SOX10 structure–function analysis in the chicken neural tube reveals important insights into its role in human neurocristopathies

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Received February 18, 2010; Revised February 18, 2010; Accepted March 18, 2010

The HMG-domain containing transcription factor Sox10 is essential for neural crest (NC) development and for oligodendrocyte differentiation. Heterozygous SOX10 mutations in humans lead to corresponding defects in several NC-derived lineages and to leukodystrophies. Disease phenotypes range from Waardenburg syndrome and Waardenburg–Hirschsprung disease to Peripheral demyelinating neuropathy, Central dysmyelination, Waardenburg syndrome and Hirschsprung disease (PCWH). The phenotypic variability can partly be explained by the action of modifier genes, but is also influenced by the mutation that leads to haploinsufficiency in some and to mutant SOX10 proteins with altered properties in other cases. Here, we used in ovo electroporation in the developing neural tube of chicken to determine which regions and properties of SOX10 are required for early NC development. We found a strict reliance on the DNA-binding activity and the presence of the C-terminal transactivation domain and a lesser influence of the dimerization function and a conserved domain in the center of the protein. Intriguingly, dominant-negative effects on early NC development were mostly observed for truncated SOX10 proteins whose production in patients is probably prevented by nonsense-mediated decay. In contrast, mutant SOX10 proteins that occur in patients were usually inactive. Any dominant negative activity which some of these mutants undoubtedly possess must, therefore, be restricted to single NC-derived cell lineages or oligodendrocytes at later times. This contributes to the phenotypic variability of human SOX10 mutations.

INTRODUCTION

The transcription factor Sox10 belongs to the family of Sox proteins and is strongly expressed during vertebrate development in the neural crest (NC) and its derivatives as well as in the central nervous system (CNS) (1,2). Using mouse as a model system, Sox10 has, furthermore, been shown to be essential for survival and maintenance of pluripotency in NC stem cells, for specification of NC cells into several derivatives, for development of melanocytes and peripheral glia and for terminal differentiation of oligodendrocytes in the CNS (3–7).

In agreement with these functions, already heterozygous SOX10 mutations cause neurocristopathies and demyelinating diseases in humans (8,9). Often, patients present with a combination of Waardenburg syndrome (i.e. sensorineural hearing problems and partial depigmentation of skin, hair and/or iris) and Hirschsprung disease (i.e. aganglionosis of the distal colon), known as Waardenburg–Hirschsprung disease (OMIM 277580) (9). Neurological symptoms due to peripheral myelinopathies and central demyelination are frequent complications and in combination with Waardenburg–Hirschsprung disease lead to Peripheral Demyelinating neuropathy, Central dysmyelination, Waardenburg syndrome and Hirschsprung disease (PCWH) syndrome (OMIM 609136) (8). However, milder or variant phenotypes have also been reported, such as cases of Waardenburg type 2 (OMIM 611584) and Yemenite deaf–blind hypopigmentation syndrome (OMIM 601706) (10,11).

These different phenotypes are believed to be caused, on the one hand, by differences in genetic background and thus by modifier genes. This assumption has indeed been supported by studies in the mouse (12–14). The other major determinant of the phenotype is the exact type of mutation in the SOX10 gene (8,15). Over 40 mutations have so far been identified...
and indeed they differ dramatically. In addition to numerous nonsense mutations that are spread over the whole open-reading frame, there are missense mutations, insertions, splice site mutations and whole-gene deletions (for a recent summary, see ref. 8,10,16).

Gene deletion and splice site mutation simply lead to loss of protein production from the affected allele (10,16). Nonsense mutations in the first two of the three coding exons (i.e. exons 3 and 4) are believed to trigger nonsense-mediated decay (NMD) of the mutant transcript and to similarly result in haploinsufficiency (8). Several other nonsense and missense mutations, however, cause production of altered proteins. The characteristics of these altered proteins probably determine the phenotypic consequences of the mutation. So far, mutations have primarily been studied biochemically for DNA binding to select sites and in tissue culture for their ability to activate a small number of target genes, either alone or in combination with other transcription factors (11,17–20). This, however, allows only limited conclusions on the global effect of these mutants on cellular gene expression and developmental processes. It is not clear, for instance, whether mutant SOX10 proteins exert their effects already in NC development or only at later times in select NC-derived lineages.

To address the effect of SOX10 mutations in early NC development, we made use of the chicken embryo and overexpressed SOX10 mutants in the neural tube by in ovo electroporation. Because SOX10 is highly conserved even at the level of the amino acid sequence, it can induce NC development in the chicken neural tube. In ovo electroporation, therefore, turned out to be a suitable system for determining the structural requirements of SOX10 in NC development and for studying the consequences of various SOX10 mutations.

RESULTS

Altered gene expression after ectopic SOX10 expression in the chick spinal cord

Ectopic expression of chicken and mouse Sox10 in the chick neural tube has previously been reported to be sufficient for ectopic NC induction (21,22). In our present study, we first repeated these experiments with human SOX10 to verify that the human protein also induces NC properties. We used the bicistronic pCAGGS-SOX10-IRES-nls-GFP expression plasmid that carries the human SOX10 open-reading frame and an IRES-linked GFP cDNA under control of the chicken β-actin promoter. This expression plasmid was electroporated in neural tubes from stage 10–11 embryos, resulting in a unilateral, somewhat variable, mosaic expression of SOX10 and GFP. An insert-free pCAGGS-IRES-nls-GFP plasmid was used for electroporation in controls. Analyses were performed 24 and 48 h after electroporation on transverse sections between fore- and hindlimbs. Similar results were obtained at both time points, but only those from 48 h post-electroporation are shown. As NC markers we used HNK-1, cadherin7, AP2α as well as Sox5, which has previously been found to play a role in the generation of NC cells by influencing RhoB expression (23), and its close relative Sox6, which is often coexpressed during embryonic development (24,25) (Fig. 1C–H).

Figure 1. Sox10 electroporation into the chicken neural tube causes the induction of NC markers. (A) The percentage of GFP-positive cells was determined, which expressed HNK-1, Sox5 and cadherin7 in control (Con) and in SOX10 electroporations. Differences between the two electroporations were statistically significant (**P < 0.001) as determined by Student’s t-test. (B) Schematic representation of the consequences of ectopic NC induction following neural tube (NT) electroporation in sections where dorsal root ganglia (DRG) are absent (left) or present (right). The electroporated side (+) shows thinning of the extracellular matrix and cell emigration. (C–I) Immunohistochemistry against HNK-1 (C), cadherin7 (D), Sox5 (E), Sox6 (F), Sox9 (G), AP2α (H) and laminin (I) (all in red) was combined with GFP autofluorescence (green) on transverse sections of chicken embryos electroporated with a pCAGG-SOX10-IRES-nGFP expression plasmid 48 h after the electroporation event. Neural tube border and lumen are demarcated by dotted lines. In the overviews (left panels), the electroporated side is oriented to the left. The right panels represent high-resolution pictures of electroporated cells. The arrows in (H) point to non-electroporated regular NC cells which in contrast to electroporated ectopic NC express AP2α. The asterisk in (D) marks the medial cadherin7-positive domain in the neural tube. Arrowheads in (I) highlight the discontinuities in the extracellular matrix around the neural tube. FP, floorplate; VR, ventral root; VZ, ventricular zone.

Of these markers, cadherin7 is also expressed in a medial region of the neural tube at the time of analysis (Fig. 1D, asterisk) (26). Therefore, cadherin7 is informative as a marker of ectopic NC induction only outside this medial region. There is also beginning expression of Sox5 and Sox6...
in ventricular zone (VZ) cells of the neural tube, and strong Sox6 expression in the floor plate (Fig. 1E and F). The VZ expression is, however, substantially weaker than that in NC cells, or in case of Sox5 in surrounding mesenchymal cells. Ectopic NC cells are thus readily identified by upregulated Sox5 and Sox6 expression.

Regular NC induction and emigration from the dorsal neural tube takes place during the period of analysis. Some of these regular NC cells become electroporated and migrate with unelectroporated NC cells along lateral and ventral pathways to their sites of destination such as peripheral ganglia and nerves. This explains why up to 4% of GFP-positive cells expressed HNK-1 and high Sox5 levels even in control electroporations (Fig. 1A). Twenty-three percent of GFP-positive cells in control electroporations were also labelled by anticadherin7 antibodies. These include regular NC cells as well as endogenously cadherin7-positive cells from the medial part of the neural tube.

Electroporation of human SOX10, in contrast, led to a widespread induction of high levels of NC markers in electroporated cells along the whole dorsoventral axis of the neural tube (Fig. 1C–F). Quantifications revealed that 75% of all GFP-positive cells expressed HNK-1, 84% expressed cadherin7 and 95% expressed high levels of Sox5 (Fig. 1A). Electroporated cells, furthermore, underwent epithelial-to-mesenchymal transition and emigrated from the neural tube at all dorsoventral levels, thereby depleting the electroporated half (Fig. 1C–H). As a consequence, the SOX10-electroporated side was usually significantly thinner and often deformed. The surrounding extracellular matrix was disrupted as evident from discontinuities in the laminin staining at sites where electroporated cells left the neural tube (Fig. 1I, arrowheads). These findings confirm that human SOX10 can function as an NC specifier in the developing chicken embryo and integrates into the corresponding regulatory network. However, not all NC markers were induced by SOX10. Neither Sox9 nor AP2α were expressed in SOX10-electroporated cells within or outside the neural tube (Fig. 1G and H). This agrees well with the studies that place Sox9 and AP2α genetically upstream of Sox10 (22,27,28).

Structure–function studies in the electroporated chicken neural tube

Having defined a set of markers to monitor the NC-specific function of SOX10, we set out to analyze which regions or properties of SOX10 are essential for NC induction. We started with the SOX10ΔDB mutant in which two amino acids were substituted in the second helix of the HMG domain (Fig. 2A) so that DNA-binding was lost (29). This SOX10ΔDB mutant failed to induce the NC markers HNK-1, cadherin7 and Sox5 (Fig. 2B, E and H). The integrity of the extracellular matrix surrounding the neural tube was also not compromised as evident from laminin staining (Fig. 2K, and data not shown), indicating that the SOX10ΔDB mutant has no NC-inducing activity. We conclude that DNA binding is one of the prerequisites for SOX10 function in the NC.

A second substitution mutant in the SOX10 HMG domain had previously been generated in which the ability to leave the nucleus was abolished (29). The DNA-binding activity, however, remained unaffected. This SOX10L138A mutant was as effective in inducing HNK-1, cadherin7 and Sox5 as the wild-type protein (Fig. 2C, F and I). The effects on laminin expression and neural tube deformation were also comparable with the wild type (Fig. 2L, and data not shown). A functional nuclear export signal and the ability to leave the nucleus are thus not essential for SOX10 function in the early NC and probably more important for termination of SOX10 function as suggested previously (29).

Another major function of SOX10 has been mapped to its C-terminal 66 amino acids. These are strongly conserved in the related SOX8 and SOX9 and function as the protein’s main transactivation (TA) domain (30). Deletion of this domain in the SOX10ΔC mutant (Fig. 2A) led to a complete...
loss of NC marker induction, delamination and emigration of cells from the electroporated side of the neural tube (Fig. 2D, G, J and M). This confirms previous results that the carboxy-terminal TA domain is indeed essential and that SOX10 primarily functions as a transcriptional activator during NC development. Apart from the DNA-binding HMG domain and the TA domain, several other regions have been defined in SOX10. The first 60 amino acids of the protein, for instance, are unique to SOX10 and set it apart from the related SOX8 and SOX9 (31). Deletion of this region in the SOX10Δ1–60 mutant (Fig. 3A) did not alter SOX10 behavior in the electroporated neural tube. NC markers were efficiently induced (88–100% depending on the marker, Fig. 3R–T) and cells emigrated from the neural tube after undergoing an epithelial-to-mesenchymal transition (Fig. 3B, F, J and N). We infer from these results that the first 60 amino acids are not essential for the early NC function of SOX10. This agrees well with the previous observation that Sox8 and Sox9, which lack this region, were as effective as Sox10 in inducing NC-like properties after electroporation into the neural tube (21).
Amino acids 61–100, on the other hand, are well conserved in SOX8 and SOX9, allow for cooperative binding of two SoxE proteins to closely spaced recognition elements and thus mediate DNA-dependent dimerization (32–34). Two mutants were tested in our study, the SOX10<sup>Δ61–100</sup> mutant, in which the complete dimerization domain was removed, and the SOX10<sup>Δ31</sup> mutant, in which alanine substitutions of amino acids 71–73 abolished the dimerization function of this conserved domain (32) (Fig. 3A). Following electroporation, most cells expressing the mutant SOX10 proteins remained in the neural tube and retained their epithelial morphology, indicating that epithelial-to-mesenchymal transition did not take place (Fig. 3C, D, G, H, K and L). Laminin staining confirmed that the neural tube boundary remained largely intact (Fig. 3O and P). The dimerization domain of SOX10 is thus essential for epithelial-to-mesenchymal transition and cell emigration. NC markers were still induced, although to a lesser extent (Fig. 3C, D, G, H, K and L). Interestingly, slight differences also became apparent between the two SOX10 mutants. Whereas both upregulated Sox5 fairly well (69% for SOX10<sup>Δ61–100</sup> and 86% for SOX10<sup>Δ31</sup> compared with 95% for wild-type SOX10, Fig. 3T) and were moderate cadherin7 inducers (61% for SOX10<sup>Δ61–100</sup> and 42% for SOX10<sup>Δ31</sup> compared with 84% for wild-type SOX10, Fig. 3S), the HNK-1 induction was selectively reduced in the SOX10<sup>Δ61–100</sup> mutant (29% for SOX10<sup>Δ61–100</sup> compared with 84% for SOX10<sup>Δ31</sup> and 75% for wild-type SOX10, Fig. 3R), suggesting that the region between amino acids 61 and 100 of SOX10 has functions additional to dimerization that are not affected by the alanine substitutions in SOX10<sup>Δ31</sup>. Another domain with high conservation among SOX10, SOX8 and SOX9 spans amino acids 234–306 in the central part of the protein. This K2 domain functions as a second, weaker, context-dependent and cell-type-specific TA domain (35). Deletion of the domain in the SOX10<sup>ΔK2</sup> mutant (Fig. 3A) reduced, but did not abolish, the NC inducing properties of SOX10. NC marker induction, delamination and emigration were less efficient (Fig. 3E, I, M and Q). Only 41% of the electroporated cells were HNK1 positive, 42% cadherin7 positive and 63% Sox5 positive (Fig. 3R–T). The K2 domain is, therefore, not strictly required to induce NC properties, but strongly modulates the efficiency of SOX10 in this process.

Consequences of human mutations on SOX10 function in the NC

Several SOX10 mutations have been identified in human patients, which lead to a wide range of neurocristopathies in the heterozygous state. The first set of SOX10 mutations that we chose to analyze for their impact on early NC development is representative of those cases where mutant transcripts are not subject to NMD (8) and where mutant proteins are generated in the patients (Fig. 4A). These include in-frame insertions and deletions as well as missense mutations and nonsense mutations in the last coding exon (8).

The SOX10<sup>482ins6</sup> mutant carries a duplication of leucine-161 and arginine-162 in the third helix of the HMG domain (9). This insertion completely abolishes DNA binding (17). Considering the results of SOX10<sup>ΔDB</sup> electroporations, the SOX10<sup>482ins6</sup> mutant was expected to have no NC-inducing activity. In ovo electroporation into the chicken neural tube indeed neither led to the induction of HNK-1, cadherin7 nor Sox5 nor were there any signs of epithelial-to-mesenchymal transition or emigration from the electroporated side (Fig. 4B, F, J and N).

Mutation of codon 377 from glutamine to a stop codon, on the other hand, produced a truncated SOX10 that completely lacks the TA domain (Fig. 4A). Therefore, we predict that this SOX10<sup>Q377X</sup> mutant should behave as SOX10<sup>ΔC</sup> in electroporations and fail to induce NC marker gene expression, epithelial-to-mesenchymal transition and cell emigration from the electroporated neural tube. Visual inspection of electroporated neural tubes confirmed this assumption (Fig. 4C, G, K and O). Quantifications revealed a slightly higher percentage of HNK-1 and Sox5 expressors among SOX10<sup>Q377X</sup> electroporated cells than among control pCAGGS-IRES-nls-GFP electroporated cells (Fig. 4R and T). However, this difference was statistically not significant. Cadherin7 was not induced above the control electroporation levels (Fig. 4S).

The SOX10<sup>Δ1355</sup> mutant carries a missense mutation in the HMG domain (Fig. 4A) that leads to a reduced DNA-binding activity, but does not abolish DNA binding completely (11). Thus, it was interesting to see how this mutation would affect SOX10 function in the early NC. Surprisingly, SOX10<sup>Δ1355</sup> induced HNK-1 and cadherin7 as efficiently as wild-type SOX10 (Fig. 4D, H, R and S). Only Sox5 induction was significantly decreased to 40% of electroporated cells when compared with 95% for the wild type, but was still significantly higher than in control electroporations (Fig. 4T). SOX10<sup>Δ1355</sup> electroporated cells had, furthermore, lost their epithelial morphology and had started to emigrate from the neural tube. Accordingly, laminin staining around the neural tube was disrupted on the electroporated side (Fig. 4P). Despite the impaired DNA-binding activity of the SOX10 mutant, differences in the wild-type SOX10 were only mild. The residual DNA binding of the SOX10<sup>Δ1355</sup> mutant is thus largely sufficient to maintain SOX10 function in the early NC.

In another set of human mutations, the regular SOX10 stop codon is removed, so that mutant proteins are generated that extend beyond their normal C terminus. The prototype is the SOX10<sup>Δ400del12</sup> mutant (36), in which 12 bp including those of the stop codon were deleted so that an additional 82 amino acids are added at the end of the protein (Fig. 4A). When this extension mutant was electroporated into the chicken neural tube, neither induction of HNK-1, cadherin7, Sox5 nor cell emigration were observed (Fig. 4E, I, M, Q, R, S and T). Instead, electroporated cells retained the identity of neural tube cells, arguing that the SOX10<sup>Δ400del12</sup> mutant is completely inactive. Recent biochemical studies had shown that the SOX10<sup>Δ400del12</sup> mutant is unable to bind DNA (18). This loss of DNA-binding activity is sufficient to explain the inactivity of the SOX10<sup>Δ400del12</sup> mutant in the early NC.

SOX10 mutations, apoptosis and dominant-negative functions

In humans, nonsense mutations in the first two coding exons of SOX10 (i.e. exons 3 and 4) probably give rise to transcripts that undergo NMD so that mutant protein is not produced (8). SOX10<sup>ΔE189X</sup> is such a mutation in which glutamate-189 in exon 4 is changed to a premature stop codon (Fig. 5A)
If translated, this mutation would lead to a truncated protein that ends shortly after the HMG domain. Furthermore, it had been shown to efficiently suppress the transcriptional activity of wild-type SOX10 as a dominant-negative in transiently transfected cells (17).

To analyze how SOX10E189X behaves in the context of NC induction, we electroporated the mutant into the chicken neural tube. HNK-1, cadherin7 and Sox5 as NC markers were not induced (Fig. 5B, D, F and L–N), and electroporated cells did not emigrate from the neural tube as indicated by the fact that the laminin staining of the neural tube border remained intact (Fig. 5H). The electroporated side of the neural tube was, however, significantly thinner than the control side. SOX10E189X-electroporated cells within the neural tube furthermore exhibited an altered morphology. The round appearance of many electroporated cells indicated that they may be dying. TUNEL staining indeed revealed that many of the SOX10E189X-electroporated cells underwent apoptosis (Fig. 5J). Rates of apoptosis were 11-fold higher than in control electroporations (Fig. 5O). Such an increased apoptosis was furthermore not observed for wild-type SOX10 or any of the aforementioned mutants (data not shown), arguing that SOX10E189X may function as a dominant-negative in the chicken neural tube.

If SOX10E189X acts as a dominant negative, it is likely to do so by interfering with the function of wild-type SoxE proteins.
into the chicken neural tube, the SOX10E189X-induced cell and emigrate from the neural tube (Fig. 5C, E, G and I). This allowed neuroepithelial cells to acquire NC characteristics (Fig. 5B, D, F, H and J) or a combination of SOX10E189X and SOX9 (C, E, G, I and L–N). Remarkably, however, NC markers were not induced and cells did not emigrate from the neural tube as indicated by laminin staining (Fig. 7C, E, G, I and L–N). The inlay in (J) combines TUNEL signal with GFP immunohistochemistry at high magnification. The electroporated side is oriented to the left. Arrows point to non-electroporated regular NC cells and arrowheads mark discontinuities in the extracellular matrix around the neural tube. (L–O) The percentage of GFP-positive cells that expressed HNK-1 (L), cadherin7 (M) and Sox5 (N) or were TUNEL positive (O) was determined for the SOX10E189X mutant. Values for SOX10E189X differed significantly from those obtained in wild-type SOX10 and control (Con) electroporations. Whether values for different groups were statistically different was determined by ANOVA with Tukey’s multiple comparison post-test. These results thus show that it is not the mere length of the region C-terminal to the HMG domain that decides whether cell death is prevented. It furthermore implicates the K2 domain in the suppression of cell death. Taking furthermore into account that the SOX10N308 mutant thus behaved similar to SOX10ΔC. It argues that residues 256–308 suppress the cell death-inducing activity.

To determine the structural basis for the cell death-inducing effect, we tested a set of additional SOX10 mutants by in ovo electroporation (Fig. 6A). First, we truncated the SOX10482ins6 protein behind the residue 189. This generates an SOX10E189X protein without DNA-binding activity. As expected, SOX10482ins6,E189X did not induce NC marker expression and cell emigration from the neural tube (Fig. 6B, E, H and K), but it also did not cause apoptosis in the electroporated cells in the neural tube (Fig. 6N and Q). We, therefore, conclude that DNA binding is essential for cell death induction.

Next, we electroporated an SOX10 mutant that was 66 residues longer than SOX10E189X (Fig. 6A). This SOX10N255 mutant behaved very similar to SOX10E189X. NC features were not induced, cells did not emigrate and cell death was prevalent throughout the electroporated half of the neural tube (Fig. 6C, F, I, L and O) with rates of apoptosis being 9-fold higher than in control electroporations (Fig. 6Q). In contrast, an SOX10 mutant with 119 more residues than SOX10E189X (Fig. 6A) did not cause apoptosis, although the NC marker expression and emigration of cells from the neural tube were still not observed (Fig. 6D, G, J, M, P and Q). This SOX10N308 mutant thus behaved similar to SOX10ΔC. It argues that residues 256–308 suppress the cell death-inducing activity.

This region strongly overlaps with the K2 domain. To address whether the K2 domain is involved in the suppression of cell death, we deleted the K2 domain in the context of the SOX10ΔC mutant (Fig. 7A). Although the resulting SOX10ΔC,N252K mutant was comparable in size with the SOX10N308 protein, it not only failed to induce NC features and cell emigration, but also increased apoptosis in electroporated cells (Fig. 7B, D, F, H and J) with rates being 7-fold higher than in control electroporations (Fig. 7O). These results thus show that it is not the mere length of the region C-terminal to the HMG domain that decides whether cell death is prevented. It furthermore implicates the K2 domain in the suppression of cell death. Taking furthermore into account that the SOX10ΔC,N252K mutant did not exhibit any signs of increased apoptosis, it appears that either the K2 domain or the C-terminal TA domain have to be present to prevent apoptosis.

The unusual features of the SOX10 Dominant megacolon mutation

In the Dominant megacolon (Dom) mouse, a single base pair is inserted into the Sox10 open-reading frame, leading to a frame shift. By conceptual translation, a mutant Sox10 protein is generated, which consists of the first 193 amino acids of Sox10 to which an additional 99 unrelated amino acids are added as a divergent C terminus (Fig. 7A) (4, 6). Previous studies had shown that this Sox10ΔDom protein is indeed produced in the Dom mouse (5).

To study the properties of the Sox10ΔDom protein, this mutant was electroporated into the chicken neural tube. As expected, NC markers were not induced and cells did not emigrate from the neural tube as indicated by laminin staining (Fig. 7C, E, G, I and L–N). Remarkably, however, Sox10ΔDom-electroporated cells did not exhibit an increased apoptosis, arguing that the Sox10ΔDom-specific extension suppresses cell death (Fig. 7K and O). This could mean that the 99 amino acid extension fortuitously functions in a manner comparable with the K2 (or

These are not only strongly expressed in the emerging NC, but also in neuroepithelial cells where Sox9 is prevalent (37). When we electroporated SOX10E189X together with SOX9 into the chicken neural tube, the SOX10E189X-induced cell death was rescued (Fig. 5K). The ectopic SOX9 even allowed neuroepithelial cells to acquire NC characteristics and emigrate from the neural tube (Fig. 5C, E, G and I).

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**Figure 5.** Truncations may lead to SOX10 mutants with dominant negative function. (A) Schematic representation of the SOX10E189X mutant protein. (B–K) Immunohistochemistry against HNK-1 (B and C), cadherin7 (D and E), Sox5 (F and G) and laminin (H and I) (red) was combined with GFP autofluorescence (green) on transverse sections of chicken embryos 48 h after electroporation with a pCAGG-IRES-nlsGFP expression plasmid for SOX10E189X (B, D, F, H and J) or a combination of SOX10E189X and SOX9 (C, E, G, I and K). Apoptotic cells were detected in these electroporations by TUNEL analysis (J and K). The inlay in (J) combines TUNEL signal with GFP immunohistochemistry at high magnification. The electroporated side is oriented to the left. Arrows point to non-electroporated regular NC cells and arrowheads mark discontinuities in the extracellular matrix around the neural tube. (L–O) The percentage of GFP-positive cells that expressed HNK-1 (L), cadherin7 (M) and Sox5 (N) or were TUNEL positive (O) was determined for the SOX10E189X mutant. Values for SOX10E189X differed significantly from those obtained in wild-type SOX10 and control (Con) electroporations. Whether values for different groups were statistically different was determined by ANOVA with Tukey’s multiple comparison post-test.

To study the properties of the Sox10ΔDom protein, this mutant was electroporated into the chicken neural tube. As expected, NC markers were not induced and cells did not emigrate from the neural tube as indicated by laminin staining (Fig. 7C, E, G, I and L–N). Remarkably, however, Sox10ΔDom-electroporated cells did not exhibit an increased apoptosis, arguing that the Sox10ΔDom-specific extension suppresses cell death (Fig. 7K and O). This could mean that the 99 amino acid extension fortuitously functions in a manner comparable with the K2 (or
TA) domain. Alternatively, the 99 amino acid extension could suppress the cell-death–inducing activity of the N-terminal 193 residues of Sox10 by interfering to the DNA-binding ability of Sox10Dom. Previous studies had shown that the

![Figure 6](https://example.com/image1.png)

**Figure 6.** Structural requirements for dominant-negative SOX10 mutants. (A) Schematic representation of the electroporated SOX10 mutants SOX10482ins6,E189X, SOX10N255 and SOX10N308. (B–P) Immunohistochemistry against HNK-1 (B–D), cadherin7 (E–G), Sox5 (H–J) and laminin (K–M) (red) was combined with autofluorescence (green) on transverse sections of chicken embryos 48 h after electroporation with pCAGG-IRES-nlsGFP expression plasmids for SOX10482ins6,E189X (B, E, H, K and N), SOX10N255 (C, F, I, L and O) and SOX10N308 (D, G, J, M and P). Apoptotic cells were detected by TUNEL analysis (N–P). The electroporated side is oriented to the left. Arrows point to non-electroporated regular NC cells. (Q) The percentage of GFP-positive cells that expressed HNK-1 (L), cadherin7 (M) and Sox5 (N) or were apoptotic (O) was determined for SOX10 mutants and compared with the corresponding values from wild-type (WT) SOX10 and control (Con) electroporations. Whether the values for SOX10482ins6,E189X and SOX10N308 differed significantly from those obtained in wild-type SOX10 electroporations (**P < 0.01) or control electroporations (##P < 0.01) was determined by ANOVA with Tukey’s multiple comparison post-test.

![Figure 7](https://example.com/image2.png)

**Figure 7.** The presence of the K2 domain prevents cell death induction by mutant SOX10 proteins. (A) Schematic representation of the electroporated mutants SOX10 DCDC2 and Sox10Dom. (B–K) Immunohistochemistry against HNK-1 (B and C), cadherin7 (D and E), Sox5 (F and G) and laminin (H and I) (red) was combined with GFP autofluorescence (green) on transverse sections of chicken embryos 48 h after electroporation with pCAGG-IRES-nlsGFP expression plasmids for SOX10DCDC2 (B, D, F, H and J) and Sox10Dom (C, E, G, I and K). Apoptotic cells were detected by TUNEL analysis (J and K). The electroporated side is oriented to the left. Arrows point to non-electroporated regular NC cells. (L–O) The percentage of GFP-positive cells that expressed HNK-1 (L), cadherin7 (M) and Sox5 (N) or were apoptotic (O) was determined for SOX10 mutants and compared with the corresponding values from wild-type (WT) SOX10 and control (Con) electroporations. Whether the values for SOX10 DCDC2 and Sox10Dom differed significantly from those obtained in wild-type SOX10 electroporations (**P < 0.001) or control electroporations (##P < 0.01) was determined by ANOVA with Tukey’s multiple comparison post-test.
Sox10\textsuperscript{Dom} protein is capable of binding to DNA, but has no TA capacity (4). However, only one particular binding site was studied that was neither a natural nor a high-affinity target site for Sox10. We therefore re-analyzed the DNA-binding activity of the Sox10\textsuperscript{Dom} protein on site B of the \textit{Mpz} promoter (33) as a bona fide high-affinity recognition element by electrophoretic mobility shift assay (EMSA) (Fig. 8A). Wild-type SOX10 bound avidly to this site, as did most of the Sox10 mutants that were generated during this study and had not been analyzed before (Fig. 8A and data not shown). The Sox10\textsuperscript{Dom} protein, in contrast, failed to form a complex on site B in significant amounts. We thus conclude that the 99 amino acid extension of Sox10\textsuperscript{Dom} interferes with the DNA-binding activity of the HMG domain on at least some Sox10 recognition sites. This reduced DNA-binding activity prevents Sox10\textsuperscript{Dom} from inducing cell death and functioning as a strong dominant negative in chicken electroporation similar to SOX10\textsuperscript{E189X}.

To address the behavior of the Sox10\textsuperscript{Dom} protein in mouse development, we compared the phenotypes of Sox10\textsuperscript{Wt/Dom} and Sox10\textsuperscript{Wt/lacZ} mice in identical genetic backgrounds. As published previously (12), more Sox10\textsuperscript{Wt/Dom} mice developed a megacolon in the first postnatal weeks than Sox10\textsuperscript{Wt/lacZ} mice, arguing that the effects of the Sox10\textsuperscript{Dom} mutation on enteric nervous system development are not simply the result of Sox10 inactivation and haploinsufficiency. Outside the developing enteric nervous system, however, very few differences were detected. Adrenal development in Sox10\textsuperscript{Wt/Dom} mice was, for instance, almost normal at both 12.5 and 18.5 dpc (Fig. 8B and data not shown) and not any more disturbed than in Sox10\textsuperscript{Wt/lacZ} mice (38). Terminal differentiation of oligodendrocytes was slightly delayed at early postnatal times in the spinal cords of Sox10\textsuperscript{Wt/Dom} mice (Fig. 8C), but the delay was comparable with that previously observed in Sox10\textsuperscript{Wt/lacZ} mice (39). We conclude that unlike the enteric nervous system, other Sox10 expressing cells are similarly affected in their development in the Sox10\textsuperscript{Wt/Dom} and Sox10\textsuperscript{Wt/lacZ} mutants. This argues for a cell lineage-specific, and against a general dominant-negative, function of the Sox10\textsuperscript{Dom} protein.

**DISCUSSION**

By performing a detailed structure–function analysis in the electroporated chicken neural tube, we have shown in this report that SOX10 function in the early NC depends on its DNA-binding activity as well as on the presence of the carboxy-terminal TA domain, thus confirming the previous results on the mouse and chicken Sox10 which have shown this protein to primarily function as a transcriptional activator in the early NC (21,22). Additionally, we have also detected a requirement for the dimerization domain and the K2 domain of the SOX10 protein in some aspects of early NC function, in particular epithelial-to-mesenchymal transition and cell emigration. Both domains also had a strong influence on the efficiency with which SOX10 induced NC markers in neuroepithelial cells.

We have previously described two hypomorphic mouse mutants in which wild-type Sox10 was replaced by Sox10\textsuperscript{aal} or Sox10\textsuperscript{MK2}, respectively (35). Neither Sox10\textsuperscript{aal/aal} nor Sox10\textsuperscript{MK2/MK2} mice exhibited severe defects in early NC development. This discrepancy between mouse studies and the chicken in vivo electroporations is probably caused by differences in the experimental setup. Whereas the Sox10 mutant is the dominant SoxE protein in electroporations as a consequence of overexpression, it co-exists in comparable amounts with other SoxE proteins such as Sox9 in the mouse mutants. Premigratory NC cells in mammals even express Sox9 before Sox10 (27). In Sox10\textsuperscript{aal/aal} and Sox10\textsuperscript{MK2/MK2} mice, Sox9 is sufficient to drive the NC induction.

It is also important to note that SOX10 mutants with lost DNA binding, dimerization, K2 or TA domain were compromised in their NC-inducing functions, but did not exhibit any
EdnrB and SOX10Q234X, SOX10Q250X and SOX10S251X mutations cause the SOX10S135T mutant. Despite the fact that this mutant with beyond the early NC stage is also supported by the behavior of activity of the mutant SOX10 proteins in the early NC.

partly from the apoptosis-inducing and dominant-negative potential, and if produced in the corresponding patients should cause the severe variants of SOX10-associated disease. The fact that this is not the case supports the hypothesis that the corresponding mutant transcripts are subject to NMD and that the proteins are never made (8). If patients with nonsense mutations in exon 4 of SOX10 do not produce the mutant protein, the disease must be caused by the fact that not enough SOX10 can be produced from the remaining functional SOX10 allele. Waardenburg–Hirsch-sprung disease as the predominant phenotype in these patients is thus a consequence of SOX10 haploinsufficiency as proposed previously (8).

Other SOX10 mutations, however, affect the DNA-binding activity or represent frame shifts and nonsense mutations in the last coding exon. These mutations probably lead to the production of mutant SOX10 proteins. We tested some of these mutations and found most of them functionally inactive in the early NC, but without dominant-negative effect. From this observation, it should be expected that these mutations cause the same disease as those that trigger NMD. However, we know that this is usually not the case as most of these mutations result in PCWH, the severe type of SOX10-associated disease. How can we explain this discrepancy? The most parsimonious explanation is that these mutations acquire dominant effects not in the early NC, but predominantly later in peripheral glia and oligodendrocytes, thus leading to the peripheral neuropathies and central myelin defects observed in PCWH patients.

Interestingly, some patient-derived truncating mutations in the last coding exon closely resemble the apoptosis-inducing SOX10N255 mutant in our study (8,15,36). Incidentally, these SOX10N234X, SOX10Q235X and SOX10S251X mutations cause a particular severe form of PCWH and lead to early postnatal death in affected patients. It is therefore tempting to speculate that disease severity in these affected patients results at least partly from the apoptosis-inducing and dominant-negative activity of the mutant SOX10 proteins in the early NC.

The fact that SOX10 mutations exert disease-related effects beyond the early NC stage is also supported by the behavior of the SOX10S135T mutant. Despite the fact that this mutant with reduced DNA-binding activity efficiently induced NC proper-

MATERIALS AND METHODS

Plasmids

The open-reading frame of human SOX10, SOX9 and rat Sox10 were inserted in wild-type and various mutant versions behind the chicken β-actin promoter and upstream of an IRES-GFP cassette into pCAGGS-ires-nls-GFP (gift of M. Cheung and J. Briscoe, NIMR, London). Already described mutants include SOX10ΔDB, SOX10Δ1–60, SOX10Δ61–100, SOX10Δ138A, SOX10Δ82ins6, SOX10S135T, SOX10C377X, SOX10K240del12, SOX10E189K, SOX10N251, SOX10ΔK2 and Sox10Dom (11,17,18,29,32,35) Using wild-type SOX10 as template, SOX10ΔK2 and SOX10N251 variants were additionally produced by polymerase chain reaction (PCR). In contrast, SOX10G82ins6, E189K and SOX10ΔK2 mutants were generated by PCR using the SOX10G82ins6 and SOX10ΔK2 mutants, respectively. PCR primers are available on request.

Chicken in ovo electroporation

Fertilized chicken eggs were obtained from Lohmann (Cuxhaven, Germany) and incubated in a humidified incubator at 37.8°C. Embryos were staged according to Hamburger and Hamilton (HH) (40). pCAGGS-ires-nls-GFP–based expression plasmids for wild-type SOX9, wild-type SOX10 and various SOX10 mutants were injected at a concentration of 2 μg/μl into the lumen of HH stage 10–11 neural tubes. Electrodes were placed at either side of the neural tube, and electroporation was carried out using a BTX ECM830 electroporator delivering five 50 ms pulses of 30 V. Transfected embryos were allowed to develop for 48 h before dissection and analysis.

Animal husbandry and genotyping

Mice with an Sox10Dom allele (4) or an Sox10lacZ allele (3) were kept as heterozygotes on a C3HeB/FeJ background. Genotyping was performed by PCR as described previously.
Germany), embryos, spinal cords and adrenal glands were embedding in Jung Tissue Freezing Medium (Leica, Nussloch, Germany), Mouse embryos were isolated at 18.5 dpc from staged pregnancies, and spinal cords were dissected from P3 animals.

Performance and quantification of in situ hybridization, immunohistochemistry and TUNEL

After 1 h to overnight fixation in 4% paraformaldehyde and embedding in Jung Tissue Freezing Medium (Leica, Nussloch, Germany), embryos, spinal cords and adrenal glands were transversely cut into 10–14 μm sections on a cryotome (Leica, Bensheim). In situ hybridizations were performed on 14 μm sections using a digoxigenin-labeled antisense probe for mouse Mbp (7). For immunohistochemistry, the following primary antibodies were used on 10 μm sections: anti-TH rabbit antisemur (1:1000 dilution, Biomol), anti-Sox9 rabbit antisemur (1:1000 dilution) (37), anti-GFP rabbit antisemur (1:3000 dilution, Molecular Probes), anti-HNK-1 mouse monoclonal (1:10000, BD Pharmingen), anti-cadherin7 mouse monoclonal (1:200 dilution, Developmental Hybridoma Bank), anti-AP2α mouse monoclonal (1:200 dilution, Developmental Hybridoma Bank), anti-laminin mouse monoclonal (1:3000 dilution, Developmental Hybridoma Bank), anti-Sox6 guinea pig antisemur (1:1000 dilution) and anti-Sox5 rabbit antisemur (1:400) (23). Detection of immunoreactivity was with secondary antibodies conjugated to Cy3 and Alexa fluor immunofluorescent dyes (Dianova and Molecular Probes). TUNEL assays were performed using the ApopTag Red In Situ Apoptosis Detection Kit (Q Biogene) after anti-GFP staining.

Immunoreactivity and GFP autofluorescence were analyzed and documented using a DMI8R inverted microscope (Leica) equipped with a cooled SPOT CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA) or an MZFLIII stereomicroscope (Leica) equipped with an Axiocam (Zeiss). For quantifications, numbers of GFP-labeled, immunoreactive cells or TUNEL-positive nuclei were counted on transverse sections of electroporated chicken embryos. Data were obtained from at least five embryos per electroporated construct. At least three sections were counted per embryo. Diagrams show mean values ± SEM. Statistical significance was determined by Student’s t-test or one-way analysis of variance (ANOVA) with Tukey’s multiple comparison post-test using GraphPad Prism4 software. P-values are given in the figure legends.

Cell culture, extract preparation, EMSA and western blot

HEK 293 cells were maintained in Dulbecco’s Modified Eagle Medium containing 10% fetal calf serum and transfected with SOX10 expression plasmids using polyethylenimine. Extracts from transfected 293 cells were prepared as described elsewhere (17). With these extracts, EMSA was performed in the presence of poly(dGdC) as unspecific competitor using 32P-labeled oligonucleotides containing the monomeric site B from the Mpc promoter (33). Extracts were also subjected to SDS–PAGE. Western blotting was performed using specific anti-sera against Sox10 (1:5000 dilution) (37) and horseradish-peroxidase-coupled secondary antibody with enhanced chemiluminescence.


