Subnuclear re-localization of SOX10 and p54NRB correlates with a unique neurological phenotype associated with SOX10 missense mutations

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Abstract

SOX10 is a transcription factor with well-known functions in neural crest and oligodendrocyte development. Mutations in SOX10 were first associated with Waardenburg-Hirschsprung disease (WS4; deafness, pigmentation defects and intestinal aganglionosis). However, variable phenotypes that extend beyond the WS4 definition are now reported. The neurological phenotypes associated with some truncating mutations are suggested to be the result of escape from the nonsense-mediated mRNA decay pathway; but, to date, no mechanism has been suggested for missense mutations, of which approximately 20 have now been reported, with about half of the latter shown to be redistributed to nuclear bodies of undetermined nature and function in vitro. Here, we report that p54NRB, which plays a crucial role in the regulation of gene expression during many cellular processes including differentiation, interacts synergistically with SOX10 to regulate several target genes. Interestingly, this paraspeckle protein, as well as two other members of the Drosophila behavior human splicing (DBHS) protein family, co-localize with SOX10 mutants in nuclear bodies, suggesting the possible paraspeckle nature of these foci or re-localization of the DBHS members to other subnuclear compartments. Remarkably, the co-transfection of wild-type and mutant SOX10 constructs led to the sequestration of wild-type protein in mutant-induced foci. In contrast to mutants presenting with additional cytoplasmic re-localization, those exclusively found in the nucleus alter synergistic activity between SOX10 and p54NRB. We propose that such a dominant negative effect may contribute to or be at the origin of the unique progressive and severe neurological phenotype observed in affected patients.

Introduction

The involvement of SOX10 in various neurocristopathies has significantly contributed to our understanding of its function during neural crest (NC) development. This member of the SOX family of transcription factors was first implicated in Waardenburg-Hirschsprung disease, also known as Waardenburg syndrome type 4 (WS4, MIM no. 613266), which is characterized by primary features of Waardenburg syndrome (i.e. sensorineural hearing...
loss and abnormal pigmentation of the hair and skin) in association with Hirschsprung disease (HSCR; intestinal aganglionosis) (1, reviewed in 2–5). Mutations were subsequently identified in about 15% of the patients presenting with WS type 2 [WS4 without HSCR, MIM no. 611584 (5,6), rare cases of isolated HSCR [MIM no. 142623 (7,8)] or neurological variants of WS2 or WS4 [i.e. peripheral demyelinating neuropathy–central dysmyelinating leukodystrophy–Waardenburg syndrome–Hirschsprung disease (PCWH), no. 690136; or PCWH without HSCR (PCW)] (9, reviewed in 5). SOX10 mutations were recently identified in patients presenting with Kallmann syndrome (hypogonadotropic hypogonadism and anosmia, MIM no. 147950) with or without deafness (10).

Most of the features associated with SOX10-related disorders result from the abnormal development of one or more NC derivatives. NC cells are a population of multipotent precursor cells that emerge at the edges of the neural tube, migrate extensively throughout the embryo and differentiate into a variety of cell types (11). SOX10 controls the maintenance of progenitor multipotency, as well as the specification and differentiation of melanocytes, Schwann cells, olfactory ensheathing cells and the enteric nervous system. To this end, SOX10 binds to the regulatory regions of numerous genes and activates transcription in association with a wide range of co-regulators (10,12–16).

About 100 SOX10 mutations have been described to date, the majority of which are truncating mutations. The more severe phenotypes (neurological variants) mostly result from mutations in the last coding exon and are thought to occur when mutant mRNAs escape the nonsense-mediated mRNA decay (NMD) pathway, leading to dominant negative effect (17). Over the last 5 years, the identification of about 20 missense mutations, associated with various phenotypes ranging from WS2 to PCWH, has enabled the analysis of the functional consequences of mutations not subjected to NMD (10,18–23). Mostly localized within the DNA-binding domain, each of these mutations has deleterious effects on the primary functions of SOX10; some of the resulting mutant proteins exhibit partial cytoplasmic redistribution and/or lose their DNA binding and/or transactivation capabilities (10,19,20,23). The observation that one missense mutant differentially influences tissue-specific gene expression was initially proposed as a general rule to explain the phenotypic variability observed (24); however, a comparative analysis of the functional consequences of a large number of them ruled out this hypothesis (20). Although the involvement of modifier genes was proposed to contribute to the phenotype variability (25), mutation-specific mechanisms remain to be identified in cases of missense mutations.

We previously observed that about half of these mutants are redistributed in nuclear foci upon transfection into various human cell lines. This subcellular re-localization appeared to correlate with different phenotype severities, depending on additional cytoplasmic redistribution (10,20). The nucleus of higher eukaryotes is functionally divided into multiple compartments or nuclear bodies that contain particular sets of proteins and nucleic acids that are involved in distinct molecular processes, ranging from DNA replication to RNA transcription and processing (26,27). To date, about ten different types of nuclear bodies have been characterized. Co-immunolabeling experiments excluded the possibility that foci observed upon transfection of SOX10 mutants corresponded to nuclear speckles, promyelocytic leukemia bodies or aggresomes (20), but the other possibilities remained.

Paraspeckles are a relatively new class of subnuclear bodies found in the interchromatin space in close proximity to nuclear speckles (28,29). The core paraspeckle proteins, PSF/SFPQ, FSPC1 and NONO/p54NRB (non-pou domain-containing octamer-binding protein), are members of the Drosophila behavior human splicing (DBHS) family. These proteins, together with the long non-coding RNA NEAT1/Men β/j, associate to form paraspeckles and maintain their integrity (reviewed in 30–34). Paraspeckles are thought to control several biological processes such as viral infection, stress responses and cellular differentiation. They are involved in the nuclear retention of RNA containing double-stranded RNA regions subject to adenosine-to-inosine editing (34–36), a sequestration mechanism mediated by the binding of p54NRB to hyperedited sequences. In addition, p54NRB and other members of the DBHS family have been implicated in a wide array of functions that encompass many aspects of transcription and RNA processing, including transcription initiation, constitutive and alternative splicing, transcriptional termination and co-activation or co-repression of transcription (30,37). Thus, p54NRB enhances the association of many DNA-binding proteins with their targets (reviewed in 30,37–40). Of interest, p54NRB physically interacts with SOX9, another member of the SOX family, enhances its transcriptional activity and controls the expression/splicing of its target genes during chondrogenesis (41).

In the present report, we demonstrate that p54NRB interacts with SOX10 and acts synergistically with it to activate various target genes. Although not required for the formation of SOX10 mutant-induced foci, p54NRB and two other DBHS family members co-localize with each mutant protein in these nuclear bodies. Remarkably, the co-transfection of wild-type and mutant SOX10 constructs led to the sequestration of wild-type SOX10 in mutant-induced foci. However, only foci-forming SOX10 mutants exclusively localized in the nucleus alter synergistic activity between SOX10 and p54NRB, a dominant negative activity that correlates with the more severe PCWH/PCW phenotypes observed in patients harboring these mutations. Thus, protein subnuclear re-localization of p54NRB and SOX10 could be the basis of a new molecular/cellular mechanism underlying the unique phenotype associated with some SOX10 missense mutations and possibly other genes involved in related or unrelated pathologies.

**Results**

**SOX10 mutant proteins co-localize with p54NRB and two other members of the DBHS family in subnuclear foci**

Although wild-type SOX10 protein is distributed in a diffuse pattern throughout the nucleoplasmin, seven of the eleven missense mutations that were previously identified in patients presenting with WS2, WS4, PCW or PCWH, namely, p.Leu145Pro, p.Lys150Asn, p.Ala157Val, p.Gln174Pro, p.Pro175Ala, p.Pro175Leu and p.Pro175Arg, induce redistribution of the resulting proteins into nuclear punctate structures or inclusions, also termed foci [Fig. 1A, and see (20) for a brief clinical description of the 11 mutations and their functional consequences]. Because we previously observed that these SOX10 mutant-induced foci were close to, but distinct from SC35-positive speckles, co-localization with p54NRB, a paraspeckle marker, was tested. To this end, HeLa cells were transfected with expression vectors containing each of the seven aforementioned SOX10 mutants. We subsequently performed co-immunolabeling experiments with SOX10 and p54NRB antibodies to observe the expression pattern of endogenous p54NRB upon expression of each SOX10 mutant protein. Although untransfected cells showed a rather homogeneous p54NRB nuclear staining with a small punctate pattern (Fig. 1A, see white arrows), expression of the SOX10 mutants induced
re-localization of p54NRB in the observed foci (Fig. 1A). This event occurred irrespective of the nuclear or nucleocytoplasmic localization of the SOX10 mutant proteins. Indeed, despite the fact that p.Leu145Pro, p.Lys150Asn and p.Ala157Val showed partial cytoplasmic re-localization, all of the nuclear foci contained both SOX10 mutant and p54NRB proteins (Fig. 1A).
In 2013, two other mutations reported in patients presenting with Kallmann syndrome, p.Phe111Val and p.Trp142Arg, were also shown to result in nuclear foci formation and cytoplasmic redistribution (10). Transfection of these two mutants followed by co-immunolabeling experiments demonstrated that p54NRB also co-localized with the SOX10 mutant proteins in the mutant-induced foci (data not shown).

In order to confirm and extend our observations, we next performed similar experiments in two other cell models: SKMel5 (melanoma cell line that endogenously expresses SOX10) and primary enteric neurospheres culture (42,43). The seven mutant constructs tested in SKMel5 cells led to SOX10 and p54NRB co-localization in all observed foci (Fig. 1B). To induce overexpression of wild-type SOX10 and selected mutants (p.Leu145Pro or p.Pro175Arg) in primary enteric neurospheres culture, the latter were infected with an SOX10-IRES-GFP retrovirus containing wild-type or mutant sequences. Co-immunolabeling experiments using GFP (to specifically identify the infected cells) and p54NRB antibodies revealed a diffuse nuclear pattern of p54NRB upon SOX10-IRES-GFP retrovirus infection and partial re-localization of p54NRB protein in the nuclear foci of most cells infected with the two SOX10 mutant constructs (Supplementary Material, Fig. S1A). The re-localization of p54NRB in enteric neurospheres, and co-localization of SOX10 mutants with p54NRB in HeLa and SKMel5 cells, suggests that the foci observed were paraspeckles or that the SOX10 mutants induced re-localization of p54NRB into new subnuclear bodies.

To test these hypotheses, we analyzed the subcellular localization of two other DBHS family members: PSF and PSPC1, also known to be paraspeckle markers. SKMel5 cells were transfected with expression vectors containing the SOX10 mutants of interest, and co-immunolabeling experiments were performed using PSPC1 and PSF antibodies. Again, the co-localization of SOX10 and PSPC1 (Fig. 1C) or PSF (Supplementary Material, Fig. S1B) was apparent in the foci, indicating that formed subnuclear bodies contained all three DBHS proteins.

p54NRB is expressed in NC derivatives and co-localizes with wild-type SOX10 when overexpressed

The co-localization of p54NRB with SOX10 mutant proteins prompted us to investigate the potential relationship between this multifunctional protein and wild-type SOX10 in various NC derivatives. To this end, we assessed SOX10 and Nono expression by reverse transcriptase–polymerase chain reaction (RT–PCR) experiments using total RNA extracted from mouse skin, melanocytes and enteric neurospheres culture. Although Nono was highly expressed in the skin as described previously (38), little or no Sox10 expression was detected (Fig. 2A). In contrast, both genes were expressed, although at different levels, in NC-derived melanocytes and enteric neurospheres culture (Fig. 2A).

To evaluate p54NRB protein expression, we performed immunofluorescence staining on melanocytes and melanoma cultures as well as dissociated enteric neurospheres, which contain enteric progenitors as well as derived neurons and glial cells. The protein was present in both melanocytes and melanoma SOX10-positive cell lines, although its expression was higher in the melanoma cell line (Fig. 2B). Co-immunolabeling of enteric neurospheres with SOX10 or GFAP, markers of enteric progenitors and glial cells, respectively, revealed p54NRB expression in both cell types (Fig. 2C). High p54NRB expression was also detected in TUJ-1-positive neurons, which are SOX10-negative (Fig. 2C, 42). Although p54NRB expression is broader, we thus found that SOX10 and p54NRB are expressed together in several NC derivatives.

Functional and physical interaction between SOX10 and p54NRB

p54NRB was previously shown to enhance the association of many DNA-binding proteins to their targets, including SOX9, and, as such, is considered a crucial transcriptional co-activator or co-repressor (31,41). To determine the functional relationship between SOX10 and p54NRB, we examined the effect of this protein on the transcriptional activity of SOX10. To this end, we tested the ability of p54NRB to activate alone or synergistically with SOX10, three well-known SOX10 target genes: MITF, GJB1 and RET. MITF and GJB1 reporter constructs containing promoter regions of each of these genes (44,45), as well as the SOX10-responsive enhancer of RET located within the first intron of this gene (46), were chosen. We transfected the three reporter constructs in HeLa cells together with p54NRB and SOX10 expression vectors alone or in combination (Fig. 3A). We observed no upregulation of the reporter gene upon transfection of p54NRB alone for any of the three constructs. However, overexpression of p54NRB markedly enhanced SOX10 transactivation of the three regulatory regions (Fig. 3A). The synergistic activation of MITF by p54NRB and SOX10 was confirmed in SKMel5 cells (Fig. 3B). In a similar manner, the synergistic activation of GJB1 by SOX10 and p54NRB was confirmed in the SH-SY5Y neuroblastoma cell line (Fig. 3C). Our results indicate that p54NRB is a new transcriptional partner for SOX10 that controls the expression of at least three genes known to play key roles in NC derivatives, namely, MITF in melanocytes, GJB1 in glial cells and RET in ENS.

To investigate the basis of the observed functional cooperation, we determined whether there was an interaction between SOX10 and p54NRB. Nuclear extracts expressing p54NRB-YFP, SOX10 or both were precipitated with anti-GFP antibody, and the precipitates were subsequently used for immunoblot analysis with the anti-SOX10 antibody. As shown in Figure 3D, co-immunoprecipitation was only apparent when the two proteins were present, indicating a protein–protein interaction. Similar experiments were conducted with a SOX10 mutant protein (p.Glu189X, Fig. 3D), which contains the HMG domain, but lacks the K2 and/or C-terminal transactivation domain (13). No interaction was detected in this case, suggesting that the HMG domain is not involved in the protein–protein interaction highlighted here.

A synergistic transcriptional activity between two proteins could require DNA binding of one or both to a promoter or enhancer region. Although the binding sites for SOX10 on the three tested target genes are well described (44–46), the DNA-binding
capacity of p54NRB is unknown. The observation that p54NRB is necessary for cAMP-dependent activation of CREB target genes in vivo (47), and the finding that the responsiveness of the MITF promoter depends upon SOX10 expression (48), led us to test the putative role of the CRE-binding site on the MITF construct in promoting the synergistic activity between SOX10 and p54NRB. We altered this site as described previously (48) and tested the resultant effects in transactivation assays. No differences between wild-type and mutant reporter constructs were observed. Indeed, both were under the synergistic regulation of SOX10 and p54NRB, suggesting that the integrity of the CRE-binding site is not required for the synergistic activation (Supplementary Material, Fig. S2).

**Analysis of WS and melanoma-associated SOX10 mutations: only foci-forming SOX10 mutants present with altered synergistic activity**

Our previous data demonstrated deleterious effects of the seven SOX10 missense mutations on DNA binding and transactivation capacities. Specifically, the p.Leu145Pro, p.Lys150Asn and p.Ala157Val mutants completely lost their DNA binding and transactivation capacities, whereas other mutants retained partial activities (20).

The synergistic activity observed between wild-type SOX10 and p54NRB led us to evaluate whether SOX10 mutants maintain this functionality. We transfected HeLa cells with GJB1 or MITF.
Inhibition of p54NRB modifies the transactivation capacity of some SOX10 mutants, but not their subcellular localization

We next attempted to determine whether inhibition of p54NRB affects the activities of wild-type and mutants SOX10. Representative of ‘foci and cytoplasmic redistribution’ (p.Leu145Pro and p.Ala157Val) and ‘foci-only’ (p.Gln174Pro, p.Pro175Ala and p.Pro175Arg) mutant classes were chosen. To this end, knockdown of NONO was performed using an siRNA-mediated strategy. After 48 h, cells were transfected again with wild-type or SOX10 mutant expression vectors and the MTF7 reporter construct. Western blot analysis confirmed inhibition of p54NRB protein expression (Fig. 5A; compare effect of Si-Ctrl and si-NONO), but no effect on wild-type SOX10 transactivation capacity was observed (Fig. 5B). The activities of the p.Leu145Pro, p.Ala157Val and p.Pro175Ala mutants were not affected either. Indeed, mutants’ transactivation potential was similar after si-control (si-Ctrl) and si-NONO transfection (Fig. 5B; note that activity is presented as fold induction ratio above activity without siRNA transfection). However, p54NRB knockdown seemed to slightly increase the residual transactivation potential of p.Gln174Pro and p.Pro175Arg mutants (Fig. 5B).

To test whether these observations were associated with modified subcellular localization of the SOX10 mutants, HeLa cells were transfected with wild-type or SOX10 mutant expression vectors 48 h after p54NRB knockdown, and co-immunolabeling experiments were performed using SOX10 and p54NRB reporter constructs and expression vectors containing p54NRB and wild-type or SOX10 mutants, alone or in combination (Fig. 4A).

The functionality of p.Leu145Pro, p.Lys150Asn, p.Ala157Val, p.Gln174Pro, p.Pro175Ala, p.Pro175SLeu and p.Pro175Arg mutants was compared with that of wild-type SOX10 and p.Asn131His, a non-foci-forming-missense mutant. As shown in Figure 4A, p.Asn131His retained its ability to act synergistically with p54NRB. In contrast, p.Leu145Pro, p.Lys150Asn and p.Ala157Val mutants retained no transactivation potential when expressed alone or together with p54NRB (data not shown). Interestingly, p.Gln174Pro, p.Pro175Ala, p.Pro175SLeu and p.Pro175Arg, which were still able to partially activate the GBJ1 and MTF7 promoters, specifically lost their synergistic activity with p54NRB (Fig. 4A). Together, these data suggest that the subnuclear re-localization of p54NRB and SOX10 mutants could alter their combined function.

In addition to SOX10 involvement in WS and related disorders, six SOX10 somatic missense mutations were previously identified in primary melanoma samples: p.Arg34Gln, p.Ala361Val, p.Gly413Asp, p.Gly413Ser, p.His414Tyr and p.Ala424Val. The functional consequences of five mutants were evaluated in vitro, but no significant differences compared with wild-type SOX10 were reported (49). Since then, c.1082c>T (p.Ala361Val) was reported at a very low frequency in the European population by the Exome Aggregation Consortium. Taking into account the described role of p54NRB on melanoma progression (50), and the regions of SOX10 that appear to mediate the protein–protein interaction (Fig. 3D), we re-evaluated the functional consequences of the following melanoma-associated mutants: p.Gly413Ser, p.His414Tyr and p.Ala424Val. As shown in Figure 4B, all three mutants retained their capacity to activate MTF7 in synergy with p54NRB. Thus, these mutations do not appear to affect the functional interaction between SOX10 and p54NRB.
antibodies. Upon p54NRB inhibition, localization of wild-type SOX10 protein remained unchanged (compare top and bottom lines of Fig. 5C), as did the localization of the five SOX10 mutants. Indeed, foci were observed after transfection of either si-Ctr or si-NONO, suggesting that p54NRB inhibition does not alter foci formation. However, p54NRB inhibition led to the upregulation of endogeneous PSPC1 (Fig. 5D, compare top and bottom lines in all cells), suggesting that the latter could compensate and/or replace p54NRB.

Together, these results suggest that p54NRB is not required for foci formation, but that its inhibition can slightly increase the transactivation capacity of some SOX10 mutants.

**Foci-forming SOX10 mutants affect wild-type SOX10 subcellular localization, but only those exclusively expressed in the nucleus affect SOX10 and p54NRB synergistic activity**

The presence of p54NRB in nuclear foci, as well as the protein–protein interaction observed between p54NRB and wild-type SOX10, led us to test the possibility that the latter could also be sequestered into nuclear bodies. To test this hypothesis, we compared the distribution of wild-type SOX10 fused to GFP upon co-transfection with myc-tagged mutants or wild-type SOX10 constructs. Cells co-transfected with plasmids containing GFP

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**Figure 4. Analysis of WS and melanoma-associated SOX10 mutations: only SOX10 foci-forming mutants present with altered synergistic activity.** (A) Activities of WS-associated SOX10 mutants. HeLa cells were transfected with GJB1 or MITF reporter constructs and expression vectors containing p54NRB and wild-type (WT) or SOX10 mutants, alone or in combination. Luciferase reporter gene activity is presented as fold induction relative to the empty expression vectors. Note that activities of non-foci-forming SOX10 mutant (p.Asn131His) and foci-forming SOX10 mutants (p.Gln174Pro, p.Pro175Ala, p.Pro175Leu and p.Pro175Arg) are presented. Although p.Asn131His retained synergistic activity with p54NRB, foci-forming SOX10 mutants lost it. (B) Activities of melanoma SOX10-associated mutants. MITF reporter constructs and expression vectors containing p54NRB and wild-type (WT) or three melanoma-associated SOX10 mutants (p.Gly413Ser, p.His414Tyr and p.Ala424Val) were transfected alone or in combination in HeLa cells. Luciferase reporter gene activity was measured and presented as fold induction relative to the empty expression vectors. Note that all melanoma-associated SOX10 mutants retained their capacity to activate MITF in synergy with p54NRB. Data are reported as mean ± standard error of three to five different experiments.
Figure 5. Inhibition of p54NRB modifies the transactivation capacity of some SOX10 mutants, but not their subcellular localization. (A) Representative western blot analysis demonstrating the efficacy of si-NONO-specific siRNA pool, compared with untransfected and Si-control (Si-Ctr)-transfected cells. Endogenous p54NRB knockdown was confirmed 48 h post-transfection. (B) Analysis of SOX10 mutants’ transactivation capacity upon p54NRB knockdown. Forty-eight hours post si-Ctr or si-NONO transfection, HeLa cells were co-transfected with the MITF reporter construct, wild-type (WT) or SOX10 mutant expression vectors (p.Leu145Pro, p.Ala157Val, p.Gln174Pro, p.Pro175Ala and p.Pro175Arg). Note that activity is presented as fold induction ratio above activity without siRNA transfection. No significant differences were observed for p.Leu145Pro, p.Ala157Val and p.Pro175Ala mutants. However, p54NRB knockdown slightly increased the transactivation capacity of p.Gln174Pro and p.Pro175Arg mutants. Data are reported as mean ± standard error of five different experiments. (C) Forty-eight hours after control (si-Ctr) or p54NRB knockdown (si-NONO), HeLa cells were transfected with wild-type (WT) or SOX10 mutant expression vectors (p.Leu145Pro, p.Ala157Val, p.Gln174Pro, p.Pro175Ala, p.Pro175Leu and p.Pro175Arg) and co-immunolabeling experiments were performed using SOX10 (green) and p54NRB (red) antibodies. Note that the re-localization of SOX10 mutant proteins in subnuclear bodies is independent of NONO expression. (D) Same experiments as in (C) followed by co-immunolabeling experiments using SOX10 (green) and PSPC1 (red) antibodies. Although the endogenous PSPC1 expression is almost undetectable in HeLa cells, comparison of the top and bottom lines highlights upregulation of PSPC1 protein (red) in si-NONO-transfected cells.
and myc-tagged wild-type SOX10 exhibited uniform nucleoplasmic staining (Fig. 6A). However, the co-transfection of wild-type and mutants SOX10 caused both wild-type (GFP staining) and mutants (myc staining) to redistribute within the foci, indicating that irrespective of their nuclear or partial cytoplasmic re-localization, all of the tested SOX10 mutants recruited the wild-type protein to the punctate foci (Fig. 6A).

To test whether such subnuclear re-localization could impact the synergistic activity of SOX10 and p54NRB, we subsequently performed competition experiments by co-transfecting GJB1 reporter plasmid together with p54NRB and wild-type SOX10 (WT) without (0×) or with (1× or 2×) SOX10 mutant-expressing vectors. Data are reported as the percentage of SOX10 and p54NRB activity upon empty vector or mutant co-transfection over the SOX10 and p54NRB activity alone and are the mean ± standard error of five different experiments. Note that the p.Gln174Pro and three p.Pro175 mutants decreased the synergistic transcriptional activity of p54NRB and wild-type SOX10 protein (*P < 0.05, **P < 0.01 or ***P < 0.001 observed depending on mutants and dose added). In contrast, no such effect was observed for p.Leu145Pro, p.Lys150Asn and p.Ala157Val mutants. Only the mutants exclusively found within the nucleus, therefore, seemed to exert a dominant negative effect.

**Discussion**

Because foci formed upon the transfection of some SOX10 mutants were in close proximity to nuclear speckles, their co-localization with p54NRB, a paraspeckle marker, was...
investigated (29,31). We showed that this multifunctional protein, together with two other members of the DBHS family (classic paraspeckle components), co-localizes with SOX10 mutants in foci. When p54NRB was overexpressed, wild-type SOX10 was also found in p54NRB-positive foci, suggesting that the observed association was not limited to SOX10 mutants. Several transcription factors, including SOX9, have been shown to co-localize with paraspeckles when overexpressed in various cell lines (33,41). In those cases, it has been suggested that the observed foci could correspond to paraspeckles or that SOX9 overexpression could re-localize to unrelated subnuclear bodies together with p54NRB (29,31). Similar mechanisms could account for the data presented here. Although the definitive nature of the foci in our experiments will require further investigation, we provide reasonable assumptions about SOX10 and p54NRB interactions in various NC derivatives and the consequences of these interactions in the context of SOX10-related disorders.

DBHS protein members dynamically shuttle between paraspeckles and the nucleoplasm. When broadly distributed in the nucleoplasm, one of their functions is to exert transcriptional activity (38,51). For example, p54NRB is expressed and localized in the nucleus of both melanoma cell lines and melanoma tissue samples, and as a ‘melanoma inhibitory activity’ target molecule, it controls the development and progression of malignant melanoma (50). Here, we confirmed the weak RNA and protein expression of p54NRB in melan-α cells and also described its expression in enteric neurospheres culture, demonstrating that SOX10 and p54NRB were co-expressed in at least two different NC derivatives. In enteric neurospheres culture, expression of p54NRB was, however, not limited to SOX10 expressing cells, as it was also highly expressed in enteric neurons. Identification of p54NRB function in the latter requires additional studies. Interestingly, expression studies performed in Xenopus revealed that Xp54NRB transcripts are expressed throughout early developmental stages, specifically in neural and sensorineural territories, indicating that Xp54NRB could be involved in anterior neural patterning (52). Thus, expression profile of this DBHS member is compatible with its function in early neural development, including NC.

The protein–protein interaction between SOX9 and p54NRB, as well as their synergistic activity, were previously demonstrated (41). In fact, p54NRB specifically stimulated the transcriptional activity of SOX9 on the Col2a1 promoter during chondrogenesis. Here, we demonstrated that p54NRB enhanced SOX10 transcriptional activity on various target genes, namely, RET, GJB1 and MITF. Co-immunoprecipitation experiments revealed a protein–protein interaction between SOX10 and p54NRB, which was not mediated by the DNA-binding domain of SOX10. Removal of the HMG domain did not affect the p54NRB and SOX9 interaction either. Although further investigations are required to map the precise interaction domain on SOX10 and SOX9, the conservation observed among the three E-group members, SOX8, SOX9 and SOX10, as well as other SOX factors, led us to speculate that physical and functional interactions with p54NRB may not be restricted to SOX10 and SOX9. Thus, p54NRB may be a new component of the transcriptional machinery triggered by SOX family members to activate the transcription of target genes in a sequential and dynamic manner (13).

To exert its activity in such complexes, p54NRB binding to DNA targets may also be required. Some studies have reported p54NRB binding to various regulatory sequences (39,53,54). In contrast, others have reported that p54NRB enhances the binding activity of protein partners without binding to the target DNA itself (40,55), making the search for putative p54NRB-DNA-binding sequences questionable. Nevertheless, several reports have suggested the existence of a close relationship between p54NRB and the transcriptional regulation of several cAMP-responsive genes. For example, cAMP-dependent activation of steroid hormone encoding genes occurs upon the release of Sin3a and histone deacetylase from the SF1/p54NRB/PSF complex (56); TORC2 and p54NRB interact on cAMP-responsive promoters and p54NRB acts as a bridge between the CREB/TORC complex and RNA polymerase II. Together with previous data suggesting that the responsiveness of the MITF promoter to cAMP (mediated by CREB binding to a CRE-binding site) depends on SOX10 (48), these data led us to evaluate whether the integrity of the MITF CRE-binding site is necessary for the synergistic activity of SOX10 and p54NRB; however, we found that this was not the case.

Knockdown of p54NRB inhibited the transcriptional activity of SOX9 on the Col2a1 promoter (41). In the present study, however, similar experiments showed no significant change. Nevertheless, p54NRB depletion seemed to partially restore the transactivation capabilities of p.Gln174Pro and p.Pro175Arg mutants. To test whether this effect was mediated by redistribution of the mutant proteins, the effect of NONO depletion on foci formation was tested. Surprisingly, p54NRB inhibition did not alter foci formation; however, upregulation of PSPC1, a functional homolog with partially interchangeable functions, was observed, leading us to speculate that the latter could at least partially compensate for the absence of NONO. These data are in accordance with previous studies showing a compensatory response involving PSPC1, which reduces the effects of NONO deficiency (57).

While seeking the functional consequences of the mutant-induced foci, we observed that only foci-forming SOX10 mutants lost their cofactor activity with p54NRB, suggesting that sub-nuclear re-localization has a deleterious impact on the residual function of SOX10 mutants. In addition, co-immunolabeling experiments demonstrated that wild-type protein also re-localized to these subnuclear bodies. By forming foci, SOX10 mutants could therefore sequester both wild-type SOX10 and its p54NRB cofactor, thereby depleting these two proteins from the nucleolus and limiting their availability to SOX10 target gene sequences. However, transactivation competition assays revealed that only foci-forming mutants exclusively located in the nucleus could exert such a dominant negative activity, whereas p.Leu145Pro, p.Lys150Asn and p.Ala157Val mutants, which have partial cytoplasmic redistribution, failed to do so (20) and this study). We propose that this could be because SOX10 mutant proteins are present at a lower amount in the nucleus than wild-type proteins, thereby limiting their adverse effects. The dominant negative effect could also result from an impairment of SOX10 self-dimerization or other interactions by SOX10 mutant proteins. NEAT1 RNA, the long non-coding RNA involved in the scaffolding of paraspeckles, was recently shown to control transcription through a similar mechanism; namely, upon viral infection, upregulation of this long non-coding RNA increased the transcription of antiviral genes such as IL8 through protein sequestration of PSF, a transcriptional repressor of IL8 (58). Along the same lines, paraspeckles were shown to be stress-inducible structures that modulate gene expression. Paraspeckle proteins are depleted from the nucleoplasm by 50% when paraspeckle assembly is enhanced, thus limiting the amount of DBHS proteins available for transcriptional activity (59). Here, we suggest that SOX10 foci-forming mutants may act through a similar sequestration mechanism, thereby mimicking dominant negative activity.

Interestingly, our in vitro observations seem to correlate with the phenotypes observed in the patients. Although patients with
mutations inducing foci and a nuclei-restricted localization of resulting proteins (residues 174 and 175) presented with severe PCW or PCWH phenotypes, patients with the other SOX10 missense mutations presented with absence or moderate neurological involvement. Patients carrying mutations of residues 174 and 175 presented with various degrees of sensorineural hearing loss (from minor unilateral hearing deficit to profound bilateral hearing loss), variable degree of pigmentation defects (from albinism to localized hypopigmented patches), constipation or intestinal pseudo-obstruction for three of them. Clinical follow-up revealed that they had central and peripheral neurological disorders with motor nerve deficiencies and sensory motor system abnormalities, which appeared at different ages but worsened over time. The three p.Pro175 patients and the affected relatives, indeed, present with an early and unusual progressive neuropathy that can also progress to dementia and cerebral atrophy. A detailed clinical description and comparison of patients harboring these mutations will be reported (C. Ceferi, V. Pingault, N. Loundon, D. Bouccara, A. Jacquette, M. Holder, F. Petit, R. Touraine, N. Garabedian, O. Sterkers and S. Marlin, manuscript in preparation). At last examination (8-year-old), the patient harboring the p.Gln174Pro mutant also demonstrated progressively more severe phenotype with lower limb spasticity and peripheral neuropathy that has made ambulation difficult, requiring use of a wheelchair. He had bilateral hip and knee flexion contractures with increased tone of the legs and the flexors of the toes. By that age, the patient had developed a pes cavus deformity on the right side. The patient was able to pull to a standing position, but could sustain his weight without support. He demonstrated a normal tone and range of motion in upper extremities. Magnetic resonance imaging follow-up showed diffuse periventricular gliosis and perivascular cystic changes with generalized hypomyelination.

The patient with the p.Lys150Asn mutation (inducing foci and cytoplasmic re-localization) was also referred as having PCWH, but the patient was young and little clinical information were available at the time of the first publication (20). Clinical re-evaluation of this 13-year-old patient has been performed. He had bilateral profound hearing loss with successful cochlear implant, fair skin and hair with a few depigmented patches and heterochromia irides and long segment Hirschsprung disease successfully operated. In addition, he had several features suggestive of a mild-to-moderate PCWH: small lower limb re.

Materials and Methods

Plasmid constructs and retrovirus production

The pCMV-SOX10Myc, pCEC-SOX10, pSOX10-GFP, pECE-1E89X, pGL3-MITFdel1718 (MITF), pGL3-Cx32 (G/J1) and RET enhancer region MSC+9.7 vectors as well as respective empty vectors have

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been described previously (6,8,20,44–46,68). p54NRB YFP-tagged (Venus-tagged) construct was kindly provided by Prof. Archa Fox (29). Untagged p54NRB clone was purchased from OriGene. All WS-associated (20) or melanoma-associated SOX10 mutations (49) were introduced within the corresponding constructs by site-directed mutagenesis using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, les Ulis, France). MITF CRE-binding site was altered by site-directed mutagenesis, as described previously (48) using the following primers: forward: 5′-GAA AAA AAG CAT CAG CTG CCA GGA GG-3′, reverse: 5′-CCC CCT GGC TTC AGC TGA TGC TTT TTT TC-3′.

The SOX10-IRES-GFP retroviral transgene was generated by subcloning the human SOX10 cDNA into the EcoRI and Xhol cloning sites of the pMX-IRES-GFP vector (42). SOX10 foci-forming mutations were introduced by site-directed mutagenesis using the Quick Change Site-Directed Mutagenesis Kit, before constructs were used to transfect PLAT-E cells, as described by the manufacturer (Cell Biolabs, Inc., San Diego, USA).

**Cell culture**

HeLa (human cervical, ATCC), SKMel5 (human melanoma, ATCC, Molsheim, France) and SH-SYSY (subclone of human neuroblastoma, kindly provided by Stephane Jamain, IMRB) cell lines were cultured at 37°C, 5% CO2 in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Saint Aubin, France). One day before transfection, cells were plated differently according to the experimental design: 75 000 cells/well in 24-well plates containing glass covers for immunohistochemistry and 1 800 000 cells/well in 12-well plates for luciferase reporter assay or siRNA experiments. Western blot experiments were performed starting from six-well plates cultures.

Neurospheres cultures were generated from E11.5 mouse fetal gut tissue, according to the protocol described previously (43). Briefly, dissected guts were dissociated using Dispase/Collagenase (Roche, Boulogne, France) and plated on fibronectin-coated dishes (20 μg/ml, Sigma, Saint Louis, MO). Cells were maintained in culture medium including 15% chicken embryo extract, basic fibroblast growth factor (20 ng/ml; R&D Systems, Minneapolis, USA) and retinoic acid. Cultures were re-fed every 2 days. Once neurospheres appeared, the latter were used to perform total RNA extraction, immunohistochemistry experiments and/or retrovirus infection.

**RNA isolation and RT–PCR analysis**

RNAs from mouse melanocytes and skin were kindly provided by Genevieve Aubin-Houzelstein (UMR955 INRA-ENVA). Total RNA from neurospheres cultures was isolated using the GeneJET RNA purification kit (Fermentas Life Sciences, Illkirch, France). About 500 ng was used to synthesize cDNA using Maxima First Strand cDNA Synthesis Kit for RT-PCR (Fermentas Life Sciences). cDNA products, diluted at 1/5 final concentration, were used to amplify the Sox10 and NONO targets, whereas the housekeeping gene Gapdh was amplified as an internal standard control. The corresponding primer sequences were as follows: nono, forward: 5′-TAC ACA GCG TAG CCG TCT TTG-3′, reverse: 5′-CCA GAG CTT TCG GAG CAG CTC GC-3′ (expected product size: 406 bp); Sox10, forward: 5′-GCT GAA CGA AAG TGA CAA GC-3′, reverse: 5′-GTG GCT GAT TTC CCC GAT GT-3′ (expected product size: 418 bp) and Gapdh, forward: 5′-GTG ATC ATC TTC GCC CCT TCT GC-3′, reverse: 5′-GAT GCC TGG TTC ACC TTC TGT-3′ (expected product size: 442 bp). PCR amplification was performed using the following conditions: 1 cycle at 94°C for 5 min; 35 + 5 cycles at 94°C for 30 s, 62°C (for Sox10)/58°C (for NONO)/60°C (for Gapdh) for 30 s, 72°C for 45 s, followed by a final elongation step at 72°C for 7 min. RT-PCR products were analyzed on 2% agarose gel electrophoresis.

**Transfection and luciferase reporter assay**

HeLa, SKMel5 and SH-SYSY cells were transfected using Lipofectamine-PLUS reagent (Invitrogen) and each of the following constructs: one plasmid containing the reporter gene (150 ng of MITF, GJB1 or MITF-CREmut or 400 ng of RET), 150 ng of one different at each set of experiment, expression vectors containing the myc-tagged wild-type SOX10 or mutants cDNAs and/or 150 ng of expression vector containing the p54NRB cDNA. The total amount of plasmid was kept constant by the addition of respective empty vectors.

For competition assays, 150 ng of mutant SOX10 plasmids (1×) or 300 ng (2×) was mixed with an equal amount of wild-type SOX10 and p54NRB expression vectors as well as the GJB1 reporter plasmid (150 ng each). The total amount of plasmid was kept constant by adding empty pECE vector.

Twenty-four hours post-transfection, cells were washed twice with phosphate-buffered saline (PBS), and extracts assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, USA) as in (6,20,44). Protein quantification and luminescence measurement were performed using the Bradford measurement and Berthold technology, respectively.

**Knockdown of NONO expression—siRNA**

The NONO siRNA pool (Fermentas Life Sciences, ON-TARGET plus SMARTpool) was composed by four target sequences: 5′-CAAGUGAUUCGAGUAUC-3′; 5′-GUCAUUUCUGUGUGUUA-3′; 5′-GGAGGGUGCAGUUGGUUA-3′; 5′-AAAGAACUGGCGAUAC-3′.

Cells were transfected using the Dharmafect Transfection Reagent (Thermo Scientific, Illkirch, France), according to the manufacturer’s protocol. Forty-eight hours after NONO siRNA pool transfection, cells were transfected again with various SOX10 expression vectors (175 ng), associated or not with MITF reporter gene (175 ng), using the Lipofectamine-PLUS reagents (Invitrogen). Twenty-four hours later, cells were harvested and used for luciferase reporter gene or immunohistochemistry experiments. p54NRB knockdown was validated by both immunohistochemistry and western blot analysis.

**Western blot analysis**

Transfected cells were dissociated using mild trypsinization, washed twice with PBS 1× and lysed using 1% RIPA-protease inhibitor lysis buffer under mechanic force on ice for 30 min. Proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane (Invitrogen) and probed with mouse anti-p54NRB antibody (1/2000, BD Bioscience, Le Pont de Claix, France) in 5% skimmed milk. Following incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1/50 000, Sigma), immunoblots were visualized using a chemiluminescence kit (Amersham System, Velizy-Villacoublay, France). Quantification was performed using GeneTool software of Syngene.
Immunocytochemistry

Transfected cells or enteric neurospheres were fixed in 4% PFA (in PBS) for 10 min at 4°C. Immunohistochemistry was performed as described previously (42). After washing twice in PBS containing 0.1% Triton X-100 (PBT), cells were maintained in blocking solution [PBT with 1% bovine serum albumin (Sigma) and 0.15% glycine] at 4°C.

Primary antibodies were diluted in blocking solution as follows: p54NRB (mouse; 1/200, BD Biosciences or rabbit polyclonal; 1/200, GeneTex), TuJ-1 (mouse; 1/1000, Covance), GFAP (rabbit; 1/200, GeneTex), TuJ-1 (mouse; 1/1000, Covance), GFAP (rabbit; 1/100, Santa Cruz), p54NRB (mouse; 1/200, BD Biosciences or rabbit polyclonal; 1/100, Sigma Aldrich) and PSPC1 (mouse; C-3 clone, 1/100, Santa Cruz). Cultures were incubated with primary antibodies at 4°C overnight. After several washes with PBT, secondary antibodies were added in blocking solution for 2 h at room temperature at the following dilutions: anti-rabbit Cy3-conjugated (sheep; 1/150, Sigma Aldrich) or anti-mouse FITC-conjugated (donkey; 1/200, Jackson ImmunoResearch, West Baltimore, USA), anti-goat FITC-conjugated (donkey; 1/150, Sigma Aldrich) or anti-mouse FITC-conjugated (goat; 1/150, Sigma Aldrich). After several washes, cover slips were mounted using Vectashield containing 4′,6-diamidino-2-phenylindole (Vector Laboratories, Les Ulis, France) solution and examined with a Zeiss Axioplan 2 confocal microscope.

Co-immunoprecipitation analysis

HEK cells grown on 100 mm diameter dishes were transfected with 5 μg of wild-type or mutant SOX10 constructs and/or Venus-tagged p54NRB (YFP-p54NRB) vector. Cells were harvested 24 h after transfection in PBS 1×, and nuclear extracts obtained were resuspended in Co-IP buffer (20 mM HEPES pH 7, 150 mM NaCl, 1 mM EDTA and 1% IGEPAL), supplemented with complete protease inhibitors (Roche Applied Sciences, Boulogne, France). After pre-clearing, the lysates were incubated with an anti-GFP antibody for 1 h at 4°C, followed by immunoprecipitation with protein A/G (Santa Cruz) overnight at 4°C. Immunoprecipitates were washed three times with Co-IP buffer and analyzed by western blotting using anti-SOX10 antibody.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 4 software. Data were compared using Student’s t-test. When more than a two-group comparison was required, the results were analyzed using analysis of variance. Data are reported as mean ± standard error of the mean. A P < 0.05 was considered significant (n.s., not significant, *P < 0.05, **P < 0.01 and ***P < 0.001).

Supplementary Material

Supplementary Material is available at HMG online.

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References


