Lack of an adrenal cortex in *Sf1* mutant mice is compatible with the generation and differentiation of chromaffin cells

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Summary

The diversification of neural-crest-derived sympatho-adrenal (SA) progenitor cells into sympathetic neurons and neuroendocrine adrenal chromaffin cells was thought to be largely understood. In-vitro studies with isolated SA progenitor cells had suggested that chromaffin cell differentiation depends crucially on glucocorticoids provided by adrenal cortical cells. However, analysis of mice lacking the glucocorticoid receptor gene had revealed that adrenal chromaffin cells develop mostly normally in these mice. Alternative cues from the adrenal cortex that may promote chromaffin cell determination and differentiation have not been identified. We therefore investigated whether the chromaffin cell phenotype can develop in the absence of an adrenal cortex, using mice deficient for the nuclear orphan receptor steroidogenic factor-1 (SF1), which lack adrenal cortical cells and gonads. We show that in *Sf1*$^{-/-}$ mice typical chromaffin cells assemble correctly in the suprarenal region adjacent to the suprarenal sympathetic ganglion. The cells display most features of chromaffin cells, including the typical large chromaffin granules. *Sf1*$^{-/-}$ chromaffin cells are numerically reduced by about 50% compared with the wild type at embryonic day (E) 13.5 and E17.5. This phenotype is not accounted for by reduced survival or cell proliferation beyond E12.5. However, already at E12.5 the 'adrenal' region in *Sf1*$^{-/-}$ mice is occupied by fewer PHOX2B^{+} and TH^{+} SA cells as well as SOX10^{+} neural crest cells. Our results suggest that cortical cues are not essential for determining chromaffin cell fate, but may be required for proper migration of SA progenitors to and/or colonization of the adrenal anlage.

Key words: Sympathoadrenal cell lineage, Neuroendocrine cells, Chromaffin phenotype, SF1 (NR5A1), Mouse

Introduction

The neural crest (NC) is a transient structure in vertebrate embryos giving rise to most cell types of the peripheral nervous system and several types of endocrine cells, melanocytes and distinct progenitor cells of the skeletal system (Le Douarin and Kalcheim, 1999). The NC and its derivatives are among the most powerful model systems for unravelling molecular and cellular cues underlying the determination of cell fate in the nervous system. A major sublineage of the NC is the sympathoadrenal (SA) cell lineage, which comprises the progenitors of sympathetic neurons, chromaffin cells and the intermediate small intensely fluorescent (SIF) cells (Landis and Patterson, 1981; Anderson, 1993; Unsicker, 1993). It has been proposed that these three derivatives of the SA cell lineage are issued from a common progenitor cell that develops in the trunk region near the dorsal aorta. In this location the SA progenitors become catecholaminergic, and then re-migrate to the sites of the secondary sympathetic ganglia and the adrenal gland. It has been hypothesized that these environments provide the essential signals for further differentiation of sympathetic neurons and chromaffin cells, respectively.

Consequently, essential cues that trigger the chromaffin cell phenotype have been assigned to the adrenal cortex. For decades, glucocorticoid hormones from the adrenal cortex were assumed to influence crucially the decision of SA progenitor cells to develop into chromaffin cells (Unsicker et al., 1978; Doupe et al., 1985; Anderson and Axel, 1986; Anderson and Michelsohn, 1989; Michelsohn and Anderson, 1992). In-vitro studies with mammalian SA progenitors had suggested that glucocorticoid signalling via the glucocorticoid receptor (GR) governs two essential steps in chromaffin cell development: (1) the suppression of neuronal traits; and (2) the induction of the adrenaline-synthesizing enzyme phenylethanolamine-N-methyltransferase (PNMT) in a majority of adrenal chromaffin cells (Wurtman and Axelrod, 1966; Bohn et al., 1981; Anderson and Axel, 1986; Michelsohn and Anderson, 1992; Anderson, 1993; Unsicker, 1993). However, studies in mice deficient for the GR have failed to support the hypothesis of an essential role of glucocorticoid signalling in chromaffin cell development: GR$^{-/-}$ mice have unaltered numbers of adrenal chromaffin cells that are phenotypically normal in all respects, with the exception of the

lack of expression of PNMT and secretogranin II (Finotto et al., 1999). Whether adrenal cortical cells might influence determination and differentiation of chromaffin cells through mechanisms other than glucocorticoid signalling has not been addressed.

The aim of the present study was therefore to determine whether the specific phenotype of 'adrenal' chromaffin cells can develop in the complete absence of an adrenal cortical anlage. Mice with a targeted mutation in the *Sf1* gene (*Nr5a1* – Mouse Genome Informatics), which codes for a nuclear orphan receptor, lack an adrenal cortex, testis and ovary (Luo et al., 1994). Our study provides evidence that chromaffin progenitors with largely normal marker expressions and the typical ultrastructure of chromaffin cells develop in *Sf1* mutant mice in a location corresponding to the site where the adrenal medulla is found in wild-type mice. A deficit in migration/colonization, rather than reduced proliferation and cell death seem to account for the 50% loss of chromaffin cells seen in $Sf1^{-/-}$ mice. Our results suggest that an adrenal cortex is not essential for determining chromaffin cell fate, but may be required for attracting and harbouring SA progenitors.

Materials and methods

Experimental animals

Sf1 mutant mice (Luo et al., 1994) were purchased from Jackson Laboratories, Bar Harbor, Maine (Strain name: D2.129P2(B6)-Nr5a1^{tm1Klp}) and backcrossed into the C57/Bl6 background. Embryos were staged considering midday of the day of the vaginal plug as embryonic day 0.5. Genotyping was carried out by PCR analysis using primers *Sf1* F (5′-ACAAGCATTACACGTGCACC-3′), *Sf1* R (5′-TGACTAGCAACCACCTTGCC-3′), and *Sf1*-Neo (5′-AGGTGAGA-TGACAGGAGATC-3′). The wild-type allele yielded a 400-bp PCR product, and the knockout allele a 600-bp PCR product.

Histology

Pregnant mice were killed by CO_2 asphyxiation. Embryos were recovered, rinsed in cold PBS (pH 7.4) and fixed in PBS containing 4% paraformaldehyde (PFA) overnight. Tissues were then rinsed three times with PBS and placed in 15% sucrose in PBS for cryoprotection. Following overnight immersion in sucrose, tissues were coated with OCT compound (Tissue Tek), frozen on dry ice and stored at –70°C until further processing. Tissues were then cut into 12 μm serial sections, mounted on Superfrost slides, and air dried for 30 minutes, before performing in-situ hybridization or immunfluorescence staining, respectively. For BrdU labelling of embryonic adrenal glands pregnant females were injected i.v. with BrdU (Sigma, 0.4 ml of an 8 mg/ml solution) at day 12.5 of gestation. Embryos were isolated 24 hours after injection of BrdU and processed further as described above.

In-situ hybridization

Non-radioactive in-situ hybridization on cryosections and preparation of digoxigenin-labelled probes for mouse neurofilament 68 (NF) (Huber et al., 2002), mouse tyrosine hydroxylase (TH) (Zhou et al., 1995), mouse RET (Pachnis et al., 1993), mouse PNMT (Cole et al., 1995) and mouse PHOX2B (Pattyn et al., 1997) were carried out using a modification of the protocol of D. Henrique (IRFDBU, Oxford, UK) as previously described (Ernsberger et al., 1997). Mouse *Sf1* (735-1336 bp) and mouse *Scg10* (550-1178 bp) were cloned by PCR using a pGEM-T vector system (Promega) following the manufacturer's instructions. The plasmids were linearized with *Not*I and transcribed with T7. If required, TH immunofluorescence staining was carried out following in-situ hybridization as described below.

Immunofluorescence staining

For immunofluorescence staining, cryosections were pretreated with 10% normal serum (corresponding to the secondary antibody) in PBS and 0.1% Triton X-100, followed by overnight incubation at 4°C with polyclonal sheep anti-TH (1:200, Chemicon International) or polyclonal guinea pig anti-SOX10 (1:1000, courtesy of Dr Michael Wegner, University of Erlangen). Sections were then rinsed in PBS and incubated for 2 hours at room temperature with Cy3- or Cy2-conjugated secondary antibody (Dianova) diluted 1:200 in 10% normal serum in PBS and 0.1% Triton X-100. Sections were then rinsed in PBS, counterstained with 4′,6-Diamidino-2-phenylindole (DAPI) diluted 1:1000 in PBS, and mounted with fluorescent mounting medium (Dako). For immunohistochemistry, endogenous peroxidase was inhibited with 3% hydrogen peroxide in PBS for 15 minutes. Following incubation with 10% normal goat serum in PBS and 0.1% Triton X-100, slides were then incubated at 4°C overnight with an anti-rabbit vesicular monoamine transporter-1 antibody (VMAT1; 1:1000, courtesy of Dr Eberhard Weihe, University of Marburg) in PBS containing 10% normal goat serum and 0.1% Triton X-100. Sections were then washed with PBS and incubated with a biotinylated goat anti-rabbit antibody (1:400, Vector Laboratories) followed by avidin and biotinylated horseradish-peroxidase-macromolecular complex (Vector Laboratories: Elite ABC reagent) and DAB according to the manufacturer's instructions. For BrdU and TH, double immunostaining cryosections were pretreated with DNAse (Roche, 1 mg/ml in 20 mmol/l Tris, 5 mmol/l $MgCl_2$ and 1 mmol/l $CaCl_2$), rinsed three times in PBS, followed by overnight incubation with polyclonal rat anti-BrdU antibody (Abcam) diluted 1:100 in 10% fetal calf serum (FCS, Gibco) in PBS and 0.1% Triton X-100. After rinsing in PBS, tissues were incubated with biotinylated rabbit anti-rat antibody (Vector Laboratories) diluted 1:100 in 10% FCS in PBS and 0.1% Triton X-100 for 2 hours at room temperature. Sections were then rinsed with PBS and incubated for 45 minutes with Cy2-conjugated streptavidin (Dianova) diluted 1:500 in PBS. Subsequently, TH immunostaining was performed as described above.

TdT-mediated dUTP nick-end labelling analysis

For detection of apoptotic chromaffin cells, TdT-mediated dUTP nick-end labelling (TUNEL) was performed on 12 μm cryosections using an ApoTag In Situ APOPTOSIS Detection Kit (Oncor) according to the manufacturer's instructions, as previously described (Finotto et al., 1999). Subsequently, TH-immunofluorescence staining was carried out as described above.

Electron microscopy

For electron microscopy, E17.5 $Sf1^{-/-}$ embryos and wild-type littermates were fixed in glutaraldehyde (1.5%) and paraformaldehyde (1.5%) in phosphate buffer at pH 7.3 for 48 hours. The embryos were subsequently cut into 500 μm thick slices using a Leica VT1000E vibratome. Slices of the suprarenal region were postfixed overnight and then processed for electron microscopy as previously described (Finotto et al., 1999).

Results

In $Sf1^{-/-}$ mice SA progenitors with features of adrenal chromaffin cells assemble at the site where adrenal chromaffin cells develop in wild-type mice

Identification of 'adrenal' chromaffin cells in the complete absence of an adrenal cortex requires markers that allow them to be distinguished from closely associated sympathetic neurons, to which they are phenotypically similar. Fig. 1C and E show that TH immunoreactivity (ir) in combination with NF mRNA in-situ hybridization can be employed as a marker to distinguish adrenal chromaffin progenitor cells (TH^+/NF^-)

from sympathetic neuron progenitors (TH^+/NF^+) as early as E13.5. At this age a well-demarcated adrenal gland has formed and is colonized by SA progenitors. In wild-type mice (Fig. 1C,E), adrenal chromaffin progenitor cells, which are intermingled with SF1 positive adrenal cortical progenitors (Fig. 1A) at this age, exhibit prominent TH immunofluorescence. NF-expressing cells are either absent or very scarce in the E13.5 adrenal gland (Fig. 1C,E). By contrast, sympathetic neurons and their progenitors in the suprarenal ganglion, which is consistently attached to the medial surface of the adrenal cortex, express both NF and TH, robust NF mRNA expression absorbing TH immunofluorescence (Fig. 1C,E).

We next employed the markers TH, NF and SF1 to investigate whether cells with a presumptive adrenal chromaffin phenotype exist in *Sf1* mutant mice in a location corresponding to the adrenal medulla in wild-type mice. Fig. 1D and F show the spatial distribution of TH-ir and NF mRNA-positive cells in E13.5 SF1 deficient mice at the axial level, where the adrenal gland develops. Similar to the wild-type situation, TH-positive, NF-negative cells are located at the lateral surface of the suprarenal ganglion, but form a compact structure due to the lack of adrenal cortical cells. Thus, presumptive adrenal chromaffin progenitors migrate to and settle at the correct site for their further development even in the absence of an adrenal cortex. Moreover, presumptive adrenal chromaffin progenitors exhibit the correct NF-negative phenotype regardless of the absence of the adrenal cortex. Fig. 2 corroborates and extends these findings by showing that TH^+/NF^- cells survive in their location associated with the suprarenal ganglion at E17.5 and 19.5 (the latter not shown). In addition, SCG10, another intermediate filament marker of sympathetic neurons, was employed and yielded identical results concerning differentiation of chromaffin progenitor cells ($SCG10^-$) and sympathetic neuron progenitors ($SCG10^+$; Fig. 3A,B). Furthermore, $Sf1^{-/-}$ chromaffin cells, like their wild-type counterparts, exhibited immunoreactivity for VMAT1 (Fig. 3C,D).

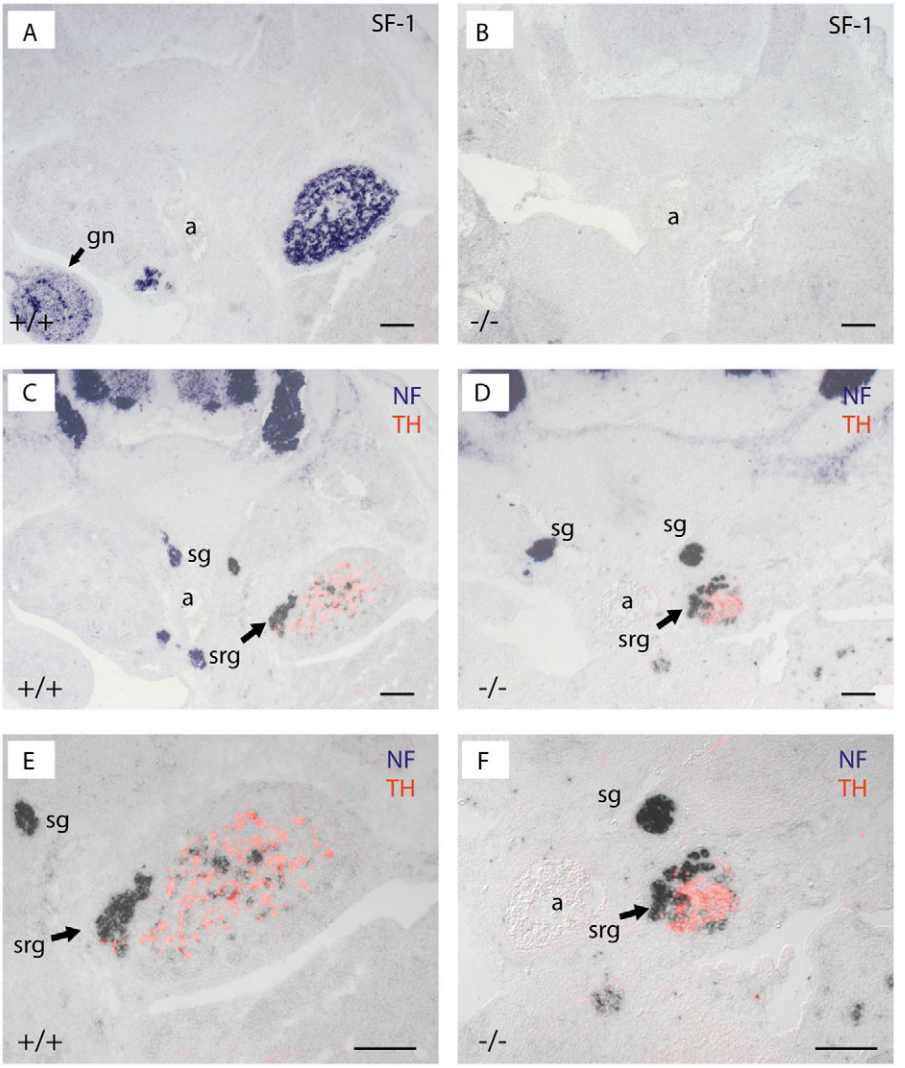

Fig. 1. Adrenal cortical cells are labelled by *Sf1* in-situ hybridization on a transverse section (dorsal, top) of an E13.5 wild-type embryo (A). *Sf1* expression is completely lacking in $Sf1^{-/-}$ littermates (B). In-situ hybridization for NF mRNA (blue) and TH immunofluorescence staining (red overlay) have been performed on a consecutive section of the wild-type (C; E, higher magnification) and the $Sf1^{-/-}$ embryo (D; F, higher magnification). Note that in $Sf1^{-/-}$ embryos TH^+/NF^- 'adrenal' chromaffin cells accumulate at a location where the adrenal gland develops in wild-type mice. Scale bars: 100 μm. a, dorsal aorta; gn, gonadal anlage; sg, sympathetic chain ganglion; srg, suprarenal ganglion.

'Adrenal' chromaffin cells in $Sf1^{-/-}$ mice exhibit all ultrastructural features of chromaffin cells

To further analyse the phenotype of 'adrenal' chromaffin cells in $Sf1^{-/-}$ mice, we used serial semithin sections and electron microscopy to reveal their precise topography and ultrastructure. Fig. 4A demonstrates the location of the right adrenal gland, the suprarenal ganglion, the upper pole of the kidney, and the crus dextrum of the diaphragm in an E17.5 wild-type embryo. Fig. 4C shows the typical ultrastructure of adrenal chromaffin cells at this age. The cells contain abundant chromaffin granules, i.e. large dense core vesicles (core diameter >100 nm), which represent the ultrastructural hallmark of chromaffin cells (Coupland, 1972; Coupland and Tomlinson, 1989). The cells are tightly packed, associated with satellite Schwann cells, and contacted by axon terminals (not shown). Adrenal cortical cells (not shown) exhibit cholesterol droplets, smooth ER and tubular mitochondria typical of steroid-producing cells (Zwierzina, 1979). The dominating cell type in the suprarenal ganglion (Fig. 4E) is a typical neuron with a large nucleus, well-developed rough ER and Golgi

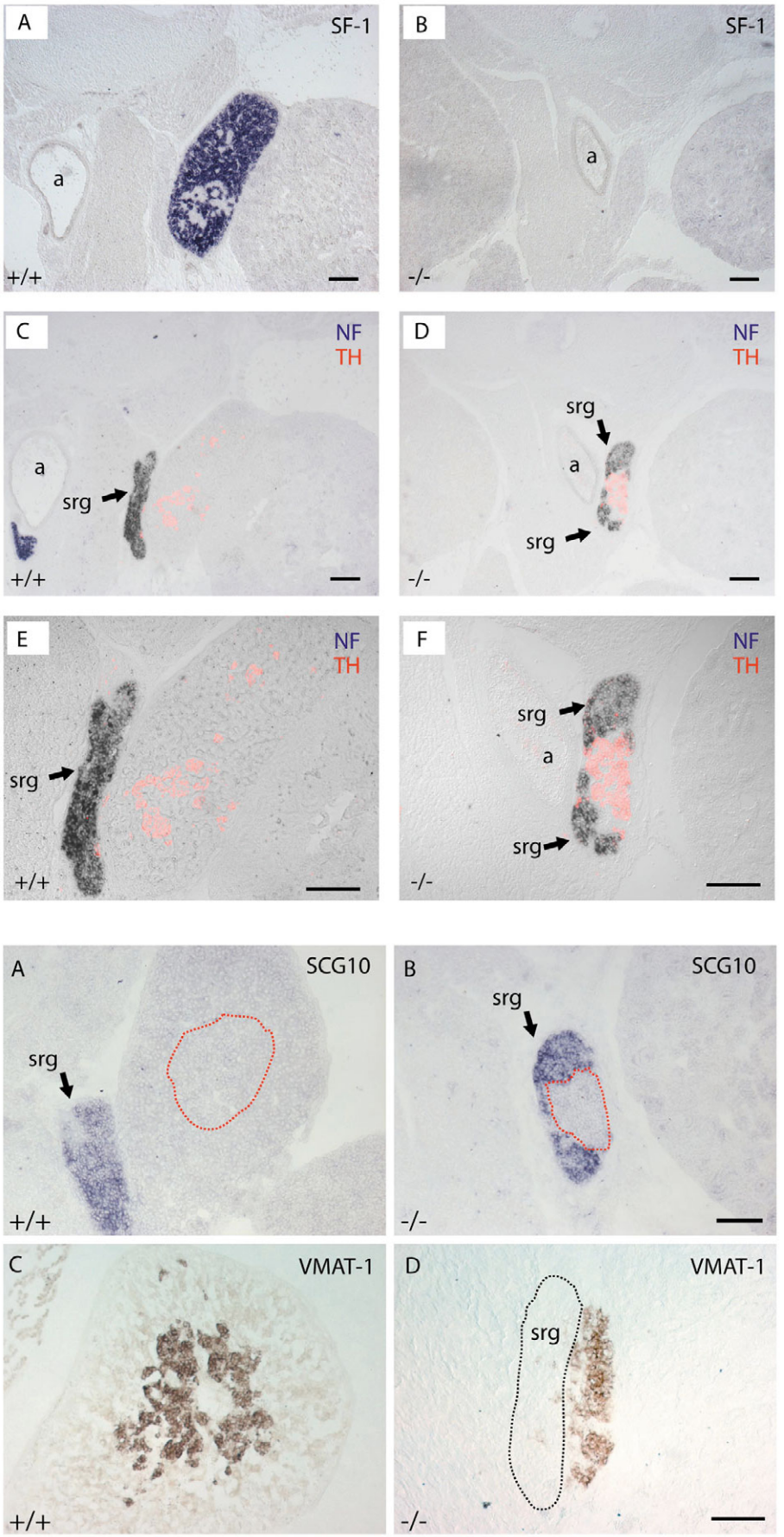

Fig. 2. In-situ hybridization for *Sf1* on transverse sections (dorsal, top) of an E17.5 wild-type embryo (A) and an $Sf1^{-/-}$ littermate (B) at the axial level of the adrenal gland. In-situ hybridization for NF mRNA (blue) and TH immunofluorescence staining (red overlay) have been performed on a consecutive section of the wild-type (C; E, higher magnification) and the $Sf1^{-/-}$ embryo (D; F, higher magnification). Note that in the $Sf1^{-/-}$ embryo TH^{+}/NF^{-} 'adrenal' chromaffin cells are still present. Scale bars: 100 μm. a, dorsal aorta; srg, suprarenal ganglion.

areas, and few small vesicles (core diameters <50 nm). In addition, there are Schwann cells, axons and axon terminals (not shown).

Fig. 4B shows the topography of the suprarenal ganglion adjacent to the crus dextrum of the diaphragm in *Sf1* mutant mice. Cells within the black demarcated area of the suprarenal ganglion are typical sympathetic neurons with exactly identical ultrastructure as described above (Fig. 4F). Laterally attached to the suprarenal ganglion is a large cell group (red demarcation) that, by location, corresponds to the NF-negative, TH-positive cell assembly previously identified. Ultrastructurally, these cells are typical chromaffin cells (Fig. 4D) comprising all features described above for wild-type adrenal chromaffin cells. We found numerous axon terminals filled with small clear (diameter approximately 50 nm) vesicles abutting on chromaffin cells (Fig. 5A) and mitotic figures (Fig. 5B). Adrenal cortical cells were not detected.

Chromaffin progenitor cells in *Sf1* mutant mice lack PNMT and maintain RET expression

A subpopulation of adrenal chromaffin cells amounting to about 50% of the total

Fig. 3. SCG10 is expressed in the sympathetic suprarenal ganglion (srg) as shown by in-situ hybridization, but is lacking in chromaffin cells (red demarcation) in both wild-type (A) and $Sf1^{-/-}$ (B) mice. Chromaffin cells of both wild-type (C) and $Sf1^{-/-}$ (D) mouse embryos are immunoreactive for VMAT1. Only very few scattered cells express VMAT1 in the sympathetic ganglion in wild-type (not shown) and $Sf1^{-/-}$ mice (black demarcation). Transverse sections are shown (dorsal, top). Scale bars: 100 μm. srg, suprarenal ganglion.

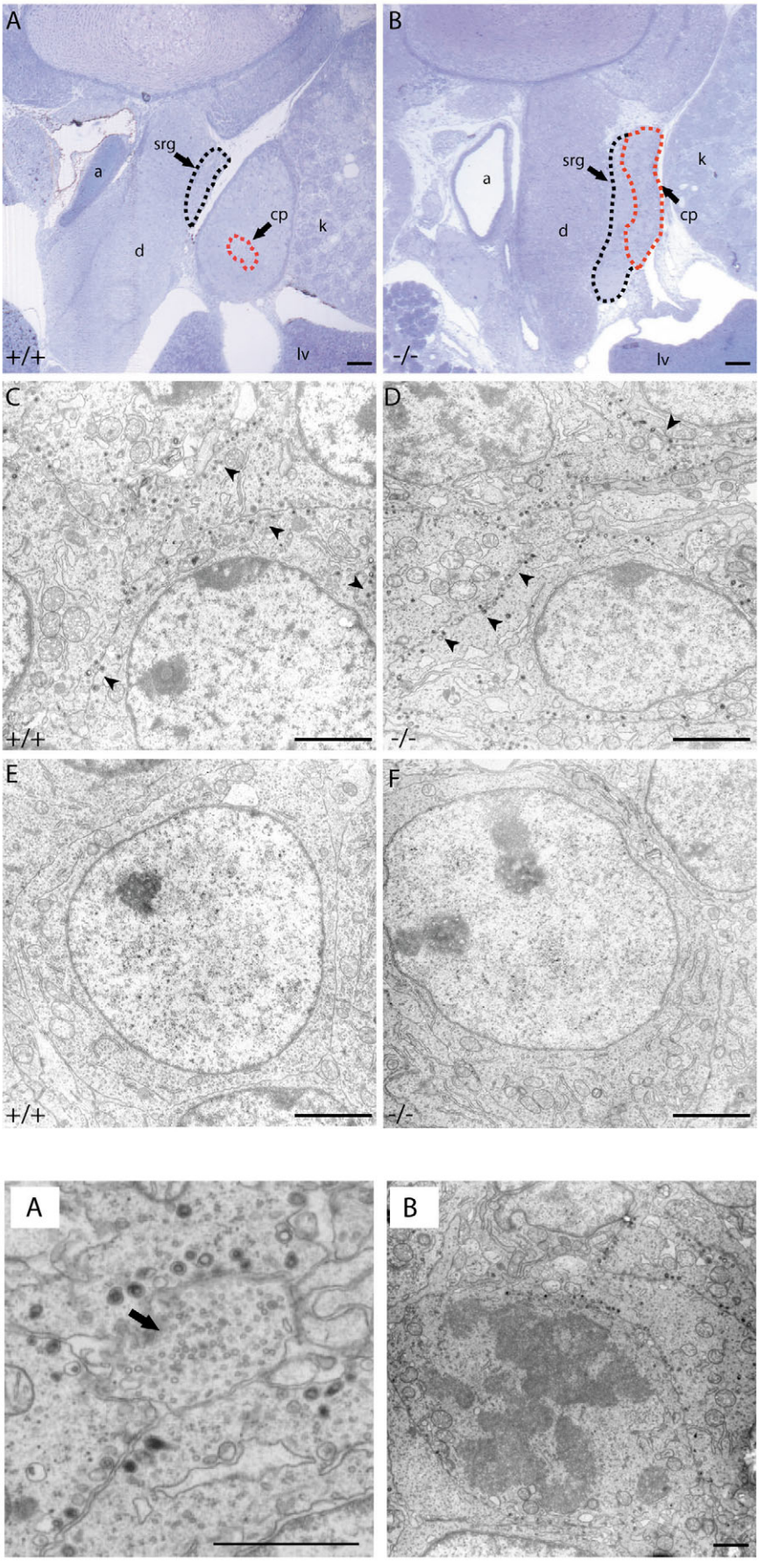

Fig. 4. Semithin transverse sections (dorsal, top) at the axial level of the adrenal gland of an E17.5 wild-type embryo (A) and an $SF^{-/-}$ littermate (B), showing the position of the right adrenal gland, the suprarenal ganglion, the upper pole of the kidney, the liver and the crus dextrum of the diaphragm. Electron micrographs have been made on tissues corresponding to the demarcated areas: red (C,D) and black (E,F). Cells with the typical ultrastructural features of chromaffin progenitor cells, which contain large dense core vesicles (arrowheads), are present in wild-type (C) and knockout (D) embryos in the areas that have been demarcated with red dashed lines (A,B). Electron micrographs (E,F) from the areas demarcated with black dashed lines (A,B) show typical sympathetic neurons of the suprarenal ganglion, which appear identical in wild-type (E) and *Sf1*$^{-/-}$ (F) embryos. Scale bars: 100 μm in A,B; and 2 μm in C-F. ag, adrenal gland; cp, chromaffin progenitor cells; d, crus dextrum of the diaphragm; k, upper pole of kidney; lv, liver; srg, suprarenal ganglion.

population at E19.5 synthesizes adrenaline and expresses the synthesizing enzyme PNMT. Induction and maintenance of PNMT essentially require glucocorticoids from the adrenal cortex (Wurtman and Axelrod, 1966; Finotto et al., 1999). As expected, *Sf1*$^{-/-}$ mice lacking adrenal cortical cells, by contrast to wild-type littermates, do not express PNMT mRNA in SA cells located near the suprarenal ganglion (Fig. 6C,G). RET is a receptor tyrosine kinase, which serves as a receptor for GDNF family ligands (Trupp et al., 1996). It is expressed by sympathetic neurons (Durbec et al., 1996) during distinct phases of their development. RET is found on adrenal chromaffin cells at E13.5 (Allmendinger et al., 2003) but is subsequently downregulated, and becomes undetectable by E16.5. Fig. 6D and H show that, unlike the wild-type situation, RET expression persists in chromaffin cells of *Sf1*$^{-/-}$ mice. However, levels of RET expression appear to be substantially lower in chromaffin cells than in the nearby located sympathetic neurons of the suprarenal ganglion.

‘Adrenal’ chromaffin progenitors in *Sf1*$^{-/-}$ mice amount to 50% of the wild-type population

We next addressed the issue of whether the

Fig. 5. Electron micrographs of ‘adrenal’ chromaffin tissue of an E17.5 *Sf1*$^{-/-}$ embryo showing axon terminals (arrow) abutting chromaffin cells (A) and a mitotic cell (B). Scale bars: 1 μm.

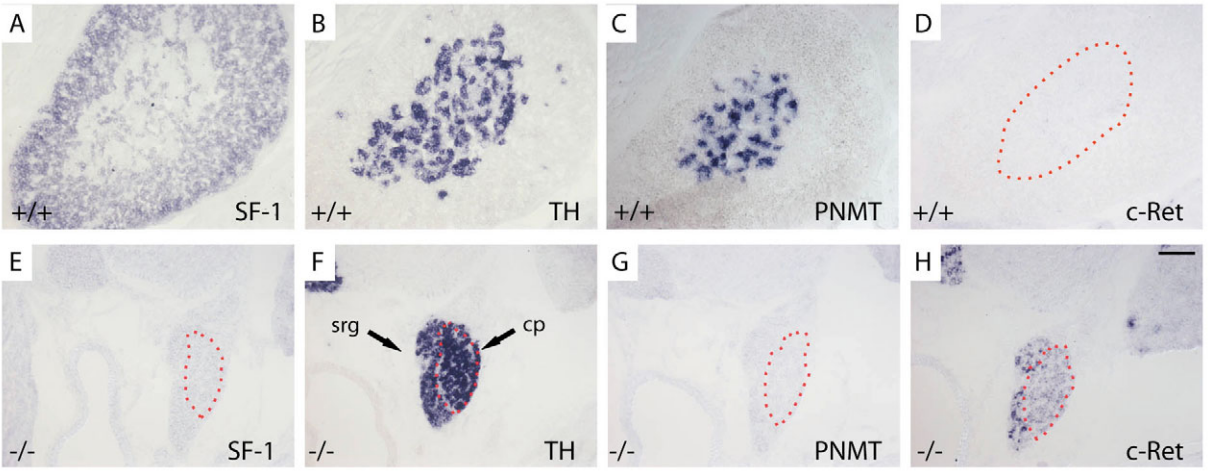

Fig. 6. In-situ hybridization for SF1 (A,E), TH (B,F), PNMT (C,G) and RET (D,H) on transverse sections (dorsal, top) of the adrenal gland of E19.5 wild-type embryos (A,B,C,D) and sections of 'adrenal' chromaffin tissue (red demarcation) of an *Sf1*$^{-/-}$ littermate (E,F,G,H). Note that PNMT expression is lacking in the *Sf1*$^{-/-}$ embryo (G), while RET expression is maintained (H). Scale bar: 100 μm.

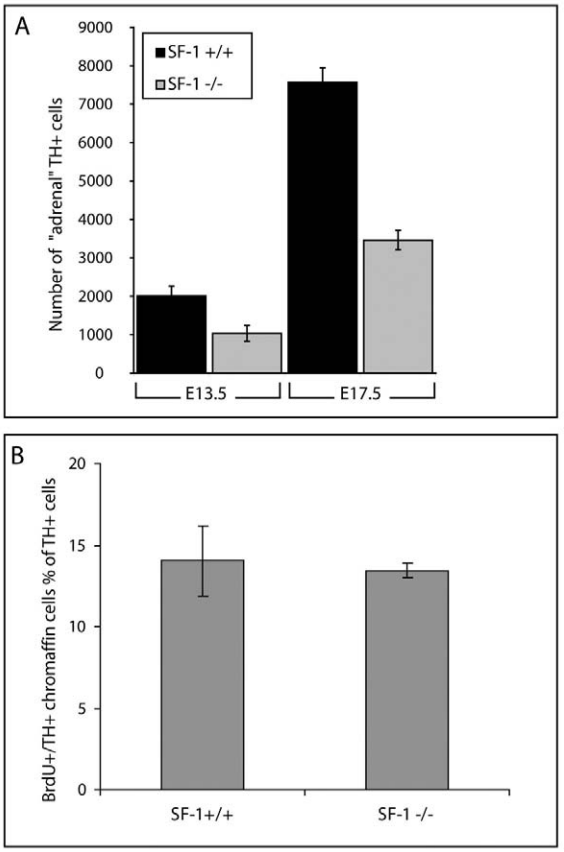

Fig. 7. (A) Quantification of TH^{+}/NF^{-} 'adrenal' chromaffin cells in E13.5 and 17.5 *Sf1*$^{+/+}$ and *Sf1*$^{-/-}$ embryos. Data are presented as mean ± s.e.m. Six 'adrenal glands' from three different animals were analysed per group. Every second section was counted.
(B) BrdU^{+}/TH^{+} adrenal chromaffin cells in *Sf1* mutant embryos and wild-type littermates at E13.5 expressed as percentage of total TH^{+} cells. BrdU was administered to pregnant females at day 12.5 of gestation. Data are presented as mean±s.e.m. Six 'adrenal glands' from three different animals were analysed per group. Every fifth section was counted.

number of chromaffin progenitor cells migrating to the site of the adrenal anlage is altered in the *Sf1* mutant compared with wild-type mice, and whether the full quantity of cells is maintained during development. On serial sections at the level of the suprarenal ganglion, we counted cells with positive TH immunofluorescence, which, by contrast to suprarenal ganglion cells, were NF-negative. Fig. 7A shows that cells identified by these criteria amounted to approximately 50% of the numbers of adrenal chromaffin cells in wild-type littermates.

TUNEL labelling was performed to investigate whether the apparent reduction in numbers of 'adrenal' chromaffin cells resulted from increased apoptosis in *Sf1* mutant mice. We did not find any evidence for the existence of TUNEL^{+}/TH^{+} cells in the E12.5, 13.5 and 17.5 'adrenal' region of *Sf1*$^{+/+}$ and *Sf1*$^{-/-}$ mice (not shown).

To investigate whether the reduced number of TH^{+} chromaffin progenitor cells in *Sf1* mutant mice was due to decreased proliferation, we investigated BrdU incorporation into chromaffin cells of wild-type and *Sf1* mutant embryos. A single pulse of BrdU was administered to pregnant females at day 12.5 of gestation. Twenty-four hours later we determined the percentage of embryonic TH^{+} cells that had incorporated BrdU. As shown in Fig. 7B, there was no significant difference in the proportion of adrenal cells encountered in the synthetic phase of the cell cycle. Together, these data suggest that an early process, taking place by the time of cell migration and/or colonization of the adrenal anlage, that is different from proliferation or apoptosis, accounts for the smaller number of chromaffin cells present in the mutants.

Numbers of SOX10-, PHOX2B-, and TH-positive cells are reduced in the 'adrenal' region of *Sf1*$^{-/-}$ mice

At E12.5, NC-derived cells have started to colonize the adrenal anlage in wild-type mice (Fig. 8B). Only a few of these cells are immunoreactive for TH, while larger numbers express PHOX2B (Fig. 8A), an early marker for SA cells, or SOX10, a marker for undifferentiated NC cells (Fig. 8A,B). Thus, at this age, most 'adrenal' chromaffin progenitors cannot be

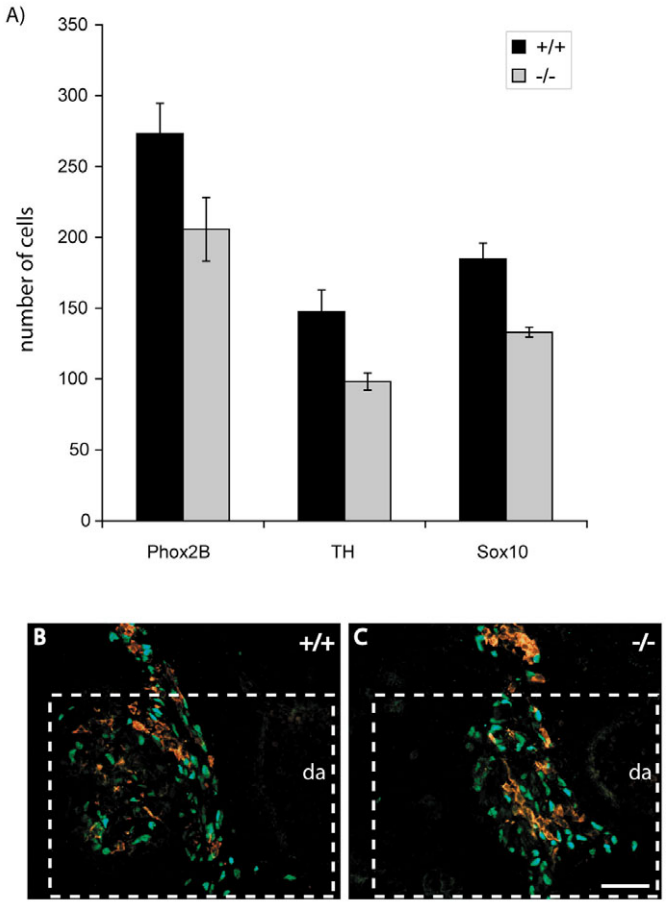

Fig. 8. (A) Quantification of SOX10-ir, TH-ir and PHOX2B mRNA-expressing cells in the region of the developing adrenal gland in E12.5 wild-type and *Sf1* mutant mice. The precise demarcations of the region analysed are described in the text. Every fifth section was counted. Four animals were analysed per group. Data are presented as mean±s.e.m. (B,C) TH (red) and SOX10 (green) double-immunolabelling on transverse sections (dorsal, top) through the region of the developing adrenal gland in wild-type and $Sf1^{-/-}$ mouse embryos. The area in which cells were counted is demarcated.

identified by combined TH and NF labelling. Note that the TH-ir and the SOX10-ir populations do not overlap in wild-type and $Sf1^{-/-}$ mice. To clarify whether chromaffin progenitor cells are numerically reduced in $Sf1^{-/-}$ mice as early as E12.5, we counted numbers of PHOX2B$^+$, TH$^+$ and SOX10$^+$ cells in wild-type and $Sf1^{-/-}$ mice in the para-aortic region within a reproducibly defined space, describing as precisely as possible the region where the adrenal anlage is located at this age. This space was demarcated in the rostrocaudal position by the caudal extremity of the lung bud and extended from there 500 μm caudally. The medial border of this space was the midline of the dorsal aorta, the lateral border a vertical plane 350 μm lateral to the midline. The dorsoventral limits were horizontal planes through the dorsal wall of the aorta and a plane 200 μm further ventrally (cf. Fig. 8B and C). Fig. 8A shows that in this region numbers of PHOX2B mRNA-positive cells, TH-ir and SOX10-ir cells are reduced by about 25% in $Sf1^{-/-}$ mice. We therefore conclude that the 'adrenal' region of $Sf1^{-/-}$ mice contains fewer chromaffin precursors as early as E12.5 and that both early progenitors and cells that are already TH-ir are affected.

Discussion

The classic model of chromaffin progenitor specification had postulated a crucial role of the local environment, in particular glucocorticoids, based on in-vitro studies (Anderson and Axel, 1986) (cf. Kandel et al., 2000; Michelsohn and Anderson 1992; Unsicker et al., 1978). A major challenge for this hypothesis was the analysis of the glucocorticoid receptor (GR) knockout (Cole et al., 1995; Finotto et al., 1999). Contrary to the prediction that loss of GR signalling would foster neuronal, and abolish chromaffin cell differentiation in the adrenal gland, GR-deficient mice had normal numbers of adrenal chromaffin cells, which resembled their wild-type counterparts in virtually all structural and chemical aspects. Although these results abrogated an essential role of GR signalling in chromaffin phenotype specification, they evidently did not rule out other contributions of the adrenal cortex to chromaffin development.

SF1 is an orphan nuclear receptor, which closely resembles fushi tarazu factor 1 isolated in *Drosophila* (Lavorgna et al., 1991). It has been implicated in the gene regulation of steroid hydroxylases and shown by recombinant gene targeting to be absolutely required for the embryonic development of the adrenal cortex and gonads (Luo et al., 1994). Initially, it had been reported that $Sf1^{-/-}$ mice completely lack adrenal glands, i.e. adrenal cortex and medulla, although *Sf1* expression is restricted to the adrenal cortex. It was therefore proposed that the NC-derived adrenal medullary chromaffin cells either fail to migrate to the appropriate position or do not survive after migration due to the lack of an adrenal cortical environment. More recently, it has been reported that in $Sf1^{-/-}$ embryos chromaffin progenitors, identified by dopamine β-hydroxylase (DBH)/LacZ, accumulate at the correct rostrocaudal location at E13.5. However, according to this study no tissue corresponding to the adrenal medulla could be detected at E15.0 (Bland et al., 2004).

Our analyses of $Sf1^{-/-}$ mice provide evidence that absence of an adrenal cortex does not substantially impair the differentiation of chromaffin cells. About half the normal number of chromaffin progenitors in *Sf1* null mice are found in the location where an adrenal medulla develops in wild-type mice, i.e. in close association with the upper pole of the kidney and with a cluster of sympathetic neurons that form the suprarenal ganglion. As in $Sf1^{+/+}$ adrenal glands, $Sf1^{-/-}$ 'adrenal' chromaffin cells express TH, but, by contrast to sympathetic neuronal progenitors in the suprarenal ganglion, virtually no NF68 mRNA. Analyses of E13.5 $Sf1^{-/-}$ mice reveal that numbers of TH$^+$/NF$^-$ cells in this location amount to about 50% of adrenal medullary chromaffin progenitor cells in $Sf1^{+/+}$ animals. Numbers of TH$^+$/NF$^-$ cells increase in both $Sf1^{-/-}$ and $Sf1^{+/+}$ mice by a factor of about 3.5 until E17.5. Together with the evidence from BrdU incorporation, these data suggest that physiologically occurring proliferation of chromaffin progenitors at this age is not impaired (cf. Finotto et al., 1999; Huber et al., 2002). The frequent detection of mitotic figures in electron microscopic samples and the absence of TUNEL-positive chromaffin progenitors in both genotypes further support this notion. Chromaffin progenitor cells in *Sf1* mutant mice display all ultrastructural hallmarks of this cell type, are

positive for VMAT1 and are contacted by preganglionic axon terminals, suggesting that preganglionic innervation is not grossly impaired. As expected, the absence of an adrenal cortex in *Sf1*$^{-/-}$ mice abrogates expression of PNMT, which in mammals essentially requires high levels of glucocorticoids (Pohorecky and Wurtman, 1968; Wurtman and Axelrod, 1966). The only other alteration that we found in the *Sf1*$^{-/-}$ chromaffin cell phenotype concerns persistent expression of RET until E19.5, which in the wild-type situation is extinguished at E15.5 (Allmendinger et al., 2003). The significance of this difference remains to be investigated.

Our analysis of chromaffin cell development in *Sf1* mutant mice provides conclusive evidence that the adrenal cortex is not an absolute prerequisite for the determination and differentiation of the chromaffin phenotype. The close association of well-differentiated chromaffin tissue and neurons in the suprarenal ganglion further argues against a crucial role of local environmental factors in the diversification of SA derivatives. This adds to an increasing body of evidence suggesting that a segregation of the two phenotypically distinct cell types, neurons and chromaffin cells, has probably occurred prior to them reaching their final destination. In support of this notion, recent studies in chick embryos have revealed an early heterogeneity of SA progenitors concerning expression of neurofilament (Ernsberger et al., 2005). Furthermore, the existence of chromaffin tissue outside the adrenal gland (extra-adrenal chromaffin cells) similarly suggests that specification of chromaffin cells is unlikely to depend crucially on the adrenal cortex. Extra-adrenal chromaffin cells are abundant in mammals at birth (cf. Coupland, 1965), but subsequently undergo massive cell death for reasons that are still unknown. Finally, comparative histology of chromaffin tissue in lower vertebrates from elasmobranchs to teleosts also suggests that the chromaffin phenotype can develop independently from adrenocortical (inter-renal) tissue (Chester and Philipps, 1986; Matty, 1985)

Generally, the segregation of subpopulations of progenitors from the multipotent NC population may take place before, during or after migration (Le Douarin and Kalcheim, 1999). As the adrenal cortex apparently does not specify the chromaffin cell phenotype, a postmigratory specification of the SA progeny into neurons and chromaffin cells seems unlikely. Therefore, signals for chromaffin cell specification probably need to be sought prior to the arrival of the progenitor cells at the adrenal target. Given the wide distribution of chromaffin cells over all cervical, thoracic and lumbar levels, and their presence in virtually all sympathetic ganglia, paraganglia, adrenal gland and in lumbar extra-adrenal chromaffin tissue, we consider it to be unlikely that distinct segmental signals, as for example provided by the Hox code (Creuzet et al., 2002), contribute to the specification of the chromaffin cell phenotype. However, we have noted in the context of previous studies that the adrenal anlage of mouse embryos is colonized at E11.5 by NC cells that express MASH1 and PHOX2B, but are still TH-negative, while nearby located sympathetic neurons already express TH. This finding might be explained by different kinetics of TH induction in sympathetic and chromaffin progenitors. Alternatively, it may suggest that chromaffin cells and sympathetic neurons are not derived from a common TH^{+} SA progenitor, but develop independently from NC cells, following different maturation schedules. Thus, chromaffin progenitors may represent a late migrating wave of NC derivatives. A time-dependent topographical segregation and fate-dependency of NC progenitors has previously been suggested by studies analysing the fate restrictions of late versus early migrating cephalic and trunk NC derivatives (Raible and Eisen, 1996). NC cells colonizing the adrenal anlage may not even require BMP-4 derived from the dorsal aorta but may receive this important signal from BMP-4 expressing peri-adrenal cells (K.H., unpublished).

Although the present study rules out an essential fate-determining role of the adrenal cortex for chromaffin progenitor cells, the missing 50% fraction of chromaffin cells in *Sf1*$^{-/-}$ embryos may suggest a role for the adrenal cortex in attracting and harbouring chromaffin cells. Our analysis of the 'adrenal' region in E12.5 *Sf1*$^{-/-}$ mice indicates that numbers of SOX10^{+} NC progenitors as well as PHOX2B- and TH-positive SA cells are reduced by 25%. The relatively modest cell loss, compared with the 50% loss of chromaffin progenitor cells at E13.5, is probably due to counting a wider range of progenitor cells, possibly including non-chromaffin progenitors, within a larger volume. Alternatively, since the cells have just started to invade the adrenal cortical anlage at E12.5, these results may indicate that fewer cells settle in their final location between E12.5 and 13.5. In light of the uniform 25% loss of SOX10-, PHOX2B- and TH-positive cells, an impairment of differentiation of SOX10-positive NC cells into PHOX2B- and TH-positive chromaffin progenitors seems unlikely. The *Sf1*$^{-/-}$ phenotype can be interpreted in several ways, including deficits in migration of NC progenitor cells to the adrenal region. We would favour, however, a hypothesis that takes into account the capacity of the adrenal anlage to provide space and an environment permissive for harbouring cells.

In conclusion, the present study suggests that the adrenal cortex is not essentially required for chromaffin phenotype specification. However, it is necessary for assembling the complete set of chromaffin cells in the correct location.

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