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SoxE Proteins Are Differentially Required in Mouse Adrenal Gland Development

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Sry-box (Sox)8, Sox9, and Sox10 are all strongly expressed in the neural crest. Here, we studied the influence of these closely related transcription factors on the developing adrenal medulla as one prominent neural crest derivative. Whereas Sox9 was not expressed, both Sox8 and Sox10 occurred widely in neural crest cells migrating to the adrenal gland and in the gland itself, and they were down-regulated in cells expressing catecholaminergic traits. Sox10-deficient mice lacked an adrenal medulla. The adrenal anlage was never colonized by neural crest cells, which failed to specify properly at the dorsal aorta and died apoptotically during migration. Furthermore, mutant neural crest cells did not express Sox8. Strong adrenal phenotypes were also observed when the Sox10 dimerization domain was inactivated or when a transactivation domain in the central portion was deleted. Sox8 in contrast had only minimal influence on adrenal gland development. Phenotypic consequences became only visible in Sox8-deficient mice upon additional deletion of one *Sox10* allele. Replacement of *Sox10* by *Sox8*, however, led to significant rescue of the adrenal medulla, indicating that functional differences between the two related Sox proteins contribute less to the different adrenal phenotypes of the null mutants than dependence of Sox8 expression on Sox10.

INTRODUCTION

The adrenal gland is composed of two ontogenetically and functionally different tissues. The adrenal cortex develops from mesoderm and produces mineralocorticoid and glucocorticoid hormones, whereas the adrenal medulla consists of neural crest-derived chromaffin cells and releases the catecholamines adrenaline and noradrenaline. To form the adrenal medulla, trunk neural crest cells that belong to the sympathoadrenal sublineage have to migrate from the dorsal aspect of the neural tube through the anterior part of the somites and past the dorsal aorta to the adrenal anlage (Loring and Erickson, 1987). At the dorsal aorta, neural crest cells are instructed by bone morphogenetic protein (BMP) signals to develop into catecholaminergic precursors, which are marked by expression of a set of transcription factors, including paired homeobox 2b (Phox2b), mammalian achaete scute homolog 1 (Mash1), Gata3, and Hand2 (for a recent review, see Goridis and Rohrer, 2002; Huber, 2006). It has been proposed that sympathoadrenal precursors represent a single homogeneous population of fate-restricted cells that give rise to both neurons in sympathetic ganglia and chromaffin cells in the adrenal gland. Recent results, however, suggest that there may be distinct precursors of sympathetic neurons and chromaffin cells already at very early times and that the precursor cell pool at the dorsal aorta as well as the neural crest cells entering the adrenal anlagen are heterogeneous (Ernsberger *et al*., 2005; Huber, 2006). After entry into the adrenal anlage, precursor cells differentiate further into chromaffin cells under the influence of local signals. What these signals are, is not fully understood after recent results have shown that glucocorticoids from the developing adrenal cortex are not required for most phases of chromaffin development as postulated for a long time from in vitro experiments (Finotto *et al*., 1999; Gut *et al*., 2005).

Transcription factors of group E of the Sry-box (Sox) protein family (i.e., Sox8, Sox9, and Sox10, jointly referred to as SoxE) are strongly expressed in the early neural crest, and they have been shown to influence neural crest development at multiple stages (Wegner, 1999; Wegner and Stolt, 2005; McCauley and Bronner-Fraser, 2006). In the mouse and in chicken, Sox9 is already expressed in the premigratory neural crest. Loss-of-function studies argue for a role of Sox9 in the survival of neural crest stem cells, whereas gain-of-function studies show that Sox9 is part of the transcriptional network that confers neural crest stem cell identity (Cheung and Briscoe, 2003; Cheung *et al*., 2005). Later roles in the epithelial-mesenchymal transition and the ectomesenchymal specification of the cranial crest have also been reported (Mori-Akiyama *et al*., 2003; Cheung *et al*., 2005).

Sox9 also induces expression of Sox10 in neural crest cells (Cheung *et al*., 2005). Whereas most neural crest cells loose Sox9 expression upon delamination, Sox10 continues to be

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Abbreviations used: β-HSD, β-hydroxysteroid-dehydrogenase; DBH, dopamine-β-hydroxylase; dpc, days postcoitum; Mash1, mammalian achaete scute homologue 1; Phox2b, paired homeobox 2b; PNMT, phenylethanolamine *N*-methyltransferase; SF1, steroidogenic factor 1; Sox, Sry-box; TH, tyrosine hydroxylase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; VMAT, vesicular monoamine transporter.

expressed in migrating neural crest cells, and it is required for their survival, the maintenance of their pluripotency, and their specification to nonectomesenchymal derivatives such as melanocytes, peripheral glia, and cells of the enteric nervous system (for review, see Wegner and Stolt, 2005; Kelsh, 2006). This important role of Sox10 is also evident from the fact that heterozygous mutations in humans lead to neurocristopathies, most frequently a combination of Waardenburg syndrome and Hirschsprung disease (Pingault *et al*., 1998).

Although Sox8 is also broadly expressed in the early neural crest and several neural crest derivatives (Sock *et al*., 2001), the function of this third SoxE protein is less clear in the mouse. In *Xenopus*, however, Sox8 has many of the neural crest functions attributed to Sox9 in mouse or chicken (O'Donnell *et al*., 2006).

Taking the wide expression of SoxE proteins throughout the neural crest and their importance into account, it is reasonable to assume that they may also influence development of the adrenal gland. However, no systematic analysis has been undertaken so far to clarify their role in adrenal development. It is only known that Sox8 and Sox10 are expressed in the embryonic adrenal medulla, that their expression is strongly down-regulated in the adult (Kuhlbrodt *et al*., 1998; Sock *et al*., 2001; Deal *et al*., 2006), and that an adrenal medulla is missing at the time of birth in homozygous dominant megacolon mice ($Sox10^{dom/dom}$) that express a truncated version of the Sox10 protein with possible dominant-negative function (Kapur, 1999). Therefore, we embarked on a study of adrenal development in mice carrying various SoxE gene mutations and clarified which SoxE protein contributed at what stage to adrenal development. This study has also implications for the genealogy of the chromaffin cell and its relation to a common sympathoadrenal precursor.

MATERIALS AND METHODS

Animal Husbandry and Genotyping

Mice were used in this study that carried the following *Sox10* or *Sox8* alleles on a mixed C3H/C57Bl6J background: $Sox10^{lacZ}$ (Britsch *et al*., 2001), $Sox10^{Sox8ki}$ (Kellerer *et al*., 2006), $Sox10^{aa1}$ (Schreiner *et al*., 2007), $Sox10^{\Delta K2}$ (Schreiner *et al*., 2007), $Sox10^{loxP}$ (Schreiner and Wegner, unpublished data), and $Sox8^{lacZ}$ (Sock *et al*., 2001). For the conditional deletion of the $Sox10^{loxP}$ allele, a *DBH::Cre* BAC transgene was used (Parlato *et al*., 2007). Genotyping was performed by polymerase chain reaction (PCR) as described previously (Britsch *et al*., 2001; Sock *et al*., 2001; Kellerer *et al*., 2006; Parlato *et al*., 2007; Schreiner *et al*., 2007).

Tissue Preparation, Immunohistochemistry, and Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling (TUNEL)

Embryos were obtained at 10.5 days postcoitum (dpc), 11.5 dpc, 12.5 dpc, and 18.5 dpc from staged pregnancies. After fixation in paraformaldehyde, cryoprotection, and freezing at −80°C in Lung Tissue Freezing Medium (Leica, Nussloch, Germany), genotyped, age-matched embryos were transversally sectioned on a cryotome. Sections (10 μm) from the region in which the adrenal gland forms or had formed were used for immunohistochemistry according to standard protocols as described previously (Stolt *et al*., 2002, 2003). The following primary antibodies were used in various combinations: anti-Sox8 guinea pig antiserum (1:1000 dilution; Stolt *et al*., 2005), anti-Sox10 guinea pig antiserum (1:1000 dilution; Maka *et al*., 2005), affinity-purified anti-Sox10 rabbit antiserum (1:7500 dilution; Stolt *et al*., 2003), affinity-purified anti-Sox9 rabbit antiserum (1:2000 dilution; Stolt *et al*., 2003), anti-p75 rabbit antiserum (1:500 dilution; Promega. Madison, WI), anti-tyrosine hydroxylase (TH) rabbit antiserum (1:1000 dilution; BIOMOL Research Laboratories, Plymouth Meeting, PA), anti-phenylethanolamine *N*-methyltransferase (PNMT) rabbit antiserum (1:500 dilution; Immunostar, Hudson, WI), anti-VMAT-1 rabbit antiserum (1:1000 dilution; Weihe *et al*., 1994), anti-3β-hydroxysteroid dehydrogenase (HSD) rabbit antiserum (1:500 dilution; gift of A. H. Payne, Stanford School of Medicine), anti-steroidogenic factor 1 (SF1) rabbit antiserum (1:1000 dilution; gift of K. Morohashi, National Institute for Basic Biology, Japan), anti-Phox2b rabbit antiserum (1:500 dilution; gift of C. Goridis, Ecole Normale Superieure, Paris), anti-β-galactosidase rabbit antiserum (1:500 dilution; MP Biomedicals, Irvine, CA), or anti-β-galactosidase goat antiserum (1:500 dilution; BioTrend, Köln, Germany). Secondary antibodies conjugated to Alexa 488, cyanine (Cy)2, and Cy3 immunofluorescent dyes (Dianova, Hamburg, Germany) were used for detection. TUNEL assays were performed according to the manufacturer's protocol (Chemicon International, Temecula, CA). Samples were analyzed and documented using a Leica inverted microscope (DMIRB) equipped with a cooled MicroMax charge-coupled device camera (Princeton Instruments, Trenton, NJ).

Quantifications

After immunohistochemistry and documentation, the number of immunoreactive cells in the adrenal gland was counted when nuclear markers were stained. For all other markers, the immunoreactive area of the adrenal gland was determined morphometrically using NIH ImageJ software (http://rsb.info.nih.gov/ij/). The size of the labeled region was measured by summarizing suprathreshold zones regardless of variations in brightness. At 12.5 dpc, the adrenal gland was identified as the region containing SF1-positive cells, and results from quantifications are shown as the actual mean number of immunoreactive cells or the actual mean size of the immunoreactive area per section. At 18.5 dpc, the complete area of the adrenal gland was calculated in parallel to the immunoreactive areas, with the outer margin of the β-HSD immunoreactivity serving to demarcate the adrenal gland. Quantifications for nuclear markers at this embryo stage are shown as mean number of positive cells per unit area of the complete adrenal gland. Immunoreactive areas for all other markers were expressed as percentage of the whole adrenal gland at 18.5 dpc. The combined immunoreactivity for β-HSD as a cortical marker, and TH as a medullary marker accounted for 80 ± 2% of the total adrenal gland area, with the remaining ~20% corresponding to intercellular space and connective tissue. To determine the proportion of cortex versus medulla, the sum of the mean values for the β-HSD labeled and the TH-labeled areas was set to 100% in each genotype. Data were obtained from at least five adrenal glands for each genotype.

RESULTS

Sox8 and Sox10, but Not Sox9 Are Present in the Developing Adrenal Medulla

To analyze the role of SoxE proteins in adrenal development, we first determined their expression pattern. At 12.5 dpc, the developing adrenal cortex became visible as an aggregate of SF1 expressing cells (Figure 1, A and F). At the same time, neural crest-derived p75- and Phox2b-positive precursor cells migrated toward the SF1-expressing cells or had already settled in the area, marking the onset of adrenal medulla formation (Figure 1, C, D, H, and I). Both Sox10 and Sox8 were expressed in cells of the migratory stream and the adrenal anlage (Figure 1, A and F).

Within the developing adrenal gland there was no overlap between Sox10 or Sox8 expression on the one side and SF1 expression on the other, arguing that neither Sox protein is expressed in the cortical precursors. However, there was a significant colocalization of both Sox proteins with p75 in the adrenal anlage as well as in the migratory population (Figure 1, C and H). In fact, all three markers seemed to label the same cell population. There was also a significant overlap between Sox8 and Sox10 expression on the one side and Phox2b occurrence on the other at 12.5 dpc. However, in addition to cells that expressed Sox8/Sox10 as well as Phox2b, there were also cells that were only positive for Sox8/Sox10 or Phox2b at 12.5 dpc (Figure 1, D and I). Phox2b is required for the expression of catecholaminergic traits in the neural crest-derived precursors of sympathetic neurons and chromaffin cells (Pattyn *et al*., 1999; Huber *et al*., 2005), and its expression precedes that of enzymes required for noradrenaline biosynthesis, such as dopamine-β-hydroxylase (DBH) or TH. As a consequence, only a fraction of the Phox2b-positive cells contained TH (data not shown). TH-expressing cells, however, lacked Sox8 or Sox10 immunoreactivity (Figure 1, E and J). We therefore conclude that Sox8 and Sox10 are down-regulated in the neural crest-derived Phox2b-positive precursors before or concomitant with the

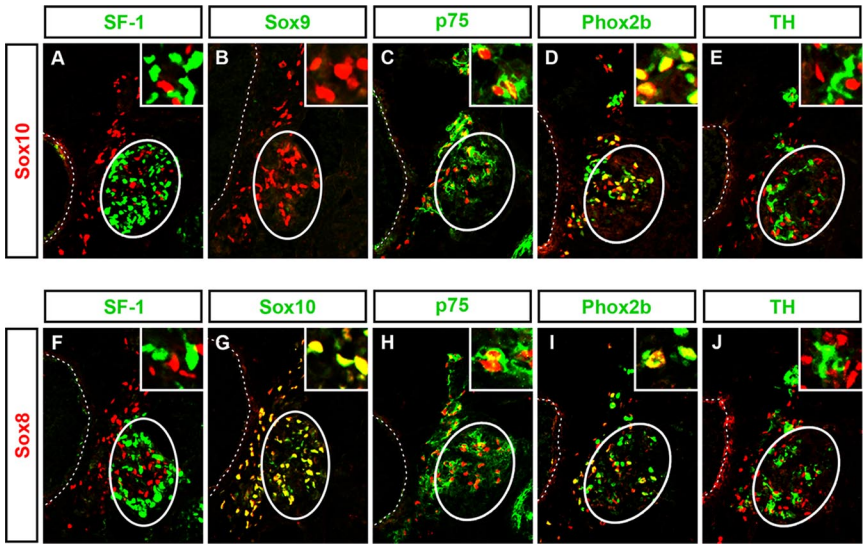

Figure 1. SoxE expression in the early adrenal gland. Expression of Sox10 (A–E) and Sox8 (F–J) (both shown in red) was compared with occurrence of SF1 (A and F), Sox9 (B), p75 neurotrophin receptor (C and H), Phox2b (D and I), TH (E and J), and Sox10 (G) (all shown in green) by coimmunohistochemistry on transverse sections of wild-type embryos at 12.5 dpc. The adrenal anlage was identified by staining for SF1 and is circled. Dorsal aortae are outlined by dashed lines. High magnifications are shown as inlays for all stainings.

onset of TH expression. Our results furthermore point to a considerable heterogeneity of neural crest-derived cells within the early adrenal gland.

The similar labeling pattern obtained for Sox8 and Sox10 argued that both Sox proteins are coexpressed during adrenal gland development at 12.5 dpc. This was confirmed by coimmunohistochemistry (Figure 1G). Sox9, in contrast, was not detected in the adrenal anlage or in the neural crest cells migrating toward it (Figure 1B). Because we also failed to detect Sox9 at later embryonic stages in the adrenal gland (data not shown), Sox9 seems not to be expressed in the adrenal gland, and it is thus unlikely to exert direct effects on its development.

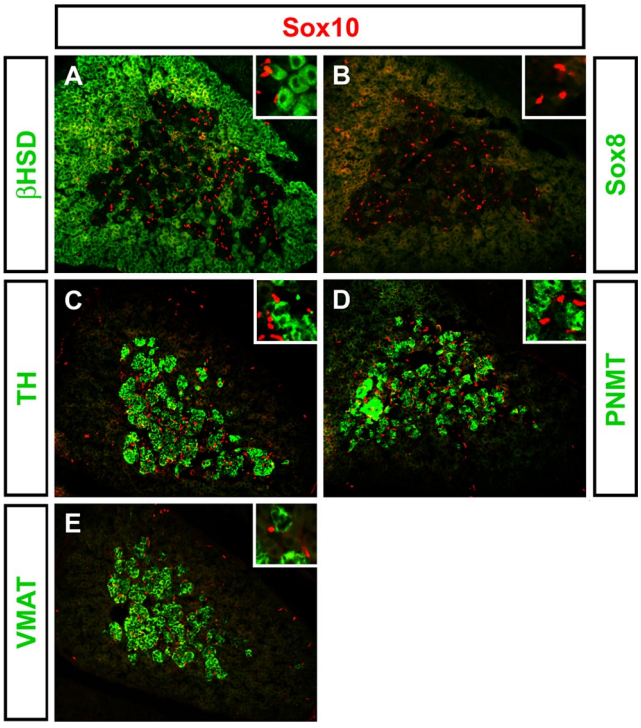

Figure 2. Sox10 expression in the late embryonic mouse adrenal gland. Expression of Sox10 (A–E) (shown in red) was compared with occurrence of β-HSD (A), Sox8 (B), TH (C), PNMT (D), and VMAT-1 (E) (all shown in green) by coimmunohistochemistry on transverse sections from wild-type embryos at 18.5 dpc. High-magnifications are shown as inlays for all stainings.

At 18.5 dpc, Sox10 expression in the adrenal gland was less prominent than at 12.5 dpc (Figure 2A). The remaining Sox10 immunoreactivity was found in the central region of the adrenal gland that was not stained with β-HSD or SF1 as two markers of the adrenal cortex (Figure 2A; data not shown). Despite its occurrence in the adrenal medulla, Sox10 was not expressed in chromaffin cells, which at this time not only express TH but also the vesicular monoamine transporter (VMAT)-1 and PNMT (Figure 2, C–E). The identity of the Sox10-expressing cells could not fully be clarified, because they were not Phox2b-positive and because p75 immunoreactivity was no longer a reliable indicator of neural crest cells at 18.5 dpc in the adrenal gland (data not shown). It is unlikely, however, that the Sox10-positive cells simply represent Schwann cells of invading nerves because most of the Sox10-expressing cells were negative for Gfap, Oct6, or Krox20 as Schwann cell markers (data not shown). From their appearance and their failure to express chromaffin or Schwann cell markers, Sox10-positive cells thus likely correspond to a residual precursor pool in the adrenal medulla.

Sox8 immunoreactivity was already strongly reduced in the adrenal gland at 14.5 dpc, and it was no longer detected at either 16.5 or 18.5 dpc (Figure 2B; data not shown), arguing that Sox8 expression is down-regulated earlier than Sox10 expression. This immunohistochemical result contrasts with previous 5-bromo-4-chloro-3-indolyl-β-D-galactoside stainings of *Sox8*$^{+/lacZ}$ mice that had still detected β-galactosidase at 18.5 dpc (Sock *et al.*, 2001). Most likely, β-galactosidase persists longer in the adrenal gland than the Sox8 protein once expression from the *Sox8* locus subsides.

Sox10 Is a Major Determinant of Early Adrenal Gland Development

To determine the role of Sox10, we analyzed adrenal gland development in constitutively Sox10-deficient (i.e., *Sox10*$^{lacZ/lacZ}$) mice. As expected there was no Sox10 immunoreactivity at 12.5 dpc throughout the embryo, including the region in which the adrenal gland started to form as an aggregate of SF1-positive cells (compare Figure 3, A and Z, with B and A′). We also failed to detect any Sox8-, β-galactosidase, or

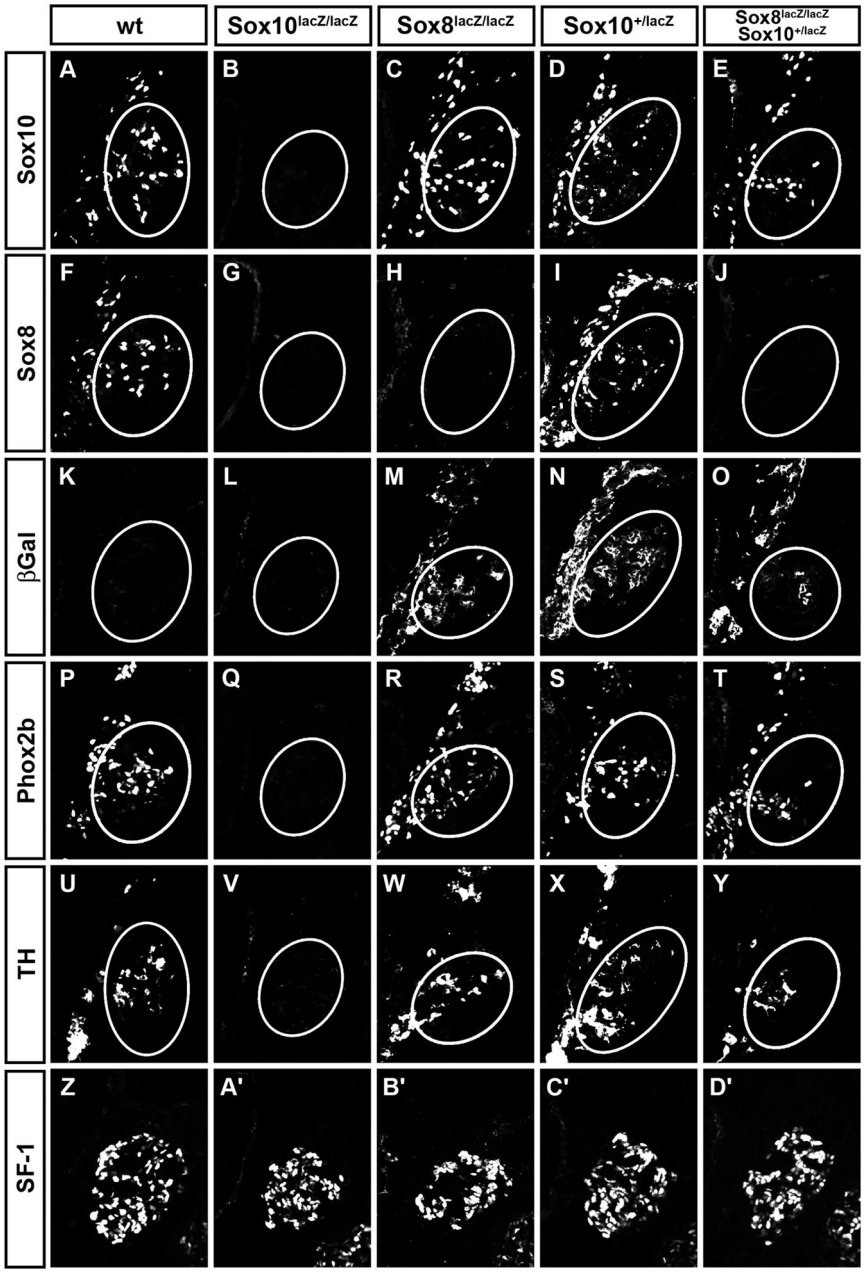

Figure 3. Early adrenal development in mice with Sox8 and Sox10 deficiencies. Immunohistochemistry was carried out on transverse sections of wild-type (A, F, K, P, U, and Z), *Sox10*$^{lacZ/lacZ}$ (B, G, L, Q, V, and A′), *Sox8*$^{lacZ/lacZ}$ (C, H, M, R, W, and B′), *Sox10*$^{+/lacZ}$ (D, I, N, S, X, and C′), and *Sox8*$^{lacZ/lacZ}$, *Sox10*$^{+/lacZ}$ (E, J, O, T, Y, and D′) embryos at 12.5 dpc by using antibodies against Sox10 (A–E), Sox8 (F–J), β-galactosidase (K–O), Phox2b (P–T), TH (U–Y), and SF1 (Z–D′). The adrenal anlage was identified by SF1 immunohistochemistry (Z–D′) and is circled in all other panels (A–Y). The dorsal aorta is to the left of the adrenal anlage.

Phox2b-positive cells in the adrenal anlage (compare Figure 3, F, K, and P, with G, L, and Q). As a consequence, TH-positive cells were also absent at 12.5 dpc (Figure 3, U and V). Furthermore, none of these markers was expressed where sympathetic ganglia normally form in the vicinity of the adrenal gland (compare Figure 4, A–C with D–F), indicating that the complete sympathoadrenal neural crest lineage was heavily affected at this trunk level. This corroborates previous reports in which a cranio-caudal gradient had been observed for defects in the sympathetic ganglion chain of Sox10-deficient embryos at 12.5 dpc with rostral ganglia hypoplastic and caudal ganglia absent (Kapur, 1999; Britsch *et al*., 2001).

TH-positive cells were still missing in the adrenal gland at 18.5 dpc (compare Figure 5, K and L) in the absence of Sox10 (Figure 5, A and B), indicating that the observed defect at 12.5 dpc not only constitutes a developmental delay. Furthermore, the lack of an adrenal medulla was confirmed by the absence of PNMT and VMAT-1 as two further independent markers of chromaffin cells (compare Figure 5, P and U, with Q and V) and the loss of β-HSD–negative cells in the center of the adrenal gland (compare Figure 5F with G). Instead, β-HSD–positive cells of the adrenal cortex had expanded into the area usually occupied by chromaffin cells. As a consequence, there was no obvious size difference between a wild-type and a *Sox10*$^{lacZ/lacZ}$ adrenal gland at the time of birth. This proves that development of the adrenal cortex is largely independent of the adrenal medulla.

Considering the already dramatic phenotype at 12.5 dpc, we analyzed neural crest development in the prospective adrenal gland region of *Sox10*$^{lacZ/lacZ}$ embryos at earlier times. By comparing β-galactosidase immunoreactivity in *Sox10*$^{lacZ/lacZ}$ embryos with Sox10 immunoreactivity in the wild-type, we detected already fewer neural crest cells in the

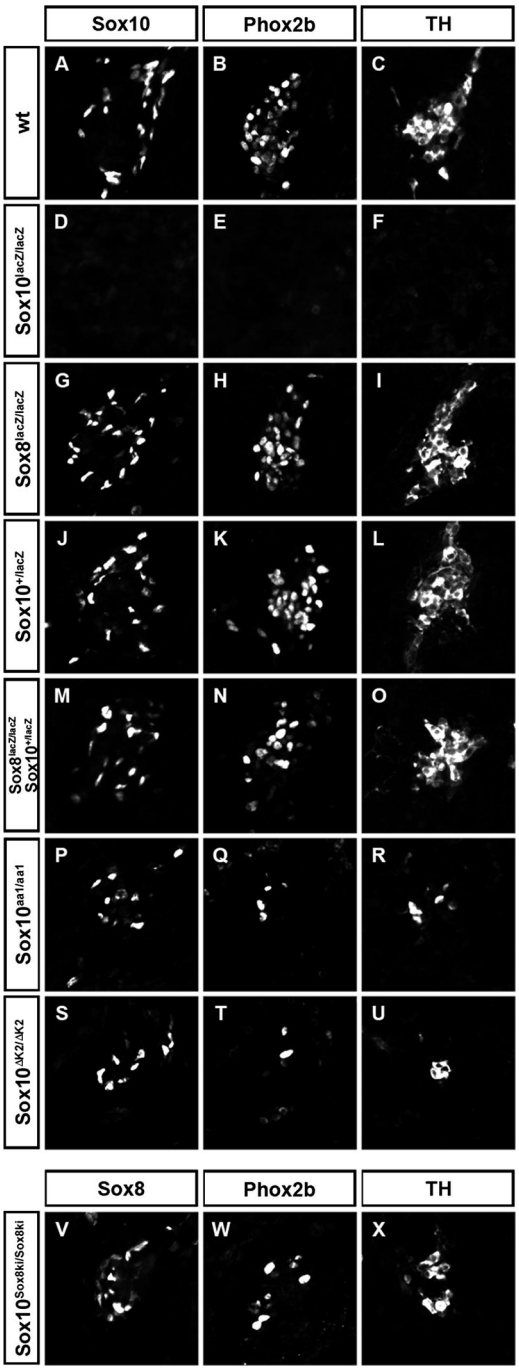

Figure 4. Development of sympathetic ganglia in the adrenal gland region in mice with *Sox8* and *Sox10* mutations. Immunohistochemistry was carried out on transverse sections of wild-type (A–C), $Sox10^{lacZ/lacZ}$ (D–F), $Sox8^{lacZ/lacZ}$ (G–I), $Sox10^{+/lacZ}$ (J–L), $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$ (M–O), $Sox10^{aa1/aa1}$ (P–R), $Sox10^{\Delta K2/\Delta K2}$ (S–U), and $Sox10^{Sox8ki/Sox8ki}$ (V–X) embryos at 12.5 dpc by using antibodies against Sox10 (A, D, G, J, M, P, and S), Sox8 (V), Phox2b (B, E, H, K, N, Q, T, and W), and TH (C, F, I, L, O, R, U, and X). Note that Sox8 staining in $Sox10^{Sox8ki/Sox8ki}$ embryos is comparable to Sox10 staining in other genotypes because of the replacement of *Sox10* by *Sox8*.

vicinity of the dorsal aorta at 10.5 dpc (Figure 6, A and E). In contrast to the wild-type, neural crest cells at the dorsal aorta were Sox8 negative and expressed Phox2b only rarely (Figure 6, B, C, F, and G). This indicated that Sox10 is needed for Phox2b induction, as reported previously for precursors of sympathetic neurons (Kim *et al*., 2003), but it also points to a so far unknown Sox10 requirement for induction and/or maintenance of Sox8 expression in the sympathoadrenal neural crest lineage. Instead, many cells at the dorsal aorta were apoptotic in $Sox10^{lacZ/lacZ}$ embryos as evident from TUNEL assays, whereas apoptosis rates were low in the corresponding wild-type controls (Figure 6, D and H). As a consequence of this increased apoptosis in $Sox10^{lacZ/lacZ}$ embryos, very few neural crest cells were found migrating toward the adrenal anlage at 11.5 dpc compared with the wild type (Figure 6, I and M). These cells did not express Sox8 nor Phox2b (compare Figure 6, J and K, with N and O). Because apoptotic cells were still present at increased rates on the migratory route in $Sox10^{lacZ/lacZ}$ embryos (Figure 6, L and P), most if not all Sox10-deficient neural crest cells die before reaching the adrenal anlage. We conclude that the absence of an adrenal medulla in $Sox10^{lacZ/lacZ}$ embryos is due to dramatically increased apoptosis in those neural crest cells destined to populate the adrenal gland. Neural crest cells furthermore fail to turn on Phox2b as an essential regulator of their development.

Sox8 Has Only Minor Influence on Adrenal Gland Development

In contrast to these dramatic consequences of a Sox10 deficiency, adrenal gland development progressed normally in the absence of Sox8. At 12.5 dpc, number and arrangement of Sox10-positive cells in the migratory stream and adrenal anlage were comparable in $Sox8^{lacZ/lacZ}$ and wild-type embryos (compare Figure 3A with C; for quantification, see Figure 7A). β-Galactosidase–positive cells in $Sox8^{lacZ/lacZ}$ embryos also resembled in number and distribution Sox8-positive cells in the wild type as expected when replacement of Sox8 by β-galactosidase is not associated with phenotypic consequences in the mutant (Figure 3, F, H, K, and M). Neural crest cells in the adrenal anlage furthermore progressed normally to Phox2b- and TH-positive cells in the absence of Sox8 (Figure 3, R and W; quantifications in Figure 7, B and C). Sympathetic ganglia also developed normally at this trunk level (Figure 4, G–I).

Even at 18.5 dpc, we could not detect any significant alterations in the adrenal gland of $Sox8^{lacZ/lacZ}$ mice. Neither the Sox10-positive putative precursor cells in the adrenal gland (Figures 5C and 7D) nor the TH-, PNMT- and VMAT-1–positive chromaffin cells (Figures 5, M, R, and W and 7, E and F) showed abnormalities in number, location, or marker expression. The β-HSD-negative area of the medulla in the center of the adrenal gland was comparable in size to the wild type (compare Figure 5, F with H; quantified in Figure 7G).

The normal adrenal development in $Sox8^{lacZ/lacZ}$ embryos may be accounted for by the ability of Sox10 to compensate for the loss of Sox8. Because lower Sox10 levels should be less likely to compensate, we also studied adrenal development in mice with Sox8 loss and simultaneously reduced Sox10 dosage. At 12.5 dpc, we consistently detected in these $Sox10^{+/lacZ}$, $Sox8^{lacZ/lacZ}$ compound mutants a decrease of neural crest-derived cells in the adrenal anlage with all available markers, including Sox10, β-galactosidase, Phox2b, and TH (compare Figure 3, A, F, K, P, and U with E, J, O, T, and Y). Slight reductions were also observed in the sympathetic ganglia at this trunk level (Figure 4, M–O). Because none of these differences was detected in age-matched $Sox10^{+/lacZ}$ *embryos* (Figure 3, D, I, N, S, and X and Figure 4, J–L), the neural crest cell reduction must be attributed to the additional loss of Sox8.

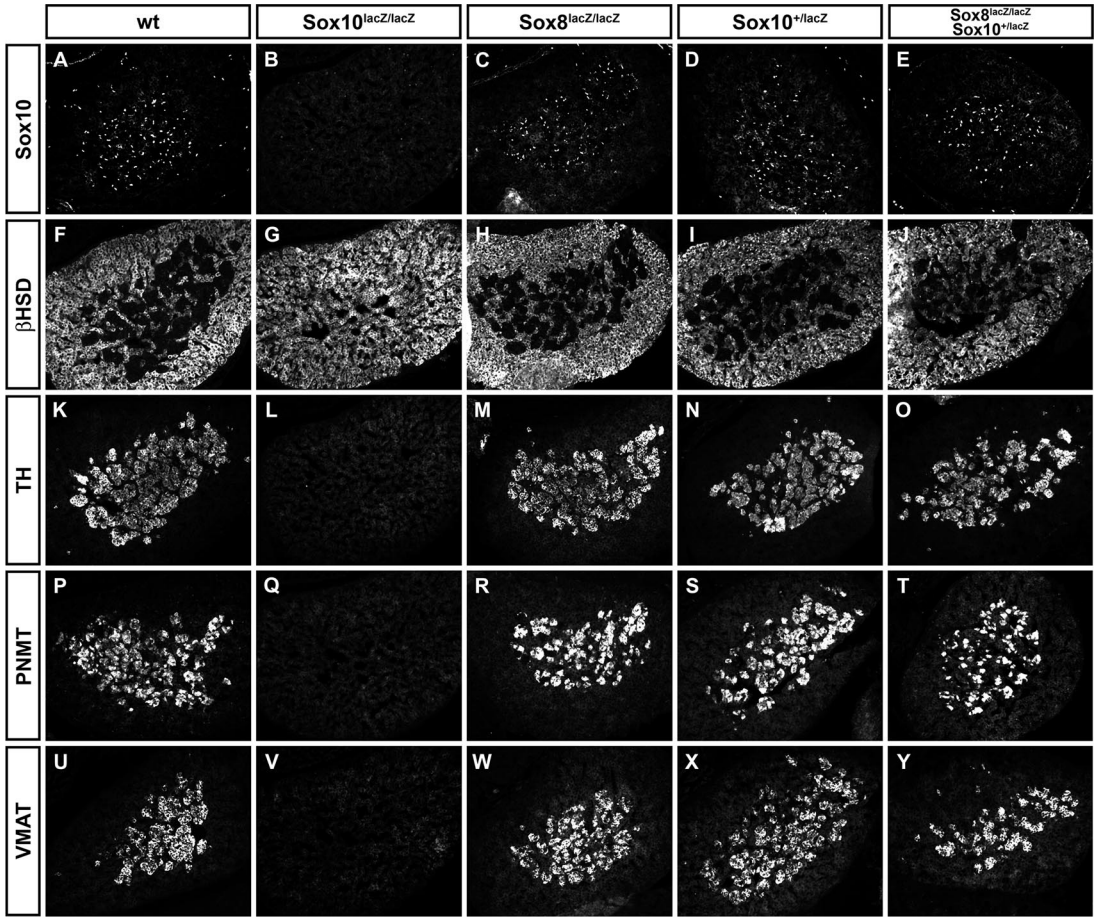

Figure 5. Development of the late embryonic adrenal gland in mice with Sox8 and Sox10 deficiencies. Immunohistochemistry was carried out on transverse sections from wild-type (A, F, K, P, and U), *Sox10*$^{lacZ/lacZ}$ (B, G, L, Q, and V), *Sox8*$^{lacZ/lacZ}$ (C, H, M, R, and W), *Sox10*$^{+/lacZ}$ (D, I, N, S, and X), and *Sox8*$^{lacZ/lacZ}$, *Sox10*$^{+/lacZ}$ (E, J, O, T, and Y) embryos at 18.5 dpc by using antibodies against Sox10 (A–E), β-HSD (F–J), TH (K–O), PNMT (P–T), and VMAT-1 (U–Y).

The phenotype in *Sox10*$^{+/lacZ}$, *Sox8*$^{lacZ/lacZ}$ compound mutants was still apparent when adrenal morphology and marker gene expression were analyzed at 18.5 dpc. The number of Sox10-positive cells was slightly reduced (compare Figure 5A with E). The area occupied by TH-positive chromaffin cells in the adrenal medulla was decreased by 5–7% compared with age-matched Sox10 heterozygotes and wild-type littermates (compare Figure 5K with N and O; quantified in Figure 7F). Similar decreases were also observed for PNMT and VMAT-1 as additional chromaffin markers (Figures 5, P, S, T, U, X, and Y, and 7E). The β-HSD–positive area of the adrenal gland was reciprocally increased by 3–5% (Figures 5, F, I, and J, and 7G). The medulla thus suffered an overall reduction from 22.5% of the total adrenal gland to 14.8% in the compound mutant (Figure 7H). We thus conclude that there is a minor role for Sox8 in adrenal gland development which is only revealed on a sensitized background with reduced Sox10 levels.

Differences in Gene Regulation and in Protein Properties Both Contribute to the Different Roles of Sox8 and Sox10 in Adrenal Gland Development

Sox10 can compensate for the loss of Sox8 in adrenal gland development, whereas Sox8 cannot compensate for the loss of Sox10 in the corresponding null mutant because of the observed dependence of its expression on Sox10. Whether there are additional functional differences between these two highly related Sox proteins in adrenal development was studied in a mouse mutant in which the *Sox10* gene was replaced by *Sox8*. These *Sox10*$^{Sox8ki/Sox8ki}$ mice express Sox8 in all cells in which Sox10 is normally expressed and in levels comparable to Sox10 (Kellerer *et al*., 2006).

Despite the absence of Sox10 (compare Figure 8, A and B), neural crest-derived precursor cells were detected in the adrenal anlage of *Sox10*$^{Sox8ki/Sox8ki}$ embryos at 12.5 dpc by expression of Sox8, Phox2b, and TH (Figure 8, G, L, and Q) as well as in sympathetic ganglia (Figure 4, V–X). This argues that Sox8 can indeed substitute for Sox10 when expressed in a Sox10-specific pattern. However, all available markers were present in fewer cells than in the wild type (Figures 7, A–C, and 8, F, G, K, L, P, and Q), indicating that the rescue in *Sox10*$^{Sox8ki/Sox8ki}$ embryos is not complete.

The precursor cell reduction also led to a proportionate decrease of chromaffin cells in the adrenal gland of *Sox10*$^{Sox8ki/Sox8ki}$ embryos at 18.5 dpc. Instead of a continuous medulla, chromaffin cells in the mutant were rather organized in islets separated by β-HSD–positive cells of the cortex (compare Figure 9, F and G). Although the remaining chromaffin cells occupied only half the area taken in the wild-type (Figure 7, D–F), they continued to express all typical chromaffin markers, including TH, PNMT, and VMAT-1 (compare Figure 9, K, P, and U, with L, Q, and V).

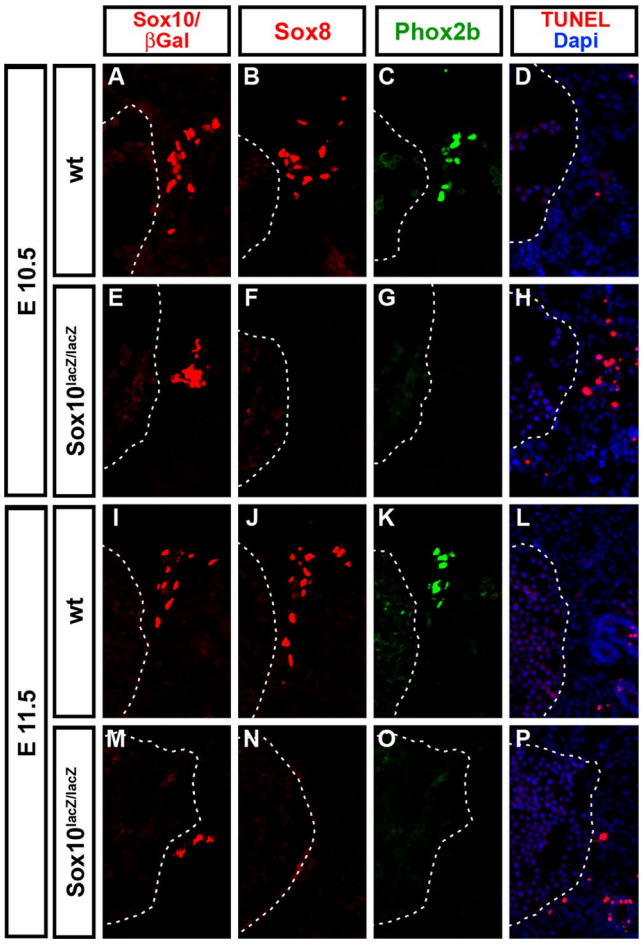

Figure 6. Sox8, Phox2b expression, and apoptosis of Sox10-deficient neural crest cells near the dorsal aorta on their way to the adrenal anlage. Immunohistochemistry (A, B, C, E, F, G, I, J, K, M, N, and O) and TUNEL (D, H, L, and P) were carried out on adjacent transverse sections from wild-type (A, B, C, D, I, J, K, and L) and *Sox10*$^{lacZ/lacZ}$ (E, F, G, H, M, N, O, and P) embryos at 10.5 dpc (A–H) and 11.5 dpc (I–P). Antibodies were directed against Sox10 (A and I), Sox8 (B, F, J, and N), Phox2b (C, G, K, and O), and β-galactosidase (E and M). Nuclei were counterstained (blue signal) in the TUNEL assays by DAPI (D, H, L, and P). Dorsal aortae are outlined by dashed lines.

Despite the reduction of the adrenal medulla from 22.5% of the adrenal gland in the wild type to 10.8% (Figure 7H), Sox8 is thus proficient for medullary development when expressed instead of Sox10. This argues that Sox8 and Sox10 are functionally similar regarding their capacity to drive adrenal development, but not equivalent. As a consequence, the different impact of Sox8 and Sox10 on adrenal gland development is caused to some extent by differences in protein properties, but more prominently by the different regulation of their expression.

Hypomorphic Sox10 Alleles Reveal an Influence of Conserved Sox10 Domains on Adrenal Gland Development

In addition to the *Sox10* null allele (*Sox10*^{lacZ} allele), several hypomorphic *Sox10* alleles are available. In the *Sox10*^{aa1} allele, a triple alanine substitution has been introduced to inactivate the protein's dimerization domain (Schlierf *et al.*, 2002), whereas the *Sox10*$^{\Delta K2}$ allele lacks a region coding for a cell-specific transactivation and putative protein–protein interaction domain in the central Sox10 portion (Schreiner *et al.*, 2007).

When adrenal gland development was studied in the two hypomorphic mouse mutants, significant defects were detected. Already at 12.5 dpc, the number of Sox10-, Sox8-, or Phox2b-positive precursor cells within the adrenal anlage was significantly reduced (Figures 7, A and B, and 8, C, D, H, I, M, and N) as was the number of TH-positive cells (Figures 7C and 8, R and S). This early reduction again translated into proportionate reductions in the size of the adrenal medulla at 18.5 dpc (Figures 7H and 9, H and I), the number of remaining precursors (Figures 7D and 9, C and D) and the number and/or occupied area of TH-, PNMT- or VMAT-1–positive chromaffin cells (Figures 7, E–G, and 9, M, N, R, S, W, and X). Interestingly, adrenal gland defects were severer in the hypomorphic mutants than in *Sox10*$^{Sox8ki/Sox8ki}$ embryos at both 12.5 and 18.5 dpc, indicating that *Sox10* replacement was less detrimental than *Sox10* mutation. Between the mutants, *Sox10*$^{\Delta K2/\Delta K2}$ embryos showed a slightly stronger phenotype than *Sox10*$^{aa1/aa1}$ embryos (compare Figure 9, C, H, M, R, and W with D, I, N, S, and X), with the remaining area occupied by chromaffin cells in the *Sox10*$^{\Delta K2/\Delta K2}$ embryos being 8–11% of the wild type (Figure 7, E and F). Taking into account that the two regions targeted in the hypomorphic *Sox10* alleles are present and highly conserved in Sox8, it is likely that their presence in Sox8 contributes to its rescue activity in the replacement mutant. Interestingly, sympathetic ganglia were also severely reduced in size at this trunk level (Figure 4, P–U), whereas ganglia of the rostral sympathetic chain are only mildly affected (Schreiner *et al.*, 2007).

Sox10 Is Not Involved in Later Stages of Chromaffin Development

The severe and early adrenal gland defect in *Sox10*$^{lacZ/lacZ}$ mice makes it impossible to analyze additional later roles of Sox10 in adrenal gland development. To rule out such later roles in chromaffin development, we used a conditional *Sox10* allele in combination with a *DBH::Cre* transgene, which deletes Sox10 efficiently from cells of the adrenal medulla at a time when these cells reach the chromaffin precursor stage and turn on catecholamine-synthesizing enzymes. Because previous analyses had shown that Sox10 expression is normally down-regulated in neural crest-derived cells of the adrenal medulla upon their acquisition of these enzymes, alterations in adrenal gland development of *Sox10*$^{loxP/loxP}$, *DBH::Cre* mice were not expected. This was indeed the case both at early (Figure 8, E, J, O, and T) and at late (Figure 9, E, J, O, T, and Y) embryonic ages thus also proving genetically that Sox10 function is restricted to the neural crest derived precursor that has not yet acquired expression of catecholamine-synthesizing enzymes.

DISCUSSION

Here, we have studied the impact of Sox8, Sox9, and Sox10 on adrenal development. Among the three SoxE proteins, Sox9 was not expressed in neural crest-derived cells migrating toward the adrenal gland or in the adrenal gland itself. It is thus unlikely to directly influence development of the adrenal medulla and its chromaffin cells.

In contrast to Sox9, both Sox8 and Sox10 occur in those neural crest cells that give rise to the adrenal medulla. Sox10 in particular is important for adrenal development because no adrenal medulla develops in the absence of Sox10. The lack of an adrenal medulla has been reported previously in

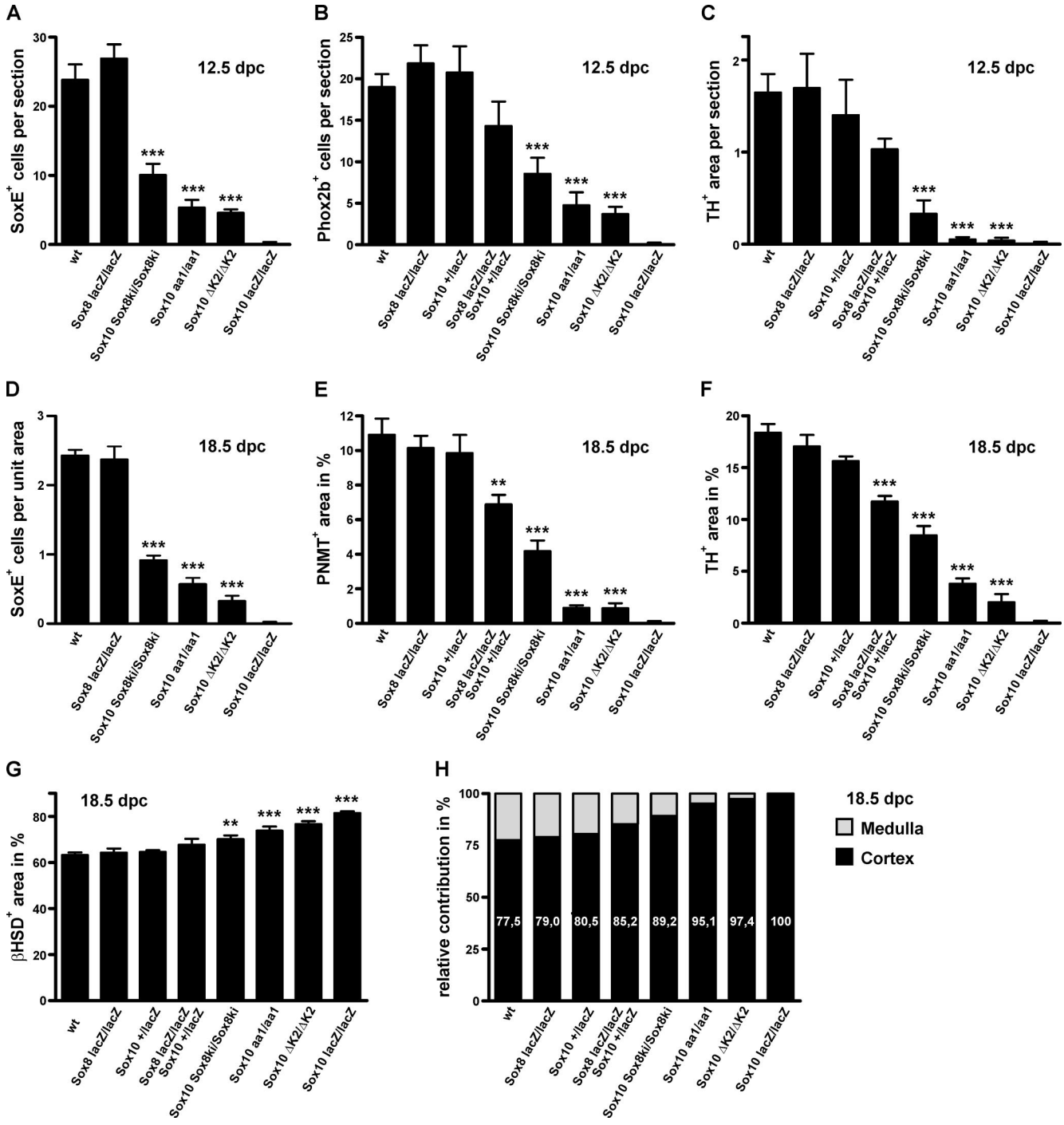

Figure 7. Quantification of marker expression in the developing adrenal gland of mice with *Sox8* and *Sox10* mutations. (A–C) 12.5 dpc; (D–H) 18.5 dpc. Depending on the marker, immunoreactive cells were counted (SoxE in A and D; Phox2b in B) or the immunoreactive area was determined (TH in C and F, PNMT in E, and β-HSD in G) in square micrometers $\times 10^3$ (C) or as percentage of the total adrenal gland (E–G) as described in *Materials and Methods*. Analyzed genotypes include wild-type, $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$, $Sox8^{lacZ/lacZ}$, $Sox10^{+/lacZ}$, $Sox10^{Sox8ki/Sox8ki}$, $Sox10^{aa1/aa1}$, $Sox10^{\Delta K2/\Delta K2}$, and $Sox10^{lacZ/lacZ}$ as indicated below the bars. Note that SoxE immunoreactivity in A and D corresponded to Sox10 labeling for all genotypes except $Sox10^{Sox8ki/Sox8ki}$ for which Sox8 immunoreactivity was counted instead. At least five adrenal glands were used for quantifications. Data are presented as mean ± SEM. Statistically significant differences of $^{**}p < 0.01$ and $^{***}p < 0.001$ are shown above each bar as determined by the Student's *t* test. (H) The raw data in F, G were used to calculate the relative contribution of cortex (black portion of the bar) and medulla (gray portion of the bar) to the adrenal gland.

$Sox10^{dom/dom}$ mice (Kapur, 1999). Because of the presence of a shortened Sox10 protein with fully functional DNA binding activity in this mouse mutant (Herbarth *et al*., 1998; Southard-Smith *et al*., 1998; Kim *et al*., 2003), it has, however, been unclear whether loss of the adrenal medulla is caused by the absence of Sox10 or by the presence of a dominant-negative Sox10 version that interferes with the action of other functionally similar proteins such as Sox8. Here, we

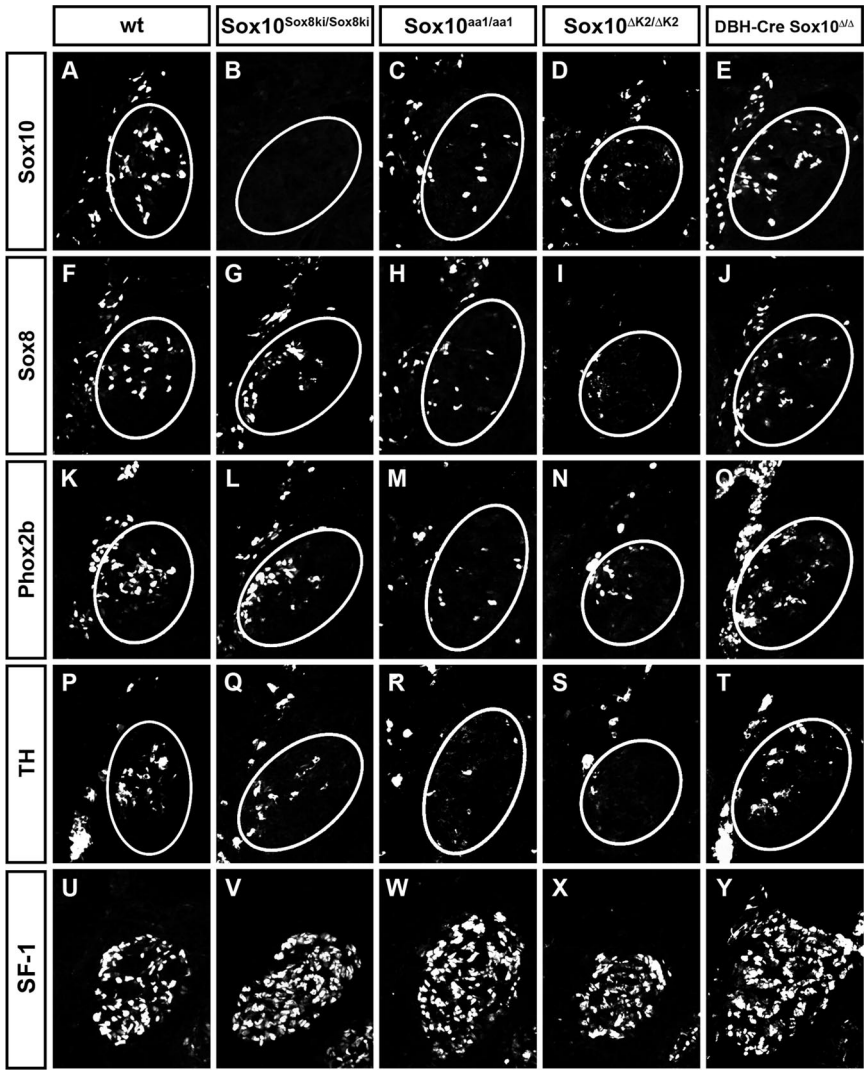

Figure 8. Early adrenal development in mice with hypomorphic or conditional Sox10 alleles. Immunohistochemistry was carried out on transverse sections of wild-type (A, F, K, P, and U), *Sox10*$^{Sox8ki/Sox8ki}$ (B, G, L, Q, and V), *Sox10*$^{aa1/aa1}$ (C, H, M, R, and W), *Sox10*$^{\Delta K2/\Delta K2}$ (D, I, N, S, and X), and *Sox10*$^{loxP/loxP}$, *DBH::Cre* (E, J, O, T, and Y) embryos at 12.5 dpc by using antibodies against Sox10 (A–E), Sox8 (F–J), Phox2b (K-O), TH (P–T), and SF1 (U–Y). The adrenal anlage was identified by SF1 immunohistochemistry (U–Y) and is circled in all other panels (A–T). The dorsal aorta is to the left of the adrenal anlage.

show that the absence of Sox10 is fully sufficient for the loss of the adrenal medulla. Sympathetic ganglia were also absent in the vicinity of the adrenal gland, arguing for a general essential role of Sox10 in the sympathoadrenal neural crest lineage at this axial level. This requirement for Sox10 is less stringent in other regions, as ganglia in the rostral sympathetic chain are hypoplastic, but nevertheless form in the absence of Sox10 (Kapur, 1999; Britsch *et al*., 2001).

The stage at which Sox10 becomes essential for adrenal development has also not been analyzed so far. Our results prove that Sox10 functions at a very early stage even before the neural crest-derived precursors have entered the adrenal anlage. Sox10 furthermore mainly functions as a survival factor. Such a role for Sox10 in survival has also been documented in other parts of the neural crest (Southard-Smith *et al*., 1998; Kapur, 1999; Britsch *et al*., 2001; Maka *et al*., 2005). Kim *et al*. (2003), however, had previously failed to detect a survival function for Sox10 in the sympathoadrenal neural crest at 9.5 dpc. Taking into account that our analysis was performed at 10.5 dpc, Sox10 may become active in its survival function between these time points. Alternatively, the sympathoadrenal neural crest cells may have been studied in different trunk regions, and the different results may simply reflect a different dependence of neural crest cell survival on Sox10 along the rostrocaudal axis.

A role of Sox10 in the induction of Phox2b expression has previously been postulated (Kim *et al*., 2003). Sympathoadrenal neural crest cells including those on their way to the adrenal anlage normally start to express Mash1 and Phox2b (Lo *et al*., 1998; Pattyn *et al*., 1999) under the influence of BMPs from the dorsal aorta (Reissmann *et al*., 1996; Schneider *et al*., 1999). The fact that sympathoadrenal neural crest cells at those axial levels where the adrenal medulla forms mostly fail to express Phox2b in Sox10-deficient embryos is compatible with the postulated role of Sox10 in Phox2b induction. In view of the large increase in cell death, the low number of Phox2b-expressing cells might, however, as well be caused by apoptosis of neural crest cells that start to express Phox2b. Increased apoptosis in Sox10-deficient sympathoadrenal neural crest, in contrast, is unlikely to be secondary to the loss of Phox2b expression, because sympathoadrenal neural crest cells are still present in significant numbers in the adrenal glands of Phox2b-deficient embryos at 13.5 dpc and later (Huber *et al*., 2005).

Although severe adrenal gland phenotypes have also been observed in Phox2b-deficient mice (Huber *et al*., 2005) or in mice with mutations in other Phox2b-dependent compo-

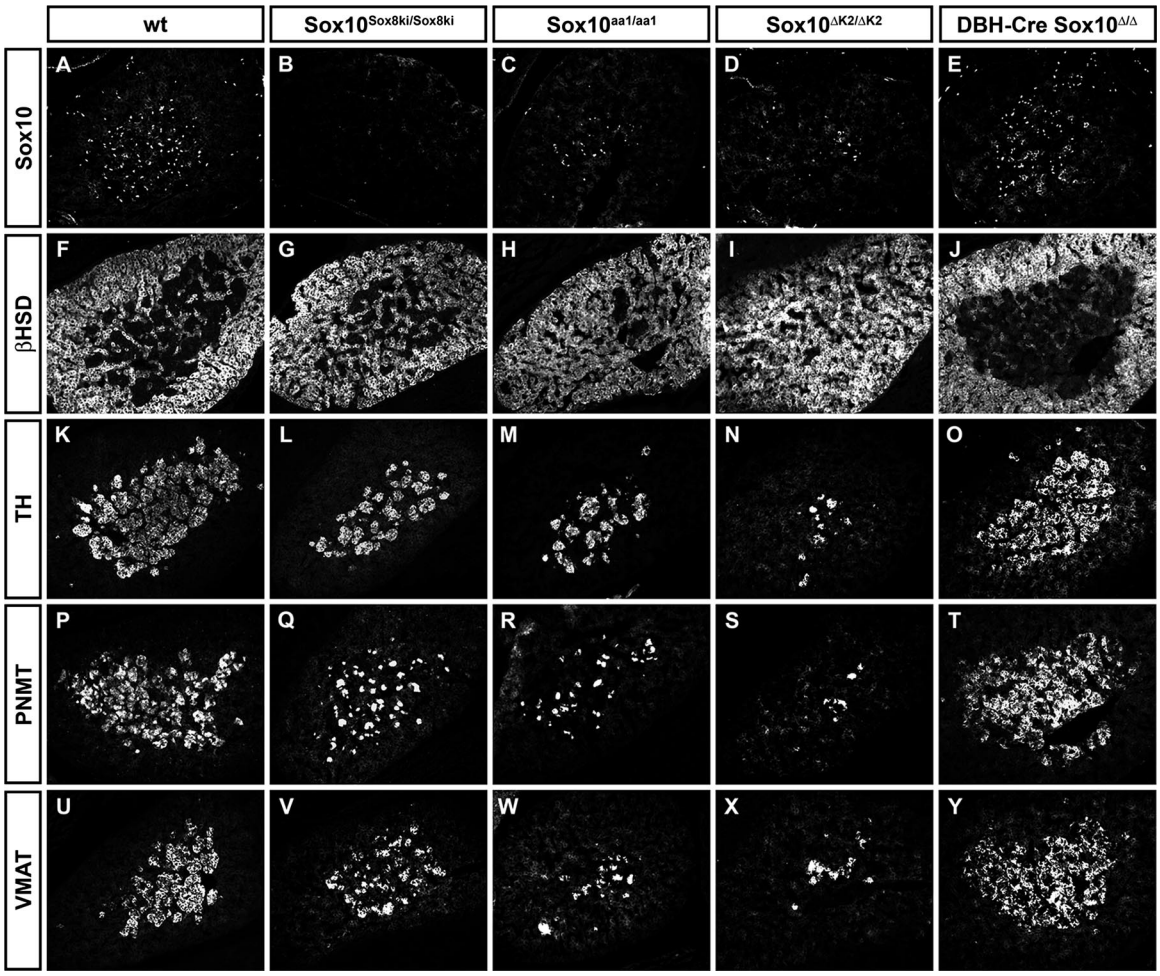

Figure 9. Development of the late embryonic adrenal gland in mice with hypomorphic or conditional Sox10 alleles. Immunohistochemistry was carried out on transverse sections from wild-type (A, F, K, P, and U), $Sox10^{Sox8ki/Sox8ki}$ (B, G, L, Q, and V), $Sox10^{aa1/aa1}$ (C, H, M, R, and W), $Sox10^{\Delta K2/\Delta K2}$ (D, I, N, S, and X), and $Sox10^{loxP/loxP}$, *DBH::Cre* (E, J, O, T, and Y) embryos at 18.5 dpc by using antibodies against Sox10 (A–E), β-HSD (F–J), TH (K–O), PNMT (P–T), and VMAT-1 (U–Y).

nents of the sympathoadrenal transcriptional network (Huber *et al*., 2002; Moriguchi *et al*., 2006), Sox10-deficient mice are the only mice in which the adrenal anlage is not colonized by neural crest cells. In all other reported cases, sympathoadrenal neural crest cells are initially present within the adrenal anlage, but they become arrested at various stages of chromaffin development and eventually die. This phenotypic difference is compatible with Sox10 being genetically the most upstream factor. Despite the complete absence of sympathoadrenal neural crest cells in the adrenal glands, the cortex forms in Sox10-deficient mice, thus proving genetically that cortical development is largely independent of signals from the neural crest.

Sox10 expression and function is furthermore restricted to early neural crest derived precursors for chromaffin cells. It looses its importance in the chromaffin cell lineage with the acquisition of catecholaminergic traits. This coincides with down-regulation of its expression. Further proof for a restricted Sox10 function in the early precursor comes from the fact that specific ablation of Sox10 in DBH-expressing cells has no consequence on adrenal development. Accordingly, Sox10 is only transiently coexpressed with the early regulators of sympathoadrenal development such as Mash1 and Phox2b (Guillemot *et al*., 1993; Pattyn *et al*., 1999) and not with later regulators such as Gata3 and Hand2 (Lim *et al*., 2000; Lucas *et al*., 2006). Sox10 should thus be limited to interactions with the early ones.

Despite its down-regulation in cells with catecholaminergic traits, Sox10-positive cells remain in the adrenal medulla at least until the time of birth. This argues for the presence of a precursor cell population in this tissue. Its characterization and developmental potential needs to be analyzed. It is also unclear whether these cells are still present in the adult adrenal medulla, because our previous failure to detect Sox10 by Northern blotting (Kuhlbrodt *et al*., 1998) may simply be due to the insensitivity of the method.

The essential requirement for Sox10 contrasts dramatically with the very minor role of Sox8 that could only be visualized as a slight decrease in neural crest and chromaffin precursor cells, when one of the two *Sox10* alleles was additionally deleted. This is surprising because Sox8 and Sox10 are coexpressed in precursor cells of the adrenal medulla during the decisive early phases of embryogenesis. Although replacement of *Sox10* by *Sox8* in the mouse reveals slight functional differences between the related proteins, one major reason for the different impact of Sox8 and Sox10 on adrenal gland development seems to lie in the fact that Sox8 expression in the sympathoadrenal neural crest de-

pends on Sox10, but not vice versa. As a consequence, *Sox10* deletion leads to a simultaneous loss of Sox8 expression, whereas *Sox8* deletion leaves Sox10 expression unaffected. This dependence of Sox8 on Sox10 is cell type specific because it has not been observed in oligodendroglial cells that also express Sox8 and Sox10 (Stolt *et al*., 2004). Furthermore, Sox10 alone is not sufficient to maintain Sox8 expression as Sox8 is down-regulated in neural crest-derived cells of the adrenal gland from 14.5 dpc onward despite the continued presence of Sox10.

In its strictest interpretation, the model of a common sympathoadrenal precursor posits that sympathoadrenal neural crest cells are uniformly specified at the dorsal aorta, and then continue their migration as Phox2b- and Mash1-positive cells to their destination to give rise to all sympathetic neurons and chromaffin cells. However, what we observe is that both during migration from dorsal aorta to adrenal anlage and within the adrenal anlage itself, the neural crest population is quite heterogenous with some cells expressing Sox10 and p75; others expressing Sox10, Phox2b, and p75; and yet others expressing Phox2b without Sox10. Although the latter may be derived from previously Phox2b- and Sox10-expressing cells that have turned off Sox10 expression in the course of their development to chromaffin precursors, the Sox10-positive, but Phox2b-negative cells are difficult to reconcile with the sympathoadrenal precursor model both at this early stage and later in the adrenal medulla at 18.5 dpc. Thus, it seems that the neural crest cells migrating into the adrenal anlage are a heterogenous population and that they may undergo specification at different times, not solely under the influence of signals from the dorsal aorta, but also driven by local signals at their final destination, the adrenal anlage, as postulated previously (Ernsberger *et al*., 2005; Huber, 2006).

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