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14. *S. cerevisiae* strains used are listed in Table 2. Strain MCY939 was provided by M. Carlson; all other strains are from our lab. All strains are congenic to S288C. FY strains are *GAL2*$^+$ derivatives of S288C [F. Winston, *et al.*, *Yeast* **11**, 53 (1995)]. Strain ABY396 is a *GAL2*$^+$ derivative of BJ2168 [E. Jones, in *Methods Enzymol.* **194**, 428 (1991)]. Standard yeast genetic methods were used [F. Sherman, G. R. Fink, C. W. Lawrence, *Methods in Yeast Genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1978)].
15. To overexpress histones H2A and H2B, the Δ16' promoter was recombined into the *HTA1-HTB1* locus, replacing the wild-type promoter, after a two-step integration protocol with the use of plasmid pUC13$^+$Δ16' (a gift from M. A. Osley) (*31*). To overexpress histones H3 and H4, the *HHT1* (H3) and *HHF1* (H4) genes were amplified by polymerase chain reaction (PCR) and subcloned under control of the Δ16' promoter to create plasmid pAB157. This integrating plasmid, which also contains the *LEU2* gene, was then integrated at *leu2Δ1* in yeast strains. We achieved expression of either *HHT1* or *HHF1* individually by dropping out the *HHT1* or *HHF1* fragments from pAB157. Sequences encoding H3Δ(4-30) were created by PCR amplification of *HHT1*, with the use of an oligonucleotide that spans the junction from codons from 4 to 30 of *HHT1*. The mutation encoding H3 (Thr^{119Ile}) was PCR-amplified from genomic DNA of strain FY889, which contains this *hht1* mutation. All PCR products were verified by sequencing. We determined the effect of histone overexpression on cell viability by shifting liquid cell cultures to the restrictive temperature (37°C) for 8 hours, after which cells were sonicated to disperse any clumps, dilutions were plated in duplicate, and plates were incubated at 30°C. Percent viability represents the fraction of viable cells at time zero that were able to grow after 8 hours of incubation at the restrictive temperature. Similar results were obtained when colony formation was monitored by light microscopy.
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21. Plasmid pGH85, encoding the GST-Spt6p fusion, is full-length *SPT6* in pEMBL-GST (*32*). GST was expressed from pSYB305. The GST and GST-Spt6p fusions were isolated from ABY396 after a 3-hour induction with 2% galactose. Proteins were routinely purified from 10 mg of protein lysate prepared with glass beads and buffer A250 [25 mM tris-HCl (pH 7.5), 15 mM $MgCl_2$, 15 mM EGTA, 10% glycerol, 0.3% Triton X-100, 0.02% NaN_3, 250 mM NaCl, and 1 mM dithiothreitol (DTT)] and the protease inhibitors chymostatin, leupeptin, pepstatin, benzamidine, and phenylmethylsulfonyl fluoride. For the column chromatography experiments, 10 mg of protein lysate was incubated with 0.5 ml of glutathione beads (Pharmacia) for 45 min in a final volume of 5 ml. The beads were then extensively washed with A250 and with A1000 (A250 with NaCl at 1 M).
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23. Acid-extracted yeast histones were prepared from purified nuclei (*6*) as described (*33*), except that no butyrate was used in extraction buffers A and B, and extraction with 0.4 N sulfuric acid was done only once. The acid-soluble protein was precipitated in 20% trichloroacetic acid and the pellet was washed in acidified acetone, air-dried, and suspended in 10 mM tris-HCl (pH 8.0). Protein microsequencing was done at the Biopolymer Facility of the Howard Hughes Institute and the Department of Genetics, Harvard Medical School.
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25. GST and GST-Spt6p proteins used in far-western analysis were purified as described above (*21*), except that 1.5 ml of glutathione beads was used for every 10 mg of protein lysate. After the beads were washed with 30 ml of 50 mM tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM DTT, the bound proteins were eluted in 2 ml of the same buffer plus 10 mM glutathione (Sigma) and concentrated on Centricon concentrators (Amicon). Far-western analysis was performed as described in the Fig. 3 legend.
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35. We are especially grateful to G. Hartzog for providing the GST-Spt6 fusion; to M. A. Osley for providing the Δ16' promoter; and to S. Brown, T. Imbalzano, and R. Kingston for the human histones and frog extracts. We also thank E. Elion for the gift of pSYB305; S. Roth for advice on far-western analysis; J. Workman for valuable discussions; and A. Dudley, G. Hartzog, and R. Kingston for critical reading of the manuscript. Supported by NIH grant GM32967.

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Inhibition of Myogenic bHLH and MEF2 Transcription Factors by the bHLH Protein Twist

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The myogenic basic helix-loop-helix (bHLH) and MEF2 transcription factors are expressed in the myotome of developing somites and cooperatively activate skeletal muscle gene expression. The bHLH protein Twist is expressed throughout the epithelial somite and is subsequently excluded from the myotome. Ectopically expressed mouse Twist (Mtwist) was shown to inhibit myogenesis by blocking DNA binding by MyoD, by titrating E proteins, and by inhibiting trans-activation by MEF2. For inhibition of MEF2, Mtwist required heterodimerization with E proteins and an intact basic domain and carboxyl-terminus. Thus, Mtwist inhibits both families of myogenic regulators and may regulate myotome formation temporally or spatially.

Members of the bHLH family of transcription factors play important roles in controlling cell type determination and differentiation in both vertebrates and invertebrates (*1*). Vertebrate skeletal muscle development is controlled by four members of this family: MyoD, Myf-5, myogenin, and MRF-4. Gene disruption in mice has established that the myogenic bHLH proteins are necessary for establishment of the myoblast cell lineage and for proper execution of the skeletal muscle differentiation program (*2*). In addition to the MyoD family, members of the MEF2 family of transcription factors also play a role in muscle differentiation. There are four members of the MEF2 family of proteins (MEF2A through MEF2D), and all share a region of homology with the MADS family of transcription factors and an adjacent region known as the MEF2 domain (*3*). MEF2 factors are highly induced during skeletal myogenesis (*4*), and many skeletal muscle–specific genes require both myogenic bHLH and MEF2 binding sites for maximal skeletal muscle expression (*3*). High-level expression of both myogenic bHLH and MEF2 family members in the somite are confined to the myotome (*5–7*).

Originally identified in *Drosophila* as a gene necessary for embryonic gastrulation and formation of mesoderm (*8*), Twist is a bHLH protein that is specifically expressed in the mesoderm (*9*). Twist homologs have been isolated from *Xenopus* (*10*), mouse (*11*), and chick (*12*). Vertebrate Twist is initially expressed throughout the somitic mesoderm. As the somites develop, Twist is excluded from the forming myotome but continues to be expressed in other mesodermal structures such as the dermomyotome, sclerotome, lateral plate mesoderm, and cranial neural crest (*10–13*). The exclusion of Twist from the myotome suggested that it may negatively regulate the myogenic bHLH proteins and that the mutually exclusive expression of these transcription factors may partition the somitic mesoderm into alternative cell fates. We now demonstrate that ectopically expressed mouse Twist (Mtwist) inhibits skeletal myogenesis by blocking the activity of both myogenic

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bHLH and MEF2 family members.

Constitutive expression of Mtwist inhibits myogenic differentiation of myoblast cell lines (*12*, *14*). To investigate the mechanism of this inhibition, we compared the ability of Mtwist to inhibit MyoD-mediated activation of a muscle creatine kinase promoter–chloramphenicol acetyltransferase (CAT) reporter construct (MCK-CAT) in cotransfected C3H10T1/2 cells with that of the dominant-negative HLH protein Id. Id inhibits MyoD function by competing with MyoD for dimerization with its bHLH binding partners, E proteins, and thereby prevents formation of active myogenic bHLH–E protein heterodimers (*15*). Both Mtwist and Id inhibited MCK-CAT expression in the presence of exogenous E12; however, Mtwist was a more efficient inhibitor than Id (Fig. 1A). With a fourfold excess of MyoD, Mtwist completely inhibited the activity of MCK-CAT, whereas this amount of Id had relatively little effect (Fig. 1A). Because this level of Id had little effect on MCK expression, we used this amount of both Mtwist and Id expression vehicles in subsequent experiments to evaluate how these proteins differentially inhibit muscle gene expression. Transfection of equivalent amounts of Mtwist or Id expression vehicles led to a steady-state level of Id protein that was greater than that of Mtwist protein (Fig. 1B), indicating that the greater efficiency of Mtwist-mediated inhibition was not the result of higher protein levels of Mtwist versus Id.

MCK gene expression is controlled by a complex muscle-specific enhancer that binds both myogenic bHLH proteins and other transcription factors (*16*, *17*). To evaluate whether Mtwist specifically inhibits MyoD function, we assayed the effect of Mtwist on MyoD trans-activation of a simplified muscle-specific promoter, containing four reiterated MyoD binding sites (termed E boxes) upstream of a minimal promoter (Ex4-CAT). Unlike Mtwist-mediated inhibition of the MCK gene, which remained repressed upon the addition of exogenous E12, Mtwist–mediated inhibition of the activity of Ex4-CAT was both less efficient and could be alleviated by addition of exogenous E12 (Fig. 1C). In contrast, Id-mediated inhibition of both promoters was approximately equivalent and in both cases was relieved by addition of exogenous E12 (Fig. 1C). Thus, Mtwist (like Id) can inhibit MyoD-mediated induction of a simplified muscle reporter (containing only reiterated E boxes) by titrating E proteins. In agreement with this finding, Mtwist and Id displayed equal affinities for E protein interaction in vitro (*12*), and electrophoretic mobility–shift assay (EMSA) of in vitro–translated proteins indicated that addition of equimolar amounts of either Mtwist or Id inhibited MyoD-E12 heterodimers equivalently from forming and binding a MyoD binding site, and this inhibition was reversed by addition of excess E12 (Fig. 1D).

In contrast to Ex4-CAT, inhibition of MCK expression by Mtwist was not relieved by addition of ectopic E proteins. This finding, together with the observation that Mtwist–E protein complexes bind only weakly to a MyoD binding site (Fig. 1D), suggested that efficient repression of MCK expression by Mtwist was the result of a mechanism other than simply E protein sequestration or MyoD binding site competition (or both). Many muscle-specific promoters, including MCK, contain binding sites for both the myogenic bHLH factors and for MEF2 proteins (*3*, *16*). Because binding sites for both factors are required for muscle-specific expression of the MCK gene (*17*), we tested whether Mtwist inhibited MEF2-mediated trans-activation. Cotransfection of E12 plus Mtwist inhibited MEF2C trans-activation of a CAT reporter construct driven by four reiterated MEF2 binding sites upstream of a minimal promoter (MEF2x4-CAT) (Fig. 2A). Maximal inhibition of MEF2 function by Mtwist required the presence of exogenous E12 (Fig. 2A), suggesting that repression of MEF2 activity by Mtwist requires heterodimerization of the latter with E proteins. In contrast, the slight inhibition of MEF2 function observed after cotransfection with Id was not augmented by the addition of exogenous E12 (Fig. 2A).

To address whether the MEF2 trans-activation domain is a target for Mtwist, we assayed the effects of Mtwist on trans-activation of a Gal4-dependent reporter by a construct containing MEF2A fused to the Gal4 DNA binding domain (Gal4-MEF2).

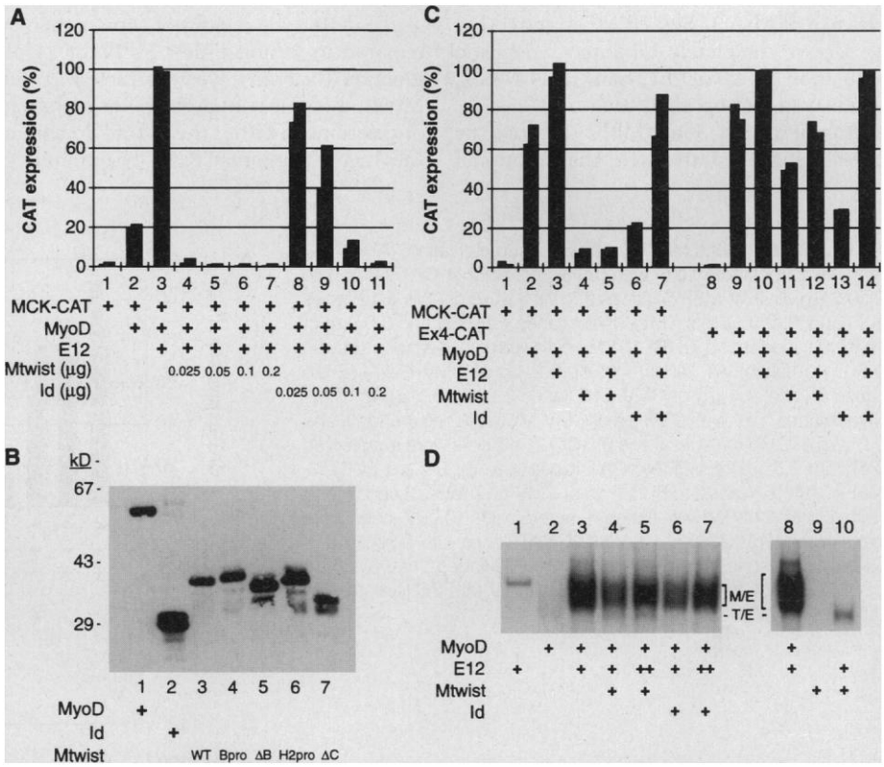

Fig. 1. Mtwist inhibits muscle gene expression by a mechanism other than E protein sequestration. (**A**) 10T1/2 fibroblasts were transfected (*26*) with 0.5 μg of MCK-CAT (lanes 1 to 11), 0.1 μg of CMV-MyoD (lanes 2 to 11), 0.1 μg of CMV-E12 (lanes 3 to 11), and a total amount of 0.2 μg of CMV expression vehicle without an insert and the indicated amount of CMV-driven Mtwist (lanes 4 to 7) or Id (lanes 8 to 11). Duplicate samples (light and dark bars) from a representative experiment are shown. (**B**) 10T1/2 cells were transfected with equal amounts of CMV expression vehicles driving expression of either MYC epitope–tagged versions of MyoD (lane 1), Id (lane 2), Mtwist WT (lane 3), or Mtwist mutants as indicated below lanes (lanes 4 to 7). After 72 hours, protein immunoblot analysis was performed on cell extracts to detect MYC-tagged proteins. (**C**) 10T1/2 cells were transfected (*26*) with 0.5 μg of MCK-CAT (lanes 1 to 7), 0.5 μg of Ex4-CAT (lanes 8 to 14), 0.1 μg of CMV-MyoD (lanes 2 to 7 and 9 to 14), 0.1 μg of CMV-E12 (lanes 3, 5, 7, 10, 12, and 14), 0.025 μg of CMV-Mtwist (lanes 4, 5, 11, and 12), and 0.025 μg of CMV-Id (lanes 6, 7, 13, and 14). (**D**) EMSA (*27*) of MEF1 binding site activity with equimolar amounts of in vitro–translated MyoD (lanes 2 to 8), E12 (lanes 1, 3 to 8, and 10), Mtwist (lanes 4, 5, 9, and 10), and Id (lanes 6 and 7). Lanes 5 and 7 contain double the amount of E12. M/E and T/E indicate the mobility of MyoD-E12 and Mtwist-E12 complexes, respectively, bound to the MEF1 oligomer.

Mtwist-E12 cotransfection repressed Gal4-MEF2 trans-activation of Gal4-CAT by ~70% (Fig. 2B). This finding, in conjunction with EMSA analysis which indicated that Mtwist does not significantly inhibit binding of MEF2C to its cognate binding site in either the absence or presence of E proteins (*12*), suggested that the transcriptional activation domain of MEF2 is inhibited by Mtwist. Myogenic bHLH proteins and members of the MEF2 family have recently been demonstrated to directly interact and thereby synergistically activate muscle gene expression (*18*). To assay whether Mtwist inhibits synergistic gene activation by MEF2 and MyoD, we assayed the effect of Mtwist on the activity of Gal4-MEF2 in the presence of MyoD-E12. MyoD-E12 cotransfection had no effect on Gal4-CAT in the absence of Gal4-MEF2; however, MyoD-E12 plus Gal4-MEF2 synergistically activated Gal4-CAT threefold over that observed with Gal4-MEF2 in the absence of MyoD (Fig. 2B). Cotransfection of Mtwist completely inhibited synergistic activation by MyoD-E12 plus Gal4-MEF2, whereas Id had no effect (Fig. 2B).

Inhibition of Gal4-MEF2 activity by Mtwist suggested that the transcriptional activation domain of MEF2 is inhibited by Mtwist. Therefore, we investigated whether addition of the VP16 transcriptional activation domain to MEF2C would render this transcription factor resistant to Mtwist-mediated inhibition. In contrast to wild-type MEF2C (Fig. 2A), the activity of MEF2C-VP16 was unabated by cotransfection of Mtwist in either the absence or presence of E12 (Fig. 2C). To test whether Mtwist repression of MCK-CAT expression was due to inhibition of MEF2 function, we evaluated whether cotransfection of MyoD with MEF2C-VP16, which is immune to inhibition by Mtwist, could reverse Mtwist-mediated repression of MCK-CAT. Indeed, addition of exogenous MEF2C-VP16, but not MEF2C, partially reversed Mtwist-mediated repression of MCK-CAT (Fig. 2D). We speculate that MEF2C-VP16 did not fully restore MCK-CAT activity in this assay because of residual titration of E proteins by Mtwist.

To define the regions of Mtwist that are required to inhibit either MEF2 or MyoD function (or both), we constructed various Mtwist mutants containing point mutations or deletions in either the bHLH domain or the highly conserved COOH-terminus of Mtwist. All of the Mtwist mutants were expressed in cells at approximately the same level as wild-type Mtwist (Fig. 1B). Introduction of a proline into the second helix (H2pro) of Mtwist, which prevents dimerization with E proteins (*12*), or deletion of either the basic domain (ΔB) or the conserved COOH-terminus (ΔC) of Mtwist reduced the ability of this protein to inhibit MEF2 trans-activation of MEF2x4-CAT (Fig. 3A). In contrast, introduction of a proline into the basic domain (Bpro) had little effect on Mtwist-mediated inhibition (Fig. 3A). These results suggest that both an intact bHLH motif and COOH-terminus are necessary for Mtwist-mediated transcriptional repression of a MEF2 reporter construct. DNA binding by Mtwist is not required to inhibit MEF2 activity because Mtwist (Bpro) is unable to bind DNA (*12*), yet inhibits MEF2 function.

Consistent with the ability of MyoD and Mtwist to either positively or negatively affect MEF2 function, both GST-MyoD and GST-Mtwist interacted with in vitro–translated MEF2, whereas GST-Id did not (*12*). Furthermore, Mtwist could be coimmunoprecipitated with MEF2C in extracts made from COS cells that had been co-

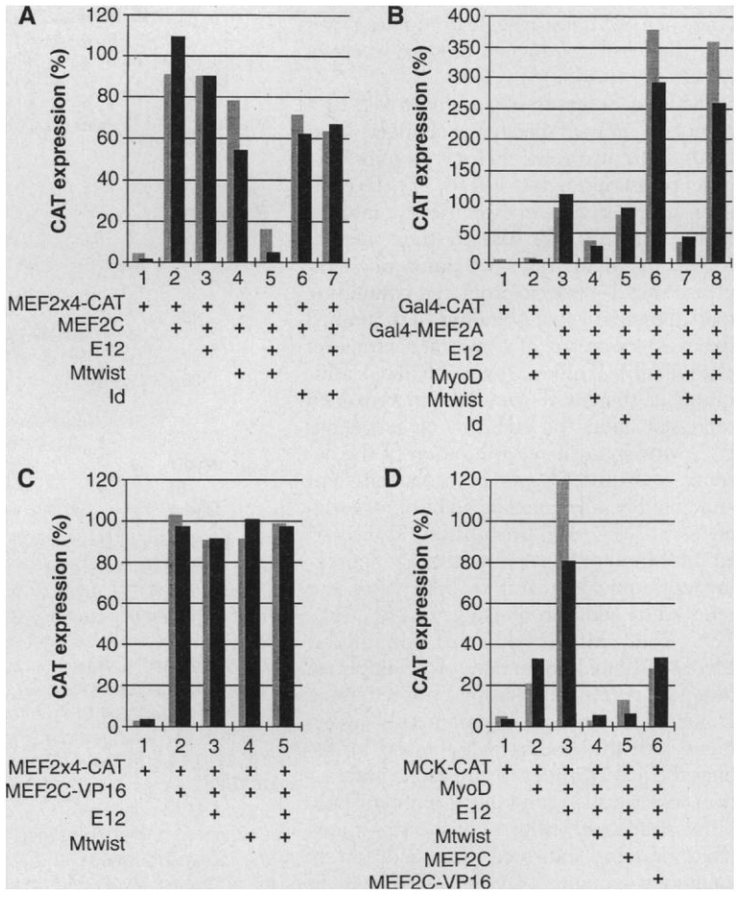

Fig. 2. Mtwist inhibits MEF2-mediated trans-activation. (**A**) 10T1/2 cells were transfected (*26*) with 0.5 μg of MEF2x4-CAT (lanes 1 to 7), 0.2 μg of CMV-MEF2C (lanes 2 to 7), 0.1 μg of CMV-E12 (lanes 3, 5, and 7), 0.05 μg of CMV-Mtwist (lanes 4 and 5), and 0.05 μg of CMV-Id (lanes 6 and 7). (**B**) 10T1/2 cells were transfected (*26*) with 0.5 μg of Gal4-CAT (lanes 1 to 8), 0.2 μg of CMV-Gal4-MEF2A (lanes 3 to 8), 0.1 μg of CMV-E12 (lanes 2 to 8), 0.1 μg of CMV-MyoD (lanes 2, 6 to 8), 0.05 μg of CMV-Mtwist (lanes 4 and 7), and 0.05 μg of CMV-Id (lanes 5 and 8). (**C**) 10T1/2 cells were transfected (*26*) with 0.5 μg of MEF2x4-CAT (lanes 1 to 5), 0.2 μg of CMV-MEF2C-VP16 (lanes 2 to 5), 0.1 μg of CMV-E12 (lanes 3 and 5), and 0.05 μg of CMV-Mtwist (lanes 4 and 5). (**D**) 10T1/2 cells were transfected (*26*) with 0.5 μg of MCK-CAT (lanes 1 to 6), 0.1 μg of CMV-MyoD (lanes 2 to 6), 0.1 μg of CMV-E12 (lanes 3 to 6), 0.05 μg of CMV-Mtwist (lanes 4 to 6), 0.2 μg of CMV-MEF2C (lane 5), and 0.2 μg of CMV-MEF2C-VP16 (lane 6).

transfected with epitope-tagged MEF2C and Mtwist (Fig. 3B). To determine whether the ability of various Mtwist mutants to inhibit MEF2 function correlated with their capacity to interact with MEF2, we immunoprecipitated epitope-tagged MEF2C and assayed for coprecipitation of the cotransfected Mtwist proteins. Both Mtwist (Bpro) and Mtwist (ΔB) interacted with MEF2C at least as well as wild-type Mtwist (Fig. 3B). In contrast, Mtwist (H2pro) and Mtwist (ΔC) were inefficiently coimmunoprecipitated with MEF2C (Fig. 3B). Thus, the HLH and COOH-terminus of Mtwist are required both for interaction with MEF2 and inhibition of MEF2 activity. The basic domain, however, is required for inhibition of MEF2 function but not for interaction with MEF2 (Fig. 3, A and B).

As another means of testing whether suppression of muscle differentiation by Mtwist correlated with inhibition of MEF2 function, we examined the effects of the Mtwist mutants on MyoD trans-activation of MCK-CAT. In the presence of exogenous E12, the same regions of Mtwist necessary for inhibition of MEF2 function (that is, the basic region, the HLH domain, and the COOH-terminus) were also required for inhibition of MCK gene expression (Figs. 3C and 4A). In contrast, in the absence of exogenous E12, only an intact Mtwist HLH domain was required to inhibit MCK transcription (Fig. 3D). Thus, when endogenous E proteins are rate-limiting, E protein titration by Mtwist is sufficient to inhibit myogenesis. However, when ectopic E proteins are supplied in excess, inhibition of myogenesis by Mtwist requires suppression of MEF2 trans-activation.

Finally, we investigated whether inhibition of myogenesis was a conserved function of Twist. In *Drosophila*, Twist has been characterized as a positive regulator of both gastrulation and mesoderm gene expression during embryogenesis (*9*, *19*); however, later in development Twist expression is specifically maintained in the precursor cells of the adult skeletal muscles before differentiation of these cells (*20*). It has recently been shown that *Drosophila* MEF2 (D-MEF2) is also expressed in these precursor cells (*21*) and that their differentiation is dependent on D-MEF2 activity (*22*). Therefore, we tested the effect of *Drosophila* Twist (Dtwist) expression on MEF2 activity using the assays described above. We found that Dtwist inhibited both MEF2C trans-activation of MEF2x4-CAT (Fig. 3A), and MyoD trans-activation of MCK-CAT in the presence of exogenous E protein (Fig. 3D), to the same extent as Mtwist, suggesting that negative modulation of both myogenic bHLH and MEF2 activities may be a conserved function of Twist.

We have shown that Mtwist, like Id, can titrate E proteins away from myogenic bHLH factors. However, in addition to E protein sequestration, Mtwist is also able to inhibit MEF2-mediated trans-activation. E protein interaction, but not DNA

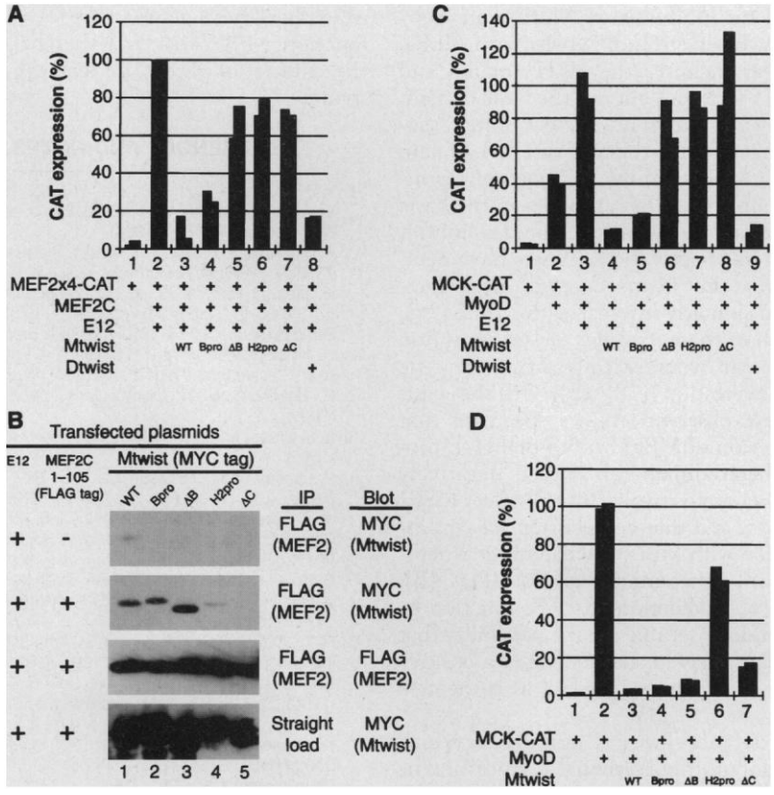

Fig. 3. The ability of Mtwist mutants to inhibit muscle gene expression in the presence of exogenous E proteins correlates with their ability to inhibit MEF2 function. (**A**) 10T1/2 cells were transfected (*26*) with 0.5 μg of MEF2x4-CAT (lanes 1 to 8), 0.2 μg of CMV-MEF2 (lanes 2 to 8), 0.1 μg of CMV-E12 (lanes 2 to 8), 0.05 μg of CMV-Mtwist WT (lane 3), CMV-Mtwist mutants (lanes 4 to 7), or CMV-Dtwist (lane 8). (**B**) COS cells were transfected with 0.5 μg of CMV-FLAG epitope–tagged MEF2C(1 to 105), 1.0 μg of CMV-E12, and 1.0 μg of CMV-MYC epitope–tagged Mtwist WT or mutants as indicated. Forty-eight hours after transfection, cell extracts were harvested, MEF2-associated proteins were immunoprecipitated with antibody to FLAG, and protein immunoblot analysis was performed to detect Mtwist (MYC tag) and MEF2 (1–105 FLAG) (*28*). The bottom panel contains total cell lysate equivalent to one-tenth the input amount of extract used in the immunoprecipitations. (**C**) 10T1/2 cells were transfected (*26*) with 0.5 μg of MCK-CAT (lanes 1 to 9), 0.1 μg of CMV-MyoD (lanes 2 to 9), 0.1 μg of CMV-E12 (lanes 3 to 9), and 0.05 μg of CMV-Mtwist WT (lane 4), CMV-Mtwist mutants (lanes 5 to 8), or CMV-Dtwist (lane 9). (**D**) 10T1/2 cells were transfected (*26*) with 0.5 μg of MCK-CAT (lanes 1 to 7), 0.1 μg of CMV-MyoD (lanes 2 to 7), and 0.05 μg of CMV-Mtwist WT (lane 3) or CMV-Mtwist mutants (lanes 4 to 7).

A

Mtwist protein		Binds to: E12	DNA	MEF2	Inhibits: MEF2-CAT	MCK-CAT
WT	bHLH	+	+	+	+	+
Bpro	P	+	-	+	+	+
ΔB		+	-	+	-	-
H2pro	P	-	-	-	-	-
ΔC		+	+	-	-	-

B

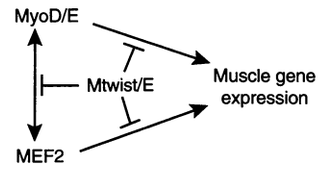

Fig. 4. Mtwist inhibits myogenesis by blocking the function of both MEF2 and myogenic bHLH transcription factors. (**A**) Summary of the activities of the Mtwist mutants. Mutants were assayed for heterodimerization with E12 (*12*), binding to a MEF1 site (*12*), binding to MEF2 protein (Fig. 3B), inhibition of MEF2x4-CAT in the presence of exogenous E12 (Fig. 3A), and inhibition of MCK-CAT in the presence of exogenous E12 (Fig. 3C). (**B**) The myogenic bHLH proteins are able to activate MEF2 transcription and synergistically activate muscle gene expression in cooperation with MEF2. Mtwist–E protein complexes act to inhibit the activity of myogenic bHLH factors and MEF2 by inhibiting DNA binding or trans-activation function, respectively. In addition, we speculate that Mtwist inhibits synergistic activation of downstream genes by myogenic bHLH and MEF2 family members by blocking the interaction between these two families of myogenic regulators.

binding, by Mtwist is necessary for both interaction with MEF2 and inhibition of MEF2 function. The basic domain and the COOH-terminus of Mtwist are also required for inhibition of MEF2 trans-activation. Interaction of Mtwist with MEF2, however, requires the HLH domain and COOH-terminus but not the basic domain of Mtwist. Together, these findings suggest that whereas the Mtwist HLH domain and COOH-terminus are required for interaction with MEF2, residues in the basic domain are specifically required to inhibit MEF2 trans-activation. Others have demonstrated that members of the MyoD family can similarly interact with MEF2 (*18*), and that these families of transcription factors can synergistically activate muscle gene expression (*18*, *23*). On the basis of these observations, we speculate that interaction of MEF2 with a bHLH–E protein heterodimer can either negatively (Mtwist) or positively (MyoD) affect MEF2 function, and that competition for this interaction with MEF2 will determine whether a cell differentiates or not (Fig. 4B). Negative modulation of MEF2 function by Mtwist does not rule out the possibility that this factor may in addition act as a positive regulator of gene expression, as is the case for Dtwist (*19*, *21*).

Vertebrate Twist is initially expressed throughout the epithelial somite and is subsequently excluded from the forming myotome (*10–13*). In contrast, high-level somitic expression of the myogenic bHLH and MEF2 family members is restricted to the myotome (*6*, *7*). Because myogenic differentiation is controlled by positive feedback regulation within and between the myogenic bHLH and MEF2 families of proteins (*3*), and low levels of the myogenic bHLH proteins are expressed in the presegmented mesoderm and epithelial somites (*6*), expression of Twist may act as a restraint to ensure that this positive regulatory loop is neither initiated prematurely nor in nonmyotomal tissues. Expression of Twist in the cranial neural crest (*10–12*) may similarly serve to restrain the activity of other bHLH proteins or MEF2 family members.

The phenotype of the Mtwist −/− mice has recently been reported; these mice die at around 10.5 to 11 days of gestation and show gross cranial neural tube defects (*24*). In addition, the somites are disorganized and show a high degree of apoptosis, consistent with a role for Mtwist in either promoting the survival or segregation of somitic cells to alternative fates (or both). Although low levels of myogenin are expressed in somites lacking Mtwist (*24*), it is not clear whether this expression is spatially normal (that is, restricted to the myotome). Furthermore, because other bHLH genes that share extensive homology to Twist are also expressed in the somite (*25*), it is possible that these genes share overlapping function with Twist and thereby temper the effects of Twist deficiency in the somite.

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26. 10T1/2 fibroblasts grown in 10% Fetal Clone II (Gibco, BRL) were transfected with lipofectamine (Gibco/BRL) according to the manufacturers directions. After 24 hours, the media was changed to one containing 2% horse serum and insulin (12 mg/ml). Forty-eight hours later, cells were harvested for CAT assay essentially as described [C. M. Gorman *et al.*, *Mol. Cell. Biol.* **2**, 1044 (1982)]. All experiments for CAT expression were repeated at least three times with similar results. Except where indicated, CAT activity is expressed as percent CAT expression observed in the absence of ectopic Mtwist or Id. Plasmid constructs containing the cytomegalovirus (CMV) promoter for transfections were as follows: MyoD, Id, Dtwist, and Mtwist derivatives were subcloned into pCS2+ or pCS2+ (6mt) containing six MYC epitope tags at the NH_2-terminus; mouse E12 was subcloned into the CMV expression vehicle pCSA [J. F. Martin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5282 (1993)]; and pCG-Gal4-XMEF2A contains *Xenopus* MEF2A subcloned into pCG-Gal4 [M.-W. Wong *et al.*, *Dev. Biol.* **166**, 683 (1994)]. Reporter plasmids contained the CAT gene driven by 3300 bp of the muscle creatine kinase promoter (MCK-CAT) [J. Janes *et al.*, *Mol. Cell. Biol.* **8**, 62 (1988)], four reiterated MEF1 sites driving the thymidine kinase promoter (MEF1x4-CAT) [H. Weintraub *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5623 (1990)], four reiterated MEF2 sites driving the minimal *Xenopus* MyoD1a promoter (MEF2x4-CAT) [M.-W. Wong *et al.*, *Dev. Biol.* **166**, 683 (1994)], and five Gal4 sites driving the E1b promoter (Gal4-CAT). Mutations were introduced into the Mtwist protein through use of the Sculptor mutagenesis kit (Amersham). Mtwist mutations: Bpro, Arg^{114} to Pro; ΔB, deletion of Gln^{113} to Arg^{124}; H2pro, Arg^{158} to Pro; ΔC, Gln^{168} to stop codon.
27. EMSAs were done as described [A. B. Lassar *et al.*, *Cell* **66**, 305 (1991)].
28. 10T1/2 fibroblasts or COS cells were transfected and cultured as described (*25*). Immunoprecipitations and protein immunoblot analysis of cell extracts were done as described [S. X. Skapek *et al.*, *Science* **267**, 1022 (1995)]. Antibodies used were a hybridoma supernatant to the MYC epitope (9E10) or a monoclonal antibody to the FLAG epitope (M2, Kodak).

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