Original Research

Chromatin immunoprecipitation-promoter microarray identification of genes regulated by PRDM16 in murine embryonic palate mesenchymal cells

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Abstract

The transcription factor PRDM16 regulates differentiation of brown adipocyte tissue in mice. Recently, however, it has been demonstrated that genetic knockout of *Prdm16* in mice leads to a complete cleft of the secondary palate in offspring. To identify genes whose promoters bind PRDM16 in mouse embryonic palate/maxillary mesenchymal cells, we have conducted a chromatin immunoprecipitation-promoter microarray analysis (ChIP-Chip). One hundred and twenty-two gene promoters were identified as capable of binding PRDM16. These could be functionally grouped to include those on genes linked to muscle development, chondrogenesis and osteogenesis, in addition to many transcription factors. These results suggest that PRDM16 may play a role in differentiation of mesenchymal cells in the embryonic secondary palate that contribute to the anterior, bony palate and posterior, muscular palate.

Keywords: Prdm16, ChIP-Chip, palate, embryo, gene regulation

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Introduction

PRDM16 (MEL1, PFM13) is a 1247 amino-acid protein (140 kDa) with six distinct domains that include the namesake PR domain (*P*RDI-BF1 and *R*IZ1 homologous), two DNA-binding domains, a proline-rich, repressor and acidic domains. The PR domain is similar to the SET domain found in many chromatin-modifying proteins. The *Prdm* gene family contains 17 members in humans (16 in mice), and the *Prdm16* gene is similar to the *Mds1/Evi1* gene and the oncogene *Evi1*.[1] *Prdm16* is also expressed as a shorter splice variant that is missing most of the PR domain.[2] The function of the shorter form is unknown.

Early studies in (1;3)(p36;q21)-positive myeloid leukemias revealed that ectopic expression of *Prdm16* led to hyperproliferation, suggesting a role in cell cycle progression.[1,2] Recently, *Prdm16* was shown to be a master gene controlling differentiation of Myf5-expressing mesenchymal cell precursors into brown adipose tissue via a transcriptional complex with C/EBP-β.[3,4]

Prdm16 is expressed in multiple embryonic[5] and adult[6] mouse tissues. We have previously demonstrated expression of *Prdm16* in the murine embryonic secondary palate,[7] suggesting a role for this transcription factor in the development of this structure. Indeed, *Prdm16* knockout mouse embryos display a completely penetrant cleft palate.[8] The mechanism by which *Prdm16* contributes to this phenotype is unknown. We have previously demonstrated that PRDM16 binds to multiple Smads and may modulate signaling through the transforming growth factor β (TGF-β) signaling pathway.[7] Signaling via TGF-β is essential for normal palate development.[9] In an effort to dissect the molecular pathways that are influenced by PRDM16 and essential for development of the secondary palate in mice, genes whose promoters bind PRDM16 in mouse embryonic palate/maxillary mesenchymal cells were identified by high-density promoter arrays following chromatin immunoprecipitation.

Materials and methods

Animals

Hsd:ICR (CD-1®) mice were obtained from Harlan Laboratories, Inc (Indianapolis, IN, USA) and Prdm16$^{+/-}$ mice (strain B6;129S5-*Prdm16*^{Gt683Lex}/Mmcd) were

purchased from the Mutant Mouse Regional Resource Center (UC-Davis, Davis, CA, USA). Both strains were housed at an ambient temperature of 22°C with a 12-h on/12-h off light cycle and access to food and water *ad libitum*. Timed matings were achieved by caging one mature $Prdm16^{+/-}$ male and two nulliparous $Prdm16^{+/-}$ female mice overnight. The presence of a vaginal plug was taken as evidence of mating and that morning was designated embryonic day 0.5 (E0.5). Pregnant mice were euthanized by carbon dioxide asphyxiation/cervical dislocation on E13.5 for preparation of primary palate mesenchymal cell cultures. All procedures for the humane use and handling of mice were approved by the University of Louisville Institutional Animal Care and Use Committee and encompass guidelines as set out in the EC Directive 86/609/EEC for animal experimentation.

Establishment and culture of primary embryonic palate mesenchymal cells

Primary cultures of mouse embryonic palate mesenchymal (MEMM) cells were established from secondary palatal tissue microdissected from E13.5 mouse embryos. The dissected tissue was dissociated by a 10-min digestion with 0.05% (w/v) trypsin and suspended in OptiMEM (Invitrogen Corp, Gaithersburg, MD, USA) supplemented with 5% fetal bovine serum (FBS; Sigma Chemical Co, St Louis, MO, USA). Cells were passed through a 70-μm filter, and 9×10^5 cells were plated in a 10-cm-diameter tissue culture dish. Cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2.

Reverse transcription and realtime polymerase chain reaction

RNA from palate tissue was isolated using the RNeasy system (Qiagen, Valencia, CA, USA). Complimentary DNAs (cDNAs) were synthesized from 400 ng of total RNA with the SuperScript First Strand cDNA Synthesis System (Invitrogen Corp). Realtime polymerase chain reaction (PCR) assays were performed with probe:primer sets obtained from Applied Biosystems (Foster City, CA, USA) on the ABI Prism 7000 Sequence Detection System platform (Applied Biosystems). The signal from 18S RNA was used as the internal control. Fold-change values were determined according to Livak and Schmittgen.[10]

Transfection

Ten culture dishes (10 cm, 60–70% confluent) were transfected (10 μg/dish pcDEF3-PRDM16-6X-myc or empty vector) using Effectene transfection reagent (Qiagen) and incubated for 48 h before crosslinking.

Crosslinking

Cells were trypsinized from each dish and like samples pooled. Protein–DNA crosslinking was effected in OptiMEM growth medium + 5% FBS by first incubating cells with 1.5 mmol/L ethylene glycol *bis*-succinimidyl succinate (EGS; Pierce Chemical Co, Rockford, IL, USA) for 20 min at room temperature, followed by 1% methanol-free formaldehyde (Polysciences, Inc, Warrington, PA, USA) for 10 min at room temperature. Crosslinking was terminated with 0.25 mol/L glycine and then cells washed two times with ice-cold Tris-buffered saline (TBS). Nuclei were isolated by washing the cell pellet three times with 10 mL ice-cold lysis buffer (10 mmol/L Tris-Cl, pH 7.5, 10 mmol/L NaCl, 3 mmol/L $MgCl_2$ and 0.5% NP-40).

Chromatin immunoprecipitation

Cells were lysed and isolated nuclei digested in nuclease digestion buffer (Active Motif, Carlsbad, CA, USA) containing protease inhibitors and 200 units/mL micrococcal nuclease for five minutes at 37°C. Digestion was terminated with ethylenediaminetetraacetic acid (EDTA). This procedure led to fragments of DNA ranging in size from 200 to 1000 bp. The sheared chromatin was immunoprecipitated with 6.7 μg/mL anti-c-Myc (mouse monoclonal clone 9E10; BD Pharmingen, Franklin Lakes, NJ, USA), 100 μL protein G magnetic beads, and protease inhibitor cocktail (Complete, EDTA-free; Roche Diagnostics, Indianapolis, IN, USA) for four hours at room temperature with constant mixing. Protein G beads were washed once with ChIP buffer 1 (Active Motif) and two times with ChIP buffer 2 (Active Motif). Bound chromatin was eluted and de-crosslinked overnight at 65°C. Samples were then digested with proteinase K (Roche Diagnostics) for two hours at 37°C and purified using Affymetrix cDNA cleanup columns (Affymetrix, Santa Clara, CA, USA). Purified chromatin was then taken through two rounds of linear amplification using the WGA2 and WGA3 genomic DNA amplification systems, respectively (Sigma, St Louis, MO, USA). During the final round of amplification, a mixture of 8 mmol/L deoxythymidine triphosphate and 2 mmol/L deoxyuridine triphosphate was used in order to prepare the samples for further fragmentation with uracil-DNA glycosylase and purinic/apyrimidinic (AP) endonuclease (Affymetrix), which yields an average fragment size of ~60 nucleotides. The integrity of the DNA at each point was determined by analysis on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and nucleic acid concentrations determined on the NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Chip hybridization

Fragmented chromatin was hybridized to Affymetrix GeneChip® mouse promoter 1.0R arrays (p/n 900890; Affymetrix) according to the manufacturer's instructions. Following hybridization, washing and staining, chips were scanned with the GeneChip® scanner 3000 (Affymetrix) and processed using GCOS1.2 software. The resultant CEL files containing the raw data were imported and analyzed using Partek Genomics Suite (Partek, St Louis, MO, USA). A cut-off of $P < 0.05$ was used to generate the list of enriched gene promoters.

PCR confirmation

To verify putative gene targets identified from the promoter array, PCR primers were designed using Primer-Blast (NCBI, NIH, Bethesda, MD, USA) and tested on control- and PRDM16-transfected MEMM cells that were immunoprecipitated with anti-c-Myc. Each reaction consisted of 2 ng DNA, 1 μmol/L each of forward and reverse primers, 10 units/mL JumpStart REDTaq (Sigma) and 100 μmol/L dNTP mix. Amplification conditions were: 95°C, five minutes; 30–35 cycles of 95, 57 and 72°C/30 s; and a final extension step of 72°C for seven minutes. Amplified products were analyzed on 2% agarose gels. The sequence of primers used for these analyses are reported in Supplementary Table 1 (please see http://ebm.rsmjournals.com/lookup/suppl/doi:10.1258/ebm.2012.011258/-/DC1).

Whole-mount *in situ* hybridization

A cDNA clone for mouse osteopontin (in the vector pCMV6-Kan/Neo) was purchased from OriGene Technologies, Inc (Rockville, MD, USA). In order to synthesize both sense and antisense riboprobes suitable for *in situ* hybridization, a 610-bp fragment of the cDNA was subcloned into pBluescript II SK (+) (Agilent Technologies). Digoxigenin-labeled riboprobes were synthesized with the DIG RNA kit (Roche Diagnostics) and purified by repeated LiCl precipitation. Wild-type and *Prdm16*$^{-/-}$ mouse fetuses were removed on E14.5 and E15.5 and the mid-facial region isolated by removing tissue anterior and posterior to the maxilla/palate. Tissue was fixed at 4°C overnight in 4% paraformaldehyde, and then prepared for *in situ* hybridization as described previously.[7]

Results

Primary cultures of MEMM cells were transfected with an empty plasmid or one that encoded Myc-tagged PRDM16 (PRDM16-6X-myc). The choice of MEMM cells was based on previous observations that these cells behave similar to cells *in vivo* with respect to several key developmental processes such as rates of proliferation, extracellular matrix production and differentiation in response to growth and differentiation factors.[11] In addition, because ChIP-grade antibodies to PRDM16 were not available, expression of a Myc-PRDM16 fusion protein was utilized because of the availability of high-quality antibodies (against c-Myc). This is critical for the success of chromatin immunoprecipitation experiments. Although it is likely that not all genes regulated by PRDM16 in the developing secondary palate can be identified by this approach, identification of candidate gene targets will provide rationale for further investigation of the role of this gene in palate development. Western blotting of nuclear lysates demonstrated that PRDM16-6X-myc was strongly expressed in transfected cells (data not shown). The cells were subjected to crosslinking with 1.5 mmol/L ethylene glycolbis and 1% formaldehyde and both control- and PRDM16-expressing MEMM cells were immunoprecipitated with anti-Myc antibodies. Sheared, purified and amplified chromatin was applied to a mouse promoter array that contained over 28,000 mouse promoters. Following data collection and analysis, a list of 122 genes was identified that were enriched in PRDM16-transfected cells ($P < 0.05$). These are reported in Table 1. The list of genes is ordered according to their MAT score, a measure of promoter enrichment.[12]

To confirm the validity of these results, 20 candidate genes were selected and PCR primers designed using Primer-Blast (NCBI). In addition, PCR primers were synthesized for *PGC1-α*, *Angiotensinogen* and *Resistin* because these three genes are directly regulated by PRDM16.[13] Each primer set was used to test chromatin immunoprecipitated with anti-Myc antibodies from control- or PRDM16-transfected MEMM cells. The results from PCR analysis of 16 gene promoters selected from Table 1 are presented in Figure 1. In each reaction, there was enrichment in chromatin from PRDM16-transfected cells demonstrating that PRDM16 bound to each promoter. Four genes either gave no enrichment or no amplification of the input material. Additionally, there was enrichment of *PGC1-α* and *Resistin*, but not *Angiotensinogen*.

Functional categorization of the genes whose promoters were bound by PRDM16 was performed using DAVID.[14] However, this analysis gave only a limited indication of functional categories: 41 of the 122 genes were shown to be linked to a category described as 'cation binding' ($P < 0.001$). Because this analysis yielded results that were difficult to interpret with respect to development of the secondary palate, we performed an exhaustive search of published literature using PubMed (PubMed.gov, NCBI) to break down the identified genes into functional groups with relevance to palate development. This analysis is presented in Table 2. Of particular interest is the identification of 10 genes potentially regulated by PRDM16 that have been (directly or indirectly) linked to muscle development. Another four genes were shown to be linked to chondrogenesis and/or osteogenesis. A significant number of PRDM16-regulated genes were associated with regulation of transcription and regulation of protein stability (via ubiquitination).

Because *Prdm16* is expressed to a greater extent in the anterior (future bony) palate,[7,8] has been demonstrated to prevent differentiation of mesenchymal precursors into a muscle phenotype,[4] and predicted to regulate genes necessary for both bone and muscle development, we hypothesized that loss of *Prdm16* expression would alter expression of genes important for these processes. Osteopontin (Opn; also known as secreted phosphoprotein-1) is a marker for bone formation,[15] and in a previous large-scale gene expression study of mouse palate development, was found to undergo dramatic upregulation from E12.5 to E14.5.[16] To test the hypothesis that *Opn* expression is regulated by *Prdm16*, secondary palates from wild-type and *Prdm16*$^{-/-}$ fetuses were isolated on days 13.5 and 14.5 of gestation, RNA purified and cDNAs synthesized. Expressions of *Opn* and a muscle cell marker, *Myf4*, were analyzed by realtime PCR, and expression levels on both days were determined and data presented as change in expression on E14.5 relative to E13.5 (Figure 2). As expected, in wild-type palate tissue, the expression of *Opn* was dramatically greater on E14.5 compared with the expression level on E13.5 (Figure 2). In contrast, the expression of *Opn* in palate tissue isolated

Table 1 Genes identified by ChIP-Chip analysis that may be regulated by PRDM16 in mouse embryonic palate/maxillary embryonic mesenchymal cells*

Transcript ID	Gene symbol	Chr†	Region start‡	Region end¶	*P* value (region)	MAT score#
NM_080847	Asb15	6	24482673	24483436	0.00475804	372.063
NM_001001178	Ccdc148	2	58960725	58961373	0.00692961	261.338
NM_001081302	Trio	15	27836104	27837312	0.00805071	230.269
NM_001164078	Tia1	6	86361005	86361795	0.00859801	212.714
NM_001113549	Ltbp4	7	28106592	28107411	0.0108843	168.981
NM_019784	Tex21	12	77305691	77306609	0.0123938	146.629
NM_032008	Slmap	14	27331006	27331609	0.0141682	129.206
NM_007400	Adam12	7	141143094	141144145	0.0149273	122.108
NM_021526	Psmd14	2	61602010	61602851	0.0157483	116.078
NM_028208	Ptar1	19	23784706	23785377	0.0167811	108.915
NM_172461	Nek11	9	105186996	105188102	0.0171431	107.141
NM_013479	Bcl2l10	9	75199360	75200134	0.0171431	107.055
NM_001105071	Vmn2r39	7	9149886	9150674	0.0175756	104.35
NM_001164099	Add3	19	53308044	53308691	0.0177168	103.714
NM_026641	Ift80	3	68775767	68776831	0.0186526	98.6448
NM_001164614	Ccdc159	9	21739146	21740246	0.0200297	91.9917
NM_177393	Nalcn	14	123857860	123858444	0.0202945	91.0125
NM_023739	Nfx1	4	40962194	40962959	0.020321	90.9378
NM_146241	Trhde	10	114205663	114206253	0.0205946	89.789
NM_009435	Tssk1	16	17893693	17894322	0.0207182	89.0963
NM_026877	Aspscr1	11	120538330	120539379	0.0212832	86.8448
NM_009109	Ryr1	7	29802829	29804006	0.021548	85.7641
NM_031182	Tcfap4	16	4550186	4550795	0.021804	85.0148
NM_001161767	Galnt6	15	100519567	100520385	0.0251849	74.1398
NM_153162	Txnrd3	6	89610296	89611074	0.0259	72.263
NM_178661	Creb3l2	6	37375061	37376080	0.0259706	72.1352
NM_029682	Stambpl1	19	34278044	34279151	0.0262089	71.5973
NM_207530	Osbpl1a	18	12963270	12964003	0.026412	71.0458
NM_025835	Pccb	9	100886396	100887430	0.0277008	68.009
NM_029640	Trappc9	15	72741174	72741768	0.0281157	67.160
NM_011631	Hsp90b1	10	86163438	86164129	0.0281157	67.136
NM_010075	Dpp6	5	27379667	27380384	0.0281598	67.004
NM_198019	Cep78	19	16029208	16030319	0.028866	65.801
NM_175268	Fam53b	7	139933196	139934052	0.0289102	65.761
NM_198106	Slc9a10	16	45605762	45606515	0.0289896	65.570
NM_030206	Cygb	11	116509669	116510742	0.029431	64.5526
NM_001045559	C920016K16Rik	17	33131802	33132513	0.0296958	64.0795
NM_181322	Ctcf	8	108193147	108194074	0.0296958	64.071
NM_177343	Camk1d	2	5218234	5218965	0.0297046	64.0057
NM_133721	Itga9	9	118689846	118690725	0.0304197	62.2819
NM_001039939	Asxl1	2	153182205	153183256	0.0309405	61.2577
NM_008173	Nr3c1	18	39611401	39612423	0.0312141	60.816
NM_001113246	Chn1	2	73461818	73462554	0.0314172	60.3894
NM_175341	Mbnl2	14	120740678	120741266	0.0314437	60.3628
NM_001081128	Mtr	13	12309977	12310611	0.0317173	59.8839
NM_008719	Npas2	1	39307177	39308208	0.031991	59.3836
NM_172436	Slc25a12	2	71167225	71167988	0.0326795	57.9863
NM_010848	Myb	10	20853078	20853964	0.033933	56.0926
NM_001172157	Dis3l2	1	88896213	88896899	0.0340213	55.8804
NM_175392	Fam73b	2	30234816	30235738	0.0345951	54.7978
NM_175549	Robo2	16	74297960	74298710	0.0350188	54.1082
NM_001111121	Ccdc6	10	69599993	69600836	0.0351865	53.9107
NM_011873	Dazap2	15	100447788	100448524	0.0352925	53.7925
NM_011082	Pigr	1	132737411	132738384	0.0358839	53.1229
NM_178692	C130074G19Rik	1	186702789	186703368	0.0363694	52.4483
NR_015351	Ipw	7	66906029	66906789	0.0368726	51.7868
NM_018761	Ctnnal1	4	56839674	56840265	0.0372786	51.2766
NR_002851	Snord82	1	88252343	88253076	0.0373493	51.2159
NM_010880	Ncl	1	88252343	88253076	0.0373493	51.2159
NM_144545	Eif3j	2	121878177	121879118	0.0376494	50.8789
NM_010210	Fhit	14	11075964	11077103	0.0376582	50.844
NM_029157	Sf3a3	4	124400687	124401320	0.0377112	50.7837
NM_025364	Sarnp	10	128297913	128299062	0.0380378	50.4115
NM_181344	C1rl	6	124455365	124455954	0.0388588	49.4644
NM_133350	Mapre3	5	31147798	31148655	0.0389029	49.3656
NM_001081152	Npat	9	53377634	53378415	0.0391236	49.1128

(Continued)

Table 1 Continued

Transcript ID	Gene symbol	Chr[†]	Region start[‡]	Region end[¶]	*P* value (region)	MAT score[#]
NM_008439	Khk	5	31227408	31228500	0.0392384	48.9549
NM_015773	Spag6	16	16791679	16792329	0.0392913	48.8872
NM_001033180	9430070O13Rik	1	158304632	158305509	0.0395473	48.5082
NM_146155	Ahdc1	4	132579814	132580857	0.0396533	48.3616
NM_029790	Mett5d1	2	109075735	109076612	0.0397415	48.2096
NM_177391	Fam109b	15	82174034	82174818	0.0399004	48.0484
NM_001114125	Dab2ip	2	35510359	35511269	0.0399181	48.0359
NM_029010	Glb1l	1	75201758	75202505	0.0399357	48.0261
NM_030174	Mctp1	13	76851082	76852018	0.0399357	48.0031
NM_001171739	Bag1	4	40885029	40885786	0.0401476	47.702
NM_027979	Chit1	1	136010779	136011539	0.0403065	47.4874
NM_011858	Odz4	7	104022113	104023108	0.0405713	47.2129
NM_030688	Il1rapl2	X	135058363	135058965	0.0415423	46.2058
NM_138599	Tomm70a	16	57150406	57151203	0.0416571	46.107
NM_001001882	Rtel1	2	181085763	181086635	0.0416571	46.0739
NM_009413	Tpd52l1	10	31106127	31106866	0.0416659	46.0642
NM_178390	Fam53a	5	33972075	33972795	0.0416659	46.0625
NM_138654	5033411D12Rik	13	17748880	17749359	0.0419837	45.673
NM_026775	Tmed10	12	86713435	86714112	0.0421691	45.4796
NM_028838	Lrrc2	9	110865334	110866439	0.042328	45.2538
NM_025911	Ccdc91	6	147433607	147434710	0.042328	45.2183
NM_029967	Adamtsl1	4	85814839	85815826	0.0424428	44.9608
NM_033327	Zfp423	8	90365778	90366763	0.0429901	44.3529
NM_170671	Mycbpap	11	94377752	94378403	0.0430077	44.3066
NM_028295	Pdia5	16	35462288	35462947	0.0430165	44.2819
NM_001165954	Phc3	3	30835728	30836660	0.043043	44.2446
NM_054094	Acsm1	7	126767612	126768365	0.0431136	44.1458
NM_001162868	Rab11fip3	17	26167961	26168579	0.0434403	43.868
NM_001012363	Slc2a9	5	38768470	38769590	0.0436168	43.6591
NM_177577	Dcdc2a	13	25192885	25193707	0.0439258	43.3334
NM_030560	Cwc22	2	77735443	77736123	0.0440935	43.2073
NM_025947	Dynlrb1	2	155072376	155072981	0.044579	42.6332
NM_001045528	Fkbp15	4	62001113	62001648	0.0447909	42.473
NM_007935	Epc1	18	6490268	6491297	0.0448262	42.4636
NM_023587	Ptplb	16	35075272	35075959	0.0453029	42.0528
NM_001081358	Lrrc7	3	157962683	157963357	0.0458502	41.5945
NM_173185	Csnk1g1	9	65867215	65868253	0.045912	41.5194
NM_010620	Kif15	9	122869843	122870640	0.0459208	41.502
NM_172816	Slc30a8	15	52126641	52127313	0.0469536	40.5018
NR_033217	BC030870	8	67624338	67624980	0.0470242	40.4386
NM_010612	Kdr	5	76341352	76341976	0.0471743	40.3323
NM_172143	Ofcc1	13	40299743	40300521	0.047192	40.3085
NM_207652	Tsc22d1	14	76824433	76825291	0.0472626	40.2537
NM_001037099	Cacnb4	2	52441471	52442132	0.0476157	40.037
NM_025412	Pycrl	15	75749979	75750561	0.0481012	39.6776
NM_001081150	Lonrf1	8	37298318	37298901	0.0483131	39.4951
NM_175013	Pgm5	19	24930928	24931622	0.0485514	39.346
NM_029609	Lhpp	7	139848019	139848615	0.0486132	39.3014
NM_027290	Mcm10	2	4928264	4929054	0.0487103	39.1862
NM_021889	Syt9	7	114556418	114557012	0.0488339	39.0655
NM_007872	Dnmt3a	12	3886534	3886929	0.0488427	39.0514
NR_015585	4933439C10Rik	11	59322948	59323569	0.049646	38.357
NM_026454	Ube2f	1	93177645	93178267	0.0496637	38.3409
NM_001081132	Upf2	2	5894995	5895505	0.0498402	38.2319
NM_021377	Sorcs1	19	50307062	50307680	0.0498844	38.175
NM_010752	Mad1l1	5	140580860	140581358	0.049955	38.1203

*Mouse embryonic palate/maxillary mesenchymal cells were transfected with PRDM16-myc-expressing plasmid and a ChIP-Chip analysis performed as described in the Materials and methods section. One hundred and twenty-two genes were identified that had a P value < 0.05

[†]Chr, chromosome number

[‡]Region start and [¶]region end are the chromosomal positions for the promoter region bound by PRDM16

[#]Model-based Analysis of Tiling-arrays (MAT) score calculated using the algorithm described by Johnson *et al.*[12]

from *Prdm16*$^{-/-}$ fetuses was significantly less compared with wild-type levels of each on E13.5 and E14.5 ($P < 0.01$). The muscle marker, *Myf-4*, was decreased on E14.5 in wild-type palates when compared with expression on E13.5, but was significantly increased in *Prdm16*$^{-/-}$ palate tissue on E14.5 (Figure 2). These data suggest that both muscle and bone development in the secondary palates of *Prdm16*$^{-/-}$ fetuses is affected.

To confirm that the expression of *Opn* was altered in *Prdm16*$^{-/-}$ palate tissue, *in situ* hybridization was conducted

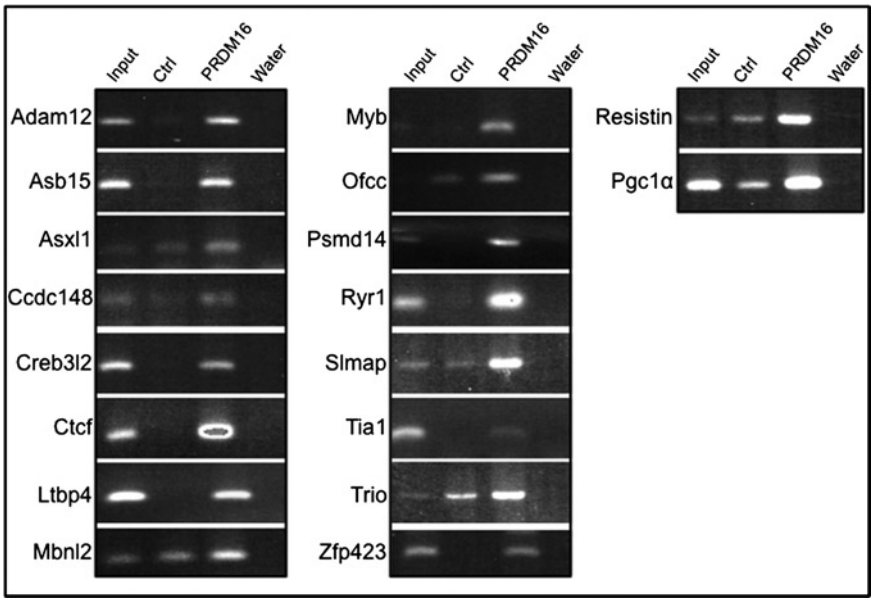

Figure 1 Polymerase chain reaction (PCR) confirmation of putative PRDM16-regulated genes in mouse embryonic palate mesenchymal (MEMM) cells. Input (1%) or immunoprecipitated, purified chromatin (2 ng) from control- or PRDM16-expressing MEMM cells was subjected to standard PCR reactions as described in the Materials and methods section. For each primer set, lane 1 represents the amplification from 1% of the amount of chromatin subjected to immunoprecipitation, lane 2 is that from chromatin from control-transfected MEMM cells, lane 3 from chromatin from PRDM16-Myc-transfected MEMM cells and lane 4, water control. In each case, there was increased amplification from PRDM16-Myc-transfected versus control-transfected MEMM cells. In some cases, the amplification from 'input' was weak, but detectable

Table 2 Functional classification of genes regulated by PRDM16 in MEMM cells*

Biological process	Genes
Muscle development	Asb15, Trio, Adam13, Ryr1, Hsp90b1, Itga9, Mapre3, Epc1, Slmap, Mbnl2
Regulation of transcription and/or chromating remodelling	Ctcf, Creb3l2, Npat, Asxl1, Myb, Nr3c1, Phc3, Tcfap4, Dnmt3a, Tsc22dl
Protein ubiquitination and/or degradation	Lonrf1, Spambpl1, Trhde, Asb15, Cirl, Nfx1, Ube2F
Intracellular protein trafficking	Aspscr1, Ccdc91, Dynlrb1, Hsp90b1, Tmed10
DNA damage response/apoptosis	Ahdc1, Nek11, Fhit, Bag, Tia1
Chondrogenesis/osteogenesis	Creb3l2, Bag1, Kdr, Tsc22d1
Cytoskeleton function/assembly	Fkbp15, Add3, Mapre3, Pgm5
Small G-protein activity	Ctnnal1, Chn1, Dab2lp

*The function(s) of the genes reported in Table 1 were obtained from searching the existing literature using PubMed Bioinformatics Resource (PubMed identification numbers and GenRIF summaries). In some cases, genes have overlapping function (e.g. Asb15). Genes were classified according to an established function

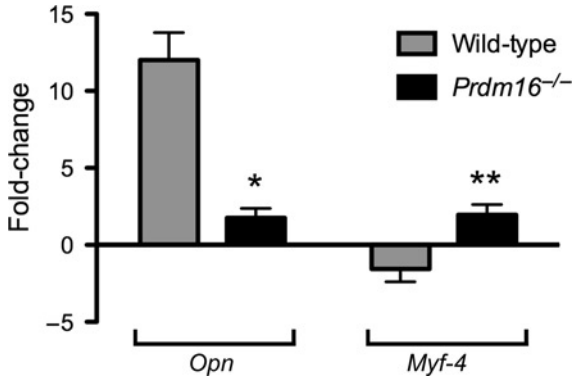

Figure 2 Expression of *Opn* and *Myf-4* in *Prdm16*$^{-/-}$ secondary palate tissue. The secondary palates from wild-type and *Prdm16*$^{-/-}$ E13.5 and E14.5 fetuses were isolated and total RNA purified, from which cDNAs were synthesized. The expression of *Opn* and *Myf-4* was assayed by semiquantitative realtime polymerase chain reaction (PCR) and normalized to the expression of *18S* RNA. The expression level on both days was determined and the data presented is the change in expression on E14.5 relative to E13.5. The expression of *Opn* was significantly upregulated on E14.5 in wild-type palate tissue; however, in the absence of Prdm16, *Opn* expression was significantly decreased ($^{*}P < 0.001$, one-way analysis of variance [ANOVA]). Conversely, the expression of *Myf-4* was decreased in wild-type tissue but increased in *Prdm16*$^{-/-}$ palate tissue ($^{**}P < 0.05$, one-way ANOVA)

on E14.5 (Figures 3a–f) and E15.5 (Figures 3g–j) maxillary and palate tissue. *Opn* has a unique expression pattern in secondary palate tissue in that it is found in small, isolated clusters of cells. In wild-type palate tissue on E14.5, these clusters were located primarily along the medial edge seam on the oral side of the palate (Figure 3a, yellow arrows). On the nasal surface of the palate, scattered *Opn*-positive areas were observed, particularly at the posterior end of the secondary palate (yellow arrow in panels b and c). As shown in Figures 3d and e, *Prdm16*$^{-/-}$ mutant fetuses have a wide cleft, and expression of *Opn* on both the oral and nasal aspects of the secondary palate was essentially absent. On E15.5, *Opn* expression was detected in the surfaces between the primary and secondary palates of wild-type fetuses (panel g, yellow arrow). On the nasal surface of wild-type fetuses, *Opn* was primarily expressed in the anterior two-thirds of the secondary palate (panel h, SP). On E15.5, *Prdm16*$^{-/-}$ fetal tissue *Opn* was found to be misexpressed in palatal rugae (panel i, red arrow) while expression in the posterior palate persisted (yellow arrowheads). There was

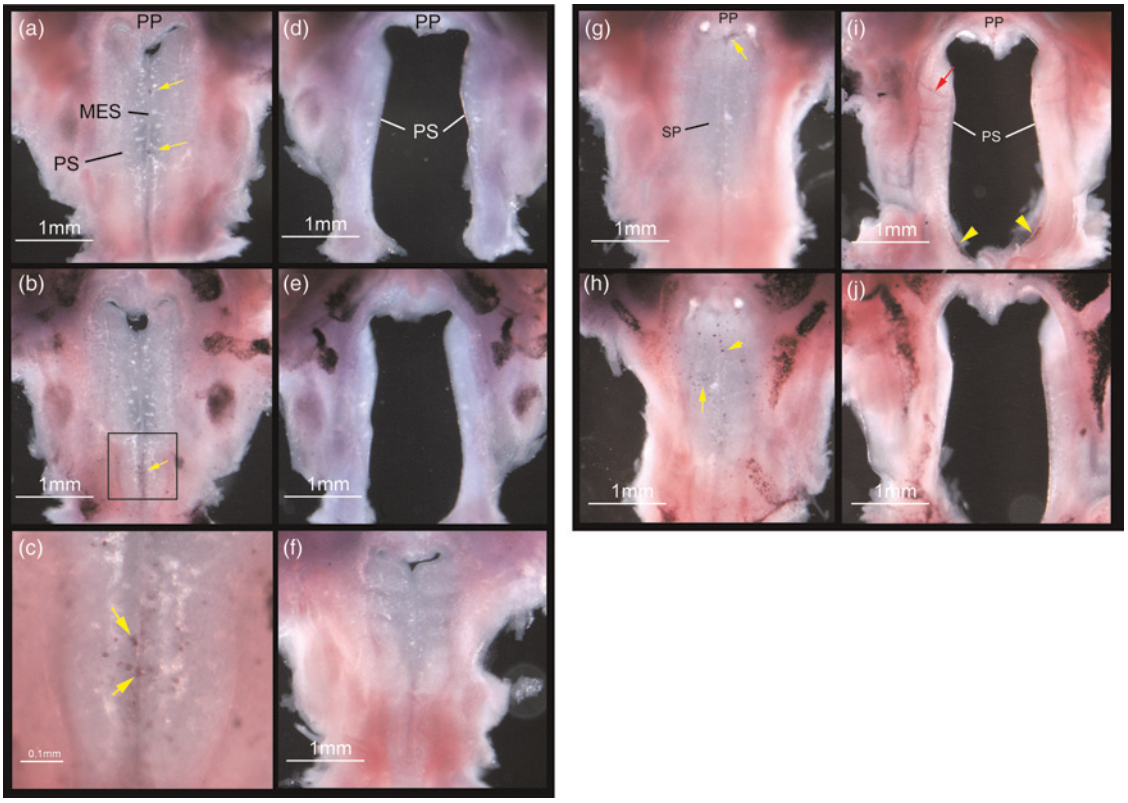

Figure 3 *In situ* hybridization analysis of *Opn* expression in wild-type and *Prdm16*$^{-/-}$ E14.5 and E15.5 fetuses. Wild-type and *Prdm16*$^{-/-}$ fetuses were isolated on E14.5 (a–f) and E15.5 (g–j), and maxillary and palatal tissues were dissected and processed for whole-mount *in situ* hybridization with a digoxigenin-labelled riboprobe specific for mouse *Opn*. Panels a and b represent E14.5 wild-type samples viewed from the oral and nasal aspect, respectively. Only a few clusters of *Opn*-positive cells were observed on the oral surface, primarily along the medial edge seam (MES, yellow arrows). On the nasal surface, scattered *Opn*-positive areas were observed, particularly at the posterior end (yellow arrow in b). Panel c is the boxed area in panel b at higher magnification. Yellow arrows indicate clusters of *Opn*-positive cells. Panels d and e are *Prdm16*$^{-/-}$ maxillary and palatal tissue viewed from the oral and nasal aspects, respectively, demonstrating a wide cleft with no detectable expression of *Opn* in the palatal shelves. Panel f is wild-type tissue hybridized to an *Opn* sense riboprobe (negative control), revealing only background staining. Panels g and h represent maxillary/palatal tissue from E15.5 wild-type fetuses viewed from the oral and nasal aspects, respectively, demonstrating *Opn* expression between the primary and secondary palates (g, yellow arrow). On the nasal surface, *Opn* was primarily expressed in the anterior two-thirds of the fused secondary palate (SP). Panels i and j are oral and nasal views, respectively, of maxillary and palate tissue from E15.5 *Prdm16*$^{-/-}$ fetal tissue. On the oral surface of the secondary palate, *Opn* was found to be misexpressed in palatal rugae (red arrow) while expression in the posterior palate persisted (yellow arrowheads). These data demonstrate that the expression of *Opn* in *Prdm16*$^{-/-}$ palate tissue is both reduced and altered in distribution. PP, primary palate; PS, palatal shelf; MES, medial edge seam

little detectable expression on the nasal surface of the secondary palate (panel j). These data demonstrate a unique expression pattern for *Opn* in the secondary palate and that the expression of *Opn* in *Prdm16*$^{-/-}$ palate tissue is both reduced in level and aberrant in localization.

Discussion

The role of the putative transcription factor PRDM16 is beginning to be elucidated in multiple tissues, most notably, in the development and differentiation of brown adipose tissue, where it drives *Myf5*-expressing (muscle)-precursor cells into the adipose lineage.[3] Interestingly, a significant number of PRDM16-regulated genes identified in the current screen have been linked to development of muscle tissue. For example, *Asb15* is primarily expressed in skeletal muscle and is important for myoblast differentiation.[17,18] *Trio* (triple functional domain protein) is a member of the Dbl-homology family of guanine nucleotide exchange factors and is also necessary for skeletal muscle development.[19] Likewise, the sarcolemma-associated protein (*Slmap*) is essential for myotube formation[20] and *Ryr1* is found predominantly in skeletal muscle where it plays a central role in excitation–contraction coupling.[21] Four genes identified have been demonstrated to contribute to chondro and/or osteogenesis (*Creb3l2*, *Bag1*, *Kdr* and *Tsc22d1*). Analysis of additional gene markers for early, mid and late bone development revealed a generalized impairment/delay (manuscript in preparation). Evidence for this hypothesis was obtained from an analysis of the expression of a marker for bone (Opn) and muscle (Myf-4) development. The expression of *Opn* was significantly reduced in palate tissue from *Prdm16*$^{-/-}$ fetuses, while that for *Myf-4* was significantly increased. *Opn* has been previously linked to human cases of orofacial clefting.[22] Thus, *Prdm16* may play an important contributory role to myo-, chondro- and/or osteogenesis in the developing orofacial region, in addition to regulating other processes important for normal development.

Development of the secondary palate in both mice and humans occurs through a series of morphogenetic events that include reorientation of the palatal processes from a

position lateral to the tongue to a position superior to the tongue, fusion of the bilateral shelves to one another and the nasal septum, and finally terminal differentiation into the bony, anterior and soft, muscular posterior palate. Only one of the PRDM16-regulated genes identified in the current screen (*Ofcc1*) is similar to a gene in humans that has been linked to orofacial clefting in humans.[23] Although the function of this gene is unknown, it has been demonstrated to be expressed in migrating neural crest cells and craniofacial bones.[24] In addition to potentially regulating both muscle and bone development in the secondary palate, PRDM16 likely regulates other, more general aspects of cellular physiology. A number of other genes were identified as PRDM16-regulated, including those linked to epigenetic regulation, protein stability and intracellular trafficking. Therefore, the role of *Prdm16* during development of the secondary palate appears to be complex, potentially regulating the expression of numerous genes that control several key processes essential for normal palate development (e.g. cell proliferation/differentiation).

The results presented in the current report suggest that *Prdm16* functions to control cell differentiation during development of the secondary palate in mice by either promoting differentiation or preventing premature differentiation. It is possible that defects in palate development seen with the *Prdm16* knockout mouse are caused by abnormal muscle and/or bone development that leads to altered morphogenesis of the nascent palatal processes leading to failure of reorientation and subsequent separation of the oral and nasal cavities.

Author contributions: All authors contributed to the design, conduct, collection and interpretation of data and to the preparation of the manuscript. Specifically, DRW and CLW conducted the experiments and collected the data; and DRW, PM, RMG and MMP analyzed the data and wrote the manuscript.

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