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Daxx Inhibits Muscle differentiation by Repressing E2A-Mediated Transcription

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ABSTRACT

The basic helix-loop-helix (HLH) E2A transcription factors bind to DNA as homodimers or as heterodimers formed with other basic HLH factors, activate gene expression, and promote differentiation of muscle, lymphoid, neuronal, and other cell types. These E2A functions can be inhibited by the Id proteins, HLH factors that sequester E2A in non-DNA binding dimers. Here we describe the direct interaction of E2A with Daxx, a broadly expressed non-HLH protein previously associated with apoptosis and transcriptional repression. Daxx inhibits E2A function, but not via an Id-like mechanism; rather, it recruits histone deacetylase activity to E2A-dependent promoters. Increased Daxx expression during muscle differentiation inhibits E2A-dependent expression of key myogenic genes and reduces myotube formation, while decreased Daxx expression promotes myotube formation. These results identify a new mechanism for limiting E2A activity and establish a link between Daxx-mediated gene regulation and control of cellular differentiation. J. Cell. Biochem. 107: 438–447, 2009.

KEY WORDS: MYOGENESIS; TRANSCRIPTIONAL REGULATION; HISTONE DEACETYLASE

E12 and E47, products of the E2A gene, are transcription factors of the basic helix-loop-helix (bHLH) protein family [Massari and Murre, 2000] that activate gene expression through binding to the E-box sequence (CANNTG) in the regulatory regions of target genes. This binding requires that E2A proteins pair with each other or with other bHLH proteins [Murre et al., 1989b]. Dimer formation is mediated by the HLH motif, so the basic regions of each protein can cooperatively bind to DNA [Murre et al., 1989a], while the potent N-terminal transactivation domains [Henthorn et al., 1990] can interact with the RNA polymerase II transcriptional complex. E2A activity is negatively regulated by interaction with the Id proteins, which contain a HLH dimerization motif but lack the basic DNA-binding region [Benezra et al., 1990]. Hence, Id-E2A heterodimers are unable to bind DNA, so E2A complexed with Id is effectively sequestered [Sikder et al., 2003].

Although the E2A proteins are broadly distributed, expression of their target genes is restricted in many instances to specific cell lineages. This is accomplished through the formation of heterodimers containing E2A and tissue-restricted bHLH factors [Massari and Murre, 2000], which activate cell-type specific gene expression and promote cellular differentiation. This lineage-selective mechanism has been described in many organs [Guillemot et al., 1993; Cserjesi et al., 1995; Hollenberg et al., 1995; Porcher et al., 1996; Naya et al., 1997; Firulli et al., 2000; Firulli, 2003; Ross et al., 2003], but is most clearly elucidated in the differentiation of skeletal muscle [Weintraub, 1993; Arnold and Winter, 1998]. During myogenesis, E2A proteins pair with the muscle-specific bHLH transcription factors MyoD, Myf5, myogenin, and MRF-4, collectively referred to as muscle regulatory factors (MRFs). These heterodimers yield a hierarchical network of transcriptional activators [Neuhold and Wold, 1993; Weintraub, 1993; Arnold and Winter, 1998] that drives the expression of key muscle-specific genes to promote the development of precursor cells (myoblasts) into multinucleated and differentiated muscle cells (myotubes). The pool of E2A proteins available for heterodimerization with the MRFs is limited by Id protein-mediated sequestration, which restricts MRF activity and muscle differentiation. Several additional factors regulate myogenesis [Puri and Sartorelli, 2000], but relatively little is known about interactions, other than the Id proteins, that target E2A function directly.

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Daxx is a protein originally identified as a regulator of cell death and cell survival. The exact nature of this Daxx function remains controversial: Daxx overexpression promotes apoptosis in some cell types in vitro [Yang et al., 1997; Chang et al., 1998; Perlman et al., 2001], but Daxx deficiency leads to early embryonic demise that points to an anti-apoptotic role essential for normal development [Michaelson et al., 1999]. More recently, Daxx has been implicated in the regulation of gene expression through interactions with the DNA-binding transcription factors Pax3, Pax5, and Ets-1. Interaction with Daxx inhibits the transcriptional activity of Pax3 and Ets-1 [Li et al., 2000b; Hollenbach et al., 2002], but potentiates the transcriptional activity of Pax5 in some cell lines [Emelyanov et al., 2002]. Although Pax and Ets proteins have been implicated in the differentiation of multiple cell types, the effect of Daxx on their transcriptional activities has not been linked to an effect on cellular differentiation.

Here, we report that Daxx interacts directly with the E2A proteins and inhibits their function as transcriptional activators. In contrast to the Id proteins, Daxx appears to interact with the E2A proteins at E2A-dependent promoter sites, recruiting HDACs to these DNA regions to repress gene activity. Notably, in C2C12 cells, we find that Daxx opposes both muscle gene expression and muscle differentiation. Consistent with chromatin immunoprecipitation (ChIP) studies, which indicate that the association of Daxx with muscle gene promoters is regulated during the course of myogenesis, increased Daxx expression reduces myotube formation and decreases expression of key E2A-dependent muscle-specific genes. Conversely, inhibition of Daxx expression by RNA interference promotes muscle differentiation. Altogether, these findings suggest that Daxx may be a physiologic regulator of E protein-mediated myogenesis, thereby providing the first example of a role for Daxx in the control of cellular differentiation.

MATERIALS AND METHODS

IDENTIFICATION OF RDAXX

rDaxx was identified through a yeast two-hybrid screen for E12-interacting proteins, as described previously [Kho et al., 1997]. In addition to LexA, E12-LexA, and E47-LexA, we characterized interactions with LexA-Id3, LexA-Max, and LexA-OCT-1 bait proteins. To improve specificity, expression of AD-rDaxx (613–730) was controlled by a galactose-inducible promoter. Yeast colonies (strain EGY48/pSH18-34) were grown at 37°C on Ura(-)His(-)Trp(-) plates, containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, X-galactose, and either 2% dextrose or 2% galactose. Colony color reflective of β-galactosidase activity was assessed after 48 h.

5′ RACE (Invitrogen, Carlsbad, CA) was performed with total rat spleen RNA as described by the manufacturer. Primer sequences used for cDNA synthesis and subsequent nested PCR reactions were: 5′-GGAGCTCTGTCCATCATTTGC-3′, 5′-TCATGGCTGGGAGAGTC-CACC-3′, and 5′-TTATCTTCCACCCACTGTCCT-3′, corresponding respectively to the reverse complements of sequences 2319–2339, 2101–2121, and 2016–2036 of the full-length rDaxx cDNA. We generated full length rDaxx, rDaxxC (rDaxx 619–730), and rDaxxΔ613–654 cDNAs by standard PCR and mutagenesis techniques; products were ligated into pCR3 (Invitrogen), sequenced in both directions, and tested by in vitro transcription/translation.

IN VITRO BINDING ASSAYS

rDaxx deletion constructs were generated by PCR-based techniques, cloned into the pGEX-2T vector (Amersham Biosciences, Piscataway, NJ), and expressed as GST fusion proteins along with the human E12-fragment (aa 477–654). [^{35}S]-labeled full-length rDaxx and E47 protein were produced with the TNT T7 System (Promega, Madison, WI). GST fusion proteins and glutathione beads were incubated with radiolabeled proteins in 50 mM NaCl/BSA for 30 min at 4°C, and washed with 0.1% Nonidet P-40 in phosphate buffered saline. Retained proteins were fractionated on 12% SDS–polyacrylamide gels, which were fixed, stained with Coomassie Blue, and exposed to Kodak XAR film.

CELL CULTURE

NIH 3T3 fibroblasts and C2C12 myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% or 15% fetal bovine serum (FBS, HyClone, Logan, UT), respectively. U266 lymphoblastoid cells were maintained in RPMI 1640 (Invitrogen) supplemented with 15% FBS.

IMMUNOPRECIPITATION

Nuclear extracts were prepared as described [Sibinga et al., 1999], and precleared nuclear protein (200 μg) was incubated with E2A antibody (1 μg, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C. After 1 h, complexes were recovered with an agarose-conjugated secondary antibody, washed with RIPA buffer, resolved by SDS–PAGE, and transferred to Immobilon-P (Millipore, Billerica, MA) filters. After blocking in TBST (Tris pH 8.0, NaCl 150 mM, and .05% Tween-20) plus 4% milk, filters were incubated overnight at 4°C with rabbit anti-Daxx antisera (0.5 μg/ml, Santa Cruz; or 1:5,000 dilution of antiserum raised against GST-rDaxx (610–730)), followed by secondary antibody at room temperature. After washing, signals were identified by enhanced chemiluminescence (Amersham).

REPORTER GENE ANALYSES

The μE-luc reporter plasmid ([μE5 + μE2 + μE3]4 TATA-luciferase) [Ordentlich et al., 1998] was provided by T. Kadesch. The MCK promoter was cloned into the pGL3 basic luciferase reporter from a −3300 MCK-CAT construct [Johnson et al., 1989] provided by S. Hauschka. NIH 3T3 fibroblasts were transfected either with the μE-luc reporter, E47 (or E12), and rDaxx (or rDaxxC), or with the MCK reporter, E12, MyoD and rDaxx (or rDaxxC). The total amount of expression plasmid was kept constant by addition of the parent vector pcDNA3 (Invitrogen). For the HDAC inhibitor experiments, trichostatin A or sodium butyrate were added 4 h after transfection to final concentrations of 100 nM and 5 mM, respectively. Cellular lysates were harvested 24 h after transfection and assayed for luciferase activity using the Glo-lysis buffer system (Promega) and a Victor 2 luminometer (Wallac/Perkin Elmer, Waltham, MA). Luciferase activities were corrected for variation in transfection efficiency relative to β-galactosidase activities. The data shown represent transfections repeated at least 3 times each.

ELECTROMOBILITY SHIFT ASSAY

rDaxx, rDaxxC, and rDaxxΔ613–654 cDNAs were cloned into the IRES-containing retrovirus vector green fluorescent protein (GFP)-RV [Ranganath et al., 1998], a gift from K. Murphy, to generate rDaxx-GFP-RV and rDaxxC-GFP-RV. The Phoenix-Eco packaging cells, provided by G. Nolan, were transfected as described on the Nolan lab website (www.stanford.edu/group/nolan/protocols/pro_helper_dep.html). NIH 3T3 cells were transduced with RDaxx, rDaxxC, or control GFP-RV retroviruses to generate stable cell lines. Nuclear proteins were extracted and assayed (5 μg) for binding to a ^{32}P-labeled double stranded probe (5′-AGAACACCTGCAGCAT-3′ plus its complement) corresponding to the μE5 E-box (underlined) [Ordentlich et al., 1998]. Specificity of E-box binding was confirmed using a 100-fold excess of unlabeled E-box competitor and supershift assays (data not shown).

C2C12 CELL DIFFERENTIATION AND ANALYSIS

C2C12 cells were transduced overnight with retroviral supernatants supplemented with 8 μg/ml polybrene, and then differentiated by replacing growth medium with DMEM supplemented with 2% or 10% horse serum (Invitrogen). Total cellular RNA was prepared with RNAeasy (Qiagen, Valencia, CA), and Northern analysis was performed with probes for MCK, MEF2C, myogenin, MyoD, and GAPDH generated by RT-PCR. For immunocytochemistry, cells were plated on Type I collagen-coated slips (Becton Dickinson, Franklin Lakes, NJ), fixed in 4% paraformaldehyde, and stained for nuclei with DAPI (Invitrogen). Myosin heavy chain and Daxx were identified with antibody MF-20 (1:200, Developmental Studies Hybridoma Bank, University of Iowa) and rabbit anti-Daxx antiserum (1:1,000, Santa Cruz Biotechnology), respectively, followed by Alexa 546-conjugated secondary antibodies (4 μg/ml, Invitrogen).

Hairpin siRNAs [Yu et al., 2002] targeted two distinct sites in mDaxx: siRNA −8 included bases −8 to +12 (GAACCCCCAUGGCCACCGAU), and siRNA +47 included bases +47 to +66 (AUGAAGCUGCUGCUCAACCA). We synthesized DNA oligonucleotides corresponding to these sequences and their reverse complements, separated by an intervening loop (GAGTACTG) bearing a *Sca* I site, and *Xho* I- and *Xba* I-compatible sequences at the 5′ and 3′ ends, respectively. Complementary oligonucleotides were annealed and ligated into pSuppressorRetro (Imgenex, San Diego, CA). The resulting plasmids were tested in transient transfection together with pBB14 (provided by L. W. Enquist), which encodes a US9-GFP fusion protein (Fig. 5A), and then packaged into retroviral particles. Immunoblotting of cellular lysates (20 μg) employed rabbit anti-Daxx antiserum (0.5 μg/ml, Santa Cruz Biotechnology) and mouse anti-tubulin (1:5,000, Sigma).

CHROMATIN IMMUNOPRECIPITATION (CHIP) ANALYSIS

ChIP was performed with a kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's directions. In brief, C2C12 cells were maintained in DMEM/15% FBS or switched to DMEM/10% horse serum for differentiation. Cellular monolayers were cross-linked with 1% formaldehyde at 37°C for 10 min and then washed with PBS supplemented with protease inhibitors. Cellular lysates from 1×10^6 cells each were sonicated to yield DNA fragments 200–1,000 bp in size, and 20% of each sample was set aside as an input control. The remaining samples were precleared by incubation with salmon sperm DNA and BSA-blocked protein A-agarose slurry and immunoprecipitated with (1) no antibody, (2) anti-histone H3 antibody (Cell Signaling, Danvers, MA, 20 μl), or (3) rabbit anti-Daxx antiserum (4 μl). Immune complexes were recovered with protein A agarose, washed extensively, and eluted. Crosslinks were reversed with addition of NaCl to 200 mM and incubation at 65°C for 4 h, and samples were treated with Proteinase K (100 μg/ml) and extracted with phenol-chloroform. DNA pellets were dissolved in 10 mM Tris pH 8.0 and used in semi-quantitative PCR reactions. Primers for amplification of the MCK enhancer (−1229/−1007) and GAPDH promoters were as described [Iezzi et al., 2002]. Primers for the MEF2C promoter, 5′-CTAACAGTGTAGAGGCTTGG-3′ and 5′-TGATCCCTCTGCACAAGTGT-3′, encompassed bases −158 to +4, including the functionally significant E-box element [Wang et al., 2001; Dodou et al., 2003]. Amplified products were analyzed in 2% agarose gels stained with ethidium bromide.

DATA ANALYSIS

Comparisons among groups were made by factorial analysis of variance followed by a Bonferroni/Dunn post hoc analysis. Statistical significance was accepted for a P value ≤ 0.05.

Results

THE INTERACTION OF DAXX AND E2A IS SPECIFIC AND DIRECT

To identify novel molecules capable of regulating E2A activity, we performed a yeast two-hybrid screen for E12-interacting proteins represented in an oligo(dT)-primed rat cDNA library. The bait, E12 (477–654), included the bHLH domain, but lacked transactivating sequences, and was fused to a LexA DNA-binding domain [Kho et al., 1997]. The cDNA sequence of one interacting clone showed a high degree of similarity to the 3′ ends of mouse and human Daxx [Yang et al., 1997], and we determined that it encoded 118 aa at the carboxyl terminus of rat Daxx (rDaxx). We used rapid amplification of cDNA ends (RACE) to obtain a full-length rDaxx cDNA (submitted to Genbank) that encodes a 730 aa protein. This deduced sequence is 73% identical and 81% similar to human Daxx, and 83% identical and 87% similar to mouse Daxx. To assess the specificity of the Daxx–E12 interaction, we fused rDaxx (613–730) to a B42 transcriptional activation domain (AD), and tested this AD-rDaxx (613–730) fusion protein for interaction in the yeast system, with the products of different LexA-bound DNA-binding bait constructs. While AD-rDaxx (613–730) interacted strongly with E12 and E47, we found no interaction with LexA itself or with other bait proteins, including the HLH protein Id3, the bHLH-Zip protein Max-1 or the POU homeodomain protein OCT-1 (Fig. 1A).

To assess whether the Daxx–E2A interaction is direct or indirect, we conducted in vitro binding assays. In vitro-translated [^{35}S]-labeled rDaxx (613–730) bound to GST-E12 (477–654), but not to GST alone (Fig. 1B, left). Reciprocal experiments with radiolabeled E2A proteins and GST-rDaxx constructs (Fig. 1B, right) further demarcated the E2A-interacting domain within rDaxx, as GST-rDaxx (613–654), but not GST-rDaxx (654–730), was able to bind to

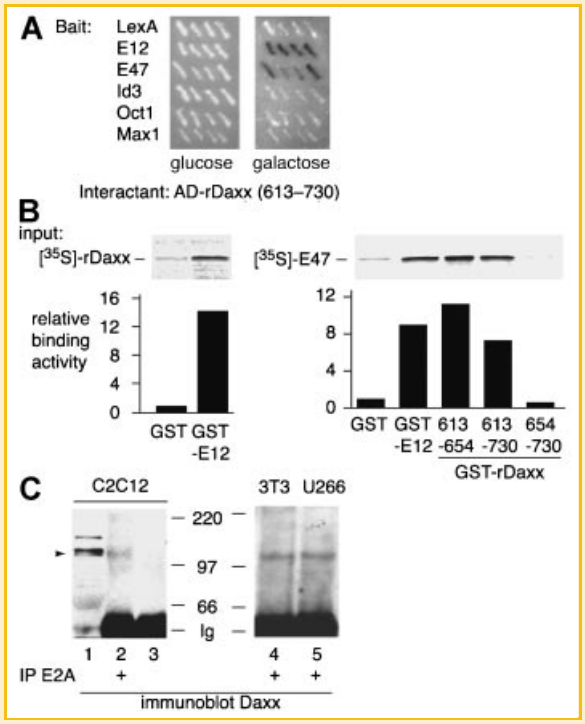

Fig. 1. The interaction of rDaxx with E2A is selective, direct, and occurs in vivo. A: Yeast two-hybrid analysis of the interaction between AD-rDaxx (613–730), a C-terminal fragment of rDaxx fused to a transcription activation domain, and a LexA DNA binding cassette or transcription factors E12, E47, Id3, Oct1, and Max1 fused to LexA. AD-rDaxx (613–730) expression was controlled by a galactose-inducible promoter, and the interactions were assessed on glucose and galactose-containing media. Dark hatches (assayed in quadruplicate) indicate β-galactosidase activity after 48 h of incubation. B: Binding of [^{35}S]-labeled proteins to GST fusion constructs retained by glutathione-Sepharose beads. Left: [^{35}S]methionine-labeled rDaxx was incubated with GST or GST-E12(477–654). Right: [^{35}S]methionine-labeled E47 was incubated with GST, GST-E12, or the indicated GST-rDaxx deletion constructs. Retained proteins were resolved by 15% SDS–PAGE, with equivalency of loading confirmed by Coomassie Blue staining (not shown). Binding activity was quantified by phosphor imaging. C: Nuclear protein extracts from C2C12 myoblasts (left), NIH 3T3 fibroblasts, and U266 B-lymphoblastoid cells (right), were analyzed by immunoprecipitation and immunoblotting. Lane 1 (immunoblotting for Daxx protein), 10% of input used for lanes 2 and 3; lanes 2–5, immunoprecipitation using control antiserum (lane 3) or anti-E47 antiserum (lanes 2, 4, 5), followed by immunoblotting for Daxx.

E47. Thus, the E2A proteins and Daxx bind directly to each other, and amino acids 613–654 of rDaxx are sufficient for this interaction.

To test for interaction of endogenous Daxx and E2A, we performed co-immunoprecipitation assays on nuclear extracts. We detected a Daxx–E2A complex in several cell types, including C2C12 myoblasts, NIH 3T3 fibroblasts, and U266 B-lymphoblastoid cells (Fig. 1C). Although we identified several Daxx isoforms with apparent molecular weights ranging from 70 to 130 kDa in conventional immunoblotting (Fig. 1C, lane 1), the major Daxx isoform that interacted with the E2A proteins had an apparent molecular weight of ~100 kDa (Fig. 1C, lanes 2, 4, and 5). The preferential co-immunoprecipitation of a specific Daxx isoform with E2A suggests that the interaction may be affected by post-translational modifications of the Daxx protein. A previous report showed that Pax3 interacts selectively with a 70 kDa Daxx isoform, and attributed the distinct mobilities of Daxx isoforms to different phosphorylation states [Hollenbach et al., 1999]. These findings suggest that different isoforms of Daxx may have distinct interaction partners.

DAXX REPRESSES E2A-MEDIATED TRANSCRIPTIONAL ACTIVITY

We next asked whether Daxx affected E12 and E47 function. The μE-luciferase (μE-luc) reporter is activated by E2A protein dimers [Ordentlich et al., 1998]. We transfected NIH 3T3 cells with μE-luc, E12 or E47, and/or rDaxx expression plasmids, and measured luciferase activity to quantify E2A-dependent transcription. Results with E47 (Fig. 2A) and E12 (data not shown) were similar. An increase in E47 expression plasmid resulted in a progressive and robust increase in reporter gene activity. Cotransfection of a fixed amount of rDaxx, however, reduced promoter activation by 50–68% (Fig. 2A). In contrast, co-transfection with the N-terminal deletion construct rDaxxC did not affect E47-mediated activation. rDaxxC, the ortholog of the mouse deletion construct DaxxC [Yang et al., 1997], contains the C-terminal 112 aa of rDaxx that harbor the E2A binding domain (Fig. 1B), but lacks N-terminal sequences including the acidic, coiled-coil, and paired amphipathic helical domains [Hollenbach et al., 1999]. In reciprocal experiments, rDaxx, but not rDaxxC, significantly limited E2A-dependent transcription across a spectrum of concentrations (Fig. 2B). At the highest dose tested, rDaxx decreased the activities of E47 by 65% (Fig. 2B, left) and E12 by 80% (data not shown). Similar findings were observed in NIH 3T3 cells transduced with retroviruses encoding Daxx or DaxxC (Fig. 2B, right). Hence, Daxx, but not DaxxC, can repress E2A-mediated transcription. Notably, while the C-terminal sequences of Daxx are sufficient for the interaction with E2A (Fig. 1), transcriptional repression also requires the N-terminal region of Daxx.

We then investigated whether Daxx could repress transcription mediated by dimers containing E2A and other bHLH proteins. Expression of muscle creatine kinase (MCK) has been studied extensively as a marker of muscle differentiation. E2A-MyoD heterodimers bind to critical E-box elements in the MCK promoter region to drive transcription, so we tested the effect of Daxx on E2A-MyoD-mediated MCK-luciferase (MCK-luc) promoter [Johnson et al., 1989] activity (Fig. 2C). While co-transfection of MyoD and E12 in NIH 3T3 cells resulted in luciferase activity that was more than 20-fold greater than baseline, promoter activity was reduced by 68–78% when rDaxx was included in the transfection. Thus Daxx, a non-HLH protein, interacts directly with E2A, and represses transcription mediated not only by E2A dimers, but also by dimers containing E2A proteins and a cell-type specific bHLH transcription factor.

REPRESSION OF E2A-DEPENDENT TRANSCRIPTION BY DAXX REQUIRES HDACS

The Id proteins inhibit E2A through a direct interaction that prevents DNA binding [Benezra et al., 1990; Jen et al., 1992]. In view of the negative effect of Daxx on E2A activity, we wanted to know if the Daxx–E2A interaction also interfered with E2A-DNA binding. To

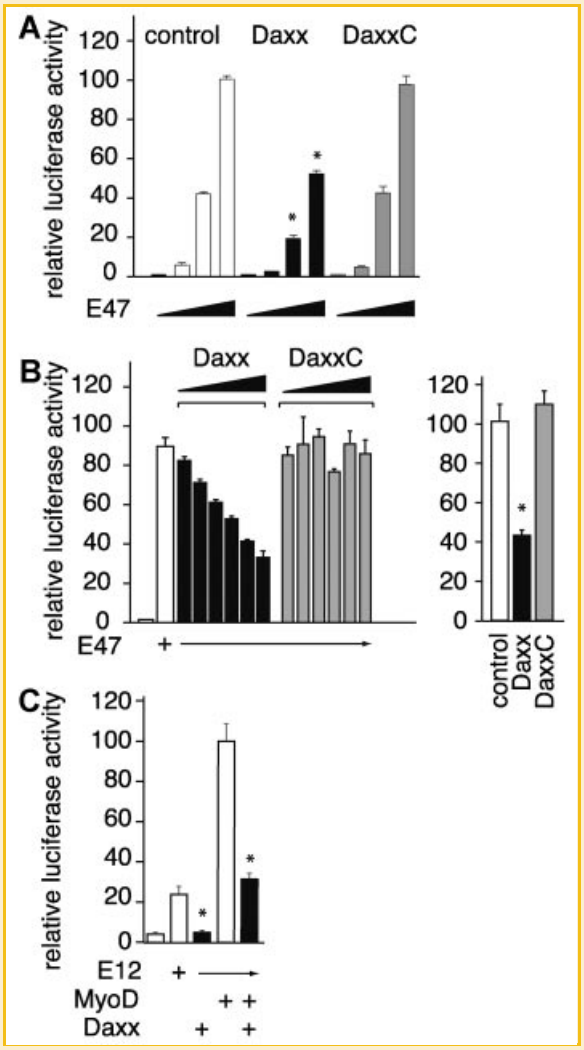

Fig. 2. Daxx represses E2A-dependent transcription. A: NIH 3T3 fibroblasts were transfected with E47 expression plasmid (0, 3, 10, or 30 ng), μE-luc (E-box) reporter construct (200 ng), and rDaxx, rDaxxC (aa 619–730), or control expression plasmids (400 ng). Bars indicate the standard error. *, $P \leq 0.0001$ vs. respective control. Maximal reporter activity in the absence of rDaxx was set to 100. B (left): NIH 3T3 fibroblasts were transfected with an E47 expression construct (10 ng), E-box reporter construct (200 ng), and a range of rDaxx or rDaxxC expression constructs (0, 3, 10, 30, 100, 200, or 400 ng). Activity with E47 alone was set to 100. Activities for rDaxx concentrations of 3 ng or greater were all significantly different ($P < 0.0005$) from E47 alone. Right: Stable NIH 3T3 lines generated with control (open bar), full-length rDaxx (black), or C-terminal fragment rDaccC (gray) retroviruses, were transfected with E-box reporter (300 ng) and E12 expression plasmid (10 ng) and subjected to luciferase assays. C: NIH 3T3 fibroblasts were transfected as indicated with expression plasmids for E12 and MyoD (100 ng each), with or without rDaxx (200 ng), along with the −3300 MCK-luc reporter (200 ng). Maximal reporter activity was set to 100. *$P \leq 0.0003$ versus activity without rDaxx.

test this possibility, we evaluated E-box binding in nuclear proteins extracted from the retrovirally transduced NIH 3T3 cells in which we had confirmed Daxx-, but not DaxxC-, mediated inhibition of E2A activity (Fig. 2B, right). Despite this functional difference between Daxx and DaxxC, gelshift assays showed that E-box-specific binding activity was similar in nuclear extracts from rDaxx, rDaxxC and control sublines (Fig. 3A). These findings indicate that Daxx inhibits E2A without decreasing E2A-DNA binding, and thus points to a mechanism of E2A regulation distinct from that used by the Id proteins.

Inhibition of histone acetylation has emerged in recent years as a major mechanism of transcriptional repression, but this mechanism has not been linked to regulation of E2A activities. Histone deacetylases (HDACs) are recruited to transcription factor complexes that are already bound to target genes. There, they remove acetyl groups from key histone amino acid residues and prevent gene expression by keeping the chromatin structure in a repressed state [McKinsey et al., 2001]. Recent reports have described interactions of Daxx with HDAC 1 [Li et al., 2000a] and 2 [Hollenbach et al., 2002]. HDAC inhibitors can limit Daxx-mediated transcriptional repression when Daxx is tethered to DNA as a fusion protein [Li et al., 2000a]. However, this effect has not been demonstrated for complexes between Daxx and native transcription factors. For example, while Daxx represses Pax3 transcription, this repression was not relieved by HDAC inhibitors [Hollenbach et al., 1999]. To determine whether Daxx-mediated repression of E2A requires HDACs or works via another mechanism, we tested its dependence on HDAC activity. Two different HDAC inhibitors, trichostatin A and sodium butyrate, decreased the inhibitory effect of Daxx on μE-luc activation by 85% and 42%, respectively (Fig. 3B). Our finding suggests that Daxx represses E2A activity, at least in part, by recruiting HDACs to E2A-dependent target sequences.

DAXX REPRESSES THE DIFFERENTIATION OF C2C12 MYOBLASTS INTO MYOTUBES

Expression of key muscle differentiation genes is driven by heterodimers formed by E2A and the MRFs [Neuhold and Wold, 1993; Weintraub, 1993; Arnold and Winter, 1998; Berkes and Tapscott, 2005]. Because Daxx repressed E2A-MyoD-mediated activation of the MCK promoter (Fig. 2C), we hypothesized that Daxx might regulate the timing or extent of muscle differentiation. To test this possibility, we used the established C2C12 myoblast culture system. Reduction of growth factor concentrations causes these cells to fuse and differentiate into multinucleate myotubes that express the key muscle-specific genes observed in differentiated muscle in vivo [Silberstein et al., 1986]. We transduced growing C2C12 cells at high efficiency (>85%) with control (GFP) retrovirus, or with retrovirus bearing full-length or mutated rDaxx sequences, and then cultured these retroviral sublines for 3–4 days in differentiation medium. In cells transduced with retrovirus encoding the C-terminal fragment rDaxxC, or rDaxxΔ613–654, which lacks the E2A-interaction domain identified through in vitro binding reactions (Fig. 1B), myotube formation was similar to that of control cells (Fig. 4A, left). In contrast, myotube formation was reduced decidedly in full-length rDaxx-transduced cells compared to control cells (Fig. 4A, left). This effect was more pronounced in cultures differentiated in 10% versus 2% horse serum (Fig. 4A, right). Staining for myosin heavy chain (MHC), a muscle-specific structural protein, also indicated decreased myotube formation in full-length rDaxx-transduced cells, as many MHC-positive cells appeared small and mononuclear (Fig. 4B). Moreover, expression of muscle-specific gene transcripts was diminished in the presence of increased full-

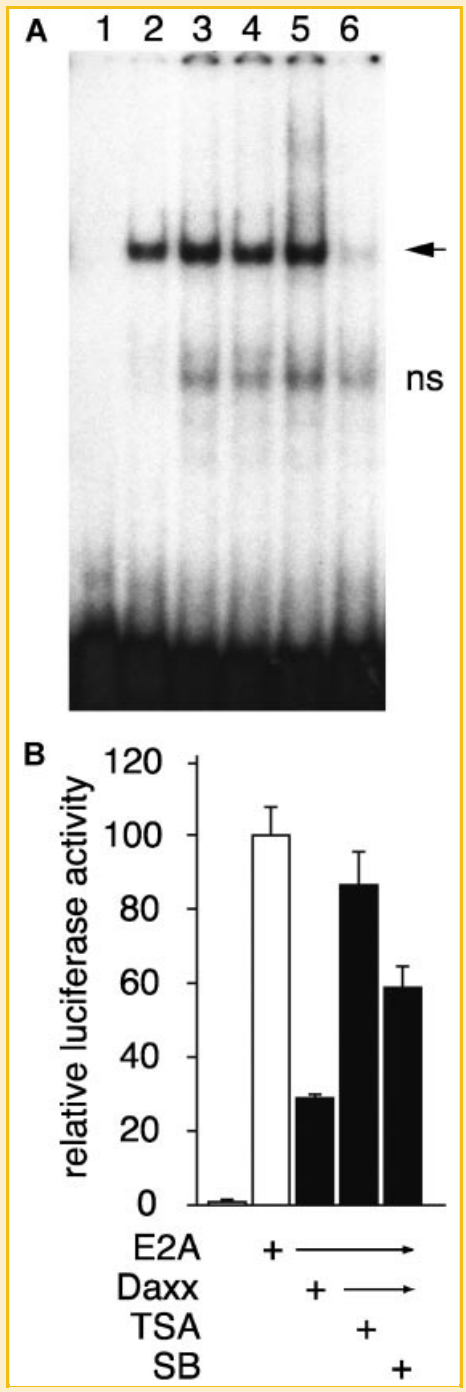

Fig. 3. Daxx inhibits transcription through a histone deacetylase-dependent mechanism. A: Nuclear extracts from the stable 3T3 lines were tested in an electromobility shift assay. Arrow indicates specific E-box binding activity; ns, a non-specific band. Lane 1, free μE5 probe; lanes 2–6, nuclear extracts from: lane 2, 3T3; lane 3, 3T3-GFP-RV; lane 4, 3T3-rDaxx-RV; lane 5, 3T3-rDaxxC-RV; lane 6, 3T3-GFP-RV, with 100-fold excess of cold E-box competitor. B: Transient transfection assays of μE-luc reporter activity in NIH 3T3 cells. Open bar, with E12 alone; solid bars, with E12 and rDaxx expression plasmids. Trichostatin A (TSA, 100 nM) or sodium butyrate (SB, 5 mM) were added 4 h after transfection, and cells were harvested after 24 h.

length Daxx expression (Fig. 4C); notably, this was not only seen for *mck*, but also with mRNAs encoding the essential myogenic transcription factors MEF2C, myogenin, and MyoD. Thus, increased Daxx expression can inhibit the phenotypic differentiation of muscle, as reflected in the reduced expression of genes critically involved in the myogenic transcriptional regulatory hierarchy.

To assess directly the effect of Daxx on E2A-dependent transcription in C2C12 cells, we determined the activity of the MCK-luc reporter in the retrovirus-transduced sublines at day 1 and day 4 of differentiation. As expected, promoter activity was very low in both control (GFP) and rDaxx-retroviral sublines at day 1 (Fig. 4D). After 4 days of differentiation, however, promoter activity had increased more than 25-fold in control cells, but only 3-fold in the rDaxx-transduced cultures (Fig. 4D, black bars). This result indicates that the reduced expression of E-box containing muscle-specific genes (Fig. 4C) results from Daxx-mediated repression of promoter activation. The effect was not due to impaired cell viability, as co-transfection of MyoD and E12 rescued MCK-luc activity in these cells (Fig. 4D, gray bars).

Together with the gel shift and HDAC inhibitor results, these findings suggested that Daxx may associate with native E2A-MRF heterodimeric complexes already bound to muscle gene promoters, including those that drive expression of MCK and MEF2C (Fig. 4C). To test this possibility, we performed chromatin immunoprecipitation (ChIP) studies throughout a time course of muscle differentiation in the C2C12 system. We crosslinked and extracted chromatin from growing myoblasts or myoblasts differentiating in culture, and used anti-Daxx antibody to recover Daxx-associated genomic DNA. PCR was performed with primers encompassing known essential E-box regions in both the MCK and MEF2C promoters [Wang et al., 2001; Iezzi et al., 2002; Dodou et al., 2003]. As shown in Fig. 4E, Daxx was associated at low levels with both promoters in growing myoblasts (day 0). These associations increased markedly in the first 24 h after exposure to reduced serum differentiation media, and then subsided at later points in the course of differentiation. These results demonstrate that Daxx does associate with native muscle-specific gene promoters, and that this association is strongly regulated during muscle differentiation.

Because the decrease in Daxx association with the MCK and MEF2C promoters in the ChIP assay (Fig. 4E) coincides with the appearance of the corresponding mRNAs in Northern analysis (Fig. 4C), we postulated that release of Daxx from muscle-specific gene promoters might be required for progression of muscle differentiation. In this case, decreasing Daxx expression below normal endogenous levels should enhance or promote myogenesis. To examine this possibility, we used small interfering RNAs (siRNAs) [Yu et al., 2002]. By immunofluorescent analysis, several siRNA expression constructs transfected into C2C12 cells were able to inhibit Daxx expression (data not shown). Two siRNA constructs were produced in retroviral form and transduced into cultures of growing C2C12 cells. Immunoblotting confirmed a significant reduction in Daxx expression levels (Fig. 5A). Cell lines with normal and reduced Daxx expression were subjected to low serum conditions to promote muscle differentiation. Quantitation of myotube formation indicated a significant increase in myotube formation in cells treated with the Daxx siRNA, but not control,

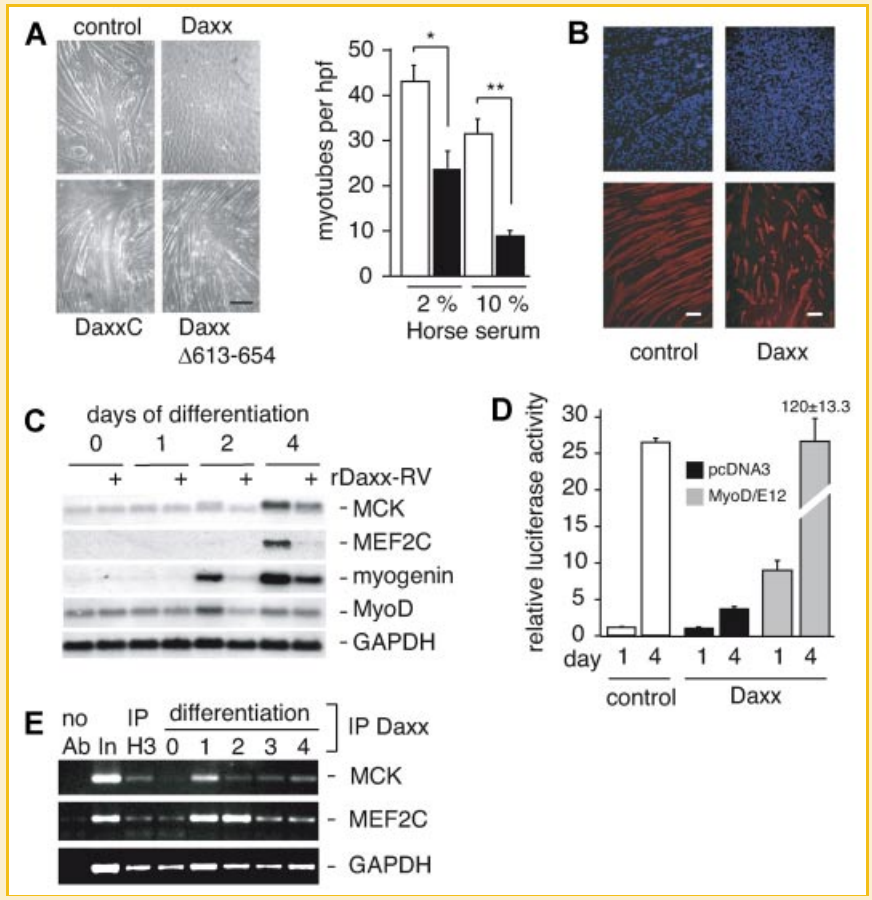

Fig. 4. Increased Daxx expression opposes myogenic differentiation and muscle gene expression. A–D: C2C12 cells were transduced with GFP-RV (control) or rDaxx-derived retroviruses (Daxx, DaxxC, and DaxxΔ613-654) and differentiated in culture. Bar, 200 μm. A (left): Phase contrast images after 4 days of differentiation. Right: Myotubes after 3 days of differentiation were counted in six randomly selected high power fields (hpf) per condition. Open bars, control; solid bars, transduced with Daxx retrovirus. $^{*}P < 0.0004$, $^{**}P < 0.0001$. B: Immunofluorescent micrographs after 4 days of differentiation. Upper panels, nuclei stained with DAPI (blue); lower panels, same fields stained for myosin heavy chain (red). C: Muscle gene expression. Total RNA from control GFP-RV or Daxx-RV (+) cells was harvested at the timepoints indicated and subjected to Northern analysis (10 μg per lane). The MCK probe identified a specific MCK band in day 2 and day 4 samples and a slightly smaller non-specific band throughout the time course. GAPDH was evaluated to assess equivalency of loading. D: Daxx inhibits activation of the MCK promoter in C2C12 cells. GFP-RV (control, left) and Daxx-transduced (right) C2C12 cells were transfected with the MCK-luc reporter (200 ng) and pcDNA3 vector (500 ng, black bars) or MyoD and E12 expression constructs (250 ng each, gray bars). Luciferase activity was determined after 1 or 4 days of differentiation. E: ChIP analysis of Daxx interaction with MCK and MEF2C regulatory elements during differentiation. C2C12 myoblasts were exposed to 10% horse serum for the indicated number of days prior to crosslinking, chromatin extraction, and immunoprecipitation. PCR was performed to identify the indicated promoter/enhancer sequences. Controls shown include no antibody (no Ab), chromatin input (1%) prior to immunoprecipitation (In), and immunoprecipitation for histone H3 (IP H3). PCR for GAPDH promoter sequences is shown as a reference. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

retroviruses (Fig. 5B). This result suggests that Daxx expressed at endogenous levels is necessary to limit the rate or extent of muscle differentiation in C2C12 cells. Given the results of the ChIP analysis (Fig. 4E), this effect is likely to be most important during the initial phase of serum withdrawal, when Daxx association with E2A-dependent muscle-specific promoters is strongest.

DISCUSSION

We have shown that Daxx interacts directly with the E2A transcription factors and represses E2A-mediated transactivation through an HDAC-dependent mechanism. Significantly, Daxx inhibits the differentiation of C2C12 myoblasts into myotubes, as demonstrated by both gain- and loss-of-function strategies. Thus, by identifying E12 and E47 as novel targets for the transcriptional repressive activities of Daxx, we define a distinctive negative regulation of E2A-driven muscle differentiation.

The Id proteins oppose differentiation of multiple cell lineages through their ability to associate with and sequester E2A proteins away from DNA [Benezra et al., 1990]. Our findings with Daxx identify a related effect on differentiation, but point to a mechanism of transcriptional repression that differs from that used by the Id proteins in two key respects. First, Daxx modifies E2A function through a novel type of protein–protein interaction. Daxx binds to E12 and E47, but not to other HLH proteins (Fig. 1A), indicating that the Daxx–E2A interaction does not depend on the HLH structural

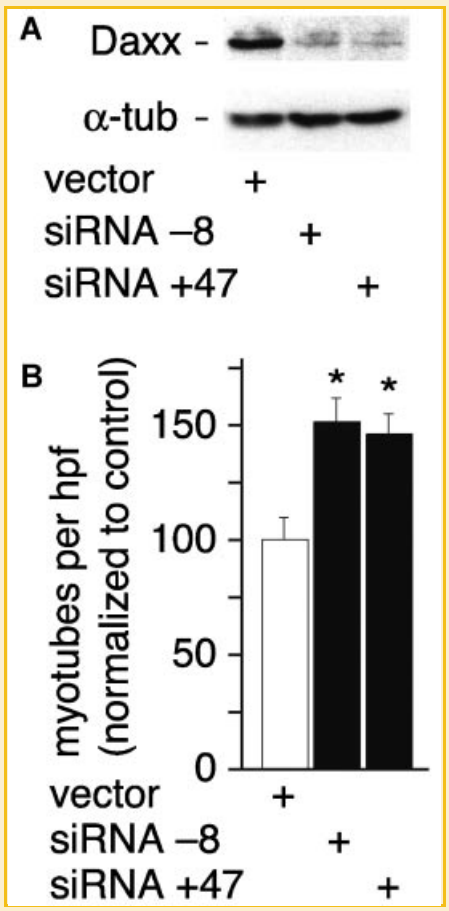

Fig. 5. Decreased Daxx expression potentiates muscle differentiation. A (left): Immunofluorescent micrographs of C2C12 cells transfected with expression plasmids pBB14 (encoding US9-GFP to identify transfected cells) and either pSupressorRetro (vector control, top) or pDaxx siRNA −8 (bottom). DAPI (Nuc), US9-GFP (GFP), and Daxx signals are indicated. Right: Immunoblot (20 μg total protein per lane) for Daxx expression in retroviral vector and pDaxx siRNA-transduced cultures. Alpha-tubulin (α-tub) is shown as a loading control. B: Myotube count after 3 days of differentiation in cells transduced with vector (open bar) or pDaxx siRNA (solid bars) retroviruses. $^{*}P < 0.0025$. Six randomly selected high power fields (hpf) were counted in each of three independent experiments.

motif common to these proteins. Second, interaction of Daxx with the E2A proteins is not sufficient for repression. The C-terminal DaxxC fragment does not repress E2A transcriptional activity (Fig. 2) or disrupt E2A-DNA binding (Fig. 3A), indicating a role for the N-terminal portion of Daxx in the mechanism of repression. In view of the requirement for HDAC activity (Fig. 3B), these findings suggest that C-terminal Daxx sequences interact with DNA-bound E2A, while N-terminal sequences recruit HDACs or HDAC-containing complexes to the promoter. Although our result (Fig. 3B) relies on a transiently introduced episomal plasmid reporter that lacks fully developed chromatin, there is support for the idea that HDAC inhibitor activities can be reliably assessed in such a system [Klan et al., 2003], as well as evidence that such findings do in fact reflect changes in histone acetylation status on native promoters with physiologic chromatin formation [De los Santos et al., 2007].

The biological relevance of this repressor function is reflected in our findings that Daxx inhibits the E2A-MRF-dependent MCK promoter in genetic reporter assays (Figs. 2C and 4D) and blocks activation of endogenous E2A-MRF-dependent muscle-specific genes, including MCK, when opposing muscle differentiation (Fig. 4C). We considered the possibility that cell death might contribute to the inhibition of muscle differentiation, because Daxx overexpression increases the susceptibility to apoptosis in several non-muscle cell types [Yang et al., 1997; Chang et al., 1998; Perlman et al., 2001]. However, DAPI-stained nuclei appeared morphologically healthy, the sub-G1 fraction did not change in the flow cytometric analysis of DNA content (data not shown), and MCK promoter activity could be rescued by co-transfection of MyoD and E12 (Fig. 4D, right). Thus, Daxx overexpression did not significantly increase apoptosis during C2C12 differentiation, indicating that Daxx-mediated reduction of myotube formation does not result from decreased myocyte viability.

Muscle gene expression is regulated by HDACs, which are recruited to muscle-specific promoter regions [McKinsey et al., 2001; Berkes and Tapscott, 2005]. Our findings indicate that Daxx recruits HDACs via E2A to such promoters, which suggests an interesting mechanism for regulating muscle-specific genes. While Daxx has been reported to associate with HDACs [Li et al., 2000a; Hollenbach et al., 2002; Kim et al., 2003; Greger et al., 2005], our studies identify a native transcription factor as a target of Daxx-medicated recruitment of HDACs and show a potential physiological consequence of such an interaction. Moreover, although HDACs can be recruited to muscle gene promoters either directly through MyoD [Bailey et al., 1999; Mal et al., 2001] or indirectly via MEF2C [Lu et al., 2000], our results indicate that such HDAC recruitment can also occur directly through the E2A proteins. Given the complex effects that HDACs exert on muscle differentiation [Iezzi et al., 2002], their precise role in Daxx-mediated repression of muscle differentiation awaits careful characterization. However, in support of the importance of such a role, many E2A/MyoD-dependent muscle-specific genes, including the MCK gene, are hypoacetylated in myoblasts, but become strongly hyperacetylated as muscle differentiation progresses [Bergstrom et al., 2002; Iezzi et al., 2002]. Moreover, hyperacetylation has been shown to be important for the activation of muscle-specific gene expression [Puri and Sartorelli, 2000; McKinsey et al., 2001].

By ChIP analysis, we found a significant but temporary increase in association of Daxx with the MCK and MEF2C promoters in the first 24–48 h after serum reduction (Fig. 4E). The timing of this increase is interesting, as it occurs during the period in which Id levels fall significantly and permanently, and E2A and MyoD are released from sequestration [Jen et al., 1992]. Hence, we postulate that Daxx and Id proteins serve complementary roles in the negative regulation of muscle differentiation. In undifferentiated myoblasts, abundant Id proteins prevent the formation of E2A/MyoD heterodimers that can bind DNA [Jen et al., 1992]. This may explain why Daxx, which in this setting depends on E2A to interact with DNA, is found at low levels on E2A-dependent promoters (Fig. 4E). As differentiation progresses, Id levels drop, E2A/MyoD binding to DNA increases [Jen et al., 1992], and Daxx becomes strongly associated with MCK and MEF2C promoters (Fig. 4E).

Because expression of MCK and MEF2C mRNA is still low during this phase, we postulate that Daxx transiently replaces Id functional effects on muscle differentiation by opposing the transcriptional activity of DNA-bound E2A-bHLH heterodimers. Through a mechanism yet to be identified, Daxx then dissociates from these promoters, allowing net histone acetylation, increased muscle-specific gene expression, and terminal differentiation. Interestingly, we found that E2A interacted primarily with one Daxx isoform; while Daxx is subject to phosphorylation [Hollenbach et al., 1999] and sumoylation [Jang et al., 2002], the precise modifications associated with specific isoforms have not been identified. We speculate that signal transduction events stemming from growth factor withdrawal trigger post-translational modifications of Daxx that may regulate its association with E2A and MCK and MEF2C E-boxes during muscle differentiation. In this regard, a recent article describes regulation of Daxx repressor activity through modification of serine 669 (human Daxx) by homeodomain interacting kinase 1 [Ecsedy et al., 2003]. However, we found no effect on Daxx-mediated repression of E2A in co-transfections with HIPK1, or using Daxx mutants in which the corresponding residue in rDaxx was replaced by either alanine or aspartic acid (data not shown). This suggests that the modifications that affect Daxx-mediated repression may vary according to target transcription factor, cell type, and/or species.

E2A proteins promote the differentiation of cell lineages other than muscle, with demonstrated roles in the formation of the heart [Cserjesi et al., 1995; Hollenberg et al., 1995; Firulli et al., 2000], nervous system [Guillemot et al., 1993; Firulli, 2003; Ross et al., 2003], blood [Porcher et al., 1996] and pancreas [Naya et al., 1997]. On the other hand, Daxx interacts with transcription factors other than E2A. Ets-1, Pax3, and Pax5 have all been implicated in the control of cellular differentiation [Urbanek et al., 1994; Bories et al., 1995; Barton et al., 1998; Chi and Epstein, 2002], but their regulation by Daxx has not been linked to this process. Our finding that Daxx represses myogenesis provides the first evidence of a role for Daxx in cell differentiation, and suggests the possibility of a related role for Daxx in other cell types, through mechanisms that may involve E2A or other Daxx-interacting transcription factors. We speculate that perturbations of differentiation in multiple cell lineages, analogous to our findings in skeletal muscle, might contribute to the early embryonic demise of Daxx null mice [Michaelson et al., 1999], which has been attributed so far to a widespread increase in apoptosis in the developing embryo. Intriguingly, E2A, Ets1, and Pax3 all bind to a region of Daxx that includes its C-terminal 115 amino acids (Fig. 1, and references [Hollenbach et al., 1999; Li et al., 2000b]). Further studies will be required to determine whether these proteins, or others yet to be identified, compete with one another for binding to Daxx, which would put Daxx in a central position from which it regulates the activities of multiple transcriptions factors important in cell differentiation.

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