

Cardiac outflow tract development relies on the complex function of Sox4 and Sox11 in multiple cell types

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Received: 29 May 2013 / Revised: 18 October 2013 / Accepted: 11 November 2013 / Published online: 6 December 2013
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Abstract Congenital heart defects represent the most common human birth defects and are often life-threatening. Frequently, they are caused by abnormalities of the outflow tract whose formation results from coordinated development of cells from mesodermal and neural crest origin and depends on the activity of many different transcription factors. However, place, time, and mode of action have only been analyzed for a few of them. Here we assess the contribution of the closely related high-mobility-group transcription factors Sox4 and Sox11 to outflow tract development and determine their function. Using cell-type-specific deletion in the mouse, we show that Sox11 is required for proper development in both mesodermal cells and neural crest cells. Deletion in either mesoderm or neural crest, or both, leads to outflow tract defects ranging from double outlet right ventricle to common arterial trunk. Sox4 supports Sox11 in its function, but has additional roles with relevance for outflow tract formation in other cell types. The two Sox proteins are dispensable during early phases of cardiac neural crest development including neural tube emigration, proliferation, and migration through the pharyngeal arches. They become essential after arrival of the neural crest cells in the outflow tract for their proper differentiation and interaction with each other as well as with the environment through regulation of cytoskeletal, cell adhesion, and extracellular matrix molecules. Our results demonstrate

that Sox4 and Sox11 have multiple functions in several cell types during outflow tract formation and may thus help to understand the basis of congenital heart defects in humans.

Keywords Sox factors · Double outlet right ventricle · Common arterial trunk · Neural crest · Cardiac gene regulatory network

Introduction

Heart development in vertebrates involves extensive morphogenetic processes and the contribution of several different cell types, and is thus highly complex. This developmental complexity partly explains the prevalence of congenital heart defects among birth defects in humans [1]. Congenital heart defects involve defects of ventricular septum, semilunar valves, or outflow tract, and frequently combinations thereof.

Initially, the outflow tract is a single vessel with a myocardial outer layer and an endocardial lining that arises at the anterior pole of the heart from a still unseptated ventricle. As heart development proceeds, this vessel undergoes extensive remodeling, including septation and rotation events. With formation of the aortopulmonary septum, the outflow tract is septated into ascending aorta and pulmonary trunk, which eventually have to join with the two different ventricles [2]. The two main cellular contributions to the outflow tract of the vertebrate heart stem from the second heart field and the cardiac neural crest. The second heart field contributes to both myocardial and endocardial lineages, while cardiac neural crest cells migrate into the conotruncal endocardial cushions and add to the existing mesenchyme, which is endocardially derived [3]. During subsequent formation of the aortopulmonary septum and outflow tract septation both cell populations interact and

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influence each other's development reciprocally [4]. Developmental defects in both cell populations can lead to outflow tract malformations and misalignments with the ventricles, including persistent common arterial trunk (CT), double outlet right ventricle (DORV), and transposition of the great arteries.

Among the genes whose mutation or deletion cause outflow tract defects are a number of transcription factors, including Pax3, AP2 α , Mef2c, Insm1, SIP1, the two Hand proteins, as well as several Gata, Tbx, Nkx, Fox, and Sox proteins [5]. Several of these have been studied in the mouse and represent excellent candidates for constituents of the transcriptional regulatory network in cardiac neural crest or second heart field-derived cells. Constitutive deletion of Sox4 in the mouse, for instance, impairs proper development of the endocardial ridges into semilunar valves and the outlet portion of the muscular ventricular septum as a consequence of which embryos develop CT and succumb to circulatory failure at embryonic day 14 [6]. Constitutive deletion of Sox11, on the other hand, leads to a wide spectrum of outflow tract phenotypes ranging from DORV to CT, usually in association with a membranous ventricular septum defect [7]. Sox11-deficient mice die shortly after birth. Disease-associated mutations in human SOX4 or SOX11 have not yet been identified.

Both Sox4 and Sox11 belong to the same SoxC subgroup of the Sox transcription factor family. They are closely related with each other and Sox12 as the third member of this subgroup, and believed to exhibit at least partial functional redundancy when co-expressed [8–10]. Nevertheless, deletion of Sox4 and Sox11 alone leads to outflow tract defects, arguing that in this context SoxC factors are functionally more diverse than expected or act in different cell types or at different times of heart development.

To investigate the role of Sox4 and Sox11 in heart development in greater detail, we deleted either one or both genes in developing mouse embryos in different cell types that contribute to the outflow tract region, and studied the consequences for cardiac development. Interestingly, we found that the two related SoxC proteins indeed do not function fully redundantly in this developmental context.

Materials and methods

Mouse husbandry, genotyping, and dissections

Mice used in this study carried the following alleles on a mixed 129SvJ/C57Bl6J background: Sox4^{loxP} [11], Sox11^{loxP} [10], and Rosa26^{stoplox-EYFP} [12]. For conditional deletion of the Sox4^{loxP}, Sox11^{loxP}, and Rosa26^{stoplox-EYFP} alleles, Wnt1::Cre, Nkx2-5^{RES::Cre} (Nkx2.5::Cre), AP2a::Cre, Tie2::Cre, Sox2::Cre transgenes were used [13–17].

Genotyping was performed by PCR. Primer sequences are available upon request. Embryos were obtained at 10.5, 13.5, and 17.5 dpc from staged pregnancies.

Histological staining procedures, immunohistochemistry, and TUNEL

For hematoxylin-eosin staining, embryos were incubated overnight in 4 % paraformaldehyde, dehydrated, embedded in paraffin, sectioned on a microtome at 3- μ m thickness, dewaxed, rehydrated, and stained in Mayer's hematoxylin and eosin according to standard protocols.

For immunohistochemistry and TUNEL, embryos underwent fixation in 4 % paraformaldehyde, were frozen at -80°C in Jung Tissue Freezing Medium (Leica, Nussloch, Germany) after cryoprotection, and then sectioned transversally on a cryotome at 10- μ m thickness. The following primary antibodies were used in various combinations: rat anti-serum against EYFP (1:1,000 dilution; GERBU Biotechnik, Heidelberg, Germany), guinea pig antisera against Sox4 (1:1,000 dilution; [18]) and Sox11 (1:1,000 dilution; [18]), and rabbit antisera against EYFP (1:2,000 dilution; Molecular Probes, Eugene, OR, USA), NG2 (1:200 dilution; Chemicon, Temecula, CA, USA), and Ki67 (1:500 dilution; Lab Vision, Fremont, CA, USA). Secondary antibodies conjugated to Alexa488 and Cy3 immunofluorescent dyes (Molecular Probes, Eugene, OR, USA, and Dianova, Hamburg, Germany) were used for detection. Phalloidin (1:5,000 dilution; Sigma-Aldrich, St. Louis, MO, USA) staining and TUNEL (Chemicon, Temecula, CA, USA) were performed according to the manufacturer's protocol.

Quantifications

Numbers of immunoreactive cells and DAPI-positive nuclei per section were counted in relevant areas. Data were obtained from four sections of at least three different embryos for each genotype and embryonic age. Diagrams show mean values \pm SEM of biological replicates. Statistical significance was determined by unpaired two-tailed Student's *t* test using GraphPad Prism6 software and is mentioned in the corresponding figure legends.

Transfections and luciferase assays

HEK293 cells and Neuro-2a cells were kept on 100-mm dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10 % FCS in the presence of penicillin and streptomycin. For luciferase assays, Neuro-2a cells were seeded on 35-mm dishes and transfected with Superfect (Qiagen, Hilden, Germany) using 400 ng of the luciferase reporter plasmid and 250 ng of the effector plasmids per dish [19]. Reporter plasmids contained the luciferase open

reading frame under control of the human *E-cadherin* promoter (Ecad 1, corresponding to positions $-1,502$ to $+98$ relative to the transcriptional start site, Ecad s, corresponding to positions -220 to $+18$, gift of J. Behrens, Erlangen) or the human *Adam19* promoter (Adam19 1, positions $-2,009$ to $+31$, Adam19 s, positions -243 to $+31$, gift of M. Kreutz, Regensburg) [20]. The Ecad s and Adam19 s promoter fragments were also inserted as a version with mutated Sox binding sites (Ecad sm and Adam19 sm). pCMV5-based effector plasmids for full-length Sox11 and Sox4 were as described [19]. The total amount of plasmid was kept constant using empty pCMV5 vector where needed. Cells were harvested 48 h after transfection, and extracts were assayed for luciferase activity [19]. For production of recombinant proteins, polyethylenimine-transfected HEK293 cells or Superfect-transfected Neuro-2a cells were used, in both cases applying 6–10 μg pCMV-based expression plasmids per 100-mm dish.

Protein extracts and electrophoretic mobility shift assay

Whole cell extracts were prepared from transfected HEK293 and Neuro-2a cells and used for electrophoretic mobility shift assays using ^{32}P -labeled oligonucleotides as probe and poly-dGdC as non-specific competitor under standard conditions [21]. Double-stranded oligonucleotides contained site B of the *Mpz* promoter [21] or predicted Sox binding sites of the human *E-cadherin* or *Adam19* promoters in wild-type version or after mutation of the Sox binding site. All oligonucleotides had a 5'-GGG overhang for labeling. Sequences were: *SiteB* CAGAGTATACAATGCCCTTA, *Ecad1* CCAGGCTAGAGGTCACGCGT, *Ecad1mut* CCAGGCTAGAGCCGCGCTCGT, *Ecad2* CACGCACCCCCTCTCAGTGCG, *Ecad2mut* CACGCACCCCCTCTGCGTGGCG, *Adam19* CGTCCACCACAAATCCCTCCTC, *Adam19mut* CGTCCACCAGCGATCCCTCCTC.

RNA preparation from heart outflow tracts for quantitative RT-PCR

Outflow tracts of the heart were dissected from mice at embryonic day 13.5, immediately frozen on dry ice, and stored at -80 °C. Five outflow tracts of each genotype were pooled and processed for RNA isolation using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed in 25- μl reactions using 2 μg RNA, oligo-dT primers, and Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Ipswich, MA, USA). Quantitative PCR using 3 μl of the obtained cDNA was performed on a Bio-Rad Lightcycler according to the manufacturer's instructions using the ABsolute

QPCR SYBR Green Mix (Thermo Scientific, Waltham, MA, USA) with an annealing temperature of 60 °C. *Rpl8* transcripts were used for normalization [22]. The following primers were used for detection: 5'-GCCTCTCTGCGACTAAGGTG-3' and 5'-CAGCAAGCCTCTGAGTCCA A-3' for *Adam9*, 5'-CCCCCTAAGAGTGTGGGTCCCG-3' and 5'-TGGCGATTCAACGCGCAGGT-3' for *Adam19*, 5'-GGAAAGGAAGCAGAAGGCTCGGC-3' and 5'-TGGGAGATGGGGAAGGCTTGGT-3' for *Cx40*, 5'-AGTGAAAGAGAGGTGCCAGACAT-3' and 5'-TCGGTCTGCGCCACTTTGAGC-3' for *Cx43*, 5'-AGCTTTTC-CGCGCTCTGCT-3' and 5'-AGAGGCAGGGTCGCGGTGGT-3' for *E-cadherin*, 5'-TGCCCAACTGTTACTGCCAA-3' and 5'-GGGCAGAAAACAACACGGG-3' for *Foxc2*, 5'-AAGGAGCGGCGCAGGACTCA-3' and 5'-CAGCCTGTCCGGCCTTTGGT-3' for *Hand2*, 5'-TGCGTCCGGCCTGCTAGAGT-3' and 5'-GCTCCACCGAAGCGAAGCGA-3' for *Insm1*, 5'-GCCTTCACGATCACCATCCT-3' and 5'-CAGGGCTCCTCTGTGTGAGA-3' for *NG2*, 5'-TCCGCACCCCACTGGTGAGA-3' and 5'-GGAGCTCTGGGCGGGTACA-3' for *Snail1*, 5'-ACTGGACACACACAGTTAT-3' and 5'-ATGGCATGGGGTCTGA AAG-3' for *Snail2*, 5'-GCTGCTCCAGCTATGTGTGA-3' and 5'-AGTGGTGCCAGATCTTTTCCA-3' for *SMA*, 5'-TGGACCACCGGCACCCAGAA-3' and 5'-CGTGGGCAGAGCCACACCTG-3' for *Sox10*, 5'-CAGTCTGGGGA CAGAGTTCC-3' and 5'-GACCAGTGGAGCAGACTTG G-3' for *Sox12*, 5'-GCAGTCGCTGAACGAGGCGT-3' and 5'-ACAATGACATCTAGGTCTCCGGCCT-3' for *Twist1*, 5'-GTTCGTGTACTGCGCAAGA-3' and 5'-ACAGGATTCATGGCCACACC-3' for *Rpl8*.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed on HEK293 or Neuro-2a cells after transfection with Sox11 expression plasmid as described previously [23]. Additionally, 16 whole hearts with attached outflow tract region or 16 dissected anterior heart poles from 13.5 dpc-old mouse embryos were used per sample as chromatin source after dissociation with a gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). After crosslinking proteins to DNA in the presence of 1 % formaldehyde, chromatin was prepared and sheared to an average fragment length of 200–500 bp using a Sonoplus HD2070 homogenizer (Bandelin, Berlin, Germany). Immunoprecipitations were performed overnight at 4 °C using guinea pig antiserum against Sox11 (1:500 dilution) [18] or guinea pig preimmune serum as control, coupled to protein A Sepharose beads. Quantitative PCR was performed on input and precipitated chromatin after crosslink reversal and purification. Primer sequences are available upon request.

Results

SoxC proteins are required for proper development of cardiac neural crest cells

To analyze the role of SoxC proteins in cardiac neural crest cells, we deleted *Sox4* or *Sox11* using floxed alleles of these genes and a *Wnt1::Cre* transgene that allows early and efficient gene deletion throughout the cranial, cardiac, and trunk neural crest. The resulting *Sox4^{fl/fl} Wnt1::Cre* and *Sox11^{fl/fl} Wnt1::Cre* mice

are henceforth referred to as *Sox4^{ΔWnt1}* and *Sox11^{ΔWnt1}*, respectively.

Sox4^{ΔWnt1} and *Sox11^{ΔWnt1}* embryos were first analyzed at 13.5 dpc (Fig. 1). All controls (i.e., embryos that were homozygous for the floxed alleles in the absence of *Cre* recombinase or embryos that carried the *Cre* transgene in combination with at least one wild-type allele) had a normally developed heart and outflow tract. The same observations were made for all other control genotypes with additional or alternative *Cre* alleles used during this study. Considering the severe outflow tract defects

Fig. 1 Early developmental defects of the cardiac outflow tract in mice with neural crest-specific SoxC gene ablations. Macroscopic appearance of the anterior heart pole (a–f) and hematoxylin-eosin stainings of corresponding consecutive serial sections (*horizontal plane*) (g–o) from wild-type embryos (Wt) (a, g, h, i) at 13.5 dpc or age-matched embryos with *Wnt1::Cre*-mediated, neural crest-specific SoxC gene ablations. Analyzed genotypes included: *Sox11^{ΔWnt1}* (*Sox11Δ*) (b, c, j, k, l), *Sox4^{ΔWnt1}* (*Sox4Δ*) (d), and *Sox4^{ΔWnt1} Sox11^{ΔWnt1}* (*Dko*) (e, f, m, n, o). Observed outflow tract abnormalities included DORV in *Sox11^{ΔWnt1}* and *Sox4^{ΔWnt1} Sox11^{ΔWnt1}* embryos (c, e, j, k, l) as well as CT in *Sox4^{ΔWnt1} Sox11^{ΔWnt1}* embryos (f, m, n, o). Ao aorta, CT common arterial trunk, LA left atrium, RA right atrium, RV right ventricle, PT pulmonary trunk. Scale bars 500 μm

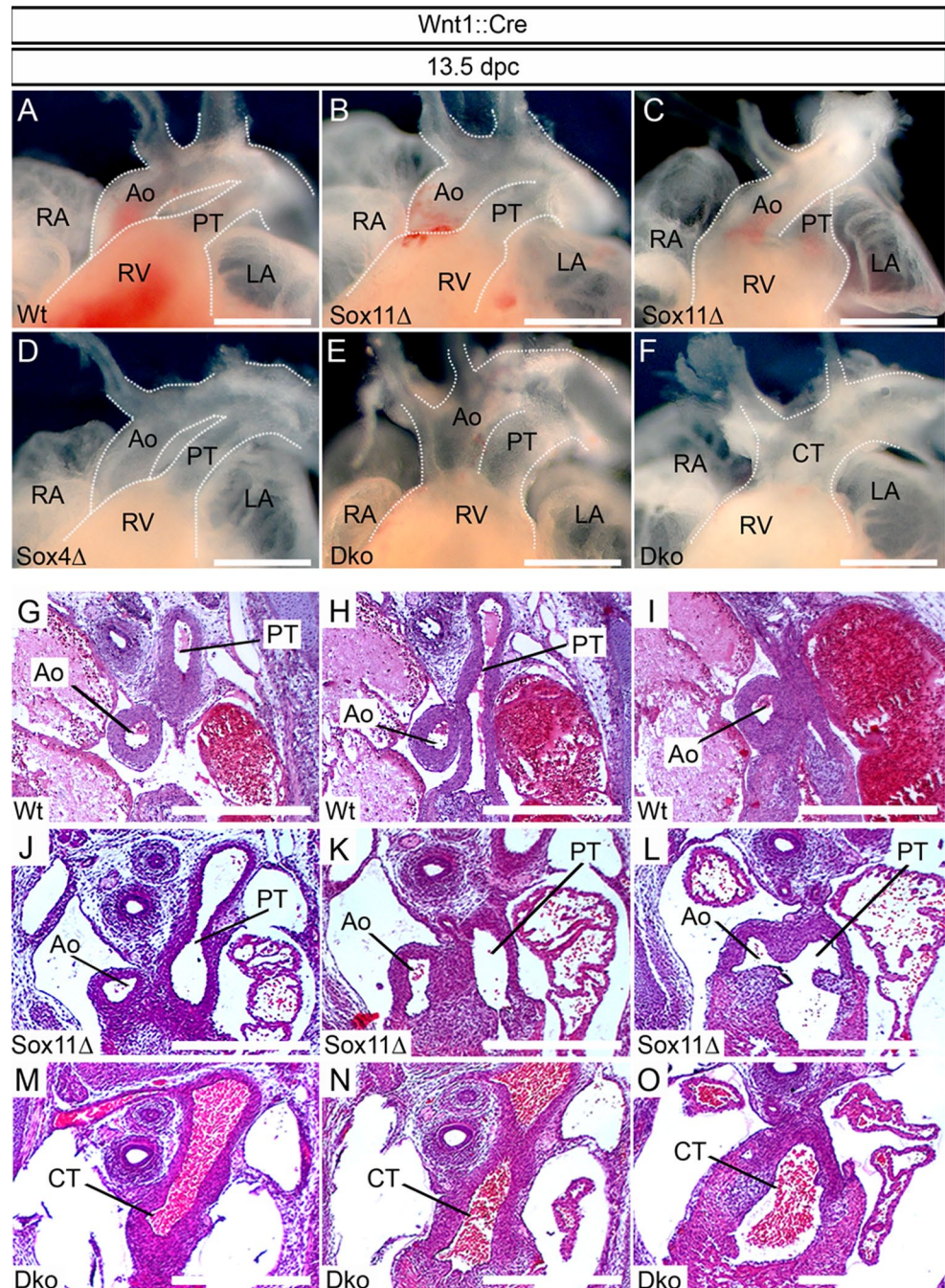
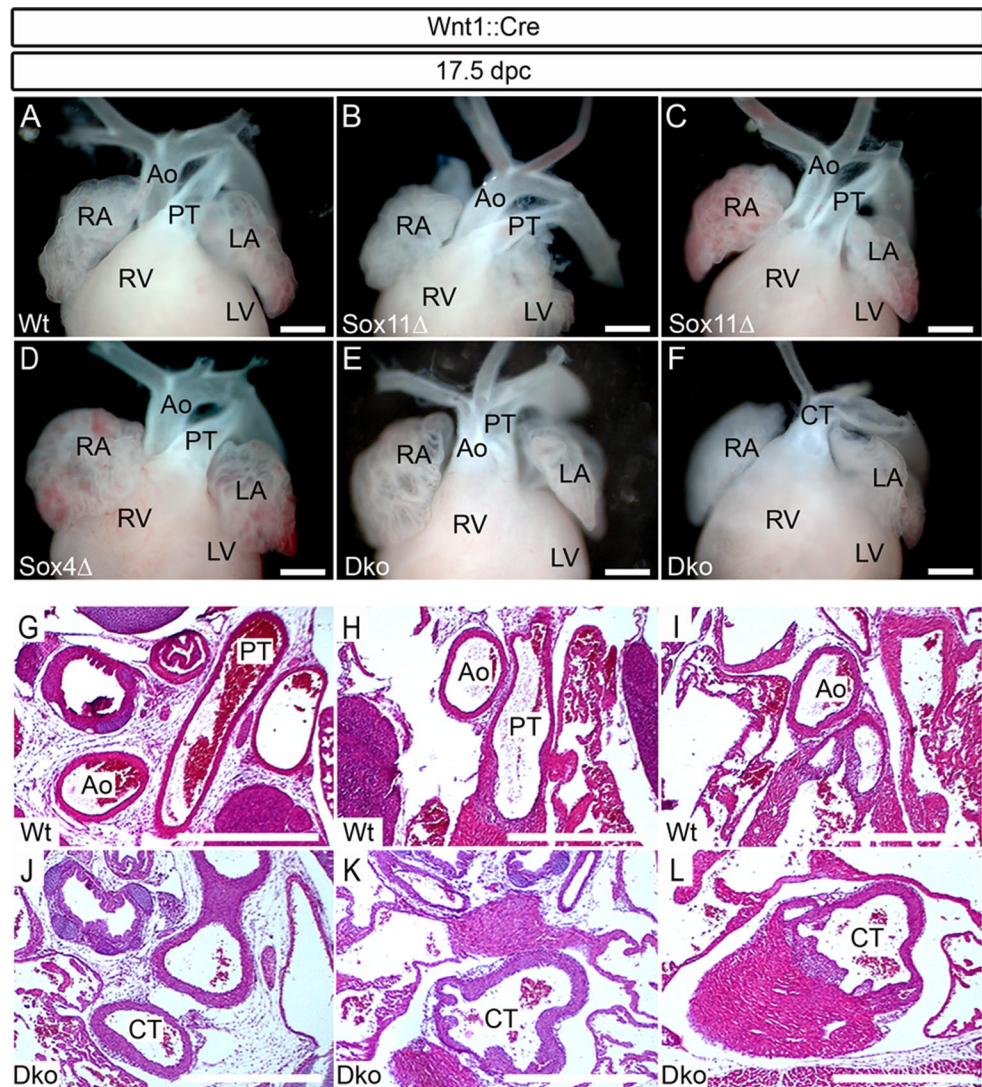


Fig. 2 Late developmental defects of the cardiac outflow tract in mice with neural crest-specific SoxC gene ablations. Macroscopic appearance of the anterior heart pole (a–f) and hematoxylin-eosin stainings of corresponding consecutive serial sections (*horizontal plane*) (g–l) from wild-type embryos (Wt) (a, g, h, i) at 17.5 dpc or age-matched embryos with *Wnt1::Cre*-mediated, neural crest-specific SoxC gene ablations. Analyzed genotypes included: *Sox11*^{ΔWnt1} (*Sox11*Δ) (b, c), *Sox4*^{ΔWnt1} (*Sox4*Δ) (d), and *Sox4*^{ΔWnt1} *Sox11*^{ΔWnt1} (*Dko*) (e, f, j, k, l). Observed outflow tract abnormalities included DORV in *Sox11*^{ΔWnt1} and *Sox4*^{ΔWnt1} *Sox11*^{ΔWnt1} embryos (c, e), whereas CT only occurred in *Sox4*^{ΔWnt1} *Sox11*^{ΔWnt1} embryos (f, j, k, l). *Ao* aorta, *CT* common arterial trunk, *LA* left atrium, *RA* right atrium, *RV* right ventricle, *PT* pulmonary trunk. Scale bars 500 μm

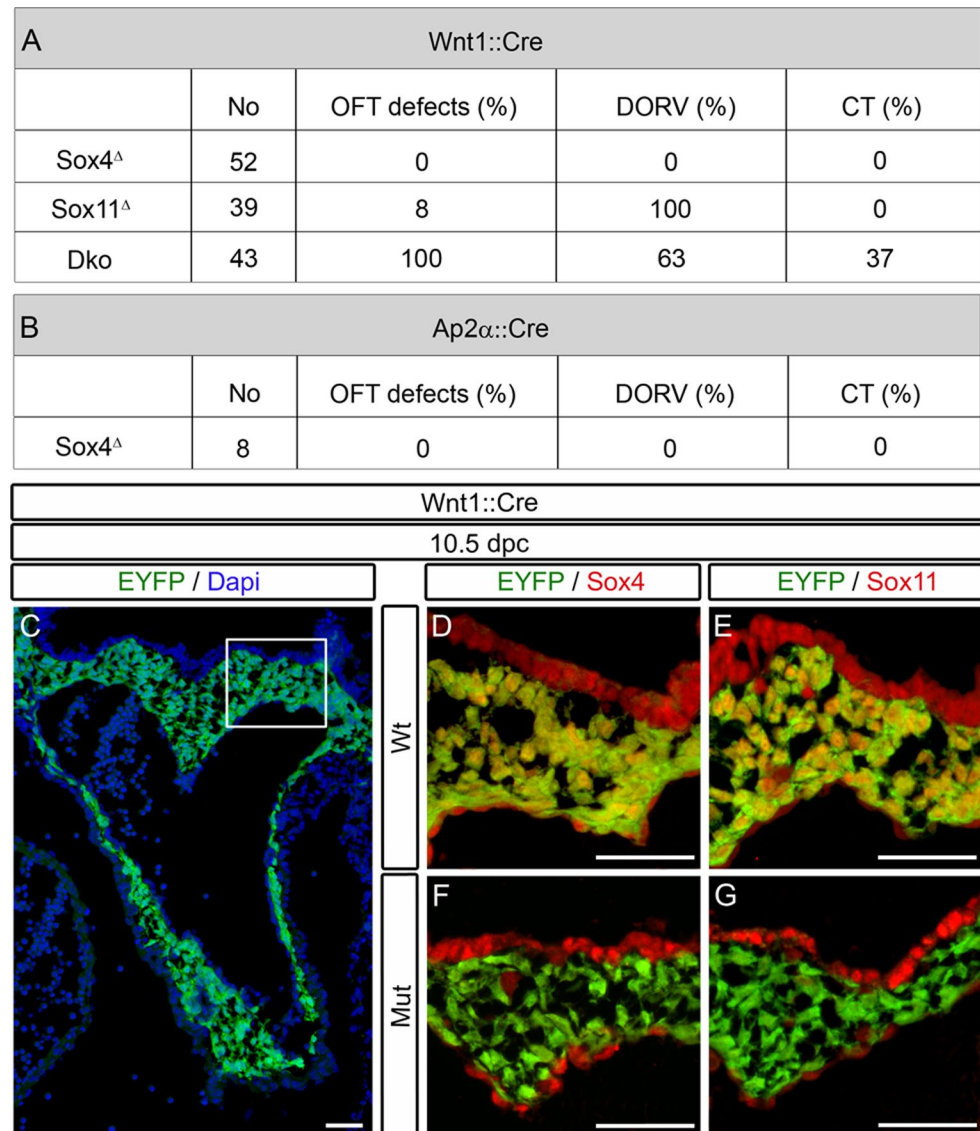


in *Sox4*^{-/-} and *Sox11*^{-/-} mice [6, 7] we were surprised to see that most *Sox4*^{ΔWnt1} and *Sox11*^{ΔWnt1} embryos exhibited a macroscopically normal outflow tract region (Figs. 1a, b, d and 3a). Even a more detailed examination of hematoxylin-eosin sections of the anterior heart pole region revealed no major conspicuous aberrations in the majority of mutant embryos in comparison to the wild type (Fig. 1g–i and data not shown). Those defects that were observed in a small minority of embryos all occurred in *Sox11*^{ΔWnt1} and not in *Sox4*^{ΔWnt1} embryos (Fig. 3a). They corresponded to a DORV phenotype (Fig. 1c, j, k, l) and were found in approximately 8 % of *Sox11*^{ΔWnt1} embryos (Fig. 3a). Phenotypic analysis at 17.5 dpc confirmed the results but failed to reveal any additional defects (Fig. 2a–d, g–i). Despite the low percentage of affected *Sox11*^{ΔWnt1} embryos the results pointed to a role of Sox11 in cardiac neural crest cells. They also showed that the outflow tract defects in

Sox11^{-/-} mice cannot be solely attributed to Sox11 functions in neural crest cells.

At first sight, the complete absence of outflow tract defects in *Sox4*^{ΔWnt1} embryos argues against a role of Sox4 in the cardiac neural crest. In general, gene deletion by the *Wnt1::Cre* transgene in cardiac neural crest is very efficient [3]. To confirm the efficiency in case of Sox4 and Sox11 deletion, we genetically labeled cardiac neural crest cells by combining *Rosa26*^{stopfloxEYFP} with *Wnt1::Cre* (Fig. 3c) on an otherwise wild-type background or in mice additionally carrying *Sox4*^{loxP} and *Sox11*^{loxP} alleles. Whereas EYFP-expressing cardiac neural crest cells were positive for both Sox4 and Sox11 in the heart region of control embryos at 10.5 dpc (Fig. 3d, e), they were almost all negative in the *Sox4*^{ΔWnt1} or *Sox11*^{ΔWnt1} mutant (Fig. 3f, g). Such high *Sox4* and *Sox11* deletion rates at early times after onset of Cre expression were observed throughout the study (data not shown).

Fig. 3 Summary of cardiac outflow tract defects and deletion efficiency of SoxC genes in cardiac neural crest. **a, b** Overview of the number of animals obtained with *Wnt1::Cre* (**a**) and *AP2α::Cre* (**b**) mediated SoxC deletions (No), and percentage of outflow tract defects (OFT defects) per genotype in total, and subdivided into DORV and CT. **c–g** Immunohistochemistry was performed at 10.5 dpc with antibodies directed against Sox4 (red in **d, f**) and Sox11 (red in **e, g**) on embryos carrying a combination of *Rosa26^{stopfloxEYFP}* and *Wnt1::Cre* alleles (Wt) (**c, d, e**) or carrying the *Rosa26^{stopfloxEYFP}* on a *Sox4^{ΔWnt1}* (**f**) or *Sox11^{ΔWnt1}* (**g**) background (Mut). *Wnt1::Cre*-dependent activation of EYFP (green) from the *Rosa26^{stopfloxEYFP}* allele was used to label cells of the neural crest lineage. Nuclei are counterstained with DAPI (blue in **c**). Pictures in panels **d–g** were taken from the region boxed in **c**. Scale bars 50 μm



The lack of an outflow tract defect in *Sox4^{ΔWnt1}* mice is thus not attributable to inefficient gene deletion. However, a previous study had shown that gene deletion by the *Wnt1::Cre* transgene in cardiac neural crest may only be achieved upon, or even after, emigration from the dorsal neural tube [24]. Deletion may then occur too late in *Sox4^{ΔWnt1}* embryos if Sox4 is required in cardiac neural crest cells at the premigratory stage. To address this issue, we combined the *Sox4^{loxP}* allele with an *AP2α::Cre* transgene to generate *Sox4^{loxP} AP2α::Cre* embryos as this *Cre* transgene is already active in premigratory neural crest cells [15, 24]. These are referred to as *Sox4^{ΔAP2α}* embryos. However, heart and outflow tract region of *Sox4^{ΔAP2α}* embryos were phenotypically indistinguishable from those of *Sox4^{ΔWnt1}* embryos and wild-type littermates (Fig. 3b). Corresponding *Sox11^{ΔAP2α}* embryos were not generated in this study.

Considering the fact that Sox4 and Sox11 are co-expressed during development in many tissues and cell types [10, 25] and share so many structural properties [19] that functional redundancy has been observed frequently [8, 10, 26, 27], we also analyzed the consequences of joint deletion of both SoxC factors in *Sox4^{ΔWnt1} Sox11^{ΔWnt1}* embryos. Intriguingly, all *Sox4^{ΔWnt1} Sox11^{ΔWnt1}* embryos that we obtained at 13.5 dpc (Fig. 1e, f, m, n, o) and at 17.5 dpc (Fig. 2e, f, j, k, l) exhibited outflow tract malformations (Fig. 3a). In addition to DORV, which we observed in 63 % of the mutant embryos (Figs. 1e, 2e), we also detected CT as the more severe phenotypic manifestation in 37 % of double-deficient embryos (Figs. 1f, m, n, o and 2f, j, k, l). The fact that additional deletion of Sox4 aggravates the phenotype obtained after deletion of Sox11 provides additional evidence that the floxed Sox4 allele is efficiently

deleted by the *Wnt1::Cre* transgene. Our results show that *Sox4* and *Sox11* are required for cardiac neural crest development, and that they function in a largely redundant manner. Of the two SoxC proteins, *Sox11* appears to be more important for cardiac neural crest development since *Sox11* ^{Δ Wnt1} embryos but not *Sox4* ^{Δ Wnt1} embryos show outflow tract malformations.

SoxC protein function is necessary for development of mesodermal cells in the outflow tract region

Phenotypic comparison of neural crest-specific and constitutive SoxC mutants argues that the cause of outflow tract malformations in constitutive SoxC mutants cannot be solely attributed to disturbances of neural crest development, and that SoxC proteins are likely active in other contributing cell types as well. To assess the requirement for *Sox4* and *Sox11* in mesodermal cells of the heart (first and second heart field), we deleted *Sox4* or *Sox11* using a *Nkx2.5::Cre* transgene (Fig. 4). The *Cre* transgene is inserted with preceding *IRE5* into the 3' UTR of the *Nkx2.5* gene so that a bicistronic allele is created that allows simultaneous *Nkx2.5* and *Cre* expression [14]. Consequently, heterozygosity for this allele does not interfere with heart and outflow tract development. Any potential phenotype in *Sox4*^{*fl/fl*} *Nkx2.5::Cre* and *Sox11*^{*fl/fl*} *Nkx2.5::Cre* embryos (referred to as *Sox4* ^{Δ Nkx2.5} and *Sox11* ^{Δ Nkx2.5} embryos) is thus caused by loss of the Sox protein. At 13.5 and 17.5 dpc, the outflow tracts of *Sox4* ^{Δ Nkx2.5} embryos were again phenotypically inconspicuous both on a macroscopic and histological level (Fig. 4a, g, j and data not shown). Among *Sox11* ^{Δ Nkx2.5} embryos, roughly half had a normally appearing outflow tract (Fig. 4b, h, j), whereas the other half exhibited outflow tract defects that all corresponded to DORV (Fig. 4c–f, i, j). We therefore conclude that *Sox11* is not only active during outflow tract formation in the cardiac neural crest but also in mesodermal cells. Whether *Sox11* is supported in this function by *Sox4* could not be analyzed as *Sox4* ^{Δ Nkx2.5} *Sox11* ^{Δ Nkx2.5} double-deficient embryos were not obtained because of early developmental defects in multiple other mesodermal lineages [10].

Extending our analysis to endothelial cell development we next deleted *Sox4* or *Sox11* using a *Tie2::Cre* transgene. In this case, however, neither the resulting *Sox4*^{*fl/fl*} *Tie2::Cre* nor the *Sox11*^{*fl/fl*} *Tie2::Cre* embryos (referred to as *Sox4* ^{Δ Tie2} and *Sox11* ^{Δ Tie2} embryos) exhibited any outflow tract anomalies at 13.5 and 17.5 dpc (Fig. 4k). Considering that even the double-deficient *Sox4* ^{Δ Tie2} *Sox11* ^{Δ Tie2} embryos were phenotypically inconspicuous (Fig. 4k), we conclude that the two SoxC factors have little cell-intrinsic impact on endothelial development.

Mesodermal and neural crest-specific defects contribute to outflow tract malformation in the absence of *Sox11*

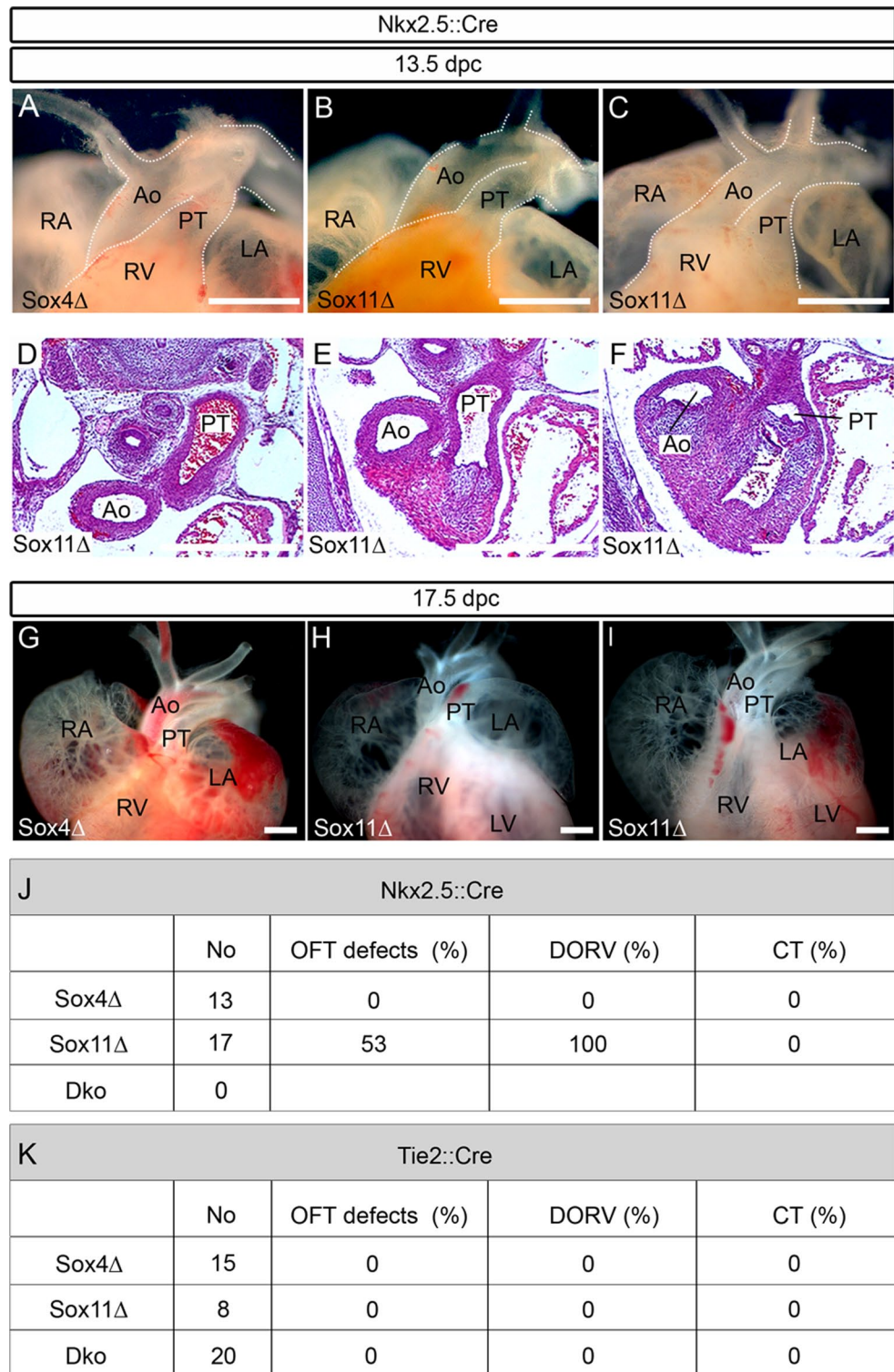
With *Sox11* loss in neural crest and mesodermal cells both leading to outflow tract defects, we next asked what would happen if *Sox11* was simultaneously deleted in both cell types. For that purpose, *Sox11* ^{Δ Wnt1 Δ Nkx2.5} embryos were generated and analyzed. As previously observed for embryos with mesodermal *Sox11* loss, approximately half exhibited outflow tract defects (Fig. 5a, b, e). Thus, the frequency was not increased upon additional *Sox11* deletion in the neural crest relative to deletion in mesodermal cells alone. However, the severity of the phenotype increased: a small percentage of *Sox11* ^{Δ Wnt1 Δ Nkx2.5} embryos exhibited CT, whereas *Sox11* ^{Δ Nkx2.5} (and *Sox11* ^{Δ Wnt1}) embryos only presented with DORV.

To our surprise, the corresponding *Sox4* ^{Δ Wnt1 Δ Nkx2.5} embryos still did not show obvious outflow tract malformations (Fig. 5c, e), thus raising the concern that the deleted *Sox4*^{*loxP*} allele may behave differently than the constitutive null allele. However, *Sox2::Cre*-dependent deletion of the *Sox4*^{*loxP*} allele throughout the epiblast fully recapitulated the outflow tract defect described for *Sox4*^{*-/-*} embryos (Fig. 5d). We thus conclude that the *Sox4* requirement for outflow tract formation cannot even be explained by its combined action in neural crest and mesodermal cells.

Generation, migration, proliferation, and apoptosis of cardiac neural crest cells are not affected by the absence of SoxC proteins

To analyze the role of SoxC proteins in cardiac neural crest cells, we labeled these cells by *Wnt1::Cre*-dependent activation of EYFP expression from a *Rosa26*^{*stop/lox-EYFP*} allele on an otherwise wild-type background or in mice carrying additionally *Sox4*^{*loxP*} and *Sox11*^{*loxP*} alleles. When the cardiac neural crest was followed via its EYFP expression and compared between both genotypes at 10.5 and 13.5 dpc, we failed to detect dramatic differences. Migration of cardiac neural crest through the pharyngeal arches occurred at the right time and in normal numbers (Fig. 6a, b, g and data not shown). Cardiac neural crest cells furthermore reached the anterior heart pole during the same time window and in similar numbers in the *Sox4*, *Sox11* double-deficient mutants as compared to the wild type (Fig. 6c, d, g and data not shown). Rates of proliferation and apoptosis were indistinguishable between the two genotypes both when analyzed on the migratory route or in the target tissue (Fig. 6h, i). We therefore conclude that SoxC proteins do not influence any of these parameters.

Fig. 4 Developmental cardiac outflow tract defects in mice with mesodermal or endothelial cell-specific SoxC gene ablations. Macroscopic appearance of the anterior heart pole (a–c, g–i) and hematoxylin-eosin stainings of corresponding consecutive serial sections (*horizontal plane*) (d–f) from embryos with *Nkx2.5::Cre*-mediated, mesodermal SoxC gene ablations at 13.5 dpc (a–f) and 17.5 dpc (g–i). Analyzed genotypes included: *Sox4*^{ΔNkx2.5} (*Sox4*Δ) (a, g) and *Sox11*^{ΔNkx2.5} (*Sox11*Δ) (b–f, h, i). For age-matched wild-type controls, see Figs. 1 and 2. DORV was observed in a fraction of *Sox11*^{ΔNkx2.5} embryos (c–f, i). *Ao* aorta, *LA* left atrium, *RA* right atrium, *RV* right ventricle, *PT* pulmonary trunk. Scale bars 500 μm. **j** Overview of the number of animals obtained with mesodermal *Nkx2.5::Cre*-mediated SoxC deletions, and the percentage of outflow tract defects (OFT defects) per genotype in total and subdivided into DORV and CT. **k** Summary of the number of animals obtained with *Tie2::Cre*-mediated, endothelial SoxC deletions, and the percentage of outflow tract defects per genotype in total, and subdivided into DORV and CT



Loss of SoxC proteins alters marker gene expression and properties of cardiac neural crest cells

As previously reported [28, 29], neural crest cells often migrate in chain-like formations, with cells contacting each other through filopodia-like processes. Such chains of

neural crest cells were regularly observed in the wild-type anterior heart pole at 10.5 dpc, and multiple filopodia-like contacts were usually observed between the cells (Fig. 6e). In age-matched double-deficient *Sox4*^{ΔWnt1} *Sox11*^{ΔWnt1} embryos, cardiac neural crest cells exhibited striking morphological alterations. The cells were no longer arranged

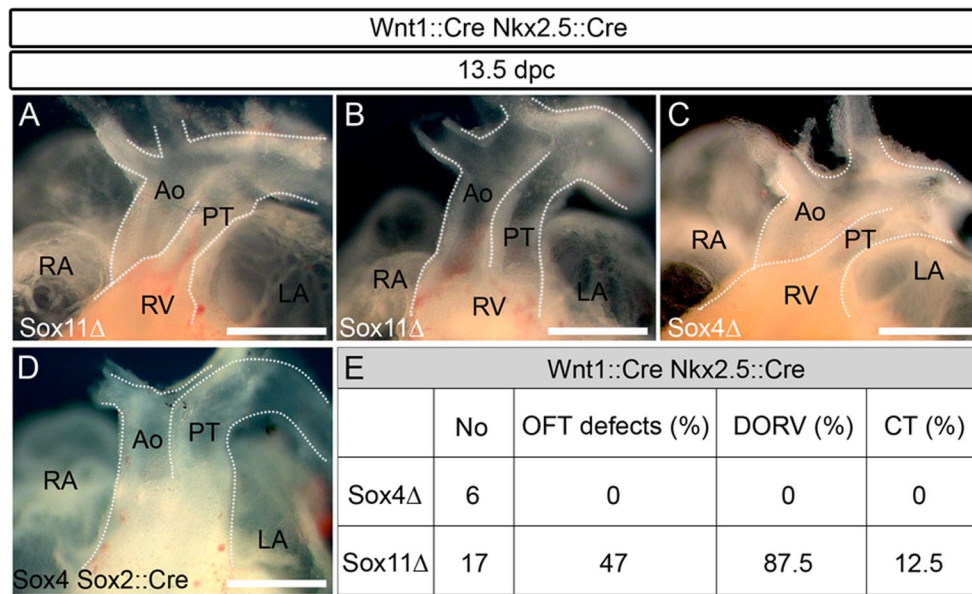


Fig. 5 Developmental cardiac outflow tract defects in mice with combined mesodermal and neural crest cell-specific SoxC gene ablations. **a–d** Macroscopic appearance of the anterior heart pole from embryos with combined neural crest and mesoderm-specific (using *Wnt1::Cre* and *Nkx2.5::Cre*), or generalized (using *Sox2::Cre*) SoxC gene ablations at 13.5 dpc. Analyzed genotypes included: *Sox11*^{ΔWnt1ΔNkx2.5} (*Sox11*Δ) (**a**, **b**), *Sox4*^{ΔWnt1ΔNkx2.5} (*Sox4*Δ) (**c**), and *Sox4*^{ΔSox2} (**d**).

DORV was observed in *Sox11*^{ΔWnt1ΔNkx2.5} and *Sox4*^{ΔSox2} embryos (**b**, **d**). *Ao* aorta, *LA* left atrium, *RA* right atrium, *RV* right ventricle, *PT* pulmonary trunk. Scale bars 500 μm. **e** Summary of the number of animals obtained with combined *Wnt1::Cre* and *Nkx2.5::Cre*-mediated SoxC deletions, and the percentage of outflow tract defects (OFT defects) per genotype in total, and subdivided into DORV and CT

in single cell chains. As a consequence, their orientation relative to each other appeared more random. Cells also exhibited an altered and often less compact morphology, and filopodial contacts were much less frequently observed (Fig. 6f).

In line with this altered morphology, cardiac neural crest cells in the double mutant also exhibited abnormalities in their cytoskeletal microfilament network as visualized by phalloidin staining. In wild-type cardiac neural crest cells, overall phalloidin staining was relatively low at 10.5 dpc, whereas it was prominent in *Sox4*^{ΔWnt1} *Sox11*^{ΔWnt1} cardiac neural crest cells (compare Fig. 7a, b to e, f). Another abnormality concerned expression of the extracellular matrix glycoprotein component NG2, which is turned on by a fraction of neural crest-derived cells in the outflow tract region as they develop into pericytes. This normally occurs well after the onset of outflow tract remodeling so that NG2 is not yet expressed in wild-type cardiac neural crest cells at 10.5 dpc as evident from the fact that *Wnt1::Cre*-induced EYFP expression does not co-localize with NG2 expression in the wild-type outflow tract at this time (Fig. 7c, d). In contrast, NG2 is detected in substantial amounts in EYFP-labeled cardiac neural crest cells of age-matched *Sox4*^{ΔWnt1} *Sox11*^{ΔWnt1} embryos at 10.5 dpc (Fig. 7g, h). By 13.5 dpc, phalloidin and NG2 staining in wild-type cardiac neural crest had caught up with mutant ones so that differences were no longer visible (data not shown; see also results

from RT-PCR in Fig. 8a). We conclude from these data that cardiac neural crest cells prematurely lose characteristics of migratory cells in the absence of Sox4 and Sox11 and turn on expression of pericyte markers in an untimely manner. It is conceivable that such misregulation during a critical time window may then prevent cardiac neural crest cells from functioning properly in outflow tract remodeling and formation of the aortopulmonary septum.

Further evidence for such a model came from the observation that several markers that are typically expressed in cardiac neural crest cells at this stage and are known to be relevant for their development exhibited strikingly altered expression levels (Fig. 8a). This was most conspicuously the case for *E-cadherin* and *Adam19*. Cadherins are not only involved in epithelial-to-mesenchymal transition at the premigratory stage and in interactions between migratory cells, they also influence the mesenchymal-to-epithelial transition post-migration [30, 31]. Expression of the metalloprotease Adam19 in cardiac neural crest cells is essential for proper outflow tract remodeling [32]. This altered expression of genes whose products influence extracellular matrix, cytoskeletal organization, or mesenchymal-to-epithelial transition, has the potential to interfere with proper outflow tract remodeling.

Interestingly, one of the genes with unaltered expression after combined deletion of Sox4 and Sox11 in cardiac neural crest cells was Sox12, the third member of

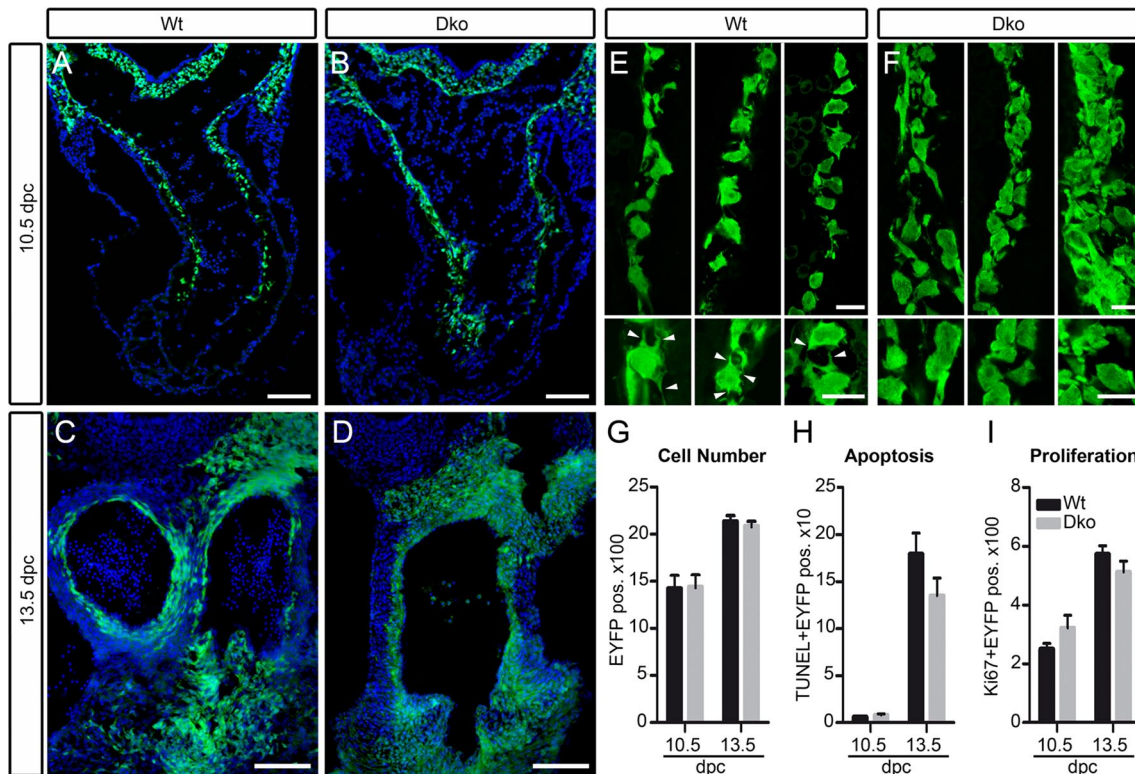


Fig. 6 Analysis of general properties and morphology of cardiac neural crest in mice with neural crest-specific SoxC gene ablations. **a–d** Immunohistochemistry for EYFP (green) was performed at 10.5 dpc (**a, b**) and 13.5 dpc (**c, d**) on embryos carrying a combination of *Rosa26^{stopfloxEYFP}* and *Wnt1::Cre* alleles (Wt) (**a, c**) or carrying the *Rosa26^{stopfloxEYFP}* on a *Sox4^{ΔWnt1} Sox11^{ΔWnt1}* background (Dko) (**b, d**). Nuclei are counterstained with DAPI (blue). **e, f** High-magnification confocal images of cardiac neural crest cells (green) at 10.5 dpc taken from the wild-type (**e**) or age-matched *Sox4^{ΔWnt1} Sox11^{ΔWnt1}* (**f**) embryos. *Wnt1::Cre*-dependent activation of EYFP from the *Rosa26^{stopfloxEYFP}* allele was used to label cells of the neural crest lineage. Lower panels show even higher magnification of single cells

shown in **e, f**. Arrowheads mark filopodia between neural crest cells. Scale bars 100 μm (**a–d**), 15 μm (**e, f**). **g–i** Quantification of the total number of cardiac neural crest cells per section (**g**), the TUNEL-positive apoptotic fraction (**h**), and the Ki67-positive proliferating fraction (**i**) at 10.5 and 13.5 dpc in outflow tract regions of embryos carrying a combination of *Rosa26^{stopfloxEYFP}* and *Wnt1::Cre* alleles (Wt, black bars) or carrying the *Rosa26^{stopfloxEYFP}* on a *Sox4^{ΔWnt1} Sox11^{ΔWnt1}* background (Dko, grey bars). At least four separate sections from three independent embryos were counted for each genotype. Data are presented as mean ± SEM. No statistically significant differences were observed between the two genotypes

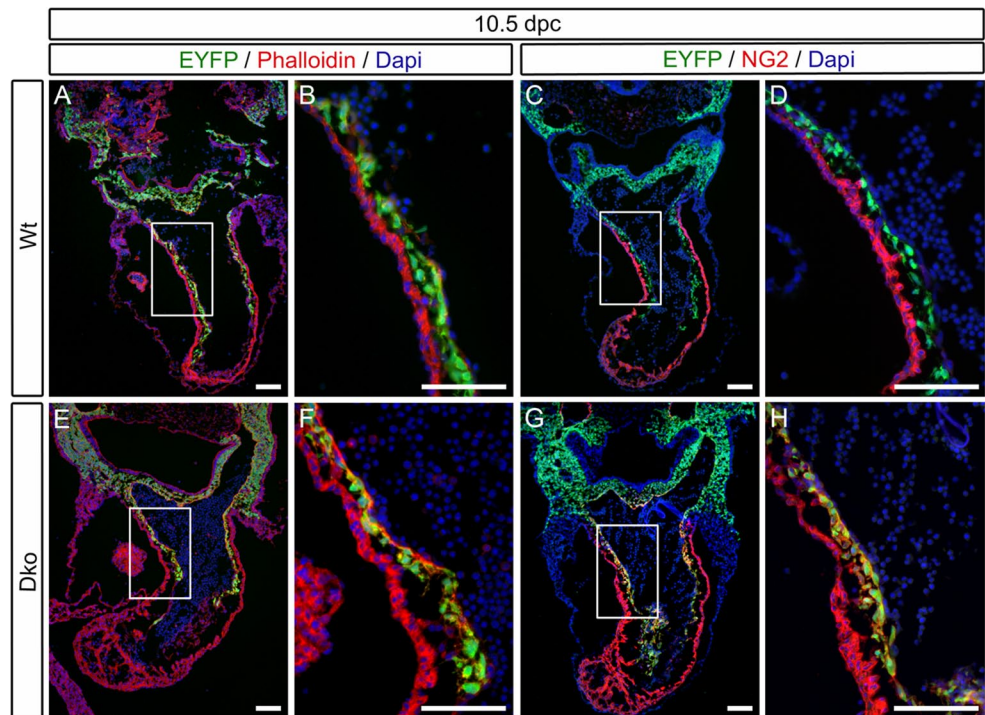
the SoxC subgroup (Fig. 8a). It thus seems that there is no substantial upregulation of the remaining SoxC member in an attempt to compensate the loss of Sox4 and Sox11.

SoxC proteins directly influence expression of *E-cadherin* and *Adam19*

To analyze whether *E-cadherin* and *Adam19* were direct target genes of SoxC proteins, we searched for the presence of potential Sox binding sites in the respective promoter regions and detected one site in the proximal *Adam19* promoter, whereas the proximal *E-cadherin* promoter contained two such sites (Fig. 8e, f). Electrophoretic mobility shift analysis revealed that Sox4 as well as Sox11 recognized these sites (Fig. 8b–d and data not shown). Considering that all oligonucleotide probes

were labeled with similar efficiencies and that similar results were obtained with extracts from transfected HEK293 and Neuro-2a neuroblastoma cells as protein source, binding of both proteins to the *Adam19* site appeared stronger than to the two *E-cadherin* sites (compare Fig. 8b, c to d). HEK293 cells were chosen because of their widespread use for protein production, while Neuro-2a cells were chosen because of their ontogenetic relationship and similarity to neural crest cells. Sox4 and Sox11 also activated both promoters in luciferase assays upon co-transfection in Neuro-2a cells (Fig. 8g, h). For both genes, the proximal promoter containing the binding sites was at least as efficiently activated as a longer promoter fragment that additionally included more distal sequences indicating that the SoxC effect is indeed mediated by the proximal promoter region. In line with observations on other target gene promoters [25, 33],

Fig. 7 Analysis of marker gene expression of cardiac neural crest in mice with neural crest-specific SoxC gene ablations. Co-immunohistochemistry was performed at 10.5 dpc on embryos carrying a combination of *Rosa26^{stopfloxed-EYFP}* and *Wnt1::Cre* alleles (Wt) (a–d) or carrying the *Rosa26^{stopfloxed-EYFP}* on a *Sox4^{ΔWnt1} Sox11^{ΔWnt1}* background (Dko) (e–h). Antibodies directed against EYFP were used to detect cells of neural crest origin (green). Actin microfilaments were detected by fluorescently labeled phalloidin (a, b, e, f) and NG2 expression by specific antibodies (c, d, g, h) (both in red). Nuclei were counterstained with DAPI (blue). The area shown at higher magnification in b, d, f, h is boxed in a, c, e, g. Scale bars 100 μm



Sox11 activated luciferase expression more efficiently than Sox4. Whether this higher activity in vitro is causally related to the higher impact of Sox11 on cardiac neural crest cell development remains to be studied in future experiments.

To further prove that promoter activation is a direct consequence of binding to these regions, we first introduced mutations in both *Adam19* promoter and *E-cadherin* promoter that prevented SoxC proteins from binding to the identified sites (Fig. 8b, c, d). As a consequence, activation of both promoters by either Sox4 or Sox11 was strongly diminished (Fig. 8g, h). Additionally, we studied by ChIP whether transfected Sox11 would associate with the endogenous *E-cadherin* and *Adam19* promoters in HEK293 and Neuro-2a cells. Both promoters were significantly enriched in chromatin precipitated with antibodies directed against Sox11 as compared to precipitates with control immunoglobulins in HEK293 cells (Fig. 8i, j) as well as in Neuro-2a cells (Fig. 8k, l). Enrichment was furthermore only observed for the promoter regions when cells were transfected with Sox11 before ChIP, and could not be obtained for other more distally located regions from the respective gene loci (Fig. 8i–l). Intriguingly, both promoters were also specifically precipitated from chromatin that was prepared from whole hearts or anterior heart poles of 13.5-dpc-old embryos as tissues that included the outflow tract region (Fig. 8m, n). Thus it appears very likely that the influence of SoxC proteins on both *E-cadherin* and *Adam19* is direct and occurs in cardiac neural crest cells.

Discussion

Congenital heart defects represent the most common type of birth defect in humans and occur with a frequency of 10 in 1,000 with three of them requiring early postnatal intervention [1]. Many of these defects represent or include malformations of the outflow tract. This region is formed by mesodermal cells from the second heart field, cardiac neural crest cells, and endothelial cells that cooperate and reciprocally influence each others' development [4].

Over the years, a number of transcription factors have been identified that are required for outflow tract formation [5]. How these factors work has only been addressed for some, and turns out to be complicated, as transcription factors may be active in any of the cell types that participate in outflow tract formation and may affect any of the numerous processes that these cells have to undergo before or while becoming part of the outflow tract [34].

Since constitutive deletion of *Sox4* or *Sox11* leads to outflow tract defects [6, 7], our first aim was the identification of the affected cell type. Our analysis clearly shows that Sox11 is required in both cardiac neural crest and mesodermal cells. The observed phenotype corresponded in all cases to DORV. Interestingly, simultaneous deletion of *Sox11* in neural crest as well as mesodermal cells led to increased phenotypic severity as a substantial number of embryos now manifested with CT instead of DORV. These findings raise the intriguing possibility that Sox11 may be involved in the reciprocal signaling that occurs between these cell types during outflow tract formation.

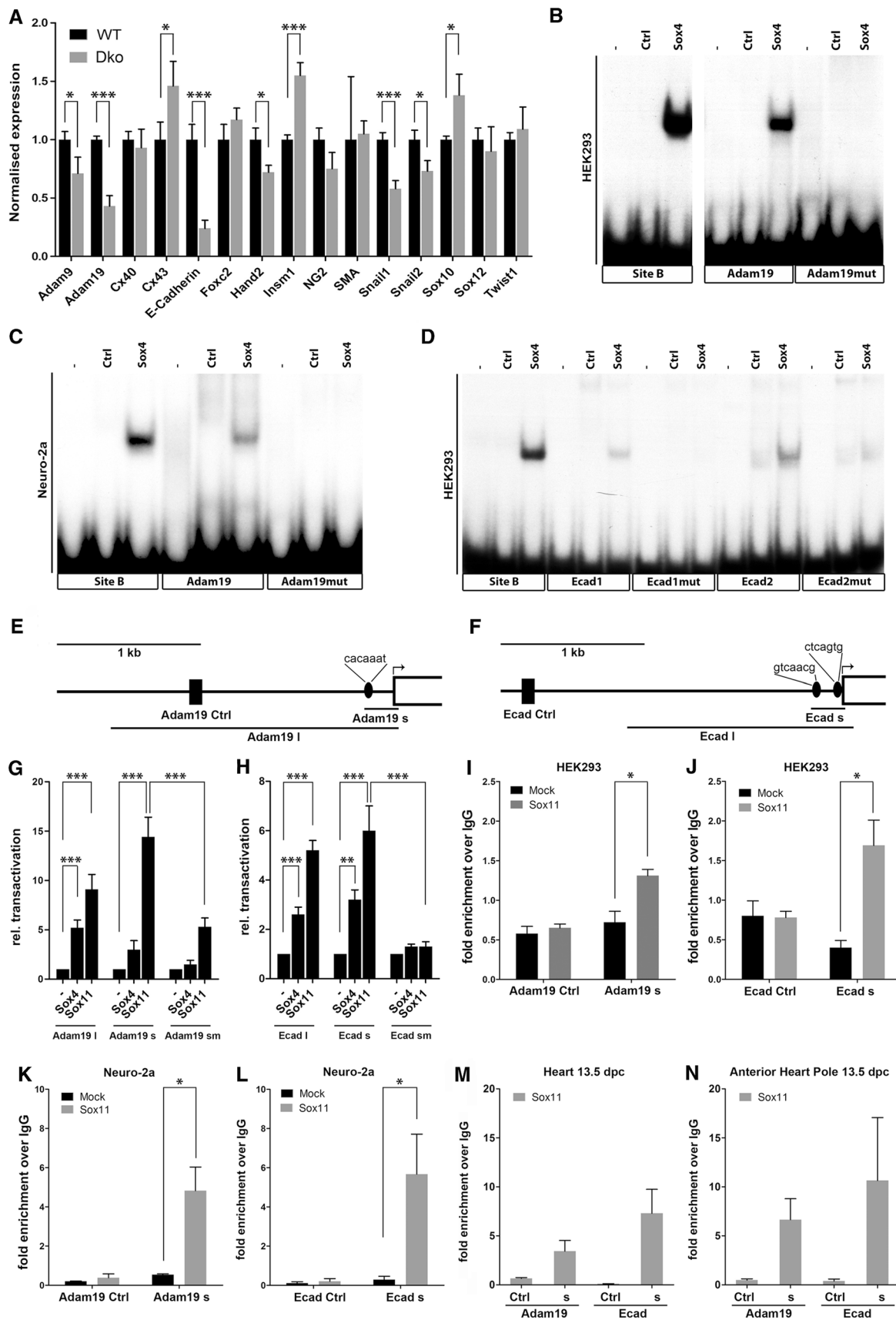


Fig. 8 Analysis of SoxC target genes in neural crest cells. **a** Expression levels were determined by quantitative RT-PCR on RNA prepared from the anterior heart pole of wild-type (Wt, *black bars*) and *Sox4* ^{Δ Wnt1} *Sox11* ^{Δ Wnt1} (Dko, *grey bars*) embryos at 13.5 dpc for several genes as indicated below the bars. Transcript levels were normalized to *Rpl8* levels in the respective samples. For each RNA source, RT-PCRs were performed on at least three independent samples. **b–d** Electrophoretic mobility shift assays with oligonucleotides encompassing potential Sox binding sites from the *Adam19* (**b**, **c**) and *E-cadherin* (**d**) promoters and the known Sox binding site B from the *Mpz* promoter. For each confirmed binding site, a wild-type (*Adam19*, *Ecad1*, *Ecad2*) and a mutant (*Adam19mut*, *Ecad1mut*, *Ecad2mut*) version were used. Oligonucleotides were incubated in the absence (–) or presence of protein extracts from mock-transfected cells (Ctrl) or cells expressing a carboxyterminally truncated Sox4 protein (*Sox4*) as indicated above the lanes. Both HEK293 (**b**, **d**) and Neuro-2A cells (**c**) were used. **e**, **f** Schematic representation of *Adam19* and *E-cadherin* upstream regions. Location of the short (*Adam19 s*, *Ecad s*) and long (*Adam19 l*, *Ecad l*) promoter regions used for luciferase assays as well as position of Sox binding sites (*ellipses*), transcriptional start sites (*arrows*) and control regions (*Adam19 Ctrl*, *Ecad Ctrl*, *black boxes*) for ChIP are indicated. The exact binding site sequences are listed above the ellipses. *Scale bar*, 1 kb. **g**, **h** Transient transfections were performed in Neuro-2a cells with luciferase reporters under control of the *Adam19* (**g**) or *E-cadherin* (**h**) promoters either in a short version encompassing the proximal promoter (274 bp for *Adam19* and 238 bp for *E-cadherin*) or a long version including additional upstream sequences (2,040 bp for *Adam19* and 1,600 bp for *E-cadherin*). Both proximal promoters were furthermore employed in the wild-type version and as a variant with inactivated Sox binding sites (*Adam19 sm*, *Ecad sm*). Empty pCMV5 expression plasmids (–) or expression plasmids for Sox4 and Sox11 were co-transfected as indicated below the bars. Luciferase activities were determined in ten experiments, each performed in triplicates. The luciferase activity obtained for a promoter in the absence of ectopic transcription factor was arbitrarily set to 1. Fold inductions in the presence of transcription factors are presented as mean \pm SEM. **i–n** ChIP was performed on chromatin from HEK293 cells (**i**, **j**), Neuro-2a cells (**k**, **l**), whole hearts (**m**), and anterior heart pole tissue (**n**) from mouse embryos at 13.5 dpc. In case of HEK293 and Neuro-2a cells, chromatin was prepared from both untransfected (mock, *black bars*) and Sox11-transfected (*Sox11*, *grey bars*) cells. Precipitation was with antibodies directed against Sox11 and control IgGs. Quantitative PCRs were carried out on immunoprecipitated chromatin to determine the relative enrichment of proximal promoters (*Adam19 s*, *Ecad s*) and upstream control regions (*Adam Ctrl*, *Ecad Ctrl*) from *Adam19* (**i**, **k**, **m**, **n**) and *E-cadherin* (**j**, **l**, **m**, **n**) gene loci in the Sox11 precipitate over control. Experiments were performed for five biological replicates in **i–l**, for three in **m**, and for two in **n** with each PCR in triplicate. Fold enrichment of PCR products from Sox11-precipitated chromatin over IgG-precipitated chromatin is presented as mean \pm SEM. Differences between samples in **a**, **g**, **h**, **i**, **j**, **k**, **l** were statistically significant as indicated according to Student's *t* test ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$). No statistical analysis was performed for **m** and **n** because of the low sample number

At least in the neural crest, Sox11 is furthermore supported in its function by the related Sox4 as only the combined deletion of both SoxC factors led to a fully penetrant outflow tract defect. Whereas neural crest-specific deletion of *Sox11* led to an incidence of outflow tract defects of 8 %, the additional deletion of Sox4 increased rates to 100 %. Both factors thus function redundantly in the cardiac neural

crest as previously seen in central nervous system and other organs throughout the developing embryo [8, 10]. From our results, we furthermore conclude that the impact of Sox11 on cardiac neural crest and mesodermal development is stronger than the one of Sox4.

Considering that the outflow tract defect in constitutive Sox4-deficient mice is more severe than the one in constitutive Sox11-deficient mice [6, 7], we were surprised that even the combined deletion of Sox4 in cardiac neural crest and mesoderm remained phenotypically inapparent. We do not think that this is due to trivial reasons, as Cre-dependent deletion of the floxed *Sox4* allele was rapid and near complete in targeted cell types and as combination of the same allele with a pan-epiblast Cre deleter yielded the same phenotype previously observed in constitutive *Sox4* null mutants. However, it is conceivable that the Sox4 requirement for outflow tract formation is based on its action in a cell type that was not hit by our deletion strategy, or at a time at which the applied Cre recombinases were not yet active. However, considering that *Wnt1::Cre* is active already very early throughout the neural crest [13], and that we obtained the same phenotype when the earlier deleting AP2 $\alpha::Cre$ [24] was applied, it appears unlikely that *Sox4* deletion in the neural crest occurred too late. The same can be argued for the heart mesoderm, as *Nkx2.5::Cre* expression sets in very early and efficiently [14]. One option therefore is that the outflow tract defect observed in constitutive Sox4-deficient mice results from effects on neuroectodermal or mesodermal development that precede neural crest or heart field formation. Alternatively, Sox4 effects on neural crest or second heart field mesoderm may be indirect and not cell-autonomous. It could for instance be envisaged that Sox4 acts in cells that provide essential cues for the second heart field or the cardiac neural crest. Pharyngeal endoderm cells are an interesting candidate as they have been shown in the past to influence outflow tract development and septation through multiple mechanisms [35, 36]. Future experiments will have to clarify this issue.

The second aim of our study was to understand how SoxC transcription factors act molecularly in the cardiac neural crest. Our studies revealed that cardiac neural crest cells deficient for both Sox4 and Sox11 emigrated normally from the neural tube. We furthermore failed to detect any gross abnormalities during the migratory phase. Rates of proliferation and apoptosis were comparable to the wild type so that normal numbers of cardiac neural crest cells reached the outflow tract region at appropriate times. We therefore conclude that SoxC factors are not required for the generation, multiplication, maintenance, or migration of the cardiac neural crest.

The changes we observed occurred after the cardiac neural crest cells had entered the outflow tract region. SoxC proteins thus affect later stages of cardiac neural

crest development. Alterations included different morphology and arrangement of cardiac neural crest cells relative to each other. Less filopodial contacts were observed between cells and their chain-like arrangement was lost so that they no longer appeared as a single cell layer along the outflow tract. This went along with alterations in cytoskeletal organization as evident from phalloidin stainings. Similar alterations in packing and morphologic appearance of cardiac neural crest cells with associated cytoskeletal alterations have been observed in neural crest-specific *Disheveled* mutants arguing that SoxC function in the cardiac neural crest may be related to non-canonical Wnt signaling and the planar cell polarity pathway [37]. Further abnormalities included a precocious expression of the pericyte marker and proteoglycan NG2 in these cells as well as reduced *E-cadherin* and *Adam19* expression. For *E-cadherin* and *Adam19*, the SoxC effect appeared to be direct. A previous study had identified *Cx43* as another direct target for SoxC proteins in the heart [38]. Although we were unable to detect a reduction of *Cx43* expression in mice with neural crest-specific SoxC deletion (likely because *Cx43* is expressed in mesodermal cells as well), it supports the notion that SoxC proteins influence a number of genes that are upregulated during differentiation and determine the interaction of cardiac neural crest cells among each other and with their environment. In the absence of SoxC proteins the differentiation program is not properly implemented and interactions are dramatically altered.

During neuronal differentiation, SoxC proteins are required for the timely establishment of neuronal properties and maturation [26, 39]. During this process, SoxC proteins affect neuronal morphology [40, 41] and influence expression of several cytoskeletal proteins including β 3-Tubulin and Doublecortin [26, 27, 42]. In analogy, SoxC proteins may coordinate the timely differentiation of cardiac neural crest cells in the outflow tract region. The role of Sox4 and Sox11 in the cardiac neural crest appears to occur later than the action of most other transcription factors that have been identified so far. These are either already active at the premigratory stage and required for neural crest cell emigration or expansion of the precursor cell pool such as SIP1 and Pax3 [43, 44], or they support survival and proper interaction with surrounding tissues during the migratory phase such as Foxc1, Foxc2, and AP2 α [45, 46]. They may also direct early specification decisions in the postmigratory phase such as Twist1 [47]. While this argues that SoxC proteins may fulfill the function of downstream effectors, future experiments will have to define the exact ways in which Sox4 and Sox11 are activated by and interact with other transcription factors known to be relevant for outflow tract formation.

Acknowledgments Jürgen Behrens and Marina Kreutz are acknowledged for the gift of *Ecadherin* and *Adam19* plasmids,

respectively. This work was supported by a grant from the DFG to E.S. (So251/3-1) and from a National Health and Medical Research Council Australia Fellowship to RPH (573705).

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