Identification of direct downstream targets of *Dlx5* during early inner ear development

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***Dlx5*, a homeobox transcription factor, plays a key role in the development of many organ systems. It is a candidate gene for human split-hand/split-foot type 1 malformation associated with sensorineural hearing loss. A deletion of one of its enhancers has been implicated in human craniofacial defects/hearing loss and it has also been associated with autism. However, little is known of how *Dlx5* exerts its regulatory effects. We identified direct targets of *Dlx5* in the mouse inner ear by gene expression profiling wild-type and *Dlx5* null otic vesicles from embryonic stages E10 and E10.5. Four hundred genes were differentially expressed. We examined the genomic DNA sequences in the promoter regions of these genes for (i) previously described *Dlx5* binding sites, (ii) novel 12 bp long motifs with a canonical homeodomain element shared by two or more genes and (iii) 100% conservation of these motifs in promoters of human orthologs. Forty genes passed these filters, 12 of which are expressed in the otic vesicle in domains that overlap with *Dlx5*. Chromatin immunoprecipitation using a *Dlx5* antibody confirmed direct binding of *Dlx5* to promoters of seven of these (*Atbf1*, *Bmper*, *Large*, *Lrrtm1*, *Msx1*, *Ebf1* and *Lhx1*) in a cell line over-expressing *Dlx5*. *Bmper* and *Lrrtm1* were up-regulated in this cell line, further supporting their identification as targets of *Dlx5* in the inner ear and potentially in other organs. These direct targets support a model in which Bmp signaling is downstream of *Dlx5* in the early inner ear and provide new insights into how the *Dlx5* regulatory cascade is initiated.**

INTRODUCTION

Dlx genes are members of an evolutionarily conserved family of homeobox transcriptional regulators. Vertebrate *Dlx* genes are orthologs of the *Distal-less* (*Dll*) gene of *Drosophila* which functions to promote distal limb development (1). Mammals possess six orthologs of *Dll* which are found in three pairs of two convergently transcribed genes—*Dlx1/2*, *Dlx3/4* and *Dlx5/6*—each linked to a *Hox* gene cluster (2–4). During mouse embryonic development, all six *Dlx* genes are expressed, primarily in the nervous system (particularly in the forebrain), head neural crest and surface ectoderm, often in overlapping domains (5–7). Their functions are numerous, and include the development of ectodermal tissues derived from head placodes (such as olfactory and otic structures), differentiation of forebrain GABA-ergic neurons as well as development of the brachial arches, cartilage, bone, limbs and inner ear.

Human chromosomal abnormalities encompassing *DLX5* and *DLX6* cause split-hand/split-foot type 1 malformation (SHFM1) associated with sensorineural deafness. Other features include ectrodactyly, cleft palate, teeth abnormalities, developmental delay and micrognathia (8–11). In addition, a paracentric inversion located ~80 kb centromeric to *DLX5* and *DLX6* [inv(7)(q21.3q35)] that includes a small (~5 kb) deletion has been shown to cause craniofacial defects and hearing loss in a three-generation human pedigree (12). The deletion appears to be in an enhancer that contains multiple conserved sequence elements, including several recognized by *DLX1* (which can regulate *DLX5* expression). This enhancer drives *LacZ* reporter gene expression in mouse embryos in some of the tissues that express *Dlx5*, including the inner ear (12).

The *Dlx5/Dlx6* double-knockout mouse is a model for SHFM1 since it recapitulates many of the human phenotypes (13,14). The *Dlx5* single knockout mouse does not exhibit

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ectrodactyly, but it does share many of the other phenotypes seen in SHFM1 and it is phenotypically very similar to humans who carry the *DLX5* enhancer deletion (12,15–17). It is interesting to note that human *DLX5* appears to be imprinted and is expressed preferentially from the maternal allele (18), whereas the mouse *Dlx5* gene is biallelically expressed and appears to escape genomic imprinting (19). This difference in imprinting may account for some of the phenotypic differences between the mouse models and the human diseases.

The *Dlx5* knockout mouse has been critical in establishing the role of the gene in normal inner ear development. *Dlx5* is normally expressed in the dorsal otic vesicle (15) and later during differentiation of the vestibular tissues (17). Vestibular morphogenesis is compromised in the null mutants such that they fail to form the endolymphatic duct (a defect visible as early as E10) as well as the anterior and posterior semi-circular canals. The lateral canal does form but is smaller, whereas the saccule appears normal and the utricle and cochlea have a mild phenotype (15–17). These defects in mutant inner ears are thought to be a result of decreased expression of *Bmp4*, a possible transcriptional target of *Dlx5* (17). Interestingly, a similar reduction in *Bmp4* expression is also observed in the developing palate of *Dlx5* null embryos (20).

The direct regulatory targets of *Dlx5* in the developing inner ear are currently unknown. However, a few *Dlx5* targets have been identified in other tissues. In particular, during bone development, *Dlx5* is known to activate *Osteocalcin* (21,22), *Collagen1A1* (23), *Secreted phosphoprotein 1* (24), *Runx2* (25) and *Alkaline phosphatase* (26). The promoters of these genes contain a short homeodomain response element (HDRE) in the form of 'ATTA' or 'TAAT' that is at least part of the binding site for *Dlx5*. Furthermore, in the developing forebrain and retina, it has been shown by chromatin immunoprecipitation (ChIP) that *Dlx1* and *Dlx2* can bind HDRE-containing sequences in the mouse intergenic enhancer located between *Dlx5* and *Dlx6* and activate reporter gene transcription (27,28), indicating that other *Dlx* genes might also bind these motifs.

In this study, we sought to identify genes regulated by *Dlx5* during early otic vesicle development as a first step in dissecting the complex *Dlx5* regulatory cascade in this and other target tissues. We employed gene chips (representing all known mouse genes) to measure gene expression levels in microdissected otic vesicles from wild-type and homozygous null *Dlx5* embryos from stages E10 and E10.5. We found 400 genes that changed in their expression levels between wild-type and mutant tissues in at least one developmental stage, including *Bmp4* which was down-regulated in mutants. We then analyzed the upstream 3 kb promoter regions of the differentially expressed genes to identify ones with previously identified *Dlx5* binding sites as well as those with other longer (12 bp) novel HDRE-containing sequence motifs shared by promoters of two or more genes, which might suggest co-regulation. After applying two additional filtering criteria, the presence of expression domains that overlap with that of *Dlx5* in the otic vesicle and a requirement for evolutionary conservation of the novel-predicted motifs, 12 genes remained as reasonable direct targets of *Dlx5*.

Immunoprecipitation of chromatin from an immortalized otic vesicle-derived cell line (IMO-2B1) over-expressing *Dlx5*, followed by PCR, confirmed direct binding of the *Dlx5* protein to the promoter regions of seven genes—*Atbf1*, *Bmper*, *Ebf1*, *Large*, *Lhx1*, *Lrrtm1* and *Msx1*. Of these, expression of *Bmper* and *Lrrtm1* was up-regulated in the transfected cell line (both were down-regulated in mutant otocysts), supporting their classification as direct downstream targets of *Dlx5* in the inner ear. These direct target genes provide the starting points for dissecting how alterations in *Dlx5* function can cause so many defects in organ development and morphogenesis.

RESULTS

Gene expression profiles of *Dlx5* wild-type and null otocysts

We chose to microdissect and transcriptionally analyze mouse otocysts from two early embryonic developmental stages; E10 and E10.5. These particular samples were selected because at E10 mutant otocysts exhibit the smallest visible signs of a defect—in the form of a deformed endolymphatic sac (Fig. 1A)—whereas at later stages much larger secondary changes occur. *Dlx5* is known to normally be expressed in otocysts at both of these early time points. Otocysts were separately microdissected and each embryo was genotyped. RNA was extracted from pools of genotype-confirmed *Dlx5* null or wild-type otocysts and analyzed on Affymetrix Genechips. Transcripts from *Dlx5* homozygous null otocysts were compared with those from wild-type otocysts. A total of 400 genes (listed in Supplementary Material, Table S1) were found to be differentially expressed by at least 1.5-fold in mutant otocysts, when compared with wild-type otocysts from E10 only, E10.5 only or from both stages. Together, these genes defined six patterns of expression—those that were up-regulated or down-regulated in mutants in both stages (11 and 20 genes, respectively), those that were up-regulated or down-regulated in mutants only at E10 (33 and 49 genes, respectively) and those that were up-regulated or down-regulated in mutants only at E10.5 (76 and 211 genes, respectively). Figure 1B shows the expression of these genes in a heat map format and Table 1 lists a sample of individual genes from all six patterns. Our working hypothesis, when we chose to analyze two stages of otocyst development, was that transcriptional changes at the early (E10) time point would more likely reflect direct effects of *Dlx5* activity and at the later time point the changes would reflect a cascade of secondary effects resulting from the earlier loss of *Dlx5* activity. Superficially, this would appear to be the case, since 113 transcripts were altered at E10 whereas 318 were altered at E10.5, of which 287 changes were unique to the later stage. However, as described below, our more detailed analyses suggest that many of the changes that are unique to the later stage also appear to result from direct interactions with *Dlx5*.

Surprisingly, in our comparative analysis, the *Dlx5* transcript was itself up-regulated in mutants at both stages (Table 1). The homozygous null animals retain the third exon of *Dlx5*, and the probe interrogating the expression of

Figure 1. Example images of microdissected otic vesicles from wild-type and *Dlx5* null embryos from E10 and E10.5 and a heat map of differentially expressed genes within them. Wild-type and *Dlx5* null otic vesicles after microdissection. Notice a lack of the endolymphatic duct (arrows) in the mutants (A). In (B), each column is a single gene chip (two for each genotype from each stage) and each row a single gene that was differentially expressed by at least 1.5-fold in mutants relative to wild-type. Expression increases from blue to white to red. The vertical line in the center separates chips from the two developmental stages. The numbers of genes following various trends of expression are shown in parentheses. For example, 20 genes were down-regulated in mutants in both stages, while 49 were down-regulated in mutants only in E10. Note that the numbers reflect unique genes and have been derived after taking overlap into account. That is, the 49 genes down-regulated in E10 are unique to this particular pattern (E10-Down) as are the 211 genes down-regulated in E10.5 (E10.5-Down). There were no genes showing opposite trends of expression in the two stages.

this gene on Affymetrix arrays is designed from this same exon. This suggested that there may be over-expression of a truncated transcript in mutant otocysts at these stages. The presence of this truncated transcript was indeed subsequently confirmed by RT-PCR (data not shown). The up-regulation of a truncated *Dlx5* transcript in mutants raises the possibility that it might produce a protein product that could interfere with gene expression. This appears unlikely since (i) more

than two-thirds of the homeodomain—the functional part of the protein that mediates various interactions—has been deleted in mutants (15), (ii) heterozygous animals are indistinguishable from their wild-type littermates suggesting an absence of dominant negative activity, and (iii) even wild-type animals appear to possess an alternatively spliced truncated transcript lacking the entire homeodomain that has been shown to fail to interact with *Msx1* and HDRE-containing DNA (29).

In agreement with previous observations, *Bmp4* was down-regulated in mutants at both stages (17), as was *Msx1* whose expression has been shown to be suppressed in the cristae when *Bmp4* signaling is blocked (30). Other genes of known relevance to the inner ear that were perturbed by *Dlx5* loss of function include two human deafness-causing genes, *Gjb2* (31) and *Ush3a* (32,33), both of which were up-regulated in mutants in at least one stage including E10. *Pax8*, one of the earliest markers of the otic placode, was down-regulated at E10, whereas *Otx2*, which is also required for normal vestibular morphogenesis (34), was down-regulated at E10.5. Interestingly, *Colla1*, a transcriptional target of *Dlx5* during bone development (23), was also down-regulated at E10.5.

Identifying conserved motifs in promoters of differentially expressed genes

Dlx5 has previously been shown to regulate the expression of several genes involved in bone development whose promoters harbor specific HDRE-containing sequence motifs (listed in Table 2). Therefore, we conducted an *in silico* motif analysis of the putative promoter regions of the 400 genes differentially expressed in *Dlx5* mutant otocysts. We extracted 3 kb of sequence immediately upstream of the transcription start site for each gene and searched these sequences for the presence of any of the HDRE-containing sequence motifs listed in Table 2. It should be noted that all of the previously described HDRE core motifs are fairly short (~8 bp) and, with the exception of the presence of either ATTA or TAAT, have little else in common. We constrained our searches to perfect matches to the 10 core motifs listed in Table 2. We were able to obtain upstream sequences for 357 well-annotated genes out of the list of 400 differentially expressed genes. Of these 357 putative promoters, there were 217 (60.7%) that satisfied this particular criterion, 57 of which were differentially expressed only in E10 or in both stages and 160 only in E10.5 (Supplementary Material, Table S2). Hence, it appears based on this criterion that there may be many direct targets of *Dlx5* whose expression is altered only in the later stage.

We were next interested in assessing whether the promoters of any of the 217 genes (each of which contained a previously described *Dlx* binding site) might also contain additional evolutionarily conserved, shared, longer HDRE-containing motifs. We initially searched for novel 12 bp long motifs shared within the promoter sequence of at least two different genes within the set of 217. The 12 bp cut-off was chosen so as to minimize discovering shorter motifs that might easily occur due to chance. Repetitive sequence elements and those unlikely to be binding sites for a *Dlx* protein (see Materials and Methods section) were not considered. Additional filtering was carried out requiring that every

Table 1. Examples of genes significantly differentially expressed between wild-type and *Dlx5* null otocysts from stages E10 and E10.5

Gene symbol	Fold-change MUT/WT	Expression pattern	Gene description
<i>Ahr</i>	-1.5	Both-DOWN	Aryl-hydrocarbon receptor
<i>Bmp4</i>	-2.0	Both-DOWN	Bone morphogenetic protein 4
<i>Chl1</i>	-6.9	Both-DOWN	Cell adhesion molecule with homology to L1CAM
<i>Msx1</i>	-1.6	Both-DOWN	Homeobox, msh-like 1
<i>Dlx5</i>	5.7	Both-UP	Distal-less homeobox 5
<i>Lhx1</i>	3.4	Both-UP	LIM homeobox protein 1
<i>Tmem169</i>	2.0	Both-UP	Transmembrane protein 169
<i>Ush3a</i>	1.8	Both-UP	Usher syndrome 3A homolog
<i>Apcdd1</i> ^a	-2.1	E10-DOWN	Adenomatosis polyposis coli down-regulated 1
<i>Myot</i>	-5.0	E10-DOWN	Myotilin
<i>Ncoa2</i>	-1.7	E10-DOWN	Nuclear receptor coactivator 2
<i>Nr6a1</i>	-2.6	E10-DOWN	Nuclear receptor subfamily 6, group A, member 1
<i>Pax8</i>	-2.2	E10-DOWN	Paired box gene 8
<i>Kcnt1</i> ^a	2.1	E10-UP	Potassium channel, subfamily T, member 1
<i>Pcdh17</i>	2.5	E10-UP	Protocadherin 17
<i>Isl1</i>	2.3	E10-UP	ISL1 transcription factor, LIM/homeodomain
<i>Gjb2</i>	1.9	E10-UP	Gap junction membrane channel protein beta 2
<i>Sfrs18</i>	1.6	E10-UP	RIKEN cDNA 5730406M06 gene
<i>Col14a1</i> ^a	-2.5	E10.5-DOWN	Procollagen, type XIV, alpha 1
<i>Dkk1</i> ^a	-3.3	E10.5-DOWN	Dickkopf homolog 1 (<i>Xenopus laevis</i>)
<i>Athf1</i>	-3.2	E10.5-DOWN	AT motif binding factor 1
<i>Col1a1</i>	-3.0	E10.5-DOWN	Procollagen, type I, alpha 1
<i>Bambi</i>	-2.9	E10.5-DOWN	BMP and activin membrane-bound inhibitor homolog
<i>Bmper</i>	-1.5	E10.5-DOWN	BMP-binding endothelial regulator
<i>Col5a3</i>	-4.0	E10.5-DOWN	Procollagen, type V, alpha 3
<i>Cryab</i>	-2.0	E10.5-DOWN	Crystallin, alpha B
<i>Dkk2</i>	-2.3	E10.5-DOWN	Dickkopf homolog 2 (<i>Xenopus laevis</i>)
<i>Dkk3</i>	-1.7	E10.5-DOWN	Dickkopf homolog 3 (<i>Xenopus laevis</i>)
<i>Foxp1</i>	-2.5	E10.5-DOWN	Forkhead box P1
<i>Large</i>	-3.3	E10.5-DOWN	Like-glycosyltransferase
<i>Lrrtm1</i>	-2.5	E10.5-DOWN	Leucine rich repeat transmembrane neuronal 1
<i>Meis1</i>	-2.0	E10.5-DOWN	Myeloid ecotropic viral integration site 1
<i>Msx2</i>	-2.1	E10.5-DOWN	Homeobox, msh-like 2
<i>Otx2</i>	-1.9	E10.5-DOWN	Orthodenticle homolog 2 (<i>Drosophila</i>)
<i>Sox17</i>	-1.9	E10.5-DOWN	SRY-box containing gene 17
<i>Tbx3</i>	-1.6	E10.5-DOWN	T-box 3
<i>Zic1</i>	-1.9	E10.5-DOWN	Zinc finger protein of the cerebellum 1
<i>Scn9a</i> ^a	2.0	E10.5-UP	Sodium channel, voltage-gated, type IX, alpha
<i>Cutl1</i> ^a	1.8	E10.5-UP	Cut-like 1 (<i>Drosophila</i>)
<i>Cbln1</i> ^a	1.8	E10.5-UP	Cerebellin 1 precursor protein
<i>Pou4f1</i>	2.4	E10.5-UP	POU domain, class 4, transcription factor 1

^aIndicates genes with at least two different probe sets that showed a differential expression in the same direction. Positive and negative fold-changes show up- or down-regulation, respectively, in mutants relative to wild-type. Supplementary Material, Table S1 contains a complete list of all 400 genes and individual probe sets that changed expression between wild-type and mutant tissues.

12 bp motif must contain an HDRE (ATTA or TAAT) and that the 12 bp motif must be 100% conserved in the promoter region of the human ortholog.

A total of 107 sequence elements satisfied all of these criteria. Table 3 lists all 107 sequences together with the individual genes (a total of 40) whose promoters they were found in. *Colla1*, which is a known transcriptional target of *Dlx5* during bone development (23), is one of the 40 genes in Table 3 as are *Bmp4* and *Msx1*, both of whose expression has been shown to be altered as a result of a change in the expression of *Dlx5* (17,30). While the inner ear functions of many of the other genes in this list are currently undefined, they represent novel candidate target genes of *Dlx5* in the developing otocyst.

ChIP and gene expression profiles in the 2B1 cell line

To test whether the *Dlx5* protein physically binds to the conserved sequence elements described above, we employed

ChIP. Ideally, this would have been conducted on otocysts. However, the very small cell numbers in these developmental structures precluded the application of ChIP as it is presently configured. Instead, we transiently transfected the 2B1 otocyst-derived cell line with a plasmid containing the *Dlx5* coding sequence under the transcriptional control of the CMV promoter and carried out ChIP using a *Dlx5* antibody. This particular cell line was derived from the E9.5 otocyst (35) and was used because we have found that its gene expression profile under differentiating conditions resembles that of the developing vestibular organ [(36), unpublished observations], suggesting that the 'founder' cell originated from this region of the otocyst. Since the *Dlx5* null otocysts begin to show defects in the vestibular region shortly after E9.5, this cell line was deemed an appropriate model system for investigating the binding of *Dlx5* to promoter regions of candidate genes.

Transfection efficiency, as determined by detecting green fluorescence protein (also contained within the expression

Table 2. Known binding sites for Dlx5 located in promoter regions (less than 3 kb upstream of transcriptional start) of several genes

Known Dlx5 binding site	Gene	Reference
CCC-CAATTAGT-CCT	<i>Osteocalcin (Bglap)</i>	(22)
CCT-TCAATTAAA-TC	<i>Bone sialoprotein (Spp1)</i>	(24)
C-TTAATTAT-AGCC	<i>Collagen 1A1 (Col1a1)</i>	(23)
GTAATTACA	<i>Chorionic gonadotropin (CGB)^a</i>	(57)
TGC-GAATTACT-GCA	<i>Runt-related transcription factor 2 (Runx2)</i>	(25)
ACA-TAATAATG-AAG	<i>Runt-related transcription factor 2 (Runx2)</i>	(25)
CA-AAAATTAAAA-AG	<i>Runt-related transcription factor 2 (Runx2)</i>	(25)
CAG-ATATTAGG-GAG	<i>Alkaline phosphatase (Alpl)</i>	(26)
CAG-GTAATTAT-CTT	<i>Located within the MI56i Dlx5/Dlx6 intergenic enhancer^b</i>	(27)
ACT-CTAATTAT-GGC	<i>Located within the MI56i Dlx5/Dlx6 intergenic enhancer^b</i>	(27)

Note that the sequence in *CGB* promoter is recognized by the Dlx3 protein and the two sequences in the *Dlx5/Dlx6* intergenic enhancer region are recognized by Dlx1 and Dlx2 proteins. Promoters (3 kb) of differentially expressed genes listed in Supplementary Material, Table S1 were investigated for the presence of HDRE-containing sequence motifs shown between dashes.

^aThis is a binding site for the Dlx3 protein in the promoter region of human *CGB* gene.

^bThese are binding sites for Dlx1 and Dlx2 proteins.

vector), was found to be ~10% (Fig. 2A and B). The expression of the Dlx5 protein was independently ascertained and confirmed by western blotting (Fig. 2C). Chromatin was then immunoprecipitated from *Dlx5*-transfected cells after 4 days of differentiation and compared with mock-transfected cells from the same time point. From the list of 40 candidate genes, 12 were selected to be tested by ChIP, based on available RNA *in situ* data from the VisiGene database of the UCSC Genome Browser. Specifically, these 12 genes have been previously shown to have domains of expression in the developing otocyst (E10–E10.5) that at least partially overlap with that of *Dlx5*. These genes were *Ahr*, *Apccd1*, *Atbfl*, *Bmp4*, *Bmper*, *Ebf1*, *Isl1*, *Large*, *Lhx1*, *Lrrtm1*, *Msx1* and *Ncoa2*. The promoter sequence motifs that were tested from these genes are marked by superscript ‘a’ in Table 3. We were unable to amplify *Apccd1* even from the input chromatin, and therefore did not attempt to amplify it from any of the immunoprecipitated chromatin. All other sequence motifs (with the exception of those in the promoters of *Ahr*, *Bmp4*, *Isl1*, two predicted motifs from *Lrrtm1* and *Ncoa2*) were enriched in chromatin that was immunoprecipitated using a Dlx5 antibody but not a normal rabbit IgG antibody (Fig. 3B), suggesting that these elements were bona fide binding targets of Dlx5.

The promoter regions that failed to be immunoprecipitated in this assay might be false positives or may reflect biological differences between the developing otocyst tissue and an immortalized cell line grown in culture. To further investigate this, we measured changes in gene expression between *Dlx5*-transfected 2B1 cells and mock/empty vector-transfected cells after 4 days of differentiation in culture. These were then compared with our *in vivo* data from null and wild-type otocysts. Our rationale in making this comparison was that bona fide Dlx5 targets might show opposite effects when the

transcription factor was over-expressed versus when it was completely absent. In conducting this comparison, we deliberately set a low threshold for significant gene expression changes in the transfected cell line (>1.2-fold). We chose this because that was the observed change in *Dlx5* mRNA levels upon transfection, despite the fact that the Dlx5 protein was easily detectable in the transfected cells by western immunoblotting (Fig. 2C). We compared differentially expressed genes and their fold-changes in the transfected cell line with the 400 differentially expressed genes from our *in vivo* otocyst comparisons. Supplementary Material, Table S3 summarizes these comparisons. There were 131 differentially expressed genes *in vitro* in the opposite direction to that detected in the mutant tissues *in vivo*. Of these, *Bmper* and *Lrrtm1* were among the list that were verified by ChIP (Fig. 3B), providing additional evidence that these are genuine direct targets of Dlx5. The assumption that direct targets might exhibit opposite trends is certainly not an absolute or foolproof predictor. Nevertheless, the remaining 129 genes that show opposite trends upon over-expression versus absence of *Dlx5* provide a smaller and more focused number of candidate direct or indirect targets in the Dlx5 regulatory cascade.

DISCUSSION

The aim of this study was to identify direct targets of Dlx5 within the early mouse otocyst and the cascade of transcriptional changes that result from the loss of *Dlx5* function. We applied four criteria to this analysis. The first was to measure gene expression differences between wild-type and knockout otocysts to identify consistent changes that might be direct or indirect targets of *Dlx5* activity. We conducted this over two early time points in inner ear development. We identified a total of 400 altered transcripts with the majority (287) being unique to the later time point (E10.5). As noted above, one hypothesis for this observation might be that the early (E10) changes reflect immediate and direct targets of the transcription factor and that the later transcriptional changes are mostly secondary downstream sequelae of the initial direct effects. Our results do not support that hypothesis. We found strong evidence for many direct targets and/or targets with convincing promoter motifs in the later E10.5 time point (see more discussion on this below) indicating that there is no obvious correlation between target prediction and early time points of *Dlx5* expression.

Dlx genes are arranged as pairs within the mouse genome and each pair shows similar patterns of expression. *Dlx5* is paired with *Dlx6* and it is likely that they exhibit some amount of overlap and redundancy in their targets and that they can partially compensate for loss of their partner gene. This is borne out by studies of *Dlx5/Dlx6* double-knockout mice. These manifest a much more severe inner ear phenotype in which there is a complete failure to form all vestibular structures (37). The expression of a few genes with known roles in inner ear development was also shown to be altered in these double mutants, including *Bmp4*, *Gbx2* and *Otx1* (down-regulated), and *Pax2* (up-regulated). This contrasts with the current study where *Bmp4*, *Otx2* (but not *Otx1*) and

Table 3. List of novel 107 unique 12 bp long motifs in promoter regions of 40 genes differentially expressed between wild-type and *Dlx5* null otocysts

12 bp motif	Gene	Mouse (Mm9)	Pattern	Human (Hg19)
ACCCCATCATT	<i>Ahnak</i>	−987(+)F	E10.5-DN	−803(−)F
AGGAAATTATTC ^a	<i>Ahr</i>	−2604(−)R	Both-DN	−1655(+)F
GTTTAATGTCGA ^a	<i>Apcdd1</i>	−2035(−)R	Both-DN	−2143(−)R
AAACAGGGATTA ^a	<i>Atbf1</i>	−1203(−)R	E10.5-DN	−1294(+)R
AAAACCTCCATTA	<i>Bmp4</i>	−1778(+)F	E10-DN	−1864(+)F
AAAATTAGTAGT ^a	<i>Bmp4</i>	−370(+)R	E10-DN	−369(+)R
AAATTAGTAGTC ^a	<i>Bmp4</i>	−369(+)R	E10-DN	−368(+)R
AACTGATGATTA ^a	<i>Bmp4</i>	−356(+)F	E10-DN	−355(+)F
AAGTAATAGAGA ^a	<i>Bmp4</i>	−414(+)R	E10-DN	−413(+)R
AATTAGTAGTCA ^a	<i>Bmp4</i>	−368(+)R	E10-DN	−367(+)R
ACCTCAAAATTA ^a	<i>Bmp4</i>	−1786(+)R	E10-DN	−1872(+)R
AGATAATGAAAC ^a	<i>Bmp4</i>	−405(+)F	E10-DN	−404(+)F
ATCTCTATTACT ^a	<i>Bmp4</i>	−402(−)R	E10-DN	−401(−)R
GAGATAATGAAA ^a	<i>Bmp4</i>	−404(+)F	E10-DN	−403(+)F
GATTAAAGTGTG	<i>Bmp4</i>	−1006(−)R	E10-DN	−1038(−)R
GTCACAAAATTA ^a	<i>Bmp4</i>	−375(+)R	E10-DN	−374(+)R
GTGTTTAAATCA ^a	<i>Bmp4</i>	−373(−)F	E10-DN	−372(−)F
AATGAATGATTA ^a	<i>Bmp4</i>	−468(+)F	E10.5-DN	−1039(+)F
AACTCTATAATA	<i>Ccdc88a</i>	−2714(−)R	E10.5-UP	−2598(+)R
AATTAACCTCTC	<i>Ccdc88a</i>	−2599(+)F	E10.5-UP	−2483(−)F
GATTACAAAGAA	<i>Ccdc88a</i>	−2180(+)F	E10.5-UP	−2128(−)F
GGGAGCATTACC	<i>Cep27</i>	−1451(−)F	E10-DN	−1420(−)F
ACTTATTACTAC	<i>Chl1</i>	−1225(−)R	E10.5-DN	−1389(−)R; −2066(−)R
AATTAAGACGCT	<i>Coll1a1</i>	−2409(+)R	E10.5-DN	−2482(−)R
ACACAGAAATTA	<i>Coll1a1</i>	−2402(+)R	E10.5-DN	−2475(−)R
AGAAATTAAGA	<i>Coll1a1</i>	−2406(+)R; −2417(+)F	E10.5-DN	−2479(−)R; −2490(−)F
ATTAAGACGTC	<i>Coll1a1</i>	−2410(+)R	E10.5-DN	−2483(−)R
GAAATTAAGAC	<i>Coll1a1</i>	−2407(+)R; −2416(+)F	E10.5-DN	−2480(−)R; −2489(−)F
GCAGAAATTA	<i>Coll1a1</i>	−2419(+)F	E10.5-DN	−2492(−)F
AAATTAGGTGAA	<i>Cpne8</i>	−875(−)R	Both-DN	−1336(−)R
ACAAATTAACCT	<i>Cpne8</i>	−1068(+)F	Both-DN	−1512(+)F
GAGAAATTAATG	<i>Cryab</i>	−1874(+)F	E10.5-DN	−2542(−)F
GGTATTAAGA	<i>Cryab</i>	−1862(+)R	E10.5-DN	−2530(−)R
ATTCTTTATTAC	<i>D3Ertd751e</i>	−1814(−)R	E10.5-UP	−2060(−)R
AATTACGGTAGA	<i>Ebf1</i>	−2447(+)R	E10.5-UP	−2281(−)R
GGATGTTAATGA ^a	<i>Ebf1</i>	−983(+)F	E10.5-UP	−713(−)F
GAAAATAATTAG	<i>Gabrb3</i>	−967(+)F	E10-DN	−1629(−)F
GGATTAATAAAA	<i>Gabrb3</i>	−955(+)R	E10-DN	−1617(−)R
GTAAAGATATTA	<i>Glcc1</i>	−2047(−)R	E10.5-UP	−2154(−)R
AACCATTAATAT ^a	<i>Isl1</i>	−1005(−)R	E10-UP	−712(+)R
AATATTAATGGT	<i>Isl1</i>	−1017(+)R	E10-UP	−724(−)R
AAATTACGGTTC ^a	<i>Large</i>	−2538(−)R	E10.5-DN	−2329(−)R
GAACATTATATG ^a	<i>Large</i>	−2482(+)F	E10.5-DN	−2273(+)F
AGAGTAATAAAC	<i>Lhfp</i>	−1863(+)R	E10.5-DN	−1992(−)R
ATTAAGTCTTCT	<i>Lhfp</i>	−2303(−)R	E10.5-DN	−2460(+)R
ACGTAATTCGCG ^a	<i>Lhx1</i>	−33(+)R	E10-UP	−448(−)R; −1706(−)R
GAATTACGTCGCG ^a	<i>Lhx1</i>	−25(−)R	E10-UP	−440(+)R; −1698(+)R
GACGTAATTCGCG ^a	<i>Lhx1</i>	−34(+)R	E10-UP	−449(−)R; −1707(−)R
GAGCGGAATTAC ^a	<i>Lhx1</i>	−20(−)R	E10-UP	−435(+)R; −1693(+)R
GCATTAAGGCGA ^a	<i>Lhx1</i>	−32(−)F	E10-UP	−447(+)F; −1705(+)F
GCGCTGCATTAA ^a	<i>Lhx1</i>	−37(−)F	E10-UP	−452(+)F; −1710(+)F
GCTGCATTAAGG ^a	<i>Lhx1</i>	−35(−)F	E10-UP	−450(+)F; −1708(+)F
GGAGCGGAATTA ^a	<i>Lhx1</i>	−19(−)R	E10-UP	−434(+)R; −1692(+)R
GGCGCTGCATTA ^a	<i>Lhx1</i>	−38(−)F	E10-UP	−453(+)F; −1711(+)F
ACAATTACATAT ^a (a)	<i>Lrrtm1</i>	−2514(−)R	E10.5-DN	−2856(+)R
ATTACACCGTTC ^a (a)	<i>Lrrtm1</i>	−2451(+)R	E10.5-DN	−2792(−)R
AGGCTGTAATTA ^a (b)	<i>Lrrtm1</i>	−2356(−)F	E10.5-DN	−2661(+)F
AATTACAGCCTA ^a (b)	<i>Lrrtm1</i>	−2366(+)F	E10.5-DN	−2671(−)F
GTATATAATGAA ^a (c)	<i>Lrrtm1</i>	−180(−)R	E10.5-DN	−499(+)R
AATTAAGTTGT	<i>Man1a</i>	−755(+)R	Both-DN	−856(+)R
AGACTTAATTTTC	<i>Man1a</i>	−759(−)F	Both-DN	−860(−)F
AATTAGGACTGA	<i>Meis1</i>	−75(−)F	E10.5-DN	−56(+)F; −2332(+)F
ATTAAGCTCCT	<i>Meis1</i>	−642(+)R	E10.5-DN	−650(−)R; −2926(−)R
GAATTAGGACTG	<i>Meis1</i>	−76(−)F	E10.5-DN	−57(+)F; −2333(+)F
AAAGTGTTAATT	<i>Msi2</i>	−1506(−)F	E10.5-DN	−1511(+)F
ACTTTAATTAC	<i>Msi2</i>	−1520(+)R	E10.5-DN	−1526(−)R
AGAGGATTAGGA	<i>Msi2</i>	−1533(+)R	E10.5-DN	−1539(−)R

Continued

Table 3. Continued

12 bp motif	Gene	Mouse (Mm9)	Pattern	Human (Hg19)
ATCCTAATCCTC	<i>Msi2</i>	-1521(-)R	E10.5-DN	-1527(+)R
GTTAATTGTGAA	<i>Msi2</i>	-1494(-)R	E10.5-DN	-1499(+)R
GAAAATGAATTA ^a (a)	<i>Msx1</i>	-1391(+)F	Both-DN	-1468(-)F
AGATTAGAGGTC ^a (b)	<i>Msx1</i>	-1257(-)R	Both-DN	-1335(+)R
GGATAATTACCC ^a (b)	<i>Msx1</i>	-1310(-)F	Both-DN	-1388(+)F
AAAGTTGATTAA ^a	<i>Ncoa2</i>	-944(+)R	E10-DN	-1096(+)R
AAGTTGATTAA ^a	<i>Ncoa2</i>	-943(+)R	E10-DN	-1095(+)R
AAAATTAGTCCC	<i>Nisch</i>	-2476(-)F	E10.5-DN	-2481(+)F
GACTAATTTTGG	<i>Nisch</i>	-2467(+)F	E10.5-DN	-2472(-)F
AAAGGATAATGA	<i>Npr3</i>	-2564(-)R	E10.5-DN	-1406(+)R; -2311(+)R
ACATTAAGTGCC	<i>Npr3</i>	-384(-)R	E10.5-DN	-189(+)R
GTTTCCTATTAC	<i>Npr3</i>	-2563(+)F	E10.5-DN	-1405(-)F; -2310(-)F
AAATTAGTCTT	<i>Nrp2</i>	-46(-)R	E10.5-DN	-119(-)R
ACTAATTTCTCC	<i>Nrp2</i>	-39(+)R	E10.5-DN	-112(+)R
GACGATTACCCC	<i>Paqr6</i>	-672(-)R	E10.5-DN	-2827(+)R
ATTAGAACCGAC	<i>Pf4</i>	-68(-)R	E10.5-UP	-165(+)R
AAAATTAGTAGT	<i>Prkag1</i>	-1675(+)F	E10.5-DN	-1642(+)F
GATGCAATTATA	<i>Prkag1</i>	-571(+)F	E10.5-DN	-582(+)F
GGAGGTAATGGG	<i>Prkag1</i>	-1563(+)R	E10.5-DN	-1530(+)R
GGGAGGTAATGG	<i>Prkag1</i>	-1564(+)R	E10.5-DN	-1531(+)R
GGGGAGGTAATG	<i>Prkag1</i>	-1565(+)R	E10.5-DN	-1532(+)R
AGGAAAACATTA	<i>Rspo3</i>	-2484(+)F	E10.5-DN	-2950(-)F
GGAAAACATTAA	<i>Rspo3</i>	-2485(+)F	E10.5-DN	-2951(-)F
ATTAAAGTCTCT	<i>Smpx</i>	-743(-)R	E10.5-DN	-792(+)R
ACAATTAAGGC	<i>Sost</i>	-344(-)F	E10-UP	-313(-)F
GAAAATTAAACAG	<i>Sost</i>	-334(-)R	E10-UP	-303(-)R
AAAAGGATTAGA	<i>Sp8</i>	-1258(-)F	Both-DN	-1094(+)F
AATTAACCTCAT	<i>Sp8</i>	-2246(-)F	Both-DN	-2093(+)F
ACTTCATTTAAT	<i>Sp8</i>	-2070(+)R	Both-DN	-1917(-)R
GAAGTAAATTAA	<i>Sp8</i>	-2071(-)F	Both-DN	-1918(+)F
GACATATAATCC	<i>Sp8</i>	-2308(-)R	Both-DN	-2155(+)R
AAAGGTTAATGA	<i>Svep1</i>	-338(+)R	E10.5-DN	-260(+)R
AAGGTTAATGAC	<i>Svep1</i>	-337(+)R	E10.5-DN	-259(+)R
AATTACTGTGGC	<i>Svep1</i>	-333(-)F	E10.5-DN	-255(-)F
ACGGTGTCTATTA	<i>Svep1</i>	-321(-)R	E10.5-DN	-243(-)R
AGAGTTATTACC	<i>Taf15</i>	-164(-)R	E10.5-UP	-177(-)R
GAGTTATTACCA	<i>Taf15</i>	-163(-)R	E10.5-UP	-176(-)R
GGCATGGTAATA	<i>Taf15</i>	-148(+)R	E10.5-UP	-161(+)R
GATTAGGTGAGA	<i>Tmed8</i>	-1781(+)R	E10.5-DN	-2030(+)R
AATCTGGATTAC	<i>Zic1</i>	-1966(+)R	E10.5-DN	-1736(-)R
ACCTAATGAATA	<i>Zic1</i>	-1962(-)F	E10.5-DN	-1732(+)F
GAAAATAATTAG	<i>Zic1</i>	-1974(-)F	E10.5-DN	-1744(+)F

All of these motifs contain an HDRE ('ATTA' or TAAT'), are also conserved in the promoters of human orthologs and are found in promoters of genes that contain at least one previously known binding site for Dlx5 (i.e. one of the motifs listed in Table 2). 'Position' is the location of the first base of the motif upstream of the transcription start site on a given strand [either (+) or (-)], and is shown for both mouse and human orthologs. The letters 'F' and 'R' denote the orientation/direction of the motif on the strand i.e. 5'-to-3' or 3'-to-5', respectively.

^aDenotes sequence elements found in promoters of genes whose expression overlaps with that of *Dlx5* in the developing otocyst and which were tested for direct binding with Dlx5 using ChIP. Motifs in *Lrrtm1* and *Msx1* were amplified from multiple PCR amplicons (Fig. 3) indicated by the letters in parenthesis next to superscript 'a'.

Pax8 (but not *Pax2*) were down-regulated in *Dlx5* mutant otocysts. These data suggest that the regulation of *Gbx2*, *Otx1* and *Pax2* may be separable from *Dlx5* downstream functions, an assertion that is supported by other studies (38,39).

Our second evaluation criterion consisted of an *in silico* analysis of promoter sequences from the initial 400 differentially expressed genes. We searched for potentially interesting promoter sequence motifs. As described above, these conservative sequence constraints resulted in the identification of 107 sequence elements that contained an HDRE, that also contained a previously known Dlx5 binding site and that were also completely conserved in human orthologous sequences. From the short list of motifs, identified in the putative promoters of

40 genes, we then chose 12 genes with domains of expression in the developing otocyst that overlapped with that of *Dlx5*. These promoter motifs were tested by ChIP in the 2B1 otocyst-derived cell line that transiently over-expressed *Dlx5*. We identified seven genes (*Athf1*, *Bmper*, *Ebf1*, *Large*, *Lhx1*, *Lrrtm1* and *Msx1*) whose promoter regions were bound directly by Dlx5. Interestingly, the majority of the motifs we discovered were in genes (27 out of the 40 total) that show alterations in gene expression only at the later E10.5 time point. Most of these (21 out of the 27 genes) were uniquely down-regulated in the E10.5 null otocysts suggesting that they may normally be positively regulated by Dlx5. This proportion of direct targets is borne out by

Figure 2. Transfection of 2B1 cell line with *Dlx5*-expression vector. Transfection efficiency, as determined by the expression of GFP, was not more than 10% using both an empty vector (A) or *Dlx5*-expression vector (B). Cells were transiently transfected when proliferating at 32°C and imaged 4 days post-differentiation at 37°C. Western blotting confirmed the over-expression of *Dlx5* and its localization in the nucleus (C) in cells transfected with the *Dlx5*-expression vector. No expression of the endogenous gene was detected.

our ChIP experiments where the majority of the confirmed direct targets (six out of the seven) were all unique to the later time point. Taken together, these data indicate that the chance of predicting *Dlx5* binding motifs or detecting a direct *Dlx5* target gene by ChIP is approximately the same at both time points (287 E10.5 unique gene expression changes versus 113 common to both). Thus, our data do not support the hypothesis that genes that exhibit earlier transcriptional changes are more likely to be direct *Dlx5* binding targets.

There is evidence from other studies that *Bmp4* is downstream of *Dlx5* (17). It has been reported that *Bmper* (BMP-binding endothelial regulator) acts to fine-tune *Bmp4* activity and is a downstream target of FoxO3a (40). Gene expression profiling of the mandibular branchial arch of a *Dlx5/Dlx6* double-knockout mouse also showed a reduction in *Bmper* expression (41), further supporting our observation that this gene is a direct downstream target of *Dlx5* in the otocyst and the 2B1 cell line. The other gene that passed all of our filtering criteria was *Lrrtm1*. The expression of this gene is particularly interesting in that it is localized in the endolymphatic duct (which is lacking in *Dlx5* nulls) during

Figure 3. ChIP of candidate binding sites of *Dlx5*. (A) shows an enrichment of *Ribosomal protein L30* (*Rpl30*) in the chromatin immunoprecipitated using histone H3 antibody relative to the normal rabbit IgG antibody and serves as a control for assessing the soundness of various steps of the entire procedure. (B) shows results of immunoprecipitation of chromatin from promoter regions of 12 candidate genes using *Dlx5* antibody in cells transfected with the *Dlx5*-expression vector. The predicted binding sites in promoter regions of *Athf1*, *Bmper*, *Ebf1*, *Large*, *Lhx1*, *Lrrtm1* and *Msx1* were enriched in the chromatin that was immunoprecipitated using the *Dlx5* antibody compared with the normal rabbit IgG antibody.

embryogenesis at E10–E10.5, making it an interesting candidate for causing that particular phenotype in the inner ear (42). The role of *Lhx1*, on the other hand, is likely to be associated with neuronal differentiation in the otocyst given that it is involved in the establishment of GABA-ergic neurons in the spinal cord (43). Since *Dlx5* is also involved in the differentiation of these types of neurons (44), this provides a potential functional link between these two genes. It may be that their roles are antagonistic within the context of otocyst development because *Lhx1* is up-regulated in *Dlx5* null inner ears. The interactions between *Msx1* and *Dlx5* appear to vary depending on the developmental process being examined. Specifically, the two genes have been shown to act independently in the mandible and the middle ear, synergistically during bone development and acting on the same processes during palatal fusion (20). In the developing otocyst, we

show here that *Dlx5* appears to up-regulate the transcription of *Msx1*. While the roles of *Athf1* and *Ebf1* have not been studied in the inner ear, their expression, as well as that of *Dlx5*, is down-regulated in the embryonic basal ganglia and cortex of *Dlx1/Dlx2* double mutants (45). However, in the developing otocyst, we observed an up-regulation of *Ebf1*, suggesting tissue- and/or stage-specific activity of *Dlx5*.

Our fourth and final filtering criterion was to identify reciprocal patterns of gene expression from the *Dlx5* null otocysts compared with the *Dlx5* over-expressing cell line. While the expression profile of the *Dlx5*-transfected 2B1 cell line *in vitro* did not significantly overlap with the *in vivo* profile of mutant tissues, the results were nevertheless interesting. Specifically, they showed that two genes (*Apcdd1* and *Socs2*) were significantly differentially expressed in the two settings in opposite directions. *Apcdd1* is noteworthy because its expression domain correlates very well with that of *Dlx5* in the otocyst (46), suggesting that it might be a direct target. Of further interest is that this gene is also a target of the canonical Wnt/B-catenin signaling (47), a pathway well known to play a role during inner ear development. Unfortunately, all of the ChIP assays we designed for the promoter of this gene failed. The *Socs2* (*suppressor of cytokine signaling 2*) gene did not pass any of our motif searching criterion and we have no independent information other than the reciprocal expression patterns on how it might intersect with *Dlx5* activity. It has not yet been studied in the inner ear but studies of knockout mice indicate that it may negatively regulate the growth hormone/IGF-I pathway (48).

These genes may also have much wider implications in the development of other human organ systems that are known to involve *DLX5*. In addition to the involvement of *DLX* genes in SHFM1 and in craniofacial/hearing defects mentioned above, changes in *DLX* genes have also been associated with autism. *DLX* transcription factors are known to regulate both the migration and differentiation of the inhibitory GABA-ergic interneurons of the cortex (49,50). Perturbing *Dlx* function can lead to an altered ratio of excitatory/inhibitory interneurons which, in turn, results in abnormal neural connectivity that is characteristic of some forms of autism (51). Polymorphisms in or near the human *DLX1* and *DLX2* genes have been associated with autism in multiplex families (52), whereas another study reported the association of three non-synonymous variants in *DLX2*, two in *DLX5*, and one in the I56i intergenic *DLX5/6* enhancer with autism (53). Recently, this latter variant (an A-to-G single nucleotide polymorphism) in a highly conserved region of the enhancer was shown to result in reduced transcriptional activity *in vivo*. Normally, this enhancer drives the expression of a reporter gene in the branchial arches and the forebrain including migrating GABA-ergic interneurons. The polymorphism is located in the first base of an 8 bp motif (ATAATTAG) that is completely conserved in 40 vertebrates. In the current study, we found this sequence to be part of a novel 12 bp long motif (GAAAATAATTAG, Table 3) shared by the promoter regions of *Gabrb3* (*Gamma-aminobutyric acid A receptor, subunit beta 3*) and *Zic1* (*Zinc finger protein of the cerebellum 1*). Both of these genes were down-regulated in *Dlx5* null otocysts.

In conclusion, our study describes gene expression differences between wild-type and *Dlx5* null otic vesicles from two developmental stages and provides novel candidate

genes that are targets of *Dlx5* in the inner ear. Further experiments, such as detailed analysis of knockout models of these target genes, as well as expression assays using *LacZ* transgenics, should provide additional information regarding the roles of the motifs and genes uncovered by this study in the early specification of inner ear structures. Furthermore, RNA *in situ* to assess whether the expression domains of the 28 untested genes from our candidate list of 40 genes overlap with that of *Dlx5* in the otic vesicle will uncover additional targets. These genes may also have much wider implications in the development of other human organ systems and in understanding the pathology of human genetic diseases that are known to involve *Dlx5*.

MATERIALS AND METHODS

Animal breeding and microdissections

The *Dlx5* null mice used in this study have been described in ref. (15). Mice breedings were carried out using standard procedures. Embryos were taken from timed-pregnant females, and the developmental stage of each embryo was assessed independently based on somite number. All inner ear dissections were carried out as described (36). For each stage and genotype, four to six otic vesicles were pooled yielding a total of 40–60 ng of total RNA and this was designated to be one biological sample (two biological samples were collected for each stage and each genotype). Total RNA was harvested using TRIzol (Invitrogen) following manufacturer's instructions.

Genotyping

A small tissue sample from each embryo was used to genotype using the REExtract-N-AmpTM Tissue PCR Kit (Sigma) following the instructions provided by the manufacturer. The genotyping primers (JL16 and JL17, and JL10 and JL11) as well as PCR conditions employed are described in ref. (15).

Cell cultures, transfections and western blots

The 2B1 cell line was grown in the chicken embryo fibroblast medium. This constituted the medium for maintaining cells in a proliferative state at 32°C. The medium for differentiation was the same except that it did not contain gamma-interferon.

Cells were grown up to 70–80% confluency in 150 mm tissue culture dishes in a proliferative state at 32°C before transfection with at least 20 µg of CMV-pTracer vector (Invitrogen) into which the *Dlx5* coding sequence was cloned. This vector also contains the coding sequence for green fluorescent protein (GFP). Transfections were carried out using the standard Lipofectamine2000 (Invitrogen) procedure in a final volume of 25 ml. After 4 h, the transfection medium containing Lipofectamine2000 was removed, cells were rinsed twice with phosphate-buffered saline (calcium and magnesium free) and 30 ml of differentiation medium were added. Cells were then incubated at 37°C for 4 days after which they were imaged for GFP fluorescence and either fixed for ChIP, processed for RNA isolation by TRIzol (Invitrogen) or lysed for making cell extracts for western blotting. Note that the transfection efficiency was

around 10% as determined by GFP fluorescence. Western blotting was carried out using cytoplasmic or nuclear extracts prepared using the Nuclear Extract Kit (ActiveMotif; Catalog no. 40010) following the manufacturer's instructions. At least 30 µg of protein were analyzed using the chicken anti-human polyclonal Dlx5 antibody (Abcam; Catalog no. ab37887) which detected a single band in the expected size range of 32–35 kDa. This antibody recognizes first 120 amino acids of human DLX5 that share 93% identity with the first 120 amino acids of mouse Dlx5. This region does not include the homeodomain, therefore avoiding cross-reactivity with other Dlx proteins. Beta-actin was probed using monoclonal antibody (Sigma; Catalog no. A5441).

ChIP and PCR

This was carried out by following the steps provided by the manufacturer of ChIP-IT enzymatic kit (ActiveMotif; Catalog no. 53007) using 3 µg of the chicken polyclonal Dlx5 antibody per reaction (Abcam; Catalog no. ab37887). For PCR, 5 ng of immunoprecipitated DNA were used as template. The following are primer sequences used to amplify PCR products (100–150 bp) from promoters of genes containing candidate Dlx5-binding motifs: *Ahr* (CTAGTCTACTCA GTGTAAGTC and CTTGCACAGAATGTCTTTAC); *Apc* *ddl* (GTTGAAAGAACTTGAGTGTCG and CTGAAATA CAGTGTATTACC); *Athf1* (GTTTGACTAAATTTGGCCTC and AGATTGCAATCGCCGTCTCC); *Bmp4* (CAGCTCCC TTCTCCATAACC and CCTGAGTTTGAATAAAGTGC); *Bmp6* (CTGCGTGGAGATTAGCGGCA and CCTTAAA GCGTGGATCGGTG); *Ebf1* (AGAGATCCCTTGCTCGC CAC and CGAGCGGCTTGTTCACAGA); *Isl1* (CCTGCTC ACGCCTTCATCT and CTATGGATCTGTTGTCCACTC); *Large* (GAACGTAGCTGCCTTCGAGC and CGTATCT GGGTTTGATGCCA); *Lhx1* (GAGGCGCTGATTGGTCA CAG and TCGGTCAGTCTCCTGGCTG); *Lrrtm1* amplicon 'a' (CTCAGAAGACTTAGAATAGC and TCTGCATGGT ATCTTTAAGC); *Lrrtm1* amplicon 'b' (GCTTAAAGATA CCATGCAGA and TCTTCTAAGTATGCCACTC); *Lrrtm1* amplicon 'c' (GCCCCGAAATAGATACAAGA and GGTG GGAAGAAAACCAACA); *Msx1* amplicon 'a' (CAACCCCT CCCTCCAGACC and CTGGCCTTTTAACTGGGCGC); *Msx1* amplicon 'b' (GCGCCAGTTAAAGGCCAG and AGGTGGAGGAGGTGATTGGC); *Ncoa2* (TTGATAAC TTCCGTAAAGC and GATTAAGGAGACGTGGAGGAG).

Micro-cDNA amplification, affymetrix target preparation, array hybridization and scanning

This was carried out in a micro-cDNA amplification system as described in ref. (36) except that the platform utilized was the Affymetrix Mouse Genome 430 2.0 expression array. All microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) database with accession number GSE22381.

Microarray data analysis

Normalized expression values from dChip (54,55) were used to filter probe sets such that those whose expression was

below 25 and which were not associated with 'Present' calls in both replicates of at least one sample were discarded. The mutant sample from each stage was compared with wild-type from the same stage in significant analysis of microarrays (SAM) (56) using the two-class unpaired option with a random number seed for a maximum number of permutations (i.e. 24) and a fold-change of 1.5 or more. The significant false discovery rate was set to 5% or less. The same comparisons were also carried out in dChip to obtain probe sets whose lower bound of fold-change was at least 1.5 after taking error into account. Finally, a probe set had to be 'Present' in the sample where it was up-regulated. Probe sets that satisfied all these criteria were considered significant. In cases where different probe sets of the same gene showed different patterns of expression, that pattern was chosen which had the majority of the probe sets and/or where the false discovery rate (FDR) and fold-changes were most significant. Analysis was also carried out using the Expression Console software (Affymetrix) by employing the Robust Multichip Analysis (RMA) algorithm. All normalized (log-base-2) expression values below 5.5 were considered 'Absent'. Those that were 'Present' were analyzed in SAM to obtain FDR values and in dChip to obtain *P*-values. The lists of significant genes and probe sets from the two methods (dChip and RMA) were then merged.

For cell line microarray data, only the RMA method was utilized and the cut-off for making 'Present' calls was set to 4.8 since this was the expression of *Dlx5* in *Dlx5*-transfected cells. Note that two independent replicates/cultures of each (i.e. *Dlx5*-transfected and empty vector-transfected cells) were profiled.

Promoter analysis

Sequences 3 kb in length immediately upstream of the transcriptional start sites of genes with identifiable transcript IDs (357 total) were obtained from the UCSC genome browser using Mm9 mouse genome version. To identify known Dlx5 binding sites, a simple search was carried out using a Perl script. To identify any 12 bp motif, the following criteria were applied also using a Perl script:

- (i) Each 3 kb promoter region was split into all possible 12 bp motifs, moving one base at a time. This was done for both strands in both directions for every promoter.
- (ii) No more than three bases of naturally occurring repeat sequence per motif were allowed.
- (iii) No more than two consecutive 3 bp long repeats were allowed.
- (iv) No more than three consecutive 2 bp long repeats were allowed.
- (v) A single base was not allowed to occur more than three times in a row.
- (vi) Each 12 bp motif had to occur at least twice in the collection of 357 promoters (i.e. at least once in two different promoters).

These criteria were chosen so as to minimize the risk of obtaining irrelevant sequence motifs with repeats. When searching for evolutionary conservation in humans, the Hg19 version of the genome sequence was used (UCSC).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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