

The MADS box transcription factor MEF2C regulates melanocyte development and is a direct transcriptional target and partner of SOX10

Pooja Agarwal^{1,*}, Michael P. Verzi^{1,*}, Thuyen Nguyen², Jianxin Hu¹, Melissa L. Ehlers¹, David J. McCulley¹, Shan-Mei Xu¹, Evdokia Dodou¹, Joshua P. Anderson¹, Maria L. Wei² and Brian L. Black^{1,3,†}

SUMMARY

Waardenburg syndromes are characterized by pigmentation and autosensory hearing defects, and mutations in genes encoding transcription factors that control neural crest specification and differentiation are often associated with Waardenburg and related disorders. For example, mutations in *SOX10* result in a severe form of Waardenburg syndrome, Type IV, also known as Waardenburg-Hirschsprung disease, characterized by pigmentation and other neural crest defects, including defective innervation of the gut. *SOX10* controls neural crest development through interactions with other transcription factors. The MADS box transcription factor MEF2C is an important regulator of brain, skeleton, lymphocyte and cardiovascular development and is required in the neural crest for craniofacial development. Here, we establish a novel role for MEF2C in melanocyte development. Inactivation of *Mef2c* in the neural crest of mice results in reduced expression of melanocyte genes during development and a significant loss of pigmentation at birth due to defective differentiation and reduced abundance of melanocytes. We identify a transcriptional enhancer of *Mef2c* that directs expression to the neural crest and its derivatives, including melanocytes, in transgenic mouse embryos. This novel *Mef2c* neural crest enhancer contains three functional SOX binding sites and a single essential MEF2 site. We demonstrate that *Mef2c* is a direct transcriptional target of SOX10 and MEF2 via this evolutionarily conserved enhancer. Furthermore, we show that SOX10 and MEF2C physically interact and function cooperatively to activate the *Mef2c* gene in a feed-forward transcriptional circuit, suggesting that MEF2C might serve as a potentiator of the transcriptional pathways affected in Waardenburg syndromes.

KEY WORDS: MEF2, MEF2C, SOX10, Melanocyte, Neural crest, Pigment, Mouse

INTRODUCTION

Neural crest cells are multipotent progenitor cells that originate from the dorsal neural tube at the border of the neural plate and the lateral epidermis to give rise to various tissues, including the skeletal elements of the face and head, dorsal root and sympathetic ganglia, the glial cells of the peripheral and enteric nervous systems, and melanocytes (Baker and Bronner-Fraser, 1997; Knecht and Bronner-Fraser, 2002; Le Douarin et al., 2004; Selleck et al., 1993; Trainor, 2005). Neural crest-derived melanocytes originate as non-pigmented precursors called melanoblasts, which migrate along characteristic pathways to various destinations, such as the dermis and epidermis, the inner ear and the choroids of the eye (Crane and Trainor, 2006; Dupin and Le Douarin, 2003; Sauka-Spengler and Bronner-Fraser, 2006; Steel and Barkway, 1989). The development of mature pigment-producing melanocytes from their neural crest precursors is a complex process involving melanoblast specification, proliferation, survival, migration and differentiation. Several transcription factors are known to be involved in melanocyte development, and mutations in the genes encoding

these factors have been linked to pigment disorders and other neurocristopathies (Baxter et al., 2004; Mollaaghababa and Pavan, 2003; Pingault et al., 2010; Spritz et al., 2003).

SOX10 is an HMG box transcription factor that is a well-established regulator of neural crest development (Hong and Saint-Jeannet, 2005; Mollaaghababa and Pavan, 2003). Loss-of-function mutations in human *SOX10* result in haploinsufficiency and are associated with Waardenburg-Hirschsprung disease, a neurocristopathy that involves hypopigmentation, deafness and aganglionic colon (Baxter et al., 2004; Inoue et al., 2004; Parisi and Kapur, 2000; Pingault et al., 1998; Pingault et al., 2010; Spritz et al., 2003). Similarly, *Sox10* heterozygous mice also exhibit hypopigmentation, as well as distal bowel aganglionosis, and, therefore, these mice serve as an excellent animal model of Waardenburg-Hirschsprung disease (Herbarth et al., 1998; Lane and Liu, 1984; Southard-Smith et al., 1999; Southard-Smith et al., 1998). *Sox10*-null mice completely lack pigmentation and fail to develop a functional peripheral nervous system, suggesting a role for *SOX10* in the specification of neural crest cells into melanocytes and glia in the peripheral and enteric nervous systems (Britsch et al., 2001; Herbarth et al., 1998; Potterf et al., 2001; Southard-Smith et al., 1998).

The MADS domain transcription factor MEF2C is important in the development of several lineages, including cardiac and skeletal muscle, hematopoietic cells, the craniofacial and axial skeletons, and the central nervous system (Black and Cripps, 2010; Potthoff and Olson, 2007). Inactivation of *Mef2c* in the germline of mice results in embryonic lethality by embryonic day (E)10 due to severe defects in cardiac and vascular development (Bi et al., 1999;

¹Cardiovascular Research Institute, University of California, San Francisco, CA 94158-2517, USA. ²Department of Dermatology, Veterans Affairs Medical Center, University of California, San Francisco, CA 94143-0316, USA. ³Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94158-2517, USA.

*These authors contributed equally to this work

†Author for correspondence (brian.black@ucsf.edu)

Lin et al., 1998; Lin et al., 1997). In the neural crest, *Mef2c* expression can be detected as early as E8.5 in the mouse in the region adjacent to the neural folds (Edmondson et al., 1994; Verzi et al., 2007). Inactivation of *Mef2c* specifically in the neural crest using a conditional knockout approach in mice results in lethality at birth due to airway obstruction and defective craniofacial development (Verzi et al., 2007).

In the present study, we have identified a novel role for MEF2C in neural crest-derived melanocyte development. Inactivation of *Mef2c* in the neural crest using *Wnt1-Cre* transgenic mice results in reduced expression of melanocyte genes during development and a significant reduction in the number of melanocytes at birth. We have also identified a highly conserved transcriptional enhancer from the *Mef2c* locus that directs expression to the neural crest and its derivatives, including the craniofacial mesenchyme, the peripheral and enteric nervous systems, and melanocytes. We demonstrate that *Mef2c* is a direct transcriptional target of SOX10 in developing melanocytes and peripheral nervous system via this evolutionarily conserved transcriptional enhancer. Finally, we show that MEF2C physically interacts with SOX10 and, together, these proteins cooperatively activate transcription. Thus, we propose that *Mef2c* has a role in melanocyte development as a transcriptional target and partner for SOX10.

MATERIALS AND METHODS

Cloning and mutagenesis

The 7039-bp *Mef2c*-F1 enhancer fragment was amplified by PCR from the mouse *Mef2c* locus using the primers F1-F: 5'-AGTGGGAAGCATAAGGCCCGGGAAGTCTGAT-3' and F1-R: 5'-ATGGTACCGTGTATGGTGGTCCCGGGAATGT-3'. The resulting PCR product was digested and cloned into the *Xma*I sites in plasmid *hsp68-lacZ* (Kothary et al., 1989) to create plasmid *Mef2c*-F1-*lacZ*. The 300-bp conserved region of *Mef2c*-F1 was generated as an *Xma*I-*Hind*III subfragment and was cloned into plasmid *hsp68-lacZ* to create plasmid *Mef2c*-F1[3-3.3]-*lacZ* for transgenic analyses or into plasmid pTK- β -gal to create plasmid *Mef2c*[3-3.3]-F1-TK-*lacZ* for transfection analyses. Mutations of cis-regulatory elements within *Mef2c*-F1 were generated using the gene SOEing method (Horton, 1997) and were confirmed by sequencing on both strands.

Generation and analysis of transgenic mice

Transgenic mice were generated by oocyte microinjection as described previously (Dodou et al., 2003; Hogan et al., 1994). *Sox10*^{Dom} mice were purchased from the Jackson Laboratories. *Wnt1-Cre*, *Mef2c*^{+/-} and *Mef2c*^{lox/lox} mice have been described previously (Danielian et al., 1998; Lin et al., 1997; Vong et al., 2005). All experiments with animals complied with federal and institutional guidelines and were reviewed and approved by the University of California, San Francisco Institutional Animal Care and Use Committee.

X-gal staining, whole-mount in situ hybridization, and immunofluorescence

β -galactosidase (β -gal) expression in *lacZ* transgenic embryos or tissues was detected by X-gal staining, as described previously (Dodou et al., 2003). Whole mount in situ hybridization was performed according to standard methods using digoxigenin-labeled antisense probes as described previously (Rojas et al., 2005). *Dct* (Image Clone ID 30539879), *Mitf* (Image Clone ID. 40047440), *Pmel17* (Image Clone ID 30541702) and *Sox10* probes were all designed by cloning the full-length cDNA into pBlueScript. Immunofluorescence labeling of cryosections was performed as described previously (Rojas et al., 2009) using the following primary antibodies at 1:100 dilution in PBS with 3% BSA and 0.1% Triton X-100: anti-SOX10 (R&D AF2864); anti-MEF2C (C-17) (Santa Cruz, sc-13268); anti-DCT (alpha-PEP8).

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed as described previously (Dodou et al., 2003). The sequences of the control SOX10 and MEF2 binding sites have been described previously (Dodou et al., 2003; Peirano and Wegner, 2000). The sense-strand sequences of the *Mef2c*-F1 oligonucleotides used for EMSA were: *Mef2c* SOX-1, 5'-GAATGCACTGACTACAAAGTGCATCCT-GAAG-3'; *Mef2c* mutSOX-1, 5'-GAATGCACTGACCCGCGTTCATCCTGAAG-3'; *Mef2c* SOX-2, 5'-GAATAGCTCTATAACAAAAGTAACTACAGAGT-3'; *Mef2c* mutSOX-2, 5'-GAATAGCTCTATCCGCGTTAACTACAGAGT-3'; *Mef2c* SOX-3, 5'-GGCCATTAGCTCA-CATGAAGGTCTGTGT-3'; *Mef2c* mutSOX-3, 5'-GGCCATTAGCTCGAGGGTAAGGTCTGTGT-3'; *Mef2c* MEF2, 5'-GGGCTC-CAACTATTATAGAAGTACTGAGTA-3'; and *Mef2c* mutMEF2, 5'-GGGCTCCAAGTACTGAGTA-3'.

Chromatin immunoprecipitation (ChIP)

B16F10 mouse melanoma cells were transfected in nearly confluent 10-cm plates with 6 μ g of pRK5-Sox10[Flag] using Lipofectamine LTX reagent (Invitrogen), according to the manufacturer's protocol. Twenty-four hours post-transfection, ChIP was performed using the ChIP Assay Kit (Upstate/Millipore) according to the manufacturer's instructions, using anti-FLAG antibody (clone M2, Sigma) and Protein G-PLUS agarose beads (Santa Cruz). Immunoprecipitated fragments and input samples were subjected to PCR using the following primers: *Mef2c*-F1-A, 5'-GTCCTGGAGTCTTGCACAG-3' and *Mef2c*-F1-B, 5'-CAGCCCAAGCTTC-CGTATGG-3' to amplify the region of *Mef2c*-F1 containing the SOX binding sites; or primers VEGFR2-F1, 5'-ATCATGTGACAGCAAGAC-CG-3' and VEGFR2-R1, 5'-TCTTGGTATGTTGGGTCCTC-3', designed to amplify a region of the *Vegfr2* promoter not expected to be bound by SOX10.

Transactivation assays

Transfections were performed in C3H10T1/2 cells in 6-cm plates using either Superfect (Qiagen) or Fugene 6 (Roche) according to the manufacturer's instructions. Each transfection contained 1 μ g of reporter and 1 μ g of expression plasmid. In transfections with two expression plasmids, the amount of each expression plasmid was adjusted to 0.5 μ g each. In transfections without an expression construct, the parent expression plasmid was added to keep the total amount of DNA in each transfection constant at 2 μ g. Cells were cultured for 48 hours after transfection, then harvested and assayed by using the Luminescent β -gal Detection System (Clontech). SOX10 and MEF2C-VP16 were each expressed from the pRK5 mammalian expression vector (BD Pharmingen).

DOPA staining and electron microscopy

Staining of 3,4-dihydroxy-L-phenylalanine (DOPA) and electron microscopy (EM) on skin samples isolated from neonatal pups from a region of the back located between the shoulders were performed as previously described (Nguyen and Wei, 2007). For EM analyses, DOPA-stained back skin was fixed in modified Karnovsky's fixative (2% paraformaldehyde, 2% glutaraldehyde and 0.06% CaCl₂ in 100 mM cacodylate buffer, pH 7.3). The tissue was then washed twice in 100 mM cacodylate buffer, fixed in 1.5% potassium ferrocyanide and 2% osmium tetroxide for 2 hours, rinsed in water, dehydrated through a series of ethanol washes, and embedded in Epon resin overnight. Epon blocks were sectioned and mounted onto formvar-coated grids. Grids were stained in 10% uranyl acetate in 50% methanol and viewed on a Zeiss 10A electron microscope at 60 kV. Melanosome density per unit area of melanocyte cytoplasm was determined by measuring the cytoplasmic area with NIH ImageJ software and counting DOPA-stained melanosomes within the cytoplasmic area.

Glutathione S-transferase (GST) pull-down and co-immunoprecipitation assays for protein-protein interaction

GST pull-down assays were performed as described previously (Chen et al., 2000). The GST-MEF2C expression plasmid was generated by inserting the entire coding sequence of MEF2C and an in-frame C-terminal FLAG epitope (Khiem et al., 2008) in-frame into the PGEX-2T expression vector (Pharmacia). SOX10 full length and SOX10 DNA binding domain

truncation proteins were generated from pCITE-Sox10[FL] and pCITE-Sox10[DBD], respectively. pCITE-Sox10[DBD] contains a 235-amino acid N-terminal fragment of SOX10 that includes the DNA binding domain cloned in-frame into pCITE. The SOX10 full length and DBD proteins were synthesized and radiolabeled with [³⁵S]-methionine in vitro using the TNT Quick Coupled Transcription/Translation System (Promega), according to the manufacturer's directions.

Co-immunoprecipitation was performed in lysates from co-transfected B16F10 mouse melanoma cells. Nearly confluent 10-cm plates were co-transfected with 3 μg each of pRK5-MEF2C-FLAG (Khiem et al., 2008) and pRK5-Sox10[HA] using Lipofectamine LTX reagent according to the manufacturer's protocol. Cells were grown for 30 hours after transfection and then harvested in NP-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, pH 7.4) containing protease inhibitors for 30 minutes at 4°C with gentle agitation followed by centrifugation to remove insoluble material. Lysates were then incubated overnight at 4°C with 3 μg of mouse anti-MEF-2 antibody (sc-17785, Santa Cruz) or mouse IgG1 antibody (ab18447, Abcam) as an isotype control. Samples were then incubated for 2.5 hours at 4°C with 100 μl of 50% Protein G Sepharose bead in NP-40 lysis buffer. Bound beads were washed 4 times with NP-40 lysis buffer and eluted in SDS sample buffer containing 100 mM DTT and boiled for 4 minutes before analysis by SDS-PAGE. Co-immunoprecipitation was determined by western blot with rabbit anti-HA antibody (sc-805, Santa Cruz) at a 1:200 dilution.

RESULTS

Identification of a *Mef2c* enhancer that directs expression to several developing neural crest lineages, including melanocytes

Although *Mef2c* function is required in the neural crest (Verzi et al., 2007), its expression in that lineage is not well defined. This is likely to be due to the strong expression of *Mef2c* in developing cardiac, skeletal muscle and vascular lineages, making the relatively weaker, but essential, expression of *Mef2c* in the neural crest difficult to visualize by in situ hybridization or immunohistochemistry (Black and Cripps, 2010; Edmondson et al., 1994). It has been observed previously that the expression of *Mef2c* during mouse embryogenesis is controlled by several discrete modular enhancers that each direct expression to a subset of the complete endogenous expression domain of *Mef2c* (Black and Cripps, 2010). Therefore, we reasoned that isolation of *Mef2c* enhancers with activity restricted to the neural crest might be used to determine the detailed expression of *Mef2c* within the neural crest without obfuscation from stronger expression in other lineages proximal to or overlying neural crest-derived tissues. Towards that goal, we identified a highly conserved enhancer that exhibited transcriptional activity in the developing neural crest and its derivatives (Fig. 1).

Mef2c-F1 enhancer activity in the neural crest was detected as early as E8.5 in the neural fold mesenchyme (data not shown). Between E9.0 and E9.5, β-gal was strongly expressed in craniofacial mesenchyme in a pattern similar to endogenous *Mef2c* at this stage (Verzi et al., 2007) (data not shown). *Mef2c*-F1 enhancer activity in the craniofacial mesenchyme began to decline by E10.5, but activity became more pronounced in the developing cranial nerves (Fig. 1B). β-Gal activity was also evident in cranial and sympathetic ganglia at E10.5 (Fig. 1B). In addition, *Mef2c*-F1-*lacZ* expression was detected in the developing heart (Fig. 1B), but the bona fide regulation of *Mef2c* in the heart via this enhancer is not a focus of the present study. By E12, β-gal expression in the craniofacial mesenchyme was no longer observed. However, activity in the cranial nerves, sympathetic chain ganglia, peripheral nerves and nerve roots, and enteric ganglia was evident (Fig. 1C). At E14, *Mef2c* enhancer activity continued throughout the

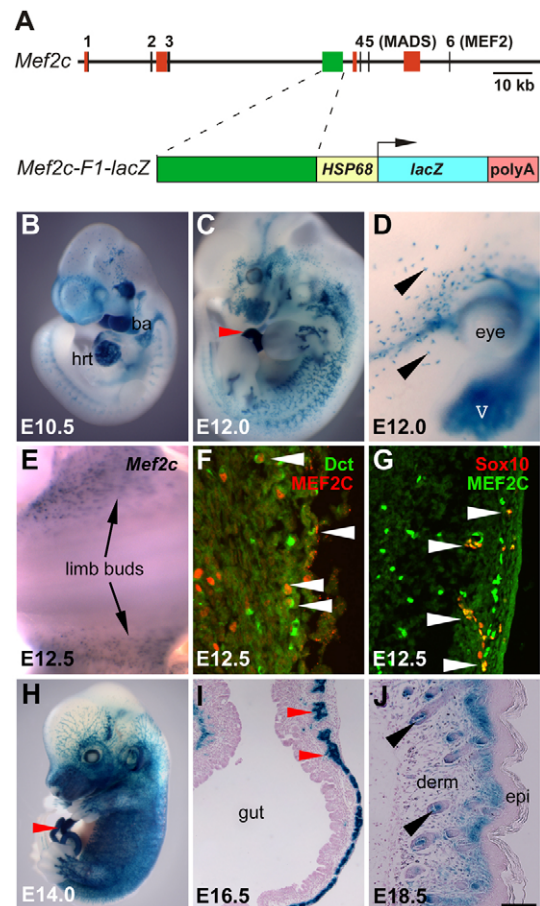


Fig. 1. Identification of *Mef2c*-F1, a neural crest enhancer from *Mef2c*. (A) The top line represents the 5' end of the mouse *Mef2c* locus. Vertical black lines depict *Mef2c* exons. Colored rectangles represent known enhancers. A schematic of the *Mef2c*-F1-*lacZ* transgene is shown below. (B-J) Whole-mount (B-E,H) or transverse sections (F,G,I,J) of X-gal-stained *Mef2c*-F1-*lacZ* transgenic (B-D,H-I) or wild-type embryos examined by in situ hybridization for *Mef2c* (E) or by immunofluorescence using antibodies against MEF2C (red in F, green in G), DCT (green in F) and SOX10 (red in G). Black or white arrowheads mark developing melanocytes. Red arrowheads mark peripheral nerves in the gut. V, fifth cranial nerve; ba, branchial arch; derm, dermis; epi, epidermis; hrt, heart. Scale bar: 100 μm.

developing peripheral and enteric nervous systems (Fig. 1H). Sections through the developing gut at E16.5 revealed that β-gal expression directed by the *Mef2c*-F1-*lacZ* enhancer was within the myenteric plexus (Fig. 1I).

Mef2c-F1-*lacZ* expression was also evident in the developing melanocyte lineage, beginning at ~E10.5 (Fig. 1B) and continuing throughout embryonic development (Fig. 1J). At E12, robust β-gal activity was apparent in single cells in the peri-ocular region of the embryo (Fig. 1D). This pattern of enhancer activity was consistent with melanocyte expression of the transgene. In perinatal skin, transgene expression was observed in follicular melanocytes and in the melanocytes that occupy the dermal-epidermal border (Fig. 1J). To determine whether the endogenous *Mef2c* gene was expressed in melanocytes, we examined *Mef2c* expression by in situ hybridization. As noted above, *Mef2c* is expressed broadly in many tissues, including the vasculature, somites and nerves (Black and Cripps, 2010; Dodou et al., 2004; Edmondson et al., 1994),

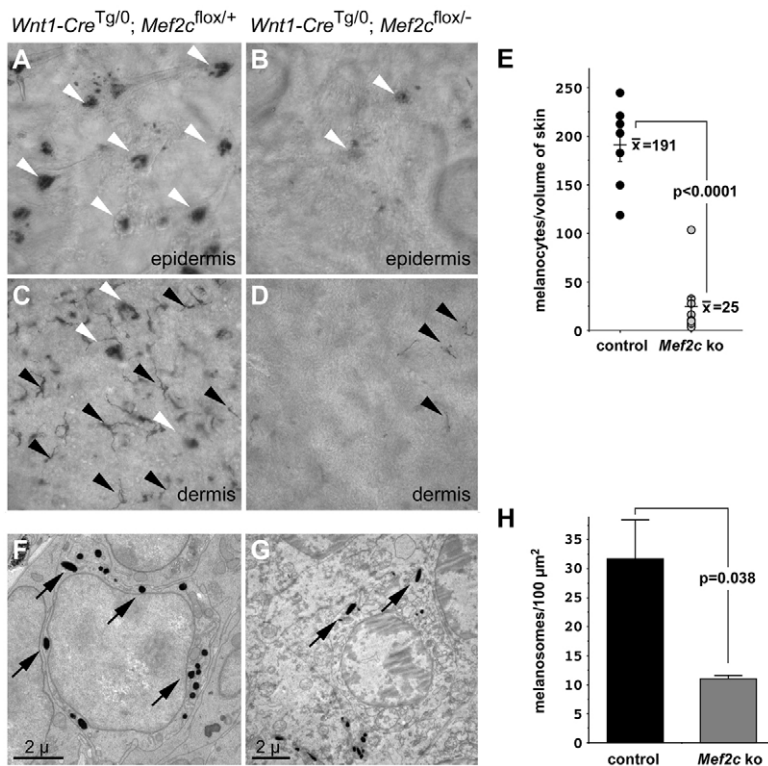


Fig. 2. *Mef2c* is required for melanocyte development in mice. (A–D) Whole-mount DOPA-stained epidermis (A,B) or dermis (C,D) from *Wnt1-Cre^{Tg/0}; Mef2c^{fllox/+}* control (A,C) and *Wnt1-Cre^{Tg/0}; Mef2c^{fllox/-}* neural crest knockout (NC KO; B,D) neonatal mice showed many fewer stained follicular (white arrowheads) and interfollicular (black arrowheads) melanocytes in *Mef2c* NC KO skin compared with controls. (E) Quantification of DOPA-stained melanocytes from *Wnt1-Cre^{Tg/0}; Mef2c^{fllox/+}* control and *Wnt1-Cre^{Tg/0}; Mef2c^{fllox/-}* NC KO epidermis. Filled circles represent individual control mice (191±16 DOPA-stained melanocytes per sample, *n*=7) and open circles represent individual *Mef2c* NC KO mice (25±10 DOPA-stained melanocytes per sample, *n*=9), *P*<0.0001. (F,G) Electron micrographs of DOPA-stained melanocytes from *Wnt1-Cre^{Tg/0}; Mef2c^{fllox/+}* control mice (F) and *Wnt1-Cre^{Tg/0}; Mef2c^{fllox/-}* NC KO mice (G) showed that *Mef2c* NC KO mice had fewer melanosomes per melanocyte (black arrows). (H) Quantification of melanosomes in individual DOPA-stained control and *Mef2c* NC KO neonates indicated the mean number of melanosomes in 100 μm² for control skin was 31.7±6.8; *n*=3 mice. The mean number of melanosomes in 100 μm² within stained melanocytes in *Mef2c* NC KO neonates was 11.0±0.6; *n*=3 mice (*P*=0.038). An average of six randomly selected melanocytes from each neonatal mouse was scored. Data are expressed as the mean number of melanosomes in 100 μm² + s.e.m.

making endogenous expression in melanocytes difficult to observe. However, *Mef2c* expression was evident at E12.5 in the dorsal region of the embryo between the hind limbs in a pattern highly reminiscent of melanocytes (Fig. 1E). To confirm that expression of MEF2C was in melanocytes, we compared the expression of MEF2C protein and the melanocyte marker dopachrome tautomerase (DCT) in the dermis at E12.5 by immunofluorescence (Fig. 1F). Numerous cells co-expressed MEF2C, which was confined to the nucleus, and DCT, which was present in the cytoplasm, confirming MEF2C expression in melanocytes. We also compared expression of MEF2C with SOX10 expression in the dermis at E12.5 and found that all SOX10-expressing cells in the nascent skin also expressed MEF2C (Fig. 1G), although, as expected, many MEF2C-expressing cells did not express SOX10. These results indicate that SOX10 and MEF2C are co-expressed in vivo in a subset of cells in the dermis.

Mice deficient in *Mef2c* exhibit pigmentation defects at birth

Because of the strong expression of *Mef2c* and robust activation of the *Mef2c*-F1 enhancer in melanocytes and in the peripheral and enteric nervous systems, we examined those lineages in detail in *Mef2c* neural crest knockout (NC KO) neonates to determine whether MEF2C might have requirements in those neural crest-derived lineages. Although *Mef2c* NC KO mice die at birth of craniofacial defects (Verzi et al., 2007), they exhibited no obvious defects in peripheral or enteric innervation at birth (data not shown). As an initial analysis of melanocyte development, we stained epidermis and dermis taken from the anterior region of the back from *Mef2c* NC KO and control neonates by incubation with DOPA, which detects pigmented cells, and examined the stained tissue by light microscopy (Fig. 2). We observed a significant reduction in the number of melanocytes in epidermis (Fig. 2, compare panels A and B) and dermis (Fig. 2, compare panels C and D) in *Mef2c* NC KO

mice compared with littermate controls. Within the dermis, we observed a strong reduction in the number of both follicular and interfollicular melanocytes (Fig. 2C,D). Indeed, the number of DOPA-stained cells per field showed that the number of melanocytes in the epidermis was reduced by 87% in *Mef2c* NC KO neonates compared with littermate controls (Fig. 2E). These observations demonstrate that MEF2C is required for establishing or maintaining the proper number of melanocytes in vivo.

Analysis of neonatal skin by electron microscopy revealed that the few remaining individual melanocytes in the dermis of *Mef2c* NC KO mice had fewer melanosomes than did the melanocytes present in littermate controls (Fig. 2, compare panels F and G). Quantification indicated a 65% reduction in the number of electron-dense melanosomes in dermal melanocytes from *Mef2c* NC KO neonates compared with their littermate controls (Fig. 2H). These data suggest that, in addition to being required for the proper number of melanocytes in the skin, MEF2C function is also required in the neural crest or in neural crest-derived cells for melanocyte function or differentiation in vivo.

Mef2c is required for proper developmental expression of melanocyte marker genes

We also examined the expression of well-established markers of melanocyte differentiation and function in *Mef2c* NC KO and control embryos (Fig. 3). Interestingly, *Mitf*, *Dct* and *Pmel17* all had reduced expression in *Mef2c* NC KO embryos compared with their littermate controls (Fig. 3). At E12.5, *Pmel17* expression was reduced, including in the trunk region around the hind limbs (Fig. 3, compare panels A and B). Similarly, *Mitf* expression was reduced in the same region at E12.5 in *Mef2c* NC KO embryos (Fig. 3, compare panels C and D). Reduced expression of melanocyte markers was apparent in multiple regions of the embryo and over several days of development in *Mef2c* NC KO embryos. For example, the expression of the melanocyte

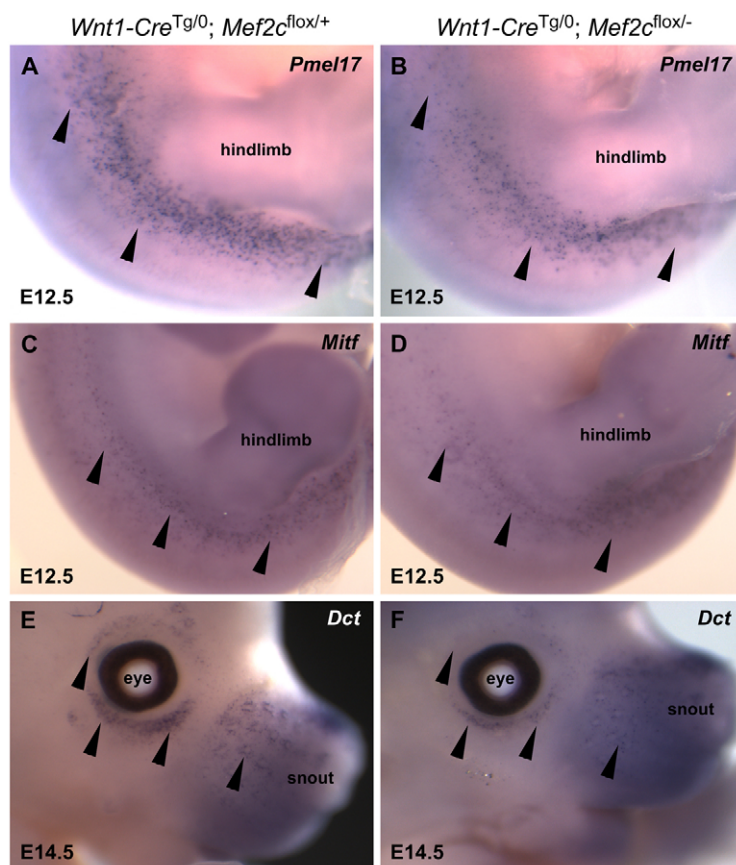


Fig. 3. *Mef2c* is required for normal expression of melanocyte marker genes during development.

(A-F) Whole-mount in situ hybridization for *Pmel17* (A,B), *Mitf* (C,D) and *Dct* (E,F) transcripts from *Wnt1-Cre^{Tg/0}; Mef2c^{flox/+}* conditional knockout embryos (B,D,F) showed reduced expression of each of the melanocyte markers during embryonic development compared with expression in *Wnt1-Cre^{Tg/0}; Mef2c^{flox/+}* control embryos (A,C,E). Black arrowheads point to melanocytes in the trunk region at E12.5 and in the sub- and supra-optic region at E14.5.

differentiation marker *Dct* was clearly reduced in the region around the eyes in *Mef2c* NC KO embryos at E14.5 (Fig. 3, compare panels E and F). Quantification of *Dct*-labeled melanocytes in the interlimb region at E12.5 showed that *Mef2c* NC KO embryos had on average only 69% as many labeled cells as control embryos (356 ± 98 labeled cells/embryo, $n=4$ embryos for *Mef2c* NC KO versus 514 ± 133 labeled cells/embryo, $n=4$ embryos for control, $P=0.0027$ by Student's *t*-test).

A small, evolutionarily conserved region that is necessary and sufficient for *Mef2c*-F1 neural crest enhancer activity in vivo contains conserved MEF2- and SOX-binding sites

To place *Mef2c* into a pathway for neural crest and melanocyte development, we next examined the *Mef2c*-F1 neural crest enhancer for essential cis-regulatory elements by generating a series of deletions within the 7-kb conserved fragment (Fig. 4A). These analyses identified a 300-bp region (3-3.3 kb) that was sufficient for *Mef2c*-F1 enhancer function in vivo and directed β -gal expression in the same pattern as the full length *Mef2c*-F1 fragment at E11.5 (Fig. 4, compare panels B and C). This 300-bp region was also required for enhancer function in the context of the full-length 7-kb *Mef2c*-F1 fragment, as deletion of this region completely abolished enhancer activity in transgenic embryos at E11.5 (Fig. 4D).

We examined the highly conserved, crucial 300-bp region from *Mef2c*-F1 for candidate cis-regulatory sequences that might be involved in neural crest expression. These analyses revealed three putative SOX binding sites and a single candidate MEF2 binding site (Fig. 4E). The presence of the conserved SOX

elements in the *Mef2c*-F1 enhancer further suggested the possibility that a SOX transcription factor might be a direct upstream activator of *Mef2c*.

Previous studies have shown that *Sox10*-null embryos have a nearly complete lack of melanocyte marker expression as early as E11 (Kapur, 1999; Potterf et al., 2001; Southard-Smith et al., 1998). The observations that *Mef2c* and *Sox10* are each required for proper melanocyte differentiation and share common downstream targets in melanocytes, including *Dct*, *Mitf* and *Pmel17*, and that the two factors are co-expressed in melanocytes in the dermis (Fig. 1G) is consistent with the idea that the two transcription factors might function in a common pathway in the neural crest and its derivatives. Based on the observation that the *Mef2c* NC KO pigmentation phenotype was similar, but substantially less severe, than the pigmentation phenotype in *Sox10*-null embryos, we hypothesized that *Mef2c* might function downstream of *Sox10* during trunk neural crest development. Moreover, we found that the expression of *Sox10* was unaffected in the peripheral nervous system in *Mef2c* NC KO embryos when compared with control embryos (data not shown), which is consistent with the notion that *Sox10* might function upstream of *Mef2c* in the trunk neural crest and its derivatives.

Mef2c-F1 is bound and activated by SOX10 and MEF2C

To determine whether the putative SOX elements in the *Mef2c*-F1 enhancer might represent bona fide cis-acting elements, each was tested for SOX10 binding in vitro by EMSA (Fig. 5A). Indeed, SOX10 bound robustly and specifically to each of the putative SOX sites from the *Mef2c*-F1 neural crest enhancer (Fig. 5A, lanes

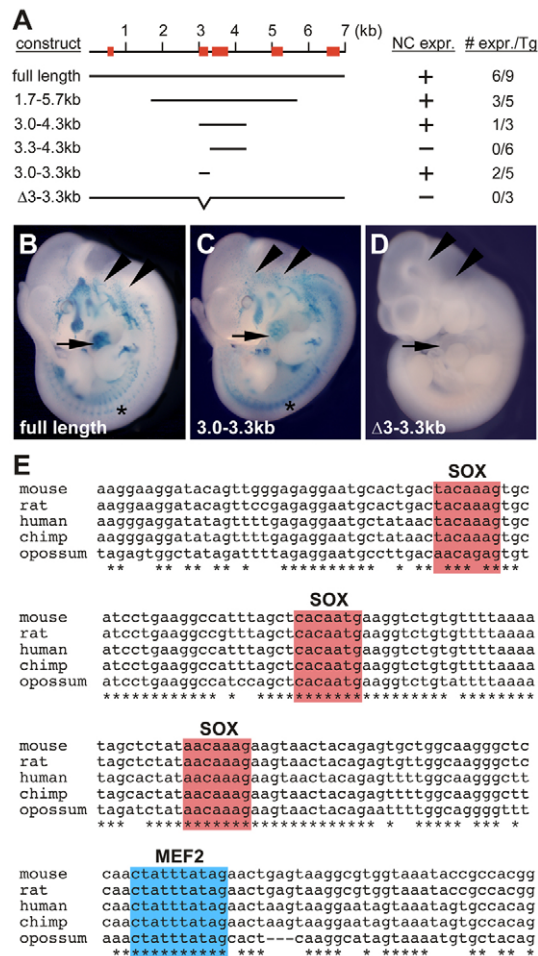


Fig. 4. A minimal fragment of the *Mef2c*-F1 neural crest enhancer is necessary and sufficient for enhancer activity and contains consensus binding sites for MEF2 and SOX10. (A) Schematic of the *Mef2c*-F1 enhancer region and the deletion constructs tested for enhancer activity in transgenic mice. Red rectangles depict regions of high conservation between the marsupial opossum and the mouse genomic sequences. The column on the left lists the regions of the *Mef2c*-F1 fragment tested in deletion analyses. The columns on the right denote the activity and the fraction of independently derived transgenic (Tg) embryos that displayed activity in neural crest (NC) derivatives. (B-D) Representative transgenic embryos for the indicated constructs. Asterisks denote transgene expression in neural crest-derivatives in the trunk. Arrows denote the location of the heart. Arrowheads indicate regions of melanocyte progenitors. (E) ClustalW analysis of *Mef2c* gene sequences in the core region of the 300-bp necessary and sufficient region of the *Mef2c*-F1 enhancer. Asterisks denote conserved nucleotides.

2, 8 and 14). We also examined the ability of SOX10 to bind to the *Mef2c*-F1 SOX binding sites in the endogenous *Mef2c* locus by ChIP *in vivo* in B16F10 mouse melanoma cells transfected with FLAG epitope-tagged SOX10 (Fig. 5B). Importantly, the DNA fragment encompassing the SOX sites in the endogenous *Mef2c*-F1 enhancer was precipitated and amplified by the anti-FLAG antibody (Fig. 5B, lane 3) but not by the isotype control IgG antibody (Fig. 5B, lane 2). Furthermore, precipitation with anti-FLAG antibody did not result in PCR amplification of a bona fide enhancer from the *Vegfr2* (*Kdr* – Mouse Genome Informatics) gene (De Val et al., 2008), which was used as a non-specific enhancer

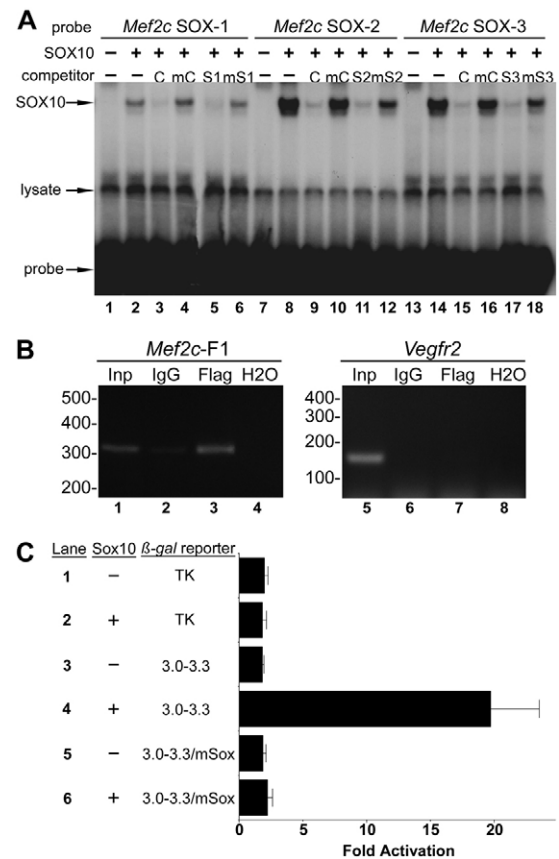


Fig. 5. SOX10 binds and activates the *Mef2c*-F1 enhancer in a SOX site-dependent manner. (A) *In vitro*-translated SOX10 was incubated with the following *Mef2c*-F1 radiolabeled probes: SOX-1 (lanes 1-6), SOX-2 (lanes 7-12) and SOX-3 (lanes 13-18). SOX10 efficiently retarded the mobility of each of the sites (lanes 2, 8, 14). Binding was competed by excess unlabeled control probe (C; lanes 3, 9, 15) but not by a mutant version (mC; lanes 4, 10, 16). Binding was also competed by excess unlabeled self probe (S1, lane 5; S2, lane 11; S3, lane 17) but not by excess mutant versions (mS1, lane 6; mS2, lane 12; mS3, lane 18). Unprogrammed reticulocyte lysates are shown in lanes 1, 7 and 13. Lysate-derived, non-specific bands and the free, unbound probe are indicated. (B) Sox10[Flag]-transfected B16F10 mouse melanoma cells were subjected to ChIP to detect SOX10 bound to the endogenous *Mef2c*-F1 enhancer using an anti-FLAG antibody. Following ChIP, the region of the *Mef2c* gene surrounding the SOX sites in the *Mef2c*-F1 enhancer (lanes 1-4), or a region of the *Vegfr2* enhancer (lanes 5-8) as a non-specific control, was amplified by PCR and analyzed by agarose gel electrophoresis. Lanes 1 and 5: amplification prior to immunoprecipitation; lanes 2 and 6: amplification following non-specific isotype-matched IgG ChIP; lanes 3 and 7: amplification following ChIP with anti-FLAG to detect SOX10-bound DNA; lanes 5 and 8: amplification without added template (H2O). Sizes in bp are shown. (C) Co-transfection of the *Mef2c*[3-3.3]-F1-TK-*lacZ* reporter plasmid with pRK5-Sox10 expression plasmid (+) or with pRK5 (-) resulted in strong activation in a SOX10-dependent manner (lanes 3, 4). Mutation of all three SOX sites abolished the ability of SOX10 to transactivate the *Mef2c*-F1 enhancer (lane 6). SOX10 did not transactivate the parental TK-*lacZ* reporter (lane 2). Data are expressed as the mean fold activation + s.e.m. from three independent transfections and analyses.

target (Fig. 5B, lane 7). These results indicate that SOX10 binds to the SOX sites in the *Mef2c*-F1 enhancer *in vitro* and *in vivo* in a relevant cell line.

To determine whether the binding of SOX10 to the *Mef2c*-F1 enhancer was functionally relevant, we tested the ability of SOX10 to trans-activate the *Mef2c*-F1 enhancer (Fig. 5C). Co-transfection of a SOX10 expression plasmid with the 300-bp *Mef2c* reporter plasmid *Mef2c*[3-3.3]-F1-TK-*lacZ* resulted in robust activation of the reporter gene (Fig. 5C, lane 4). This activation was dependent upon the presence of the SOX binding sites, as mutation of these elements in the *Mef2c* enhancer eliminated SOX10-dependent activation (Fig. 5C, lane 6). Taken together with the EMSA results shown in Fig. 5A and the ChIP results shown in Fig. 5B, these trans-activation studies strongly support the notion that the SOX binding sites in the *Mef2c*-F1 enhancer represent bona fide SOX response elements and that *Mef2c* is a direct transcriptional target of SOX10.

We also examined the MEF2 site in the *Mef2c*-F1 neural crest enhancer for binding by MEF2C in EMSA (Fig. 6A). MEF2C bound robustly to the bona fide MEF2 element from the myogenin promoter (Dodou et al., 2003) and to the MEF2 site from *Mef2c*-F1 (Fig. 6A, lanes 2 and 8). The MEF2 element in the *Mef2c*-F1 enhancer was also responsive to MEF2-dependent activation in C3H10T1/2 cells (Fig. 6B). Co-transfection of a MEF2C-VP16 expression plasmid with the 300-bp *Mef2c*-F1 enhancer plasmid *Mef2c*[3-3.3]-F1-TK-*lacZ* resulted in significant trans-activation (Fig. 6B, lane 4). The activation of the *Mef2c*-F1 enhancer by MEF2C was dependent on the MEF2 site in the enhancer as mutation of the site completely abolished activation (Fig. 6B, lane 6). Together, the results of Fig. 6A,B supports the notion that the MEF2 element in the *Mef2c*-F1 enhancer represents a bona fide binding site, and suggest that *Mef2c* is subject to auto-regulation by itself or cross-regulation by other members of the MEF2 family in developing neural crest lineages.

MEF2C and SOX10 physically interact and cooperatively activate transcription

Based on the observation that MEF2C and SOX10 each trans-activated the *Mef2c*-F1 enhancer (Fig. 5C and Fig. 6B), we tested whether the two could cooperatively activate the enhancer (Fig. 7A). Indeed, when SOX10 and MEF2C-VP16 expression plasmids were co-transfected with the 300-bp *Mef2c*-F1 reporter plasmid, the two transcription factors synergistically activated the enhancer (Fig. 7A, lane 7). The strong transcriptional synergy by MEF2C and SOX10 suggested that the two transcription factors might physically associate. We tested this possibility in GST pull-down assays in vitro and co-immunoprecipitation assays in vivo (Fig. 7B,C). In control reactions, neither SOX10 nor an N-terminal fragment of SOX10, which includes the HMG DNA-binding domain, interacted with GST alone (Fig. 7B, lanes 2 and 4). However, full length SOX10 and the HMG domain of SOX10 each specifically interacted with GST-MEF2C fusion protein (Fig. 7B, lanes 1 and 3). Interaction of GST-MEF2C with the N-terminal portion of SOX10 was more robust than the interaction with full length SOX10 protein (Fig. 7B, compare lanes 1 and 3), which is consistent with the interaction that has been observed previously between another SOX protein, SOX18, and MEF2C (Hosking et al., 2001). SOX10 and MEF2C also interacted in vivo (Fig. 7C). B16F10 mouse melanoma cells were transfected with MEF2C and HA-tagged SOX10 expression constructs and were harvested and subjected to immunoprecipitation with anti-MEF2C antibody. Following immunoprecipitation, cells were subjected to western blot analyses with anti-HA to detect co-precipitated SOX10. HA-SOX10 was specifically detected only in the presence of anti-MEF2C antibody (Fig. 7C, lane 4) and not by isotype-matched IgG

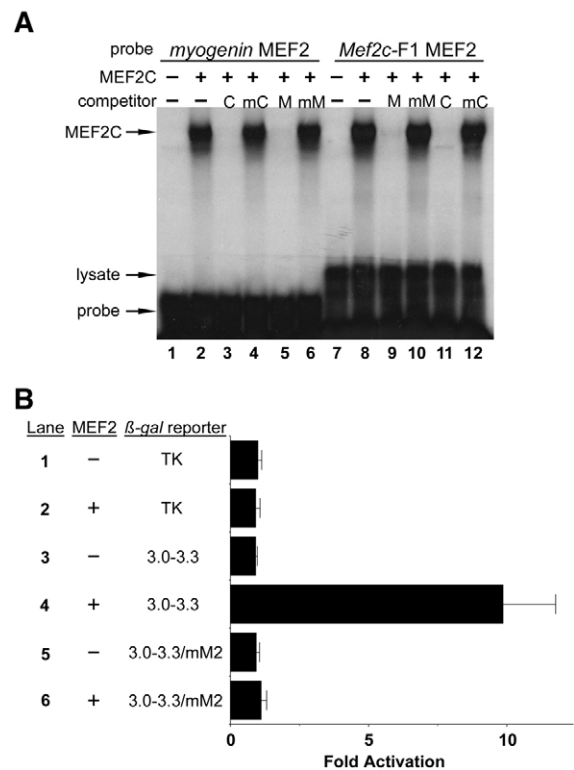


Fig. 6. MEF2C binds the MEF2 site in the *Mef2c*-F1 enhancer. (A) In vitro translated MEF2C was incubated with radiolabeled probes corresponding to a consensus bona fide MEF2 site from the myogenin promoter (lanes 1-6) or from the *Mef2c*-F1 enhancer (lanes 7-12). MEF2C bound the *Mef2c*-F1 MEF2 (lane 8) and myogenin control MEF2 (lane 2) sites with similar affinity. Excess unlabeled *Mef2c*-F1 MEF2 site (M; lanes 5, 9) and myogenin control (C; lanes 3, 11) probes each competed efficiently for binding. Mutant versions of the control probe (mC) or the *Mef2c*-F1 probe (mM) failed to compete for binding to either labeled probe (lanes 4, 6, 10, 12). Unprogrammed reticulocyte lysate was used in EMSA with each probe (lanes 1, 7) and no specific binding was observed. Lysate-derived, non-specific binding and the free, unbound probe are indicated. (B) Co-transfection of the *Mef2c*[3-3.3]-F1-TK-*lacZ* reporter plasmid with a MEF2C-VP16 activator plasmid (+) or with pRK5 (-) demonstrates that MEF2 can activate the *Mef2c*-F1 enhancer (lanes 3, 4). Mutation of the MEF2 site in *Mef2c*-F1 completely abolished activation by MEF2C-VP16 (lane 6). No activation of the parental minimal promoter-containing reporter plasmid TK-*lacZ* by MEF2C-VP16 was observed (lane 2). Data are expressed as the mean fold activation + s.e.m. from three independent transfections and analyses.

(Fig. 7C, lane 3) or by beads alone (Fig. 7C, lane 2), indicating that SOX10 was specifically precipitated by MEF2C co-expression in B16F10 cells. Taken together, these results show that MEF2C and SOX10 cooperatively activate the *Mef2c*-F1 neural crest enhancer, and suggest that this transcriptional synergy might be dependent on a physical association between the two transcription factors.

Mef2c is a direct transcriptional target of SOX10 in developing neural crest lineages

To test the requirement for the MEF2 and SOX cis-regulatory elements in the *Mef2c*-F1 enhancer in vivo, the MEF2 site or all three SOX sites were mutated in the context of the full-length 7-kb *Mef2c*-F1 enhancer, which was then tested for activity in transgenic embryos (Fig. 8). Mutation of the MEF2 site in the enhancer had

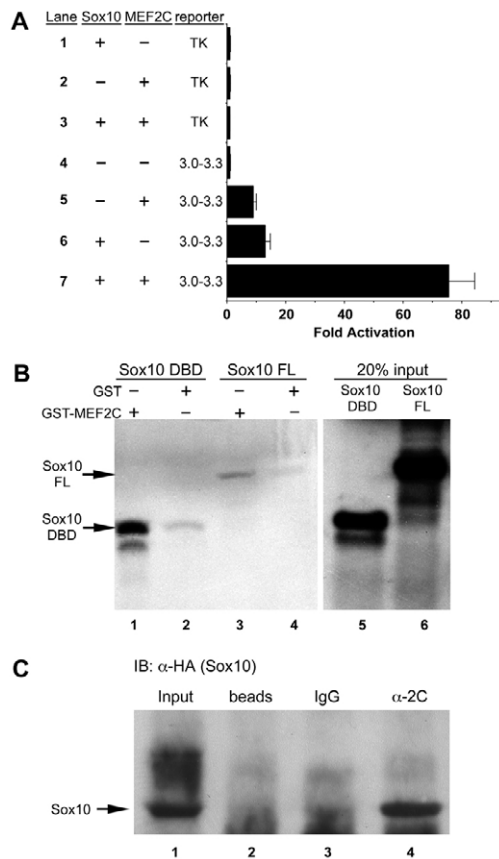


Fig. 7. MEF2C and SOX10 physically interact and synergistically activate the *Mef2c-F1* enhancer. (A) Co-transfection of expression plasmids for MEF2C-VP16 and SOX10 with the *Mef2c*[3-3.3]-F1-TK-*lacZ* reporter plasmid resulted in synergistic activation of the *Mef2c-F1* enhancer (lane 7) compared with transfection of the reporter with MEF2C-VP16 (lane 5) or SOX10 (lane 6) alone. No activation of the reporter with empty expression vectors or of the parental TK-*lacZ* reporter was observed (lanes 1-4). Data are expressed as the mean fold activation \pm s.e.m. from three independent transfections and analyses. (B) Agarose conjugated to either GST-MEF2C (lanes 1, 3) or GST alone (lanes 2, 4) were used as bait in pull-down assays with radiolabeled DNA binding domain (DBD) of SOX10 (lanes 1, 2) or with radiolabeled full length (FL) SOX10 (lanes 3, 4). Twenty percent of the input amount of radiolabeled SOX10 DBD and SOX10 FL are included in lanes 5 and 6, respectively. (C) MEF2C and SOX10 interact in vivo in B16F10 melanoma cells. MEF2C- and SOX10[HA]-transfected cells were subjected to immunoprecipitation with anti-MEF2C followed by western blot analysis with anti-HA (SOX10). SOX10 was only detected following precipitation with anti-MEF2C (α -2C, lane 4) but not with isotype-matched IgG (lane 3) or with beads alone (lane 2). Lane 1 contains input sample only for western blot (no prior immunoprecipitation) as a positive control for SOX10[HA] detection.

only a partial effect on the activity of the enhancer in the nascent neural crest at E8.5 (Fig. 8, compare panels A and B, arrowheads) but resulted in a nearly complete loss of transgene expression by E11.5 (Fig. 8, compare panels C and D). These observations suggest that the initial activation of *Mef2c-F1* does not require the MEF2 site but that maintenance of *Mef2c* expression in the neural crest via the *Mef2c-F1* enhancer requires auto- and cross-regulation by MEF2C and other MEF2 factors.

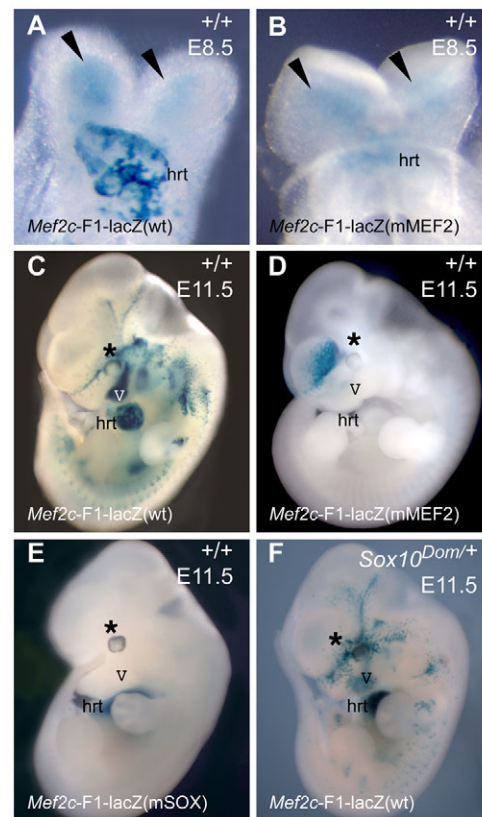


Fig. 8. The *Mef2c-F1* neural crest enhancer is regulated by MEF2 and SOX10 in vivo. (A,C) The wild-type *Mef2c-F1* transgene in an otherwise wild-type background ($+/+$) directs expression to the nascent neural crest adjacent to the neural folds at E8.5 (A) and in neural crest derivatives, including melanocytes, at E11.5 (C). The same pattern was observed in six out of nine independent transgenic lines examined at both E8.5 and E11.5. (B,D) Mutation of the MEF2 site in *Mef2c-F1* completely abolished transgene expression in the neural crest and its derivatives at E11.5 (D) but not at E8.5 (B). Note that the expression in the forebrain region in D was not reproducible and represents ectopic transgene expression. Similar activity of the MEF2 mutant transgene was observed in each of three independent transgenic lines examined at E8.5 and E11.5 and an additional three F0 transient transgenic embryos examined at E11.5 only. (E) Mutation of all three SOX sites in the *Mef2c-F1* enhancer abolished transgene expression in neural crest derivatives, but not in the heart (hrt), in each of five independent mSOX transgenic lines examined. (F) Crossing the wild-type *Mef2c-F1-lacZ* transgene into a *Sox10* heterozygous (*Sox10*^{Dom/+}) background resulted in a diminution of transgene expression in neural crest derivatives at E11.5 but did not affect transgene expression in the heart. Arrowheads indicate neural crest cells. Asterisks indicate melanocytes in the supraocular region at E11.5. hrt, heart; V, fifth cranial nerve.

Mutation of the three SOX sites in the *Mef2c-F1* enhancer also abolished expression in all neural crest-derived lineages at all stages of embryonic development, including at E11.5 (Fig. 8, compare panels C and E). Interestingly, the SOX sites were not required for *Mef2c-F1* enhancer activity in the heart at E11.5 (Fig. 8E), demonstrating the specificity of those sites for neural crest-specific activity of the enhancer in vivo. To test specifically the role of SOX10 as a possible upstream activator of *Mef2c* via the *Mef2c-*

F1 enhancer, we crossed mice harboring the wild-type *Mef2c*-F1-*lacZ* transgene into a heterozygous *Sox10*-mutant background (*Sox10^{Dom/+}*). *Mef2c*-F1 enhancer activity was significantly reduced in all neural crest lineages when one functional copy of *Sox10* was missing (Fig. 8, compare panels C and F), further supporting a possible role for SOX10 specifically as an upstream regulator of *Mef2c* in the neural crest.

DISCUSSION

A role for MEF2C in melanocyte development

MEF2C is a well-known regulator of gene expression in skeletal and cardiac muscle, the central nervous and immune systems, and the axial and craniofacial skeletons (Black and Cripps, 2010; Potthoff and Olson, 2007). Here, we show that loss of *Mef2c* in the neural crest results in a profound deficit in pigmentation due to a reduction in the number of melanocytes in the skin and impairment of melanocyte differentiation and function. Interestingly, in other lineages in which MEF2C function is required, such as in cardiomyocytes and endothelial cells, initial lineage specification occurs, but the differentiated phenotype fails to stabilize and eventually development of the heart and vasculature collapses in the absence of MEF2C (Bi et al., 1999; Lin et al., 1998; Lin et al., 1997). The phenotype that we observed here is similar in that markers of the melanocyte lineage, including *Mitf*, *Dct* and *Pmel17* are reduced but still clearly present in *Mef2c* NC KO fetuses (Fig. 3). However, by birth, pigmentation was much more severely impaired (Fig. 2). We considered the possibility that the observed dramatic loss of pigmentation might be caused by loss of melanocytes due to apoptosis or impaired proliferation between E13.5 and birth, but we observed no differences in TUNEL staining or in BrdU incorporation from E11.5 to E18.5 (data not shown). It is possible that proliferation or apoptosis were affected but below the level of detection of our assays. However, we favor the hypothesis that MEF2C function is required to maintain the differentiation phenotype in melanocytes. The observation that MEF2C function was required for appropriate melanosome number (Fig. 2F-H) supports this notion.

MEF2C and SOX10 function as a positively reinforcing transcriptional circuit

Feed-forward transcriptional regulation is a common theme in developmental gene regulation, in which this positive-acting mechanism helps to reinforce developmental decisions to differentiate (Amin et al., 2010; Penn et al., 2004; Sandmann et al., 2007; Tapscott, 2005; Verzi et al., 2007). MEF2 transcription factors are known to participate in this type of self-reinforcing transcriptional circuitry in other systems, in which they often function to potentiate the activity of other transcription factors in a feed-forward fashion (Black and Cripps, 2010). Here, we identified MEF2C as a direct transcriptional target of SOX proteins and demonstrate that it can function as a partner of SOX10. The function of SOX10 in different neural crest-derived lineages is spatially and temporally modulated by its interaction with other transcriptional factors, including PAX3, MITF, KROX-20 (EGR2 – Mouse Genome Informatics) and SP1, and the functional interaction of these transcriptional partners with SOX10 is often facilitated by direct physical association (Jiao et al., 2004; Kuhlbrodt et al., 1998; Lang and Epstein, 2003; Ludwig et al., 2004; Melnikova et al., 2000). Consistent with this cofactor-based role for SOX10 in neural crest development, we observed a direct physical association between SOX10 and

MEF2C (Fig. 7). We also observed co-expression of MEF2C and SOX10 in the same cells during development (Fig. 1G), further supporting the notion that SOX10 might function as a bona fide regulator and cofactor of MEF2C. However, we cannot rule out the possibility that other SOX factors, such as SOX9, might also be involved in *Mef2c* regulation during neural crest development. Based on the direct regulation of *Mef2c* by the novel *Mef2c*-F1 enhancer identified here, the synergistic activation of transcription by MEF2C and SOX10, and the direct physical interaction and co-expression of the two factors, we propose a model in which SOX10 cooperates with MEF2 to activate *Mef2c* expression in melanocytes in a feed-forward transcriptional pathway.

MEF2C as a candidate gene involved in neural crest disorders

Mutations in human *MEF2C* are strongly associated with neuronal disorders, including seizures, mental retardation and epilepsy (Le Meur et al., 2010; Novara et al., 2010; Nowakowska et al., 2010), demonstrating the important role for MEF2C in the development of neuronal lineages in humans. Mutations in human *SOX10* often cause a severe form of Waardenburg syndrome (Type IV, also known as Waardenburg-Hirschsprung disease), which involves aganglionic colon and severe pigmentation defects (Parisi and Kapur, 2000; Pingault et al., 1998; Pingault et al., 2010). In addition to *SOX10*, mutations in genes encoding other transcription factors known to function as SOX10 cofactors or to function in a common pathway with SOX10, also cause various forms of Waardenburg syndromes, including *PAX3*, *MITF* and *SNAI2* (Pingault et al., 2010). Interestingly, only 50% of human patients with classic symptoms of Waardenburg-Hirschsprung disease have mutations in known disease loci, and even in cases with known genetic causes, the severity or the symptoms are often highly variable (Parisi and Kapur, 2000; Pingault et al., 1998; Pingault et al., 2010; Southard-Smith et al., 1999). Distinct mutations in human *SOX10* are associated with different severity of disease, depending on the effect of the various mutations on SOX10 protein function (Inoue et al., 2004). It has also been postulated that some of the variability observed in Waardenburg-Hirschsprung patients with *SOX10* mutations is due to modifier genes, which are largely hypothetical, because few modifiers of *SOX10* have been defined in humans (Parisi and Kapur, 2000; Pingault et al., 2010). In mice, however, several modifiers of *Sox10* function have been identified, including genes encoding endothelin signaling components and the related SOX protein SOX8 (Maka et al., 2005; Stanchina et al., 2006). It is important to note that neither heterozygous *Mef2c* mice nor *Mef2c* neural crest conditional knockout mice exhibit aganglionosis of the bowel (Lin et al., 1997; Verzi et al., 2007), a hallmark of Waardenburg-Hirschsprung disease, and we did not observe any exaggeration of the *Sox10^{Dom/+}* phenotype when crossed into a *Mef2c* heterozygous background (data not shown). However, we do show here that loss of *Mef2c* function in the neural crest results in hypopigmentation at birth, which is characteristic of many forms of Waardenburg syndromes; therefore, the *MEF2C* gene should be considered for a direct or modifier role in congenital neural crest disorders in humans.

Acknowledgements

The authors thank Ivy Hsieh and Jillian Jarrett for excellent technical assistance. This work was supported by Grant #6-FY08-311 from the March of Dimes and R01 DE019118 from the NIDCR to B.L.B. and by grants from the ACS and the Melanoma Research Alliance to M.L.W. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

References

- Amin, N. M., Shi, H. and Liu, J.** (2010). The FoxF/FoxC factor LET-381 directly regulates both cell fate specification and cell differentiation in *C. elegans* mesoderm development. *Development* **137**, 1451-1460.
- Baker, C. V. and Bronner-Fraser, M.** (1997). The origins of the neural crest. Part I: embryonic induction. *Mech. Dev.* **69**, 3-11.
- Baxter, L. L., Hou, L., Loftus, S. K. and Pavan, W. J.** (2004). Spotlight on spotted mice: a review of white spotting mouse mutants and associated human pigmentation disorders. *Pigment Cell Res.* **17**, 215-224.
- Bi, W., Drake, C. J. and Schwarz, J. J.** (1999). The transcription factor MEF2C-null mouse exhibits complex vascular malformations and reduced cardiac expression of angiopoietin 1 and VEGF. *Dev. Biol.* **211**, 255-267.
- Black, B. L. and Cripps, R. M.** (2010). Myocyte enhancer factor 2 transcription factors in heart development and disease. In *Heart Development and Regeneration*, Vol. 2 (ed. N. Rosenthal and R. P. Harvey), pp. 673-699. Oxford: Academic Press.
- Britsch, S., Goerich, D. E., Riethmacher, D., Peirano, R. I., Rossner, M., Nave, K. A., Birchmeier, C. and Wegner, M.** (2001). The transcription factor Sox10 is a key regulator of peripheral glial development. *Genes Dev.* **15**, 66-78.
- Chen, N., Baudino, T., MacDonald, P. N., Green, M., Kelley, W. L., Burnett, J. W. and Buller, R. M.** (2000). Selective inhibition of nuclear steroid receptor function by a protein from a human tumorigenic poxvirus. *Virology* **274**, 17-25.
- Crane, J. F. and Trainor, P. A.** (2006). Neural crest stem and progenitor cells. *Annu. Rev. Cell Dev. Biol.* **22**, 267-286.
- Danielian, P. S., Muccino, D., Rowitch, D. H., Michael, S. K. and McMahon, A. P.** (1998). Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr. Biol.* **8**, 1323-1326.
- De Val, S., Chi, N. C., Meadows, S. M., Minovitsky, S., Anderson, J. P., Harris, I. S., Ehlers, M. L., Agarwal, P., Visel, A., Xu, S. M. et al.** (2008). Combinatorial regulation of endothelial transcription by Ets and Forkhead transcription factors. *Cell* **135**, 1053-1064.
- Dodou, E., Xu, S. M. and Black, B. L.** (2003). *mef2c* is activated directly by myogenic basic helix-loop-helix proteins during skeletal muscle development in vivo. *Mech. Dev.* **120**, 1021-1032.
- Dodou, E., Verzi, M. P., Anderson, J. P., Xu, S. M. and Black, B. L.** (2004). *Mef2c* is a direct transcriptional target of ISL1 and GATA factors in the anterior heart field during mouse embryonic development. *Development* **131**, 3931-3942.
- Dupin, E. and Le Douarin, N. M.** (2003). Development of melanocyte precursors from the vertebrate neural crest. *Oncogene* **22**, 3016-3023.
- Edmondson, D. G., Lyons, G. E., Martin, J. F. and Olson, E. N.** (1994). *Mef2* gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. *Development* **120**, 1251-1263.
- Herbarth, B., Pingault, V., Bondurand, N., Kuhlbrodt, K., Hermans-Borgmeyer, I., Puliti, A., Lemort, N., Goossens, M. and Wegner, M.** (1998). Mutation of the Sry-related Sox10 gene in Dominant megacolon, a mouse model for human Hirschsprung disease. *Proc. Natl. Acad. Sci. USA* **95**, 5161-5165.
- Hogan, B., Beddington, R., Costantini, F. and Lacy, E.** (1994). *Manipulating the Mouse Embryo*. Plainview, NY: Cold Spring Harbor Laboratory Press.
- Hong, C. S. and Saint-Jeannet, J. P.** (2005). Sox proteins and neural crest development. *Semin. Cell Dev. Biol.* **16**, 694-703.
- Horton, R. M.** (1997). In vitro recombination and mutagenesis of DNA: SOEing together tailor-made genes. In *PCR Cloning Protocols*, Vol. 67 (ed. B. A. White), pp. 141-149. Totowa, NJ: Humana Press.
- Hosking, B. M., Wang, S. C., Chen, S. L., Penning, S., Koopman, P. and Muscat, G. E.** (2001). SOX18 directly interacts with MEF2C in endothelial cells. *Biochem. Biophys. Res. Commun.* **287**, 493-500.
- Inoue, K., Khajavi, M., Ohyama, T., Hirabayashi, S., Wilson, J., Reggin, J. D., Mancias, P., Butler, I. J., Wilkinson, M. F., Wegner, M. et al.** (2004). Molecular mechanism for distinct neurological phenotypes conveyed by allelic truncating mutations. *Nat. Genet.* **36**, 361-369.
- Jiao, Z., Mollaaghababa, R., Pavan, W. J., Antonellis, A., Green, E. D. and Hornyak, T. J.** (2004). Direct interaction of Sox10 with the promoter of murine Dopachrome Tautomerase (Dct) and synergistic activation of Dct expression with Mitf. *Pigment Cell Res.* **17**, 352-362.
- Kapur, R. P.** (1999). Early death of neural crest cells is responsible for total enteric aganglionosis in Sox10(Dom)/Sox10(Dom) mouse embryos. *Pediatr. Dev. Pathol.* **2**, 559-569.
- Khiem, D., Cyster, J. G., Schwarz, J. J. and Black, B. L.** (2008). A p38 MAPK-MEF2C pathway regulates B-cell proliferation. *Proc. Natl. Acad. Sci. USA* **105**, 17067-17072.
- Knecht, A. K. and Bronner-Fraser, M.** (2002). Induction of the neural crest: a multigene process. *Nat. Rev. Genet.* **3**, 453-461.
- Kothary, R., Clapoff, S., Darling, S., Perry, M. D., Moran, L. A. and Rossant, J.** (1989). Inducible expression of an hsp68-lacZ hybrid gene in transgenic mice. *Development* **105**, 707-714.
- Kuhlbrodt, K., Herbarth, B., Sock, E., Hermans-Borgmeyer, I. and Wegner, M.** (1998). Sox10, a novel transcriptional modulator in glial cells. *J. Neurosci.* **18**, 237-250.
- Lane, P. W. and Liu, H. M.** (1984). Association of megacolon with a new dominant spotting gene (Dom) in the mouse. *J. Hered.* **75**, 435-439.
- Lang, D. and Epstein, J. A.** (2003). Sox10 and Pax3 physically interact to mediate activation of a conserved c-RET enhancer. *Hum. Mol. Genet.* **12**, 937-945.
- Le Douarin, N. M., Creuzet, S., Couly, G. and Dupin, E.** (2004). Neural crest cell plasticity and its limits. *Development* **131**, 4637-4650.
- Le Meur, N., Holder-Espinasse, M., Jaillard, S., Goldenberg, A., Joriot, S., Amati-Bonneau, P., Guichet, A., Barth, M., Charollais, A., Journel, H. et al.** (2010). MEF2C haploinsufficiency caused by either microdeletion of the 5q14.3 region or mutation is responsible for severe mental retardation with stereotypic movements, epilepsy and/or cerebral malformations. *J. Med. Genet.* **47**, 22-29.
- Lin, Q., Schwarz, J., Bucana, C. and Olson, E. N.** (1997). Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. *Science* **276**, 1404-1407.
- Lin, Q., Lu, J., Yanagisawa, H., Webb, R., Lyons, G. E., Richardson, J. A. and Olson, E. N.** (1998). Requirement of the MADS-box transcription factor MEF2C for vascular development. *Development* **125**, 4565-4574.
- Ludwig, A., Rehberg, S. and Wegner, M.** (2004). Melanocyte-specific expression of dopachrome tautomerase is dependent on synergistic gene activation by the Sox10 and Mitf transcription factors. *FEBS Lett.* **556**, 236-244.
- Maka, M., Stolt, C. C. and Wegner, M.** (2005). Identification of Sox8 as a modifier gene in a mouse model of Hirschsprung disease reveals underlying molecular defect. *Dev. Biol.* **277**, 155-169.
- Melnikova, I. N., Lin, H. R., Blanchette, A. R. and Gardner, P. D.** (2000). Synergistic transcriptional activation by Sox10 and Sp1 family members. *Neuropharmacology* **39**, 2615-2623.
- Mollaaghababa, R. and Pavan, W. J.** (2003). The importance of having your SOX on: role of SOX10 in the development of neural crest-derived melanocytes and glia. *Oncogene* **22**, 3024-3034.
- Nguyen, T. and Wei, M. L.** (2007). Hermansky-Pudlak HPS1/pale ear gene regulates epidermal and dermal melanocyte development. *J. Invest. Dermatol.* **127**, 421-428.
- Novara, F., Beri, S., Giorda, R., Ortibus, E., Nageshappa, S., Darra, F., Bernardina, B. D., Zuffardi, O. and Van Esch, H.** (2010). Refining the phenotype associated with MEF2C haploinsufficiency. *Clin. Genet.* **78**, 471-477.
- Nowakowska, B. A., Obersztytn, E., Szymanska, K., Bekiesinska-Figatowska, M., Xia, Z., Ricks, C. B., Bocian, E., Stockton, D. W., Szczaluba, K., Nawara, M. et al.** (2010). Severe mental retardation, seizures, and hypotonia due to deletions of MEF2C. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* **153B**, 1042-1051.
- Parisi, M. A. and Kapur, R. P.** (2000). Genetics of Hirschsprung disease. *Curr. Opin. Pediatr.* **12**, 610-617.
- Peirano, R. I. and Wegner, M.** (2000). The glial transcription factor Sox10 binds to DNA both as monomer and dimer with different functional consequences. *Nucleic Acids Res.* **28**, 3047-3055.
- Penn, B. H., Bergstrom, D. A., Dilworth, F. J., Bengal, E. and Tapscott, S. J.** (2004). A MyoD-generated feed-forward circuit temporally patterns gene expression during skeletal muscle differentiation. *Genes Dev.* **18**, 2348-2353.
- Pingault, V., Bondurand, N., Kuhlbrodt, K., Goerich, D. E., Prehu, M. O., Puliti, A., Herbarth, B., Hermans-Borgmeyer, I., Legius, E., Matthijs, G. et al.** (1998). SOX10 mutations in patients with Waardenburg-Hirschsprung disease. *Nat. Genet.* **18**, 171-173.
- Pingault, V., Ente, D., Dastot-Le Moal, F., Goossens, M., Marlin, S. and Bondurand, N.** (2010). Review and update of mutations causing Waardenburg syndrome. *Hum. Mutat.* **31**, 391-406.
- Potter, S. B., Mollaaghababa, R., Hou, L., Southard-Smith, E. M., Hornyak, T. J., Arnheiter, H. and Pavan, W. J.** (2001). Analysis of SOX10 function in neural crest-derived melanocyte development: SOX10-dependent transcriptional control of dopachrome tautomerase. *Dev. Biol.* **237**, 245-257.
- Pothoff, M. J. and Olson, E. N.** (2007). MEF2: a central regulator of diverse developmental programs. *Development* **134**, 4131-4140.
- Rojas, A., De Val, S., Heidt, A. B., Xu, S. M., Bristow, J. and Black, B. L.** (2005). Gata4 expression in lateral mesoderm is downstream of BMP4 and is activated directly by Forkhead and GATA transcription factors through a distal enhancer element. *Development* **132**, 3405-3417.
- Rojas, A., Schachterle, W., Xu, S. M. and Black, B. L.** (2009). An endoderm-specific transcriptional enhancer from the mouse Gata4 gene requires GATA and homeodomain protein-binding sites for function in vivo. *Dev. Dyn.* **238**, 2588-2598.

- Sandmann, T., Girardot, C., Brehme, M., Tongprasit, W., Stolc, V. and Furlong, E. E.** (2007). A core transcriptional network for early mesoderm development in *Drosophila melanogaster*. *Genes Dev.* **21**, 436-449.
- Sauka-Spengler, T. and Bronner-Fraser, M.** (2006). Development and evolution of the migratory neural crest: a gene regulatory perspective. *Curr. Opin. Genet. Dev.* **16**, 360-366.
- Selleck, M. A., Scherson, T. Y. and Bronner-Fraser, M.** (1993). Origins of neural crest cell diversity. *Dev. Biol.* **159**, 1-11.
- Southard-Smith, E. M., Kos, L. and Pavan, W. J.** (1998). Sox10 mutation disrupts neural crest development in Dom Hirschsprung mouse model. *Nat. Genet.* **18**, 60-64.
- Southard-Smith, E. M., Angrist, M., Ellison, J. S., Agarwala, R., Baxevanis, A. D., Chakravarti, A. and Pavan, W. J.** (1999). The Sox10(Dom) mouse: modeling the genetic variation of Waardenburg-Shah (WS4) syndrome. *Genome Res.* **9**, 215-225.
- Spritz, R. A., Chiang, P. W., Oiso, N. and Alkhateeb, A.** (2003). Human and mouse disorders of pigmentation. *Curr. Opin. Genet. Dev.* **13**, 284-289.
- Stanchina, L., Baral, V., Robert, F., Pingault, V., Lemort, N., Pachnis, V., Goossens, M. and Bondurand, N.** (2006). Interactions between Sox10, Edn3 and Ednrb during enteric nervous system and melanocyte development. *Dev. Biol.* **295**, 232-249.
- Steel, K. P. and Barkway, C.** (1989). Another role for melanocytes: their importance for normal stria vascularis development in the mammalian inner ear. *Development* **107**, 453-463.
- Tapscott, S. J.** (2005). The circuitry of a master switch: MyoD and the regulation of skeletal muscle gene transcription. *Development* **132**, 2685-2695.
- Trainor, P. A.** (2005). Specification of neural crest cell formation and migration in mouse embryos. *Semin. Cell Dev. Biol.* **16**, 683-693.
- Verzi, M. P., Agarwal, P., Brown, C., McCulley, D. J., Schwarz, J. J. and Black, B. L.** (2007). The transcription factor MEF2C is required for craniofacial development. *Dev. Cell* **12**, 645-652.
- Vong, L. H., Ragusa, M. J. and Schwarz, J. J.** (2005). Generation of conditional Mef2*loxP/loxP* mice for temporal- and tissue-specific analyses. *Genesis* **43**, 43-48.