Loss-of-function mutations in SOX10 cause Kallmann syndrome with deafness.

Veronique Pingault, Virginie Bodereau, Viviane Baral, Severine Marcos, Yuli Watanabe, Asma Chaoui, Corinne Fouveaut, Chrystel Leroy, Odile Vérier-Mine, Christine Francannet, et al.

Loss-of-function mutations in *SOX10* cause Kallmann syndrome with deafness

Veronique Pingault,[1,2,3*] Virginie Bodereau,[3] Viviane Baral,[1,2] Severine Marcos,[4] Yuli Watanabe,[1,2]AsmaChaoui,[1,2]Corinne Fouveaut,[5] Chrystel Leroy,[5] Odile Vérier-Mine,[6] Christine Francannet,[7] Delphine Dupin-Deguine,[8] Françoise Archambeaud,[9] François-Joseph Kurtz,[10] Jacques Young,[11] Jérôme Bertherat,[12]Sandrine Marlin,[13]Michel Goossens,[1,2,3]Jean-Pierre Hardelin,[14]Catherine Dodé,[4,5]Nadege Bondurand[1,2]

[1] INSERM, U955,Equipe 11, F-94000,Créteil, France ;

[2] Université Paris-Est, UMR_S955, UPEC, F-94000,Créteil, France ;

[3] Hôpital Henri Mondor, Laboratoire de Biochimie et Génétique, F-94000,Créteil, France ;

[4] INSERM, U1016, Institut Cochin, Département de génétique et développement, Université Paris-Descartes, F-75014,Paris,France ;

[5] Laboratoire de biochimie et génétique moléculaire, APHP, Hôpital Cochin, F-75014, Paris, France ;

[6] Service d'endocrinologie, Centre hospitalier Valenciennes, F-59322, France ;

[7] Service de génétique médicale, Hôtel Dieu, F-63058,Clermont Ferrand,France ;

[8] Service de génétique médicale, Hôpital Purpan, F-31059,Toulouse, France ;

[9] Service de médecine et d'endocrinologie, Hôpital du Cluzeau, F-87042,Limoges,France ;

[10] Service de pédiatrie, Hôpital Bel Air, F-57126,Thionville, France ;

[11]Service d'endocrinologie, Hôpital Bicêtre, F-94275,Le Kremlin-Bicêtre, France ;

[12]Service d'endocrinologie, Hôpital Cochin, F-75014, Paris, France ;

[13]Service de Génétique, Centre de référence «Surdités génétiques», INSERM, U587, Hôpital Armand Trousseau, APHP, F-75012, Paris, France ;

[14] INSERM, U587, Département de neuroscience, Institut Pasteur, Université Pierre et Marie Curie, F-75015, Paris, France

*Correspondence: veronique.pingault@inserm.fr

Abstract

The SOX10 transcription factor plays a role in the maintenance of progenitor cellmultipotency, lineage specification, cell differentiation, and is a major actor in the development of the neural crest. Ithas been implicated in Waardenburg syndrome (WS), a rare disorder characterized by the association of pigmentation abnormalities and deafness, but *SOX10*mutations cause a variable phenotype that spreads over the initial limits of the syndrome definition. Based on recent findings of olfactory bulb agenesis in WS individuals, we suspected*SOX10*was also involved in Kallmann syndrome (KS).KS is defined by the association of anosmia and hypogonadotropic hypogonadismdue to incomplete migration of neuroendocrine GnRH (gonadotropin-releasing hormone)-cells along the olfactory, vomeronasal, and terminal nerves. Mutations in any of thenine genes identified to date account for only 30% of the KS cases. KS can be either isolated or associated with a variety of other symptoms includingdeafness.This studyreports*SOX10* loss-of-function mutations in approximately one-third of KS individualswith deafness, indicating a substantial involvement in this clinical condition.Study of SOX10-null mutant mice revealeda developmental role of SOX10 in a subpopulation of glial cells called olfactory ensheathing cells. Thesemice indeed showed an almost complete absence of these cells along the olfactorynerve pathway, as well as defasciculation and misrouting of the nerve fibers, impaired migration of GnRH-cells,and disorganization of the olfactory nerve layer of the olfactory bulbs.

Introduction

SOX10 (MIM 602229) belongs to the SOX family of transcription factors, whose members are involved ina multitude of developmental and cellular processes.[1] First identified as a glial cell transcription factor, it wassoon revealed as a major playerin the development of neural crest (NC) cells. NC cells are a population of multipotent precursor cells that emerge at the borders of the neural tube,migrate extensively throughout the embryo, and differentiate into a variety of cell types includingskin pigment cells, and neurons and glia of the peripheral and enteric nervous systems.[2]SOX10 plays a role in the maintenance of progenitor multipotency, specification, and differentiation of numerous cell types through the regulation of several transcriptional targets.[1,3-5]Its involvement in Waardenburg syndrome (WS) contributed significantly to the understanding of its function in NC in general and in the melanocytic and enteric lineages in particular.[6]

WS is a clinically and genetically heterogeneous condition that manifests with sensorineural congenital deafness and abnormal pigmentation of the hair, skin, and iris. Four subtypes (WS1 to WS4 [MIM 193500, 193510, 148820, 277580]) as well as neurological variant (PCWH, for Peripheral demyelinating neuropathy-Central dysmyelinating leukodystrophy-Waardenburg syndrome-Hirschsprung disease [MIM 609136]) have beendescribed.[6] Since 1998, approximately 100 heterozygous point mutations or deletions of *SOX10* have been reported, first in WS4 (WS with Hirschsprung disease/megacolon),[7] then in its neurological variant,[8]and finally in WS2 (WS without Hirschsprung disease)[9] (see also the WS gene mutation database at LOVD[Leiden Open Variation Database]).[6]

To delineate the range of temporal bone abnormalities associated with impaired SOX10 function, we recently performed a study of MRI and/or CTscans of inner ears from 15 WS individuals. Incidentally, this study also revealed an unexpectedly high frequency of olfactory

bulb agenesis (88% of the caseswho could be analyzed),[10] a finding that hadbeen described only once before in association with a *SOX10* mutation.[11]Notably, one of the maleincluded in our radiological study hadpreviouslybeen reported as having anosmia and hypogonadism.[9] The association of anosmia and hypogonadotropic hypogonadism is known as Kallmann syndrome (KS[MIM 147950, 244200, 308700, 610628, 612370, 612702]) and explained by a pathological sequence in embryonic development. Premature interruption of the vomeronasaland terminal nerve fibers in the peripheral olfactory system have been shown to result in incomplete migration of the neuroendocrine GnRH (gonadotropin-releasing hormone)-cells along these nerves, precluding them from penetrating into the forebrain and reaching their final destination in the preoptic and hypothalamic region.[12]The prevalence of KShas been estimated at 1/8,000 in males and 1/40,000 in females. Nine genes have been implicatedto date, namely *KAL1*,*FGFR1*, *FGF8*, *PROKR2*, *PROK2*, *WDR11*, *HS6ST1*, *CHD7*, and*SEMA3A*(MIM 300836, 136350, 600483, 607123, 607002, 606417, 604846, 608892, and 603961, respectively), but mutations in any of these genes have been identified inonly approximately 30% of KS individuals.[13] KS can beassociated witha variety of non-olfactory, non-reproductivesymptoms including deafness, but the association of KS withdeafness has so far received littlegeneticexplanation.

Here, we asked whether personsaffected byKScarrymutations in *SOX10*. We found a notable prevalence(about 38%) of *SOX10* mutations in a groupof individualspresenting with the clinical association of KS and hearing impairment, thereby shedding new light on this clinical association. We showed that these mutations affect SOX10 function in vitro. Homozygous *Sox10* mutant mice show an almost complete absence of olfactory ensheathing cells (OECs) along the olfactory, vomeronasal,and terminal nerves, abnormal fasciculation and axonal misrouting of the olfactory neurons, impaired GnRH-cell migration, and disorganization of the olfactory nerve layer of the olfactory bulbs.

Subjects and Methods

Subjects

This study was approved by the national research ethics committee (Agence de Biomedicine, Paris, France).Seventeen cases(9 males and 8 females) presenting with KS plus at least one WS-like featurewere investigated, as well as 86"random" cases (67 males and 19 females)addressed for genetic exploration of KS, including 20 withvarious non-olfactory, non-reproductive associated anomalies (including eight with cleft lip or palate).Hypogonadism was diagnosed based on clinical and hormonal evaluation, while diagnosis of anosmia/hyposmia was based on the individual's interview and confirmed by olfactory tests with increasing concentrations of odorant molecules (olfactometry) and/or MRI showing agenesis of the olfactory bulbs. Informed consent for genetic testing was obtained.Genomic DNA was extracted from peripheral blood leukocytes using standard protocols.These individuals did not carry mutations in the coding sequences of previously analyzed genes, specifically,*KAL1*, *FGFR1*, *FGF8*, *PROKR2*, and*PROK2*, all involved in KS.

Mutation screening

The coding exons of *SOX10*were analyzed by Sanger sequencing of the PCR products as previously described.[9] Mutations were named according to the international nomenclature based on RefSeqaccession number NM_006941.3 for the *SOX10* cDNA. *SOX10* deletions or rearrangements were sought using QMF-PCR with a method slightly modified from Bondurand et al., 2007.[9]The three coding exons were amplified in fiveamplicons sorted in two different reaction mixes. The sequences of the primers used are available upon

request.Individuals carrying a *SOX10* mutation were further tested for the presence of mutations in the other genes involved in KS orin non-syndromic congenital hypogonadotropic hypogonadism, specifically, *CHD7*, *WDR11*, *HS6ST1*, *SEMA3A*, *GNRHR*, *GNRH1*, *TACR3*, *TAC3*, *KISS1R*, and *KISS1*. To confirm the *de novo* occurrence of the mutation, comparison with the parental DNA (when available) was conducted through analysis of six microsatellites located on different chromosomes, using the linkage mapping set (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's instructions.

Animals and genotyping

The generation and genotyping of *Sox10*^{lacZ}mice (Sox10^{tm1Weg}) have been described previously.[14] Experiments were performed in accordance with the ethical guidelines of the Institut National de la Santéet de la Recherche Médicale. Embryos at E12.5 and E14.5 were obtained from staged pregnancies, fixed in 4% paraformaldehyde (PFA) at 4°C, and frozen in OCT (Optimal Cutting Temperature)compound.Head sections (16 µm thick) were cutusinga Leica CM3050S cryostat. Alternatively, embryo heads were fixed in 1% PFA and 0.2% glutaraldehyde and used for X-Gal staining.

Immunostaining and X-Gal staining

X-Gal staining followed standard procedures. Immunostaining ofmouse embryo sections wasperformed as described.[15]The primary antibodies used wereanti-SOX10 (N-20 or D-20) (goat, 1/50 Santa Cruz), anti-TUJ1 (mouse, 1/1,000, Eurogentec), anti-BLBP/BFABP (rabbit, 1/5,000, kindly provided by T. Müller),[16]anti-P75 (rabbit, 1/500, Promega), anti-S100 (rabbit, 1/400, Dako Cytomation), anti-GnRH (rabbit, 1/500, Abcam), and anti-β-galactosidase(chicken, 1/500, Abcam).Secondary antibodies wereanti-goat-FITC, anti-rabbit-Cy3, anti-mouse-Cy3,anti-goat-AlexaFluor555, anti-rabbit- AlexaFluor488, anti-mouse-

AlexaFluor647,anti-guinea pig-Cy3, and anti-chicken-AlexaFluor488 (1/200, Invitrogen or Jackson ImmunoResearch). TUNEL staining was performed using the In Situ Cell Death Detection kit, fluorescein (Roche) according to the manufacturer's instructions. The total number of GnRH-cells was estimated by counting the GnRH-positive cell bodies in more than two-thirdsof the sections, from the vomeronasal organ to the end of their migration pathway. The number of GnRH-cells on the missing slides was scored as the mean of the previous and following ones. Preparations were mounted in Vectashield and examined using an Olympus SZH10 stereo-microscope coupled to the Visilog capture program or a Zeiss Axioplan 2 confocal microscope coupled to the Metamorph software package.

Human tissues

A human fetus was obtained from medically terminated pregnancyat 8 weeks of embryonic development, with parents' written informed consent. After fixation in a 4% formaldehyde solution,the head was embedded in paraffin.Serial sagittal sections (4 μm thick) were cut using a Microm HM340E microtomeand collected on Superfrost Plus slides (Thermo Scientific). Immunochemistry analysis was conducted as described[17] using the antibodies against SOX10, TUJ1, and S100 cited above. Due to the limited number of slides, double SOX10/S100 staining was performed on the nasal mesenchyme only.

Plasmids

The pECE-SOX10, pCMV-SOX10Myc, pECE-PAX3, pECE-EGR2, pGL3-MITFdel1718, and pGL3-Cx32 vectors have been describedpreviously.[18-20] The mutations identified were introduced within the pECE-SOX10 (in the case of c.2T>G mutation) or pCMV-SOX10Myc constructs by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene, Netherlands). The pGL3-MPZ construct was kindly provided by J. Svaren.[21]

Cell culture, transfection, and reporter assays

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and transfected using Lipofectamine Plusreagent (Invitrogen, Carlsbad, CA, USA). Cells were plated on 12-well plates and transfected 1 day later with 0.175 μg of each effector and reporter plasmid for the *MITF* and *Cx32/GJB1* promoter study. In the*MPZ*enhancerstudy, 0.250 μg of reporter plasmid was used. As far as the competition assays are concerned, increasing amounts of mutant SOX10 plasmids (0.175, 0.350, or 0.525μg) were mixed with a fixed amount of wild-type SOX10 plasmid (0.175 μg) and reporter constructs.In each case, the total amount of plasmid was kept constant by the addition of empty pECE or pCMV-Myc vectors.Twenty-four or 48 hours post-transfection, cells were washed twice with PBS, lysed, and the extracts were assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI, USA) as described previously.[9,19,22] Production of SOX10 was assessed by immunoblot analysis using standard protocols.[23] Quantification was performed using GeneTools v3.05 software. Alternatively, cultures were fixed in 4% PFAfor 10 min at room temperatureand immunocytochemistry was performed as described above usingSOX10 N-20 andD-20 antibodies.

In silico analysis of the mutations

Mutationswere analyzed using software packages including Polyphen-2, SIFT, Alamut2.0.2 software for splicing, dbSNP, 1000 Genome Project, and Exome Variant Server. The conformation files for SOX17/DNA wereimported from the Protein Data Bank (accession code 3F27) and visualized using the Swiss-Pdb Viewer software.[24,25]

Results

SOX10 mutations are frequentin individuals with KS and deafness

We first searched for *SOX10*point mutations and deletions in individualsdiagnosed withKS, who also presented one or severalof the known features of *SOX10*-linked WS. We screened 17cases(9 males and 8 females) previously found negative for mutations in the main genes involved in KS, 13of them presenting with deafness (unilateral in threecases), twowith pigmentation defects (hair and skin hypopigmentation; early graying in a deaf), twowith intellectual disability and onewith psychomotor delay(both features that can be found in the neurological variant of WS [PCWH]). Eleven casesalso had additional symptoms unrelated to WS.

Six new*SOX10*nucleotidic changes were identified in this cohort: a mutation of the initiation codon (c.2T>G, p.?), two nucleotidechanges predicting missense mutations within the HMG domain (c.331T>G, [p.Phe111Val]; c.424T>C, [p.Trp142Arg]), a splice site mutation (c.698-1G>C), a single base-pair deletion predicted to result in a frameshift (c.1290del, [p.Ser431Argfs*71]), and finally a nucleotidechange predicted to result in a missense mutation located in the transactivation domain (c.1298G>A, [p.Arg433Gln]). These changes were not found in at least 50 control individuals (100 chromosomes) of the same origin and the relevant databases (dbSNP, 1000 Genome Project, Exome Variant Server) did not provide evidence that any of these variations are found in control populations.

Clinical and molecular findings in persons carrying *SOX10* mutations are summarized in Table 1 (cases A to F). All sixindividuals had anosmia as well asabsentor delayed spontaneous puberty. Two maleshad cryptorchidism, and one also had micropenis. One of them demonstrated early grayingof the hair.Interestingly, all but one were deaf.Pedigrees of the two familial cases are represented in Figure S1A. A sample from the clinically affected

mother of case D (anosmia and deafness) (see Table 1 and Figure S1A) wasobtained, and cosegregation of the mutation and the disease was confirmed.Parents could also be analyzed fortwo of the sporadic cases (caseB and E) and the mutation was proved to be de novo in both cases.

Five mutations result in impaired SOX10 function

In silico prediction suggested likely pathogenic consequences of all sixmutations. The splice site mutation c.698-1G>C was predicted to result incomplete loss of the last splice acceptor site using several programs, with consequences similar topreviously identified splice site mutations at the same location (c.698-2A>C, c.698-2A>T).[26,27]The Polyphen2 and SIFT programsrespectively predicted a "likely damaging" and a "not tolerated" effect of the threemissense substitutions. Functional effects of the mutations weretested through in vitro analysis.

The p.Phe111Val, p.Trp142Arg, c.1290del, and p.Arg433Gln mutationswere tested using a construction allowing production of SOX10 with a Myctagat its aminoterminus. After confirmation of the protein production by immunoblot analysis (Figure 1A), we first comparedthetransactivation capacities of the mutant proteins on *MITF*(Microphthalmia-asssociated transcription factor) and *MPZ*(Myelin protein zero; P0) promoters/enhancers, alone or in synergy with known SOX10 partner transcription factors, PAX3 (Paired-box 3) and EGR2 (Early growth response 2) respectively (Figure 1B and C).[19,21]The p.Phe111Val, p.Trp142Arg, and c.1290del mutant SOX10 were unable to transactivate the *MITF* reporter construct alone or in synergy with PAX3, whereas the p.Arg433Gln mutant SOX10 behaved like the wild-type protein. On the *MPZ* enhancer reporter construct, the p.Phe111Val, p.Trp142Arg, and c.1290del mutant SOX10 also lost their transactivation capacities alone and demonstrated variable synergistic activity with EGR2 (specifically, conserved for

p.Phe111Val and p.Trp142Arg, lost for c.1290del). Again, the p.Arg433Gln mutant protein showed no change compared with wild-type SOX10. Together, these findings indicated loss of function of the first threemutations and no pathogenic effect of the p.Arg433Gln mutation in vitro. These findingsareconsistent with the observation that Arg433 is located in the transactivation domain, where no WS-causing missense mutations have been characterized thus far, while Phe111 and Trp142 are located within the highly conserved HMG domain,as are the aminoacid residues modified by the previously identified SOX10 missense mutations.[24]

Due to its location in the translation initiation codon, the c.2T>G mutation was tested using a *SOX10* construction allowing production of an untagged protein. Although not detected in immunoblot analysis performed under standard conditions (data not shown), the mutant proteinwas visualized by immunocytochemistry.Use of an antibody directed against the carboxy-terminal portionof SOX10 revealed that amutant protein wasproduced and localized within the nucleus, as was the wild-type protein (Figure 1D, D20-Ab). Notably, we failed to detect the mutant proteinwith another antibody directed against the aminoterminus of SOX10 (Figure 1D, N20-Ab), which supportsthe use of a downstream methionine as an alternative initiation codon. This mutant protein showed either absent or reduced transactivation capacity on the *MITF*,*MPZ*,or *GJB1*(another SOX10 target gene, encoding connexin 32) reporter constructs(Figure 1E). Synergistic activity was lost with PAX3,while it was retained with EGR2 on *MPZ* but not on *GJB1*(Figure 1E).

The six mutations were also tested in a competition assay on the *MITF* promoter. They did not show any dominant negative effect at a ratio of 1:1 and 2:1 to the wild-type protein (Figure S1B). Only at a ratio of 3:1 did the mutations(except p.Arg433Gln)induce a decrease of the luciferase induction.

Functional analysis of *SOX10* missense mutations previously identified in the context of WS allowed us to show that aminoacid substitutions in close contact with the main hydrophobic core of the HMG domain alter subcellular localization of the protein, leading to its accumulation in nuclear foci.[24]The hydrophobic core is composed of four highly conserved residues, two tryptophans and two phenylalanines, which are located at the intersection of the three α-helices that compose the HMG domain and maintain their structural shape. The p.Phe111Val and p.Trp142Arg mutationsdescribed here bothremove one of the hydrophobic core components (Figure 2A). We therefore analyzed the effects of the mutations on the subcellular localization of SOX10 by immunocytochemistry. Whereas the c.1290del and p.Arg433Glnmutant proteins showed a nuclear localization pattern similar to that observed for the wild-type protein (Figure 2B), the p.Phe111Val and p.Trp142Arg proteins showed partial cytoplasmic relocalization (consistent with the location ofPhe111 and Trp142 within the bipartite nuclear localization signals (NLS) and the nuclear export signal (NES), respectively) as well as subnuclear accumulation in foci.

Together, these results stronglyindicatea pathogenic effect of five mutationsamong the six identified, all found in individuals presenting withthe clinical association of KS and deafness.

SOX10 mutations are rare in KS individuals without hearing impairment

We then aimedto determine whether*SOX10* is also involved in other clinical forms ofKS, and particularly in KSwithout deafness. We screened 86random KS individuals, including66 KScases without associated signs and 20 cases with non-olfactory, non-reproductiveadditional symptoms, for *SOX10* point mutations ordeletions. We identifiedtwoadditional mutations (c.323T>C, [p.Met108Thr]and c.451C>T, [p.Arg151Cys]) (Table1, cases G and H; see Figure S1C for case G pedigree)affecting aminoacid residuesofthe HMG domain. Notably,

theindividualwho carriedthe p.Met108Thr mutation showed hypoacusis, whereasthe absence of hearing impairment was confirmedin the individualcarryingthe p.Arg151Cys mutation. Arg151is located close to the hydrophobic core of the protein (Figure 2C, upper panel) whereas the Met108 residuecontactsboth the hydrophobic core and the DNA target (Figure 2C, lower panel), and is located in the bipartite NLS of SOX10. Its substitution couldthus affect SOX10 structure,binding to DNA and/or nuclear localization as the primary defect. Immunocytochemistry experiments were performed to analyze the subcellular location of the mutant proteins. Both p.Met108Thr and p.Arg151Cys missense mutations resulted in partial cytoplasmic relocalization of SOX10 and subnuclear accumulation in foci (Figure 2D). Luciferase assays confirmed theloss-of-function effect of these mutations on the *MITF* promoter, both alone and in the presence of the cofactor PAX3 (Figure S1D).

The seven likelypathogenic mutations found in this study are shownalong with the main functional domains of SOX10 (Figure 2E).Additional screening for mutations in the genes involved in KSor congenital non-syndromic forms of hypogonadotropic hypogonadism was performed in the individuals with a *SOX10* mutation (A to H, Table 1) and only yieldeda *SEMA3A* heterozygous missense variant (p.Val435Ile) in caseE.This variant has been found at similar frequencies in KS individuals and control subjects, although it has been reported to have some deleterious functional consequences in vitro.[28]

These results indicated that *SOX10* mutations are rare in KS individuals without associated signs and confirmed that the clinical association of KS and hearing impairment is more specific. We then used a mouse model to explore the embryonic defect leading to KS as a result of impaired SOX10 function.

SOX10 is expressed in embryonic olfactory ensheathing cellsin mice and humans

OECs, peculiar glial cells involved in the growth and guidance of the olfactory axons, have recently been shown to derive from NC in mouse and chick. SOX10 was used as a marker of NC-derived cells in these studies,and its pattern of expression indeed suggested that it is expressed by OECs.[29-31]As OECs are heterogeneous in the pattern of markers they express,[32] wefully characterizedthe SOX10 expression profile during development of the olfactory structures using antibodies directed against SOX10 and several markers of OECs.

In E12.5 mouse embryo, the olfactory, vomeronasal, and terminalnerve fibers can be seen in the mesenchyme surrounding the olfactory epithelium and converge on the migratory mass, a heterogeneous group of cells located in the fronto-nasal mesenchyme beneath the presumptive olfactory bulbs. OECs are detected all along the nerve pathway, where their cytoplasms ensheathe the axon bundles, and begin to invade the olfactory nerve layer (ONL) of the developing olfactory bulb at this stage (Figure 3A). Immunostaining on coronal sections of E12.5 embryo head (Figure3A) revealed that SOX10 is coexpressed with the OEC marker BLBP/BFABP,whereas it showed mutually exclusive expression with the neuronal marker TUJ1 (β3-tubulin). More specifically, SOX10 (nuclear staining, red) and BLBPwere coexpressed along the olfactory, vomeronasal, and terminal nerve pathway, in the fronto-nasal mesenchyme, and in the migratory mass below the presumptive olfactory bulb (Figure3B and data not shown). SOX10-positive cells also expressedthe OEC markers P75/NGFR (note that this marker is not specific to OECs and also labels most cells in the nasal mesenchyme)andS100along the nervefibers and in the nasal mesenchyme(Figure 3B). By contrast,theydid not express P75and showed variable S100 immunostaining within the migratory mass(Figure3B).

At E14.5, the olfactory epithelium has developed. SOX10 demonstrated the same pattern of expression in OECs (defined asBLBP+, P75+, S100+, TUJ1- cells) of the nasal mesenchyme and along the nerve pathway (Figure3C and data not shown).At this stage, the olfactory bulb

had formed from the rostral telencephalon,and a continuous outer layer of SOX10-expressing cells was observed, which corresponds to the ONL (Figure 3D). These cells were BLBP-positive and P75-negative, while only the outermost cellswere S100-positive (Figure 3D and E).Apart from OECs, we observed some SOX10expression in the nasal glands (Figure S2A and B). We also confirmed the presence of a few non-neuronal SOX10-expressing cells in the olfactory epithelium at this stage (Figure S2C), as previously reported.[29,31]

Finaly, we studied SOX10 expression in the peripheral olfactory system of a human embryo at 8weeks of embryonic development (equivalent stage to mouse E14.5).Immunostaining experiments revealed that SOX10-expressing cells (nuclear, green) were located along the olfactory, vomeronasal, and terminal nerve trajectory (TUJ1 staining, red) in the nasal mesenchyme (Figure 4A), in the migratory mass, and in the olfactory bulb (Figure 4B). Double staining of SOX10 with the OEC marker S100 along the olfactory nerves confirmed that SOX10-expressing cells are OECs (Figure 4C).

Together, these results confirmed SOX10 expression in OEC during early development of the peripheral olfactory system in miceand humans.

TheSox10 mutant mousehas olfactory ensheathing cell defects

We then used mice bearing a *Sox10*mutation (*Sox10*^{lacZ})[14]to explore the embryonic defect leading to KS as a result of impaired SOX10 function. We first performed X-Gal stainingto detect β-galactosidase (*lacZ*expression) inheterozygous and homozygous mutant E14.5embryos. The olfactory bulbshad formed in both genotypes, but an abnormal colonization of the olfactory bulbsby X-Gal-stained (blue) OECswas apparent in homozygotes (Figure5A). We then compared β-galactosidase expression with various markers by immunohistochemistry on coronal sections. A general overview of the olfactory system showed that the olfactory, vomeronasal, and terminal nerves had formed in the homozygous

mutant embryos, but OECs (β-galactosidase-positivecells)were almost absent in the nasal mesenchyme and along the nerve pathway (Figure5B and higher magnification in Figure 5C). Only a few OECs were observed in the upper part of the fronto-nasal mesenchyme, under the migratory mass. By contrast, numerous OECswere present in the migratory mass and the ONL, but they appeared to encircle the olfactory bulbs incompletely, forming athinner, disorganized, and discontinued layer.

We then determined the expression pattern of OEC markers in the absence of SOX10. In the upper part of the fronto-nasal mesenchyme, the marker profile of the few β-galactosidase-positive cells present in the mutant homozygotesdiffered fromthat of cells in heterozygotes,with fainter BLBPand absentS100labeling in most of these cells,while P75staining was preserved(Figure5D). In the olfactory bulb ONL, BLBP staining was preserved, and S100 staining (normally found in the outermost cell layer of the ONL) was lost,but most if not all OECswere now P75-positive (Figure5E).

In summary, these results indicated an almost complete absence of OECs in the nasal mesenchyme of the *Sox10*homozygous mutant embryos, defective colonization of theolfactory bulbs, and abnormal profiles of the remaining OECs in both the nasalmesenchyme and the ONL of the olfactory bulbs.

To explainthese defects, we compared the development of OECs in heterozygous and homozygous *Sox10* mutant embryosat an earlier stage. At E12.5, the absence of OECs in most of the nasal mesenchyme was already visible inthe mutant homozygotes (Figure S3A). The migratory mass had formed; however, defective colonization of the olfactory bulb anlage by OECs was already detectableon more rostral sections (Figure S3B). To determine if the absence of OECs in the nasal mesenchyme was due to apoptosis of *Sox10*-defective cells, we counted the number of TUNEL-positive cells in the nasal mesenchyme. We did not find asignificant difference in the number of apoptotic cells between heterozygous and

homozygous *Sox10*^{lacZ}embryos (Figure S3C), indicating that the defect responsible for the low number of OECs in the homozygousmutant embryos occurred earlier or by a non-apoptotic mechanism.

The OEC defect impacts the development of olfactory, vomeronasal, and terminal nerves and the migration of neuroendocrine GnRH-cells

OECs ensheathe the olfactory, vomeronasal, and terminal nerve fibersand also contribute to axonal pathfinding, fasciculation, and defasciculation.[32]We thus looked at the trajectory of these nerve fibers in the *Sox10* mutantembryos. TUJ1 staining on E14.5 heads showed that thenerves hadformed in mutant homozygotesdespitethealmost complete absence of OECs in the nasal mesenchyme (Figure 6A). However, axons wereabnormallyrouted; some of them did not target the olfactory bulb and instead contactedaxons from the other side, dorsally to the nasal septum (red arrowhead in Figure 6A).Furthermore, as a result of the absence of OECs, axons were not ensheathed along their most ventraltrajectory, and their defasciculation could be observed near the nasal septum (red arrow on Figure 6A; defasciculation is also visible on Figure 5D).

Hypogonadism in KS results from the so-called olfacto-genital fetopathological sequence, whereby incomplete embryonic migration of the neuroendocrine GnRH-cells from the nose to the brain arises from the disruption of vomeronasal and terminal nerve fibers.[12] We therefore analyzed the migration of these cellsin the *Sox10* mutantembryo. In E14.5 wild-type and heterozygous embryos, most GnRH-cells had already left the extracerebral nerve pathway and were migrating withinthe two cerebral hemispheres. In homozygous mutant embryos, GnRH-cells were found to accumulate on the vomeronasal and terminal nerve trajectory in the nasal mesenchyme (Figure 6B and data not shown). GnRH-cell count along the nerve pathwayconfirmed this observation by showing a significantly larger number of GnRH-cell

bodies in the *Sox10*^{laZ}homozygotesthan in wild-typeand heterozygous embryos(Figure 6C). Althoughsome GnRH-cellsdidreach the migratory massin the homozygous mutant embryos, few cellshadpenetrated into the forebrain en route tothe hypothalamic region (Figure 6D). The overall number of GnRH-cells appeared unchanged (estimated 969 in wild-type, 949 in heterozygous and 937 in homozygous mutant embryos).

These results show that the *Sox10* homozygous mutant mice, which have a complete lack of SOX10, also undergo a pathological sequence in embryonic development similar to that responsible for KS. The defects we observed in the *Sox10* homozygous mutantmice are schematized in Figure 7.

Discussion

KS is genetically heterogeneous, with various modes of transmission: autosomal recessive, autosomal dominant with incomplete penetrance, X chromosome-linked, and oligogenic. However, only 30% of cases havemutations in any of the nine genesidentifiedthusfar.[13,28]Deafness is one of various non-olfactory, non-reproductive anomalies that are sometimesfound together with KS.[13]Its prevalence in KS individuals has been estimated at approximately 5%.[33]As our understanding of the molecular basis of KShas progressed, deafness has been reported in individuals with *KAL1*, *FGFR1*,*FGF8*or *CHD7*mutations, but these cases remain relatively rare.[34-38] Taking into account the high prevalence of hearing impairment in the general population, the association of KS with deafness may sometimes be coincidental, as wasshown in a family where deafness cosegregated neither with KS nor with the *FGF8* mutation.[39]Comparatively, the remarkably

large penetrance of deafness in the KS individuals who carry mutations in*SOX10* appears highly significant.

We characterized seven different *SOX10* mutations in persons affected by KS. As in WS, themutations were found inthe heterozygous state together with a dominant mode of inheritance.Some phenotypic variation was observed. In family D, the mother, who also carries the mutation, suffered from deafness and anosmia but had a normal puberty and spontaneous pregnancy. Although mosaicism cannotbe excluded, this finding most likely refers to the usual variability of phenotypic expression that is frequentlyobserved in developmental disorders, and in particular in KS or SOX10-linked WS. Stochastic events and modifier genes are often proposedto explain at least part of the phenotypic variability.In this respect, the SEMA3A sequence variant that was found in caseE may influence the expression of the disease.

In the first part of our study, five mutations were found among 13individuals withthe clinical association of KS and deafness, corresponding to a *SOX10* involvement of 38%. This rate may be slightly overestimated, because a few KS individuals with deafness and already known mutations in other genes had been excluded. However, to date *SOX10* stands as the main gene involved in this specific clinical association.

The hearing loss was sensorineural, profound or total in most cases. The audiograms of cases D and E are shown in Figure S4, illustrating a total loss of hearing on the two ears at all frequencies. In contrary, caseG showed a mild sensorineural hearing loss (30 dB at 1000 and 2000 Hz on the left ear, 40 dB from 250 to 1000 Hz on the right ear).A striking finding is the high proportion of femalesamong persons carrying*SOX10* mutations in this series (4 femalesand 3 males), while KS has been estimated to be three tofive times more frequent in males than in females.[13]However, an approximately 1:1 sex ratio was also found in the group

of KSplusdeafness individuals (7 females out of 13 cases), an observation that needs to be confirmed by further studies.

Sevenof the 8 *SOX10* sequence variations identified (four missense substitutions, one frameshift mutation, one splice site mutation, and onemutation of the initiation codon, schematically summarized in Figure 2E) result, or are predicted to result, in an altered production or function of SOX10. We performed transactivation assays on two target genes of SOX10, namely *MITF* and *MPZ*.[19,21]*MITF* was chosen due to its critical rolein melanocyte development, whereas *MPZ*was selected based on its known expression in OECs.[40]Our results do not support a deleterious effect of the p.Arg433Gln mutation. The other 7 mutations were found to alter subcellularlocalization and/or transactivation capacities, thus validating their deleterious effect. The fourmissense mutations located in the HMG domain showed a peculiar aspect of relocalization tonuclear bodies,whichwe had previously observed for aminoacid substitutions in close contact with the main hydrophobic core of the HMG domain.[24]Whether these foci are a cause or a consequence of the pathogenic effect is still unclear.

The c.2C>T substitution affects the *SOX10*initiation codon. However, immunostaining of cells transfected with cDNA carrying the mutation,using antibodies directed against the SOX10 aminoorcarboxy terminus, showed thatan in-frame protein is produced. The first in-frame ATG codon corresponds to Met90 and, if used, would produce a protein conserving the HMG and transactivation domains, but lackingmost of the dimerization domain.[41]As Met90 is the only in-frame methionine more proximal to the two NLS, this hypothesisis consistent with our observation that the protein produced doesnot relocalize tothe cytoplasm.[42]

Significantly, one of the KS individuals had early graying and deafness and therefore fulfilled the diagnosis criteria for type 2 WS, but no pigmentation defects were reported for the other cases. For some persons, clinical reevaluation was possible after the identification of the *SOX10* mutation. The absence of WS-like pigmentation disturbance was confirmed in caseD

and her mother as well as in caseB and C, whilecaseE had lately developed a frontal white forelock at approximately age 25 years. No evidence at this point indicates what makes agiven*SOX10* mutation a KS- or WS-causing mutation. As a whole, the mutations we identified here were not typically different from the mutations previously identified in WS except for their relative frequency: we found mostly missense mutations of the HMG domain and few truncatingmutationsin our group of KS individuals (while most *SOX10* mutations are predicted to result in a truncated protein in WS), a finding that mustbe confirmed in additionalstudies. In regard to previous clinical findings in WS (very rare reportsof anosmia and hypogonadism/cryptorchidism among almost 100 reportedpersons with different*SOX10* mutations), as well as the recent observation that 88% of WS individuals with a *SOX10* mutation and inner ear morphological abnormalities also have olfactory bulbagenesis, it remains possible that anosmia and hypogonadism have been underestimatedin WS. This phenomenon could possibly be explained in part because people usually do not spontaneously complain ofanosmia and by the fact that WS is often diagnosed in childhood.

The involvement of SOX10 in mouse olfactory development was not suspected until recently, whenits expression was reported in OECs.[29-31]Here, we showed that SOX10 plays a major role in the development of these peculiar NC-derived glial cells. In Sox10-null mutant embryos, a large part of the peripheral embryonic olfactory system lacked OECs at the embryonic stages analyzed.

Our results showed that the OEC deficiency occurs prior to E12.5 in the mouse. Based on current knowledge about SOX10 function in other tissues, the defect likely occurs between the emergence of NC cells from the neural tube and their arrival at the olfactory placode. SOX10 is thought to play a role in sustaining the survival of multipotent NC cells. In the absence of SOX10, a dramatic increase in cell death has been reported for vagal NC cells prior to their entry into the foregut as well as in various other NC derivatives.[46-50]

The remaining OECs were located in the uppermostpart of the fronto-nasal mesenchyme, the migratory mass, and the ONL. They hadan abnormal expression profile and formed a discontinued and disorganized layer around the olfactory bulbs, indicating that SOX10 is alsoprobably involved in their identity and function at several locations. Several explanations can be proposed as to why some OECs persisted in the mutant mice. One possibility is that not all OECs are SOX10-dependent. Another hypothesis is a partial functional redundancy between SOX10 and another protein of the SOX family. SOX8, which together with SOX10 and SOX9 forms the E subgroup of SOX transcription factors, is a good candidate based on its expression in the ONL during embryogenesis and the described redundancy between SOXE subfamily members in other tissues.[43-45]

Despite the markedly reduced number of OECs, the olfactory, vomeronasal, and terminal nerves had formed in the Sox10-null embryos, as had the olfactory bulbs. This finding may be surprising since OECs not only ensheathe and fasciculate the nerve fibers, but also extend processes ahead of the pioneer olfactory axons they ensheathe, suggesting that they orchestrate their growth and guidance.[32,51] However, although the olfactory axons were formed and extended some processes toward the migratory mass and the olfactory bulb in the Sox10-null mutant embryos, we found that they were partially misrouted and defasciculated, thus confirming the role of OECs in these processes.

In the mouse, neuroendocrine GnRH-cells begin to leave the epithelium of the olfactory pit at approximatelyE11.5. They migrate in close association with growing fibers of the vomeronasal and terminal nerves, then penetrate into the rostral forebrain and continue their migration toward the hypothalamus along the terminal nerve or a branch of the vomeronasal nerve.[52]In E14.5 Sox10-null embryos, the latest embryonic stage we could examine, GnRH-cells accumulatedoutside the brain, along the vomeronasal and terminal nerve pathway, in the regions devoid of OECs. Few GnRH-cells were seen in the forebrain of these mice, which

could reflect an impaired or delayed migration.To the extent thatmice and humans are comparable, it remains to be determined whetherthe defective GnRH-cell migration in this particular genetic form of KS is primarily due to the absence of OECs on the vomeronasal and terminal nerve trajectory or to the defective structure of these nerves,as previously suggested in other genetic forms.[12]

All the abnormalities we found in the *Sox10*mutant mouse were observed in homozygous embryos, and embryonic lethality of these mice precludes further testing of late olfactory developmentand fertility. In the normal ONL, OECs contribute to the defasciculation, sorting, and refasciculation of axonsand are therefore crucial to olfactory bulbglomeruli formation and the establishment of the olfactory topographic map.[32]They are also involved in the renewal of olfactory receptor neurons and their axonal growth throughout life. Accordingly, it is tempting to speculate that the defect in the number and function of OECs in the *Sox10*mutant mouse could have drastic consequences on the maturation of the olfactory bulbs and the ability of olfactory nerve fibers to renew throughout life. The use of a conditional knock-out mouse model to avoid lethalitywould be of interest to explore the late consequencesof the absence of SOX10 in the peripheral olfactory system.

SOX10 mutations have been found in the heterozygous state in both KS and WSindividuals.No reproduction defect has been reportedin the *Sox10* heterozygous mutant mice,suggesting that both olfaction and GnRH neurosecretion are not strongly affected, althoughit remains possible that subtle, as yet unrecognized defects in OECs have some functional consequences in adult mice. Bycontrast, depigmentation and megacolon are found inheterozygous miceas in WS4 individuals. Such differences in sensitivity to genetic diseasesbetween miceand humansare not rare. Alternatively, oligogenicity has been described in KS and mutations in unknown genes may contribute to the disease in some instance. Notably, the inner ear morphological defects associated with *SOX10* mutations are also quite

markedand have a high penetrancein humans, while they are much less evident in the *Sox10*homozygous mouse mutant.[10,53]

*Sox10*is expressed in the melanocytic intermediate cells of the cochlear stria vascularis,which produce the endocochlear electric potential essential to the hearing process.[54] It is also widely expressed duringearly inner ear development before being restricted to the cochlear and vestibular ganglia and to supporting cells of the sensory epithelium. In addition to a specific role in glial development of the ganglia, SOX10 has been shown to promote the survival of cochlear progenitors during otocyst formation and differentiation of the organ of Corti.[53-55] In the *Sox10* homozygous mutant mice, the structure and cellular organization of the organ of Corti appear normal, but the cochlear duct is shortened,with no apparentmalformation of the vestibule.[53] In humans, apart from the cochlear degeneration thought to take place in WS in the absence of intermediate cells of the stria vascularis, a proportion of WS individuals with *SOX10* mutations had an enlarged vestibule, agenesis or hypoplasia of semi-circular canals, and an abnormally shaped cochlea.[10]Together,these results suggest that the high penetrance of deafness among persons who carry *SOX10* mutations is likely the consequence of several different defects in the development of the inner ear.

MRI or CT scans of the temporal bone have been performed in 4 of the 7 individuals. Abnormal images were not found in caseB (not all the temporal bone structures could be analyzed but the semi-circular canals were present and normally shaped). A vestibulo-cochlear dysplasiawas reported incaseD. The temporal bone CT scan of caseC showed a bilateral hypoplasia of the lateral and posterior semi-circular canals as well as a vesicular vestibule on one side and an enlargement of the vestibular aqueduct on the other side. The CT scan of caseE showed bilateral enlargement of the vestibule and hypoplasia of the lateral semi-circular canals.Interestingly, the association of semi-circular canal hypoplasia or agenesis with olfactory bulb agenesis and hypogonadotropic hypogonadism is also found in

CHARGE syndrome (Coloboma, Heart defects, Choanal atresia, Retardation, Genital and Ear anomalies) due to mutationsin*CHD7*.[56]Individuals affected by typical CHARGE syndrome are not difficult to distinguish from persons carrying *SOX10* mutations, but inindividuals affected by mild forms of CHARGE syndrome,who do not show all the cardinal signs of the disease,this condition may be difficult to differentiate in the absence of a complete clinical description.[56-58]From now on, the existence of semi-circular canalhypoplasia or agenesis in a person affected by KS should be considered an indication for both *CHD7* and *SOX10* molecular analyses.Isolated or minorisolated signs of CHARGE syndrome, such as coloboma, heart defects, but alsofacial asymmetry, middle or external ear malformations, hypoplastic vestibulemay point towards *CHD7*, while some depigmentation features or enlarged vestibule may point to a *SOX10* defect.

Our findings implicate NC-derived OECs in the pathogenesis of KS, thus defining the *SOX10*-related genetic form of the disease as a new neurocristopathy. The peripheral olfactory nervous system is unique in that it renews throughout life, a property attributed to the presence of the OECs, which makes them strong candidates for cell-mediated repair of a variety of neural lesions.[32,51] Despite this major medical relevance, the consequences of the absence or depletion of OECs in the peripheral olfactory system have been poorly characterized. In this respect, the *Sox10* knock-out mouse may prove to be a valuable model.

In summary, we characterized a new role of *SOX10* in human pathology, 14 years after the cloning and first implicationof this gene in WS. We found *SOX10*loss-of-function mutations in individuals demonstratingthe clinical association of KS with hearing impairment and more rarely in individuals demonstrating KS without associated signs. From now on, *SOX10* is the first gene to test for the presence of mutations in the KS plus hearing impairment association. Based on a mouse model study, we suggest that this particular genetic form of KS resultsfrom

a previously unreported primarydefect affecting OECs during early embryonic developmentof the peripheral olfactory system.

Supplemental data

Supplemental data include fourfigures, S1, S2, S3 and S4.

Acknowledgments

We thank the index casesand family members for their contribution to the study.We thank Fabien Guimiot for providing sections of human fetuses, Michael Wegner for providing us with the *Sox10*^{lacZ}mice.We thank the Hôpital Henri Mondor sequencing facility, Marjorie Collery for animal husbandry, and Xavier Ducrouy for confocal imaging. This work was supported by the Institut de la Santé et de la Recherche Médicale (Inserm) and the Agence Nationale de la Recherche (ANR-JCJC-2010 to NB and ANR-2009-GENOPAT-017 to CD). AC is a recipient of a fellowship from the Fondation pour la Recherche Médicale (FRM). SM is receiving a salary on theANR-2009-GENOPAT-017 grant.

Web ressources

1000 genome project: http://www.1000genomes.org/

dbSNP: http://www.ncbi.nlm.nih.gov/projects/SNP/

exome variant server: http://evs.gs.washington.edu/EVS/OMIM:

http://www.ncbi.nlm.nih.gov/omim

Polyphen-2: http://genetics.bwh.harvard.edu/pph2/

SIFT:http://sift.jcvi.orgWS gene mutation database at LOVD:

http://grenada.lumc.nl/LOVD2/WS/home.php?select_db=SOX10

References

1. Wegner, M. (2005). Secrets to a healthy Sox life: lessons for melanocytes. Pigment Cell Res *18*, 74-85.

2. Le Douarin, N.M., and Kalcheim, C. (1999). The neural crest.(Cambridge: Cambridge University press).

3. 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.

4. 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.

5. Zhu, L., Lee, H.O., Jordan, C.S., Cantrell, V.A., Southard-Smith, E.M., and Shin, M.K. (2004). Spatiotemporal regulation of endothelin receptor-B by SOX10 in neural crest-derived enteric neuron precursors. Nat Genet *36*, 732-737.

6. 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.

7. 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.

8. 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.

9. Bondurand, N., Dastot-Le Moal, F., Stanchina, L., Collot, N., Baral, V., Marlin, S., Attie-Bitach, T., Giurgea, I., Skopinski, L., Reardon, W., et al. (2007). Deletions at the SOX10 gene locus cause Waardenburg syndrome types 2 and 4. Am J Hum Genet *81*, 1169-1185.

10. Elmaleh-Bergès, M., Baumann, C., Noël-Pétroff, N., Sekkal, A., Couloigner, V., Devriendt, K., Wilson, M., Marlin, S., Sebag, G., and Pingault, V. (In press). Spectrum of temporal bone abnormalities in patients with Waardenburg syndrome and SOX10 mutations. Am J Neuroradiol.

11. Barnett, C.P., Mendoza-Londono, R., Blaser, S., Gillis, J., Dupuis, L., Levin, A.V., Chiang, P.W., Spector, E., and Reardon, W. (2009). Aplasia of cochlear nerves and olfactory bulbs in association with SOX10 mutation. Am J Med Genet A *149A*, 431-436.

12. Teixeira, L., Guimiot, F., Dode, C., Fallet-Bianco, C., Millar, R.P., Delezoide, A.L., and Hardelin, J.P. (2010). Defective migration of neuroendocrine GnRH cells in human arrhinencephalic conditions. J Clin Invest *120*, 3668-3672.

13. Dode, C., and Hardelin, J.P. (2009). Kallmann syndrome. Eur J Hum Genet *17*, 139-146.

14. 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.

15. Bondurand, N., Natarajan, D., Thapar, N., Atkins, C., and Pachnis, V. (2003). Neuron and glia generating progenitors of the mammalian enteric nervous system isolated from foetal and postnatal gut cultures. Development *130*, 6387-6400.

16. Kurtz, A., Zimmer, A., Schnutgen, F., Bruning, G., Spener, F., and Muller, T. (1994). The expression pattern of a novel gene encoding brain-fatty acid binding protein correlates with neuronal and glial cell development. Development *120*, 2637-2649.

17. Schwanzel-Fukuda, M., Bick, D., and Pfaff, D.W. (1989). Luteinizing hormone-releasing hormone (LHRH)-expressing cells do not migrate normally in an inherited hypogonadal (Kallmann) syndrome. Brain Res Mol Brain Res *6*, 311-326.

18. Bondurand, N., Girard, M., Pingault, V., Lemort, N., Dubourg, O., and Goossens, M. (2001). Human Connexin 32, a gap junction protein altered in the X-linked form of Charcot-Marie-Tooth disease, is directly regulated by the transcription factor SOX10. Hum Mol Genet *10*, 2783-2795.

19. Bondurand, N., Pingault, V., Goerich, D.E., Lemort, N., Sock, E., Le Caignec, C., Wegner, M., and Goossens, M. (2000). Interaction among SOX10, PAX3 and MITF, three genes altered in Waardenburg syndrome. Hum Mol Genet *9*, 1907-1917.

20. Sanchez-Mejias, A., Watanabe, Y., R, M.F., Lopez-Alonso, M., Antinolo, G., Bondurand, N., and Borrego, S. (2010). Involvement of SOX10 in the pathogenesis of Hirschsprung disease: report of a truncating mutation in an isolated patient. J Mol Med (Berl) *88*, 507-514.

21. LeBlanc, S.E., Jang, S.W., Ward, R.M., Wrabetz, L., and Svaren, J. (2006). Direct regulation of myelin protein zero expression by the Egr2 transactivator. J Biol Chem *281*, 5453-5460.

22. Zhang, H., Chen, H., Luo, H., An, J., Sun, L., Mei, L., He, C., Jiang, L., Jiang, W., Xia, K., et al. (2012). Functional analysis of Waardenburg syndrome-associated PAX3 and

SOX10 mutations: report of a dominant-negative SOX10 mutation in Waardenburg syndrome type II. Hum Genet *131*, 491-503.

23. Peirano, R.I., Goerich, D.E., Riethmacher, D., and Wegner, M. (2000). Protein zero gene expression is regulated by the glial transcription factor Sox10. Mol Cell Biol *20*, 3198-3209.

24. Chaoui, A., Watanabe, Y., Touraine, R., Baral, V., Goossens, M., Pingault, V., and Bondurand, N. (2011). Identification and functional analysis of SOX10 missense mutations in different subtypes of Waardenburg syndrome. Hum Mutat *32*, 1436-1449.

25. Guex, N., and Peitsch, M.C. (1997). SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis *18*, 2714-2723.

26. Jiang, L., Chen, H., Jiang, W., Hu, Z., Mei, L., Xue, J., He, C., Liu, Y., Xia, K., and Feng, Y. (2011). Novel mutations in the SOX10 gene in the first two Chinese cases of type IV Waardenburg syndrome. Biochem Biophys Res Commun *408*, 620-624.

27. Sznajer, Y., Coldea, C., Meire, F., Delpierre, I., Sekhara, T., and Touraine, R.L. (2008). A de novo SOX10 mutation causing severe type 4 Waardenburg syndrome without Hirschsprung disease. Am J Med Genet A *146A*, 1038-1041.

28. Hanchate, N.K., Giacobini, P., Lhuillier, P., Parkash, J., Espy, C., Fouveaut, C., Leroy, C., Baron, S., Campagne, C., Vanacker, C., et al. (2012). SEMA3A, a Gene Involved in Axonal Pathfinding, Is Mutated in Patients with Kallmann Syndrome. PLoS Genet *8*, e1002896.

29. Barraud, P., Seferiadis, A.A., Tyson, L.D., Zwart, M.F., Szabo-Rogers, H.L., Ruhrberg, C., Liu, K.J., and Baker, C.V. (2010). Neural crest origin of olfactory ensheathing glia. Proc Natl Acad Sci U S A *107*, 21040-21045.

30. Forni, P.E., Taylor-Burds, C., Melvin, V.S., Williams, T., and Wray, S. (2011). Neural crest and ectodermal cells intermix in the nasal placode to give rise to GnRH-1 neurons, sensory neurons, and olfactory ensheathing cells. J Neurosci *31*, 6915-6927.

31. Katoh, H., Shibata, S., Fukuda, K., Sato, M., Satoh, E., Nagoshi, N., Minematsu, T., Matsuzaki, Y., Akazawa, C., Toyama, Y., et al. (2011). The dual origin of the peripheral olfactory system: placode and neural crest. Mol Brain *4*, 34.

32. Ekberg, J.A., Amaya, D., Mackay-Sim, A., and St John, J.A. (2012). The migration of olfactory ensheathing cells during development and regeneration. Neurosignals *20*, 147-158.

33. Quinton, R., Duke, V.M., Robertson, A., Kirk, J.M., Matfin, G., de Zoysa, P.A., Azcona, C., MacColl, G.S., Jacobs, H.S., Conway, G.S., et al. (2001). Idiopathic gonadotrophin deficiency: genetic questions addressed through phenotypic characterization. Clin Endocrinol (Oxf) *55*, 163-174.

34. Dode, C., Fouveaut, C., Mortier, G., Janssens, S., Bertherat, J., Mahoudeau, J., Kottler, M.L., Chabrolle, C., Gancel, A., Francois, I., et al. (2007). Novel FGFR1 sequence variants in Kallmann syndrome, and genetic evidence that the FGFR1c isoform is required in olfactory bulb and palate morphogenesis. Hum Mutat *28*, 97-98.

35. Dode, C., Levilliers, J., Dupont, J.M., De Paepe, A., Le Du, N., Soussi-Yanicostas, N., Coimbra, R.S., Delmaghani, S., Compain-Nouaille, S., Baverel, F., et al. (2003). Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. Nat Genet *33*, 463-465.

36. Falardeau, J., Chung, W.C., Beenken, A., Raivio, T., Plummer, L., Sidis, Y., Jacobson-Dickman, E.E., Eliseenkova, A.V., Ma, J., Dwyer, A., et al. (2008). Decreased FGF8 signaling causes deficiency of gonadotropin-releasing hormone in humans and mice. J Clin Invest *118*, 2822-2831.

37. Massin, N., Pecheux, C., Eloit, C., Bensimon, J.L., Galey, J., Kuttenn, F., Hardelin, J.P., Dode, C., and Touraine, P. (2003). X chromosome-linked Kallmann syndrome: clinical heterogeneity in three siblings carrying an intragenic deletion of the KAL-1 gene. J Clin Endocrinol Metab *88*, 2003-2008.

38. Soderlund, D., Canto, P., and Mendez, J.P. (2002). Identification of three novel mutations in the KAL1 gene in patients with Kallmann syndrome. J Clin Endocrinol Metab *87*, 2589-2592.

39. Trarbach, E.B., Abreu, A.P., Silveira, L.F., Garmes, H.M., Baptista, M.T., Teles, M.G., Costa, E.M., Mohammadi, M., Pitteloud, N., Mendonca, B.B., et al. (2010). Nonsense mutations in FGF8 gene causing different degrees of human gonadotropin-releasing deficiency. J Clin Endocrinol Metab *95*, 3491-3496.

40. Lee, M.J., Calle, E., Brennan, A., Ahmed, S., Sviderskaya, E., Jessen, K.R., and Mirsky, R. (2001). In early development of the rat mRNA for the major myelin protein P(0) is expressed in nonsensory areas of the embryonic inner ear, notochord, enteric nervous system, and olfactory ensheathing cells. Dev Dyn *222*, 40-51.

41. 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.

42. Malki, S., Boizet-Bonhoure, B., and Poulat, F. (2009). Shuttling of SOX proteins. Int J Biochem Cell Biol *42*, 411-416.

43. 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.

44. Reiprich, S., Stolt, C.C., Schreiner, S., Parlato, R., and Wegner, M. (2008). SoxE proteins are differentially required in mouse adrenal gland development. Mol Biol Cell *19*, 1575-1586.

45. Stolt, C.C., Lommes, P., Friedrich, R.P., and Wegner, M. (2004). Transcription factors Sox8 and Sox10 perform non-equivalent roles during oligodendrocyte development despite functional redundancy. Development *131*, 2349-2358.

46. Dutton, K.A., Pauliny, A., Lopes, S.S., Elworthy, S., Carney, T.J., Rauch, J., Geisler, R., Haffter, P., and Kelsh, R.N. (2001). Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates. Development *128*, 4113-4125.

47. 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.

48. Paratore, C., Goerich, D.E., Suter, U., Wegner, M., and Sommer, L. (2001). Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. Development *128*, 3949-3961.

49. Sonnenberg-Riethmacher, E., Miehe, M., Stolt, C.C., Goerich, D.E., Wegner, M., and Riethmacher, D. (2001). Development and degeneration of dorsal root ganglia in the absence of the HMG-domain transcription factor Sox10. Mech Dev *109*, 253-265.

50. 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.

51. Su, Z., and He, C. (2010). Olfactory ensheathing cells: biology in neural development and regeneration. Prog Neurobiol *92*, 517-532.

52. Wray, S. (2010). From nose to brain: development of gonadotrophin-releasing hormone-1 neurones. J Neuroendocrinol *22*, 743-753.

53. Breuskin, I., Bodson, M., Thelen, N., Thiry, M., Borgs, L., Nguyen, L., Lefebvre, P.P., and Malgrange, B. (2009). Sox10 promotes the survival of cochlear progenitors during the establishment of the organ of Corti. Dev Biol *335*, 327-339.

54. Watanabe, K., Takeda, K., Katori, Y., Ikeda, K., Oshima, T., Yasumoto, K., Saito, H., Takasaka, T., and Shibahara, S. (2000). Expression of the Sox10 gene during mouse inner ear development. Brain Res Mol Brain Res *84*, 141-145.

55. Breuskin, I., Bodson, M., Thelen, N., Thiry, M., Borgs, L., Nguyen, L., Stolt, C., Wegner, M., Lefebvre, P.P., and Malgrange, B. (2010). Glial but not neuronal development in the cochleo-vestibular ganglion requires Sox10. J Neurochem *114*, 1827-1839.

56. Janssen, N., Bergman, J.E., Swertz, M.A., Tranebjaerg, L., Lodahl, M., Schoots, J., Hofstra, R.M., van Ravenswaaij-Arts, C.M., and Hoefsloot, L.H. (2012). Mutation update on the CHD7 gene involved in CHARGE syndrome. Hum Mutat *33*, 1149-1160.

57. Bergman, J.E., de Ronde, W., Jongmans, M.C., Wolffenbuttel, B.H., Drop, S.L., Hermus, A., Bocca, G., Hoefsloot, L.H., and van Ravenswaaij-Arts, C.M. (2012). The results of CHD7 analysis in clinically well-characterized patients with Kallmann syndrome. J Clin Endocrinol Metab *97*, E858-862.

58. Verloes, A. (2005). Updated diagnostic criteria for CHARGE syndrome: a proposal. Am J Med Genet A *133A*, 306-308.

Figure titles and legends

Figure 1. Functional analysis of the mutant SOX10 proteins

(A) Immunoblotting analysisshowing wild-type (wt) or mutant SOX10 proteins usingan antibody directed against the carboxyterminal portion of the protein.

(B and C) Luciferase reporter gene analysis. HeLa cells were co-transfected with the wild-type (wt) or mutant SOX10 expression vectorandareporter construct containing the *MITF* promoter (B) or *MPZ* intronic enhancer (C) and known SOX10 cofactors expression vectors, i.e., PAX3 (B) or EGR2 (C). Reporter gene activation is presented as luciferase fold induction relative to the empty vector. Results are the mean ± s.e.m of at least threedifferent experiments, each performed in duplicate.

(D) Detection and localization of the c.2T>G mutant protein. HeLa cells transfected with wild-type (left panels) or mutant c.2T>G (right panels) SOX10 constructs. Nuclei were counterstained with TO-PRO-3 iodide (blue). Transfected cells were immunostained with anti-SOX10 antibodies (red) directed against either the aminoterminal (N20) or the carboxyterminal (D20) of the protein, as indicated. The merged images are presented below. A higher magnification of SOX10 labeling is also shown in gray (bottom panels).

(E) Luciferase reporter gene analysis. HeLa cells were co-transfected withthe wild-type (wt) or mutant c.2T>G SOX10 expression vectorsandareporter construct containing the *MITF* promoter (left panel), *MPZ* intronic enhancer (central panel), or the *GJB1*promoter (right panel) and the PAX3 (left panel) or EGR2 (central and right panels) expression vector. Reporter gene activation is presented as fold induction relative to the empty vector. Results are the mean ± s.e.m of at least threedifferent experiments, each performed in duplicate.

Figure 2. Subcellular localization of the mutant SOX10 proteinsand summary of all SOX10 mutations found in this study

(A) Three-dimensional representation showing the location within the HMG domain of the residues affected by the identified missense mutations. Three-dimensionalview of the HMG domain of SOX17 (backbone in red) that forms threeα-helices (white ribbons), bound to its DNA target (blue). Upper panel: the four residues that form the hydrophobic core areshown in yellow. Lower panel: the residues corresponding to Phe111 and Trp142 of SOX10, both belonging to the hydrophobic core, are indicated in green and pink, respectively.

(B) Subcellular localization of wild-type and mutant proteins by immunostaining. HeLa cells transfected with wild-type (left panels) or mutant (other panels) SOX10 constructs. Nuclei were counterstained with TO-PRO-3 iodide (blue). Transfected cells were immunostained with anti-SOX10 antibodies (red). Images shown are with the D20 antibody, but similar results were obtained with N20directed against the amino terminus of the protein. The merged images are presented below. A higher magnification of SOX10 labeling is also shown in gray (bottom panels).

(C) Three-dimensional representation of the location of the HMG domain residues affected by themissense mutations identified in "random" KS individuals, as in (A). Upper panel: the residue corresponding to Arg151, in contact withthe hydrophobic core, is indicated in green. Lowerpanel: the residue corresponding to Met108, in contact withthe DNA target, is indicated in green.

(D) Subcellular localization of the Arg151Cysand Met108Thr mutant proteins by immunostaining, as in (B).

(E) Schematic representation of the SOX10 protein and mutations found in this study.

D: dimerization domain; K2: K2 domain; HMG: HMG domain; TA: transactivation domain.

Figure 3. SOX10 expression in the mouse embryonic olfactory system

(A) Doubleimmunolabeling of E12.5 embryo head (coronal sections) forSOX10 (red), the neuronal marker TUJ1 (blue),or the OEC marker BLBP (green). The boxed regions are the migratory mass and the nasal mesenchyme magnified in (B).

(B) Higher magnification of the vomeronasal nerve (left panel), nasal mesenchyme (middle panel), and migratory mass (right panel). Triple labeling forSOX10 (red), the neuronal marker TUJ1 (blue), and anOEC marker (green;BLBP, P75,or S100 as indicated). The dotted lines represent the limit between the olfactory epithelium and the mesenchyme.

(C) Double labeling of E14.5 embryos olfactory epithelium and mesenchyme (coronal sections) forSOX10 (red), the neuronal marker TUJ1 (blue), or the OEC marker BLBP (green).

(D) Triple labeling of E14.5 embryo olfactory bulbs (coronal sections) forSOX10 (red), the neuronal marker TUJ1 (blue), or the OEC marker BLBP or P75 (green) as indicated. The boxed regions in the medial ONL are magnified in (E).

(E) Higher magnification at the level of the medial ONL.Triple labeling forSOX10 (red), the neuronal marker TUJ1 (blue), and an OEC marker (green;BLBP, P75, or S100 as indicated).

M: mesenchyme; MM: migratory mass; OB: olfactory bulb; OE: olfactory epithelium; VNO: vomeronasal organ.

Figure 4. SOX10 expression in the human embryonic olfactory system

(A and B) Immunostaining of the head of a human fetus at 8 weeks of embryonic development (sagittal sections), at the level of the nasal mesenchyme (A) or migratory mass and olfactory bulb (B). Double labeling for SOX10 (green) and the neuronal marker TUJ1 (blue),and counterstaining with DAPI (blue).

(C) Immunostaining showing an olfactory nerve at higher magnification. Double staining for SOX10 (green) and the OEC marker S100 (red) and counterstaining with DAPI.

M: mesenchyme; MM: migratory mass; OB: olfactory bulb; OE: olfactory epithelium.

Figure 5.OEC defectinthe E14.5*Sox10* mutant mice

(A)Whole-mount X-Gal staining of the head in heterozygous (*Sox10*$^{lacZ/+}$, left panel) and homozygous (*Sox10*$^{lacZ/lacZ}$, right panels) mutant embryos, facial view. The skin was removed to allow visualization of the olfactory bulbs, indicated by the black arrowheads.

(B) General overview of the olfactory system upon triple labeling for β-galactosidase (green), the neuronal marker TUJ1 (blue), and the OEC marker BLBP (red) in *Sox10*heterozygous (left panel) and homozygous (right panels) mutant embryos.

(C) Higher magnification of the regions boxed in (B), showing the presence of OECs ensheathing the vomeronasal nerve fibers in the heterozygous *Sox10*mutant embryos and the absence of these cells in the homozygous embryos.

(D and E) Higher magnification of the nasal mesenchyme (D) or ONL (E) immunostained for anOEC marker (BLBP, P75, or S100; red), β-galactosidase (green), and the neuronal marker TUJ1 (blue), as indicated in the figure, in *Sox10*heterozygous (left panels) and homozygous (right panels) mutant embryos.

MM: migratory mass; NS: nasal septum; OB: olfactory bulb; OE: olfactory epithelium.

Figure 6. Abnormal nerve fasciculation, axonal pathfinding, and GnRH-cell migration in *Sox10* mutant mice

(A) TUJ1 (upper panel) and β-galactosidase (middle panel) immunostaining and merged image (lower panel) over the nasal septum at asimilar level of heterozygous (*Sox10*$^{lacZ/+}$) and homozygous (*Sox10*$^{lacZ/lacZ}$) mutantE14.5 embryos, as indicated. The arrow and arrowhead in

the homozygote indicate the defasciculation of sensory axons and their misrouting over the nasal septum, respectively.

(B) GnRH-cells along the vomeronasal nerve trajectory in wild-type (left panel) and homozygous *Sox10*mutant (right panel) E14.5 embryos. The GnRH immunostaining is in red, and DAPI staining is blue. Insets show higher magnifications.

(C) Box plots showing quantification of the number of GnRH-cells along the trajectory of the vomeronasal nerve in wild-type, heterozygous, and homozygous mutant mouse embryos. Each dot corresponds to the number of GnRH-positive cell bodies counted on one side of a section. The top and bottom of each box represent the 25^{th} and 75^{th} percentiles, respectively. The middle line is the median. Statistical significance was tested with Student's ttest. n.s., not significant; *** indicates a P value <0.0001.

(D) DAPI (upper panel) and GnRH (lower panel) staining of sections at the level of the preoptic area in the wild-type (left), heterozygous (center) and homozygous (right) E14.5 embryos.

OB: olfactory bulb; VNO: vomeronasal organ; NS: nasal septum; OE: olfactory epithelium.

Figure 7. Summary of the defects observed in the *Sox10* mutant embryo

(A) Schematization of the cut planes (a and b) drawn in (B) on the head of an E14.5 mouse embryo.

(B) Summary of the defects observed in the *Sox10* homozygous mutant embryos ($Sox10^{lacZ/lacZ}$, right panel) compared to wild-type embryos (left panel). The OECs are shown in green, the nerve fibers in blue, and the GnRH-synthesizing cells in red.

OE: olfactory epithelium; OB: olfactory bulb; POA: preoptic area; VNO: vomeronasal organ.

Table 1. Phenotypes of the KS individuals carrying*SOX10* mutations

Case	Age (year)	Gender	Occurrence	FSH (UI/L) basal-peak	LH (UI/L) basal-peak	E2 (pg/ml)	T (ng/ml)	Sponta-neous puberty	Sense of smell	Olfactory bulb (MRI)	Hearing	Other clinical signs	Mutation (DNA)	Mutation (protein)	In silico evidence	In vitro evidence
Clinically selected KS individuals																
A	26	M	familial	0.2-2.0	0.4-2.1		<1.7	no	anosmia	agenesis	unilateral deafness	micropenis, cryptorchidism, white hair (22 years old)	c.2T>G	p.?	pathogenic	pathogenic
B	18	F	sporadic	2.8-ND	0.3-ND	<10		no	anosmia	agenesis	prelingual deafness	ptosis	c.331T>G (de novo)	p.Phe111Val	pathogenic	pathogenic
C	39	F	sporadic	<0.5-ND	0.87-ND	42		no	anosmia	ND	prelingual deafness	obesity (BMI=51)	c.424T>C	p.Trp142Arg	pathogenic	pathogenic
D	25	F	familial (anosmia + deafness in mother)	2.1-7.9	0.8-8	20		delayed	anosmia	agenesis	prelingual deafness		c.698-1G>C	p.spl?	pathogenic	
E	20	M	sporadic					delayed	anosmia	ND	prelingual deafness	cryptorchidism *SEMA3A*: p.Val435Ile (+/-)	c.1290del (de novo)	p.Ser431Argfs*71	pathogenic	pathogenic
F	20	M	sporadic						anosmia	ND	normal	intellecual disability, dysmorphy, polymalformation	c.1298G>A	p.Arg433Gln	pathogenic	not pathogenic
Random KS individuals																
G	33	M	familial? (a brother likely anosmic)	0.8-0.9	0.41-0.6		0.23	no	anosmia	ND	hypoacusis	ptosis	c.323T>C	p.Met108Thr	pathogenic	pathogenic
H	19	F	sporadic	<1-4.4	0.5-8	<0.1		no	anosmia	agenesis	normal	macroscelia	c.451C>T	p.Arg151Cys	pathogenic	pathogenic

Age is indicated at time of DNA sampling, usually age of diagnosis. The following abbreviations were used: BMI, body mass index; E2, estradiol; FSH, follicle stimulating hormone; LH, luteinizing hormone; MRI, magnetic resonance imaging; ND, not determined; nt, nucleotide; T, testosterone.Normal range: FSH/LH: 2-20 UI/L; E2: >20 pg/mL; T: 2-10 ng/mL.