Genome-wide discovery of Pax7 target genes during development

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White RB, Ziman MR. Genome-wide discovery of Pax7 target genes during development. Physiol Genomics 33: 41–49, 2008. First published January 15, 2008; doi:10.1152/physiolgenomics.00256.2007.—Pax7 plays critical roles in development of brain, spinal cord, neural crest, and skeletal muscle. As a sequence-specific DNA-binding transcription factor, any direct functional role played by Pax7 during development is mediated through target gene selection. Thus, we have sought to identify genes targeted by Pax7 during embryonic development using an unbiased chromatin immunoprecipitation (ChIP) cloning assay to isolate cis-regulatory regions bound by Pax7 in vivo. Sequencing and genomic localization of a library of chromatin-DNA fragments bound by Pax7 has identified 34 candidate Pax7 target genes, with occupancy of a selection confirmed with independent chromatin enrichment tests (ChIP-PCR). To assess the capacity of Pax7 to regulate transcription from these loci, we have cloned alternate transcripts of Pax7 (differing significantly in their DNA binding domain) into expression vectors and transfected cultured cells with these constructs, then analyzed target gene expression levels using RT-PCR. We show that Pax7 directly occupies sites within genes encoding transcription factors Gbx1 and Eya4, the neurogenic cytoskeleton channel Kcnk2, and the signal transduction kinase Camk1d in vivo and regulates the transcriptional state of these genes in cultured cells. This analysis gives us greater insight into the direct functional role played by Pax7 during embryonic development.

PAX7 PLAYS DIVERSE ROLES DURING development, functioning in the developing central nervous system, neural crest, dermo-neuro-myotome, and myotome and in specification of satellite cells, critical for adult muscle regeneration (4, 30, 65). Pax7 knockout mice exhibit skeletal muscle and craniofacial deformities (44, 65); however, the full function of Pax7 is almost certainly obscured in this analysis by partial functional redundancy with its paralog Pax3 (59).

In the developing central nervous system, Pax7 is broadly expressed throughout the dorsal alar plate prior to differentiation (30), becoming further restricted as differentiation proceeds to the superior colliculus/tectum (69, 70). Misexpression of Pax7 in the diencephalon generates an ectopic tectum that becomes innervated by retinal neurons (46), thus defining Pax7 as vital for superior collicular development and innervation. Importantly, Pax7 remains strongly expressed in the adult where it is thought necessary for neural cell maintenance (68, 70).

In the developing neural tube, Pax7 is strongly expressed dorsally (31, 59), and later in commissural neurons of the spinal cord and dorsal root ganglia (43, 51), and in the absence of both Pax7 and Pax3, cells within the dorsal spinal cord acquire a ventral fate (43). Pax7 also specifies a subset of cephalic neural crest cells that migrate rostrally and laterally from fore-, mid-, and hindbrain regions to the nasal prominence and the pigmented epithelium around the eye (4, 33), with Pax7 expression remaining in the nasal neuroepithelium (30). Pax7−/− knockout mice display craniofacial malformations involving the nose and maxilla consistent with neural crest cell defects (44).

Pax7 and Pax3 function in the specification of somatic skeletal muscle precursors during embryogenesis (15, 23, 28, 59). Pax7 is initially expressed uniformly throughout the dorsolateral half of nascent somites (23) becoming concentrated in the medial dermomyotome (15, 32, 59) with expression continuing in almost all myotome cells, often concomitantly with the myogenic differentiation factor MyoD (31). While Pax7−/− mice appear to have normal skeletal muscle at birth, their postnatal growth, predominantly driven by satellite cells, is heavily retarded (44) since these cells are severely depleted in Pax7−/− mice (64). Significantly, quiescent and activated satellite cells, defined by their expression of Pax7, downregulate this gene during their differentiation into myoblasts (65, 79, 80). It is now apparent that Pax7 is involved in both proliferation and survival of these cells; Pax7−/− satellite cell cultures show 25–30% reduced proliferation, and Pax7 dominant negative infected satellite cells display significantly increased apoptosis (58).

Pax7 is a sequence-specific DNA-binding transcription factor containing two distinct DNA binding domains, the paired domain and the homeodomain. To date, an accurate description of the function of this transcription factor has not been obtained through an understanding of its target genes. Here we have applied a chromatin immunoprecipitation (ChIP) cloning strategy to identify genetic loci bound by Pax7 in murine embryos and detected physiologically relevant downstream targets including several of known significance in the development or function of nervous and muscular systems. We have then used Pax7 transfection and semi quantitative RT-PCR to assess regulation of targeted genes.

Complicating this analysis, the Pax7 paired domain is subject to two alternate splicing events resulting in the alternate inclusion of a glutamine residue (Q+) at position 75 and/or a glycine-leucine dipeptide (GL+) at position 118, producing four alternate isoforms labeled Pax7a (Q+/-GL+), Pax7b (Q+/ GL+), Pax7c (Q+/-GL−), and Pax7d (Q+/GL−) (83). These alternate isoforms differ in consensus DNA target sequence binding in vitro (19), so here we have sought to ascertain whether this translates to differences in target gene selection/ regulation.

These strategies provide a powerful approach for identification of target genes regulated by a transcription factor during embryogenesis, and clarify the functional role played by Pax7 during development.
MATERIALS AND METHODS

ChIP. ChIP was conducted as previously described (74) using a modification of the Upstate ChIP-kit protocol (Upstate Biotechnology). All experimental procedures were ratified by the Edith Cowan University (ECU) Animal Research Ethics Committee and conformed to Australian National Health and Medical Research Committee regulations. Pregnant female C57BL/6J mice were killed by Lethobarb injection (15 mg/100 g body wt ip) followed by cervical dislocation. Chromatin from whole embryos was used for all ChIP-cloning and target gene identification. Latter experiments involving ChIP-PCR were conducted using chromatin isolated from either whole embryos or isolated whole brains. Whole embryonic day 16 (E16) embryos, individually killed by being placed in a −20°C freezer for 5 min followed by decapitation, or whole brains dissected from E16 embryos, were minced to ~1 mm-sized pieces, immediately cross-linked in 1% (wt/vol) formaldehyde in phosphate-buffered saline (PBS) pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 10 mM NaH₂PO₄, and 1.4 mM KH₂PO₄, for 15 min at room temperature then quenched by addition of 0.125 M glycine, washed once in PBS with 0.125 M glycine and twice with PBS. Minced, fixed tissue was Dounce (Bellco Glass, Vineland, NJ) homogenized in cell lysis buffer pH 7.2, containing 10 mM Tris, 10 mM NaCl, 0.2% (vol/vol) Nonidet P-40 and protease inhibitors; 1 mM phenyl methane sulfonyl fluoride (PMSF) and 1 μg/ml protease inhibitor cocktail (Roche). After centrifugation, the pellet was homogenized in nuclei lysis buffer pH 8.1, containing 50 mM Tris-HCl, 10 mM EDTA, 1% (wt/vol) SDS, 10 mM sodium butyrate, 1 mM PMSF, and 1 μg/ml protease inhibitor cocktail, and chromatin was sonicated on ice to an average length of 800 bp using a Branson 450 Sonifier (power output 2, 10% duty cycle), and processed as per Upstate ChIP-kit protocol (Upstate) using anti-Pax7 monoclonal antibody (Santa Cruz Biotechnology) per sample, using chromatin that equated to either one whole E16 embryo per eight ChIP samples (for ChIP cloning and latter ChIP-PCR) or one whole E16 brain per two ChIP samples. After immunocomplex elution, ChIP samples (for ChIP cloning and latter ChIP-PCR) or one whole protein G-agarose (Santa Cruz Biotechnology) per sample, using experiments (74), or no antibody for control treatment, and 120 μl DNA was pelleted (calf intestinal phosphatase), column purified, and target gene identification. Chromatin from whole embryos was used for all ChIP-cloning reactions. Cellular location of Pax7 targets was determined via densitometric quantification using QuantityOne software (Bio-Rad). Fold enrichment was determined (expressed as +Pax7-specific antibody/no antibody) and subjected to unpaired two-tailed t-tests using StatistiXL v1.3.

Construction of Pax7 alternate transcript vectors. Full-length cDNAs encoding alternate isoforms of Pax7 (a–d) were isolated via nested RT-PCR from RNA of C57BL/6J mouse gastrocnemius muscle. RNA (1.8 μg) was reverse transcribed using Omniscript reverse transcriptase (Qiagen) and oligo-dT₁₈ primers in a 20 μl standard reaction. CDNA solution (2 μl) was PCR amplified in a 25 μl reaction using ProofStart DNA polymerase (Qiagen) for 35 cycles of 3-step PCR with primers F1 (5′-GAGGTTTATTCGCGCGAAGTCTG-3′) and R1 (5′-AGGGTTGGCTGCGCACTTGGG-3′), with an annealing temperature of 55°C and 2 min extension times. This reaction (1 μl) was then used as input for the nested PCR reaction using primers F2 (5′-CACCGACTCGGTTCCTGCACCAGG-3′) and R2 (5′-GTAGCTGTGTCGGTCATGAGG-3′) with above conditions cloned into pcDNA3.1/D/N-His-TOPO (Invitrogen) and transformed into OneShot TOP10 E. coli cells using the manufacturer’s instructions. Clones were PCR analyzed and those containing inserts of the correct size (~1.5 kb) in the correct orientation were screened by restriction digest (10 μl PCR product with BstXI and EcoRI, Fermentas) (83). Cloned Pax7 alternate transcripts were purified using QIAquick columns (Qiagen) and sequenced (as above) in both orientations with vector primers T7 and BGH Reverse (Invitrogen). Plasmids were isolated using QIAfilter Plasmid Midi kits (Qiagen), isopropanol precipitated, and quantified both spectrophotometrically and by agarose gel analysis.

Cell culture and transfection. Regulation of ChiP-identified target genes was assessed via transfection of pcDNA-Pax7(a–d) (or pcDNA3.1 control vector: Invitrogen) into cultured cells followed by RT-PCR analysis for expression levels of target genes. Murine P19 and NIH3T3 cells (American Type Culture Collection) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (vol/vol) fetal bovine serum and 1% (vol/vol) 1-glutamine (Invitrogen) at 37°C and 4% CO₂. All transfections were performed in triplicate in six-well plates using Lipofectamine 2000 (Invitrogen) and 4 μg plasmid vector. Transient transfections were maintained for 48 h, and RNA was isolated using the PureLink RNA isolation kit (Invitrogen) with residual DNA removed via DNase treatment using the DNA-free kit (Ambion).

RT-PCR. DNase-treated RNA was reverse transcribed with Omniscript reverse transcriptase (Qiagen) and oligo-dT₁₈ primers, and cDNA (2 μl) was then amplified with Taq polymerase (Qiagen) using primers and conditions contained in Supplementary Table S2; optimal cycle number was empirically determined for all reactions. The RNA level of all Pax7 targets was determined via densitometric quantification as above, normalized to Gapdh levels, and data were subjected to unpaired two-tailed t-tests (comparison of Pax7 and control pcDNA transfectants) or full-factorial ANOVA with post hoc Tukey multiple-comparison testing (comparison of Pax7 alternate transcript transfectants) using StatistiXL v1.3.

Bioinformatics. Full-length cloned regions of 34 ChIP-identified Pax7 target genes (mouse) (Table 1) and orthologous human and rat sequences were retrieved from Entrez with cross-species megaBLAST (NCBI) and used for bioinformatics analysis. Sequences were aligned using LAGAN/mVISTA (8) and input into Compare Protec-tors (40) to search for conserved overrepresented motifs. Comparative mouse/human conserved noncoding sequence maps were generated using mVISTA.

RESULTS

ChIP identification of 34 genes targeted by Pax7. We cloned and sequenced a library of chromatin-DNA immunoprecipitated with anti-Pax7 and analyzed the genomic location of

1 The online version of this article contains supplemental material.
We next sought to confirm Pax7 association with a selection of identified target genes. ChIP experiments were conducted in quadruplicate both with and without Pax7-specific antibody and analyzed for the presence of identified target regions using semiquantitative PCR with primers designed to flank target regions of selected genes, ciliary neurotrophic factor receptor (CntfR), gastrulation brain homeobox 1 (Gbxi), eyes absent 4 homolog (Eya4), and ciliary neurotrophic factor receptor. MSI analysis of PCR products revealed specific enrichment of Pax7-bound sequences and highlighting the usefulness of ChIP-PCR as a screening device for initial analysis of ChIP-cloning results.

We then performed PCR upon ChIP DNA immunoprecipitates using primers designed to the identified sites. We observed association of Pax7 with identified sites within first introns (11/34), other introns (9/34), and proximal promoter regions (8/34) of identified target genes.

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Significantly greater specific enrichment of the sequences associated with these genes was observed in chromatin immunoprecipitates using Pax7-specific antibody over no-antibody immunoprecipitates (Fig. 1). This analysis failed to show association of Pax7 with Gbxi; no enrichment was observed, indicating that this fragment’s cloning was likely an artifact and highlighting the usefulness of ChIP-PCR as a screening device for initial analysis of ChIP-cloning results.

We then performed PCR upon ChIP DNA immunoprecipitated from whole embryonic mouse brains (E16) to assess Pax7 occupation of identified loci specifically during brain development. We observed association of Pax7 with identified sites within CntfR, Eya4, Gbxi, and Cnk2, while no association was detected with Rasa3 or Prrx1 loci (Fig. 1B).

Bioinformatic prediction of Pax7 ChIP consensus site. We next searched ChIP-identified sequences of the 34 identified targets and orthologous human and rat sequences to define overrepresented motifs that may be indicative of Pax7 binding sites. Compare Prospector analysis successfully defined a consensus sequence for Pax7 (Fig. 2A) present in ChIP-identified Pax7 target genes, including CntfR that contains a core motif GTCAC previously shown to bind Pax7 in vitro (19). This GTCAC motif is highly enriched within ChIP-identified Pax7 target genes; for example the identified region of CntfR contains a core motif GTCAC previously shown to bind Pax7 in vitro (19). Two sites are present within the first 200 bp of intron one, one is palindromic and the other, present at TSS −58 bp, contains an optimal paired plus homeodomain binding site (TAATTA)

<table>
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<th>Target ID</th>
<th>Gene Name</th>
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<tr>
<td>CntfR</td>
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<td>Eya4</td>
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<td>Gbxi</td>
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<td>Ca²⁺/calmodulin-dependent protein kinase 1d</td>
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<td>PDZ and LIM domain containing 4</td>
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<td>potassium channel K2 (aka TREK1)</td>
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Identification of target genes based on cloned chromatin immunoprecipitation fragments being located within 100 kb of a coding sequence by BLAST analysis.
Pax7 regulation of target genes in transiently transfected cells. Having identified a suite of genes targeted by Pax7 during embryonic development, we next sought to test the capacity of Pax7 to regulate their expression by perturbing Pax7 levels and measuring target gene RNA expression. We designed intron-flanking primer sets to assay DNase-treated RNA from eight ChIP-identified target genes and conducted semiquantitative RT-PCR on P19 embryonal carcinoma and NIH3T3 cells transiently transfected with pcDNA-Pax7b expression vectors 48 h posttransfection, with all normalized to expression from the housekeeping gene Gapdh.

Our results show that Pax7b acts as a transcriptional activator of CntfR (11-fold change, \( P = 0.004 \)), Gbx1 (25-fold change, \( P = 0.003 \)), Kcnk2 (25-fold change, \( P = 0.003 \)), calcium/calmodulin-dependent protein kinase 1D (CntfR, 8-fold change, \( P = 1.2 \times 10^{-6} \)), and UDP-N-acetyl-\( \alpha \)-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 13 (Galnt13, 6-fold change, \( P = 9.6 \times 10^{-5} \)) (Fig. 4) in transfected cells. Interestingly, this activation was almost entirely limited to P19 cells, with small but insignificant upregulation observed in NIH3T3 cells; Kcnk2 and CamkId displayed a 2.5-fold and a 2-fold induction, respectively (Fig. 4A). By contrast and still more intriguingly, Pax7 acts as a transcriptional repressor of Eye4 (10-fold change, \( P = 5.5 \times 10^{-6} \)) in NIH3T3 cells (Fig. 4).

Pax7 isoform specificity. Pax7 is expressed as four isoforms (Pax7a–d), differing by one, two, or three amino acids in its critical paired DNA binding domain. To assess the capacity of individual isoforms to regulate expression of ChIP-identified target genes, we cloned cDNAs of alternate transcripts into expression vectors and transfected these into cultured P19 and NIH3T3 cell lines. Functional differences in target gene selection and/or transactivation potential of each isoform of Pax7 were assessed by full-factorial ANOVA upon data from Gapdh normalized RT-PCR analyses of transfected cells. This analysis uncovered a significant difference in the effects of Pax7 isoforms on CntfR RNA levels (F = 5.89, P = 0.010); post hoc testing (Tukey’s) revealed that isoform Pax7b displayed a significantly greater activation of CntfR gene expression than both Pax7c (\( q = 5.60, P = 0.009 \)) and Pax7d (\( q = 5.43, P = 0.033 \)) isoforms (Fig. 4B). Eye4, Gbx1, Rasa3, Kcnk2, Sclt1, CamkId, and Galnt13 were similarly analyzed and found not to be differentially expressed following forced expression of Pax7 isoforms (see Supplementary Fig. S1).

Lastly, we wished to determine whether transfected Pax7 exerted autoregulatory effects, as these have been previously documented for other Pax genes. To do this we conducted RT-PCR using primers specific for endogenous Pax7 RNA (using a forward primer complementary to the Pax7 5’ untranslated region not present in the cloned transcripts). Endogenous Pax7 was not expressed at detectable levels in either cell line transfected with pcDNA control vector; however, strong endogenous Pax7 expression is observed in all cells following transfection with Pax7(a–d), irrespectively (Fig. 4C).

**DISCUSSION**

Here we have utilized an unbiased approach to discover genes directly targeted by Pax7 in vivo during development. This analysis has, for the first time, identified a suite of genes...
directly occupied by Pax7 during embryonic development and furthermore has confirmed the capacity of Pax7 to regulate expression of a cohort of identified target genes. Cataloguing the known functions of the diverse variety of Pax7 target genes has delineated genetic and biochemical pathways that may function downstream of Pax7, providing new insight into the function of this transcription factor.

Pax7 regulates the expression of CntfR, Eya4, Gbx1, Kcnk2, Camk1d, and Galnt13 in cultured cells and directly occupies these genes during development. Interestingly, although transient Pax7 transfection showed some similarity in target gene regulation between cell lines (e.g., Kcnk2 and Camk1d), striking differences were observed. Pax7 acts predominantly as a transcriptional activator in P19 cells, and either as a mild activator or a strong repressor in NIH3T3 cells. A likely explanation for the functional difference in the behavior of Pax7 at different loci between cell lines is the presence of different cofactors in each cell line. Pax3 and Pax7 have long been considered very impotent transcriptional activators, and the most common reason cited for this is the requirement for unidentified cofactors (5, 19).

Pax7 target genes. Pax7 specifically targets genes involved in myogenesis, neurogenesis and neural activity, providing a direct mode of action for the developmental functions of Pax7. Gbx1 and Eya4 are both developmentally important transcriptional regulators and are both direct regulatory targets of Pax7. Gbx1 encodes an antennapedia class homeobox transcription factor expressed during gastrulation throughout the primitive streak and later in the brain, neural crest, dorsal spinal cord, and the developing dermomyotome (29, 73), and its single paralog, Gbx2, dictates neurulation and defines the midbrain-hindbrain organizer (47a). Gbx1 and Pax7 are also both involved in the formation of interneurons in the dorsal spinal cord (42, 43, 48) and remain expressed in the adult (29).

Likewise, Pax7 and Eya4 share significantly similar developmental expression patterns; both play critical roles in neurogenesis and myogenesis and are highly expressed in developing brain, craniofacial mesenchyme, dermomyotome and...
Fig. 4. Regulation of target genes by transient Pax7 transfection. A: RT-PCR analysis of target gene mRNA expression in P19 cells and NIH3T3 cells (3T3) transiently transfected with Pax7b-pcDNA vector (gray bars) or pcDNA control vector (white bars). Experiments were conducted in quadruplicate and normalized to Gapdh mRNA levels (data shown are means ± SE, n = 4, unpaired t-test, **P < 0.01). B: Pax7 alternate isoforms differentially regulate CntfR expression. Alternate transcript vectors Pax7a−d-pcDNA were transiently transfected into P19 cells and RT-PCR was conducted for CntfR mRNA levels. Experiments were conducted in quadruplicate and normalized to Gapdh mRNA levels (data shown are means ± SE, n = 4, full-factorial ANOVA, P = 0.010. Tukey’s post hoc testing *P < 0.05, **P < 0.01). C: autoregulation of endogenous Pax7. Pax7a–d-pcDNA alternate transcript vectors or pcDNA control vector were transiently transfected into NIH3T3 and P19 cells and DNase-treated RNA was analyzed by RT-PCR using primers specific for transfected Pax7 (Pax7Tc), endogenous Pax7 (Pax7end), and the housekeeping gene Gapdh, also included is a no-DNA PCR control (black).

limb during embryogenesis (6, 25, 49), and both Eya4 and Pax7 are robustly expressed in brain and skeletal muscle in adult mouse tissues (65, 70, 82). Eya family members function as transcriptional coactivators controlling cell fate specification, cell survival and apoptosis, proliferation, differentiation, and morphogenesis (34, 49, 57).

Pax and Eya proteins function in complex interactive pathways across phylogeny. Drosophila eye development is regulated by several Pax6 homologs (eyeless, eyegone, twin-of-eyeless, and twin-of-eyegone) (18) that function in synchrony with eyes absent, sine oculus (so or six), and dachshund (Dach) proteins (54). Conservation of this pathway by paralogous genes has been observed in murine myogenesis, where Pax3 and Eya2 function upstream of myogenic regulatory factors to induce myogenesis (60). Eya1 and Eya2 are functionally redundant during myogenesis and act upstream of Pax3 during dermomyotome development (25). Retroviral misexpression of Eya2 (with either six1 or Dach2) initiates Pax3 expression in somite explant cultures; conversely, retroviral misexpression of Pax3 upregulates Eya2 expression (26). Likewise, Pax3 overexpression initiates, and Pax3 dominant negative expression inhibits, Eya2 expression during induced skeletal myogenesis of P19 cells in vitro (60). Eya1, 2, and 4 are coexpressed in the dorsal somite, medial and lateral dermomyotome, and myogenic precursors, where all three are speculated to display functional redundancy (6, 76).

Our analysis has also identified multiple receptors and signaling molecules critical to muscular and nervous system development. Pax7 targets and regulates Camk1d, which functions in calmodulin-dependent signaling, a critical pathway for the activation of myogenesis (22, 50, 77). Calmodulin-dependent kinase activity mediates myogenic differentiation in part through the activation of myogenin, and interestingly components of this pathway, Calml and Camk1g, have been shown to be directly bound by MyoD and myogenin using ChIP analysis of cultured myoblasts (9).

CntfR and Rasa3 are both critical regulators of signaling pathways regulating proliferation and differentiation of neural cells during development. Rasa3 is a negative regulator and downstream effector of Ras (72) and is specifically expressed at high levels in neurons and oligodendrocytes of the developing and adult brain (3). CntfR is an essential component of the receptor complex necessary for signaling by ciliary neurotrophic factor (Cnft), cardiotoxhin-like cytokine, and neuropoietin; is expressed on neuronal precursors, neurons, and astrocytes; and is upregulated during in vitro neural differentiation (56). CntfR plays a vital role in modulating a cell’s responsiveness to its environment via Cntf-mediated signaling, affecting neuronal stem cell fate, survival, and differentiation of neurons (66, 67) and promoting self-renewal and expansion of ventricular zone neural precursors (24). Interestingly, CntfR is also expressed within developing and adult skeletal muscle (16), where it regulates myogenic differentiation; application of Cntf to regenerating muscles accelerates differentiation (45), and perhaps contradictorily, Cntf induces in vitro dedifferentiation of adult skeletal myoblasts into multipotent progenitor cells (13).

Pax7 also targets genes specifically functioning in differentiated neurons. Kcnk2 (aka TREK1) encodes a two-pore domain potassium channel (K2P) expressed throughout the central
nervous system, which regulates neuronal potassium leak, neuron excitability, and resting membrane potential (12, 21, 27). As well as targeting a potassium channel, Pax7 also targets Scitl, which possesses protein domains thought responsible for the aggregation of sodium channels at nodes of Ranvier (39). Galt13 encodes a neuron-specific glycosphingolipid involved in signal transduction and cell-cell signaling (81), and vomeronasal receptor Vlr7c is a G-protein signaling pheromone receptor exclusively expressed on sensory neurons of the apical zone of the vomeronasal organ vomeronasal epithelium (17), and although colocalization is necessary to verify coexpression, Pax7 is expressed throughout the nasal cavity and processes (44) and olfactory epithelia (36).

Pax7 regulation of target genes. Of the fragments of DNA chromatin immunoprecipitated by Pax7 that occur within 100 kb of a gene’s coding region, >80% (5/6) of selected sites analyzed were confirmed by independent quantitative ChiP-PCR analysis, and the expression levels of 75% (6/8) of selected target genes were significantly altered following forced Pax7 expression. It is worth noting that this analysis is preliminary, being carried out only in two cell lines and at one time point, 48 h posttransfection, thus an extension of this analysis could provide further insight into the capacity of Pax7 to regulate other genes identified by ChiP.

Furthermore, we analyzed Pax7-ChiP identified genomic regions and orthologous rat/human sequences using Compare Prospector analysis and successfully retrieved a consensus site containing a core motif, GTCAC, identical to the core of a motif initially uncovered as a Pax3 consensus binding site containing a core motif, GTCAC, identical to the core of a region and orthologous rat/human sequences using Compare. Performing this experiment in a background would help elucidate this further.

Role for Pax7 in regulating cellular responsiveness. A large proportion of the target genes isolated by this research implicates Pax7 as an upstream regulator of genes functioning in signaling pathways and provides a compelling explanation for experimental observations of Pax7 expression in cell types that are poised to respond to environmental cues, including myogenic satellite cells (14, 52, 78) and neural crest cells (4).

Pax7 activates expression of Cnfr, an inducer of Jak/STAT and Ras/ MAPK signaling pathways, and Camk1d, a component of calcium/calmodulin-dependent signaling, and binds to Rasa3, a GTPase activator and negative regulator of Ras intracellular signaling cascades. Other target genes including Pdlim4 and Fyn-related kinase (FrK) also function in intracellular signaling cascades, where Pdlim4 is involved in glutamate receptor signaling (61) and FrK is known to regulate cell survival, differentiation, and progression through the cell cycle (1). In fact, Pax7 is often ascribed as having the ability to regulate proliferation and apoptosis, an attribute that may be a direct result of the action of Pax7 upon its targets Cnfr (a positive regulator of proliferation and survival factor), FrK (a negative regulator of proliferation and cell cycle progression), and/or Rasa3 (a regulator of both proliferation and apoptosis).

In summary, we have identified a suite of genes that are directly targeted by Pax7 during embryonic development. Interestingly, many of these genes act as regulators of neurogenic and myogenic differentiation, proliferation, prevention of apoptosis, and responsiveness to signaling.

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