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Mutation update: Waardenburg Syndrome

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Abstract

Waardenburg syndrome (WS) is characterised by the association of pigmentation abnormalities, including depigmented patches of the skin and hair, vivid blue eyes or heterochromia irides, and sensorineural hearing loss. However, other features such as dystopia canthorum, musculoskeletal abnormalities of the limbs, Hirschsprung disease or neurological defects are found in a subset of patients and used for the clinical classification of WS.

Six genes are involved in this syndrome: *PAX3* (encoding the paired box 3 transcription factor), *MITF* (microphthalmia-associated transcription factor), *EDN3* (endothelin 3), *EDNRB* (endothelin receptor type B), *SOX10* (encoding the Sry bOX10 transcription factor) and *SNAI2* (snail homolog 2), with different frequencies.

In this review we provide an update on all WS genes and set up mutation databases, summarize molecular and functional data available for each of them and discuss the applications in diagnostics and genetic counseling.

Introduction

Waardenburg syndrome (WS) is named after the Dutch ophthalmologist who first described the association between deafness, depigmentation features and dysmorphology that is now known as type I WS (MIM193500) [Waardenburg, 1951]. Its prevalence is estimated 1/42.000 and it is responsible for 1-3 % of total congenital deafness [Read and Newton, 1997]. It is often described as an autosomal dominantly inherited disorder of neural crest cells (NCC), but we now know that WS is clinically and genetically heterogeneous and that not all forms are dominantly inherited.

Following the involvement of *PAX3* (paired box 3) in type I WS as well as in type III WS (also called Klein-Waardenburg syndrome [Klein, 1947; Klein, 1983], MIM148820) [Baldwin et al., 1992; Hoth et al., 1993; Tassabehji et al., 1992], it appeared that another gene, *MITF* (microphthalmia-associated transcription factor), was altered in a subset of patients who did not present dystopia canthorum (an outward displacement of the inner canthus of the eyes typical type I WS) and were therefore classified into type II WS (MIM193510, [Arias, 1971]) [Tassabehji et al., 1994b]. Shortly after, the endothelin pathway (*EDN3*, endothelin 3; *EDNRB*, endothelin receptor type B) was found to be involved in the fourth subtype of WS (also called Shah-Waardenburg syndrome [Shah et al., 1981], MIM277580), characterized by the association of deafness, depigmentation and intestinal aganglionosis (called Hirschsprung disease (HD)) [Edery et al., 1996; Hofstra et al., 1996; Puffenberger et al., 1994a; Shah et al., 1981]. In 1998, the study of a mouse model led to the identification of another type IV WS gene: *SOX10* (Sry bOX10) [Pingault et al., 1998]. Mutations in this gene are also responsible for an extended syndrome involving peripheral and central neurological phenotypes, referred to as PCWH (peripheral demyelinating neuropathy, central dysmyelinating leucodystrophy, Waardenburg syndrome, Hirschsprung disease) [Inoue et

al., 2004]. More recently, *SOX10* mutations were also found in type II WS with or without neurological impairment [Bondurand et al., 2007]. For each of these genes, mutations are mostly private and a high intra- and inter-familial variability is reported. We set up mutation databases for these genes at http://grenada.lumc.nl/LOVD2/WS/. However, despite the increasing number of genes involved in this syndrome and the molecular overlap between subtypes, a number of cases remain unexplained at the molecular level.

Clinical features and classification

During development, the pluripotent NCC migrate from the neural tube throughout the embryo along several pathways and give rise to different cell types, including melanocytes of the skin and inner ear, glia and neurons of the peripheral and enteric nervous systems, and some of the craniofacial skeletal tissue [Le Douarin and Kalcheim, 1999]. Defects in neural crest (NC) development are a significant cause of human disease. The term neurocristopathies collectively refers to these NC disorders [Bolande, 1974]. The association of hearing loss and pigmentary abnormalities characteristic of WS results from an abnormal proliferation, survival, migration or differentiation of NC-derived melanocytes. The four subtypes of WS were defined on the basis of the presence or absence of additional symptoms [Read and Newton, 1997], type I and II being the most frequent: Type I WS (WS1): dystopia canthorum; Type II WS (WS2): no additional feature; Type III WS (WS3): dystopia canthorum and musculoskeletal abnormalities of the upper limbs; Type IV WS (WS4): HD.

The sensorineural hearing loss is the most frequent feature (from 60% in WS1 to 90% in WS2) [Newton, 1990]. Its severity is very variable within and between families, ranging from profound deafness to a progressive postlingual hearing loss [Hageman and Delleman, 1977; Hildesheimer et al., 1989; Newton, 1990]. Bilateral deafness is more frequent than unilateral and can be asymmetrical [Newton, 1990]. In WS2, the hearing defect is progressive in 70% of cases [Hildesheimer et al., 1989]. There is no typical audiogram shape. The reported prevalence of

temporal bone abnormalities varies from 0 to 50% [Madden et al., 2003; Oysu et al., 2001]. The most frequent inner ear malformations are vestibular aqueduct dilatations and semicircular canal malformations (dilatation to absence) [Higashi et al., 1992; Madden et al., 2003]. The prevalence of vestibular dysfunctions is not well established [Black et al., 2001; Hageman and Oosterveld, 1977]. Vertigo is rare but vestibular function abnormalities at caloric or rotation tests were described [Black et al., 2001; Hageman and Delleman, 1977; Hildesheimer et al., 1989]. A walking delay can occur in cases of vestibular bilateral areflexia associated with semicircular canal malformations.

A white forelock is present in at least one third of both WS1 and 2. It is located in the middle of the forehead and continues posteriorly [da-Silva, 1991; Hageman and Delleman, 1977]. In absence of forelock, one third of the WS patients present with premature greying of the hair before 30 years. White eyebrows or eyelashes can be observed. Hypoplastic irides, particularly brilliant blue eyes, are present in 10% of WS patients. The heterochromia, observed in about 30% of WS1 and 2 cases, can be complete (one normal irid and one hypoplastic) or segmental (with a part of hypoplastic irides) [Ohno et al., 2003]. Fundus pigmentation varies with the iris pigmentation without visual impairment. In case of heterochromia, the fundus is also heterogeneous, being blond or albinoid in the regions corresponding to hypoplastic irides [Ohno et al., 2003]. The skin pigmentary disorders are less frequent (10-20%), mostly represented by patchy depigmented areas [Liu et al., 1995a] that are preferentially located on limbs extremities, abdomen and thorax.

WS1 and 3 are characterized by a peculiar facial finding: dystopia canthorum (or telecanthus), a lateral displacement of inner canthus of the eyes, considered as the most reliable feature for WS1 classification due to its very high penetrance [Read and Newton, 1997]. The W index use biometric measurement to estimate dystopia canthorum, with a threshold reevaluated in light of the molecular data [Arias and Mota, 1978; Farrer et al., 1994; Read and Newton, 1997]. The nasal root is frequently high and broad in WS1 and 2. Synophrys is much more frequent in WS1 (85%) than in WS2 (25%) [Liu et al., 1995b].

Musculoskeletal anomalies are found in WS3. Patients present with flexion contractures and muscle hypoplasia of the upper limbs with a broad range of severity [Klein, 1947; Klein, 1983; Read and Newton, 1997]. Other signs such as camptodactyly may be associated [Hoth et al., 1993; Sheffer and Zlotogora, 1992]..

WS4 is defined by the association with HD [Meire et al., 1987; Shah et al., 1981]. The extent of the intestinal aganglionosis and the phenotypic manifestations are variable and could lead to severe occlusion in neonates [Karaca et al., 2009]. A few patients present with an intestinal phenotype not related to HD (chronic intestinal pseudo-obstruction: obstruction without an identified cause or obstacle) [Valenzuela et al., 1995].

Neurological features are found in a subset of WS4. They include peripheral neuropathy, mental retardation, cerebellar ataxia and spasticity [Kawabata et al., 1987; Touraine et al., 2000], leading to the definition of a specific entity called PCWH [Inoue et al., 2004]. More recently, these neurological features are also been found in WS2 patients [Bondurand et al., 2007].

PAX3: the paired box 3 transcription factor / WS1, WS3

PAX3 encodes a transcription factor (TF) belonging to the family of paired-containing proteins, named from the Drosophila *paired* (*prd*) gene and playing a role in the maintenance of stem cell pluripotency, cell-lineage specification, proliferation, migration, apoptosis and inhibition of terminal differentiation. Among the 9 members of this family, several are involved in human genetic diseases. Alterations in *PAX3* are found in human malignancies, i.e. rhabdomyosarcoma, melanoma, neuroblastoma [Kubic et al., 2008; Wang et al., 2008] and in WS.

PAX3 is involved in the development of the central nervous system, somites, skeletal muscle, NC-derived lineages including cardiac tissue, melanocytes and enteric ganglia. It is required to expand a pool of committed melanoblasts or restricted progenitor cells early in development but prevents their terminal differentiation [Kubic et al., 2008]. In cooperation with several other TF, it activates or represses the expression of several markers of melanocyte development: MITF,

DCT/TRP2, TRP1 [Galibert et al., 1999; Lang et al., 2005; Watanabe et al., 1998]. It is also necessary for induction, proliferation and migration of muscle cell precursors in the dorsal dermomyotome while preventing their terminal differentiation. In line with this function, it regulates the expression of the muscle-specific TF, myoD and myf-5 [Bajard et al., 2006; Bendall et al., 1999]. PAX3 also controls key regulators of NC development, such as c-RET, TGF-β2 and WNT1 [Fenby et al., 2008; Lang and Epstein, 2003; Mayanil et al., 2006]. The dependence on PAX3 function for proper NC development is highlighted by the phenotype induced by PAX3 mutations in human and mice.

The *Splotch* locus and *Pax3*, closely mapped on mouse chromosome 1, were identified as the same gene in 1991 [Epstein et al., 1991]. Six *Splotch* mutants, resulting from different spontaneous or radiation-induced mutations of *Pax3*, as well as targeted mutants, present a range of phenotypes including defects of NC derivatives (melanocytes, peripheral nervous system, thyroid, cardiac NCC), limb musculature hypoplasia, neural tube anomalies (spina bifida, exencephaly), and central nervous system abnormalities [Kubic et al., 2008]. Most notable in heterozygous *Splotch* mice is the patchy loss of pigmentation, especially on abdomen, tail and feet. Homozygosity results in death during gestation or shortly after birth.

PAX3 is composed of 10 exons on chromosome 2q35. An alternative acceptor splice site in exon 3 generates isoforms containing or lacking Glutamine 108, referred to as Q+ and Q- isoforms, that have distinct DNA-binding properties [Vogan et al., 1996]. The major PAX3 protein, with 479 amino acids in its Q+ form, results from the use of a stop codon in exon 8 and contains two DNA binding domains (the paired domain and a homeodomain), a conserved octapeptide located in between (mediating protein-protein interactions), and a Ser/Thr/Pro-rich carboxy-terminal transactivation domain [Lalwani et al., 1995; Read and Newton, 1997]. More recently, minor transcripts have been described, resulting from an alternative splicing before the exon 8 stop codon and use of exons 9 and 10 to generate an alternative C-terminal end of the protein [Barber et al.,

1999]. Their physiological and physiopathological significances are unknown but in vitro studies suggest that some of them have functional capacities similar to the major isoform.

The *PAX3* gene was found to be involved in WS1 following the identification of a patient with a de novo inversion (inv(2)(q35q37.3)) of a region syntenic to the mouse *Splotch* locus. The first heterozygous mutations were discovered in an incomplete form of the gene, referred to as *HuP2* and containing only exons 2, 3 and 4 [Baldwin et al., 1992; Tassabehji et al., 1992]. Since then, about 70 different *PAX3* point mutations have been identified, very few of them being recurrent. Missense and nonsense mutations, frameshifts, small in-frame insertions or deletions and splice alterations have been described. Altogether, about half of the mutations reported are missense and half are truncating variations. Partial or total gene deletions have also been described and may represent 10% of cases without identified point mutations [Lu-Kuo et al., 1993; Milunsky et al., 2007; Pasteris et al., 1993; Soejima et al., 1997; Tassabehji et al., 1994a; Wu et al., 1993]. About 95% of *PAX3* mutations are located throughout exons 2-6, but this observation might be slightly biased due to incomplete analysis performed by several groups (exons 1, 7 and 8 are not included in all the screenings performed). The highest rate of mutation is found in exon 2, accounting for the frequency of missense mutations in the paired domain, then in exons 5-6, which both encode the homeodomain. We did not find any mutation in the recently described exons 9 and 10. The previously published and 13 new *PAX3* mutations are reported in Table 1.

Heterozygous *PAX3* mutations are expected to be responsible for most, if not all, WS1 cases. They are found either as de novo variations in sporadic cases, or inherited on a dominant mode with variable expressivity. The disease is thought to be fully penetrant when considering at least one sign, but the penetrance of each feature is not complete. Homozygous or compound heterozygous *PAX3* mutations have been described in severe cases of WS3, with extended depigmentation and upper limb defects, sometimes leading to death in early infancy or in utero [Bottani et al., 1999; Wollnik et al., 2003; Zlotogora et al., 1995]. In these cases, heterozygous relatives present with

WS1. However, *PAX3* mutations have also been found at the heterozygous state in moderate cases of WS3 [Hoth et al., 1993; Read and Newton, 1997; Tassabehji et al., 1995; Tekin et al., 2001].

There is no obvious correlation between the deleted, truncating or missense nature of the mutation, its location, and the severity of the disease [Baldwin et al., 1995; Tassabehji et al., 1995], arguing that gene dosage plays an important role in the physiopathology of the syndrome and that point mutations abolish PAX3 ability to bind and activate its transcriptional targets [Baldwin et al., 1995]. However, a statistical study of 271 patients from 48 independent families showed a slight correlation between truncation mutations / deletions and the penetrance of white forelock or skin pigment abnormalities [DeStefano et al., 1998]. Although incompletely penetrant, deafness seem to be clustered in some families, supporting the hypothesis that stochastic events do not solely account for its expression and that genetic factors and/or the environmental background influence the phenotype [Morell et al., 1997a].

A few patients present with spina bifida, but no correlation with the nature or location of the mutation is apparent [Baldwin et al., 1995]. The only WS2 patient carrying a *PAX3* mutation [Tassabehji et al., 1993] was subsequently reclassified as WS1 [Farrer et al., 1994]. One mutation was described in a patient with Craniofacial-Deafness-Hand Syndrome (CDHS, OMIM #122880), that shares several clinical characteristics of WS1/3 associated with specific dysmorphic features [Asher et al., 1996]. Another mutation was found in a WS1 family among whom one patient also exhibited Septo-Optic Dysplasia (SOD) but the connection between WS1 and SOD was not established [Carey et al., 1998].

Missense mutations are almost exclusively located within the two DNA binding domains. The 3D crystal structure of a paired domain revealed two structurally independent globular structures, a N-terminal domain (containing a short region of antiparallel β sheets and three α helices) that made extensive DNA contacts and a C-terminal domain, whose contribution to DNA binding is less critical in vitro but may vary according to the PAX protein and/or the model used to achieve target specificity [Vogan and Gros, 1997; Xu et al., 1995]. When compared to this structure, most of the

paired domain missense mutations (15/20) concerned invariant residues of the N-terminal domain involved in direct contact with the DNA target. On the other hand, missense mutations located in the homeodomain may be classified into two categories: mutations affecting the stability of the domain fold by disrupting the hydrophobic core that maintains its conformation, and mutations affecting residues involved in sequence-specific or non specific DNA binding [Birrane et al., 2009; Chi, 2005]. Among the latter, several are located in two hotspots of homeodomain protein mutations [Chi, 2005], corresponding to Arg223 and Arg270/Arg271 of human PAX3.

A few mutations have been tested for their functional consequences. The tests mostly included DNA-binding activity and transactivation capabilities [Chalepakis et al., 1994; Corry and Underhill, 2005; Fortin et al., 1997; Watanabe et al., 1998]. Interestingly, several missense mutations of the paired domain or the homeodomain showed a reciprocal effect on the DNA-binding properties [Fortin et al., 1997], thereby explaining identical phenotypic consequences. More recently, a study of mutant protein dynamics and subnuclear localization concluded that altered nuclear dynamics might be the primary defect for some of the mutations, possibly through an impaired ability to form functional complexes during transcription [Corry et al., 2008]. The only two missense mutations located outside the DNA-binding domains but occurring at intron-exon junctions, Ala196Thr and Gln391His, did not show any subnuclear localization abnormality, consistent with the idea that they may rather contribute to PAX3 dysfunction by aberrant splicing [Corry et al., 2008].

MITF: the microphthalmia-associated transcription factor / WS2

The microphthalmia-associated transcription factor belongs to the Myc supergene family of b-HLH-Zip (basic helix-loop-helix leucine zipper) proteins that function as homo- or heterodimeric TF. Mutations and aberrant expression of *MITF* are reported in human melanoma [Steingrimsson et al., 2004].

MITF is known as the key TF of melanocyte development. Through binding on DNA sequences called E-boxes and especially to a more specific sequence called the M-box, it regulates

melanocyte differentiation and transcription of several melanocyte-specific genes, including tyrosinase (*tyr*) and the tyrosinase-related proteins *trp1* and *Dct/trp2* [Bertolotto et al., 1998; Yasumoto et al., 1994]. But MITF more extensively regulates many aspects of melanocyte development: cell survival, cell cycle progression and proliferation through Bcl2, c-Met, tbx2, ink4A/P16, p21 and CDK2 [Hou and Pavan, 2008; Steingrimsson et al., 2004]. Finally, it provides a link between several TF and signaling pathways that regulate melanocyte development, including PAX3 and SOX10 [Bondurand et al., 2000; Lee et al., 2000; Potterf et al., 2000; Verastegui et al., 2000]. MITF is also involved in the development of other cell types, such as osteoclasts and mast cells [Steingrimsson et al., 2004].

Spontaneous, chemical, radiation or insertional mutagenesis have provided an abundance of mouse *mitf* alleles, either semi-dominant or recessive. All have reduced (either patchy or diluted) or absent pigmentation due to a loss of melanocytes. Some of them also present with deafness, small or absent eyes due to a failure of RPE (retinal pigment epithelium) development, osteopetrosis, mast cells defects [Moore, 1995; Steingrimsson et al., 2004]. Sequestering of wild type MITF (or other TF) in inactive dimers by some of the mutant proteins has been proposed to explain part of the phenotypic variability and differences observed in the mode of inheritance [Hemesath et al., 1994; Moore, 1995; Takebayashi et al., 1996].

The *MITF* gene contains nine alternative promoters and first exons that produce differentially expressed isoforms following a complex pattern of splicing [Hershey and Fisher, 2005]. The M (for melanocytic) isoform, composed of 9 exons (1M, 2-9), encodes a protein of 419 amino acids [Tassabehji et al., 1994b]. Although differing by their amino terminus, all isoforms contain a basic DNA binding domain (b), followed by a helix-loop-helix (HLH) motif and a leucine zipper (Zip) involved in homo- or heterodimerisation with the related TF TFE3, TFEB and TFEC [Hemesath et al., 1994]. Several transactivation domains are described, the best characterized being N-terminal to the bHLH-Zip motif [Hou and Pavan, 2008; Steingrimsson et al., 2004]. The use of alternative splice acceptor sites at the beginning of exon 6 generates two proteins that differ by six internal

amino acids, called (+) and (-) isoforms, the longest being used as the reference [Tachibana et al., 1994]. Their expression has been studied in mouse but their respective roles have not been fully characterized, though are possibly related to cell proliferation and transcriptional activities [Bismuth et al., 2005; Murakami et al., 2007].

Soon after *PAX3* involvement in WS1 and exclusion in WS2 [Farrer et al., 1994], WS2 was linked to 3p12-14.1 in two large families [Hughes et al., 1994]. According to the observation that the *microphthalmia* homologue mapped within the interval, and on the basis of the mouse mutants phenotype [Asher and Friedman, 1990], *MITF* mutations were soon identified in human [Tassabehji et al., 1994b]. Here, we report 7 new mutations in addition to the 14 previously described (Table 2). Only the M isoform is routinely studied, but except for two exon 1M splice alterations, mutations are expected to affect all MITF isoforms. Point mutations are not equally scattered along the gene: most of them are located in exons 7 and 8 that correspond to the b- HLH-Zip motifs. Interestingly, several reproduce the exact equivalent of mouse mutants. Except for p.Ser298Pro (see functional tests below), missense mutations located out of the basic domain lie within alpha helices of the functional domains (p.Ile224Ser, p.Ser250Pro, p.Tyr253Cys in the HLH domain, p.Asn278Asp in the leucine zipper) ([Tassabehji et al., 1995] and this paper). A small proportion of gene deletions is also described [Milunsky et al., 2007; Schwarzbraun et al., 2007].

It is estimated that *MITF* mutations occur in about 15% of WS2 [Read and Newton, 1997], which has long been reported as genetically heterogeneous [Hughes et al., 1994]. *MITF*-linked WS is therefore called WS2A. Mutations are found at the heterozygous state, resulting from dominant inheritance or occurring de novo [Read and Newton, 1997]. The variable phenotype and incomplete penetrance of each feature result in families in which some affected patients present with an isolated sign of the disease, which would be insufficient to make the diagnosis on its own [Lautenschlager et al., 1996]. Digenic inheritance of WS with ocular albinism has been suggested in two families segregating a *MITF* mutation as well as a significant *TYR* (tyrosinase) polymorphism (Arg402Gln,

R402Q) or a new *TYRP1* (tyrosinase-related protein 1) missense variation (Pro513Arg, P513R) [Chiang et al., 2009; Morell et al., 1997b].

Consistent with the observation that eye defects and osteopetrosis in mice occur only in homozygotes, neither microphthalmia nor osteopetrosis have been observed in human with *MITF* heterozygous mutations [Steingrimsson et al., 2004]. Of note, we recently found a *MITF* mutation in a patient with multiples osteochondromas (see Table 2) but whether this particularity may be attributed to the *MITF* mutation remains to be explored. The phenotype variability observed in a single family suggests caution in analyzing genotype/phenotype correlations. So far there is a noteworthy relationship between non-truncating mutations of the basic domain and Tietz syndrome (characterized by a uniform dilution of pigmentation instead of the patchy depigmentation seen in WS) ([Izumi et al., 2008; Smith et al., 2000; Tassabehji et al., 1995] and this paper). It has been hypothesized that these mutations exert a dominant negative effect, whereas truncated proteins or mutations disrupting dimerization result in WS2 through haploinsufficiency [Smith et al., 2000]. However, a full *MITF* deletion was recently described in a patient presenting with a phenotype intermediate between Tietz Syndrome and WS2, leading to the hypothesis that WS2-causing *MITF* point mutations keep a certain amount of functionality [Schwarzbraun et al., 2007]. The specificity of the phenotype observed in this case may also result from genetic modifiers or a contiguous gene syndrome, as we recently found two other full *MITF* gene deletions in patients with the classical form of WS2 (unpublished results).

Few functional tests have been performed on human *MITF* mutations. Two nonsense mutations were shown to abrogate DNA binding and transactivation of the tyrosinase promoter, with no evidence of a dominant negative effect on the wild type MITF protein [Nobukuni et al., 1996]. They are therefore most likely loss-of-function mutations that result in haploinsufficiency. It appears that mutation of the Ser298 impairs phosphorylation by GSK-3β in vitro and decreases MITF DNA-binding [Takeda et al., 2000] although the existence and significance of such a phosphorylation during development is not established [Steingrimsson et al., 2004]. In addition,

several human mutations are identical to mouse *mitf* alleles with known functional effects. The *mi* allele (Arg217del in human) impairs nuclear localization of the resulting protein and has an inhibitory effect on nuclear localization potential of wild-type MITF [Takebayashi et al., 1996]. The mi^{or} allele (Arg216Lys in human) abrogates DNA binding to E-boxes and precludes binding of wild type MITF homodimers [Hemesath et al., 1994].

EDN3 and *EDNRB*: the endothelin pathway / WS4 (±WS2)

The endothelins are a group of 3 peptides (ET1, ET2, ET3) that mediate their effect through two G protein-coupled heptahelical receptors called ETA and ETB. Although ETB can bind all three endothelins with comparable affinities, the observation of similar phenotypes in ET3 and ETB human and mouse mutants indicates that ET3 is the main physiological ligand for ETB [McCallion and Chakravarti, 2001].

A large number of studies have established that the signaling mediated by endothelins plays an essential role in the development of NC-derived cell lineages. In vertebrates, *Ednrb* (encoding the endothelin receptor type B, ETB) is first expressed at the dorsal tip of the neural tube, then in NCC on both dorso-ventral and dorso-lateral pathways [Parichy et al., 2000]. ETB function has been shown to be required between E10 and E12.5 [Shin et al., 1999], a period that corresponds to the migration / differentiation of enteric nervous system (ENS) precursors within the gut and to the migration of melanoblasts along the dorso-lateral pathway. Thus, this signaling pathway seems to be important for the survival, proliferation and/or migration of mouse melanoblasts, but not for their determination or proliferation before migration (in the migrating staging area; MSA). ET3 also prevents the premature differentiation of enteric neuroblasts [Bondurand et al., 2006; Nagy and Goldstein, 2006]. ETB signaling in cultured human melanocytes influence MITF expression and posttranslational modifications [Sato-Jin et al., 2008], indicating the existence of common pathways between WS genes (see also "biological relevance").

Identification of the critical role of ET3/ETB in melanocytes and enteric development, via targeted disruption of the mouse genes *Edn3* and *Ednrb* was somewhat unexpected [McCallion and Chakravarti, 2001; Pla and Larue, 2003; Tachibana et al., 2003]. Targeted deletion of *Ednrb* resulted in an autosomal recessive phenotype with white spotting and aganglionic megacolon. By phenotypic non complementation and molecular analysis, it was then showed that *Ednrb* was allelic to two other well known hypopigmentation mutations in mouse (*piebald*, *s*, hypomorphic mutation; and *piebald-lethal*, s^l, gene deletion) [Hosoda et al., 1994]. In a similar manner, targeted deletion of the *Edn3* gene was generated and found allelic to the mouse *lethal spotting* (*ls*, missense mutation) [Baynash et al., 1994]. The extent of white spotting is precisely dependent on the dosage of *Ednrb* expression, while the megacolon phenotype occurs almost only in s^l/s^l mice among *Ednrb* genotypes. This is compatible with the idea that the two NC-derived cell lineages require different minimal threshold levels of *Ednrb* expression [Baynash et al., 1994; Hosoda et al., 1994]. Since then, other mouse models have been generated [Druckenbrod et al., 2008; Matera et al., 2007; Matsushima et al., 2002; Shin et al., 1999]. Of note, the NC-specific excision of the receptor is sufficient to produce a WS4 phenotype.

Endothelins are 21 amino acid peptides derived from preproendothelins by a 2-step procedure: first, a step of cleavage to proendothelin (also called “big endothelin”) by a non-specific endopeptidase, then second cleavage to mature endothelin by an endothelin-converting enzyme (ECE1) [Kurihara et al., 1999]. Mature endothelins contain four cysteines involved in two disulfide bonds. A domain called the ET-like peptide (as its sequence is homologous to the sequence of the mature endothelin) is located in the carboxy-terminal part of the preproendothelin that is removed by the first cleavage step (see Fig. 4). Preproendothelin3 is encoded by a five exon *EDN3* gene located at 20q13.2-13.3. Several isoforms result from alternative splicings at the level of exons 4 and 5 [O'Reilly et al., 1992; Onda et al., 1990]. As they are all localized distal to the ET-like peptide in a early-cleaved region, their functional consequences are unclear.

The endothelin receptor type B is encoded by the *EDNRB* gene that encompasses seven exons on 13q22 [Arai et al., 1993]. ETB is a 442-residue protein belonging to the G protein-coupled heptahelical superfamily, with an extracellular amino-terminal part and an intracytoplasmic carboxy-terminus. Although several isoforms have been described, their expression and function during development are unknown [Elshourbagy et al., 1996; Shyamala et al., 1994; Tsutsumi et al., 1999]. Some are predicted to result in a different amino-or carboxy-terminus end. To our knowledge, their involvement in WS has not been tested so far.

As described above, knock-out of the *Edn3* and *Ednrb* genes in mouse revealed unexpected depigmentation and megacolon phenotypes [Baynash et al., 1994; Hosoda et al., 1994]. Concomitantly, linkage analysis performed on a large inbred Menonnite family segregating HD with associated WS features in a subset of patients led to demonstrated the involvement of the 13q22 region, then analysis of the *EDNRB* coding sequence revealed the presence of a missense mutation (Trp276Cys) that was neither necessary nor sufficient to explain HD [Puffenberger et al., 1994a; Puffenberger et al., 1994b]. Heterozygous and homozygous mutations of both *EDNRB* and *EDN3* were subsequently identified in isolated HD (heterozygous) and WS4 (mostly homozygous) patients; only the mutations involved in WS will be reviewed here. Regarding *EDNRB*, missense mutations have mostly been identified and are located throughout the protein (see Fig. 3). A few truncating mutations and several deletions are also characterized. Few *EDN3* mutations have been characterized so far, either truncating or missense (see Fig. 4). They are located in exons 2 and 3 (exons 1, 4 and 5 correspond to the cleaved amino and carboxy terminus, respectively). All the published mutations associated with WS, along with new identified ones, are reported in Table 3 and Table 4. As the phenotype of heterozygous *EDNRB* and *EDN3* carriers clearly overlap that of WS2 in some families, we also screened these two genes in a cohort of 30 WS2 and found only one heterozygous *EDNRB* mutation in a family segregating three patients over two generations (see Table 3, and manuscript in preparation), indicating that, although *EDNRB* (and likely *EDN3*) mutations could be involved, they are not a major cause of WS2.

A homozygous mutation in *EDNRB* was also characterized in an autosomal recessive syndrome called ABCD and characterized by Albinism, Black lock, Cell migration disorder of the neurocytes of the gut and Deafness. This phenotype clearly overlaps that of WS4, allowing the conclusion that ABCD should not considered a separate entity, but "as Shah-Waardenburg syndrome" [Verheij et al., 2002]. Indeed, the presence of a black occipital lock on a white scalp, instead of the classically described white forelock of WS, is sometimes found associated to *EDN3* or *EDNRB* mutations in the homozygous state ([Sangkhathat et al., 2005] and unpublished data). Very recently, Tuysuz and al described patients with heterozygous proximal 13q deletions (including *EDNRB*) and one or more WS4 features as well as a review of the literature on similar cases [Tuysuz et al., 2009]. Apart from developmental delay and mental retardation, it appears that this so-called 13q deletion syndrome also includes hypertelorism and epicanthus which are important to notice and should not be mistaken for the dystopia observed in WS1. For *EDN3* as well as for *EDNRB*, there is no obvious correlation between the type of mutation involved and the phenotype of the patients. Most mutations are private, and even if a few of them are recurrent, we can often identify the origin of the several families affected to the same country or region, indicating that they are possibly distal branches of the same, original family.

Detailed analysis of the Mennonite family showed that this *EDNRB* mutation was associated with an incomplete and dosage sensitive penetrance of the phenotype [Puffenberger et al., 1994a]. Following the subsequent observations of heterozygous mutations of *EDNRB* (and to a lesser extent, of *EDN3*) in isolated HD and homozygous mutations in WS4, it was suggested that WS4 was inherited as a recessive condition and HD as a dominant condition with incomplete penetrance. Since then, however, several cases of dominant transmission with incomplete penetrance have been described in WS4, associated with *EDN3* or *EDNRB* mutations [Pingault et al., 2001; Pingault et al., 2002; Syrris et al., 1999] and this paper). Extensive review of the published mutations and our new observations reveal the following points. Among the *EDNRB* homozygous (or compound heterozygous, either proved or suspected) cases, about 70% seem to segregate with a fully recessive

transmission, while in the remaining families, some heterozygous relatives present with isolated HD, constipation or depigmentation features. At the same time, 3 families of dominant with incompletely penetrant WS4 have been described, as well as heterozygous mutations associated with isolated, usually sporadic, HD (see Table 3 for WS references; [Amiel et al., 2008] for review on HD). A similar situation is observed among *EDN3* homozygous (or compound heterozygous) mutations, except that affected heterozygous relatives present with depigmentation and deafness but no HD. Consistently, very few heterozygous mutations of *EDN3* have been reported in isolated HD (see Table 4 for WS references; [Amiel et al., 2008] for review on HD). One case of dominant WS4 associated with an *EDN3* mutation has been described. Whether phenotypic discrepancies result from the mutation involved or from the genetic background remains to be determined. However, the observation that heterozygous relatives may present with some features on one side of the family and not the other, or that the same mutation in different families results in different phenotypes in heterozygotes, argue for an influence of the genetic background. As a result, the overall transmission of *EDNRB* and *EDN3* mutations is complex, but it can be considered that homozygotes have a high probability of developing severe phenotypes, while heterozygotes may, in some instances, present one or more features of the disease with low or incomplete penetrance. To include all these data, we favor the use of not fully recessive-not fully dominant transmission.

Missense *EDNRB* mutations are scattered along the protein, in the transmembrane, intracytoplasmic or extracellular domains. They can result in an overall destabilization of the protein and diminution of the number of receptors on the cell surface, impaired ligand binding, or alteration of the transduction signal. Twelve *EDNRB* missense mutations, involved in WS or HD, have been studied at the functional level [Abe et al., 2000; Fuchs et al., 2001; Tanaka et al., 1998]. For two of them, immunofluorescence experiments showed abnormal cellular distribution compared to wild type. Several mutant receptors resulted in an abnormal intracellular Ca2+ mobilization [Abe et al., 2000; Tanaka et al., 1998]. In contrast, others presented a defect in the guanine nucleotide binding protein (G protein) Gi signaling pathway despite their normal coupling to Gq [Fuchs et al.,

2001]. However, both endothelin receptors are coupled to Gq and G12/G13 families of G proteins. Transgenic mouse lines lacking Gq/ G11 or G12/ G13 in NCC were therefore generated, but none of them showed pigmentation and ENS defects [Dettlaff-Swiercz et al., 2005]. This suggests that ET3/ETB signaling involves other G proteins, and renders part of the biochemical data difficult to extrapolate. Importantly, among the mutations initially described in isolated HD, several are now known as polymorphisms, highlighting the difficulty of interpretation. Other rare, subpopulation-specific polymorphisms may have been missed in control studies. On the other hand, due to the low penetrance in heterozygotes, the identification of a sequence variation in one control does not allow its definitive exclusion as a causative mutation. The pathogenicity of some *EDNRB* variations is therefore difficult to assess.

EDN3 missense mutations are easier to classify. Interestingly, several of them are thought to impair the processing of preproendothelin by removing or creating cysteine residues in the critical region lying between the mature peptide sequence and the ET-like peptide. A functional in vitro assay showed that one of these mutations, Cys159Phe, indeed resulted in a virtual absence of the mature ET3 product (Yanagizawa, cited in [Hofstra et al., 1997]). Other missense mutations are located in the mature endothelin sequence or at cleavage sites.

SOX10: the SRY box 10 transcription factor / WS4, PCWH, WS2

SOX10 belongs to the SOX family of TF along with about 20 other proteins involved in cell fate determination and cell lineage development. SOX is for SRY box, named from the testis determining factor whose mutations lead to sex reversal. SOX10 is closely related to SOX8 and SOX9, the latter being involved in campomelic dysplasia [Wegner, 1999]. SOX10 has also been studied in melanoma [Cronin et al., 2009; Flammiger et al., 2009] and linked to the development of schizophrenia [Maeno et al., 2007].

SOX10 is involved in the early development of NC. Its expression starts in late pre-migratory NC and is maintained in most migratory NCC. Firstly, SOX10 has been shown to play a role in

promoting cell survival prior to lineage commitment [Kapur, 1999]. Indeed, the multipotent postmigratory NCC that migrate in its absence undergo apoptosis before reaching maturation stage. Secondly, SOX10 was shown to play a role in maintaining multipotency of NC stem cells [Kelsh, 2006; Kim et al., 2003; Stolt and Wegner, 2009]. Besides these early functions, SOX10 also influences fate decisions of several NC derivatives. Its role in specification of the melanocyte lineage was highlighted by its capacity to regulate the *MITF/Mitf* gene in synergy with PAX3 [Bondurand et al., 2000; Lee et al., 2000; Potterf et al., 2000; Verastegui et al., 2000]. Since it can also directly regulate expression of genes important for melanin synthesis such as *TRYP2/Dct* or tyrosinase, a role in melanocyte differentiation was suggested [Elworthy et al., 2003; Jiao et al., 2004; Ludwig et al., 2004; Wegner, 2005]. SOX10 seems crucial for the maintenance of pluripotency of migrating enteric progenitors and their differentiation [Bondurand et al., 2006; Britsch et al., 2001; Herbarth et al., 1998; Paratore et al., 2002; Southard-Smith et al., 1998]. Indeed, forced SOX10 expression resulted in inhibition of neuronal differentiation in NC and ENS cultures [Bondurand et al., 2006; Kim et al., 2003].These functions correlate with the fact that it regulates the expression of *EDNRB* and the RET protooncogene, a tyrosine kinase receptor involved both in isolated HD and in some NC-derived tumors [Lang et al., 2000; Yokoyama et al., 2006b; Zhu et al., 2004]. Finally, SOX10 is essential for fate specification and differentiation of glial cells of the peripheral nervous system and for terminal differentiation of oligodendrocytes [Britsch et al., 2001; Hong and Saint-Jeannet, 2005; Paratore et al., 2001; Pevny and Placzek, 2005; Wegner and Stolt, 2005]. The SOX10 target genes within these cell types include genes important for glia development and identity such as ErbB3, myelin proteins (P0, MBP, PLP), and connexins 32 and 43 [Mollaaghababa and Pavan, 2003; Stolt and Wegner, 2009; Wegner, 2009].

Demonstration of the importance of SOX10 in these three NC derivatives also relied on mouse model studies. Mouse work has focused on two alleles: the *Dominant megacolon* mice (*Dom*) and a knock-in mice in which the *Sox10* coding region was replaced by the LacZ gene [Britsch et al., 2001; Herbarth et al., 1998; Southard-Smith et al., 1998]. *Sox10* heterozygous mice

show enteric aganglionosis and hypopigmentation phenotype characteristic of WS4 (white feet and a white belly spot) [Lane and Liu, 1984; Southard-Smith et al., 1999]. Homozygotes die during gestation or at birth, but the primary reason of this early lethality is unknown. Homozygous *Sox10* embryos are severely deficient in several NC derivatives such as dorsal root ganglia, several cranial ganglia, sympathetic and enteric ganglia. A complete lack of melanocytes has also been reported.

SOX10 is composed of 4/5 exons located on chromosome 22q13.1. Three exons code for a 466 amino acids protein. The 5'UTR contains one or more non-coding exons, but its structure is not fully characterized in human [Pingault et al., 1998; Pusch et al., 1998]. SOX10 contains an HMG (High mobility group) DNA binding domain, a dimerisation region right upstream of the HMG domain, a transactivation domain at the carboxy end and a conserved domain in the center of the protein which is described as a putative transactivation domain in some species [Wegner, 2009].

It was identified as the deleterious gene by positional cloning in the *Dom* mouse mutant [Herbarth et al., 1998; Southard-Smith et al., 1998] and then incriminated in the corresponding human disease [Pingault et al., 1998]. Following its initial recognition as a WS4 gene, several works described an increasing variety of phenotypes associated with *SOX10* mutations. The most important, by its frequency and severity, is referred to as PCWH syndrome (peripheral demyelinating neuropathy, central dysmyelinating leucodystrophy, Waardenburg syndrome, Hirschsprung disease) [Inoue et al., 2004; Inoue et al., 1999; Pingault et al., 2000; Touraine et al., 2000]. The peripheral demyelinating neuropathy, associated to a diminution in the NCV (nerve condution velocity) measurement, is characterized by muscle wasting or atrophy and areflexia. The central demyelinating leukodystrophy results in cerebellar ataxia, nystagmus, spasticity, mental retardation, neonatal hypotonia, hypoventilation and seizure. These patients also frequently show alacrima, asialia and reduced sweating. We recently found that *SOX10* mutations account for about 15% of WS2 as well [Bondurand et al., 2007] and that the phenotype sometimes includes neurological features reminiscent of PCWH, therefore delineating a new extended PCW phenotype. In this regard, the so-called mild form of the Yemenite syndrome, due to a missense *SOX10*

mutation [Bondurand et al., 1999], can in fact be considered as a WS2 (possibly with a very mild neurological involvement, as nystagmus was reported). Other groups confirmed the occurrence of point mutations in WS2 or in PCW [Barnett et al., 2009; Iso et al., 2008; Sznajer et al., 2008]. Apart from the neurological signs of PCW/PCWH, other non specific features are associated with *SOX10* mutations. A few patients suffer from intestinal disturbances but without any aganglionosis. They present instead with a chronic intestinal pseudo-obstruction [Pingault et al., 2002; Pingault et al., 2000]. Inner ear abnormalities at the MRI or CTscan, mostly semi-circular canals defects, have been reported. In one case, aplasia of the olfactory bulbs was observed [Barnett et al., 2009]. Among the few adult patients, several also present with hypogonadism ([Bondurand et al., 2007; Touraine et al., 2000] and this work).

Only the coding exons of *SOX10* are routinely studied. Among the 30 private mutations published, only one was found more than once. They are displayed in Table 5 along with 8 new mutations. The very large majority are truncating mutations, either frameshift or nonsense, scattered along the gene. Four missense or small in frame insertions are described in the HMG domain. Remarkably, there is a significant number of non-stop mutations (ie, mutations of the stop codon that lengthen the protein), which are thought to exert a dominant negative effect due to the extended tail [Chan et al., 2003; Inoue et al., 1999; Sham et al., 2001]. Partial or full gene deletions represent a significant proportion of SOX10 mutations [Bondurand et al., 2007].

SOX10 is the main gene of WS4, accounting for about half of the cases (including classical forms of WS4 and PCWH). The mutations are present at the heterozygous state and most often occur de novo. In a few instances, they are observed in a healthy parent suspected of having mosaicism [Southard-Smith et al., 1999; Touraine et al., 2000]. There is a high phenotypic variability in the few familial cases described [Pingault et al., 1998; Pingault et al., 2002; Southard-Smith et al., 1999]. The penetrance of each feature is incomplete, sometimes leading to phenotypes lacking cardinal signs and therefore departing from typical WS.

The absence of large families hampers the study of genotype/phenotype correlation. It is also possible that, due to frequent *de novo* mutations, mosaicism participates in incomplete penetrance of some features in subsets of patients. So far, there is no obvious explanation or genotype/phenotype correlation between the presence of absence of these signs and the mutation involved, except for neurological defects. These defects have long been suggested to result from a dominant negative effect [Inoue et al., 1999; Touraine et al., 2000], until Inoue and coworkers incriminated the NMD pathway (non sense-mediated RNA decay) [Inoue et al., 2004]. When a truncating mutation occurs in any exon but the last one, the mutant mRNA is recognized by the NMD pathway and degraded. This leads to haploinsufficiency and results in the classical form of WS4. On the contrary, when the mutation is located in the last coding exon (or the very last nucleotides of the penultimate exon), the mRNA is not recognized, the mutant protein is synthesized and acts as a dominant negative protein impairing function of the wild type SOX10, therefore resulting in the more severe phenotype PCWH. The earlier the mutation in the last exon, the more severe the phenotype as the result of a stronger dominant negative effect. In agreement, mutations located in the first part of the last exon (Gln234X, Gln250X, Ser251X) result in severe symptoms ranging from neonatal distress at birth, coma, hypoventilation/respiratory failure, and death in the postnatal period [Inoue et al., 2002; Pingault et al., 2002; Touraine et al., 2000]. The reasons why *SOX10* deletions resulting in haploinsufficiency lead to PCW/PCWH in some patients are not explained in this model [Bondurand et al., 2007].

Apart from NMD studies, functional tests showed a decreased transcriptional activity of SOX10 mutants relative to wild-type. A few mutations, such as Ser135Thr, differentially influenced expression of target genes, an observation that might account for the phenotypic differences observed [Chan et al., 2003; Lang and Epstein, 2003; Yokoyama et al., 2006a]. When cotransfected in vitro with wild-type SOX10 in a dose-dependent manner, truncated SOX10 proteins, irrespective of their associated phenotypes (WS4 or PCWH), displayed a similar dominant-negative effect.

However, such tests are inappropriate when performed on truncating mutations subjected to NMD-dependent mRNA degradation.

SNAI2: the snail homolog 2 transcription factor / WS2

The Snail-related zinc-finger transcription factor SNAI2 (Slug) is a member of the SNAIL family of zinc-finger TF that share an evolutionary conserved role in mesoderm formation in invertebrates and vertebrates. On binding to E-boxes, members of this family function as transcriptional repressors [Cobaleda et al., 2007].

SNAIL factors are best known for inducing the so-called epithelial-mesenchymal transition (EMT), an effect partly due to the direct repression of E-cadherin transcription during both development and tumor progression. Various studies over recent years have described its expression in a wide range of cancers. Transcriptional targets have been identified in mesenchymal cells, belonging to the following main categories: self-renewal, EMT, survival, cell cycle/DNA damage control [Cobaleda et al., 2007]. *Snai2* is expressed in migratory NCC and is necessary for melanoblast migration and/or survival, but not for NC formation. Additional sites of embryonic expression are the craniofacial mesenchyme, proliferating chondrocytes, outflow tract and endocardial cushions of the heart, and the mesenchymal components of the lungs, kidneys, and gut. In line with these observations, *Snai2*-null mice are viable but small, with minor craniofacial defects, pigmentary abnormalities (diluted coat with additional areas of depigmentation on the tails and feet and the characteristic white forehead blaze), macrocytic anemia and infertility [Jiang et al., 1998; Perez-Losada et al., 2002]. Hyperactivity and circling was also observed in some animals, suggesting hearing impairment [Sanchez-Martin et al., 2002]. Altogether these results suggest an essential role for SNAI2 in germ cells, melanocytic and hematopoietic stem cells [Cobaleda et al., 2007].

The *SNAI2* gene is located on 8q11 and contains three coding exons. In 2002, one group described two unrelated patients with homozygous *SNAI2* deletions that lead to a recessive form of

WS2 [Sanchez-Martin et al., 2002]. The unaffected parents were not studied. The same authors also described heterozygous deletions in piebaldism [Sanchez-Martin et al., 2003]. As no other group confirmed these results to our knowledge, we used the cohort of WS2 patients described in [Bondurand et al., 2007] to search for *SNAI2* point mutations (direct sequencing of coding exons and intron-exon boundaries) and deletions (QMF-PCR), and found none. Therefore, *SNAI2* has a minor involvement in WS2.

Biological relevance

Melanoblasts originate from multipotent NCC and migrate along several pathways to various destinations such as the skin, the iris and the choroids of the eye, and the inner ear. In the skin, they differentiate into melanin-producing cells that protect the organism from UVs and determine skin color [Hou and Pavan, 2008]. Piebaldism is a disorder characterised by patches of white skin and hair due to the absence of melanocytes in the depigmented areas. The association of such pigmentary disturbance with deafness in WS is thought to be due to the lack of melanoblast-derived intermediate cells of the stria vascularis, a small cochlear structure which plays an important role in the production of endolymph. A lack of strial intermediate cells leads to degeneration of the organ of Corti, and thus hearing loss, in several animal models [Tachibana et al., 2003]. However, early and wide *SOX10* expression during the inner ear development [Breuskin et al., 2009; Dutton et al., 2009; Watanabe et al., 2000] suggests that other mechanisms may account for deafness associated with SOX10 mutations. A shortening of the cochlea due to a reduced sensory progenitor survival has recently been described in the *Sox10* knock-out mouse [Breuskin et al., 2009]. These results are consistent with the observation that *SOX10* mutations in human lead to morphological abnormalities of the inner ear at the MRI or CT-scan ([Barnett et al., 2009; Inoue et al., 2004; Pingault et al., 2002; Sznajer et al., 2008; Vinuela et al., 2009] and manuscript in preparation).

All the WS genes are involved in a complex network, not fully understood, that takes place in NCC and derivatives (summarized in Fig. 6). A striking correlation links their function and

regulation to the physiopathology of WS and related diseases. Studies have shown that the TF PAX3 and SOX10 cooperate upon binding to the *MITF* promoter to modulate its expression [Bondurand et al., 2000; Potterf et al., 2000]. Besides this regulation, SOX10 and MITF proteins synergistically activate expression of melanocyte enzymes such as *Dct* [Jiao et al., 2004; Ludwig et al., 2004]. MITF regulates SNAI2 expression in vitro [Sanchez-Martin et al., 2002]. However, MITF is not sufficient to induce *Tyr* expression and full melanocyte differentiation in the absence of a functional SOX10 [Hou et al., 2006]. Recently, SOX10 was also described as able to regulate *Ednrb* expression during both ENS and melanocyte development [Yokoyama et al., 2006b; Zhu et al., 2004]. Finally, epistatic relationships between the ET3/ETB pathway and MITF were recently described [Sato-Jin et al., 2008]. ET3/ETB signaling regulates MITF in two ways: 1) direct phosphorylation through MAPK and 2) up-regulation of *MITF* gene expression. Mitf is also associated with feedback loop of *EDNRB* expression. Although there are still questions about the occurrence and exact significance of such events in melanocyte development in vivo, these regulations loops highlight strong epistatic connections and feedback loop regulations between WS genes. Full description of such interactions could provide a deep insight into the pathogenesis of WS as well as the biological mechanisms underlying hyper- or hypopigmentary disorders.

Clinical and diagnostic relevance

While the molecular findings in WS1 and WS3 appear relatively simple, the other WS subtypes are clearly genetically heterogeneous, with several genes implicated (Fig. 7) and different modes of transmission. In WS2, *MITF* mutations are involved in about 15% (dominant transmission), *SOX10* in another 15% (dominant transmission; possibility of neurological features, i.e., PCW), *EDNRB* (and possibly *EDN3* as well) in a small percentage (dominant with incomplete penetrance) and *SNAI2* (recessive) in another small percentage. In WS4, about 50% are due to *SOX10* mutations (dominant; possibility of neurological features, ie, PCWH), 20-30% to *EDN3* or *EDNRB* (not fully recessive, not fully dominant inheritance). While *PAX3*, *MITF*, *EDN3* and

EDNRB mutations are found in patients presenting with typical forms of different WS subtypes, *SOX10* mutations are associated with a larger range of phenotypes possibly not completely delineated.

For all these genes, the high inter and intra-familial phenotype variability strongly suggests that, besides some variability due to the mutation itself, there is an interplay between genetic modifiers, environmental factors and stochastic events. Mouse models have been useful to identify genetic modifiers of the phenotype. In case of *Sox10* for example, loci on mouse chromosomes 3, 5, 8, 11 and 14 have been shown to produce distinct effects on the penetrance and severity of the aganglionosis [Owens et al., 2005]. Genetic studies in mouse double mutants also highlighted genetic interactions between *Sox10* and *Sox8* or *Edn3*/*Ednrb* [Maka et al., 2005; Stanchina et al., 2006]. In human, the observation of different phenotypes in families with the same mutations, such as differences in heterochromia penetrance associated with a *PAX3* mutation [Lalwani et al., 1998], or presence versus absence of WS features in *EDN3* heterozygous relatives (see Table 4), also argues for the influence of the genetic background. In this regard, the large Mennonite family described by Puffenberger is of interest. Analysis of modifier loci showed influence of the 21q22 and RET loci, as well as a higher penetrance of HD in heterozygous males than in females [Puffenberger et al., 1994a; Puffenberger et al., 1994b]. Of note, a common hypomorphic allele of the RET gene has not been found to be a modifier of the WS4 phenotype [de Pontual et al., 2007].

Importantly, the incomplete penetrance of each feature leaves open the possibility that a small percentage of isolated deafness, isolated depigmentation features, or isolated HD, are due to mutations in the WS genes. On the other hand, features like deafness or early greying are relatively common, which makes the clinical diagnosis difficult, especially for WS2. The respective part of phenotypic variability, mutation-specific effect, and the additional possibility of mosaicism associated to de novo mutations, is difficult to assess. As a consequence, boundaries between the clinically well-defined WS2 and WS4 are becoming fuzzy from the molecular point of view. In families segregating a WS2-associated (or PCW-associated) heterozygous *SOX10* or *EDNRB*

deletion or point mutation, we are currently unable to define the risk of HD in case of recurrence in a newborn.

Apart from confirming the diagnosis, molecular analysis is sometimes necessary to determine the mode of transmission and predict the risk of recurrence. Both intestinal and neurological features can threaten vital prognosis in some patients, even with appropriate medical care (homozygous *EDN3* and *EDNRB* mutations, severe PCWH associated to *SOX10* mutations). Prenatal diagnosis may be considered in a few of these cases.

Molecular analysis is complicated by the observation that mutations of the genes involved in WS are mostly private and scattered along most, if not all, exons. Given the high allelic heterogeneity observed and the frequency of the de novo mutations (in *PAX3*, *MITF* and *SOX10*), it is likely that the vast majority of the mutations arose as single events. Recurrent variations may, in some instances, be found in remote branches of the same family. Haplotype analysis showed that two identical *MITF* mutations were carried by the same chromosomal haplotype, suggesting the occurrence of a single mutational event [Pandya et al., 1996]. In another case, two identical *PAX3* mutations were carried by different haplotypes, suggesting that the mutation occurred twice independently [Lalwani et al., 1998].

Although non-sense, frameshift or splice mutations are usually easy to incriminate, the validation of missense mutations is not straightforward. In *PAX3* or *MITF*, most of them arise in specific domains and can be shown either to occur de novo or to co-segregate with the disease in the family. The situation is more difficult with *EDN3* and *EDNRB* as mutations are inherited and penetrance is low or incomplete in heterozygotes. In addition, functional tests are not easy to perform in routine diagnosis, impairing the assessment of the pathogenicity of some of the missense variations.

Future prospects

Following the characterization of *SOX10* mutations in patients with WS2/PCW and the observation that few WS2 present with heterozygous *EDNRB* mutations, about 70% of WS2 still remain unexplained at the molecular level. Also, 15-35 % of WS4 cases have no identified mutation in *SOX10*, *EDN3* or *EDNRB*. It may be that some other genes remain undiscovered, especially in WS2, but it may also be that our methods of screening may fail to identify all the mutations in the known genes. As a consequence, non coding regions as well as newly identified regulatory regions of each of these genes should be screened. Apart from WS2A (*MITF*), WS2D (*SNAI2*) and WS2E (*SOX10*), two loci, WS2B and WS2C, have been localised on chromosomes 1p21-p13.3 and 8p23, respectively (MIM 600193; MIM 606662) [Lalwani, 1994; Selicorni et al., 2002].

The last fifteen years have been rich in the identification of WS genes. In addition to detecting new mutations and identifying new genes of WS, further studies should clarify the respective role of private mutations, genetic background, environment or stochastic events in the variability of the WS patients phenotype.

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Legends to figures

Figure 1. Localisation of the PAX3 mutations characterized in Waardenburg syndrome. PD, paired domain; o, octapeptide; HD, homeodomain; TA, transactivation domain. For recurrent mutations, the number of occurrences is indicated between brackets. Mutations in bold have been described at the homozygous (or compound heterozygous) state in WS3. Heterozygous mutations are associated to WS1 except when underlined (WS3) or italicized (CDHS).

Figure 2. Localisation of the MITF mutations characterized in Waardenburg syndrome and the related Tietz syndrome. AD1-3 : transactivation domains; b, basic domain; HLH, helix-loop-helix domain; LZ, leucine zipper domain. Hatches represent the alternative splice of six amino acids. Mutations leading to Tietz syndrome are underlined. For recurrent mutations, the number of occurrences is indicated between brackets.

Figure 3. Localisation of the EDNRB variations characterized in Waardenburg syndrome. PS, peptide signal; TM, transmembrane domains; e1-4, extracytoplasmic regions; i1-4, intracytoplasmic regions. Mutations in bold were found at the heterozygous state (the others at the homozygous or compound hererozygous state, either proved or suspected). The mutation underlined was found in WS2. It is possible that some of the missense variations correspond to rare, uncharacterized, population-specific polymorphisms. The (?) refers to doubts regarding the pathogenicity of this mutation (see table 3). For recurrent mutations, the number of occurrences is indicated between brackets.

Figure 4. Localisation of the EDN3 mutations characterized in Waardenburg syndrome. PS, peptide signal; ET, mature endothelin 3; l, ET-like peptide; white arrows, endopeptidase cleavage sites;

black arrow, endothelin conversion enzyme cleavage site. The mutation in bold was found at the heterozygous state (the others at the homozygous or compound hererozygous state). For recurrent mutations, the number of occurrences is indicated between brackets.

Figure 5. Localisation of the SOX10 mutations characterized in Waardenburg syndrome. D, dimerisation domain; HMG, HMG domain; E, conserved domain of SOX8/9/10; TA, transactivation domain; ex3-5 : exons 3 to 5. Mutations associated to neurological features (PCW/PCWH) are in bold, and mutations associated to WS2 (or PCW) are underlined. One mutation has been found twice (indicated between brackets).

Figure 6. The WS genes interplay during melanocyte differentiation.

Figure 7. Current knowledge of the molecular basis of WS. *SNAI2*, *EDN3*, *EDNRB* mutations in WS2 are <5% but not frequent enough for an acute determination of frequency.