The Pax3–FKHR oncoprotein is unresponsive to the Pax3-associated repressor hDaxx

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The Pax3–FKHR fusion protein is present in alveolar rhabdomyosarcoma and results from the t(2;13) (q35;q14) chromosomal translocation. Its oncogenic activity is dependent on a combination of protein–DNA and protein–protein interactions mediated by the Pax3 homeodomain recognition helix. In this report we demonstrate that human Daxx (hDaxx) interacts with Pax3 *in vivo* and with DNA-bound Pax3 *in vitro*. This interaction is mediated primarily through the homeodomain recognition helix with the additional involvement of the octapeptide domain and its N-terminal flanking amino acids. Through this interaction hDaxx represses the transcriptional activity of Pax3 by ~80%. The Pax3–FKHR fusion is unresponsive to this repressive effect despite an observed endogenous interaction with hDaxx in a rhabdomyosarcoma tumor cell line. Therefore, these data support the model that fusion of FKHR to Pax3 not only adds a strong transactivation domain, but also deregulates transcriptional control of Pax3 by overriding the natural repressive effect of hDaxx.

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Introduction

Alveolar rhabdomyosarcoma, a malignant tumor of skeletal muscle, is characterized by the t(2;13) (q35;q14) translocation (Shapiro *et al.*, 1993; Galili *et al.*, 1995) that results in the fusion of Pax3, a member of the paired class homeodomain family of transcription factors, to FKHR, a member of the forkhead family of transcription factors. The Pax3–FKHR fusion, which has been demonstrated to be a more potent transcriptional activator than Pax3 (Fredericks *et al.*, 1995; Bennicelli *et al.*, 1996; Lam *et al.*, 1999), maintains the integrity of the Pax3 DNA-binding motifs (paired domain and homeodomain). However, the Pax3 transactivation domain is replaced by the bisected, nonfunctional FKHR DNA-binding domain (DBD) and the FKHR transactivation domain. A recent structure–function analysis of Pax3–FKHR demonstrated that, in conjunction with a small region of the FKHR activation domain, the integrity of the recognition helix of the Pax3 homeodomain (the third α -helix) is necessary for the oncogenic activity of Pax3–FKHR in NIH 3T3

fibroblasts (Lam *et al.*, 1999). In this report it is demonstrated that point mutants in the third α -helix of the homeodomain diminish homeodomain-mediated Pax3–FKHR DNA binding with a correlative reduction in the oncogenic activity of the fusion protein. However, point mutants in the paired domain that completely disrupt Pax3–FKHR DNA binding ability while retaining an intact homeodomain maintain a wild-type oncogenic activity. These results suggest that the oncogenicity of Pax3–FKHR is independent of paired domain-mediated DNA binding but instead requires both protein–DNA and protein–protein interactions mediated through the Pax3 homeodomain recognition helix (Lam *et al.*, 1999).

Two recent reports have identified proteins that interact with the homeodomain of Pax3. The retinoblastoma protein (Rb) was demonstrated to repress Pax3 transcriptional activity 2- to 3-fold by a direct interaction with the first two α -helices of the homeodomain (Wiggin *et al.*, 1998). HIRA, a mammalian homologue of a *Saccharomyces cerevisiae* transcriptional co-repressor, was demonstrated to interact with the Pax3 homeodomain (Magnaghi *et al.*, 1998). However, it remains to be determined whether Pax3 is regulated by HIRA and whether the Pax3–FKHR fusion protein is affected by the regulatory actions of these proteins. The Pax3–FKHR fusion protein may alter the regulation of transcription of Pax3 target genes in one of two ways. First, after the Pax3 homeodomain mediates the binding of Pax3–FKHR to the promoter regions of Pax3 target genes the presence of the more potent FKHR transactivation domain may overcome the natural regulatory actions of proteins that interact with the Pax3 homeodomain recognition helix. Alternatively, the FKHR portion of the fusion may create steric hindrance and prevent these regulatory proteins from interacting with Pax3. In either case it is important to identify additional regulatory proteins that may be involved.

The murine homologue of hDaxx was previously identified as a putative Fas receptor binding protein, implicated in the enhancement of Fas-mediated apoptosis (Yang *et al.*, 1997; Chang *et al.*, 1998, 1999). Human Daxx (hDaxx) was also isolated using a yeast one-hybrid screen to detect transcription factors that regulate a steroidogenic factor 1-like element from the human steroidogenic acute regulatory protein gene promoter (Kiriakidou *et al.*, 1997). In addition, hDaxx has been demonstrated to interact with the centromeric protein CENP-C in a cell cycle-dependent manner (Pluta *et al.*, 1998). In this report we demonstrate that hDaxx is also involved in the regulation of Pax3 transcriptional activity. This regulation is mediated by the direct interaction of hDaxx with the Pax3 homeodomain recognition helix and the N-terminally extended octapeptide domain. We also show that hDaxx is associated endogenously with the Pax3–FKHR fusion protein. However, Pax3–FKHR does not respond to the repressive

action of hDaxx. These results provide some of the first evidence for a mechanism that explains, in part, the oncogenic activity of Pax3-FKHR.

Results

Isolation and structural characteristics of hDaxx

In order to identify proteins that interact with Pax3 and that may contribute to the oncogenicity of Pax3-FKHR, the Pax3 moiety of Pax3-FKHR was fused to the LexA DNA-binding domain and used in a yeast two-hybrid analysis (Gyuris *et al.*, 1993). One of 11 initial clones isolated from an oligo(dT)-primed HeLa cell cDNA library was identical to the C-terminal 120 amino acids of hDaxx. hDaxx encodes a protein of 740 amino acids and contains a Ser/Pro/Thr-rich C-terminus, which we found to interact with Pax3, and a region rich in acidic amino acids (Figure 1A), both motifs commonly found in transcriptional regulators (Mitchell and Tjian, 1989). hDaxx was also demonstrated to contain a region predicted to form a coiled coil like structure (Pluta *et al.*, 1998). In addition, we observed that hDaxx contains two regions predicted to form paired amphipathic helices (PAHs) (Figure 1B) that share conserved amino acids with the four PAHs present in the yeast transcriptional co-repressor Sin3 and its mammalian homologue Sin3a (Figure 1C) (Wang *et al.*, 1990; Ayer *et al.*, 1995).

hDaxx is expressed in all tissue types (data not shown; Kiriakidou *et al.*, 1997), suggesting that hDaxx may have an alternative function in cells not expressing the Fas receptor. The reported interaction between Daxx and the Fas receptor would indicate that hDaxx should localize to the cytoplasm of cells. However, overexpressed six-histidine epitope-tagged hDaxx localized exclusively to the nucleus of transfected COS1 cells, consistent with the presence of two nuclear localization signals in the hDaxx sequence (Kiriakidou *et al.*, 1997). In direct agreement with this observation, we have also noted a strict nuclear staining for hDaxx, which was identical to the cellular localization of hemagglutinin (HA)-Pax3, indicating a co-localization of hDaxx and Pax3 to the nucleus (data not shown). We have also confirmed a strict nuclear localization of both endogenous and overexpressed hDaxx in all cell lines examined (COS1, NIH 3T3 and Jurkat; data not shown).

In all cell lines examined, a C-terminal antibody against hDaxx identified three distinctly migrating species with apparent molecular weights of 70, 97 and 110 kDa (Figure 2, lane 2). To rule out the possibility that the three species of hDaxx are N-terminal degradation products of the full-length protein, hDaxx was N-terminally FLAG-epitope tagged and overexpressed in NIH 3T3 fibroblasts. A Western analysis of these cell lysates, utilizing both the C-terminal hDaxx antibody (Figure 2, lane 2) and the N-terminal FLAG antibody (Figure 2, lane 1), identified the same three species of hDaxx. This confirms the presence of both the N-terminus and the C-terminus of hDaxx in all three species and that these species do not result from degradation of hDaxx. Northern analysis also demonstrated that the hDaxx mRNA exists as a single transcript of 2.4 kb (data not shown; Kiriakidou, 1997) indicating that the three species of hDaxx are not the result of alternatively spliced transcripts. Instead, the three

Fig. 1. Schematic representation of hDaxx structural characteristics. (A) The domains of hDaxx are presented as follows: the Ser/Pro/Thr-rich domain (S/P/T) is depicted by the shaded box, the acidic rich domain (D/E) by the horizontal lines, the region of coiled-coil (C-C) by the cross-hatched box, and the two paired amphipathic helices (PAHs) by the diagonal stripes. The vertical lines above the structure indicate predicted phosphorylation sites and the region of hDaxx required for the interaction with Pax3 is indicated. (B) Amino acid sequences of helices A and B of the two PAHs represented as Edmundson helical wheels. The amino acid sequence of PAH1 is closest to the wheel and hydrophobic residues are indicated. (C) Sequence similarity of hDaxx and yeast Sin3 (Wang *et al.*, 1990) and mammalian Sin3a (Ayer *et al.*, 1995) PAHs. Amino acids are represented by the single letter code.

forms of hDaxx may arise from different post-translational modifications of the protein. Consistent with this, a PROSITE analysis of hDaxx (Bairoch *et al.*, 1997) predicted 34 phosphorylation sites located near key structural regions of the protein (Figure 1A). Incubation of NIH 3T3 fibroblasts with [32 P]orthophosphate specifically labeled the apparent 110 kDa species, but not the 97 or 70 kDa species (Figure 2, lane 3). A Western analysis of the immunoprecipitated protein demonstrated the ability of the FLAG antibody to precipitate all forms of hDaxx (Figure 2, lane 4). In addition, incubation of hDaxx containing cell lysates in the absence of phosphatase inhibitors resulted in the presence of only the 70 kDa form of hDaxx (Figure 2, lane 5). These results directly demonstrate that the different species of hDaxx result, in part, from a post-translational hyperphosphorylation.

Fig. 2. hDaxx is present in all cell lines as three distinctly migrating species. NIH 3T3 cells overexpressing an N-terminal FLAG epitope tagged hDaxx (FLAG-hDaxx) were lysed and separated by SDS-PAGE, and the presence of FLAG-hDaxx was detected with either an N-terminal anti-FLAG antibody (lane 1) or a C-terminal anti-hDaxx antibody (lane 2) as described in the Materials and methods. A schematic of the structural characteristics of hDaxx and the location of the respective epitopes is presented. To examine the phosphorylation status of hDaxx, FLAG-hDaxx was directly labeled with [32 P]orthophosphate and immunoprecipitated from COS1 cell lysates (lane 3). The FLAG antibodies precipitated all forms of hDaxx as demonstrated by a direct Western blot using the anti-hDaxx antibody (lane 4). In addition, Jurkat cells overexpressing full-length hDaxx were lysed in either the presence (lane 6) or absence (lane 5) of phosphatase inhibitors, incubated at 37°C for 12 h, and the presence of hDaxx was detected using the C-terminal hDaxx antibody.

Physical interaction between Pax3 and hDaxx

To demonstrate a direct physical interaction between Pax3 and hDaxx *in vivo*, COS1 cells were co-transfected with influenza virus HA epitope tagged Pax3 (HA-Pax3) and full-length hDaxx, followed by co-immunoprecipitation. Immunoprecipitation of HA-Pax3 from cell lysates expressing similar levels of both proteins (Figure 3A, lanes 3 and 4) co-precipitated the 70 kDa species of hDaxx (Figure 3A, lane 2) consistent with an interaction between Pax3 and non-phosphorylated hDaxx. This interaction was specific for HA-Pax3, since no hDaxx was detected when the identical co-immunoprecipitation was carried out on cells overexpressing hDaxx alone (Figure 3A, lane 1). To examine the interaction between hDaxx and DNA-bound Pax3, full-length Pax3 and the interacting region of hDaxx (hDaxx₆₃₅₋₇₄₀) were expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* and subjected to electrophoretic mobility shift analysis. GST-Pax3 shifted the radiolabeled Pax3-specific PRS-9 oligonucleotide probe, which contains both Pax3 paired and homeodomain recognition sequences (Figure 3B, lane 3). This complex was supershifted by the addition of Pax3-specific antiserum and could be specifically competed by a molar excess of unlabeled probe (Figure 3B, lanes 4 and 5). Increasing amounts of GST-hDaxx₆₃₅₋₇₄₀, added prior to DNA binding, resulted in a titratable supershift of the Pax3-DNA complex (Figure 3B, lanes 6–8). The addition of purified GST to the binding reaction was unable to supershift the Pax3-DNA complex (Figure 3B, lane 9), demonstrating that the observed supershift is specific for hDaxx and not a result of non-specific binding of GST to GST-Pax3. In the same manner GST-hDaxx₆₃₅₋₇₄₀ alone was unable to shift the Pax3-specific probe (Figure 3B, lane 10). Therefore, we conclude that hDaxx binds directly to Pax3 both *in vivo* and to DNA-bound Pax3 *in vitro*.

Fig. 3. The 70 kDa species of hDaxx interacts with Pax3 *in vivo* and to DNA-bound Pax3 *in vitro*. (A) Pax3 interacts with the 70 kDa species of hDaxx *in vivo*. COS1 cells overexpressing equivalent amounts of hDaxx and HA-Pax3 (lanes 3 and 4) were lysed in the presence of protease and phosphatase inhibitors and immunoprecipitated with an anti-HA antibody followed by detection with an anti-hDaxx antibody (lane 2). As a negative control cells overexpressing hDaxx alone were subjected to an identical treatment (lane 1). The mobilities of HA-Pax3 and hDaxx are indicated by the solid and open arrows, respectively. (B) hDaxx interacts with DNA-bound Pax3 *in vitro*. Bacterially expressed GST-Pax3 and the indicated amounts of bacterially expressed GST-hDaxx₆₃₅₋₇₄₀ or GST were subjected to an electrophoretic mobility shift assay (EMSA) with an end labeled PRS-9 oligonucleotide probe, which contains both the Pax3 paired and homeodomain recognition sequences. The mobilities of DNA bound Pax3 and the Pax3-hDaxx complex are indicated by the solid and open arrows, respectively.

Specificity of the Pax3 and hDaxx interactions

In order to identify the domains of Pax3 required for the interaction with hDaxx, deletion mutants targeting key structural domains of Pax3 (Figure 4) were used as the bait and hDaxx₆₃₅₋₇₄₀ was used as the interacting protein in the LexA yeast two-hybrid system (Gyuris *et al.*, 1993). The interaction was detected by a quantitative liquid β -galactosidase assay (Adams *et al.*, 1997). The introduction of the deletion mutants did not affect the stability of the proteins as determined by immunoprecipitation of metabolically labeled protein from NIH 3T3 fibroblasts (data not shown). Deletion of the N-terminal Pax3 domain responsible for transcriptional inhibition (Δ NH₂)

Bait	Library	β -galactosidase activity
pSH17-4 Positive control	----	7.98 ± 0.89
pRFHM1 Negative control	----	0.03 ± 0.02
----	Daxx ₆₃₅₋₇₄₀	0.02 ± 0.01
Pax3	Daxx ₆₃₅₋₇₄₀	2.07 ± 0.13
Δ NH ₂	Daxx ₆₃₅₋₇₄₀	2.21 ± 0.19
PDND	Daxx ₆₃₅₋₇₄₀	2.69 ± 0.07
Δ PD	Daxx ₆₃₅₋₇₄₀	2.35 ± 0.16
Δ PD2	Daxx ₆₃₅₋₇₄₀	1.31 ± 0.28
Δ PDOD2	Daxx ₆₃₅₋₇₄₀	0.41 ± 0.07
Δ PDOD	Daxx ₆₃₅₋₇₄₀	0.45 ± 0.13
HD3D	Daxx ₆₃₅₋₇₄₀	0.08 ± 0.07
Δ HD	Daxx ₆₃₅₋₇₄₀	3.01 ± 0.13
Pax7	Daxx ₆₃₅₋₇₄₀	6.39 ± 0.81
Pax4	Daxx ₆₃₅₋₇₄₀	0.01 ± 0.01

Fig. 4. The Pax3 homeodomain recognition helix and the N-terminally extended octapeptide domain are required for interaction with hDaxx. Pax3, the indicated Pax3 deletion mutants, Pax7 and Pax4 were fused in-frame to the LexA bait vector. The Pax3 and Pax7 paired domains are represented by the diagonal stripes, the octapeptide domains by the shaded box, and the homeodomains by the dark hashed box. The Pax4 paired domain is represented by the horizontal lines and the Pax4 homeodomain by the cross-hatched box. The ability of the mutants to interact with the partial hDaxx clone, hDaxx₆₃₅₋₇₄₀, was determined by the yeast two-hybrid liquid β -galactosidase assay (see Materials and methods). The results are presented as β -galactosidase activity corrected for the background activity of the deletion mutants in the absence of hDaxx₆₃₅₋₇₄₀. Errors represent the standard deviation from four independent determinations.

(Chalepakakis *et al.*, 1994), the region of the paired domain involved in making DNA contacts (PDND) (Xu *et al.*, 1995) or the entire paired domain (Δ PD) had no effect on hDaxx binding, indicated by wild-type β -galactosidase activity (Figure 4). However, deletion of an additional 20 amino acids past the paired domain (Δ PD2) or deletion of the paired and octapeptide domains (Δ PDOD2) resulted in ~2- and 5-fold decreases in β -galactosidase activity, respectively, with no additional decrease in activity upon further deletion (Δ PDOD) (Figure 4). This is consistent with a role of the octapeptide domain and its N-terminal flanking amino acids, which will be referred to as the N-terminally extended octapeptide domain, in the interaction of Pax3 with hDaxx. In the same manner, deletion of the recognition helix of the homeodomain (HD3D) resulted in no significant β -galactosidase activity above background (Figure 4). When the HD3D mutant was used in independent experiments with PAPI, a calcium-binding protein was shown to interact primarily with the Pax3 paired domain (data not shown), wild-type β -galactosidase activity was seen providing additional evidence that the HD3D deletion mutant does not significantly affect the

stability of the protein. From this we conclude that the homeodomain recognition helix in conjunction with the N-terminally extended octapeptide domain are necessary for the interaction between Pax3 and hDaxx. Surprisingly, the homeodomain deletion mutant (Δ HD) retained an activity slightly higher than full-length Pax3. This enhanced activity may result from the removal of a *cis*-acting inhibitory action associated with the Pax3 homeodomain (Bennicelli *et al.*, 1996), or more simply removal of the homeodomain may make paired domain- and octapeptide domain-mediated interactions more accessible.

Because different members of the Pax family of transcription factors show variable sequence and domain conservation (Stuart *et al.*, 1995), Pax4 and Pax7 were chosen to determine the specificity of hDaxx interactions with other members of the Pax family of transcription factors. Pax4, which does not contain an octapeptide domain and is only 45% identical to Pax3 in the homeodomain, had no detectable binding ability (Figure 4). Conversely, Pax7, which contains an octapeptide domain and is 97% identical to Pax3 in the homeodomain (Stuart *et al.*, 1995), resulted in a β -galactosidase activity nearly

three times higher than that for Pax3 (Figure 4). This would suggest that hDaxx₆₃₅₋₇₄₀ binds to Pax7 with a higher affinity than that of Pax3. This result is further consistent with the role of the octapeptide domain and the homeodomain recognition helix in the interaction of hDaxx with both Pax3 and Pax7.

hDaxx acts as a transcriptional repressor

The presence of transcriptional regulatory domains in hDaxx and the sequence similarity of the PAHs in hDaxx to the co-repressor Sin3 suggest that hDaxx may act as a transcriptional regulator. To test this hypothesis full-length hDaxx was fused to the GAL4 DBD and the effect of GAL4-hDaxx on the constitutively active reporter construct 1X-CRE-TK-CAT, which contains one GAL4 DNA-binding site, was determined. Transient co-transfection of NIH 3T3 fibroblasts with the reporter construct and increasing amounts of GAL4-hDaxx or hDaxx (pcDNA3-hDaxx) resulted in an ~70% decrease in transcriptional activity (Figure 5A, shaded bars). This repression was dependent on the tethering of hDaxx to DNA through the GAL4 DBD since hDaxx alone was unable to repress transcription in this system (Figure 5A, open bars). This result directly demonstrates the ability of hDaxx to act as a transcriptional repressor in this experimental setting. Despite the sequence similarity between the PAHs of hDaxx and Sin3, domains responsible for the recruitment of histone deacetylase activity by Sin3 (Alland *et al.*, 1997; Hassig *et al.*, 1997; Heinzel *et al.*, 1997; Kadosh and Struhl, 1997; Nagy *et al.*, 1997; Zhang *et al.*, 1997), we were unable to demonstrate a relief of repression when the transient transfections were repeated in the presence of the histone deacetylase inhibitor, Trichostatin A (data not shown). This suggests that the inhibitory effect of hDaxx involves mechanisms other than the recruitment of histone deacetylases.

To determine the effect of hDaxx on Pax3 transcriptional activity, NIH 3T3 cells were transiently transfected with Pax3, increasing amounts of full-length hDaxx, and the reporter construct (PRS-9)TK-CAT, which contains both Pax3 paired and homeodomain recognition sequences (Chalepakakis *et al.*, 1994). Increasing amounts of hDaxx caused a titratable inhibitory effect on Pax3 transcription resulting in ~80% repression of Pax3 transcriptional activity (Figure 5B, shaded bars). This is a level of repression similar to that seen for the action of the co-repressors Sin3a and Sin3b on Ume6 and Myc respectively (Allend *et al.*, 1997; Kadosh and Struhl, 1997). To confirm that overexpression of hDaxx has no effect on the protein levels of Pax3, similar transcription experiments were repeated using co-infection of cells with Pax3 and hDaxx retrovirus, a system that allows a higher level of overexpression of Pax3 and hDaxx. Under these conditions it was found that overexpression of hDaxx represses Pax3 transcription just as well as in transient co-transfections but it had no detectable effect on the level of Pax3 protein expression, as determined by Western analysis (data not shown). The observed repression of reporter gene expression was also not a result of hDaxx-induced apoptosis since TUNEL assays and visual inspection of morphology of cells overexpressing both Pax3 and hDaxx, determined by immunofluorescence, confirmed the absence of any detectable apoptosis in all transcription assays. In fact we

Fig. 5. Transcriptional regulation by hDaxx. **(A)** hDaxx represses transcription when tethered to DNA through the GAL4 DBD. The indicated amounts of the GAL4-hDaxx fusion (pM2-hDaxx, shaded bars) or hDaxx alone (pcDNA3-hDaxx, open bars) were co-transfected into NIH 3T3 cells with a chloramphenicol acetyl transferase (CAT) reporter construct containing one GAL4 DNA binding site. Forty-eight hours post-transfection CAT activity was determined as described previously (Ausubel *et al.*, 1996). All values were normalized for co-transfected secreted alkaline phosphatase activity (Bram *et al.*, 1993) and are presented as the percentage of CAT activity in the absence of hDaxx (pM2 only). Error bars represent the standard deviation from four independent determinations. **(B)** hDaxx represses Pax3 but not Pax3-FKHR transcriptional activity. Pax3 (shaded bars) or Pax3-FKHR (open bars) and increasing amounts of hDaxx were co-transfected into NIH 3T3 cells with the (PRS-9)TK-CAT reporter construct, which contains both the Pax3 paired and homeodomain recognition sequences. After 48 h CAT activity was determined as described (Ausubel *et al.*, 1996). All values were normalized for co-transfected secreted alkaline phosphatase activity (Bram *et al.*, 1993) and are presented as the percent maximal activity relative to Pax3 or Pax3-FKHR transcriptional activity in the absence of hDaxx. Error bars represent the standard deviation from eight independent determinations.

were unable to detect any hDaxx-induced apoptosis in either NIH 3T3 fibroblasts overexpressing co-transfected Fas receptor and hDaxx or in Jurkat cells overexpressing transfected hDaxx (data not shown). Therefore we conclude that under the conditions used in this study hDaxx acts as a Pax3-associated transcriptional repressor.

Fig. 6. Pax3-FKHR interacts with hDaxx. (A) Pax3-FKHR interacts with hDaxx₆₃₅₋₇₄₀ in yeast. hDaxx₆₃₅₋₇₄₀ was fused in-frame to the LexA bait vector and Pax3-FKHR was fused in-frame to the LexA library vector. The results are presented as β -galactosidase activity corrected for the background activity of hDaxx₆₃₅₋₇₄₀ in the absence of Pax3-FKHR. Errors represent the standard deviation from four independent determinations. (B) *In vivo* interaction between Pax3-FKHR and hDaxx. NIH 3T3 cells overexpressing both hDaxx and Pax3-FKHR were lysed in the presence of protease and phosphate inhibitors and subsequently incubated with magnetic beads containing the Pax3-specific oligonucleotide, e5 (Pax3 site resin), which contains both paired and homeodomain recognition sequences, or the control beads that contained no oligonucleotide (resin). Proteins retained by the beads were separated by 10% SDS-PAGE and the presence of Pax3-FKHR (right panel) or hDaxx (left panel) were determined by Western analysis. (C) Endogenous interaction between Pax3-FKHR and hDaxx. Rh30 cells (5×10^6) that naturally express both Pax3-FKHR and hDaxx were lysed in the presence of protease and phosphatase inhibitors and submitted to the Pax3 site resin analysis as described for (B).

Pax3-FKHR is unresponsive to repression by hDaxx

When the Pax3-FKHR fusion protein was used in the identical transcription assays as for Pax3, we found that hDaxx exerted only a minimal repressive action on Pax3-FKHR transcriptional activity (Figure 5B, open bars). The inability of hDaxx to repress Pax3-FKHR transcriptional activity may be caused by the additional FKHR sequences preventing hDaxx from binding to Pax3. To determine whether the presence of FKHR prevents the interaction between Pax3 and hDaxx, Pax3-FKHR was used as the

interacting protein in a yeast two-hybrid analysis. In this system Pax3-FKHR alone was transcriptionally active when used as the bait protein. Therefore, Pax3-FKHR was cloned into the library plasmid, pJG4-5, and the hDaxx partial clone hDaxx₆₃₅₋₇₄₀ was cloned into the bait plasmid, pEG202. Co-expression of the hDaxx₆₃₅₋₇₄₀ clone and Pax3-FKHR resulted in β -galactosidase activity far above background levels (Figure 6A), demonstrating an interaction between hDaxx and Pax3-FKHR in yeast.

To demonstrate an interaction between Pax3-FKHR and hDaxx *in vivo*, both proteins were overexpressed in NIH 3T3 cells and the interaction was determined by using a Pax3-specific oligonucleotide affinity resin (Figure 6B). A Western blot of the resulting proteins retained by the Pax3 site-specific resin demonstrate that in addition to binding overexpressed Pax3-FKHR, primarily the 110 kDa species of hDaxx was also retained (Figure 6B). The retention of hDaxx by the resin was directly dependent on the binding of Pax3-FKHR since neither Pax3-FKHR nor hDaxx bound to the resin alone (Figure 6B). Despite the retention of overexpressed Pax3 by the Pax3 site-specific resin we were unable to demonstrate the retention of hDaxx from cell lysates overexpressing both Pax3 and hDaxx (data not shown). This suggests that the affinity of hDaxx for Pax3 is not nearly as great as the affinity of hDaxx for Pax3-FKHR.

Finally, to show an endogenous interaction between Pax3-FKHR and hDaxx the Pax3-specific oligonucleotide affinity resin was used to isolate Pax3-FKHR and associated proteins from Rh30, a rhabdomyosarcoma cell line that endogenously expresses both Pax3-FKHR and hDaxx (data not shown). Once again a Western blot analysis of the endogenous proteins retained by the Pax3 site-specific resin demonstrated the retention of Pax3-FKHR and primarily the 110 kDa species of hDaxx, and to a minor extent the 97 and 70 kDa species of hDaxx (Figure 6C). The retention of hDaxx by the resin was directly dependent on the binding of Pax3-FKHR to the Pax3-specific oligonucleotide since neither Pax3-FKHR nor hDaxx bound to the resin alone (Figure 6C). The observation that hDaxx and Pax3-FKHR interact in yeast, when overexpressed in NIH 3T3 cells and endogenously in the Rh30 cell line (Figure 6) indicates that the unresponsiveness of Pax3-FKHR to the repressive action of hDaxx is not caused by steric hindrance of FKHR sequences preventing hDaxx from binding to Pax3-FKHR. Instead our data support a model in which the fusion protein is unresponsive to the transcriptional regulation of hDaxx on Pax3.

Discussion

Regulation of Pax3 transcriptional activity by hDaxx

The Pax3-FKHR fusion protein is a hallmark of the skeletal muscle tumor alveolar rhabdomyosarcoma. The oncogenic activity of this fusion protein is due, in part, to protein-protein interactions mediated through a combination of the Pax3 homeodomain recognition helix and a small region of the FKHR transactivation domain (Lam *et al.*, 1999). The goal of this study was to identify proteins that interact with and regulate Pax3 and to determine how the Pax3-FKHR fusion protein alters this regulation. Using a yeast two-hybrid analysis with Pax3

as the bait we have identified that hDaxx interacts with Pax3 and acts as a transcriptional repressor. Furthermore, we have demonstrated that hDaxx represses the transcriptional activity of Pax3 through direct protein–protein interactions involving primarily the Pax3 homeodomain recognition helix with the potential involvement of the N-terminally extended octapeptide domain.

The conclusion that hDaxx can act as a transcriptional repressor that directly interacts with Pax3 is based on the following information. First, hDaxx contains a Ser/Pro/Thr-rich domain and an acidic rich domain. Both domains are consistent with its role as a transcriptional repressor since these motifs are commonly found in transcriptional regulators. Secondly, immunoprecipitation of Pax3 from cell lysates overexpressing both Pax3 and hDaxx specifically co-precipitated the 70 kDa species of hDaxx (Figure 3A) and purified GST–hDaxx could specifically supershift a Pax3–DNA complex (Figure 3B). Thirdly, deletion analysis identified the requirement of primarily the homeodomain recognition helix with the additional involvement of the N-terminally extended octapeptide domain of Pax3 for the interaction with hDaxx. The octapeptide domains of both Pax2 and Pax8 have been demonstrated to act as transcriptional repressor domains (Lechner and Dressler, 1996), which would suggest a similar function for the Pax3 octapeptide domain. Another report has identified a *cis*-acting repression activity associated with the Pax3 homeodomain believed to work through protein–protein interactions with an unidentified inhibitor protein (Bennicelli *et al.*, 1996). Clearly, hDaxx would be a good candidate to moderate this repression, although we can not exclude the possibility that other as yet unidentified repressors could be responsible. Finally, hDaxx can act as a transcriptional repressor when tethered to DNA through the GAL4 DBD (Figure 6A) and most importantly, co-expression of hDaxx with Pax3 results in an ~80% repression of Pax3 transcriptional activity (Figure 6B). Therefore we conclude that hDaxx acts as a transcriptional repressor, which through a direct interaction with Pax3 represses its transcriptional activity.

Consistent with the role of hDaxx as a transcriptional repressor is the observed strict nuclear localization of hDaxx (Kiriakidou *et al.*, 1997; Pluta *et al.*, 1998; data not shown). This seems to be in direct contrast to previous reports implicating hDaxx as a mediator of Fas-dependent apoptosis through a direct interaction with the cytoplasmic death domain of the Fas receptor (Yang *et al.*, 1997). Two scenarios have been proposed to explain this apparent inconsistency (Pluta *et al.*, 1998). First, the interaction of hDaxx with the Fas receptor death domain could occur by default after nuclear envelope breakdown at prometaphase. Alternatively, hDaxx may shuttle between the nucleus and cytosol, perhaps in response to activation of Fas. However, induction of massive apoptosis by activation of the Fas receptor with anti-Fas antibodies in Jurkat cells that express endogenous FasR did not alter the nuclear localization of hDaxx (data not shown). Furthermore, we were unable to observe cell membrane co-staining in α FasR and α hDaxx double-staining immunofluorescence experiments (data not shown). We also did not observe any significant amounts of hDaxx in the cytoplasm of various cell lines (COS1, NIH 3T3, C2C12) with overexpressed or endogenous hDaxx. Finally, we did not observe any

significant apoptosis when hDaxx was overexpressed in Jurkat cells, nor did we observe apoptosis when the Fas receptor and hDaxx were simultaneously overexpressed in NIH 3T3 fibroblasts. Although we cannot explain this discrepancy with published observations, we are confident that the repressive effects of hDaxx in transient transfection assays are not caused by apoptosis of the transfected cells.

Alternative roles for hDaxx

Since Pax3 and Fas both have restricted patterns of expression, the ubiquitous nature of hDaxx, determined by both Northern (Kiriakidou *et al.*, 1997) and Western analysis (data not shown), would predict alternative regulatory actions for hDaxx in tissues not expressing Pax3 or FasR. Our observation that hDaxx exists as three distinct species may provide an explanation for the potential alternative roles of hDaxx. Other investigators have also noted that several different migrating species of hDaxx are present in cell lysates overexpressing hDaxx (Kiriakidou *et al.*, 1997; Pluta *et al.*, 1998) and in longer exposures of *in vitro* translated hDaxx (Pluta *et al.*, 1998). However, these different forms of hDaxx were attributed to degradation of the full-length protein. Instead we have conclusively demonstrated by Western analysis that both the N- and C-terminus of hDaxx are present in all three migrating species and the apparent 110 kDa hDaxx species results, at least in part, from a direct phosphorylation event (Figure 2). Since a single mRNA is detected on Northern blots, this strongly suggests that the full-length protein is present in all three distinct species and that the alternative mobilities of hDaxx must arise from different post-translational modifications of the protein.

The observation that hDaxx exists as three distinctly migrating species resulting from different post-translational modifications may be important for the interaction of essentially the same region of hDaxx with Pax3, the centromere protein CENP-C (Pluta *et al.*, 1998) and possibly FasR. Post-translational modifications of transcription factors or proteins that interact with transcription factors is a common mechanism of altering protein–protein interactions and thereby regulating transcriptional activity (Hunter and Karin, 1992). It is conceivable that post-translational events on hDaxx may regulate its interaction with Pax3, CENP-C, FasR and other as yet unidentified proteins. Our own data provide precedence for this possibility since we found that Pax3 interacts with the 70 kDa species of hDaxx, whereas Pax3–FKHR seems to bind preferentially the 110 kDa species.

Potential role of hDaxx in Pax3–FKHR oncogenicity

Based on the data presented here we can not conclusively define the exact role that hDaxx may play in the oncogenic activity of Pax3–FKHR, nor can we determine whether any additional proteins may be necessary for this oncogenic activity. However, the data presented here allow us to put forth several possible mechanisms that include a potential role for hDaxx in the oncogenic activity of Pax3–FKHR. Despite an observed interaction between Pax3 and hDaxx by co-immunoprecipitation (Figure 3A), electrophoretic mobility shift analysis (Figure 3B) and yeast two-hybrid analysis (Figure 4), we were unable to detect an interaction with either the Pax3-specific oligonucleotide affinity resin or the yeast two-hybrid switch-around (data not shown).

These were conditions with which an efficient interaction was observed between Pax3-FKHR and hDaxx (Figure 6). Although not conclusive this would suggest that under these conditions hDaxx has a higher affinity for Pax3-FKHR than Pax3. This apparent higher affinity of Pax3-FKHR for hDaxx may act to sequester endogenous hDaxx from other natural targets resulting in an improper regulation of additional genes unrelated to Pax3 but necessary for the normal functioning of the cell. This model is also consistent with the previous observation that paired domain point mutants of Pax3-FKHR that are unable to bind DNA are able to transform NIH 3T3 cells with wild-type efficiency, presumably through protein-protein interactions (Lam *et al.*, 1999).

More importantly we have demonstrated that the Pax3-FKHR fusion protein is unresponsive to the repressive action of hDaxx (Figure 5B). This result is consistent with a previous report demonstrating that the Pax3-FKHR protein was less susceptible to the *cis*-acting inhibitory action associated with the Pax3 homeodomain (Bennicelli *et al.*, 1996). This observation suggests that alternative mechanisms may be involved that work either in conjunction with or in addition to the potential sequestering of hDaxx, and it allows us to propose a model that explains, in part, the oncogenic activity of Pax3-FKHR. The oncogenicity of Pax3-FKHR has been demonstrated to depend on both protein-DNA interactions, mediated through the Pax3 homeodomain, and protein-protein interactions, mediated through a combination of the Pax3 homeodomain recognition helix and a small region of the FKHR transactivation domain (Lam *et al.*, 1999). In our model Pax3 homeodomain-mediated protein-DNA interactions would be responsible for directing the binding of Pax3-FKHR to the promoter regions of Pax3 target genes. After binding of Pax3-FKHR to DNA the fusion protein would then interact with hDaxx and a variety of other as of yet unidentified regulatory proteins. In the case of hDaxx this interaction would occur primarily through the Pax3 homeodomain recognition helix with the additional involvement of the N-terminally extended octapeptide domain and influence by the FKHR transactivation domain. Despite this interaction the Pax3-FKHR protein would no longer be responsive to the natural regulatory action of hDaxx and possibly other regulatory mechanisms. This would result in an improper regulation of Pax3 target genes, which would have an important role in the oncogenic activity of Pax3-FKHR.

Several mechanisms could explain the inability of Pax3-FKHR to respond to the repressive action of hDaxx despite an apparently strong interaction. First, because Pax3-FKHR contains a much stronger transactivation domain than Pax3, the binding of hDaxx to the Pax3 homeodomain and octapeptide domain may not be sufficient to displace the transcriptional machinery recruited by the FKHR transactivation domain. Secondly, the presence of the FKHR moiety, while not preventing the interaction of hDaxx with the octapeptide domain and homeodomain of Pax3, may cause steric hindrance of hDaxx, preventing the recruitment of additional factors required for effective repression. Thirdly, the tighter binding of hDaxx by Pax3-FKHR, possibly mediated through the additional role of the small region of the FKHR transactivation domain, may cause the binding of hDaxx in a nonfunctional

conformation. Finally, Pax3-FKHR binds the 110 kDa alternatively post-translationally modified form of hDaxx, rather than the 70 kDa form bound by Pax3. Although we can not exclude the possibility that this difference in binding simply occurs as a result of DNA-bound Pax3-FKHR versus unbound Pax3, it is possible that this alternatively modified form of hDaxx may no longer contain repressive activity. Further experiments are required to determine which of these mechanisms may be important.

Recently it has been reported that Rb is able to repress Pax3 transcriptional activity through a direct interaction with the Pax3 homeodomain (Wiggin *et al.*, 1998). It has also been reported that HIRA, a histone-associated transcriptional co-repressor, specifically interacts with the Pax3 homeodomain (Magnaghi *et al.*, 1998). However, it remains to be established whether the Pax3-FKHR fusion protein is also refractive to the regulatory effects of these proteins. Presently it is not known how and if hDaxx cooperates with either Rb or HIRA in the repression of Pax3, but biochemically their interactions are different: hDaxx binding involves the octapeptide domain whereas HIRA and Rb do not. We propose that evasion from a natural repressor such as hDaxx and possibly other proteins such as HIRA and Rb may contribute to the deregulated transcriptional control by the Pax3-FKHR protein during tumor formation.

Materials and methods

Yeast two-hybrid screen and cloning of hDaxx

A PCR fragment encoding amino acids 1–347 of Pax3 was subcloned into plasmid pEG202 in-frame with the yeast LexA DBD to generate the pEG202-Pax3 bait plasmid. The yeast two-hybrid screen using pEG202-Pax3 was performed essentially as described previously (Gyuris *et al.*, 1993; Ausubel *et al.*, 1996). Of 9×10^5 initial transformants, 11 expressed β -galactosidase, grew on SD synthetic medium lacking leucine and were demonstrated to be specific for Pax3. One of these clones encoded a partial cDNA (HeLa 5-6) identical to hDaxx. The ~700 bp insert from the library plasmid pJG4-5/HeLa5-6 was radiolabeled and used as a probe to screen 6×10^5 plaques from an unamplified oligo(dT)-primed human rhabdomyosarcoma cDNA library. This resulted in the isolation of the complete hDaxx cDNA, which was subsequently cloned into the *EcoRI* site of Bluescript and pcDNA3.

Pax3 deletion mutants and quantitative β -galactosidase assays

The internal deletion mutants PDND and HD3D were generated by PCR amplification from previously established Pax3-FKHR internal deletion constructs (Lam *et al.*, 1999). The remaining deletion mutants were generated by PCR amplification and standard cloning techniques. The paired and homeodomains of Pax7 and Pax4 were generated by PCR amplification from cDNAs kindly provided by M. Busslinger and B. Sosa-Pineda, respectively, using primers generated from published sequences (Schafer *et al.*, 1994; Inoue *et al.*, 1998). All mutants were subcloned into plasmid pEG202 in frame with the yeast LexA DBD and confirmed by sequence analysis.

To quantitatively determine β -galactosidase activity Pax3, the indicated Pax3 deletion mutants, Pax7, Pax4 or Pax3-FKHR, were co-transformed into the yeast strain EGY48 with the partial hDaxx clone (HeLa 5-6) and the LacZ reporter plasmid pSH18-34 (Gyuris *et al.*, 1993). A quantitative liquid β -galactosidase assay was performed as described previously (Adams *et al.*, 1997) for four to six independent transformants of each construct.

Antibody production and immunoprecipitation

Anti-hDaxx antibodies were produced by rabbit immunization with a bacterially expressed GST-hDaxx_{635–740} (Rockland Laboratories). The rabbit anti-hDaxx antibody was affinity purified by first pre-clearing the antiserum over a GST-Sepharose column followed by GST-hDaxx_{635–740}

affinity chromatography using the AminoLink® Plus immobilization kit (Pierce), according to the manufacturer's specifications. The Pax3 antiserum was as described previously (Lam *et al.*, 1999) and was used without further purification.

To co-immunoprecipitate Pax3 and hDaxx, COS1 cells (1×10^5) were plated in 60 mm dishes and co-transfected the following day with mammalian expression vectors encoding the HA1 epitope tagged Pax3 (pCMV-HA-Pax3, kindly provided by P.Hamel) and full-length hDaxx (pcDNA3-hDaxx), by the Superfect™ method (Qiagen) according to the manufacturer's specifications. After 48 h the cells were harvested and lysed on ice in EBC dissociation buffer [50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 2 µg/ml aprotinin, 1 mM β-glycerophosphate, 1 mM NaF and 0.1 mM NaVO₄] for 1–2 h. The cell debris was removed by centrifugation at maximum speed in a Sorvall RMC 1Y microfuge for 15 min at 4°C. The resulting supernatants were incubated with a mouse monoclonal anti-HA antibody (12CA5, Boehringer Mannheim) overnight at 4°C on a rotary shaker. Immune complexes were collected with protein A-Sepharose (Pharmacia) and washed four times with ice-cold EBC dissociation buffer. The pellets were resuspended in 40 µl of SDS-PAGE buffer and boiled for 5 min. Supernatants were resolved by 10% SDS-PAGE, blotted to nitrocellulose and hDaxx was detected by Western analysis using the rabbit polyclonal anti-hDaxx antibody. To confirm overexpression of HA-Pax3 and hDaxx, 50 µg of cell lysate were resolved by 10% SDS-PAGE, blotted to nitrocellulose, and protein was detected by Western analysis using either the anti-hDaxx or the anti-HA antibodies.

Phosphorylation status of hDaxx

Jurkat cells (1×10^5 /ml) were transfected with the mammalian expression vector pcDNA3-hDaxx by the Superfect™ method (Qiagen) according to the manufacturer's specifications. After 48 h the cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 0.1 mM PMSF, 10 µg/ml leupeptin and 2 µg/ml aprotinin) for 1–2 h at 4°C, in either the absence or presence of phosphatase inhibitors (1 mM β-glycerophosphate, 1 mM NaF and 0.1 mM NaVO₄). The cell debris was removed as described above and the resulting lysates were incubated at 37°C overnight. 50 µg of the cell lysates were resolved by 10% SDS-PAGE, blotted to nitrocellulose and protein was detected by Western analysis using the rabbit anti-hDaxx polyclonal antibody.

For direct [³²P]orthophosphate labeling of hDaxx, COS1 cells (1×10^5) were plated in 60 mm dishes and transfected the following day with a mammalian expression vector encoding the FLAG-epitope tagged hDaxx (pFlex-hDaxx) by the Superfect™ method (Qiagen) according to the manufacturer's specifications. After 48 h the medium was replaced with phosphate free Dulbecco's modified Eagle's medium containing 200 µCi of [³²P]orthophosphate (Amersham) and incubated for 3 h in 10% CO₂ at 37°C. Cells were harvested and lysed in RIPA buffer containing protease and phosphatase inhibitors, as described above, immunoprecipitated with monoclonal anti-FLAG antibody, M2 (Eastman Kodak), collected with protein A-Sepharose and the immune complex separated by 10% SDS-PAGE. The dried gel was visualized by autoradiography. To confirm that all forms of hDaxx were precipitated by the anti-FLAG antibody, unlabeled immune complexes were separated by 10% SDS-PAGE and the presence of hDaxx was determined using the C-terminal anti-hDaxx antibody.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift analysis of Pax3 and hDaxx was performed as previously described (Lam *et al.*, 1999). Protein-DNA binding reactions included bacterially expressed GST-Pax3 (0.5 µg) or GST (0.5 µg). Where indicated, GST-Pax3 and either bacterially expressed GST-hDaxx_{635–740} (1.0, 2.5 or 5.0 µg) or GST (5.0 µg) were incubated for 10 min at room temperature before the addition of radiolabeled oligonucleotide probe PRS-9, which contains both the paired domain and the homeodomain recognition sequences. Incubation of GST-hDaxx_{635–740} (5 µg) with labeled PRS-9 probe was performed in an identical manner. Incubation of GST-Pax3 with competitor (1500-fold molar excess of unlabeled PRS-9 probe) or with the Pax3 rabbit antiserum and separation of the DNA-protein complexes were performed as described previously (Lam *et al.*, 1999). Gels were fixed in 10% acetic acid, dried, and labeled complexes were visualized by autoradiography.

Pax3-specific oligonucleotide affinity resin

The creation of the Pax3-specific oligonucleotide resin and the binding of either Pax3 or Pax3-FKHR to this affinity resin were carried out

essentially as previously described (Glass *et al.*, 1988). Briefly, a biotinylated e5 oligonucleotide, which contains both paired and homeodomain recognition sequences (1 µg), was incubated with 80 µl of streptavidin MagneSphere beads (Promega) at room temperature for 15 min followed by three washes with affinity resin binding buffer [15 mM Tris-HCl pH 7.5, 60 mM KCl, 7.5% glycerol, 0.5 mM DTT, 4 mM spermidine, 0.025% bovine serum albumin (BSA) and 0.05 µg/µl poly dIodC] resulting in ~90% coupling efficiency. Either NIH 3T3 cells overexpressing both Pax3-FKHR and hDaxx or the rhabdomyosarcoma cell line Rh30 (1×10^7) were resuspended in the affinity resin buffer and lysed by four rounds of freeze-thaw. After clearing the debris 750 µg of total cell lysate were incubated with either the Pax3 site resin or empty resin at room temperature for 30 min. The samples were then washed three times with the affinity resin buffer and once with phosphate-buffered saline (PBS). The retained proteins were released by adding SDS-PAGE loading buffer and boiling the mixture for 5 min. The supernatant was separated by 10% SDS-PAGE, transferred to Immobilon-P and analyzed by Western blot.

Transient transfections and CAT assays

NIH 3T3 cells (1×10^5) were plated in 60 mm dishes and transfected the following day by the lipofectAMINE™ method (Gibco-BRL), according to the manufacturer's specifications. The lipofectAMINE™/DNA precipitate consisted of 1 µg of the 1X-GAL4-TK-CAT reporter plasmid containing one GAL4 DNA-binding site, 500 ng of the secreted alkaline phosphatase (SEAP) control plasmid under control of the MAP1 promoter, and the indicated amounts of either pM2-hDaxx or pcDNA3-hDaxx. After 48 h the medium was assayed for SEAP activity as described previously (Bram *et al.*, 1993), and the cells were harvested and assayed for CAT activity as described by Ausubel *et al.* (1996). The percentage of [¹⁴C]chloramphenicol acetylation was quantitated from thin-layer chromatography plates using a PhosphorImager (Molecular Dynamics). The transfection efficiency was normalized relative to the SEAP activity and each experiment was repeated two times in duplicate plates.

To examine the effect of hDaxx on Pax3 and Pax3-FKHR transcriptional activity NIH 3T3 cells (1×10^5) were plated in 60 mm dishes and transfected the following day by the lipofectAMINE™ method (Gibco-BRL), according to the manufacturer's specifications. The lipofectAMINE™/DNA precipitate consisted of 20 fmol of pcDNA3-Pax3 (85 ng) or 20 fmol of pcDNA3-Pax3-FKHR (100 ng), increasing amounts of pcDNA3-hDaxx [20 fmol (91 ng), 50 fmol (226 ng) or 100 fmol (450 ng)], 1 µg of the (PRS-9)pTK-CAT reporter plasmid, containing the Pax3 paired and homeodomain recognition sequences, and 500 ng of the secreted alkaline phosphatase (SEAP) control plasmid under control of the MAP1 promoter. Because the increasing amounts of input hDaxx alter the total amount of input DNA it was necessary to normalize all samples to 2 µg by the addition of pcDNA3-antisense hDaxx. Analysis of CAT activity was performed as described (Ausubel *et al.*, 1996). All samples were corrected for the basal transcriptional activity in the absence of either Pax3 or Pax3-FKHR (20 fmol pcDNA3) and each experiment was repeated twice in quadruplicate plates.

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References

- Adams, A., Gottschling, D.E., Kaiser, C.A. and Stearns, T. (1997) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*. Cold Spring Harbor Laboratory Press, Plainview, NY.

- Alland,L., Muhle,R., Jr, Potes,J., Chin,L., Schreiber-Agus,N. and DePinho,R.A. (1997) Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature*, **387**, 49–55.
- Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1996) *Current Protocols in Molecular Biology*. John Wiley & Sons, Boston, MA.
- Ayer,D.E., Lawrence,Q.A. and Eisenman,R.N. (1995) Mad–Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. *Cell*, **80**, 767–776.
- Bairoch,A., Bucher,P. and Hofmann,K. (1997) The PROSITE database, its status in 1997. *Nucleic Acids Res.*, **25**, 217–221.
- Bennicelli,J.L., Edwards,R.H. and Barr,F.G. (1996) Mechanism for transcriptional gain of function resulting from chromosomal translocation in alveolar rhabdomyosarcoma. *Proc. Natl Acad. Sci. USA*, **93**, 5455–5459.
- Bram,R.J., Hung,D.T., Martin,P.K., Schreiber,S.L. and Crabtree,G.R. (1993) Identification of the immunophilins capable of mediating inhibition of signal transduction by cyclosporin A and FK506: roles of calcineurin binding and cellular location. *Mol. Cell. Biol.*, **13**, 4760–4769.
- Chalepakis,G., Jones,F.S., Edelman,G.M. and Gruss,P. (1994) Pax-3 contains domains for transcription activation and transcription inhibition. *Proc. Natl Acad. Sci. USA*, **91**, 12745–12749.
- Chang,H.Y., Hishitoh,H., Yang,X., Ichijo,H. and Baltimore,D. (1998) Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. *Science*, **281**, 1860–1863.
- Chang,H.Y., Yang,X. and Baltimore,D. (1999) Dissecting Fas signaling with an altered-specificity death-domain mutant: Requirement of FADD binding for apoptosis but not Jun N-terminal kinase activation. *Proc. Natl Acad. Sci. USA*, **96**, 1252–1256.
- Fakan,S. (1994) Perichromatin fibrils are *in situ* forms of nascent transcripts. *Trends Cell Biol.*, **4**, 86–90.
- Fredericks,W.J., Galili,N., Mukhopadhyay,S., Rovera,G., Bennicelli,J., Barr,F.G. and Rauscher,F.J., III (1995) The PAX3–FKHR fusion protein created by the (t(2;13) translocation in alveolar rhabdomyosarcomas is a more potent transcriptional activator than PAX3. *Mol. Cell. Biol.*, **15**, 1522–1535.
- Galili,N., Davis,R.J., Fredericks,W.J., Mukhopadhyay,S., Rauscher,F.J., III, Emanuel,B.S., Rovera,G. and Barr,F.G. (1995) Fusion of a fork head domain gene to PAX3 in the solid tumour alveolar rhabdomyosarcoma. *Nature Genet.*, **5**, 230–235.
- Glass,C.K., Holloway,J.M., Devary,O.V. and Rosenfeld,M.G. (1988) The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. *Cell*, **54**, 313–323.
- Gyuris,J., Golemis,E., Chertkov,H. and Brent,R. (1993) Cdi1, a human G₁ and S phase protein phosphatase that associates with Cdk2. *Cell*, **75**, 791–803.
- Hassig,C.A., Fleischer,T.C., Billin,A.N., Schreiber,S.L. and Ayer,D.E. (1997) Histone deacetylase activity is required for full transcriptional repression by mSin3A. *Cell*, **89**, 341–347.
- Heinzel,T. *et al.* (1997) A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature*, **387**, 43–48.
- Hunter,T. and Karin,M. (1992) The regulation of transcription by phosphorylation. *Cell*, **70**, 375–387.
- Inoue,H., Nomiyama,J., Nakai,K., Matsutani,A., Tanizawa,Y. and Oka,Y. (1998) Isolation of full-length cDNA of mouse PAX4 gene and identification of its human homologue. *Biochem. Biophys. Res. Commun.*, **243**, 628–633.
- Kadosh,D. and Struhl,K. (1997) Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. *Cell*, **89**, 365–371.
- Kiriakidou,M., Driscoll,D.A., Lopez-Guisa,J.M. and Strauss,J.F., III (1997) Cloning and expression of primate Daxx cDNAs and mapping of the human gene to chromosome 6p21.3 in the MHC region. *DNA Cell Biol.*, **16**, 1289–1298.
- Lam,P.Y.-P., Sublett,J.E., Hollenbach,A.D. and Roussel,M.F. (1999) The oncogenic potential of the Pax3–FKHR fusion protein requires the Pax3 homeodomain but not DNA binding. *Mol. Cell. Biol.*, **19**, 594–601.
- Lechner,M.S. and Dressler,G.R. (1996) Mapping of Pax-2 transcription activation domains. *J. Biol. Chem.*, **271**, 21088–21093.
- Magnaghi,P., Roberts,C., Lorain,S., Lipinski,M. and Scambler,P.J. (1998) HIRA, a mammalian homologue of *Saccharomyces cerevisiae* transcriptional co-repressors, interacts with PAX3. *Nature Genet.*, **20**, 74–77.
- Mitchell,P.J. and Tjian,R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science*, **245**, 371–378.
- Nagy,L., Kao,H.-Y., Chakravarti,D., Lin,R.J., Hassig,C.A., Ayer,D.E., Schreiber,S.L. and Evans,R.M. (1997) Nuclear receptor repression mediated by a complex containing SMRT, mSin3A and histone deacetylase. *Cell*, **89**, 373–380.
- Pluta,A.F., Earnshaw,W.C. and Goldberg,I.G. (1998) Interphase-specific association of intrinsic centromere protein CENP-C with HDaxx, a death domain-binding protein implicated in Fas-mediated cell death. *J. Cell Sci.*, **111**, 2029–2041.
- Shafer,B.W., Czerny,T., Bernasconi,M., Genini,M. and Busslinger,M. (1994) Molecular cloning and characterization of a human PAX-7 cDNA expressed in normal and neoplastic myocytes. *Nucleic Acids Res.*, **22**, 4574–4582.
- Shapiro,D.N., Sublett,J.E., Li,B., Downing,J.R. and Naeve,C.W. (1993) Fusion of PAX3 to a member of the forkhead family of transcription factors in human alveolar rhabdomyosarcoma. *Cancer Res.*, **53**, 5108–5112.
- Stuart,E.T., Yokota,Y. and Gruss,P. (1995) PAX and HOX in Neoplasia. *Adv. Genet.*, **33**, 255–274.
- Wang,H., Clark,I., Nicholson,P.R., Herskowitz,I. and Stillman,D.J. (1990) The *Saccharomyces cerevisiae* SIN3 gene, a negative regulator of HO, contains four paired amphipathic helix motifs. *Mol. Cell. Biol.*, **10**, 5927–5936.
- Wiggin,O.N., Taniguchi-Sidle,A. and Hamel,P.A. (1998) Interaction of the pRB-family proteins with factors containing paired-like homeodomains. *Oncogene*, **16**, 227–236.
- Xu,W., Rould,M.A., Jun,S., Desplan,C. and Pabo,C.O. (1995) Crystal structure of a paired domain–DNA complex at 2.5 Å resolution reveals structural basis for Pax developmental mutations. *Cell*, **80**, 639–650.
- Yang,X., Khosravi-Far,R., Chang,H.Y. and Baltimore,D. (1997) Daxx, a novel Fas-binding protein that activates JNK and apoptosis. *Cell*, **89**, 1067–1076.
- Zhang,Y., Iratni,R., Erdjument-Bromage,H., Tempst,P. and Reinberg,D. (1997) Histone deacetylases and SAP18, a novel polypeptide, are components of a human Sin3 complex. *Cell*, **89**, 357–364.

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