1* enhancer, four different fragments were amplified with primers containing KpnI and XhoI restriction sites and ligated into pTK109-luc vector. Mouse *MyHC-I* promoter fragments were cloned from mouse genome DNA. Digested by Kpn I and Xho I, these promoters were linked to the promoter region of the firefly luciferase gene carried in pGL3-Basic vector containing a modified coding region for firefly luciferase and lacking eukaryotic promoter and enhancer sequences (Promega). The kit for plasmid DNA mutation was purchased from Toyobo Life Science Department (SMK-101). GMR-miR miRNA mimics and inhibitors for miR-133a were obtained from Shanghai GenePharma. The sequences of TEAD1 siRNAs were as follows. siTEAD1: 5′-CGUGUACCGAAUAAACC-GCtt-3′ (sense) and 5′-GCGGUUUAUUCGGUACACGtt-3′ (antisense).

### Northern blot analysis of miRNAs
Total RNA from skeletal muscle was extracted using Trizol reagent (Invitrogen) according to the protocol. Total RNA (40 µg) was resolved in a denatured 15% polyacrylamide gel and transferred to a nylon membrane (GeneScreen Plus; PerkinElmer). Synthetic oligonucleotides labeled at the 5′ end with $\gamma$-[$^{32}$P]ATP were used as probes. After UV cross-linking, the membrane was hybridized with probes overnight, and exposed to a phosphorimager. The sequences of probes are shown in Table S2.

### Real-time RT-PCR and Western blot analysis

Total RNA was extracted from tissues and cells by using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was reverse-transcribed by using a PrimeScript RT reagent kit (Takara Bio Inc.), and small RNA polyadenylation was performed (Zhang et al., 2012). Real-time PCR was performed on a real-time PCR system (ABI 7900; Applied Biosystems). The primer sequences are provided in Table S3. Mouse monoclonal anti–TEF-1 (610922; BD), rabbit polyclonal anti-MyoD (sc-760; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-MEF2 (sc-313; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-myogenin (sc-12732; Santa Cruz Biotechnology, Inc.), mouse monoclonal anti–α-tubulin (T6199; Sigma-Aldrich), mouse monoclonal anti–β-actin (A5316; Sigma-Aldrich), mouse monoclonal anti–glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH; KC-5G4; KangChen Bio-tech Inc.), rabbit polyclonal anti-TEAD2 (21159-1-AP [Proteintech]; 33900 [Signalway Antibody]), rabbit polyclonal anti-TEAD3 (13120-1-AP; Proteintech), and rabbit polyclonal anti-TEAD4 (12418-1-AP) antibodies were used for Western blot analysis.

### Chromatin immunoprecipitation assay

Chromatin immunoprecipitation was performed according to the Magna ChIP manufacturer's instructions (EMD Millipore). In brief, each 10-cm cell culture dish of human HEK293T or mouse C2C12 cells was used for the appropriate antibody pull-downs. The primers for ChIP assays are provided in Table S3. Mouse anti-TRα1/TRβ1 (C4; sc-740; Santa Cruz Biotechnology, Inc.), anti-TEF1 (610922; BD), and anti-SRC-1 (C-20; sc-6096) antibodies were used for the ChIP assay.

### Immunostaining and metachromatic ATPase staining

Immunostaining of C2C12 myotubes was performed as described previously (Matsuoka and Inoue, 2008; Zhang et al., 2012). After transfection, myoblasts were induced into myotubes for the indicated time on sterile cover glass (Nest Biotechnology), and then were fixed with cold methanol. Myotubes were treated with 0.1% Triton X-100 in PBS for 10 min. After blocking with 2% BSA in PBS for 30 min, the expressions of MYH, MyHCs, and MyHCf in C2C12 myotubes were detected with rabbit polyclonal MyHC (1:200, sc-20641; Santa Cruz Biotechnology, Inc.), mouse monoclonal skeletal myosin-fast (1:2,000; M4276; Sigma-Aldrich), or mouse monoclonal myosin-slow (1:2,000; M8421; Sigma-Aldrich) antibodies. Alexa Fluor 488 goat anti–mouse IgG1 and Alexa Fluor 594 goat anti–rabbit IgG1 (1:1,000; Invitrogen) were added as secondary antibodies. The cells were incubated for 1 h at room temperature. The nuclei were stained with DAPI (Thermo Fisher Scientific). Representative images of cells were taken by fluorescence microscope, and MHC, myosin-fast, and myosin-slow expression was quantified (the number of MHC and myosin-fast– or MHC and myosin-slow–positive myotubes per field). Quantitative values and the percentage of myosin-fast– or myosin-slow–positive cells were determined in four random fields for each group. Values shown in Table S1 represent the mean ± SD of three independent experiments.

Metachromatic MyHC ATPase staining was conducted as described previously (Hintz et al., 1984). In brief, muscle tissues from mice and rats were frozen in isopentane near its freezing point. Muscle fiber type was determined by the ATPase staining for preincubation at either pH 10.6 or 4.2.

### Image acquisition

All digital images were visualized at room temperature using a microscope (BX61; Olympus), a cooled charge-coupled device camera (QICAM Fast; QImaging), and the software package Q-Capture (version 2.9.11; QImaging) with a UPlan-Apochromat 10×/0.40 NA (Olympus) objective lens. To reduce the background of images, brightness and contrast were adjusted with Photoshop software (Adobe).

### Statistical analysis

GraphPad Software was used for statistical analysis. All data are expressed as means ± SD and analyzed with an unpaired Student's $t$ test for statistical significance. P-values <0.05 were considered to be significant. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

### Online supplemental material

Fig. S1 shows the effect of T3 on the levels of miR-1 and miR-133a in SOL muscle of mice or in cultured mouse primary myotubes. Fig. S2 shows the effect of T3 on the levels of primary transcripts encoding miR-1-1, miR-1-2, miR-133a1, or miR-133a2 in SOL muscle of hypothyroid mice or in C2C12 myoblasts. Fig. S3 shows the differential expression of MyomiRs in the SOL and GAS muscle of mice. Fig. S4 shows the effect of ant-133a on T3-induced alteration of MyHC isoform expression in SOL muscle of hypothyroid mice. Fig. S5 shows the effect of miR-133a on TEADs and TEAD1 protein expression in TR isoform–specific knockout mice after T3 treatment. Table S1 shows the effect of miR-133a mimics or inhibitors on the subtype of C2C12 myotubes. Tables S2 and S3 contain the information of oligos used for cloning, Northern blot analysis, and primers used for qRT-PCR and ChIP assays. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201406068/DC1. Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201406068.dv.

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