CHARLES UNIVERSITY IN PRAGUE

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Developmental and Cell Biology

The role of transcriptional factor Tcf7l1 and Wnt/β-catenin signaling pathway during differentiation of the head ectoderm.

Ph.D. Thesis

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Declaration

I declare that I wrote the thesis independently and that I cited all the information sources and literature. This work was not presented to obtain another academic degree or equivalent.

Prague, 10.2.2016   Jan Mašek

Acknowledgments

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List of abbreviations

4-OHT	4-hydroxitamoxifen
aa	amino acid
ANF	anterior neural fold
ANF	anterior neural fold
A-P	anterio-posterior
APC	adenomatous polyposis coli
APC	adenomatous polyposis coli
Axin	axis inhibition
Axud	Axin-1 up-regulated gene protein
BAT-gal	β-catenin activated transgene driving expression of nuclear β-galactosidase reporter
Bcl	B-Cell CLL/Lymphoma
BMP	bone morphogenic protein
BrDU	5-Bromo-2'-deoxyuridine
Brna3a	POU class 4 homeobox 1
BSA	bovine serum albumin
CBP	CREB binding protein
Cdkn1a	cyclin dependent kinase inhibitor 1
CK	casein kinase
cMaf	V-maf musculoaponeurotic fibrosarcoma oncogene homolog
c-Myc	avian myelocytomatosis viral oncogene homolog
CNS	central nervous system
C-term	carboxy-terminus
Dkk	dickkopf
Dlx	distal-less homeobox
Dvl	dishevelled
Eya	eyes absent
EYFP	enhanced yellow fluorescent protein
FGF	fibroblast growth factor
FLP	LoxP-flippase
Fox	forkhead box
Fzd	frizzled

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Gbx	gastrulation brain homeobox
GRG	groucho-related gene
GRN	gene regulatory network
GSK3	glycogen synthase kinase 3
H&E	hematoxylin & eosin
H3-K4me3	tri-methylation of lysine 27 on histone H3
Hesx	homeobox expressed in ES cells
ICAT	inhibitor of β-catenin and Tcf
Irx	iroquois homeobox
Lef	lymphoid enhancer-binding factor
LoxP	locus of crossover in P1
Lrp	low density lipoprotein receptor-related protein
MEF	mouse embryonic fibroblast
Meis	myeloid ecotropic viral integration site homeobox
MHB	midbrain-hindbrain boundary
MLL	methyltransferases mixed-lineage-leukemia
MO	morpholino
NC	neural crest
Neo	neomycine resistance
NeuroD	neuronal differentiation
NNE	non-neural ectoderm
NPB	neural plate border
Oct	octamer-binding protein
Otx	orthodenticle homeobox
p21	protein 21
p57	protein 57
Pax	paired box
PBS	phosphate buffer solution
PCNA	proliferating cell nuclear antigen
PFA	paraformaldehyde
PGK	phosphoglycerate kinase promoter
PP	protein phosphatase
PPR	pre-placodal region

Prox	prospero homeobox
ROSA	reverse orientation splice acceptor
Rx	retina and anterior neural fold homeobox
SFRP	secreted frizzled-related protein
Six	sine oculis homeobox
Sox	SRY(Sex Determining Region Y)-Box
Tcf	T Cell-specific factor
Tfap2α	transcription factor AP-2 Alpha
TLE	transducin-like enhancer of split
Wif	Wnt inhibitory factor
Zic	zinc finger protein of the cerebellum
β-TrCP	F-box containing β-transducin repeat containing

Abstract (English)

Differentiation of the head ectoderm is crucial for the evolutionary diversification of vertebrates. Expression of the genes responsible for this process is orchestrated troughout complex gene regulatory networks that are induced and modulated by Wnt, FGF and BMP signaling pathways. In addition, Wnt/β-catenin signaling, in combination with expression of the Wnt antagonists from the rostral-most part of the head ectoderm, represent a key source of information for the regionalization of the tissue along the antero-posterior axis. This allows the differentiation of the anterior ectoderm that gives rise to the anterior neural fold (ANF) and anterior part of the presumptive placodal region (PPR), and more posterior ectoderm where higher levels of active Wnt/β-catenin signaling promote differentiation into the neural crest (NC) and posterior PPR. Although the requirement of Wnt/β-catenin signalling for ANF, PPR and NC development has been intensively studied in non-mammalian vertebrate model organisms, we lack a clear picture about the situation in mammals. Furthermore, current knowledge in mammals has been gathered via experiments on the level of β-catenin and very little is known about the individual roles of the Tcf/Lef transcription factors. Thereby, we decided to manipulate the Tcf7l1, member of the Tcf/Lef family with unique ability to act as a strong transcriptional repressor even in the presence of β-catenin. Through conditional deletion of the *Tcf7l1* gene in neural plate border of developing mouse embryo, we managed to describe function of the Tcf7l1 protein as well as the role of the Wnt/β-catenin signaling in the process of mammalian head ectoderm specification. Absence of Tcf7l1 led to aberrant activation of the Wnt/β-catenin signaling in the anterior neuroectoderm and its conversion into the NC. As a result, cell populations of the developing prosencephalon and anterior PPR were restrained. Importantly, this phenotype was not accompanied by changes of the anterior-posterior neural character. Thus, Tcf7l1 defines the border between the region of prospective forebrain and anterior placodes on one hand and the neural crest on the other, via restriction of the Wnt/β-catenin signaling gradient.

Abstrakt (Czech)

Jedním z klíčových momentů v evoluci obratlovců bylo bezpochyby rozrůznění funkcí hlavového ektodermu. Genové regulační sítě využívané během tohoto procesu, jsou tvořeny množstvím genů jejichž exprese je spouštěna, a dále modulována pomocí signalizačních drah Wnt, FGF a BMP. K tomu je třeba přičíst i vliv signalizace Wnt/β-kateninu a jejích antagonistů přítomných v rostrální části hlavového ektodermu, kteří jsou klíčoví pro regionalizaci tkání v rámci anterio-posteriorní osy embrya. Kombinace těchto faktorů umožnila diverzifikaci anteriorního ektodermu na anteriorní neurální val (ANV) a rostrální preplakodální oblast (PPO), zatímco v posteriornějším ektodermu vznikají působením signální dráhy Wnt buňky neurální lišty (NL) a posteriorní PPO. Přestože byl vliv signalizační dráhy Wnt/β-katenin detailně studován v kontextu vývoje ANV, PPO a NL u nesavčích modelových organizmů, její vliv na vývoj těchto tkání u savců je stále nejasný. Navíc bylo současných poznatků u savců dosaženo pomocí experimentálních zásahů na úrovni β-kateninu, v důsledku čehož zůstává úloha transkripčních faktorů z rodiny Tcf/Lef v těchto procesech takřka neznámá. Z těchto důvodů jsme se rozhodli sudovat transkripční represor *Tcf7l1*, který je členem rodiny Tcf/Lef a vyznačuje se schopností potlačovat genovou expresi i za přítomnosti β-kateninu. Pomocí cílené delece genu *Tcf7l1* v oblasti lemující neurální ploténku se nám podařilo objasnit funkci proteinu Tcff7l1 během specifikace hlavového ektodermu a zároveň charakterizovat význam, který při tomto procesu hraje signalizační dráha Wnt/β-katenin. Nepřítomnost proteinu Tcf7l1 vedla k zvýšené aktivaci signalizace Wnt/β-katenin v buňkách předního neuroektodermu, která měla za následek jejich přeměnu v buňky NL. Tento zásah negativně ovlivnil vývoj buněčných populací ANV a PPE. Je důležité zmínit, že embrya s tímto fenotypem nevykazovala změny v anterio-posteriorním charakteru neurální tkáně. Souhrnem lze říct, že Tcf7l1 definuje hranici mezi budoucím předním mozkem a anteriorními plakodami na jedné straně a neurální lištou na straně druhé, a to pomocí restriktivní kontroly gradientu aktivity signalizační dráhy Wnt/β-katenin.

Introduction

Wnt/β-catenin signaling pathway

The first mammalian gene coding for Wnt ligand was identified more than 30 years ago (Nusse and Varmus, 1982) and since then the Wnt signaling pathway became one of the most extensively studied phosphorylation cascade in general. The term "Wnt signaling" does not imply a single biochemical signal transduction pathway. Rather, it refers to a collection of signaling cascades triggered by Wnt ligand-receptor interactions that can affect key cellular processes: cell polarity, movement, proliferation, differentiation, survival and self-renewal. Due to its important role in maintaining homeostasis during adulthood, deregulation of the Wnt signaling activity is often connected with carcinogenesis and therefore it is of great interest for the whole field of biomedical research (reviewed in Clevers and Nusse (2012); Holland et al. (2013); Krausova and Korinek (2013)). Wnt/β-catenin (also mentioned as Wnt Canonical pathway signaling) is being referred when the signal from phosphorylation cascade is integrated at the level of cytoplasmic β-catenin protein. Changes in the concentration of β-catenin subsequently affect the gene expression via transcription factors from the Tcf/Lef protein family.

Wnt/β-catenin signaling pathway occurs in all the phyla of the animal kingdom, including metazoan animals with primitive body plan such as *Trichoplax* from Placozoa (Srivastava et al., 2008). Despite various differences between individual species, the core mechanism of the signal transduction of the Wnt/β-catenin signaling pathway remains highly conserved. In the absence of the Wnt ligand, cytosolic β-catenin is marked for degradation by the so-called "destruction" complex of proteins, consisting of two serine/threonine kinases, namely casein kinase 1 alpha (CK1α) and glycogen synthase kinase 3 (GSK3) and two tumor suppressors, the axis inhibition (Axin) and the adenomatous polyposis coli (APC) proteins (Ikeda et al., 1998; Rubinfeld and Martin, 1993; Siegfried et al., 1994). As a result, N-terminally phosphorylated β-catenin is ubiquitinated by the F-box containing β-transducin repeat containing (β-TrCP) E3 ubiquitin protein ligase and subsequently degraded by the proteasome (Aberle et al., 1997; Kitagawa et al., 1999). Without nuclear β-catenin, Tcf/Lef proteins associate with the transcriptional repressors from the Groucho/transducin-like enhancer of split (TLE) family (Roose and Clevers, 1999; Roose et al., 1998) that block expression of Wnt-responsive genes.

When the Wnt ligand (19 in mammals) is present, it binds to a seven-pass-transmembrane receptor from the Frizzled family (11 in mammals) and its co-receptor

- low density lipoprotein receptor-related protein 5/6 (Lrp5/6) (Schweizer and Varmus, 2003). Ligand-receptor interaction triggers a complex cascade of intracellular events leading to the stabilization of the β-catenin. This process involves phosphorylation of the adaptor protein Disheveled (Dvl) by casein-kinase 1 γ/ε (CK1 γ/ε) (Bernatik et al., 2011). Additionally, the intracellular portion of the Lrp 5/6 is phosphorylated by CK1γ and GSK3, recruiting Axin from the cytosol to the intracellular part of the plasmatic membrane (Bilic et al., 2007). This can be further potentiated by additional phosphorylation of the PPPS/TP motif on the intracellular part of the Lrp5/6 by mitogen activating protein kinases (MAPKs) (Cervenka et al., 2011). Simultaneously, the Axin protein becomes dephosphorylated by the protein phosphatase 1 (PP1) (Kim et al., 2013; Luo et al., 2007). The subsequent change in its conformation prevents further phosphorylation, leading to β-catenin accumulation in the cytoplasm and its translocation to the nucleus (Kim et al., 2013) (Fig. 1).
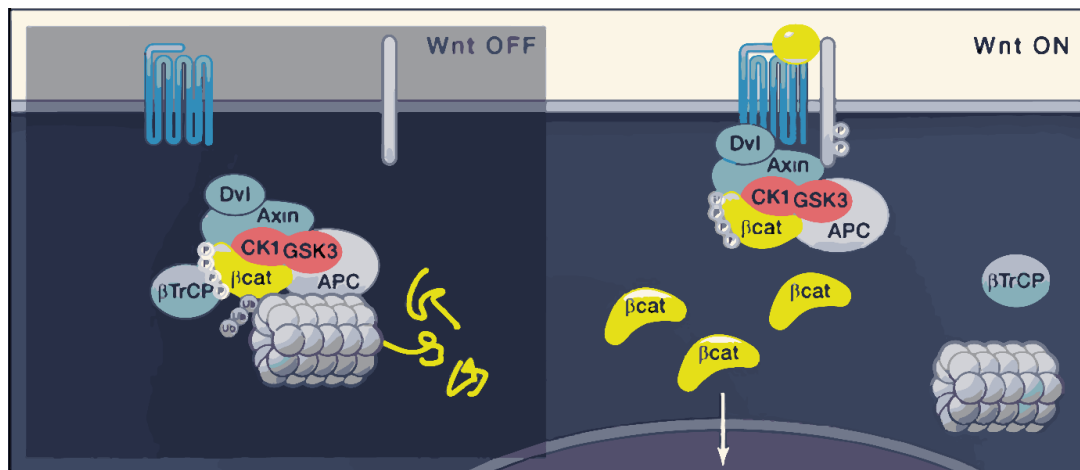
Figure 1: Model of the current view on the Wnt/β-catenin signaling pathway. Left: Without presence of the Wnt ligand β-catenin is constitutively marked by destruction complex for degradation in the proteasome. Right: Binding of the secreted Wnt proteins to their Frizzled receptors and Lrp5/6 co-receptors recruits the destruction complex to the plasmatic membrane, allowing stabilization of the β-catenin. Stabilized β-catenin accumulates in the cytoplasm and enters the nucleus where it binds to the Tcf/Lef family of transcription factors, thereby regulating the expression of Wnt target genes. (Adapted from Clevers and Nusse (2012)).

Wnt/β-catenin signaling in the nucleus and transcription factors Tcf/Lef

Nuclear β-catenin does not only compete out the Groucho/TLE corepressors from Tcf/Lefs, but its carboxy-terminus (C-term) acts also as a transcriptional activation domain (van de Wetering et al., 1997). Additionally, it recruits several modulators of transcription. Among these, B-Cell CLL/Lymphoma 9 (Bcl9) and Pygopus, were found to be indispensable for Wnt/β-catenin signaling in *Drosophila* (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002), however knock-out studies in mice suggest that these proteins do not play equally important role in mammals (Brack et al., 2009; Deka et al., 2010; Schwab et al., 2007). β-catenin also serves as a scaffold protein for histone modifiers, such as CREB binding protein (CBP) and its close relative p300, both linked to activation of Wnt target gene expression (Mosimann et al., 2009; Teo and Kahn, 2010). Presence of the CBP/p300 on Wnt target gene chromatin positively correlates with an increase in H3 and H4 acetylation (Kioussi et al., 2002; Parker et al., 2008; Sierra et al., 2006). Furthermore, active Wnt/β-catenin signaling causes an elevation of the H3-K4me3 at various target promoters (Blythe et al., 2010; Chen et al., 2010; Parker et al., 2008). The methyltransferases mixed-lineage-leukemia proteins (MLL1/2), catalyzing this modification, seems to be required for complete activation of several Wnt targets (Chen et al., 2010; Sierra et al., 2006) (Fig. 2) (Reviewed in Cadigan and Waterman (2012); Clevers and Nusse (2012); Mosimann et al. (2009)).
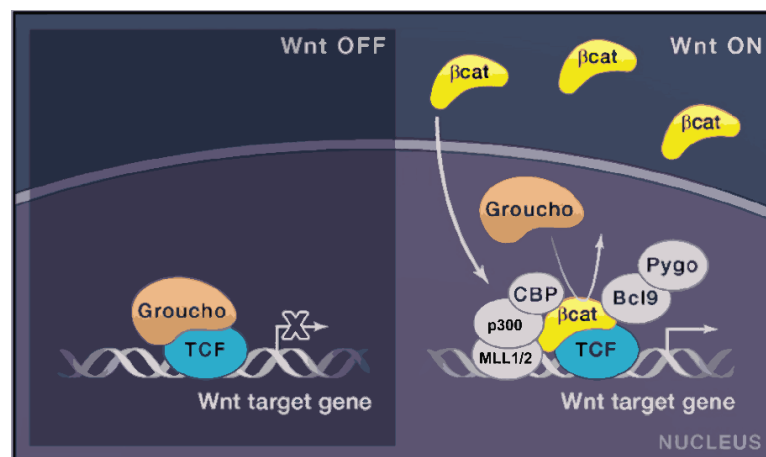
Figure 2: Model of the Wnt/β-catenin signaling in the nucleus. Left: Without presence of the β-catenin, Tcf/Lef proteins are bound by co-repressor Groucho/TLE and act as transcriptional repressors. Right: Binding of the β-catenin to the Tcf/Lef family of transcription factors prevents the binding of co-repressor Groucho/TLE, promotes the recruitment of chromatin remodeling proteins, transcription modulators and induces Tcf/Lef mediated expression of the Wnt target genes. (Adapted from Cadigan and Waterman (2012); Clevers and Nusse (2012)).

Four Tcf/Lef genes have been identified in vertebrates; *Lef1* (alias *Lef1*) (Travis et al., 1991), *Tcf7* (alias *Tcf1*) (van de Wetering et al., 1991), *Tcf7l1* and *Tcf7l2* (alias *Tcf3* and *Tcf4*) (Castrop et al., 1992). Tcf/Lefs share several characteristic protein features, such as the N-terminal β-catenin binding motif, Groucho-related gene (GRG) motifs for binding of Groucho/TLE co-repressors and the HMG box containing the DNA binding domain. The consensus TCF cognate motif for vertebrate and *Drosophila* Tcf/Lef is AGATCAAAGG. Moreover, Tcf7 and Tcf7l2 contain a C-clamp, i.e. a second DNA binding motif that further modulates gene target specificity (Arce et al., 2006; Cadigan and Waterman, 2012; van de Wetering et al., 1997).

Unlike invertebrates, where a single TCF is typically present, individual Tcf/Lefs in vertebrates have different, context-dependent potential to activate or repress target genes. In *in vivo* experiments in mouse and *Xenopus* Lef1 and Tcf7 identified as activators of the Wnt target genes (Galceran et al., 1999; Kratochwil et al., 2002; Liu et al., 2005; Reya et al., 2000), Tcf7l2 can act both as a repressor or an activator depending on the developmental context (Korinek et al., 1997; Liu et al., 2005; Nguyen et al., 2009; Tang et al., 2008), while Tcf7l1 represents the least potent activator but the strongest repressor from the whole family (Cole et al., 2008; Liu et al., 2005; Ombrato et al., 2012). Taken together, distinct expression patterns of individual Tcf/Lefs and their unequal capability to activate/repress Wnt target genes, represent the ultimate regulatory step of the Wnt/β-catenin signaling pathway.

Differentiation of the head ectoderm

Diversification of the developing epiblast cells into the three primary germ layers is one of the key developmental features of the Bilateria. It takes place during gastrulation (embryonic day E6.5 to E7.5 in mouse) and is directly followed by formation of the neural plate (E7.5-E8.5), and finally neurulation (E8.5-E9.25). During this time, emergence of the prospective head from the head ectoderm and underlying mesoderm takes place (Fossat et al., 2012). Correct differentiation of this region is achieved through differential gene expression orchestrated in complex gene regulatory networks (GRNs) that are responsive to stimuli from Wnt, FGF and BMP signaling pathways (Garnett et al., 2012). As a result, head ectoderm gives rise to four distinct cell populations: placodes, neural plate, non-neural ectoderm, and neural crest (NC) cells, that later form sensory organs; brain; epidermis; and sensory neurons, cartilage and bone of the skull, respectively (Fig. 3) (Groves and LaBonne, 2014; Litsiou et al., 2005; Simoes-Costa and Bronner, 2015; Steventon et al., 2009).
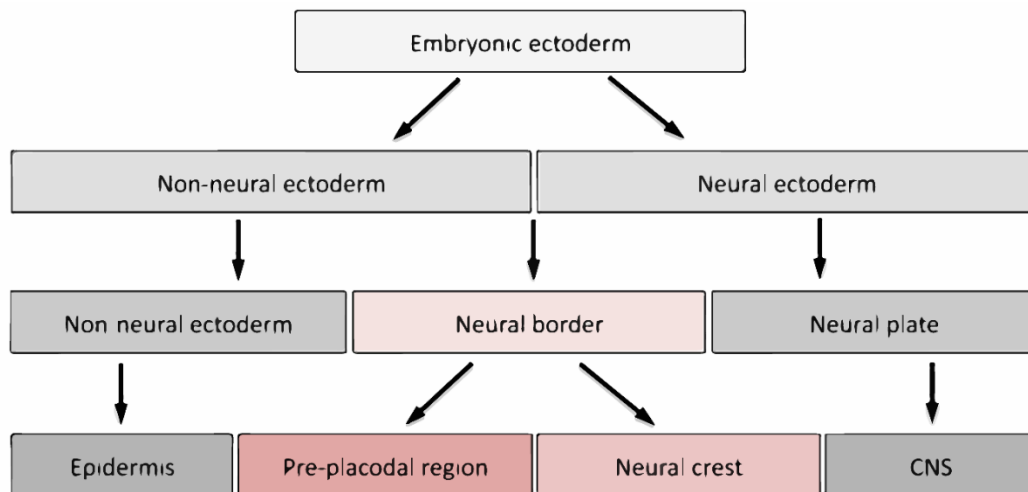
Figure 3: **Scheme of the embryonic ectoderm differentiation.** During gastrulation, embryonic ectoderm is subdivided into the non-neural ectoderm, later forming the epidermis, and the neural ectoderm, which gives rise to the central nervous system (CNS). A neural border zone appears at their boundary and gives rise to cell populations of the neural crest and pre-placodal region (PPR). Adapted from (Saint-Jeannet and Moody, 2014).

Anterior neural fold and Wnt/β-catenin signaling

The anterior neural fold (ANF), the most rostral region of the neural plate, serves as a regional organizer secreting signaling molecules that generate the spatial patterning of the forebrain. Wnt/β-catenin signaling controls A-P identity during body plan formation in most bilaterally symmetric animals, as has been demonstrated both in deuterostomes and protostomes (reviewed in (Petersen and Reddien, 2009). Unlike the rest of the body, the anterior patterning appears to proceed correctly only in the absence of the Wnt stimuli. Ectopic overactivation of the Wnt/β-catenin signaling pathway results in aberrant expansion of the posterior character of the embryonic tissue anteriorly, compromising or completely diminishing head formation, as has been demonstrated in mouse, zebrafish and *Xenopus* (Fossat et al., 2011; Kiecker and Niehrs, 2001; Kim et al., 2000; Popperl et al., 1997). On the contrary, depletion of the Wnt/β-catenin pathway showed the opposite phenotype i.e. expansion of the head neuroectoderm (Galceran et al., 1999; Kiecker and Niehrs, 2001; Shimizu et al., 2005). A-P gradient of the Wnt/β-catenin pathway activation can be nicely visualised using BAT-gal reporter mouse strain (Maretto et al., 2003) (Fig.

4). Collectively, tight control of the Wnt/β-catenin signaling activity is a key prerequisite for proper development of the ANF.
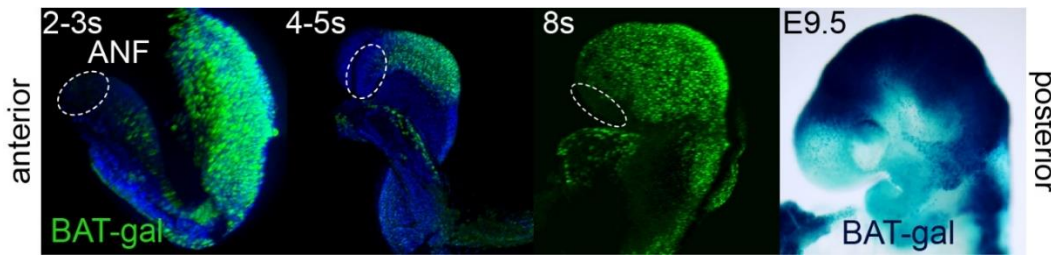
Figure 4: **Gradient of Wnt/β-catenin signaling in developing head of the mouse embryo.** With the progress of the embryogenesis, a wave of Wnt/β-catenin signaling activity proceeds from posterior towards anterior portion of the embryo, as can be visualized using Wnt reporter mouse strain BAT-gal (Maretto et al., 2003). The ANF region, however, stays devoid of the BAT-gal signal. More precise staging in somite pairs(s) has been used between E8.0-E8.5. (Adapted from Masek *et al.*,in revision) ANF – anterior neural fold (marked with dashed ellipse)

Wnt signaling in the ANF is restrained through expression of a set of Wnt antagonists, acting at different levels of the signal transduction cascade. Secreted molecules, including secreted frizzled-related protein (SFRP), Dickkopf-1 (Dkk1), Wnt inhibitory factor-1 (Wif1) or Cerberus, negatively regulate Wnt signaling activity by preventing the ligand-receptor binding (Kawano and Kypta, 2003). SFRPs diminish the signal activity presumably by competing with Wnt ligands for physical interaction with the Fzd receptor (Kawano and Kypta, 2003), while Dkk1 interferes with the interaction of Lrp5/6 co-receptor with Fzd and Wnt ligands, disrupting the activation of the receptor complex (Mao et al., 2001; Semenov et al., 2001).

The inhibitor of β-catenin and Tcf (ICAT), an 81aa protein, is another antagonist of the Wnt signaling pathway, acts in the nucleus, and intervenes in the interaction between β-catenin and Tcf/Lef (Tago et al., 2000). Whole body deletion of the *Dkk1* (Mukhopadhyay et al., 2001) and *ICAT* (Satoh et al., 2004) results in defects in A-P patterning and exencephaly, which results from aberrant Wnt/β-catenin signaling. Several other genes, such as *sine oculis homeobox 3* (*Six3*) (Lagutin et al., 2003) or *homeobox expressed in ES cells 1* (*Hesx1*) (Andoniadou et al., 2007), which are required for proper development of the ANF, are probably also involved in repression of the Wnt/β-catenin signaling, since their deletion results in upregulation of the Wnt reporter and/or increased expression of the Wnt target genes along with severe reduction of the ANF tissue. This further demonstrates distinct requirement of

the Wnt/β-catenin signaling during the ANF patterning and the A-P patterning of the whole body.

Role of the Wnt/β-catenin signaling in neural crest formation

Neural crest (NC) cells are vertebrate-specific, multipotent, highly migratory cells originating from the ectoderm of the neural plate border (NPB) (Fig. 3). NC cells possess the ability to differentiate into a wide range of cell types, including the bone and cartilage of the skull, sensory neurons, outflow tract of the heart, enteric ganglia and pigment cells. NC originate from a border region of the neural plate and surrounding non-neuronal ectoderm along the dorsal part of the embryo.

Experiments in *Xenopus*, zebrafish and chicken have shown a requirement for Wnt/β-catenin signaling even from the earliest steps of neural crest development. During gastrulation, Wnt/β-catenin signaling drives the expression of the NC specifier genes *paired box 3* (*Pax3*) (Bang et al., 1999; Garnett et al., 2012; Taneyhill and Bronner-Fraser, 2005; Zhao et al., 2014), *forkhead box D3* (*FoxD3*) (Janssens et al., 2013) and *gastrulation brain homeobox 2* (*Gbx2*) (Li et al., 2009). Later on, during NC maintenance (Kleber et al., 2005; Steventon et al., 2009) and the epithelial-to-mesenchymal transition (EMT), it triggers the *Slug* gene expression (Burstyn-Cohen et al., 2004; Sakai et al., 2005; Vallin et al., 2001). Compared to non-mammalian experimental models, no clear picture about the role of Wnt/β-catenin signaling in early NC cell populations in mammals is shaped. This is partly caused by a lack of appropriate Cre drivers for the early pre-migratory NC lineage. The widely used mouse strain Wnt1-Cre targets the NC population after its induction and specification (Danielian et al., 1998; Lewis et al., 2013). Depletion of β-catenin using Wnt1-Cre leads to malformations of the craniofacial structures, accompanied with a deficiency in sensory neurons (Brault et al., 2001; Gay et al., 2015). Similar phenotypic changes have been observed in compound Wnt1/Wnt3a mutants that also show impaired formation of sensory neurons and defects in several bones and cartilage originating from the NC (Ikeya et al., 1997). Ectopic Wnt1-Cre driven expression of stabilized β-catenin (β-cat$^{Ex3fl/+}$) results in excessive differentiation of NC progenitors into sensory neurons expressing neuronal differentiation (NeuroD) and POU class 4 homeobox 1 (Brn3a) proteins, thus limiting multipotency of the NC cells (Lee et al., 2004). Collectively, these data confirm the requirement of Wnt/β-catenin signaling in NC maintenance and differentiation, nevertheless, they do not clarify the role of Wnts in NC induction and specification. The apparent inconsistency may be explained in two ways. Either the signaling pathway network governing NC induction and specification differs between mouse and other species (Barriga et al., 2015) or, more likely, the

Wnt1-Cre driver targets the already specified NC, thus not allowing the investigation of the earlier events of NC development.

Wnt/β-catenin signaling in placodal development

Patches of thickened ectoderm, called placodes, recruit originate from U-shaped domain of the head ectoderm that lines the outer edge of the anterior NPB. This domain, called pre-placodal region (PPR), is characterized by expression of members of the *six homeobox* (*six*), *eyes absent* (*eya*) and *distal-less homeobox* (*dlx*) gene families (Schlosser and Ahrens, 2004). At the onset of neurulation Pre-placodal region could be divided into the anterior and posterior PPR; the former gives rise to adenohypophyseal, olfactory, and lens placodes and the latter forms the cranial sensory ganglia (together with NC cells), lateral line (in fishes), epibranchial, and otic placodes (Reviewed in Grocott et al. (2012); Saint-Jeannet and Moody (2014)).

Similarly to the case of ANF and NC, spatio-temporal differenct levels of Wnt, FGF and BMP signaling activity are responsible for expression of specific sets of genes that define PPR induction, A-P polarization and later differentiation into individual placodal subtypes. Experiments in *Xenopus* (Ahrens and Schlosser, 2005; Pieper et al., 2012), zebrafish (Bhat et al., 2013), and chicken (Litsiou et al., 2005) revealed that high levels of FGF but low levels of BMP and Wnt/β-catenin signaling are necessary for expression of the genes o*rthodenticle homeobox 2* (*otx2*), *paired box 6* (*pax6*), *six3, six6* that define anterior PPR. On the other hand *gbx2* and *iroquois homeobox* (Irx1-3) positive posterior PPR fate is induced by combination of high levels of the FGF and Wnt/β-catenin but low BMP signaling during onset of neurulation (Ahrens and Schlosser, 2005; Schlosser and Ahrens, 2004; Steventon et al., 2012).

Far less is known about involvement of the Wnt/β-catenin signaling during placodal development in mammals. Conditional loss of β-catenin using Pax2-Cre results in the otic placode size reduction while activation of the pathway through aberrant stabilization of the β-catenin, using the same cre-line, causes the opposite phenotype (Ohyama et al., 2006). The different size of the otic placode, is accompanied with changes in expression of the *Dlx5*, *Pax8*, results of the changes in ratio of ectodermal differentiation into placodal and epidermal fates (proliferation and apoptosis were not affected) (Ohyama et al., 2006). Conversely, β-catenin deletion in lens placode using the LR-Cre, where three copies of Pax6 lens enhancer were cloned upstream of the Cre gene (Kreslova et al., 2007; Smith et al., 2005), results in formation of ectopic lenses (Machon et al., 2010). β-catenin stabilization, using the same driver of recombination, prevents the lens placode formation through

downregulation of the lens specifiers Meis2 and Pax6 expression (Machon et al., 2010). Transcription factor *Pax6* activates expression of the Wnt antagonists *Dkk1* and *Sfrp2*, thus its absence further potentiates the phenotype in Lens-Cre;β-cat$^{Ex3fl/+}$ mutants (Machon et al., 2010). Based on additional studies focusing on NC development in other vertebrate model organisms, we can conclude, that Wnt inhibition through the soluble antagonists secreted from the cells of the anterior neural plate and underlying mesoderm during ANF development, such as Dkk1, Sfrps, and Cerberus, is also important for the anterior PPR formation in mouse (Carmona-Fontaine et al., 2007; Kawano and Kypta, 2003; Litsiou et al., 2005; Monaghan et al., 1999). Figure 5 summarizes the effects of Wnt/β-catenin signaling pathway on differentiation of the head ectoderm, with focus on ANF, NC, PPR regions and genes involved in their specification.
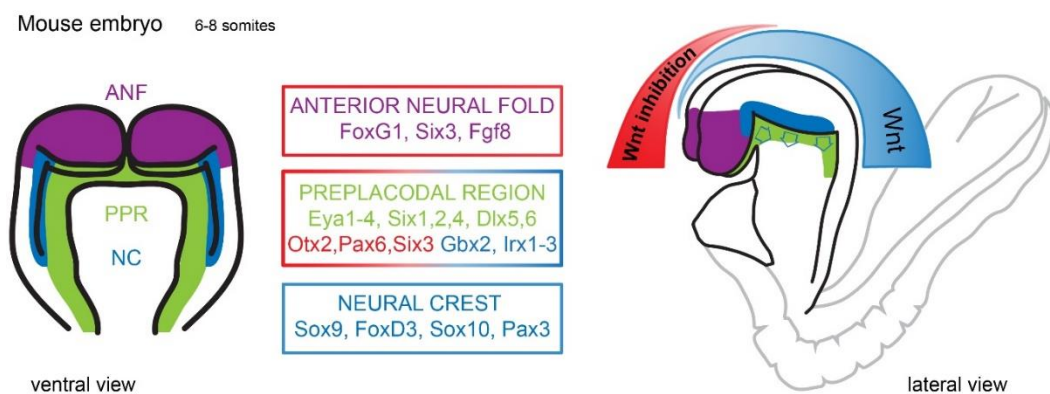
Figure 5: Schematic overview of the embryonic head ectoderm differentiation in relation to Wnt/β-catenin signaling. Ventral (left) and lateral (right) view of the head region of the whole developing mouse embryo at 6-8 somite stage. A→P gradient of the Wnt antagonists promotes differentiation of the most anterior ectoderm into *foxg1*, *six3*, *fgf8* expressing ANF and *otx2*, *pax6* and *six3* anterior PPR. A←P gradient of the active Wnt/β-catenin signaling induces formation of the *gbx2*, *irx1-3* positive posterior PPR and *pax3*, *sox9*, *foxd3* and *sox10* expressing NC.

ANF-anterior neural fold, PPR-preplacodal region, NC- neural crest

Tcf7l1: a potential regulator of the Wnt activity during mouse embryonic development

As mentioned above, Tcf7l1 represents the least potent activator but the strongest repressor from the whole family (Cole et al., 2008; Liu et al., 2005; Ombrato et al., 2012). This has been verified *in vivo*, using genetically modified mice that express only the N-terminally truncated form (lacking β-catenin binding domain) of the Tcf7l1. The mutant mice display defective eyelid closure and develop only four fingers, they do not, however, show any defects in differentiation of the anterior ectoderm or A-P patterning in general (Wu et al., 2012). Tcf7l1 deficient mice, on the other hand, die shortly after gastrulation displaying elevated levels of the Wnt/β-catenin signaling

activity, duplicated axis and a severely reduced anterior part of the embryo (Merrill et al., 2004), suggesting that Tcf7l1 function lays predominantly in repression of the Wnt/β-catenin signaling.

The repressor activity of Tcf7l1 has been shown to be crucial for maintaining the balance between pluripotency and differentiation in various stem cells. For instance in ES cells, Tcf7l1 reduces the levels of transcription factors responsible for pluripotency, such as Oct4, Sox2 and Nanog (Cole et al., 2008; Cole and Young, 2008). In Tcf7l1 mutant ES cells (or after *tcf7l1* mRNA knockdown), Nanog expression is increased and self-renewal of ES cells is enhanced at the expense of cell differentiation. This phenotype can be rescued using small molecular inhibitors of the Wnt/β-catenin pathway (Pereira et al., 2006; Tam et al., 2008; Yi et al., 2008).

During mouse embryogenesis, Tcf7l1 is expressed in the anterior ectoderm at E7.5 and the ANF at E8.5 (Korinek et al., 1998; Merrill et al., 2004). Tcf7l1 inactivation seems to allow spreading of Wnt/β-catenin signaling towards the anterior neural fold in both zebrafish and mouse. This leads to a deficiency in expression of the ANF specific genes accompanied with severe defects in head formation (Andoniadou et al., 2011; Dorsky et al., 2003; Kim et al., 2000). All these data have been interpreted in a manner consistent with the fact that repression of Wnt signaling in the ANF is required for A-P axis determination of the neural tube. However, complete understanding of on the role of Tcf7l1 in the differentiation of the mammalian head ectoderm is still missing.

Aims of the study

Although the requirement of Wnt/β-catenin signalling for ANF, PPR and NC development has been studied intensively in non-mammalian vertebrate model organisms, we lack a clear picture about the situation in mammals. Current knowledge was collected via experiments at the level of β-catenin and very little is known about the individual roles of the Tcf/Lef transcription factors. Therefore, we decided to abolish Tcf7l1, member of the Tcf/Lef family with unique ability to act as transcriptional repressor even in the presence of β-catenin. Through conditional deletion of the *Tcf7l1* gene in mouse, we would attempt to describe the function of both the Tcf7l1 and Wnt/β-catenin signaling in the context of the head ectoderm differentiation in mammals.

According to available data, the signaling pathway network governing NC induction and specification differs significantly between mouse and other species (Barriga et al., 2015). There is a high probability that this inconsistency is due to the wide usage of the Wnt1-Cre driver of recombination. Wnt1-Cre targets already specified NC and thereby is not suitable for studying early events of the NC formation (Danielian et al., 1998; Lewis et al., 2013). In order to investigate the role of Wnt/β-catenin signaling in the earliest possible steps of the NC development, we decided to conditionally delete Tcf7l1 using Ap2α-Cre line. This mouse Cre-expressing mouse strain was derived from a NC inducer gene Tfap2α and delivers recombination during NC induction (Macatee et al., 2003). Since the expression pattern of this Cre-line covers entire NPB (and therefore a significant part of the anterior ectoderm) resulting phenotype should provide information not only about the Tcf7l1 function in NC development, but also about its role during specification of the ANF and PPR.

Tight control of Wnt/β-catenin signaling is also essential for the formation of the vertebrate eye. In mouse, down-regulation of the Wnt signaling in the lens surface ectoderm is a key prerequisite for lens placode specification; aberrant activation of the pathway in that stage completely disrupts eye development (Machon et al., 2010). Tcf/Lef transcription factors, differentially expressed in the embryonic eye structures, may further fine-tune the transcriptional response to Wnt/β-catenin signaling, yet their function during mouse eye development remains unknown. The most abundant members of the Tcf/Lef family in developing lens tissue are Tcf7l1 and Tcf7 (Liu et al., 2006). In order understand the role of Tcf7l1 during lens placode development, we specifically deleted *Tcf7l1* by employing a lens placode specific Cre expressing mouse strain, namely Lens-Cre (Ashery-Padan et al., 2000).

Specific aims:

1) Manipulation of the Wnt/β-catenin signaling in the mouse neural crest and anterior neural fold via conditional deletion of *Tcf7l1* gene and subsequent analysis of the phenotype

2) Investigation of the effects following *Tcf7l1* conditional inactivation on the anterior pre-placode, with the focus on lens placode development in mouse

Materials and Methods

Mouse strains

Housing of mice and *in vivo* experiments were performed in compliance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and national and institutional guidelines. All procedures involving experimental animals were approved by the Institutional Committee for Animal Care and Use (permission PP-071/2011, PP-281/2011). This work did not include human subjects. LR-Cre and Rx-cre were produced in our laboratory (Klimova et al., 2013; Kreslova et al., 2007). AP2α-IRESCre (AP2α-Cre) was described previously (Macatee et al., 2003), Cre reporter lines Rosa26$^{EYFPfl/+}$ (B6.129X1-*Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$*/J, stock No: 00614) and ROSA26 (B6.129S4-Gt(ROSA)26Sor^{tm1Sor}/J), stock No: 003474) were purchased from The Jackson Laboratory. Tcf7l1$^{fl/+}$ strain (Tcf7l1$^{tm1a(EUCOMM)Wtsi}$) was purchased from the EUCOMM. Transgenic mouse strain BAT-gal (Maretto et al., 2003) was kindly provided by S. Piccolo. The Lens-Cre was kindly provided by Dr.Ashery-Padan (Ashery-Padan et al., 2000). Mice with a conditional "floxed" β–catenin (β-cat$^{Ex3fl/+}$) were kindly provided by Dr. M. M. Taketo (Harada et al., 1999). Generation of Rosa26$^{dnTCF4fl/fl}$ was described in (Janeckova et al., in press). Briefly, human TCF7L2 coding sequence lacking N-terminal domain (aa 1-31) was cloned into the ROSA26 targeting vector containing arms for homologous recombination into ROSA26 locus in a same way as when generating the Rosa26$^{ctβcat-Tcf7l1fl/+}$. Details about the Rosa26$^{ctβcat-Tcf7l1fl/+}$ mouse line generation are present in Fig. S4. A C-terminal part of β-catenin (aa 696-781) was fused with the mouse full length Tcf7l1 and inserted into ROSA26 locus using homologous recombination in ES cells. The construct carries tdTomato coding sequence flanked with the LoxP sites serving as a stop cassette for ctβcat-Tcf7l1. Excision of the tdTomato cassette via Cre-mediated recombination, allows transcription of ctβcat-Tcf7l1 mRNA (Fig. 5A). PGK-Neo cassette was removed using FLP-FRT recombination, F1 animals were crossed to the Actb:FLPe strain (Rodriguez et al., 2000) and kept on a C57BL/6J background. Primers for genotyping Rosa26$^{ctβcat-Tcf7l1fl/+}$ allele: JM21F GAGGAGCCATAACTGCAGAC, JM23R CCTGGCTATCCTAGATAGAAC and JM27R GTACTGTTCCACGATGGT the expected size of the PCR product is 965bp in the mutant and 300bp in the wild-type.

Morpholino mediated knock-down in zebrafish

Zebrafish wild-type, single-cell embryos were injected with combination of previously described Tcf7l1a (5′ CTCCGTTTAACTGAGGCATGTTGGC 3′) and Tcf7l1b (5′ CGCCTCCGTTAAGCTGCGGCATGTT 3′) morpholinos (Dorsky et al., 2003), in 1:1 ratio at a final concentration of 1.6ng/nl (1-2nl injected) (Gene Tools). Embryos were raised at 28°C and harvested at 12hpf.

Immunohistochemistry and RNA in situ hybridization

At least three embryos of each genotype and similar developmental stage were analyzed per staining. All used primary antibodies are listed in Table 1. Secondary antibodies: anti-mouse (-chicken, -rabbit, -goat) Alexa Fluor488 or 594 (dilution 1:500, Life Technologies), Biotinylated anti-rabbit (Vector Laboratories), Vectastain ABC Elite kit and ImPACT DAB substrate (all Vector Laboratories). For wholemount immunohistochemistry we used the protocol published by Sandell et al. (Sandell et al., 2014). Briefly, embryos were fixed overnight in 4% PFA, permeabilized using Dent's bleach and 100% methanol and rehydrated. Blocking for 2 hours and antibody incubation overnight were performed in 3% BSA in 0.1M Tris-Cl, pH 7.5, 0.15 M NaCl, 0.03% Triton X-100. After 5 times of 1-hour washing in PBS, overnight incubation with secondary antibody in the blocking buffer followed. The next day, embryos were stained for nuclei using Hoechst (dilution 1:1000, Life technologies), washed three times in PBS (20 minutes each) and stored in 2% formaldehyde (Sigma). Whole-mount immunofluorescence was captured using binocular microscope Apotome.2 Ax10 zoom.V16 (Zeiss). Fluorescence and bright-field light images were acquired in Nikon Diaphot 300 with objectives 4x/0.1 and 10x/0.25 and confocal Leica SP5.

In case of staining on section, following protocol was utilized. Embryos of desired age were disected, fixed in 4% paraformaldehyde (PFA) from for up to 4 hours at 4°C, washed with PBS, cryopreserved in 30% sucrose and frozen in OCT (Sakura). The cryosections (10-12 µm) were permeabilized with PBT (PBS with 0.1% Tween), blocked with 10% BSA in PBT and incubated with primary antibody (1% BSA in PBT) overnight at 4°C. Sections were washed with PBS, incubated with fluorescent secondary antibody (Life Technologies, 1:500) for one hour at room temperature, washed with PBS, counterstained with DAPI and mounted in Mowiol. When paraffin sectioning was employed, embryos were fixed ON at 4-8% PFA, dehydrated, embedded in paraffin, sectioned (4-8 µm) rehydrated and antigens were retrieved by boiling in Citrate Buffer (Citric acid monohydrate, final concentration 0.1mM, pH 6.0) for 15 minutes in high pressure pot, prior staining as described above for cryosections.

Pregnant mice were injected with 100ug of 5-Bromo-2'-deoxyuridine (BrDU) into peritoneal cavity 1 hour prior sacrifice. Samples were processed by paraffin sectioning and stained using anti-BrDU antibody (Abcam).

Whole-mount *in situ* hybridization was performed using standard protocols. Riboprobes were cloned into pGEM-T-easy vector (Promega) using primers specified in the Table 1.

ISH probes		
Gene	forward	reverse
FoxD3	GGACCGCAAGAGTTCGCGGA	TCCGGAGCTCCCGTGTCGTT
Sox9	GAGCACTCTGGGCAATCTCAG	CTCAGGGTCTGGTGAGCTGTG
Gbx2	Gift from Peter Rathjen	Adelaide University
Sp5	CGTGAAGACGCACCAAAATA	TATTTTCACGCTGCCAACTG
Fgf8	CAGGTCCTGGCCAACAAG	GAGCTCCCGCTGGATTCCT
Sox10	Gift from Anthony Firulli	Indiana University, USA
Sox2	Open Biosystems	BC057574
FoxG1	Gift from Stefan Krauss	OUS, Oslo, Norway
Six3	Gift from Guillermo Oliver	St. Jude Hospital, Memphis, USA
Wnt1	Gift from Andy McMahon	USC, USA
Tcf7l1	Open Biosystems	BC128306
Tcf7l2	Open Biosystems	BC052022
Antibodies		
Gene	Company	Dilution
Sox1	RαD AF3369	1:1000
Tfap2α	Santa Cruz Biotech SC-184	1:1000
Sox10	Santa Cruz Biotech SC-17342	1:1000
Sox9	Millipore AB5535	1:1000
N-cadherin	BD Transduction Lab. 610920	1:2000
GFP	Life Technologies A11122	1:1000
β-galactosidase	Abcam ab9361-250	1:1000
Lef1	Cell Siganling C12A5	1:1000
a-BrdU	Abcam AB6326	1:100
E-cadherin	Invitrogen 13-1900	1:400
Pax6	AF-1 custom made	1:4000
FoxE3	Gift from Peter Carlsson	1:500
Prox1	Abcam 11941	1:1000

PCNA	DSHB	1:1000
cMaf	Bethyl Laboratories BL662	1:500
cleaved caspase 3	Cell Signaling 9664	1:350
Tcf7l1	Santa Cruz Biotech SC-M-20	1:2000
γ-crystalline	Gift from Hisato Kondoh	1:400
p21	Santa Cruz Biotech SC-471	1:1000
plasmids		
SuperTopFlash	AddGene	12456
Renilla pRL-SV40P	AddGene	27163
pCI-neo	Promega	E1841
Tcf7l1	Open Biosystems	BC128306
Tcf7l2	Open Biosystems	BC052022
Tcf7	Open Biosystems	BC057543
Lef1	Open Biosystems	BC138596
Dn-b-catenin	modified gift from V. Korinek	IMG, Prague
β-gal	Clontech	631719
p21Luc	Gift from H.Clevers	Hubrecht Institute, Netherlands

Table 1: list of used reagents

SuperTop Flash reporter assay

HEK293 cells (30 000 cells/well/96-well plate) were transiently transfected using Lipofectamine 2000 (Invitrogen) with 30 ng of Super8X TopFlash and 1 ng of Renilla luciferase constructs and indicated amount of the expression vector, filled with mock DNA to final concentration of 200ng DNA per well. Luciferase assays were performed 48h later with a Dual-Glo luciferase kit (Promega). The luciferase signal was normalized to a co-transfected Renilla readout. Experiments were performed on at least 6 biological replicates per condition.

P21-luc reporter assay

MEF cells from Ert2-Cre;Tcf7l1$^{fl/fl}$ embryos at E13.5 were isolated using protocol described by Bryja *et al.* (Bryja et al., 2006). After first two passages were cells seeded on 10cm dish and treated with 4-*hydroxitamoxifen* (4-OHT). Cells were detached by trypsin treatment (1% in DMEM without serum) and transfected in GT porator solution

(Biologicals) by Amaxa Nucleofector (Lonza). 2×10^6 cells and 3ug of total DNA (p21-Luc, Tcf7l1, β-gal) were used per condition. Cells were re-plated at 6-well dishes and after 36 hours lysed subjected for luciferase assays were One-Glo luciferase kit (Promega), absorbance of the β-gal was used for normalization. Experiment was repeated at least three times on biological duplicates.

Results

1) Manipulation of the Wnt/β-catenin signaling in the mouse neural crest and anterior neural fold via conditional deletion of *Tcf7l1* gene and subsequent analysis of the phenotype

Results presented in this chapter were included in a manuscript entitled "**Tcf7l1 protects the anterior neural fold from adopting the neural crest fate**" by **Jan Mašek, Vladimír Kořínek, M. Mark Taketo, Ondřej Machoň, and Zbyněk Kozmik;** submitted to the journal *Development*, potentially accepted after major revision

AP2α-Cre mediated deletion of *Tcf7l1* during mouse NC specification results in exencephaly.

Tcf7l1 is detected in the developing mouse ectoderm prior to gastrulation, and later, at E7.5, it is expressed in the anterior ectoderm and mesoderm (Korinek et al., 1998; Merrill et al., 2004). Expression of *Tcf7l1* during neurulation was characterized using whole mount *in situ* hybridization. *Tcf7l1* mRNA was detected in the anterior neural plate at the 1 somite pair (s) stage, being further restricted to the ANF region and along the anterior NPB with the progress of neurulation 3-4s (Fig. 6A). We next wanted to relate the expression pattern of *Tcf7l1* to that of known NC-specific genes and the area of Wnt/β-catenin signaling. *Tfap2α* is one of the key genes involved in the NC induction, while *FoxD3, Sox9, Sox10* have been associated with the NC specification (Groves and LaBonne, 2014; Simoes-Costa and Bronner, 2015). During neurulation, the Tfap2α protein is continuously expressed along the whole NPB (Fig. 6B). *Sox9* and *FoxD3* transcripts were initially detected in the neural plate, as early as at the 1-2s stage (Figs 6C, 7A), while at 3-4s they were expressed robustly in characteristic stripes along the anterior neural plate border (Figs 6C, 7A). The onset of *Sox10* mRNA expression was detected later (4-5s) and slightly more caudally (Fig. 7B), in a very similar pattern as the expression of *Sox9* (Fig. 6C). To map the activity of the Wnt/β-catenin pathway, we used the BAT-gal reporter mice (Maretto et al., 2003). Starting from the 2-3s stage, the BAT-gal signal, spread across the prospective mesencephalon and metencephalon, but did not reach the ANF at the 4-5s stage (Fig. 6D). In conclusion, *Tcf7l1* expression in the ANF is reciprocal to that of *Sox9, FoxD3,*

Sox10 and BAT-gal, which reside in the caudal areas of the neural plate, but overlaps with Tfap2α in the anterior part of the NPB.
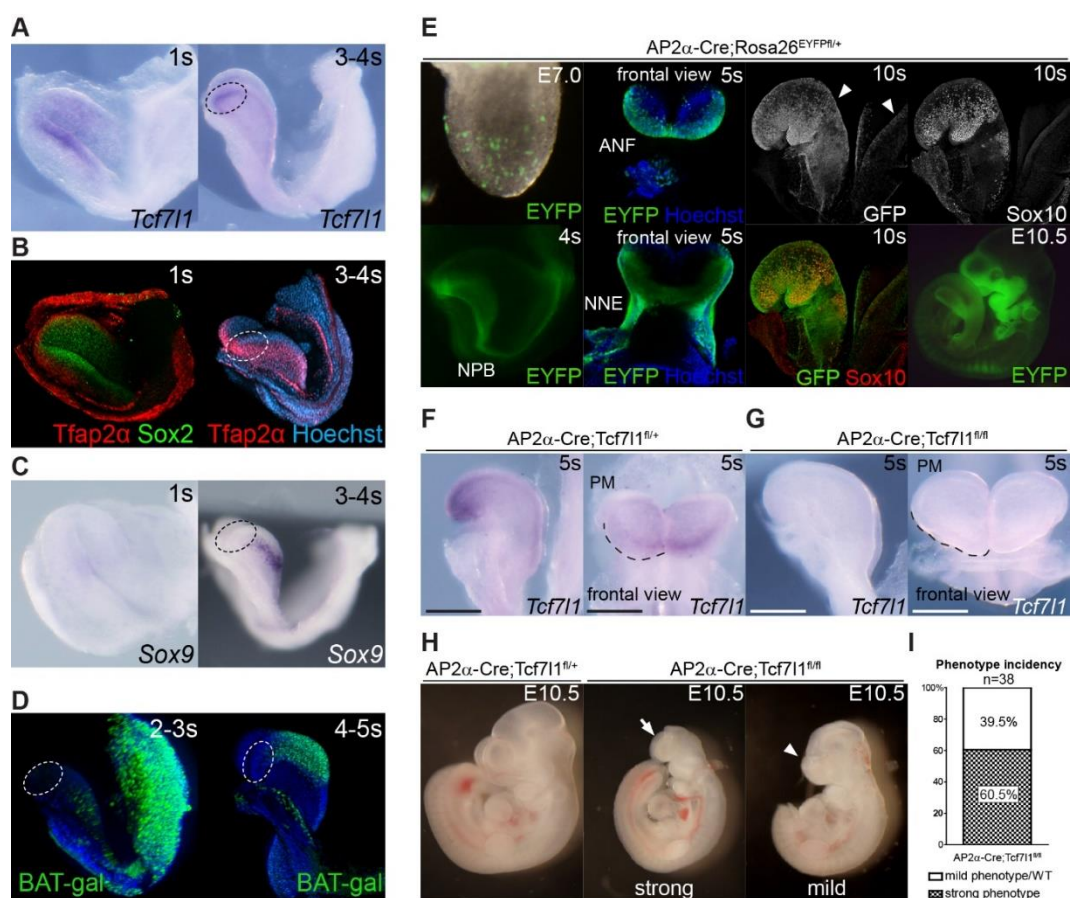
Figure 6: Tcf7l1 is expressed in a mutually exclusive manner with markers of specified NC and is required for proper forebrain development. (A-D) Expression of (A) *Tcf7l1*, (B) Tfap2α, early NC marker, (C) *Sox9*, marker of specified NC and (D) Wnt reporter BAT-gal, in the NPB and the ANF (encircled) of developing mouse embryo. Please note the overlap of *Tcf7l1* transcript and Tfap2α protein, and of *Sox9* mRNA and BAT-gal signal. **(E)** Mapping of recombination delivered by AP2α-Cre, using Rosa26[EYFPfl/+] reporter mouse at various stages (E7.0-E10.5). EYFP was detected either directly or using anti-GFP antibody. The recombination occurred along the entire NPB (arrowheads, 10s stage). Co-staining of GFP with anti-Sox10 antibody revealed recombination in the whole cranial NC population at 10s stage. **(F-G)** *In situ* hybridization shows loss of *Tcf7l1* mRNA expression in AP2α-Cre;Tcf7l1^{fl/fl} mutants. ANF in mutant is wider at PM, but narrower towards anterior tip of the ANF (dashed line) than in the control at 5s stage (scale 30μm). **(H)** Control embryo (left) and typical examples of strong (middle) and mild (right) AP2α-Cre;Tcf7l1^{fl/fl} phenotype. **(I)** Quantification of strong and mild phenotype incidence between E10.5-E13.5. ANF (encircled) - anterior neural fold, NPB-neural plate border, NC-neural crest, NNE-non-neural ectoderm, PM-prosencephalic-mesencephalic boundary, s-somite pairs. All embryos are shown from a lateral view if not stated otherwise. Nuclei were stained with Hoechst.

In order to study the function of *Tcf7l1* during NC induction and specification, Tcf7l1 was inactivated, by crossing Tcf7l1 conditional knock-out mice (EUCOMM) with AP2α-IRESCre (AP2α-Cre) transgenics (Macatee et al., 2003). Tracing of the AP2α-Cre-driven recombination using the Rosa26[EYFPfl/+] reporter strain revealed the

first EYFP-positive cells scattered throughout the embryo at E7.0 (Fig. 6E). The EYFP signal became focused along the entire NPB at the 4s stage, while the highest levels were detected rostrally, in the neural- and non-neural ectoderm of the ANF, at the 5s stage. AP2α-Cre most probably mediates the recombination in the entire NC population, as the recombined EYFP-positive cells included the entire Sox10-positive NC population of the AP2α-Cre;Rosa26$^{EYFPfl/+}$ embryos at the 10s stage (arrowheads, Fig. 6E). At E10.5, the EYFP signal was present in NC derivatives (brachial arches, dorsal root ganglia and sensory neurons), the head ectoderm, lens placodes, and in the anterior telencephalon (Fig. 6E, Fig. 7C).

In AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants, *Tcf7l1* mRNA expression was completely abolished, whereas high levels were detected along the anterior NPB, including the ANF, in control embryos at the 5s stage (Fig. 6F-G). At this stage, phenotypical changes following *Tcf7l1* deletion became apparent; the shape of neural folds changed from rounded into more a narrow morphology and the neural plate expanded laterally at the prosencephalic-mesencephalic boundary (dashed lines in frontal views in Fig. 6F,G). At later stages (E10.5), we observed a variability in the penetrance of morphological changes among individual mutants. Therefore, we decided to classify the defects into two categories: (i) a strong phenotype, represented by a severe reduction of the telencephalic tissue and exencephaly (arrow, Fig. 6H) and (ii) a mild phenotype, with reduced telencephalon and bilateral anopthalmia (arrowhead, Fig. 6H). Quantification of phenotype incidence, between E10.5-E13.5, revealed that the strong phenotype was present in 60.5% and the mild phenotype in 39.5% of the analyzed embryos (n=38) (Fig. 6I). Mutant embryos with the strong phenotype exhibited massive exencephaly and severe defects in craniofacial structures and did not survive beyond E13.5 (Fig. 7D, E). These data demonstrate that *Tcf7l1* function in the ANF is crucial for the development of the mouse forebrain.
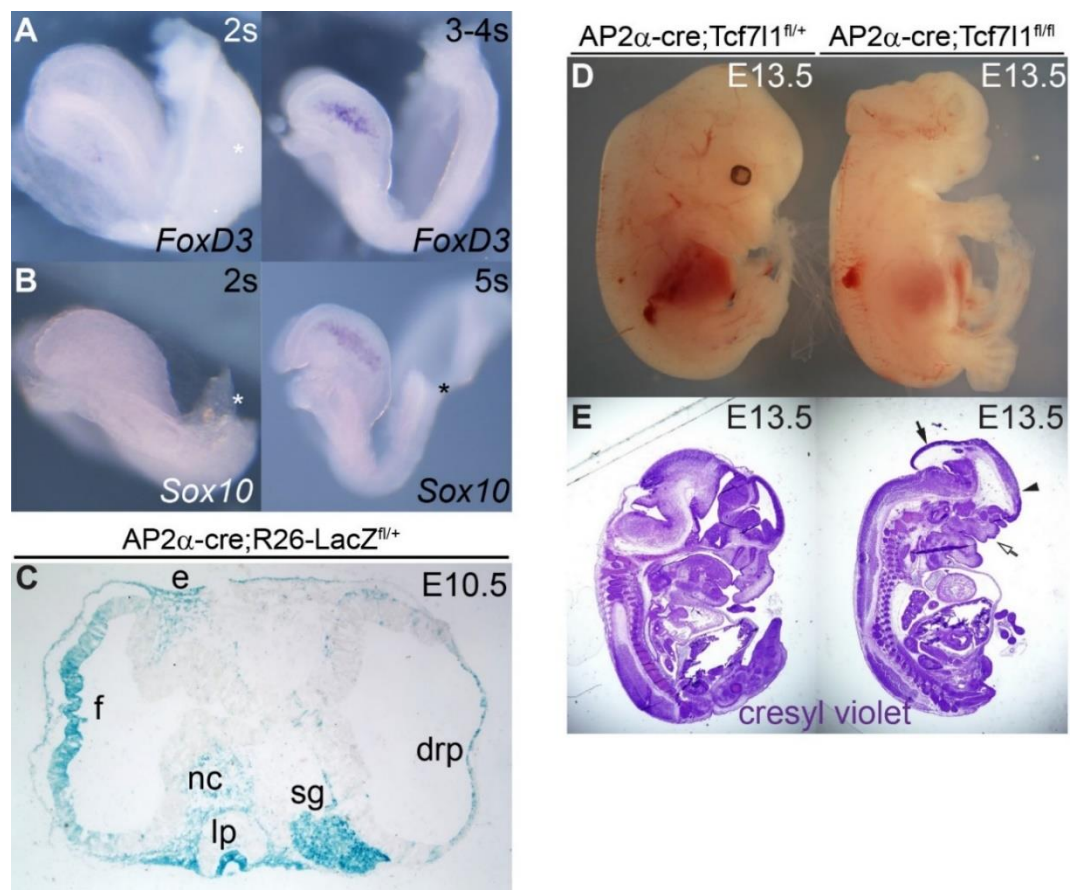
Figure 7: Expression of NC genes during early mouse embryogenesis. (A-B) RNA *in situ* hybridization of *FoxD3* and *Sox10* transcripts, at 2s and 3-4s, and at 2s and 5s stages, respectively. Asterisks mark artifacts caused by sample preparation. **(C)** Mapping of AP2α-Cre delivered recombination. Coronal section of AP2α-Cre;Rosa26^LacZfl/+, at E10.5 stage. Recombination was detected enzymatically with β-galactosidase. drp-dorsal roof plate, e-ectoderm, f-forebrain, lp-lens pit, nc-neural crest, sg-sensory ganglion. **(D, E)** RNA *in situ* hybridization of *FoxD3* and *Sox10* transcripts, at 2s and 3-4s, and at 2s and 5s stages, respectively Histological analysis of sagittal sections, stained with cresyl violet, from the AP2α-Cre;Tcf7l1^fl/fl 'strong' mutant at E13.5, shows severe defects in the splanchnocranium (white arrow), forebrain (black arrowhead) and midbrain (black arrow) of the mutant embryo.

Tcf7l1 is required for the expression of anterior neural markers

Tcf7l1 has been implicated in A-P patterning of the developing brain in both zebrafish and mouse, since its loss-of-function mutations result in severe reductions of anterior neural regions, accompanied with apparent expansion of midbrain and hindbrain markers towards the anterior (Dorsky et al., 2003; Merrill et al., 2004). In contrast to published data, we did not observed any A-P shift in mRNA expression of the core regulators of the midbrain-hindbrain boundary (MHB), namely *Fgf8*, *Gbx2* and *Wnt1*, between Tcf7l1 conditional mutant embryos and controls at E9.0 (E9.0 in the figure) (arrowheads Figs 8A-B´, 12D). Remarkably, we observed a progressive loss of the posterior prosencephalon marker *Tcf7l2* (Tcf4) mRNA (Fig. 8C-C´´),

accompanied by gradual loss of anterior neural tissue in AP2α-Cre;Tcf7l1fl/fl mutants with mild (C´) or strong (C´´) phenotype at E9.0. Furthermore, the expression of the ANF specific transcripts, *Six3* (Lagutin et al., 2003), *FoxG1* (Hanashima et al., 2004) and *Fgf8* (Meyers et al., 1998) was lost or strongly reduced in the mutants when compared with controls of appropriate stage (Fig. 8D-F´). Next we tested whether *Tcf7l1* deletion affected the neural character the ANF cells. RNA *in situ* hybridization revealed reduced expression of the neural marker *Sox2,* and an expansion of *Sox2* negative cells along the whole lateral border of the ANF in AP2α-Cre;Tcf7l1fl/fl mutants (Fig. 8G,G´), suggesting that the cells had lost their neural identity.
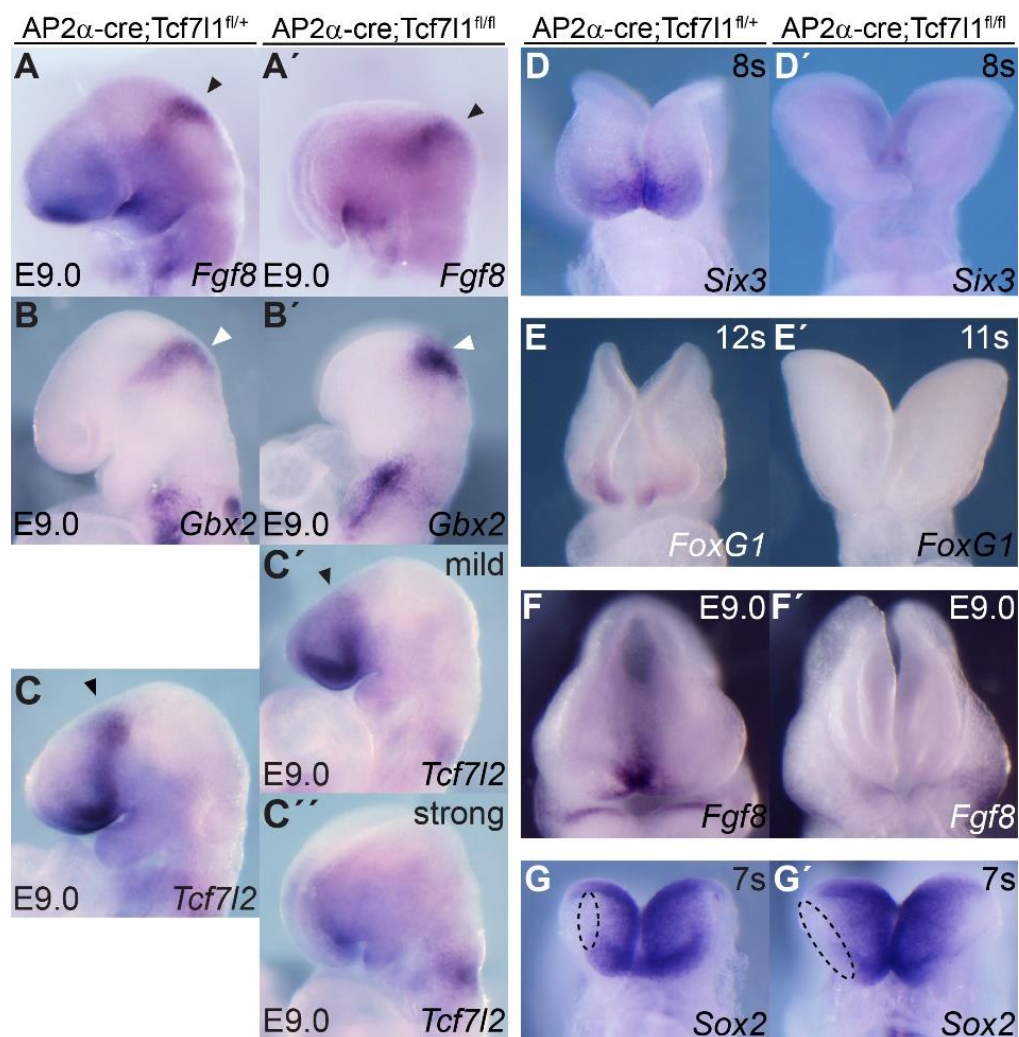
Figure 8: *Tcf7l1* deletion induces loss of ANF specifiers, without affecting more posterior brain structures. (A-B´) RNA *in situ* hybridization shows unchanged expression of *Fgf8* and *Gbx2* in MHB (arrowheads) at E9.0, all lateral view. **(C-C´´)** *Tcf7l2* expression in the posterior prosencephalon of control and AP2α-Cre;Tcf7l1fl/fl embryos displaying mild and strong phenotype at E9.5, all lateral view. **(D-G´)** *Tcf7l1* deletion resulted in marked reduction of endogenous expression of *Six3* and *Sox2* at 7-8s stage, of *FoxG1* at 11-12s stage and of *Fgf8* at E9.0 in the ANF of AP2α-Cre;Tcf7l1fl/fl embryos, all frontal view.

Tcf7l1 deletion results in aberrant trans-differentiation of the ANF cells into NC

NC cells need to lose their neuroepithelial character in order to undergo EMT and migrate towards their target destinations. NC specification, which precedes the EMT in *Xenopus* and chicken, is regulated by transcription factors FoxD3, Sox9 and Sox10 (Cheung et al., 2005; Dottori et al., 2001; Liu et al., 2013). Therefore, we decided to investigate expression of these three factors in the absence of Tcf7l1.

As can be seen in the whole-mount immunostaining, Sox9 positive NC cells expanded into the Sox1 positive ANF region in AP2α-Cre;Tcf7l1^{fl/fl} mutants compared with controls at the 5s stage (Fig. 9A). Examination under higher magnification revealed decreased neural Sox1 staining towards the anterior part of the mutant embryo (Fig. 9A). A frontal view on the same embryos clearly showed ectopic Sox9-positive NC cells along the ANF in mutants (Fig. 9B). This was further confirmed by *in situ* hybridization of *Sox9* mRNA (arrowhead Fig. 10A). Consistently, the expression of *FoxD3* mRNA also shifted anteriorly in mutant embryos compared to controls at the 10s stage (Fig. 9C). Later at E9.0, aberrant *Foxd3* mRNA was detected in the rostral part of the head in mutants (arrowhead, Fig. 10B), while it was completely absent in controls. Expression of *Sox10* mRNA was apparently delayed in the absence of *Tcf7l1*, being barely detectable at the 6s stage (Fig. 9D). Two days later, at E9.5, however, RNA *in situ* hybridization revealed robust ectopic expression of *Sox10* in the anterior part of the head of mutant embryos (arrowhead, Fig. 9D). Interestingly, we also observed a cell fate shift, characterized by the change from Sox1 to Sox9, in caudal neural tissue. Coronal sectioning of the developing hindbrain at E9.0 also uncovered aberrant Sox9-positive/Sox1-negative cells in the mutants (arrowheads, Fig. 10C).
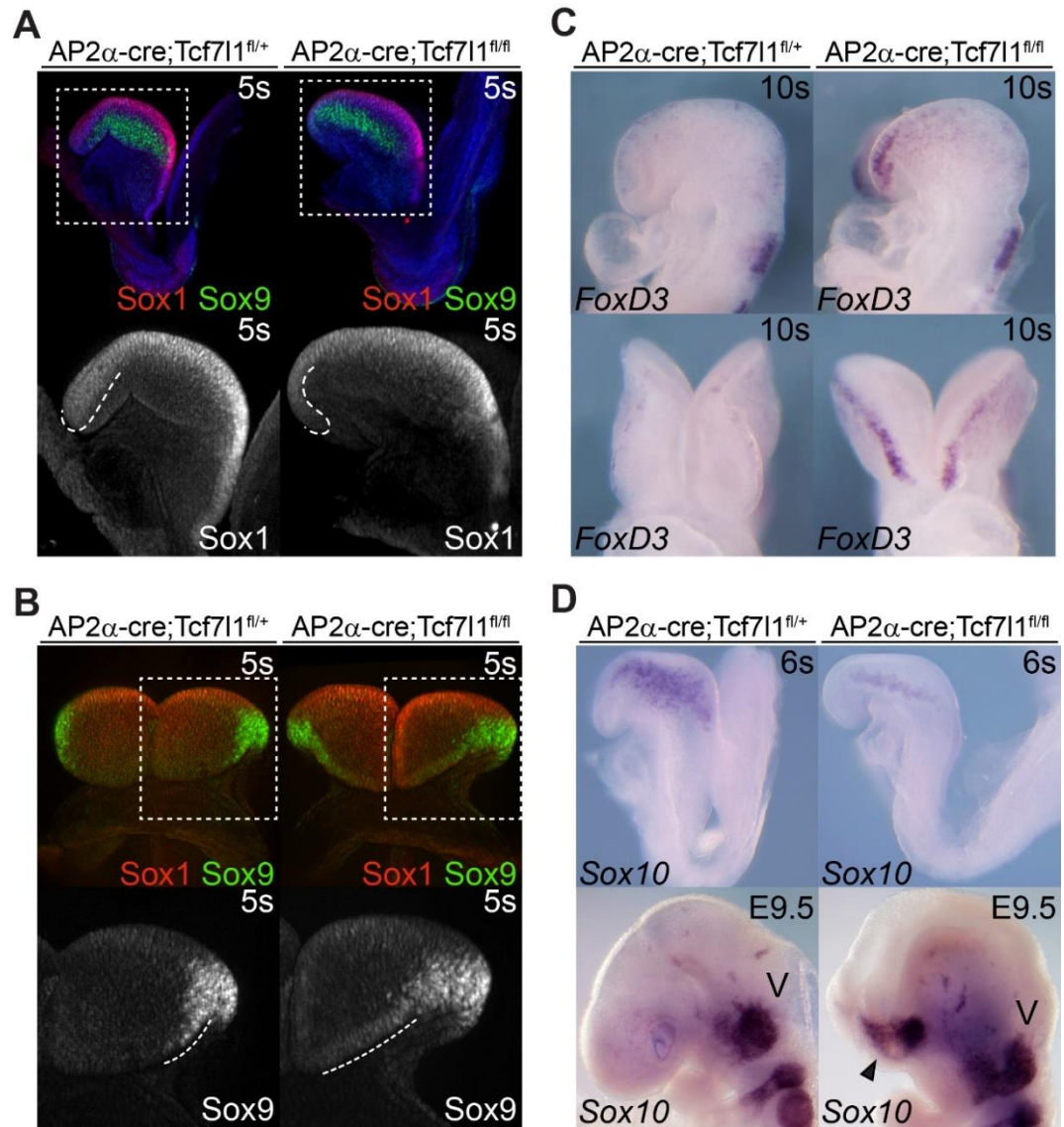
Figure 9: The NC population expands anteriorly in the absence of *Tcf7l1*. (A) Whole mount immunohistochemical staining of Sox1 and Sox9 protein at 5s stage, lateral view. Higher magnification reveals reduction of Sox1 staining in the ANF (dashed line) of the AP2α-Cre;Tcf7l1^fl/fl^ mutants. Nuclei were stained with Hoechst. **(B)** Same embryos as in (A), frontal view; higher magnification (bottom) documents the expansion (dashed line) of Sox9 positive cells along the ANF border in mutants. **(C)** Lateral (top) and frontal (bottom) views revealed aberrant expression of the NC marker *FoxD3* along the ANF in the mutants, while it is absent in the control embryos at 10s stage. **(D)** *Sox10* mRNA is less abundant in the Tcf7l1 mutants at 6s stage but becomes ectopically expressed later in anterior head (arrowhead) of AP2α-Cre;Tcf7l1^fl/fl^ embryos at E9.5. V-trigeminal ganglion.

Analogously to the NC expansion in AP2α-Cre;Tcf7l1^{fl/fl} mice mutants, we observed ectopic expression of *FoxD3*, *Pax3* and *Sox9* mRNA along the entire anterior NPB, upon MO-mediated knock-down of both Tcf7l1a and Tcf7l1b in zebrafish embryo (arrowheads, Fig. 10D), this fact denotes the conservation of *Tcf7l1* function across vertebrate species. Taken together, the expression of several genes responsible for the NC specification and EMT initiation is expanded towards ANF in the AP2α-Cre;Tcf7l1^{fl/fl} mutants, suggesting that Tcf7l1 maintains the neuroepithelial character of the ANF and restricts spreading of NC fate within the anterior head.
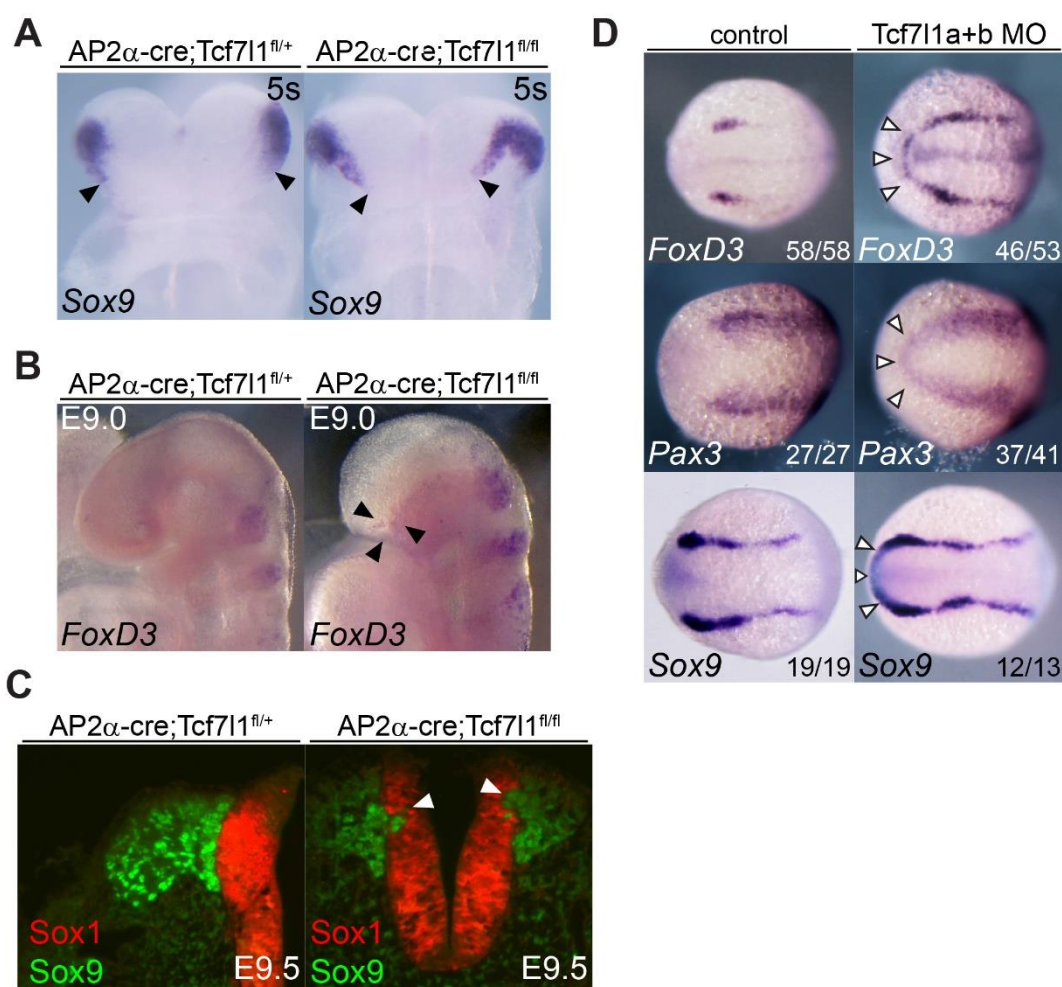
Figure 10: Expansion of NC markers follows *Tcf7l1* deletion in mouse and zebrafish. (A-C) Expression of *Sox9* mRNA expanded rostrally in AP2α-Cre;Tcf7l1^{fl/fl} mutant at 5s stage. Abberant *FoxD3* mRNA expressing cells are present in the Tcf7l1 mutant at E9.0. Immunnohistological staining of coronal sections from the E9.0 hindbrain reveled ectopic Sox9-positive/Sox1-negative cells in AP2α-Cre;Tcf7l1^{fl/fl} mutants. **(D)** Expression of *FoxD3*, *Pax3* and *Sox9* mRNA expands along the anterior NPB upon MO-mediated knock-down of both *Tcf7l1a* and *Tcf7l1b* variants in zebrafish at 12h stage.

Trans-differentiation of the ANF is driven by aberrant Wnt/β-catenin signaling

Tcf7l1 is a very efficient repressor of Wnt/β-catenin signaling target genes, as has been shown both in mouse embryonic stem cells and *in vivo* (Cole et al., 2008;

Shy et al., 2013; Wu et al., 2012). Gain- and loss-of-function experiments in non-mammalian vertebrates have shown that the Wnt/β-catenin pathway is required for physiological, and sufficient for ectopic induction and expansion of the NC (Garcia-Castro et al., 2002; Heeg-Truesdell and LaBonne, 2006; Steventon et al., 2009; Taneyhill and Bronner-Fraser, 2005; Wu et al., 2005). In our transient reporter assays, using the SuperTopFlash reporter in HEK293 cells, *Tcf7l1* was able to compete out other Tcf/Lefs, significantly reducing Wnt reporter signal (Fig. 12A). Therefore we questioned whether the expansion of NC cells in AP2α-Cre;Tcf7l1^{fl/fl} mutants was caused by upregulation of Wnt/β-catenin signaling. We crossed BAT-Gal reporter mice with Tcf7l1 conditional mutants and stained whole-mount embryos with anti-galactosidase and anti-Sox9 antibodies.

As expected, the Wnt reporter BAT-gal immunofluorescence expanded anteriorly in the AP2α-Cre;Tcf7l1^{fl/fl} mutants (arrow, Fig. 11A). This was accompanied with a high number of ectopic Sox9-positive cells, as clearly visible from the frontal view of the 8s stage embryos (white arrowheads, Fig. 11A). The co-expression of Sox9 and BAT-gal positive cells does not seem to be aberrant, as cross-sections from the 8s old wild-type embryos showed large overlaps between the Sox9 and BAT-gal immunofluorescence (Fig. 12B), indicating that Sox9 may be induced by Wnts. Furthermore, expression of *Sp5*, a known Wnt/β-catenin target gene, was also upregulated and expanded rostrally in the mutant embryos at 6s stage (arrowheads, Fig. 11B), confirming the ectopic increase of the Wnt/β-catenin signaling activity in the anterior neuroectoderm. Apart from its role in MHB formation (McMahon and Bradley, 1990; Thomas and Capecchi, 1990), Wnt1 is also required during NC development (Ikeya et al., 1997; Saint-Jeannet et al., 1997). Interestingly, we found aberrant *Wnt1* expression in the ANF of the *Tcf7l1* mutants at the 5-6s stage (Fig. 11C). The ectopic pattern of *Wnt1* mRNA expression along the NPB of the prosencephalon was even more obvious at E9.0 (arrowheads, Fig. 11C). We observed no difference in *Wnt1* mRNA expression in the MHB between controls and *Tcf7l1* mutants (arrowheads, Fig. 12D) at the same stage, suggesting that the aberrant expression of *Wnt1* reflects the NC phenotype, rather than changes in A-P pattering.
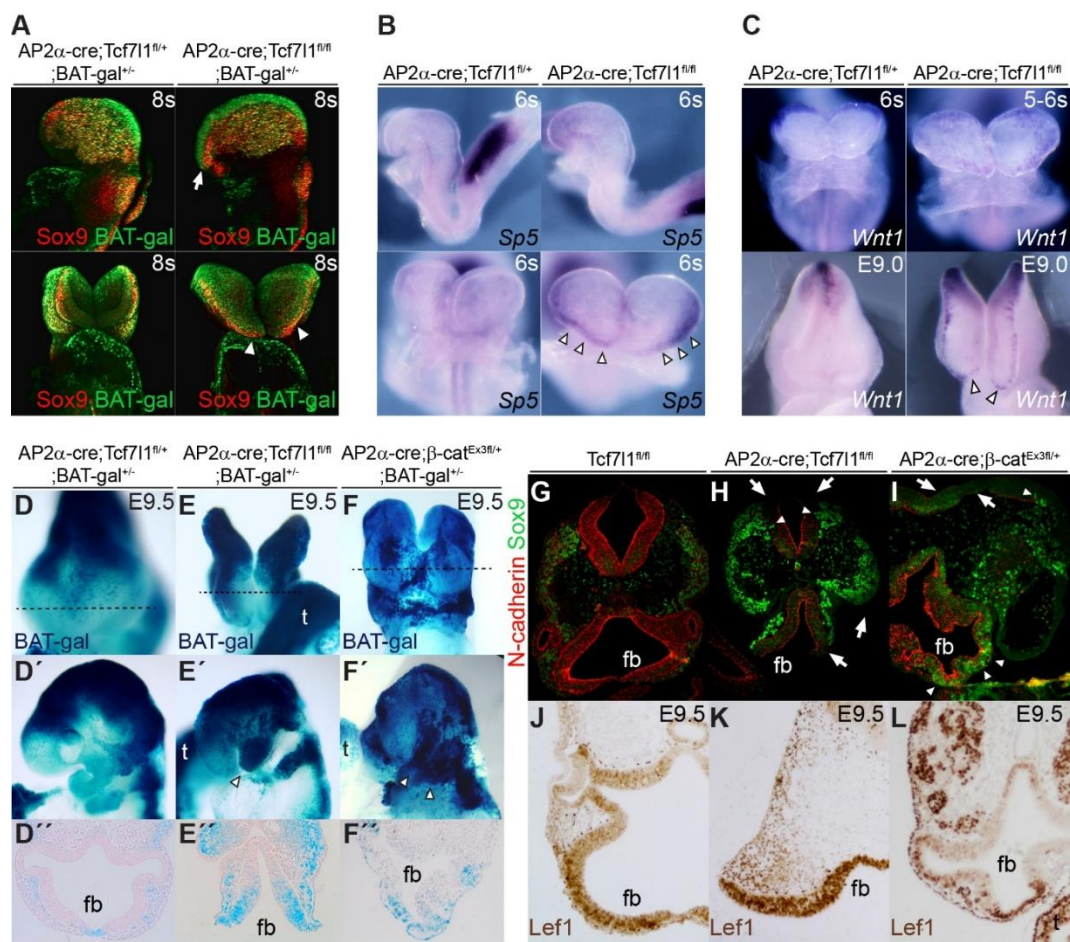
Figure 11: The ectopic activation of Wnt/β-catenin pathway promotes NC formation in mouse. (A) BAT-gal signal (detected with β-galactosidase antibody) and Sox9 staining expands anteriorly in AP2α-Cre;Tcf7l1^{fl/fl} mutants at the 8s stage.. **(B, C)** *In situ* hybridization shows upregulation of the Wnt target *Sp5* and Wnt ligand *Wnt1* along the NPB of the ANF in Tcf7l1 mutants at 5s and E9.5 stage**. (D-F´)** Frontal (top) and lateral (middle) view on the whole mount BAT-gal staining of the control, AP2α-Cre;Tcf7l1^{fl/fl} and AP2α-Cre;β-cat^{Ex3fl/+} embryos at E9.5. **(D´´-F´´)** Coronal sections in the dashed line shown in **(D-F)**. Different embryos were used in F, F´ and F´´. **(G-I)** Coronal sections immunostained with antibodies against N-Cadherin and Sox9 at E9.5. Sox9+ population is increased and N-Cadherin staining decreased in the AP2α-Cre;Tcf7l1^{fl/fl} and AP2α-Cre;β-cat^{Ex3fl/+} mutants, but not in control embryos. **(J-L)** Lef1 immunostaining of coronal sections of the control **(J)**, AP2α-Cre;Tcf7l1^{fl/fl} **(K)** and AP2α-Cre;β-cat^{Ex3fl/+} **(L)** embryos at E9.5. fb-forebrain, t-tail.

To confirm that the phenotype observed in AP2α-Cre;Tcf7l1^{fl/fl} mutants resulted from an aberrant activation of the Wnt/β-catenin pathway, we activated the signaling using β-cat^{ΔEx3fl/fl} mice (Harada et al., 1999), in which a truncated, non-degradable form of β-catenin is produced upon AP2α-Cre-mediated recombination. The AP2α-Cre;β-cat^{ΔEx3fl/+} embryos exhibited neural tube closure defects and did not survive beyond E10.5 (data not shown). The crossing of the BAT-gal reporter strain with the AP2α-Cre;Tcf7l1^{fl/fl} revealed activation of the reporter in the forebrain; this was even more pronounced in AP2α-Cre;β-cat^{ΔEx3fl/+};BAT-gal^{+/-} mutants that also showed a strong over-activation of the signal in most of the head ectoderm when

compared with controls (Fig. 11D-F). A lateral view of the Tcf7l1 and β-cat$^{\Delta Ex3fl/+}$ mutants clearly showed an aberrant population of BAT-gal-positive cells migrating towards the branchial arches (arrowheads, Fig. 11D´-F´). Cross sections of these embryos uncovered aberrant BAT-gal staining in the forebrain of both Tcf7l1 and β-cat$^{\Delta Ex3}$ mutants (Fig. 11D´´-F´´).

Next we assayed the EMT using immunofluorescence of neuroepithelial cells marked with N-cadherin and migrating NC cells labelled with Sox9 antibodies. In control embryos, only a thin layer of Sox9 positive cells between the N-cadherin positive forebrain tissue and the epidermis was observed. The hindbrain in controls labeled with N-cadherin was found to be completely devoid of Sox9 positive cells at E9.5. (Fig. 11G). In contrast, AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants clearly displayed aberrant Sox9-positive cells in the hindbrain prior to EMT (arrowheads, Fig. 11H). Concomitantly, N-cadherin expression was weaker in the anterior forebrain and the dorsal hindbrain mutants with "strong" phenotype (arrows, Fig. 11H). Intriguingly, the same analysis of AP2α-Cre;β-cat$^{Ex3fl/+}$ embryos revealed that almost the whole mutant forebrain converted into Sox9 positive NC (arrowheads, Fig. 11I). Similarly to Tcf7l1 conditional mutants, we also detected aberrant Sox9 expressing cells in the hindbrain, together with a massive decrease in the levels of N-cadherin (arrows, Fig. 11I). Furthermore, protein levels of Lef1, the Wnt/β-catenin transcriptional activator and target gene (Galceran et al., 1999; Kratochwil et al., 2002; Liu et al., 2005), were increased in the forebrain of the Tcf7l1 mutants in comparison with controls (Fig. 11J, K). β-cat^{Ex3} mutants displayed very strong upregulation of Lef1 in the mesenchyme adjacent to the hindbrain, as well as in the forebrain (Fig. 11L). Whole-mount immunofluorescence of wild-type embryos revealed co-localization of Lef1 and Sox10-positive cells at the 8s stage (arrowhead, Fig. 12C). It should be noted that in addition to the effects on NC population, the strong activation of the Wnt/β-catenin pathway in the AP2α-Cre;β-cat$^{Ex3fl/+}$ mutants disorganized both the A-P patterning of the neural tissue and the non-neural ectoderm surrounding the NC. This was clearly visible from the expansion of *Gbx2* and *Fgf8* mRNA expression domains at E9.5 (Fig. 12E,F). Collectively, deletion of *Tcf7l1* resulted in the ectopic activation of the Wnt/β-catenin pathway, suppression of neuroepithelial character and premature initiation of NC cell fate. Similar observations were made in conditional AP2α-Cre;β-cat$^{Ex3fl/+}$

mutants that yielded even more profound changes in the expression of described cell markers and in more extended areas than in Tcf7l1 mutants.
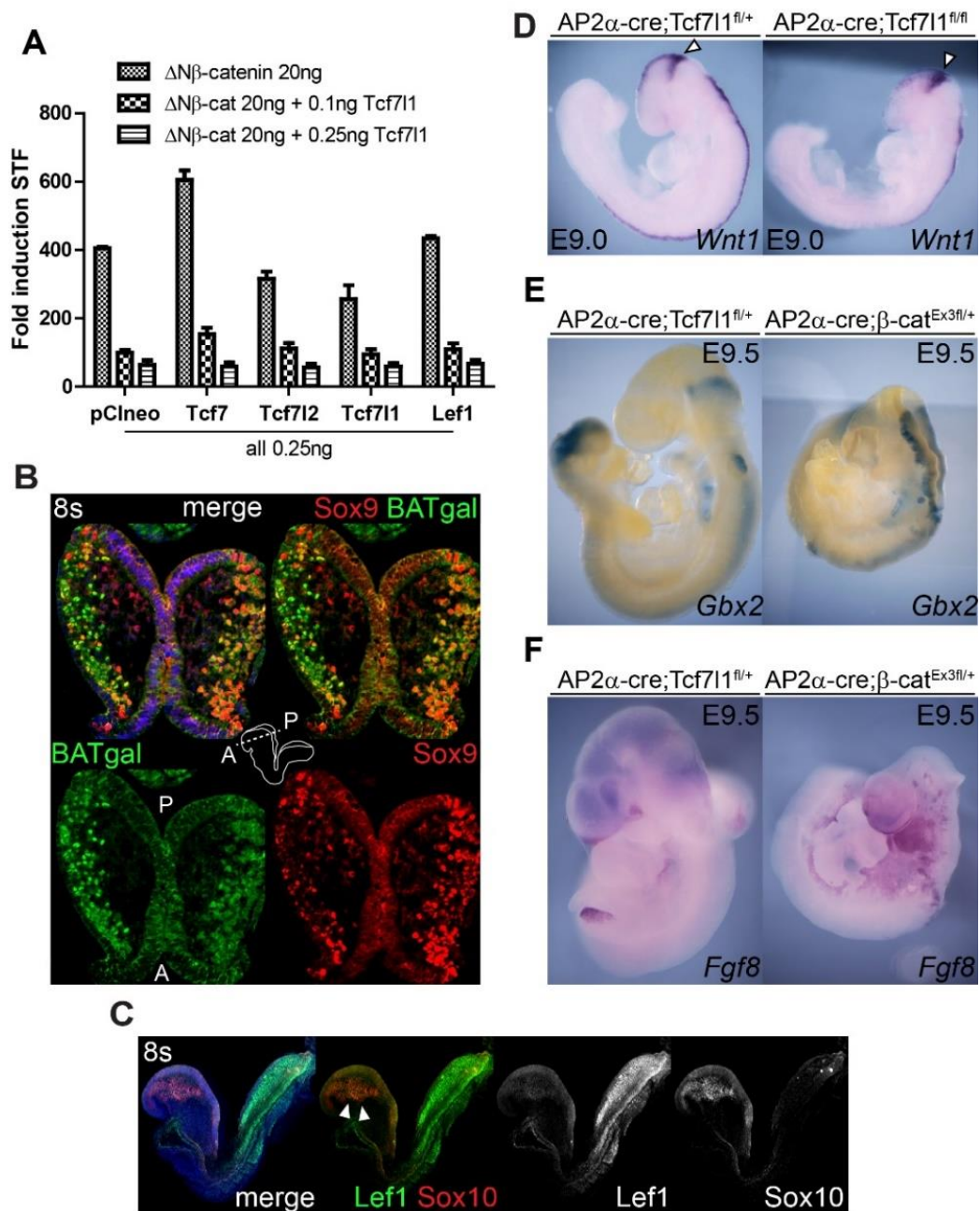
Figure 12: Balance between Wnt/β-catenin pathway activation and Tcf7l1 delivered repression regulates NC specification. (A) SuperTopFlash luciferase reporter assay in HEK293 cells documents Tcf7l1 ability to repress Tcf/Lef driven gene expression. Activation of the pathway was achieved by co-transfection with non-degradable form of β-catenin (ΔNβ-catenin). **(B)** Immunostaining of coronal sections from 8s stage wild-type embryos, A-anterior, P-posterior. Please note a large overlap between the Sox9-positive and BAT-gal-positive delaminating cells. Nuclei were stained with DAPI. **(C)** Whole mount immunofluorecence revealed overlap of Lef1 and Sox10-positive cells in wild-type embryo at 8s stage (arrowheads). Nuclei were stained with Hoechst. **(D)** The expression of *Wnt1* transcripts is unchanged in the MHB of AP2α-Cre;Tcf7l1^fl/fl mutants at E9.0 (arrowheads). **(E-F)** RNA *in situ* hybridization of the MHB markers *Gbx2* (E) and *Fgf8* (F) in controls and the AP2α-Cre;β-cat^Ex3fl/+ mutants at E9.5.

The phenotype can be rescued with a truncated form of TCF4 but not with Tcf7l1 fused with β-catenin

Our data suggest that the loss of Tcf7l1-mediated repression of Wnt targets is the primary cause of NC cell fate expansion. In order to verify this hypothesis, we performed rescue experiments using two mouse strains, conditionally expressing either dominant Tcf repressor or activator. As a dominant repressor we used the mouse strain Rosa26$^{dnTCF4fl/+}$, which contains the N-terminally truncated form of the human TCF7L2 (TCF4) targeted into the ROSA26 locus, 3´ to the lox-STOP TdTomato (TdT) cassette (originally referred to as Rosa26$^{+/tdTomato}$). This form cannot bind β-catenin and thus acts as dominant repressor of Wnt/β-catenin targets (Janeckova et al., in press). Secondly, we generated the mouse strain Rosa26$^{ctβcat\text{-}Tcf7l1fl/+}$, to allow for conditional expression of full-length mouse Tcf7l1 fused with the transactivation domain of β-catenin (aa696-781), analogous to the described Cat-Lef fusion (Hsu et al., 1998). This construct (ctβcat-Tcf7l1), that mimics the β-catenin binding to Tcf7l1, was able to deliver activation of the Wnt reporter (Fig. 13A). Fig. 13B represents a scheme of the Rosa26 locus and targeted construct, where primers for genotyping and Southern blot analysis are also depicted. We have confirmed correct targeting of the construct into mES cells using Southern blotting. Autoradiograph showed bands of predicted size for short arm (5.9kb) and long arm (9.9kb) (Fig. 13C). We have confirmed production of the ctβcat-Tcf7l1 fusion protein using Western blot analysis on mouse embryonic fibroblasts (MEFs) isolated from R26$^{ERT2\text{-}cre}$;Rosa26$^{ctbcat\text{-}Tcf7l1fl/+}$ (Fig. 13D). Addition of 4-*hydroxitamoxifen* resulted in expression of the protein of the desired size (aprox. 110 kda) that is detected both by Tcf7l1 antibody and a-Flag antibody (Fig. 13D). Furthermore, we see ubiquitous expression of the fluorescent protein tdTomato in the Tcf7l1$^{fl/+}$;Rosa26$^{ctbcat\text{-}Tcf7l1fl/+}$ mice (Fig. 13E), yet the expression disappears in the AP2α-Cre;Tcf7l1$^{fl/fl}$; Rosa26$^{ctβcat\text{-}Tcf7l1fl/+}$ compound mutants in the regions where recombination occurs (Fig. 13G); AP2α-Cre;Tcf7l1$^{fl/fl}$ serves as a negative control (Fig. 13F). In brief, following Cre recombination, the TdT cassette is excised, allowing production of either dnTCF4 or ctβcat-Tcf7l1 mRNA (Fig. 14A).
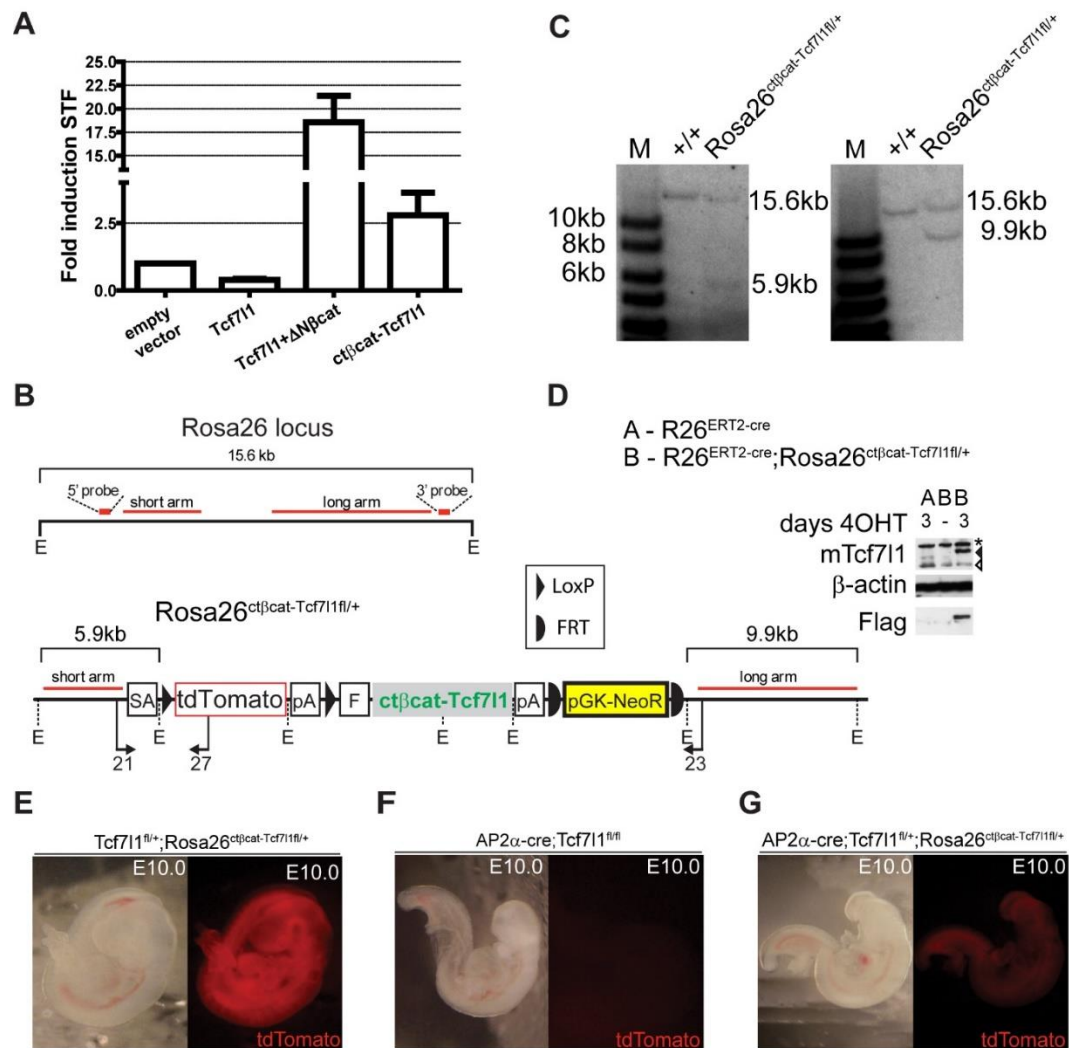
Figure 13: Generation and validation of the mouse strain Rosa26$^{ct\beta cat\text{-}Tcf7l1fl/+}$. (A) SuperTopFlash assay in HEK293 cells showed ctβcat-Tcf7l1 mediated activation of the Tcf/Lef driven luciferase reporter. **(B)** Scheme of the Rosa26 locus and the targeting vector, homology arms are depicted in red. E-EcoRI cleavage site, SA-splice acceptor, pA-poly-adenylation signal, pGK-NeoR–neomycin expressing cassette, F-Flag tag sequence, primers for genotyping 21-JM21F, 23-JM23R and 27-JM27R. **(C)** Southern blotting analysis confirms correct homologous recombination into mES cells, short arm is on the left. **(D)** Western blotting of lysates from mouse embryonic fibroblasts (MEFs) isolated from the R26$^{ERT2\text{-}cre}$;Rosa26$^{ctbcat\text{-}Tcf7l1fl/+}$ embryos revealed presence of the ctβcat-Tcf7l1 fusion protein (black arrowhead aprox. 115kDa), 3 days after administration of 4-OHT (4-*hydroxitamoxifen*). Black and white arrowhead marks endogenous Tcf7l1(aprox. 80kDa). * marks nonspecific binding of the antibody. **(E-G)** Tcf7l1$^{fl/+}$;Rosa26$^{ctbcat\text{-}Tcf7l1fl/+}$ mice ubiquitously express fluorescent protein tdTomato, the expression is lost in the regions where recombination occurs, visible in the AP2α-Cre;Tcf7l1$^{fl/fl}$; Rosa26$^{ct\beta cat\text{-}Tcf7l1fl/+}$ compound mutants. AP2α-Cre;Tcf7l1$^{fl/fl}$ serves as a negative control.

We crossed the Tcf7l1$^{fl/fl}$;Rosa26$^{dnTCF4fl/fl}$ and AP2α-Cre;Tcf7l1$^{fl/+}$ strains and evaluated the strength of the phenotype in AP2α-Cre;Tcf7l1$^{fl/fl}$;Rosa26$^{dnTCF4fl/+}$ compound mutants at E10.5 stage (scheme Fig. 14A). Intriguingly, overexpression of the dnTCF4 was able to reduce the occurrence of the strong phenotype by more than

half (27.3%), compared with the 60.5% incidence in the AP2α-Cre;Tcf7l1$^{fl/fl}$ mutant (Fig. 14D). The effect of the rescue on NC population was further analyzed by *in situ* hybridization showing no or very little ectopic *Sox10* mRNA in the AP2α-Cre;Tcf7l1$^{fl/fl}$;Rosa26$^{dnTCF4fl/+}$ 'rescued' mutants (arrowheads, Fig. 14B,C). On the contrary, the βcat-Tcf7l1 fusion protein showed significantly (P=0.049, Fishers test) decreased capability to rescue the phenotype than the dnTCF4; 85.7% of analyzed embryos displayed the strong phenotype in the AP2α-Cre;Tcf7l1$^{fl/fl}$; Rosa26$^{ctβcat-Tcf7l1fl/+}$ compound mutants (Fig. 14D). Expression of ctβcat-Tcf7l1 was also unable to reduce the aberrant *Sox10*-positive NC population (Fig. 14B,C). In summary, using transgenic mice strains, we have genetically confirmed that Tcf7l1, acting as a repressor of Wnt/β-catenin signaling, protects anterior neural tissue from conversion to a NC cell fate.
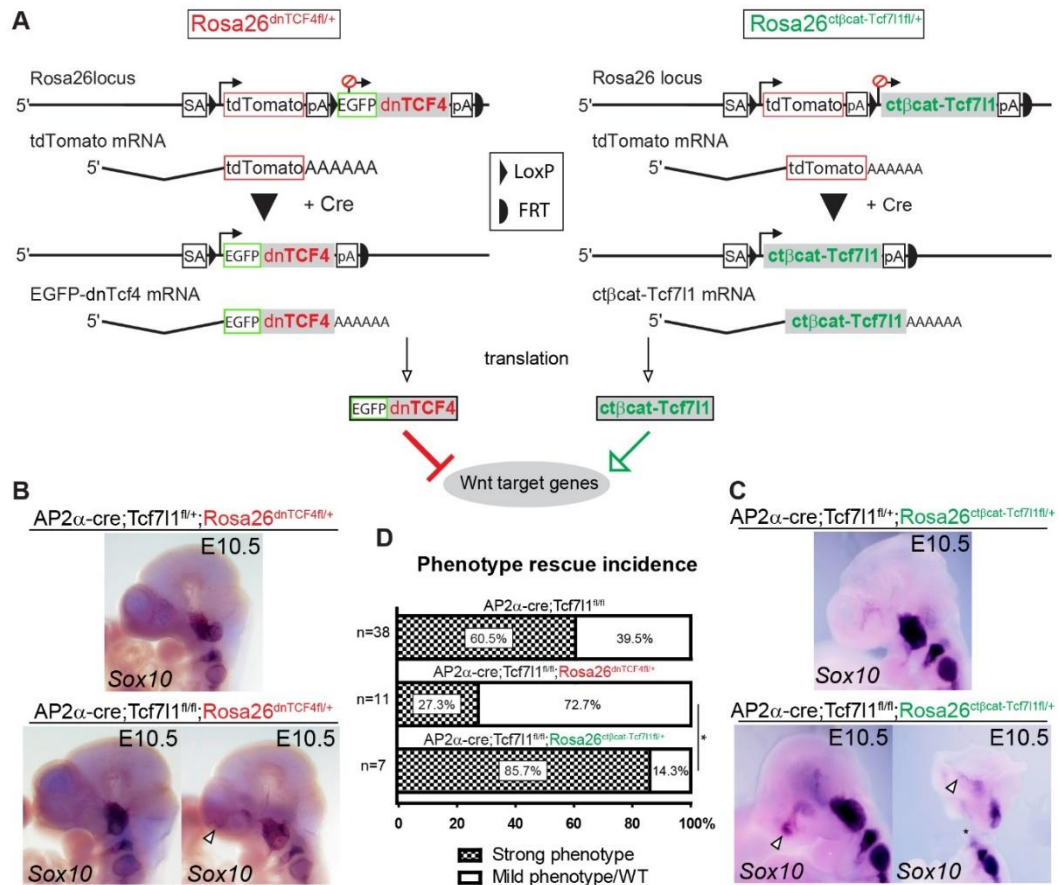
Figure 14: A constitutively expressed repressive form of Tcf is able to rescue the ANF absence in AP2α-Cre;Tcf7l1^{fl/fl} mutants. (A) Diagram describing Rosa26^{dnTCF4fl/+} and Rosa26^{ctβcat-Tcf7l1fl/+} mouse strains used in the rescue experiments. SA-splice acceptor, pA-poly-adenylation signal. **(B,C)** Expression of *Sox10* transripts was either unaffected or only mildly affected in the AP2α-Cre;Tcf7l1^{fl/fl};Rosa26^{dnTCF4fl/+} compound mutants (arrowheads), confirming the rescue capability of dnTCF4. AP2α-Cre;Tcf7l1^{fl/fl}; Rosa26^{ctβcat-Tcf7l1fl/+} compound mutants displayed no rescue or even stronger phenotype. Asterisk (*) marks artifact caused during sample preparation. (D) Quantification of the strong and mild phenotype incidence and rescue capability of dnTCF4 and ctβcat-Tcf7l1, scored at E10.5-13.5. *(P=0.049, Fishers test), n-number of embryos analyzed.

2) Investigation of the effects of *Tcf7l1* deletion on the anterior pre-placode, with the focus on lens placode development in mouse

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Results presented in this chapter are unpublished data

Tcf7l1 positively regulates expression of the anterior PPR and ANF specifiers

In order to better characterize the global effects of *Tcf7l1* deletion, we employed differential gene expression analysis. We took advantage of the EYFP fluorescence of the Rosa26$^{EYFPfl/+}$ mouse strain and isolated the NPB cells from the 6-8s Rosa26$^{EYFPfl/+}$;AP2α-Cre;Tcf7l1$^{fl/fl}$ mutant and Rosa26$^{EYFPfl/+}$;AP2α-Cre;Tcf7l1$^{fl/+}$ control embryos using FACS sorting (scheme of the experimental workflow is in Fig. 15A). RNA isolated from sorted cells was amplified and processed on Illumina MouseRef-8v2.0 Expression BeadChip, while collected data were analyzed in R-environment using Bioconductor packages.

Expression profiling (Fig. 15B) revealed downregulation of several Wnt antagonists, among which *Dkk1* and *Tcf7l1,* while various components of the Wnt/β-catenin pathway, such as *Wnt1* and *Lef1*, were upregulated, confirming the whole mount RNA *in situ* hybridization and BAT-gal reporter data shown in previous section of the results (Fig. 11). Analysis also confirmed the previously shown (Fig. 8A) downregulation of *Six3* and *Fgf8* and enriched the list of the compromised ANF specifiers with *Zic1*, *Zic5*, and *Hesx1* genes, whose expression was more than 4 times downregulated. We did not observe many changes in the expression of the NC specifiers (with the exception of a 1.84 and 0.76 fold increase case of *Foxd3* and *Pax3*, respectively). This could be attributed to the fact that their relatively high expression along the entire NPB masks the effects of *Tcf7l1* deletion in the most anterior part of the NPB.

Next, we focused on genes connected with PPR development. Unfortunately, combination of variability between different stages, penetrance of the phenotype and the subsequent amplification step introduced inconsistency into the data set that hampered the statistical significance of the accumulated data. Nevertheless, the overall changes in the expression levels of several key regulators of the placodal

development from the Six, Eya and Dlx gene families as well as like *Pax6* and *Otx2* (Fig. 15B), suggest an important role of *Tcf7l1* in expression of the anterior PPR genes The most pronounced changes were observed for those genes that specify anterior PPR (normally devoid of active Wnt/β-catenin siganling), i.e. *six5*, *otx2*, *pax6*, *six4*, *six3*, *eya2* and *eya3*, which were downregulated in Rosa26$^{EYFPfl/+}$;AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants. On the other hand, the expression of more posteriorly localized genes, such as *foxi1*, *dlx1*, and *pitx2*, which are normally exposed to intermediate levels of Wnt/β-catenin signaling activity, remained unchanged.

It has been shown that Pax6 and genes from Six and Eya families play important role in development of adenohypophyseal, nasal and lens placodes during mouse embryogenesis (Chen et al., 2009; Dellovade et al., 1998; Grindley et al., 1995; Hogan et al., 1986; Lagutin et al., 2003; Purcell et al., 2005). Gross morphology of the AP2α-Cre;Tcf7l1$^{fl/fl}$ "strong" mutants (Fig. 6H) at embryonic day 9.0-9.5 prevented analysis of these structures, so we decided to score the placodal defects of the "mild" mutants. EYFP signal in the Rosa26$^{EYFPfl/+}$;AP2α-Cre;Tcf7l1$^{fl/fl}$ "mild" mutants and Rosa26$^{EYFPfl/+}$;AP2α-Cre;Tcf7l1$^{fl/+}$ control embryos at E10.5 stage allowed us to identify recombined tissue and compare its morphology (Fig. 16A,B). Mutant embryos did not developed nasal structures and eyes, the trigeminal ganglion was not formed properly, but the otic vesicle appeared to be without obvious defects (Fig. 16B) when compared with the control embryos from the same stage (Fig. 16B). Immunohistochemical staining of the sections from the AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants confirmed expected absence of Tcf7l1 protein in the region of future lens placode (arrows, Fig. 16D), while it is present in the controls at embryonic stage E9.5 (arrows, Fig. 16C). Immunostaining with antibody for ectodermal marker Tfap2α revealed complete absence of ectodermal thickening and failure in lens pit formation in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutant (Fig. 16F,F´), when compared to AP2α-Cre;Tcf7l1$^{fl/+}$ controls (Fig. 16E,E´) at E10.5 stage. Even though these results looked promising, low overall incidence of the "mild" mutants (39.5%) (Fig. 6I) and substantial variability in observed defects caused by broad region of AP2α-Cre activity, led us to employ different Cre

expressing mouse strain and focus on the effects of *Tcf7l1* deletion during lens placode development.

A

AP2α-Cre;Rosa26^EYFPfl/+

NPB
EYFP

↓

FACS

⇓

aprox. 2000
EYFP⁺ cells/
embryo

⇓

RNA isolation

⇓

Reverse transcription,
amplification

⇓

DNA microarray
analysis

B

SYMBOL	GENENAME	logFC	FDR
Negative regulators of the Wnt/-catenin pathway			
Crebbp	CREB binding protein	-1.35	0.056
Nkd2	naked cuticle 2 homolog (Drosophila)	-1.17	0.055
Dkk1	dickkopf homolog 1 (Xenopus laevis)	-0.82	0.17
Nkd1	naked cuticle 1 homolog (Drosophila)	-0.81	0.17
Tcf7l1	transcription factor 7 like 1 (T cell specific, HMG box)	-0.72	*0.05
Components of Wnt pathway			
Dvl1	dishevelled, dsh homolog 1 (Drosophila)	0.58	0.16
Lrp6	low density lipoprotein receptor-related protein 6	0.72	0.065
Tcf7l2	transcription factor 7 like 2, T cell specific, HMG box	0.86	0.2
Wnt1	wingless-type MMTV integration site family, member 1	1.1	0.14
Lef1	lymphoid enhancer binding factor 1	2.11	0.056
ANF genes			
Zic1	zinc finger protein of the cerebellum 1	-2.26	*0.017
Hesx1	homeobox gene expressed in ES cells	-2.12	*0.047
Zic5	zinc finger protein of the cerebellum 5	-0.6	*0.038
Fgf8	fibroblast growth factor 8	-0.97	0.059
Six3	sine oculis-related homeobox 3	-0.76	0.21
Sox2	SRY (sex determining region Y)-box 2	-0.71	0.22
NC genes			
Foxd3	forkhead box D3	0.92	0.16
Pax3	paired box 3	0.38	0.12
PPR genes			
Six5	sine oculis-related homeobox 5	-1.47	0.19
Otx2	orthodenticle homolog 2	-1.19	0.16
Pax6	paired box 6	-1.13	0.11
Six4	sine oculis-related homeobox 4	-0.85	0.16
Six3	sine oculis-related homeobox 3	-0.76	0.21
Eya2	eyes absent 2 homolog (Drosophila)	-0.46	0.11
Eya3	eyes absent 3 homolog (Drosophila)	-0.77	*0.048
Foxi1	forkhead box I1	-0.2	0.16
Dlx1	distal-less homeobox 1	-0.19	0.13
Pitx2	paired-like homeodomain transcription factor 2	-0.15	0.14

A
WNT
P

Figure 15: Tcf7l1 *deletion* results in reduced expression of the ANF and anterior PPR genes. (A) Scheme of the experimental workflow. RNA isolated from sorted EYFP⁺ cells of the Rosa26^EYFPfl/+;AP2α-Cre;Tcf7l1^fl/fl mutants (n=4) and Rosa26^EYFPfl/+;AP2α-Cre;Tcf7l1^fl/+ controls (n=4) at 6-8s stage was amplified and subjected to processing on Illumina MouseRef-8v2.0 Expression BeadChip. **(B)** Data from the DNA microarray analysis were analyzed in R-environment using Bioconductor packages. Elimination of the most outlying values for mutant and control datasets was performed. Red and blue colors represent upregulated and downregulated genes in the mutants, respectively. A-anterior, P-posterior, FDR-False discovery rate.
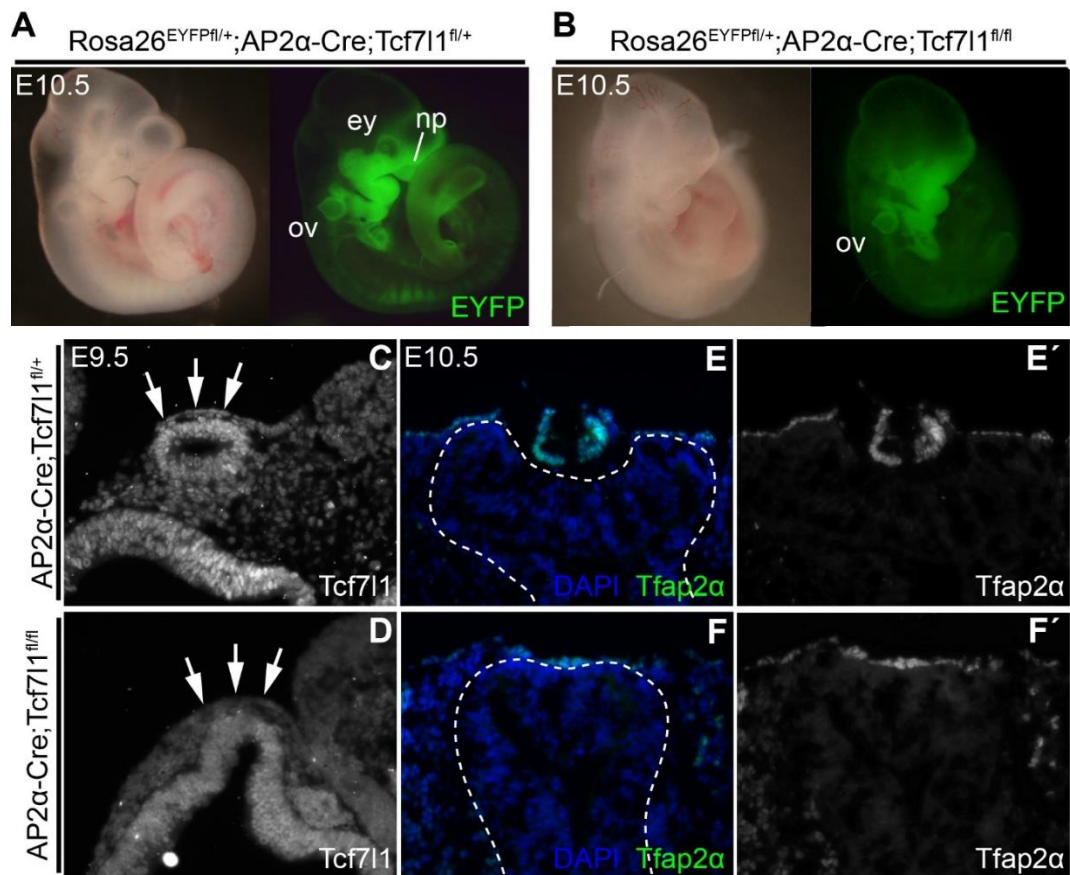
Figure 16: Placodal defects resulting from *Tcf7l1* deletion. (A,B) Comparison of morphology between Rosa26^EYFPfl/+;AP2α-Cre;Tcf7l1^fl/+ controls (A) and Rosa26^EYFPfl/+;AP2α-Cre;Tcf7l1^fl/fl mutants (B) at E10.5 stage. **(C,D)** Immunohistochemical analysis of the developing lens placode. Tcf7l1 protein is present in controls (C) and disappears in presumptive lens ectoderm of the AP2α-Cre;Tcf7l1^fl/fl mutants (D) at E9.5. **(E-F´)** Tfap2α positive lens pit, present in controls (E,E´) is completely missing in AP2α-Cre;Tcf7l1^fl/fl mutants (F,F´) at E10.5. Dashed line marks the developing retina. ov-otic vesicle, ey-eye, np-nasal placode.

Conditional deletion of *Tcf7l1* in the lens placode causes severe defects in lens development

In order to delete *Tcf7l1* during early events of the lens placode development, we have crossed the Tcf7l1 conditional knock-out mouse strain (EUCOMM) with Lens-Cre (Ashery-Padan et al., 2000). This Cre expressing mouse strain has been widely used in studies focused on early lens development, due to its ability to deliver recombination specifically in lens placode ectoderm from E9.0 onward (Ashery-Padan et al., 2000).

Histochemical staining on cryosections from the developing eye revealed that deletion of critical exons in *Tcf7l1* results in loss of Tcf7l1 protein expression (Fig. 17A´,B´), along with reduction of the lens size in the Lens-Cre;Tcf7l1$^{fl/fl}$ mutants, when comparing with the control embryos of the same developmental stage (E13.5) (Fig. 17A,B). Adult Lens-Cre;Tcf7l1$^{fl/fl}$ animals were viable, but displayed severe microphthalmia. Sectioning and histochemical analysis at post-natal day 21 revealed severe reduction of the lens size (black arrowhead, Fig. 17D´), together with reduced or missing pupillary openings (black and white arrowhead, Fig. 17D´) in the mutants (Fig. 17C-D´).
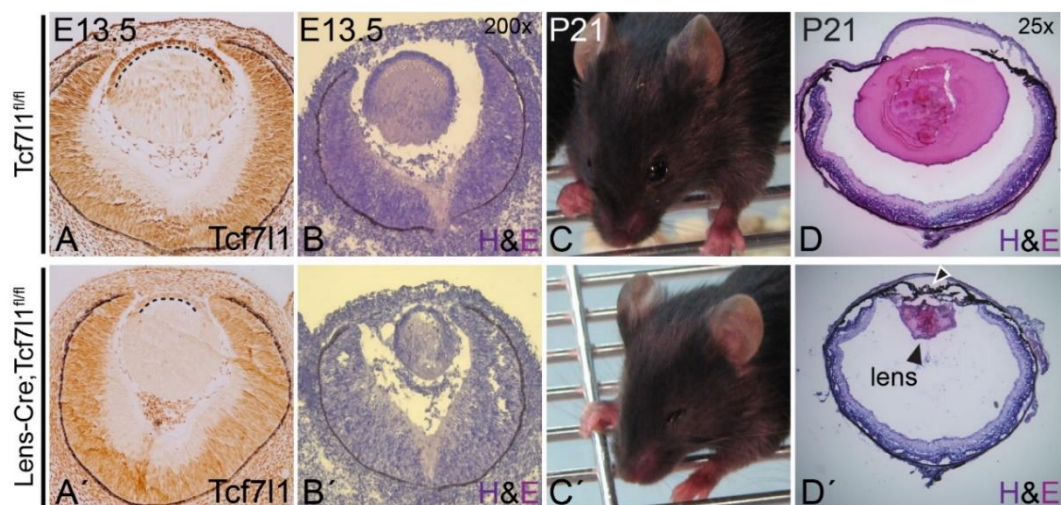
Figure 17: *Tcf7l1* is required for proper lens formation. (A,A´) immunohistochemical staining of Tcf7l1 protein on cryosections from developing eyes of control and Lens-Cre;Tcf7l1$^{fl/fl}$ mutant embryos at E13.5 stage. Note the reduction in the length of lens epithelium (dashed line). **(B-D´)** Hematoxylin & eosin (H&E) staining revealed progressive reduction of the lens size in the mutants (B´,D´) when compared with corresponding controls from E13.5 to P21 stage (B,D). Macroscopically noticeable microphthalmia of Lens-Cre;Tcf7l1$^{fl/fl}$ in pups at P21 stage (C´) is not present in controls of a same stage (D).

Tcf7l1 is not required during early differentiation of the developing lens

It has been shown that aberrant activation of the Wnt/β-catenin signaling pathway in the lens surface ectoderm arrests lens placode specification and completely disrupts eye development (Machon et al., 2010; Smith et al., 2005). However, when activation of the pathway occurs in later stages of the embryonic development (E10.5), lens develop, but display defects in differentiation of the lens epithelium or lens fiber cells. This has been shown either through stabilization of the β-catenin using MLR10-Cre (Martinez et al., 2009) or using transgenic mice expressing constitutively active form of *Lef1* (αA-CLEF) under the control of αA-crystallin promoter (Antosova et al., 2013).

To detect changes in the lens fiber cell differentiation, we performed immunohistochemical analysis on cryosections from developing eyes and compared the expression of differentiation markers in fiber cell of the Lens-Cre;Tcf7l1^{fl/fl} mutants and Tcf7l1^{fl/fl} controls at E13.5. Surprisingly, staining for Prox1 and C-Maf as well as γ-crystallin revealed no changes between mutant and control lenses (Fig. 18A-C´). We repeated this type of analysis, this time focusing on proteins that are involved in the maintenance of the lens epithelium. Staining of the sections, using antibodies against Pax6, FoxE3 and E-cadherin, also revealed no difference between mutant and control lenses at E13.5 (Fig. 18D-F´).
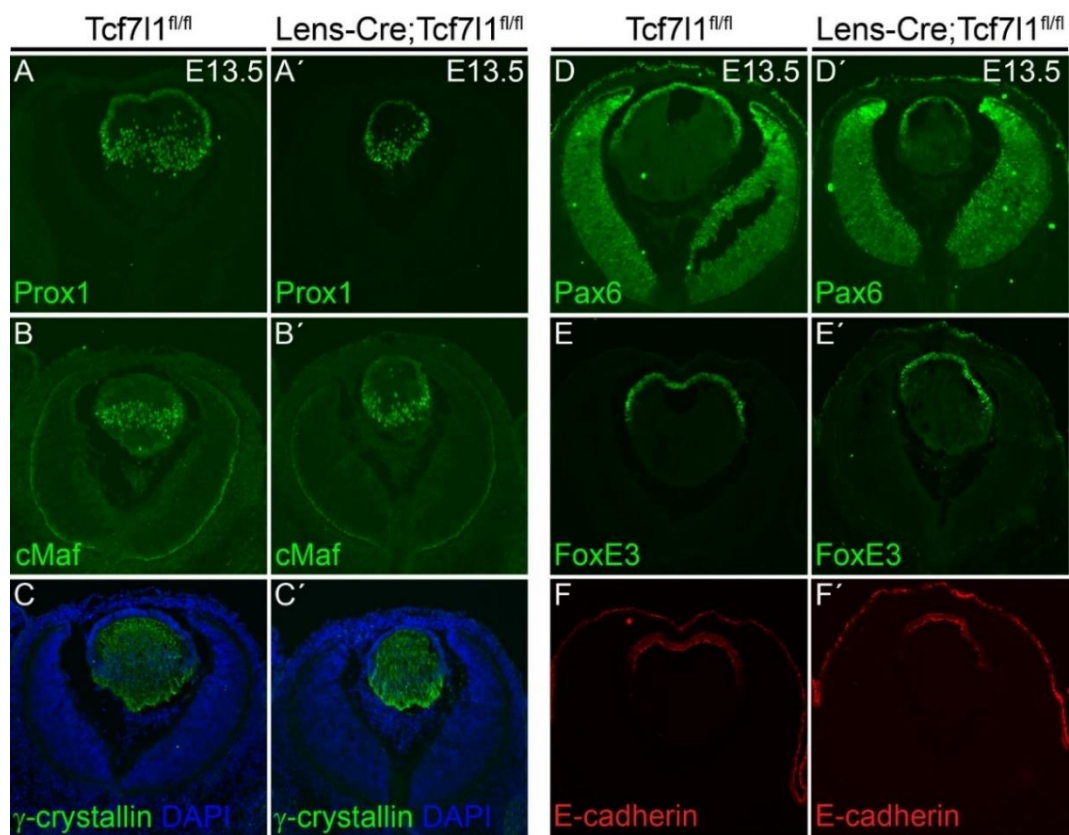
Figure 18: Deletion of *Tcf7l1* does not affect lens differentiation. (A-F´) Immunohistochemical staining of cryosections from embryonic eyes at E13.5 stage revealed no changes between Lens-Cre;Tcf7l1^{fl/fl} mutants and controls, in regard to the expression of the early lens marker Prox1 (A,A´), markers of fiber cells cMaf (B,B´) and γ-crystallin (C,C´), as well as of markers of lens epithelium, namely Pax6 (D,D´), FoxE3 (E,E´), and E-cadherin (F,F´).

Absence of *Tcf7l1* results in the cell cycle misbalance and apoptosis of the fiber cells

Wnt/β-catenin signaling pathway is known to regulate cell cycle progression (reviewed in Niehrs and Acebron (2012)). There were no changes in lens differentiation following *Tcf7l1* deletion, yet the size of the lens appeared to be smaller at E13.5, therefore we decided to evaluate putative changes in proliferation or cell death.

Quantification of the BrdU incorporation assay revealed no significant difference in the total amount of BrdU$^+$ cells between the mutant and control lenses at E13.5 stage (Fig.19C). Surprisingly, we found statistically significant (P<0,05) increase in ectopic BrdU$^+$ fiber cells in Lens-Cre;Tcf7l1$^{fl/fl}$ mutants (arrows Fig. 19A´), that were not present in controls, since these cells are post-mitotic (Fig. 19A,B). To further confirm the presence of proliferating cells in the fiber cell compartment, we performed double immunohistochemistry using antibodies against PCNA and Pax6, markers of proliferating and epithelial cells, respectively (Fig. 19D-E´). Concordantly, we found aberrant PCNA$^+$ fiber cells present in the mutants, when compared with controls (arrows Fig. 19D,D´). Quantification confirmed there is a significantly (P<0,001) higher number of the Pax6$^-$/PCNA$^+$ cells in the mutants than in controls at the E13.5 stage (Fig. 19E-F).

Disruption of the cell cycle often leads to cell death via apoptosis. Immuno-histochemical analysis showed Caspase-3 staining among fiber cells (E-cadherin$^-$) in the mutants but not in the controls at E13.5 (Fig. 19G,G´). More detailed quantification of the Caspase-3$^+$ cells revealed a significant increase of the apoptosis in the lenses of Lens-Cre;Tcf7l1$^{fl/fl}$ mutants. The highest number of the ectopic Caspase-3$^+$ cells was observed in the mutants at E11.5 (p<0.01) and was still present (to lesser extent) at E13.5 stage (p<0.05) (Fig. 19G-I). Comparison of the total amount of cells (DAPI$^+$) in mutant and controls, at E13.5 stage, revealed more than 30% decrease (P<0,001) of the cell numbers in the mutant lenses (Fig. 19J). The Pax6$^-$ and E-cadherin$^-$ population of fiber cells showed similar decrease in numbers of DAPI$^+$ nuclei, suggesting that the loss of cells is equally spread among epithelial and fiber compartment (Fig. 19J). This data illustrate that *Tcf7l1* deletion in the lens placode will not allow lens epithelial cells to terminate their cell cycle progression during maturation into fiber cells, and are eventually driven to apoptosis.
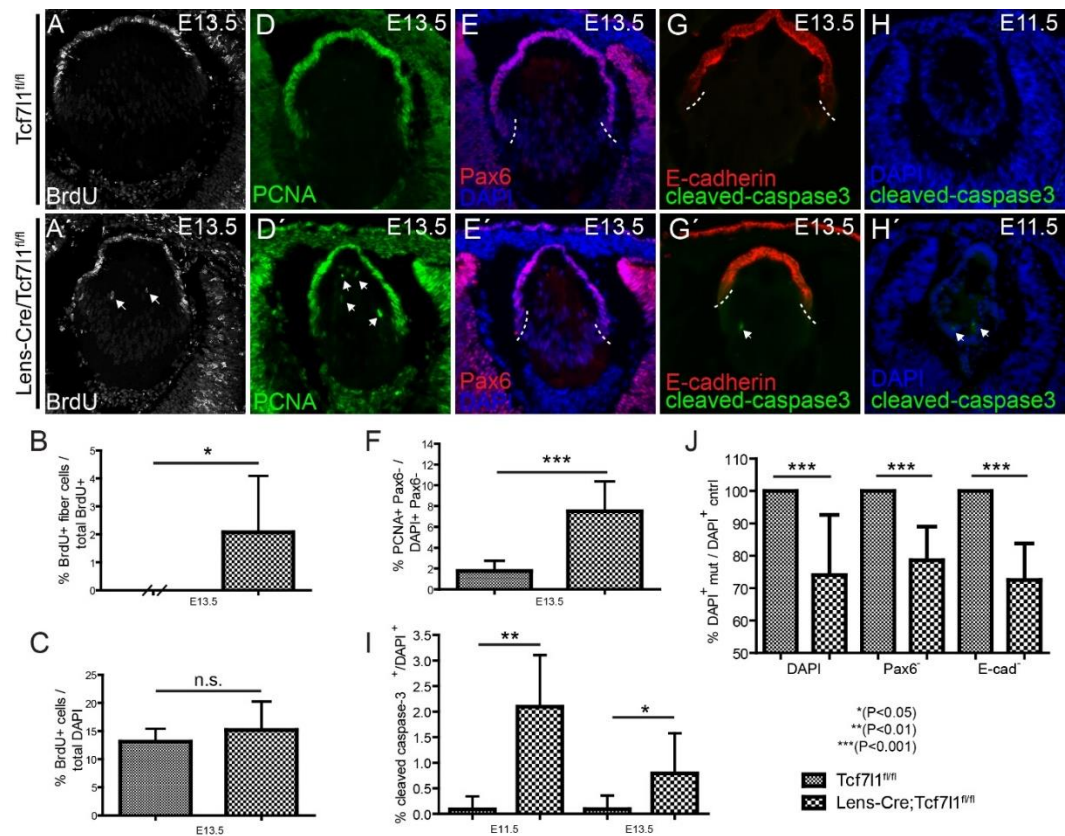
Figure 19: Absence of Tcf7l1 causes aberrant proliferation of fiber cells. (A,A´) Confocal images of cryosections from E13.5 lenses after 1h BrdU pulse, stained with anti-BrdU antibody. Arrows in (A´) indicate BrdU+ fiber cells in lenses of Lens-Cre;Tcf7l1fl/fl mutants. **(B,C)** total ratio of BrdU+ nuclei per total nuclei at E13.5 was not significantly changed (n=7) (C). Significantly higher number of BrdU+ fiber cells in E13.5 mutant lenses in comparison with controls (B). **(D-F)** cryosections of control (D,E) and mutant (D´,E´), stained with antibody against the marker of proliferation PCNA and the marker of epithelial cells Pax6. PCNA+ cells among the fiber cells (Pax6-) in Lens-Cre;Tcf7l1fl/fl mutants are marked by arrows (E´). Significant increase of PCNA+ cells were observed in the mutants (p<0.001, T-test) when compared with controls (nmut =16, ncntrl=11) (F). **(G-J)** Immunohistochemical staining, using the marker of epithelial cells E-cadherin and the marker of apoptosis cleaved caspase-3, revealed apoptotic cells among the fiber cells of the Lens-Cre;Tcf7l1fl/fl mutants (G´,H´) but not in the controls (G,H) at E11.5 and E13.5 stage. Quantification of the cleaved caspase 3 staining showed significant increase in apoptosis in the mutants at E11.5 (p<0.01, T-test) and E13.5 stage (p<0.05, T-test) (I). E13.5 mutant lenses are significantly hypo-cellular (p<0.001, T-test) (J). Nuclei were stained with DAPI.

Tcf7l1 is negative regulator of the Cdkn1a[P21/Waf] expression during lens development

Regulation of the cell cycle progression is achieved through interaction of many proteins forming multiple feedback loops. In order to identify which particular genes might be responsible for the proliferation defects we observed in the lenses of Lens-Cre;Tcf7l1^{fl/fl} mutants, we employed differential gene expression analysis.

We have dissected 22 lenses from Lens-Cre;Tcf7l1^{fl/fl} mutants and 28 lenses from control animals at embryonic stage E16.5-17.0. We have divided them randomly into 3 mutant and 3 control groups (lenses?) and isolated the RNA. Extracted RNA was processed on Illumina MouseRef-8v2.0 Expression BeadChip and collected data were analyzed in R-environment, using Bioconductor packages. Among the 38 significantly ($p < 0.05$ & $|logFC| \geq 1$) altered genes, 30 were upregulated and 8 downregulated (Fig. 20). The fact that more genes were up- rather than down-regulated, is in line with previous research, suggesting that *Tcf7l1* functions predominantly as a transcriptional repressor (Cole et al., 2008; Liu et al., 2005; Ombrato et al., 2012). One of the most upregulated genes (4.76 fold) was *cyclin dependent kinase inhibitor 1* a (*Cdkn1a*), also known as p21[Cip1/Waf]. At low levels, p21 promotes cell cycle progression; under stress conditions, is its expression increased in a p53-dependent manner, leading to cell cycle arrest in G1 phase (reviewed in Jung et al. (2010)).
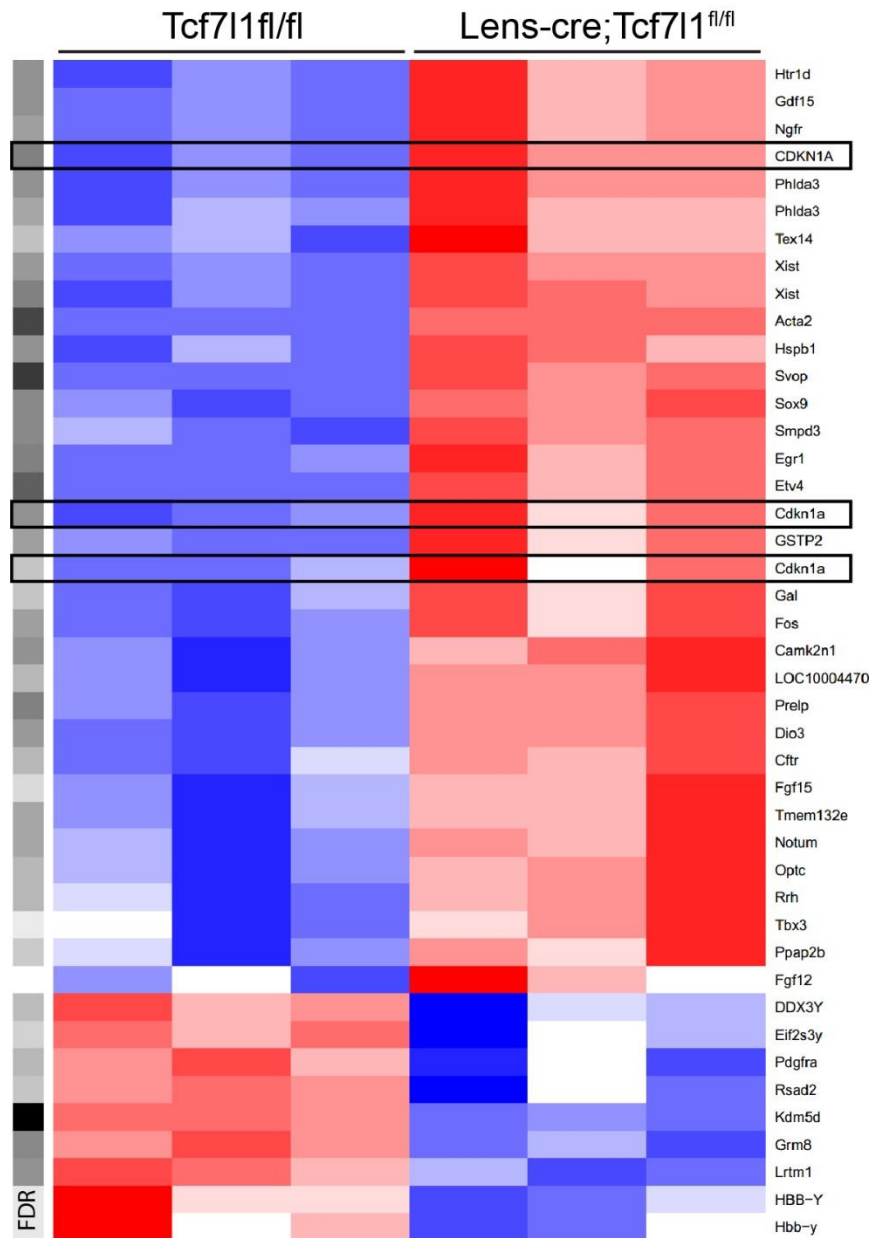
Figure 20: Heatmap showing patterns of differentially expressed genes in mutant and control, based on Z-score of probe intensity. RNA was isolated from dissected lenses of Lens-cre;Tcf7l1^{fl/fl} mutants and Tcf7l1^{fl/fl} controls at E16.5-17.0 stage. Analysis was performed on biological triplicates (p<0.05 & |logFC|≥1). Data from Illumina MouseRef-8v2.0 Expression BeadChip were analyzed in R-environment, using Bioconductor packages. Red represents genes upregulated and blue represents genes downregulated in the Tcf7l1^{fl/fl} x Lens-Cre ;Tcf7l1^{fl/fl} lenses. FDR-False discovery rate

Immunohistochemical staining confirmed the p21 upregulation in eye sections of Lens-Cre;Tcf7l1^{fl/fl} mutants (arrows, Fig. 21B,D), while it was not detectable in the control lenses (Fig. 21A,C) at E11.5 and E13.5 stages. Furthermore, co-staining with E-cadherin, the marker of epithelial cells, revealed delay in formation of the lens vesicle in the mutant embryos, at embryonic stage E11.5 (Fig. 21A´, B´). This is also manifested also by the presence of a lens stalk in Lens-Cre;Tcf7l1^{fl/fl} mutants (arrowhead Fig. 21D´) not present in controls, at E13.5 (Fig. 21C´).

It was shown recently, that, when crossed to certain genetic backgrounds, mice hemizygous for Lens-Cre transgene can develop various eye abnormalities, including smaller lens size (Dora et al., 2014). To exclude the possibility that phenotype observed in Lens-Cre;Tcf7l1^{fl/fl} mutants is caused by Lens-Cre transgene, we crossed our Tcf7l1^{fl/fl} strain with transgenic line LR-Cre. In this transgene, expression of the Cre-recombinase is driven by three copies of a lens-specific element derived from *Pax6* and starting its expression both in lens placode and retina, between the E9.0-9.5 stage (Kreslova et al., 2007). LR-Cre;Tcf7l1^{fl/fl} mutant mice also exhibit disrupted lens development, presence of the lens stalk and high number of p21^{+} cells at E13.5 (Fig. 21F,F´), when compared with LR-Cre;Tcf7l1^{fl/+} controls from the same stage (Fig. 21E,E´). Control crossing of Tcf7l1^{fl/fl} mouse strain with early retina specific Cre-recombinase expressing transgenic mouse strain Rx-Cre (Klimova et al., 2013) was performed to exclude possible contribution of the Tcf7l1 depletion in retina in the LR-Cre;Tcf7l1^{fl/fl} mutants. Histological analysis of the Rx-Cre;Tcf7l1^{fl/fl} mutants showed no obvious difference in lens and retina morphology between the mutant and control embryos at postnatal stage P0 (Fig. 21G,G´).

Ultimately, we wanted to test the effects of *Tcf7l1* on p21 expression, using p21-Luc luciferase reporter (van de Wetering et al., 2002) in tissue cultures. In this construct, a 2.3 kb fragment of the P21[CIP1/WAF1] promoter sequence was cloned into a luciferase reporter vector. We isolated MEFs from Ert2-Cre;Tcf7l1^{fl/fl} embryos. In this cell line, administration of the 4-*hydroxitamoxifen* (4-OHT) induces recombination, generating a Tcf7l1 KO cell line. We transiently transfected p21-Luc into Tcf7l1 KO MEFs, 3-fold increase in activity of the promoter was observed, however this activity was abolished by co-transfection of Tcf7l1 expressing plasmid (Fig. 21H), confirming that p21 is directly repressed by Tcf7l1.
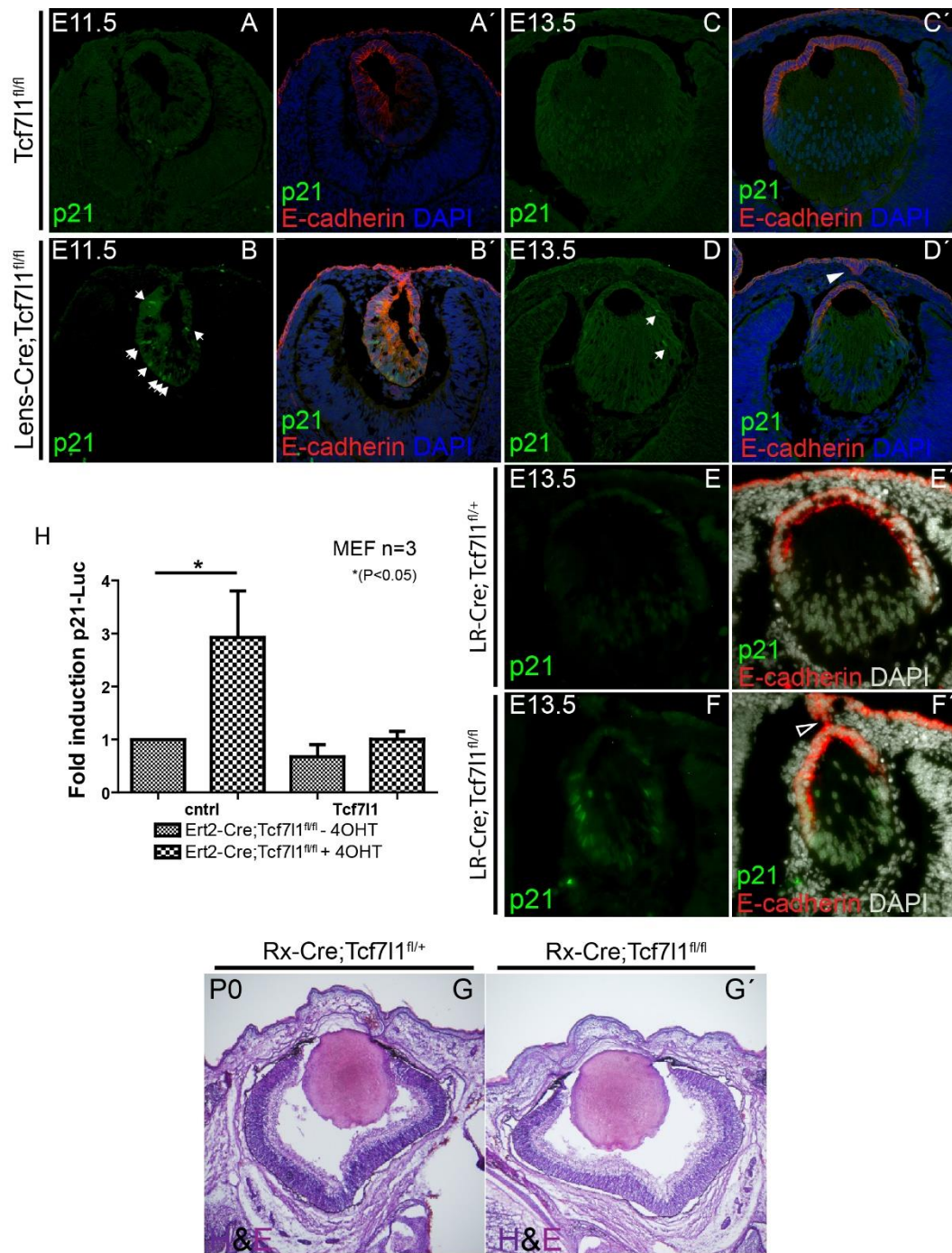
Figure 21: Cdkn1a$^{P21/Waf}$ is negatively regulated by *Tcf7l1*. (A-D) immunohistological staining of p21 and E-cadherin proteins on eye cryosections from Lens-Cre;Tcf7l1$^{fl/fl}$ mutants and controls at E11.5 and E13.5 stages. **(E-F)** immunohistological staining of p21 and E-cadherin proteins on eye cryosections from LR-Cre;Tcf7l1$^{fl/fl}$ mutants and controls at E13.5 stage confirms the upregulation of p21 expression and delay in progression of lens development observed in Lens-Cre;Tcf7l1$^{fl/fl}$ mutants. **(G,G´)** Hematoxylin and eosin staining of the control and Rx-Cre;Tcf7l1$^{fl/fl}$ mutants at P0 stage reveal no obvious difference in lens and retina development. **(H)** Transient transfection of the p21-Luc into mouse embryonic fibroblasts (MEFs) isolated from Ert2-Cre;Tcf7l1$^{fl/fl}$ and treated with 4-*hydroxitamoxifen* (4-OHT) revealed 3 fold induction of the reporter (p<0,05, n=3), this activation was lost in the case of co-transfection with Tcf7l1 expressing plasmid.

Discussion

How does the progressive activation of Wnt/β-catenin pathway affect development of the anterior neural tissue, preplacodal region and neural crest?

Spatio-temporal regulation of Wnt/β-catenin pathway activation, which is required for proper development of the prosencephalon, is secured by expression of several ANF specific negative regulators of the Wnt/β-catenin signaling. Our data, together with evidence from others (Cole et al., 2008; Liu et al., 2005; Ombrato et al., 2012; Wu et al., 2012) characterize *Tcf7l1* as a Wnt/β-catenin target gene repressor. Aberrant activation of the Wnt/β-catenin pathway, due to Tcf7l1 systemic deficiency, is considered the cause of strong A-P patterning defects both in zebrafish (Dorsky et al., 2003; Kim et al., 2000) and mouse (Merrill et al., 2004). In zebrafish, the Tcf7l1a loss-of-function in headless (*hdl*) mutants, as well as MO-mediated knock-down of Tcf7l1a and Tcf7l1b variants, result in expansion of the expression of the MHB markers *Pax2.1* and *Gbx1* anteriorly, accompanied by the loss of *Six3* and *Rx3* expression (Dorsky et al., 2003). The Tcf7l1 mice mutants also displayed expansion of the midbrain marker *Engrailed,* joined with the downregulation of *Hesx1* expression (Merrill et al., 2004). Similarly, deletion of *Dkk1* (Mukhopadhyay et al., 2001), *Six3* (Lagutin et al., 2003), *Hesx1* (Andoniadou et al., 2007) and *ICAT* (Satoh et al., 2004) in mouse is followed by the loss of anterior neural tissue, a fact that has been interpreted as a defect in A-P patterning induced by aberrant Wnt/β-catenin signaling. We think that this is a rather generalized view and that, in the context of the ANF phenotypes, is only partially true. In *Xenopus*, low levels of ectopic Wnt/β-catenin activation enrich the NC population without affecting A-P patterning, while high doses affect both (Wu et al., 2005). This might be also valid for mammals, as mice carrying ENU-induced activating point-mutations in Lrp6 and β-catenin display a gradual loss of the prospective prosencephalon, depending on the strength of the Wnt/β-catenin pathway overactivation, but do not manifest a shift of posterior markers towards the anterior part of the embryo (Fossat et al., 2011). Furthermore, compound deletion of one *Hesx1* allele and both alleles of *Tcf7l1* in mouse, using prosencephalon-specific Hesx1-Cre, results in the aberrant activation of Wnt/β-catenin signaling, accompanied with loss of anterior neural tissue and no effect on the A-P patterning (Andoniadou et al., 2011). Similarly, we did not detect any changes in the expression of the MHB markers *Fgf8* or *Gbx2* in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants. Nevertheless, we did observed a gradual loss (depending on the strength of the phenotype) of the posterior prosencephalon marker *Tcf7l2* and the ANF markers *Six3, Fgf8* and *FoxG1*. This was

accompanied by the expansion of *Sox9, FoxD3* and *Sox10*-positive NC populations along the NPB anteriorly. We therefore conclude that the loss of Tcf7l1 mediated repression of Wnt target genes in the AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants allows the gradient of Wnt ligands to influence cell fate in a wider region of the ANF than in controls, without having an effect on A-P patterning. This is not the case with AP2α-Cre;β-cat$^{Ex3fl/+}$ mutants, in which ectopic stabilization of β-catenin facilitates strong over-activation of Wnt/β-catenin driven transcription, affecting both NC and A-P. Notably, we observed effects on the population of undifferentiated Sox9-positive NC cells in AP2α-Cre;β-cat$^{Ex3fl/+}$ mutants at E9.5 and earlier. In contrast, the imbalance in NC differentiation observed in Wnt1-Cre/β-cat$^{Ex3fl/+}$ mutants occurred one day later, at E10.5 (Lee et al., 2004). This merely reflects the earlier onset of Cre recombination in AP2α-Cre (Macatee et al., 2003) than in the widely used Wnt1-Cre (Danielian et al., 1998; Lewis et al., 2013), rendering AP2α-Cre a more suitable tool for studying early events of NC development. Our data provide solid evidence for earlier role of the Wnt/β-catenin pathway in NC development in regard to previously published findings from the mouse model showing the requirement of the Wnt/β-catenin pathway in NC maintenance and differentiation (Brault et al., 2001; Gay et al., 2015; Lee et al., 2004).

The effects of the Tcf7l1 deficiency in mice and zebrafish on NC development were not, until now, characterized in detail. Analogously to the NC expansion in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutant mice, we observed ectopic expression of *FoxD3, Pax3* and *Sox9* mRNA along the entire anterior NPB, upon Tcf7l1a MO-mediated knock-down in zebrafish, implicating the conservation of Tcf7l1 function across vertebrate species. In accordance with this, MO-mediated knock-down of *Dkk1* in *Xenopus* is followed be the enrichment of NC population, marked by the expansion of *Snail2* mRNA expression towards the anterior portion of the embryo (Carmona-Fontaine, 2007). *Hesx1* mutant mice display expansion of Wnt target genes, as well as of the NC markers *Pax3* and *FoxD3* anteriorly (Andoniadou et al., 2007). Furthermore, loss of the Wnt inhibitor Dkk1 has been shown to cause expansion of the NC mRNA marker *Sox10* (Carmona-Fontaine et al., 2007), being in agreement with the interpretation of the data presented here. Finally, mutual functional redundancy of these factors was demonstrated in zebrafish, where injection of either *Six3* or *Hesx1* mRNA rescued the *hdl* phenotype (induced by Tcf7l1a deletion) (Andoniadou et al., 2011; Lagutin et al., 2003).

Our analysis of the AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants identify Tcf7l1 as a negative regulator of the NC population. Given the fact that NC are positively regulated by

Wnt/β-catenin pathway in all studied vertebrates, other Tcf/Lefs should be responsible for activation of Wnt/β-catenin targets in the NC. It is likely that *Lef1*, *Tcf7* and *Tcf7l2* are redundant in NC cells, since whole body double knock-outs of Lef1/Tcf7 and Tcf7/Tcf7l2 in mice do not display any obvious NC phenotype (Galceran et al., 1999; Gregorieff et al., 2004). Ectopic Sox9-positive cells in AP2α-Cre;β-cat$^{\Delta Ex3fl/+}$ mutants spatially overlap with the elevation of the BAT-gal reporter, Lef1, Tcf7 but not Tcf7l2 proteins, suggesting that Lef1 and Tcf7 are positively regulated by Wnt/β-catenin signaling and probably participate in the expression of Wnt responsive NC specific genes. This is further supported by our microarray data showing more than 4 times increase in expression of *Lef1* in the NPB of the AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants. *Sp5*, another well-defined Wnt target gene and transcriptional repressor (Fujimura et al., 2007), was strongly upregulated in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants spreading its expression across the entire ANF. Work in *Xenopus* has identified *Sp5* as a novel NC-specific inducer (Park et al., 2013). MO-mediated knock-down of *Sp5* leads to the upregulation of *Zic1*, making it a candidate target of Sp5 repression. *Zic1* acts as a NC inducer only when expressed in appropriate balance with *Pax3*, another NC inducer (Hong and Saint-Jeannet, 2007; Park et al., 2013). *Pax3* has been recently identified as a direct target of Tcf/Lefs (Zhao et al., 2014), providing an additional link to the GRN driving NC induction. Aberrant induction of *Sp5*-mediated repression of *Zic1*, in combination with the ectopic expression of Wnt1 ligand which enhances the β-catenin/Tcf/Lef driven transcription of *Pax3*, might serve as an artificial positive feedback loop, further potentiating the effects on NC induction. Additionally, the aberrant Wnt/β-catenin signaling present in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants might upregulate the expression of the transcription factor *Axud1*, a novel Wnt-dependent, positive regulator of NC specification (Simoes-Costa et al., 2015).

Analysis of the microarray data from the Tcf7l1 mutant embryos at the 6-8s stage uncovered downregulation of PPR genes involved in specification and maintenance of the nasal and lens placodes, namely members of the Six and Eya families (more specifically *Six*3-5, *Eya2,3*), of the master regulator of eye development *Pax6* and of *Otx2*, which is also involved in the establishing the anterior character of the head. It has been shown that Pax6 deficiency causes arrest of nasal, lens and adenohypophyseal placode development in mouse (Dellovade et al., 1998; Grindley et al., 1995; Hogan et al., 1986). Similar defects were described after *Six3* deletion in mouse, displaying anopthalmia and reduced nasal structures (Lagutin et al., 2003). Transcription factor *Six3* appears to be indispensable for *Pax6* expression during lens induction (Liu et al., 2006) forming in this way a core auto-regulatory

feedback loop of the PPR GRN. This loop is further maintained by *Pax6*-dependent expression of *Six3*, *Dach1*, and *Eya1* (Purcell et al., 2005). In nasal placode, *Pax6* expression is retained thanks to transcription factors *Six1* and *Six4*, whose absence results in arrest of the placodal development (Chen et al., 2009). Interestingly, transcription factor FoxG1, generally not implicated in the placode development, is also expressed in developing nasal placode and its deficiency leads to severe arrest of nasal placode development in mouse (Duggan et al., 2008). In the first part of results, we show complete loss of both *Six3* and *FoxG1* mRNA expression in the AP2α-Cre;Tcf7l1^{fl/fl} mutants, thus we demonstrate requirement of *Tcf7l1* for the expression of the genes governing the development of anterior placodes. This was further confirmed by the fact that the Tcf7l1 "milder" mutants did not developed nasal and lens placodes. Recently published work (Gaston-Massuet et al., 2016) revealed requirement of the Tcf7l1-mediated control of Wnt/β-catenin signaling activity during development of pituitary gland (also PPR derivative) in mouse, further backing our hypothesis. Notably, Gaston-Massuet et al. also managed to link two different point mutations *TCF7L1* to congenital hypopituitarism in humans.

Our data from mRNA *in situ* hybridization, showing expression of Tcf7l1 both in the anterior neuroectodem and surrounding non-neural ectoderm of the early mouse neurula (1-5s), are in line with previous studies at pre-somitic stages (E7.5), where Tcf7l1 was detected in the anterior portion of all three germ layers (Merrill et al., 2004). This suggest that hampering of the Tcf7l1 function should affect only the most anterior part of the PPR. Concomitantly, the strong aberrant activation the BAT-gal signal in the most anterior tip of the neural- and non-neural-ectoderm at 8s stage in the AP2α-Cre;Tcf7l1^{fl/fl} mutants allows us to interpret the resulting phenotype as an effect of aberrantly activated Wnt/β-catenin signaling in developing PPR. First part of the results describes aberrant expansion of the NC population on expanse of the ANF tissue in Tcf7l1 deficient embryos, induced by increased activity of the Wnt/β-catenin pathway. The exact mechanism with which Wnt/β-catenin pathway promotes NC fate is still under intensive investigation, but according to available data (described above), it appears that several NC specific genes are direct targets of Wnt/β-catenin-dependent transcription. On the other hand, despite the few reported examples of genes negatively regulated by β-catenin:Tcf/Lef complex, reviewed in Hoverter and Waterman (2008), there is no evidence of Tcf/Lef interfering with transcription factors involved in the GRN of the anterior PPR. Similarly to the situation in ANF, it rather appears that anterior PPR is influenced by indirect effects of the ectopic Wnt/β-catenin signaling pathway activation in the NPB. We believe that over-abundant signaling

from excessively formed NC cells, which is associated with reduction of the pool of PPR progenitor cells at the NPB, is the major cause of the multiple placodal phenotypes in AP2α-Cre;Tcf7l1^{fl/fl}.

It is well known from zebrafish, *Xenopus* and chicken, that BMP (a member of TGF-β superfamily) and Wnt/β-catenin signaling inhibit the placodal development, while medium activity of the BMP pathway and high activity of the Wnt/β-catenin signaling is crucial for NC specification and maintenance during neurulation (Bhat et al., 2013; Kwon et al., 2010; Litsiou et al., 2005; Steventon et al., 2009). Under physiological conditions, secretion of these morphogens by migrating NC cells plays important role in restricting the spatial distribution of the placodes. This has been nicely demonstrated in chicken, where TGF-β1, Activin-βA and Activin-βB secreted from migrating NC cells in the mesoderm induced Smad3-dependent inhibition of *Pax6* expression in periocular ectoderm, which was further potentiated by activation of the Wnt/β-catenin signaling thanks to TGF-β-induced expression of Wnt2 ligand (Grocott et al., 2011). Studies in mouse confirmed the negative effect of nuclear β-catenin on *Pax6* expression, using Lens-Cre or LR-Cre driven stabilization of the β-catenin (β-cat[Ex3fl/+] strain) (Kreslova et al., 2007; Smith et al., 2005). Decreased levels of the *Pax6* in presumptive lens ectoderm further potentiate the phenotype, as the expression of *Dkk1* and *Sfrp2* genes is dependent on transcriptional factor *Pax6* (Machon et al., 2010). The "placode suppressing loop", initiated by ectopic Wnt/β-catenin signaling, is in the AP2α-Cre;Tcf7l1^{fl/fl} mutants potentiated to even higher

extent as the other negative regulators of the Wnt/β-catenin pathway, e.g. Six3, Hesx1 and FoxG1, become downregulated after Tcf7l1 ablation.
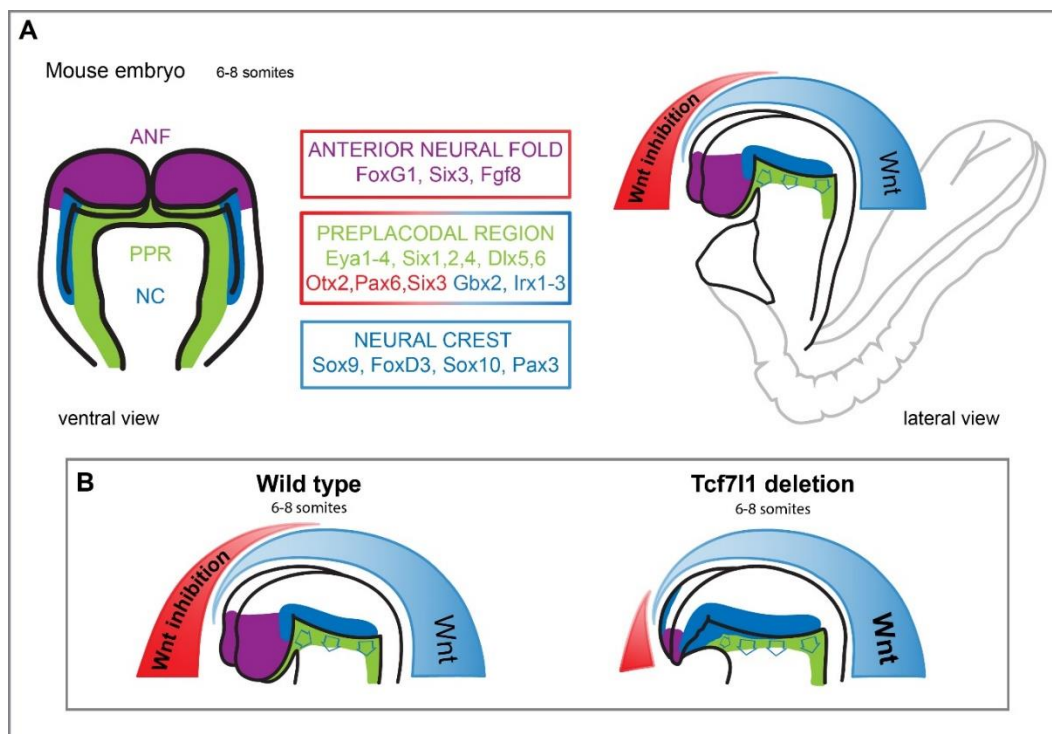
Figure 22: Schematic overview of the embryonic head ectoderm differentiation in relation to Wnt/β-catenin signaling and Tcf7l1 deletion. (A) (shown previously as Fig. 5) Ventral view of the head region (left) and lateral view of the whole developing mouse embryo (right) at 6-8 somite stage. A→P gradient of the Wnt antagonists promotes differentiation of the most anterior ectoderm into *foxg1*, *six3*, *fgf8* expressing ANF and *otx2*, *pax6* and *six3* characteristic for the anterior PPR. While A←P gradient of the active Wnt/β-catenin signaling induce formation of the *gbx2*, *irx1-3* positive posterior PPR and *pax3*, *sox9*, *foxd3* and *sox10* expressing NC. **(B)** Summary of the phenotypical changes resulting from the absence of Tcf7l1 in AP2α-Cre;Tcf7l1^{fl/fl} mutants. These mutants display expansion of the sox9, foxD3, and sox10 expressing NC population, accompanied with combined reduction in expression of the ANF specific genes *foxG1*, *six3*, *fgf8*, *zic1*, and *hesx1* and PPR specifiers *eya3*, *pax6*, *six3*, and *foxG1*, caused by overabundance of the Wnt/β-catenin pathway activation in the anterior ectoderm. ANF-anterior neural fold, PPR-preplacodal region, NC- neural crest

Not surprisingly, deletion of *Tcf7l1* in lens placode, using either Lens-Cre expressed in presumptive lens ectoderm (Ashery-Padan et al., 2000) or LR-Cre delivering recombination in presumptive lens ectoderm and neuroectoderm of the developing retina (Kreslova et al., 2007), results in considerably milder lens phenotype than the one observed in AP2α-Cre;Tcf7l1^{fl/fl}, supporting our hypothesis that the main reason behind the AP2α-Cre;Tcf7l1^{fl/fl} phenotype is the ectopic signaling from aberrantly formed NC cells. It is necessary to mention that the onset of Cre-recombination in Lens-Cre and LR-Cre occurs approximately one day later than in AP2α-Cre, however it should still be early enough, since ectopic stabilization of β-catenin, using the same Lens- and LR-Cre expressing lines, blocks expression of

Pax6 and completely disrupts lens placode development (Machon et al., 2010; Smith et al., 2005). The morphological defects of the Lens-Cre;Tcf7l1^{fl/fl} mutants share, to some extent, common features with the phenotype observed in the MLR10-Cre; β-catenin[ex3/+] mutants (Martinez et al., 2009). In these mice, the onset of β-catenin stabilization is triggered one day later (E10.0) than in Lens-Cre; β-catenin[ex3/+] mutants (Martinez et al., 2009; Smith et al., 2005; Zhao et al., 2004). In Lens-Cre;Tcf7l1^{fl/fl} mutants, apart from the fact that the lenses look less defective and with no changes in differentiation, an increase in BrDU staining and proliferation markers has been observed, as well as increase in apoptosis in fiber cells. Similarly, MLR10-Cre; β-catenin[ex3/+] mutants display enhanced proliferation but to a much higher extent and in both epithelial and fiber cells, whereas apoptosis is also present, predominantly in fiber cells (Martinez et al., 2009). Martinez and colleagues also showed upregulation of cell cycle proteins connected with cell cycle progression, namely CyclinD1, p57 and phopshorylated-histone 3 (PH3). Levels of these proteins should be also analyzed in Lens-Cre;Tcf7l1^{fl/fl} mutants, especially since CyclinD1 has been reported to be a target of the Wnt/β-catenin-dependent transcription factor c-Myc (Daksis et al., 1994). Another way of how Wnt/β-catenin pathway induces proliferation through expression of the transcription factor c-Myc is that it represses p21[Cip1/ Waf] gene, presumably in cooperation with transcriptional repressors Sp1 and Sp3 (Gartel et al., 2001). Interestingly, p21[Cip1/ Waf] transcriptional activation is also induced in the presence of Tcf/Lef factors, but only of those isoforms that contains the C-clamp binding domain (Tcf7 and Tcf7l2) (Hoverter et al., 2012). This effect is probably β-catenin-independent, as the induction of the p21-Luc reporter occurs even when β-catenin insensitive forms (dnTcf7 and dnTcf7l2) were used (Hoverter et al., 2012; van de Wetering et al., 2002). Here, we report for the first time the Tcf/Lef-dependent negative regulation of p21[Cip1/ Waf] expression. The fact that Tcf7l1 on one hand does not have a C-clamp and on the other hand acts as a transcriptional repressor would not make it a good candidate for Tcf/Lef responsible for c-Myc expression in lens ectoderm. Therefore, the upregulation of p21, observed during early lens development in the mutants, might be simply attributed to p21 promoter de-repression caused by absence of the Tcf7l1. This is however an assumption that would require further experimental confirmation. Collectively, Wnt/β-catenin driven regulation of p21[Cip1/ Waf] expression appears to be complex and context-dependent, as individual Tcf/Lefs display different potential to affect its transcription.

In summary, collective evidence point towards the fact that Tcf7l1 is one of the several inhibitors of Wnt/β-catenin pathway in the anterior neural and non-neural

ectoderm, which, under physiological conditions, define a spatial border to the expansion of the NC. Similarity in the phenotypes following deficiency of individual Wnt antagonists signifies that the anterior NPB region is very close to a threshold of the NC inducing Wnt/β-catenin activation. Therefore, from the evolutionary perspective, attenuation of the sensitivity to Wnt/β-catenin signaling in the anterior ectoderm by fine-tuning of the expression levels of Wnt signaling inhibitors may represent a very sensitive tool that allows simultaneous modification of three prominent features of the vertebrates, the sensory placodes, the neural crest and the forebrain.

Conclusions

This thesis is focused on the involvement of Tcf7l1 and Wnt/β-catenin signaling pathway during specification of the head ectoderm in mouse. The key conclusions are:

· Tcf7l1 is required as repressor of Wnt/β-catenin-driven gene expression in the anterior head ectoderm.

· Tcf7l1 is a negative regulator of the neural crest.

· Conditional inactivation of Tcf7l1 in the neural plate border leads to Wnt/β-catenin dependent expansion of the NC cell population.

· Evidence of the inductive capability of the Wnt/β-catenin pathway on early NC development eliminates most of the ambiguity in the interpretation of previous findings about NC development in mouse and other vertebrate model organisms.

· Tcf7l1 proved to be indispensable for proper development of the ANF and PPR probably due to abnormal signaling from the aberrantly formed NC cells.

· Our data also provide a model, which explains the phenotypes previously acquired in mouse after de-repression of the Wnt/β-catenin signaling pathway during early brain development.

· During lens induction Tcf7l1 is employed in control of proliferation and its absence leads to arrest in cell cycle progression and apoptosis.

· The balanced proliferation of the lens epithelial cells and fiber cells appears to be maintained by Tcf7l-dependent negative regulation of p21$^{Cip1/Waf}$ expression.

List of publications

Machon, O., <u>Masek, J.</u>, Machonova, O., Krauss, S., and Kozmik, Z. (2015). Meis2 is essential for cranial and cardiac neural crest development. *BMC Dev Biol* **15**, 40.

Manuscript in revision:

Tcf7l1 protects the anterior neural fold from adopting the neural crest fate

Jan Mašek, Vladimír Kořínek, M. Mark Taketo, Ondřej Machoň, and Zbyněk Kozmik; submitted to the journal *Development*, currently under revision

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Appendix

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Meis2 is essential for cranial and cardiac neural crest development

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Abstract

Background: TALE-class homeodomain transcription factors Meis and Pbx play important roles in formation of the embryonic brain, eye, heart, cartilage or hematopoiesis. Loss-of-function studies of Pbx1, 2 and 3 and Meis1 documented specific functions in embryogenesis, however, functional studies of Meis2 in mouse are still missing. We have generated a conditional allele of Meis2 in mice and shown that systemic inactivation of the Meis2 gene results in lethality by the embryonic day 14 that is accompanied with hemorrhaging.

Results: We show that neural crest cells express Meis2 and Meis2-defficient embryos display defects in tissues that are derived from the neural crest, such as an abnormal heart outflow tract with the persistent truncus arteriosus and abnormal cranial nerves. The importance of Meis2 for neural crest cells is further confirmed by means of conditional inactivation of Meis2 using crest-specific AP2α-IRES-Cre mouse. Conditional mutants display perturbed development of the craniofacial skeleton with severe anomalies in cranial bones and cartilages, heart and cranial nerve abnormalities.

Conclusions: Meis2-null mice are embryonic lethal. Our results reveal a critical role of Meis2 during cranial and cardiac neural crest cells development in mouse.

Keywords: Meis2, Neural crest, Persistent truncus arteriosus, Craniofacial skeleton, Cranial nerves

Background

Neural crest cells (NCC) represent a multi-potent embryonic cell population that generates a very diverse range of cell types including cranial nerves, neurons and glia of the peripheral nervous system, enteric neurons, melanocytes, cranial bones and cartilages [1, 2]. The first NCC appear at the neurula stage in the neural plate border region. As the neural tube closes in mouse, NCC delaminate from the regions of neural plate border and ectomesenchyme after epithelial-to-mesenchymal transition (EMT) and migrate to various developing organs. The very broad differentiation potential of NCC provides a complex model of cell type specification and migration and the gene regulatory network determining the spatiotemporal control of NCC diversification has been extensively studied. For instance, the NCC population is specified by the set of transcription factors Sox9, Sox10, FoxD3, Snai2 together with Msx1, Pax3/7 or Zic1 in the

neural plate border [3]. These effector genes are regulated by coordinated action of signaling pathways such as Wnt, Bmp and Fgf from the adjacent paraxial mesoderm and non-neural ectoderm [2, 4]. The differentiation potential of NCC is spatially determined by their position along the rostrocaudal axis. In a simplified view, cranial NCC coming from mesencephalic and rhombencephalic regions generate head bones, cartilages, cranial nerves and selected connective tissues [5, 6]. Vagal NCC from the area of somites 1-7 are destined to the enteric nervous system. Cardiac NCC (somites 1-4) are involved in septation of the cardiac outflow tract [7] and trunk NCC form sensory and sympathetic ganglia. The current debate, however, favors the scenario proposing that originally multi-potent NCC stem cells are exposed to different environmental cues along the rostrocaudal axis that spatiotemporally restrict their differentiation potential [1, 8].

Meis proteins are transcription factors that are orthologous to the *Drosophila* homothorax (Hth) protein. They contain a TALE (three-amino-acid loop extension) sub-class of the homeodomain that binds to DNA. In

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humans and mice, three homologues Meis1, Meis2 and Meis3 have been identified [9] and it has been shown that they directly bind to Pbx proteins [10–12]. The Meis/Pbx protein complex binds to DNA through respective Meis- and Pbx-consensus binding sites thereby regulating transcription. The Meis/Pbx complex plays important roles during development of several organs including limbs [13, 14], heart [15, 16], lens [17], pancreas [18] and hindbrain [19–22]. Hox genes are among the target genes of Meis-Pbx control via modulation of histone acetylation indicating recruitment of Hox proteins as cofactors of Meis-Pbx complex [23, 24].

Mice lacking Meis1 display liver hypoplasia, hemorrhage, impaired erythropoiesis and eye defects, and die by the embryonic day (E) 14.5 [25, 26]. Although a substantial amount of data have been reported on the role of Meis1 in organogenesis, hematopoiesis and leukemia induction, the function of the other homologs, Meis2 and Meis3, is much less clear. Chicken Meis2 has a specific role in determining cell fate in the midbrain-hindbrain boundary by controlling the expression of Otx2 [22] and it also affects proliferation of retinal progenitor cells [27]. Several recent reports in various model systems indicated that Meis2 may play a role in neural crest cells. Meis2 was identified as one of the key transcription factors in the gene regulatory network driving differentiation of human embryonic stem cells towards cardiovascular cell types, and this was further confirmed by knock-down experiments in zebrafish [16]. Morpholino-based screens in zebrafish revealed the importance of Meis1 and Meis2 factors during craniofacial development [28]. Moreover, gene expression analysis of EMT in endocardial cushions identified Meis2 among enriched genes [29]. In this context it is very interesting that some human disorders displaying cleft palate and heart developmental defects have been linked to mutations in the Meis2 locus [30–33]. Nonetheless, a clear picture of the Meis2 function based on a genetic mouse model is still missing.

In the present study, we examined the role of Meis2 during embryogenesis by generating conditional knock-out mice. We studied morphological defects after either zygotic inactivation of the Meis2 allele or NCC-specific conditional knock-out using AP2α-IRES-Cre. We conclude that hemorrhaging most probably causes embryonic lethality. Further, many embryonic defects in the tissues derived from neural crest in systemic Meis2-nulls were recapitulated upon conditional deletion of Meis2 in NCC suggesting an indispensable role of Meis2 in NCC.

Results
Meis2-/- embryos are lethal and display hemorrhaging
A conditional mutant allele of the *Meis2* gene (Meis2 cKO) was created by inserting LoxP sites in the introns

2 and 6 that flank exons 3 and 6 in the *Meis2* gene (Fig. 1a). To generate mutant mice lacking functional Meis2 in the whole organism, Meis2 cKO were at first crossed with the Hprt1-Cre mice (Jax Mice and Services) that exert a zygotic expression of *Cre* recombinase facilitating gene excision in all tissues. The first generation mice, which were heterozygous for the Meis2 gene (Meis2+/-), were intercrossed to obtain Meis2-null (Meis2-/-) animals. Animals were genotyped using primers flanking loxP sites as depicted in Fig. 1a. The loss of exons 3-6 in Meis2-/- embryos was tested by PCR (Fig. 1a, left). The absence of Meis2 protein was verified using Western blot analysis of protein extracts from E12.5 embryos (Fig. 1b, right). Meis2-/- mice displayed embryonic lethality between E13.5-E14.5 and suffered from hemorrhaging (Fig. 1c). The size of mutant embryos was smaller at E14.5 as mutant embryos stopped growing approximately at E13.5 when hemorrhaging became prominent. Although the severity of this phenotype varied among Meis2-/- (n = 29 litters, 51 mutants), all mutants displayed bleeding and a small liver size (Additional file 1: Figure S1B).

A detailed inspection of internal organs in mutant embryos revealed that the liver was the most impaired organ with a destructed cellular organization in large regions (Additional file 1: Figure S1C-D). These impaired regions contained almost no erythrocytes labelled with Ter119 but many apoptotic cells as shown by TUNEL assay (Additional file 1: Figure S1E-F). Surprisingly, Meis2 was not found to be expressed in the fetal liver while Meis1 was readily detectable (Additional file 1: Figure S1A). Based on this we suggest that the observed cell death in the liver is a consequence of strong hemorrhaging in the whole embryo that leads to anemia and apoptosis primarily in the liver and may be a cause of the embryonic lethality. Having observed anemia in Meis2-/- embryos we further pursued the possibility that Meis2 may influence embryonic hematopoiesis similarly to Meis1 that controls proliferation of hematopoietic stem cells in the fetal liver and is also essential for megakaryocyte viability [25, 26, 34]. We therefore mapped the expression of Meis2 and Meis1 in the area of the aorta-gonad-mesonephros (AGM), the site of origin of embryonic hematopoietic stem cells. As shown in Additional file 1: Figure S2B, neither Meis2 nor Meis1 were observed in endothelial cells labelled with CD31 but both proteins were abundant in the mesenchyme surrounding the endothelial wall of the dorsal aorta. We further found that circulating hematopoietic progenitors labeled with anti-Runx1 antibody did not express Meis2 (Additional file 1: Figure S2C). Finally, we carried out erythroblast cultures derived from the fetal liver and found no differences in the growth and differentiation of liver erythroid progenitors between Meis2-/- and

Fig. 1 Generation of Meis2 -/- mice. **a** Scheme of the *Meis2* gene with 11 exons (black rectangles) and position of loxP sites for Cre recombination. The homeodomain (HD) is encoded by exons 7-9. **b** Meis2-null (-/-) embryos were obtained by crossing Meis2 cKO with Hprt1-Cre mice. Left: Genotyping of Meis2-/- embryos using primers loxF, frtF and frtR illustrated in (**a**) (see also Methods). Right: Western blot analysis of E12.5 whole-embryo protein extracts with an anti-Meis2 antibody. **c** Mutant Meis2 mouse embryos at E14.5 display severe hemorrhaging. **d** The embryonic heart at E13 with persistent truncus arteriosus (PTA) in which the aortic and pulmonary arteries are not separated in Meis2-/-. A dashed line depicts the crossing of the separated aorta and pulmonary artery in the wild-type heart while this is not visible in the mutant outflow tract. **e** Immunohistochemical staining of Meis2 in the embryonic heart at E13 (transverse sections) illustrating its strong expression in the aortic (av) and pulmonary valves (pv). Aortic and pulmonary valves are missing in Meis2 mutants (arrowhead). **f** α-Smooth muscle actin (SMA) staining on transverse sections showing persistent truncus arteriosus with the fused aorta (ao*) and pulmonary artery (pa*) in Meis2-/-. Ao, ascending and descending aorta; pa, pulmonary artery; PTA, persistent truncus arteriosus

controls (data not shown). As Meis2, in our hands, was not detected in hematopoietic progenitors neither in the AGM nor in the fetal liver, we hypothesize that the anemia in the mutants originates from extensive bleeding or defective circulation rather than defects in hematopoiesis.

The lack of Meis2 results in fetal heart malformation

In the heart of Meis2-/- embryos at E12.5, we observed incomplete septation of the outflow tract that normally separates the aorta from the pulmonary artery. This defect is known as persistent truncus arteriosus (PTA) and was observed in all analyzed mutants (n = 8 litters, 14 mutants) (Fig. 1d). To correlate the observed defects with the expression of Meis2, we carried out immunohistochemistry on sections of embryonic heart at E13 using anti-Meis2 antibody. As shown in Fig. 1e, a

remarkably strong presence of Meis2 was observed in the aortic and pulmonary valves. Strikingly, these valves were lost in the Meis2-/- heart (arrowheads). To visualize the PTA in the Meis2-/- outflow tract, we used antibody against α-smooth muscle actin (SMA). Stained heart sections confirmed the PTA but SMA appeared normally expressed in the mutant heart (Fig. 1f).

Widespread expression of Meis2 was observed in the developing embryo including the central nervous system, in the upper and lower jaw and in the lumen of the intestinal tract (Fig. 2a). Specifically in the heart, Meis2 protein was detected in the myocardium (arrow) and pericardium (arrowhead) (Fig. 2a') in which Meis2-positive cells also expressed sarcomeric actin (Fig. 2f-f", arrows). Remarkably strong expression of Meis2 was found in the valves (aortic, pulmonary, tricuspid and mitral) as well as in the atrioventricular cushion (Fig. 2b-f").

Fig. 2 Mapping of Meis2 expression in the mouse embryo at E13. Meis2 protein is present in many developing tissues including the forebrain, midbrain, hindbrain, spinal cord, myocardium, cardiac cushions, cardiac outflow tract, valves (aortic, pulmonary, tricuspid, mitral). (**a**) A sagittal section of the whole embryo with a detail of the heart (A') documenting Meis2 presence in the myocardium (arrow) and pericardium (arrowhead). (**b-e**) Transverse sections of the heart at E13 from rostral to caudal with details of the framed areas (**b'-e'**) illustrating strong Meis2 expression in cardiac valves. (**f**) Immunofluorescent labeling of sarcomeric actin in the wild-type heart at E13. (**f'-f''**) Double labeling of sarcomeric actin (red) and Meis2 (green), a higher magnification view on the framed area in (**f**) showing Meis2 in the myocardium (arrows) and in the atrioventricular cushion. ao, aorta; av, aortic valve;, pv, pulmonary valve; mv, mitral valve; avc, atrioventricular cushion

To document heart defects in Meis2-/- embryos in detail, we performed immunofluorescence on transverse and sagittal sections from embryos at E12.5 using antibodies against Meis2, α-smooth muscle actin (SMA) and heart-specific myosin Myl7. Figure 3a-c illustrates that the aortic and pulmonary arteries were not separated (ao*, pa*, pv* in Fig. 3b) and the aortic valve was absent (arrowhead in Fig. 3a). These regions strongly expressed Meis2 in the controls and the signal was lost in the mutants (Fig. 3a). Both the aortico-pulmonary spiral septum and the aortic and pulmonary valves are formed from from cardiac neural crest cells [7] which can be visualized by anti-Sox9 and anti-Twist1 antibody [35]. In Meis2-/- embryonic hearts, we observed less Sox9-

positive cells in the outflow valves. The number of Twist1-positive cells was not significantly changed though its expression level was reduced and the organization of these cells was altered compared to controls (Fig. 3d). Clear morphological defects in the septation of the truncus arteriosus and in the outflow valves (arrowheads) strongly suggest that the defective outflow tract in Meis2 nulls arises from impaired cardiac neural crest cells.

Neural crest cells express high levels of Meis2
Based on the heart abnormalities of potential neural crest origin, we decided to map the expression of Meis2 at the embryonic stages that are critical for generation and migration of NCC. At E8.5 before the neural tube

Fig. 3 Immunohistochemistry of transverse and sagittal sections at E13.5 from the embryonic heart in Meis2-/- mice and controls. **a** Anti-Meis2 antibody staining (brown) show strong expression in the pulmonary and aortic valve. The aortic valve is absent in the mutants lacking Meis2 (arrowhead). **b** α-Smooth muscle actin (SMA) staining showing the persistent truncus arteriosus (PTA) with the fused aorta and pulmonary artery (presumptive ao* and pa*). Sagittal sections document a shortened heart length and a malformed pulmonary valve. **c** Heart-specific myosin light chain 7 (Myl7) staining shows normal cell specification in the myocardium in Meis2 nulls and, again, absent aortic and abnormal pulmonary valve on transverse and sagittal sections, respectively. **d** Left: Decrease of Sox9-positive cells in the valves in the outflow tract in Meis2 nulls. Right: The level of Twist1 expression in the condensed mesenchyme in the pulmonary valve and in the tricuspid valve appears normal in the mutants but it clearly reveals severe morphological changes in OFT. av, aortic valve; pv, pulmonary valve; tv, tricuspid valve; ao, aorta; pa, pulmonary artery

closure, abundant Meis2 expression was noticed in the neural plate that weakened towards the plate border from which the premigratory NCC delaminate (Fig. 4a). At E9.5, the Meis2 protein was found in the neuroepithelium of the hindbrain (Fig. 4b-b") in which the signal became diminished towards the roof plate. Meis2 was also seen in migrating cells in the mesenchyme lateral to the hindbrain and in the pharyngeal arches 1-2 (PA1 and PA2) (Fig. 4b') and in the outflow tract (OFT) (Fig. 4B"). Many of these cells especially in the PA co-express Tfap2, a marker of NCC [36, 37] (Fig. 4c-c" and Additional file 1: Figure S3C-D"). At E10.5, Meis2 protein was detected in the developing tissues that are derived from NCC, such as the trigeminal and facial nerve ganglions, in the the otic vesicle and its surrounding mesenchyme, in the maxillary and mandibular component of the PA1, in the cardiac outflow tract cushion and in the

PA2 (Fig. 4d-d'" and Additional file 1: Figure S3A-B"). The expression pattern revealed by our immunohistochemistry thus corresponds to in situ hybridization of Meis2 mRNA reported by Cecconi and co-workers [38]. Apart from the neuroectoderm, many of Meis2-positive cells displayed a mesenchymal character as revealed by co-staining with the anti-Twist1 antibody (Fig. 2E and Additional file 1: Figure S3E).

Conditional deletion of Meis2 in neural crest cells leads to a defective heart outflow tract
Because some developmental defects in Meis2 nulls may arise from affected NCC, we aimed to verify this assumption by means of conditional inactivation of Meis2. We employed AP2α-IRES-Cre (AP2α-Cre) transgenic mouse in which Cre recombinase was inserted into the Tfap2 (AP2α) gene enabling loxP recombination specifically

Fig. 4 (See legend on next page.)
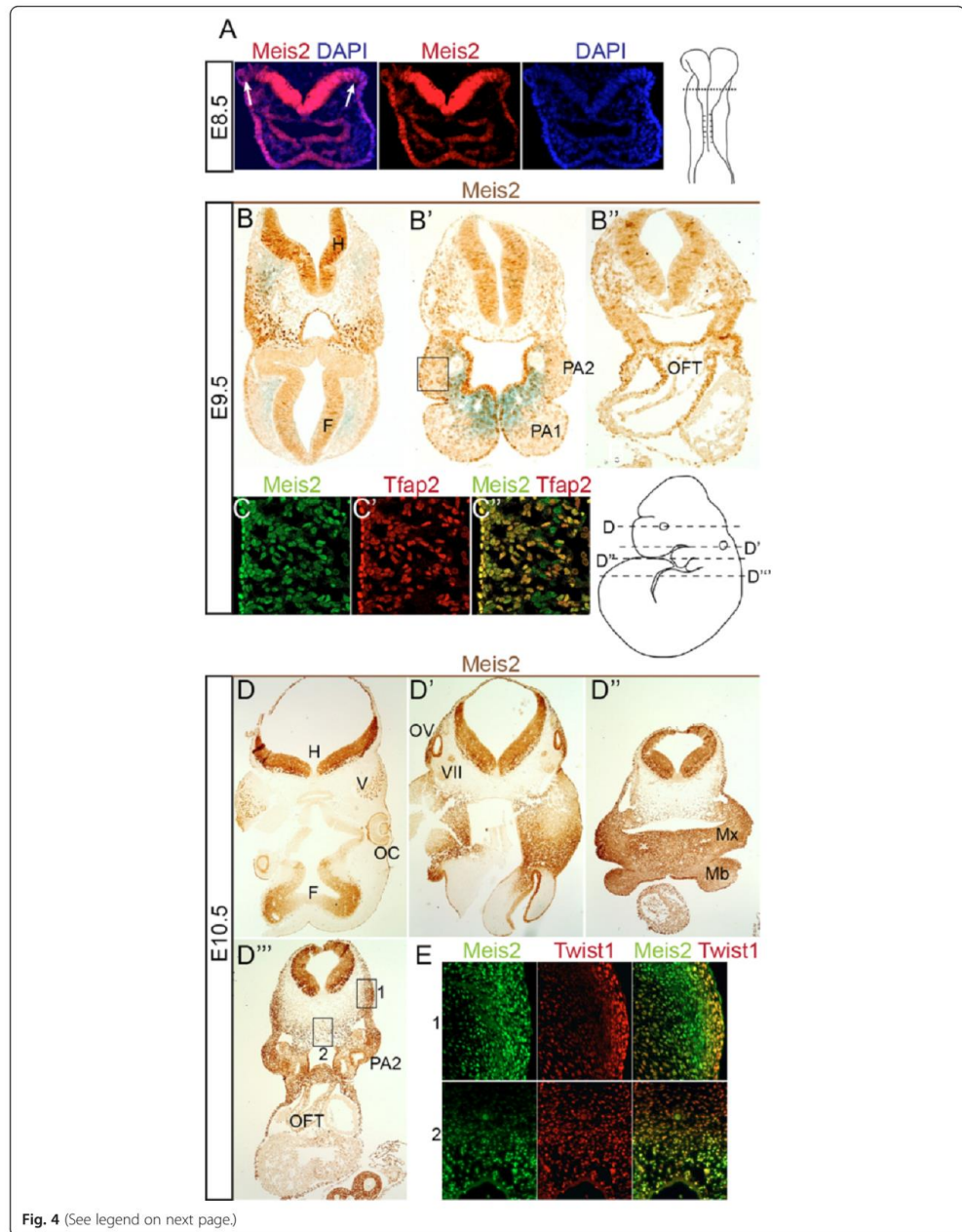
(See figure on previous page.)
Fig. 4 Meis2 is expressed in migrating NCC. (**a**) Meis2 expression in the neural plate stage at E8.5 becomes weak towards the neural plate border (arrows). Right: schematic embryo at E8.5 with a depicted section plane (dashed). (**b-b″**) Sections from rostral (**b′**) to caudal (**b″**) of wild-type embryos at E9.5 revealing Meis in pharyngeal arches (PA), hindbrain (H) and in the cardiac outflow tract (OFT). (**c-c″**) Immunofluorescent double-labeling of Meis2 (green) and Tfap2 (red) in the region approximately depicted by a black rectangle in A′. (**d-d‴**) Transverse sections from rostral to caudal at E10.5 with immunostained Meis. Sections levels correspond to the illustrated cartoon. Meis2 was detected in the hindbrain (H), forebrain (F), optic cup (OC), trigeminal nerve ganglion (V), otic vesicle (OV), facial nerve ganglion (VII), maxillary (Mx) and mandibular (Mb) process of PA1, in PA2 and outflow tract (OFT). (**e**) Immunofluorescence of Meis2 (green) and Twist1 (red) shows their co-expression in two areas labelled with rectangles in D‴. Upper panels (1) belong to the lateral zone (1) and lower panels (2) to the midline area (2)

in NCC [39]. Conditional mutants AP2α-Cre/Meis2 cKO survived the critical stage E14, at which systemic Meis2 nulls die, but they were lethal around the time of birth. This cKO approach also allowed us to analyze older embryos. To perform lineage tracing experiments and examine migratory routes of NCC, conditional mutants AP2α-Cre/ Meis2 cKO were crossed to ROSA26 reporter mice [40]. This allele allows detection of Cre-mediated recombination using β-galactosidase enzymatic assay and tracing of all cells derived from AP2α-Cre positive NCC. Figure 5a-a′ illustrates lineage tracing of NCC in normal and AP2α-Cre/ Meis2 cKO embryos. 75 % of mutants (n = 8 litters, 8 mutants) displayed aberrant distribution of β-galactosidase-positive cells along the neural tube (arrow in 5A′), and all mutants had abnormalities in the PA2, which appeared thinner and misshaped, (arrowheads in 5A-A′) and also all mutants had smaller otic vesicles (asterisks). Sectioning of AP2α-Cre/Meis2 cKO/ROSA26 E10.5 embryos revealed poor colonization of the OFT by cardiac NCC (arrows in 5B-B′). Conditional deletion of Meis2 effectively erased the Meis2 protein in the regions of the AP2α-Cre activity, however, many surrounding cells still express normal levels of Meis2 (arrows in Fig. 5c-c′). Next we studied the OFT defects at later stages using immunohistochemistry on cryosections from AP2α-Cre/Meis2 cKO/ROSA26 embryos. At E11, anti-Sox9 antibody labelled all β-galactosidase-positive cells in the OFT and we observed a lower density and disorganization of double-labelled cells in conditional mutants (Fig. 5d-d′). 90 % of mutants had malformed OFT valves at E12 (n = 5 litters, 9 mutants) (such as av in Fig. 5e-e′). However, the septation of the truncus arteriosus appeared normal in conditional mutants as the aorta and pulmonary arteries were normally separated (Fig. 5 f-f′), which was never seen in systemic nulls all displaying the PTA. Of note, we detected still high levels of Meis2 in non-recombined cells, including the smooth muscle of artery walls and in the vicinity of valves, which may provide explanation for less severe defects in conditional mutants compared to systemic ones. Finally, Fig. 5g-g′ shows an example of valve malformation at E14 known as double outlet right ventricle (DORV) labelled with anti-Sox9. In summary, tissue-specific deletion of Meis2 using AP2α-Cre resulted in various valve defects in the heart outflow tract documenting its critical role in cardiac NCC.

Meis2 affects cranial nerve development

As Meis2 expression is abundant in the head region, we next explored other NCC derivatives such as cranial nerves. Formation of their neuronal projections in Meis2-/- embryos was followed using whole-mount immunostaining of neurofilaments with the 2H3 antibody. As seen in Fig. 6a-b′, the trigeminal (cranial nerve V), facial (VII) and vestibulocochlear (acoustic) nerves (VIII) were severely impaired in Meis2 nulls at E10.5 (asterisks in Fig. 6b′). An analogous analysis was performed in AP2α-Cre/Meis2 cKO. We again crossed conditional mutants with ROSA26 reporters to map the activity of Cre. β-galactosidase signal in AP2α-Cre/ROSA26 controls was seen in trigeminal ganglions, the lens, the periocular mesenchyme and the surface ectoderm. A similar spatial pattern in this head region was found in the mutants AP2α-Cre/Meis2 cKO/ROSA26 and the size of the trigeminal ganglion V was smaller and misshapen (Fig. 6c′). 2H3 neurofilament staining showed that the cranial nerves VII and VIII were damaged in conditional mutants (asterisks in Fig. 6d′) but the trigeminal nerve V again less affected than in Meis2-/- (n = 3 litters, 3 mutants). Thus, the phenotype in cranial nerves appeared weaker in conditional mutants in comparison with systemic ones. We further noticed that the cornea and eyelids were not developed properly (Fig. 6e-e′) (similarly in Meis2-/- embryos at E13.5, not shown). The cornea was much thinner (arrow) while eye lids (el) did not grow and close over the eye bulbs. The cornea was reported to originate from NCC [41–43] as also indicated by our lineage tracing data showing AP2α-Cre activity in the periocular mesenchyme (Fig. 6c). In summary, tissue-specific deletion of Meis2 in NCC and the surface ectoderm resulted in malformed cranial nerves, the cornea and eyelids.

Development of cranial cartilage is perturbed in Meis2-null embryos

Next, we examined the development of the craniofacial skeleton that is derived from NCC. Mapping of AP2α-Cre/ROSA26 showed complete recombination in PA1 and otic vesicle at E10 and this was further verified at E13 on sagittal view in which recombined cells resided in jaws and the nasal cartilage (Fig. 7a). Immunostaining
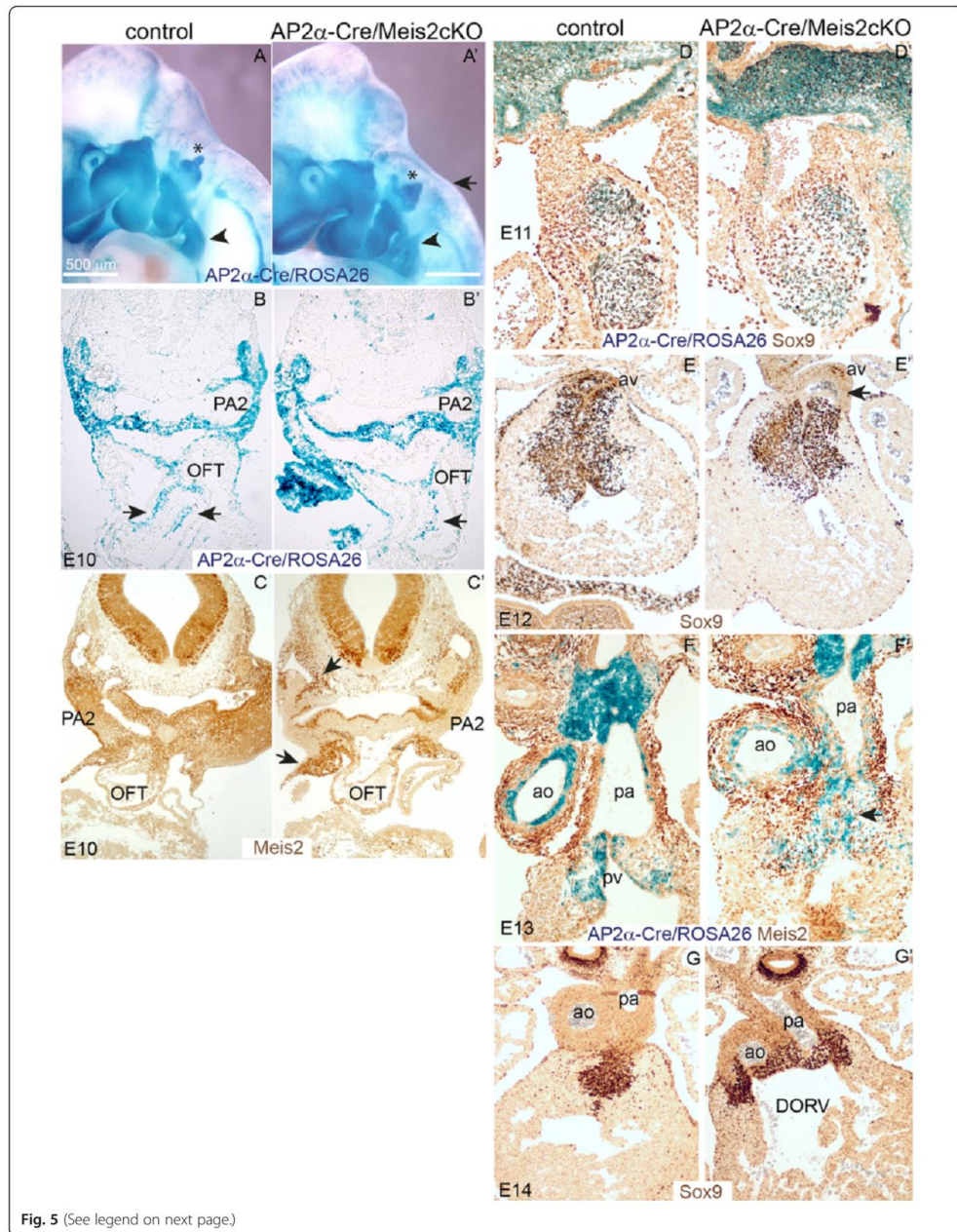
Fig. 5 (See legend on next page.)

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Fig. 5 Heart defects upon conditional inactivation of Meis2 in NCC. (**a-a'**) Lineage tracing of NCC in AP2α-Cre /Meis2 cKO/ROSA26 embryos at E10.5 (**a'**) and controls (**a**). (**b-b'**) Sections of embryos shown in (**a**) at the level of PA2 and OFT, compare position and number of β-galactosidase-positive cells in the control and the mutant cKO (arrows in B'). (**c-c'**) Immunohistochemical staining of Meis2 in cKO (**c'**) shows the areas of its deletion. (**d-d'**) Cryosetions of the OFT from AP2α-Cre /Meis2 cKO/ROSA26 stained for β-galactosidase and Sox9, note cells disorganization in cKO (**d'**). (**e-e'**) Paraffin-embedded sections of E12 heart stained with anti-Sox9 defective aortic valve in cKO (**e'**). (**f-f'**) Meis2 and β-galactosidase double-labeling of E13 OFT shows a number of Meis2-positive cells in cKO (**f'**) that are excluded from Cre recombined areas. The pulmonary valve does not develop properly. (**g-g'**) anti-Sox9 immunohistochemical labeling of severed valves in cKO at E14 (**g'**). Ao, aorta; av, aortic valve; OFT, outflow tract; PA2, pharyngeal arch 2; pa, pulmonary artery, DORV, double outlet right ventricle
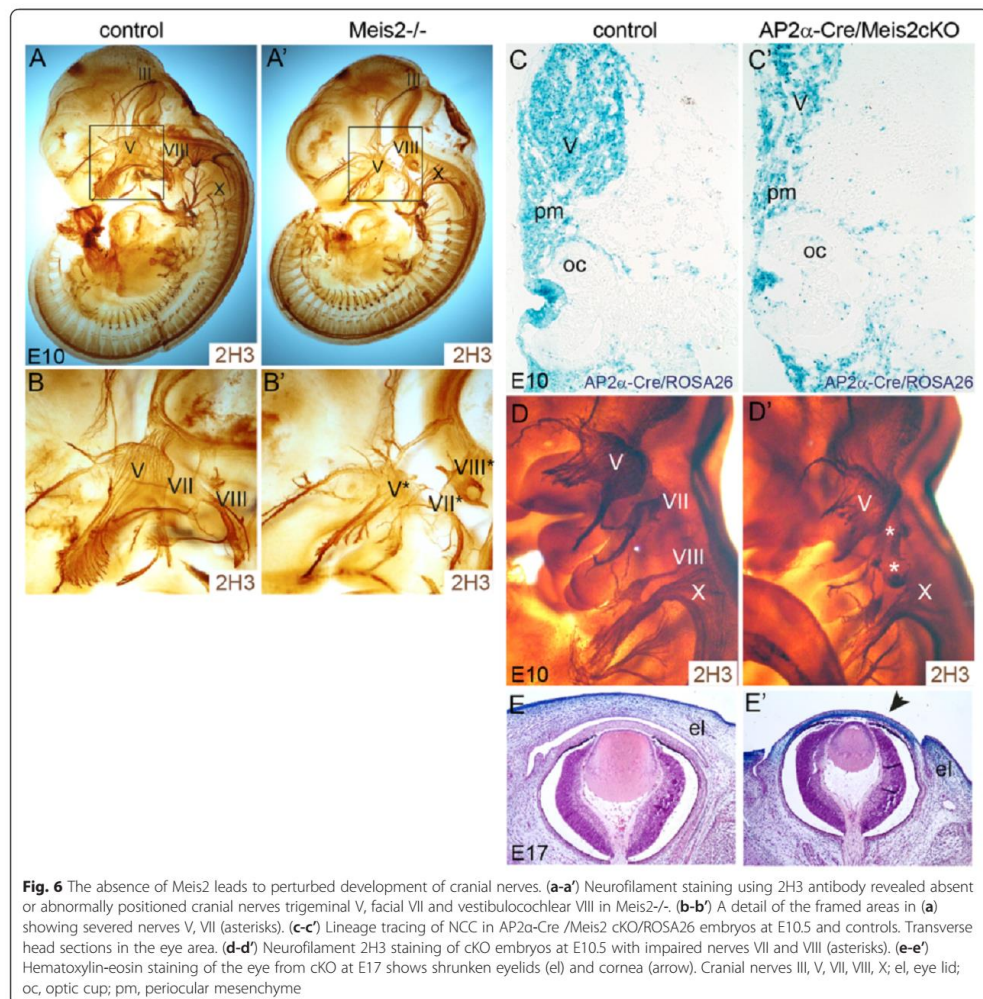
Fig. 6 The absence of Meis2 leads to perturbed development of cranial nerves. (**a-a'**) Neurofilament staining using 2H3 antibody revealed absent or abnormally positioned cranial nerves trigeminal V, facial VII and vestibulocochlear VIII in Meis2-/-. (**b-b'**) A detail of the framed areas in (**a**) showing severed nerves V, VII (asterisks). (**c-c'**) Lineage tracing of NCC in AP2α-Cre /Meis2 cKO/ROSA26 embryos at E10.5 and controls. Transverse head sections in the eye area. (**d-d'**) Neurofilament 2H3 staining of cKO embryos at E10.5 with impaired nerves VII and VIII (asterisks). (**e-e'**) Hematoxylin-eosin staining of the eye from cKO at E17 shows shrunken eyelids (el) and cornea (arrow). Cranial nerves III, V, VII, VIII, X; el, eye lid; oc, optic cup; pm, periocular mesenchyme

Fig. 7 Conditional inactivation of Meis2 in NCC affects osteochondrogenesis in the head. (**a-a′**) Illustration of the zone of Cre activity visualized in AP2α-Cre/ROSA26 embryos, left: transverse section of E10.5 embryo, right: sagittal view on E13.5. (**b-b**) Meis2 immnunostaining of AP2α-Cre/ Meis2cKO embryos at E10.5, transverse sections in the region of hindbrain and the first pharyngeal arch. Compare the area of Meis2 deletion with a comparable cryosection from AP2α-Cre /Meis2 cKO/ROSA26 in the left panel (**a**). (**c-c′**) Sagittal sections from cKO at E14, hematoxylin-eosin (H&E) staining. Note the abnormal palate (p*) and tongue (t*). (**d-d′**) A higher magnification of the tongue on transverse sections at E14 documents its much reduced size and tissue disorganization. (**e-e′**) An example of severely disrupted palate (p*) in some cKO embryos, transverse section H&E staining. Alcian staining of cartilage at E16 with no otic capsule in cKO. (**g-g′**) Submandibular gland is almost absent and tongue muscle severed, transverse sections. (**h-h′**) Alizarin Red/Alcian Blue staining of E17.5 heads. Side view with remarkably shorter mandible (arrow), absent interparietal bone, abnormal hyoid bone and otic capsule cartilage. Ossification frontline of the parietal bone is severed in the mutants (arrowhead). (**i-i′**) Ventral views showing an abnormal hyoid bone (asterisk). oc, otic capsule cartilage; ip, interparietal bone; hy, hyoid bone; mb, mandible, p, palate; pr, parietal bone; smg, submandibular gland; t, tongue

of the Meis2 protein confirmed efficient deletion in AP2α-Cre /Meis2 cKO in the regions of Cre recombination (asterisk in Fig. 7b′, compare with the panel 7a). We found abnormal palate (p) and tongue (t) in sagittal sections of E14 conditional mutants (Fig. 7c-c′). The size of tongue was greatly reduced and muscle fibers looked disorganized, as shown in a detailed view on transverse sections (Fig. 7d-d′). In 33 % of mutants (n = 6 litters, 4 mutants), severely malformed developing cartilage were observed in the palate (asterisk in Fig. 7e′), 67 % of

mutants also displayed abnormal palates though less severe. Further, the otic capsule cartilage was absent as shown by Alcian staining at E16 (asterisk in Fig. 7 f'). Submandibular gland (smg) was missing in 33 % mutants and 66 % had much smaller smg (Fig. 7g-g') (n = 6 litters, 4 and 8 mutants, respectively). We carried out whole-mount Alcian Blue/Alizarin red staining at E17.5 that confirmed our observations from tissue sections: The mandibular bone was poorly developed and its length was much shorter in the mutants (black arrow in Fig. 7h'). The interparietal bone and the cartilage of the otic capsule were absent and the boundary of ossification of the parietal bone was abnormal (arrowhead in Fig. 7h'). The hyoid bone was severely malformed (asterisk in Fig. 7i'). These defects clearly document that Meis2 is essential for the head bones and cartilages that originate from cranial NCC.

Meis2 does not affect proliferation and expression of main determinants of NCC

Migrating NCC are exposed to a number of environmental factors that can alter their properties including fate restriction or proliferation. In order to class Meis2 into the gene regulatory network determining NCC development we first examined the levels of expression of well-known genes specifying NCC. Trigeminal ganglions stained with anti-Pax3 were found reduced in size or completely missing in Meis2-/- embryos at E10 (Fig. 8a-A'). Conditional mutants showed normal levels of Pax3 and a normal size of ganglions V (not shown). Pax3 was reported to control colonization of the OFT by cardiac NCC [44] and the promoter of Pax3 is directly controlled by Pbx1 [45]. As Pbx1-/- embryos display similar heart defects as Meis2-/- embryos [15], we checked the expression of Pax3 in the OFT and PA2 of our mutants.

Fig. 8 Expression of NCC markers in the absence of Meis2. (**a-a'**) Pax3 immnostaining of trigeminal ganglions in systemic Meis2-/- E10.5 embryos showing greatly reduced or even absent ganglions V. (**b-b'**) Pax3 in the second pharyngeal arch (PA2). (**c-c'**) Tfap2 immunostaining in Meis2-/- embryos at E10.5. (**d-d'**) Sox10 immunofluorescence of ganglions V at E10.5. (**e-e'**) Melanocytes stained with anti-Mitf were detected normally in Meis2 nulls. (**f-f'**) Sox10 in dorsal root ganglia and the peripheral nervous system was not changed in mutants. DRG, dorsal root ganglia; H, hindbrain; V, trigeminal ganglion; PA2, the second pharyngeal arch;, sc, spinal cord

However, we found no change in Pax3-positive cells in PA2, moreover, Pax3 was not detected in the OFT even in wild-type controls (Fig. 8b-b'). Next we assayed the level of expression of Tfap2, Sox10 and Mitf, a marker of melanocyte precursors. As shown in Fig. 8c-f', their expression was not found to be changed in the absence of Meis2.

We also analyzed the number and spatial distribution of migrating NCC using anti-Sox9 antibody at E10.5. Sox9-positive NCC are abundant in the embryonic head mesenchyme and pharyngeal arches, however, their quantification did not reveal significant changes between controls and mutants (Additional file 1: Figure S4A). Further, we examined cell proliferation using antibodies against PCNA and PH3 on head sections. Again, quantification of all proliferating cells (all PCNA+ or PH3+) or proliferating NCC (Sox9+/PCNA+) did not differ between controls and mutants (Additional file 1: Figure S4B-D). Finally, apoptosis, as measured by anti-Cas3 immunohistochemistry at E10.5, was not significantly altered in the mutants (Additional file 1: Figure S4E). In summary, Meis2 absence did not result in dramatic changes in cell proliferation and viability at E10.5.

Systemic Meis2 mutants show aberrant position of FoxD3 and Sox9

As the defects in NCC derivatives in the absence of Meis2 were not caused most probably by insufficient cell proliferation or changed expression of main NCC specifiers we performed whole-mount in situ hybridization to map the position of migrating NCC. At first, E9.5 embryos from Meis2-/- embryos and control littermates were stained with riboprobes for *FoxD3* and *Sox9* mRNA [46, 47]. As shown in Fig. 9A-B, both *FoxD3* and *Sox9* were expressed in Meis2-/- embryos at the levels that were comparable to control littermates, indicating again that the overall differentiation program of NCC was not affected. Interestingly, we observed an aberrant position of FoxD3-positive cranial NCC in Meis2 nulls (arrows in Fig. 9a-a'). Expression of *FoxD3* mRNA in the trigeminal ganglion confirmed its abnormality as also seen in Fig. 6b'. *Sox9* mRNA revealed that otic vesicles were consistently smaller in mutants (arrow in Fig. 9b'). Next, we carried out the same analysis in E9.5 embryos from conditional mutants AP2α-Cre/Meis2 cKO. However, the levels and distribution *FoxD3* and *Sox9* transcripts were not altered in conditional mutants (n = 5 litters, 8 mutants) (Fig. 9c-d'). Thus, the changes in the expression pattern of NCC specifying genes Sox9 and FoxD3 were found to be more profound in systemic Meis2 mutants compared to conditional ones which may explain their stronger phenotypic defects.

Discussion

In this study, we showed that the transcription factor Meis2 is abundant in NCC and it is essential for their function. The embryonic lethal phenotypes of mutant
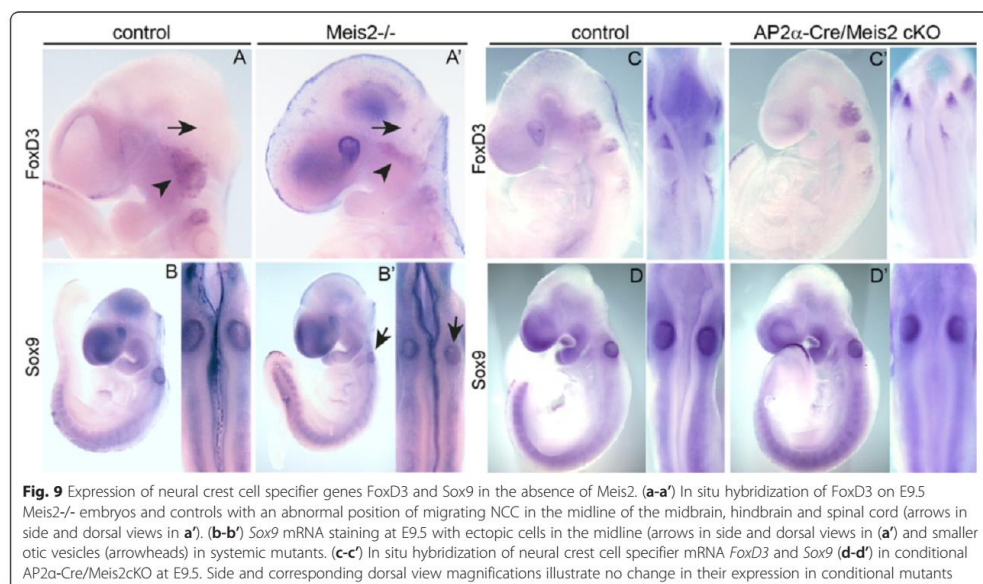

Fig. 9 Expression of neural crest cell specifier genes FoxD3 and Sox9 in the absence of Meis2. (**a-a'**) In situ hybridization of FoxD3 on E9.5 Meis2-/- embryos and controls with an abnormal position of migrating NCC in the midline of the midbrain, hindbrain and spinal cord (arrows in side and dorsal views in **a'**). (**b-b'**) *Sox9* mRNA staining at E9.5 with ectopic cells in the midline (arrows in side and dorsal views in (**a'**) and smaller otic vesicles (arrowheads) in systemic mutants. (**c-c'**) In situ hybridization of neural crest cell specifier mRNA *FoxD3* and *Sox9* (**d-d'**) in conditional AP2α-Cre/Meis2cKO at E9.5. Side and corresponding dorsal view magnifications illustrate no change in their expression in conditional mutants

mouse embryos of Meis2 and its paralogue Meis1 are similar in the timing of death and in strong hemorrhaging. Meis1 is expressed hematopoietic stem cells in the fetal liver, the primary organ of hematopoiesis at E13. Various assays showed defects in erythropoiesis providing explanation for anemia in Meis1 mutants [25, 26]. In contrast, we did not detect Meis2 in the fetal liver and in hematopoietic progenitors in the dorsal aorta. We therefore speculate that hemorrhaging in Meis2-/- embryos caused destructive changes primarily in the fetal liver that is highly sensitive to low oxygen. Impaired function of the liver may lead to failure in erythropoiesis and thus bleeding rather than defects in hematopoiesis in Meis2 mutants may cause lethality. Despite 83 % sequence identity of Meis1 and Meis2 and the almost identical homeodomain, they acquired different functions during evolution probably due to distinct expression patterns.

Stankunas and colleagues [15, 45] showed that various combinations of mutant alleles of Pbx1/2/3 or Meis1 exhibit cardiac anomalies in the outflow tract or in the septation of ventricles implying that cardiac NCC require transcriptional control by combinatory Meis/Pbx complexes. Along this line, zebrafish Meis2 genes influence development of heart and cranial skeleton [16, 28]. The data presented here extend our knowledge about the role of Meis/Pbx complexes controling the fate of NCC. Apart from the previously proposed function in cardiac neural crest, we suggest that a similar transcriptional control network takes place in cranial NCC including chondrogenic and neuronal lineages. Our data represent the first loss-of-function study of Meis2 in mouse and may in fact serve as a disease model for certain human developmental disorders. Intriguingly, several reports describe patients with Meis2 mutations who display disorders such as the cleft palate, septal defects in the heart or intellectual disabilities [30–33].

Pax3 is expressed in the neural plate border and plays an important role in cardiac NCC as documented by conditional knock-out studies in mouse [44]. A specific Pbx1/Meis1 complex has been reported to directly regulate Pax3 in premigratory NCC in rhombomeres, in which cardiac NCC originate, linking Pbx1-Pax3 regulatory hierarchy with the OFT defects in Pbx1-/- embryos [45]. We did not see reduced Pax3 expression in Meis2-/- at E10.5 although Pax3-positive trigeminal ganglions were shrunken in systemic mutants. Nor could we detect Pax3 in the wild-type OFT at E10.5. It is possible that Meis2 and Pax3 are co-expressed in earlier NCC in which these factors may cooperate during NCC differentiation.

Our experiments utilizing conditional mutants AP2α-Cre/Meis2 cKO confirm defects in tissues derived from cardiac and cranial NCC such as the OFT or craniofacial cartilage. However, the phenotypes in cKO appeared in some cases weaker than in systemic mutants. PTA, for instance, was never found in conditional mutants while all systemic mutants displayed defective septation of the OFT. It is important to note that we still found a substantial amount of Meis2 protein after conditional deletion in the areas that are in close vicinity of AP2α-Cre targeted regions (mapped in the ROSA26 reporter) in which we observed effective inactivation. Standby Meis2-positive cells may be involved in forming the truncus septum. Although AP2α-Cre mouse is the earliest driver for NCC [44], we cannot exclude the possibility that a minor NCC population forms before AP2α-Cre mediated recombination and thus it is not targeted. Another possibility is that neighboring non-NCC cells expressing Meis2 participate in the truncus septation. In this context it is interesting to note that systemic Pax3 KO display the PTA while Wnt1-Cre or AP2α-Cre cKOs of Pax3 show valve defects but normal separation of aorta and pulmonary arteries [44]. Moreover, the PTA defects were also seen in systemic Pbx1-3 mutants; the data from Pbx cKOs are not available [15, 45]. The fact that the truncus septation may partially originate from non-NCC population is supported by the report of Bai and colleagues [48] who observed PTA in Mef2c-Cre/Bmp4/7, a Cre driver not normally used for NCC targeting.

Altered proliferation, apoptosis or significant changes in expression of transcription factors determining NCC (Sox9, Sox10, Tfap2, Pax3, Mitf) were not detected between critical stages E10.5-E11.5 in our hands. Meis2 may influence late phases of NCC differentiation (including cell proliferation and viability) that is required for proper formation of the OFT or for differentiation of osteochondral progenitors.

We hypothesize that Meis2 absence may be compensated by Meis1 which may rescue some defects during earlier phases of NCC development. Meis2 deficiency is thus reflected in the areas and stages in which the Meis2 function is unique, e.g. during craniofacial development. Meis proteins may diversify in their expression pattern after the initial NCC specification and acquire unique function, for instance, during formation of the OFT and cartilage. In order to test this hypothesis it will be necessary to generate and analyze Meis1 and Meis2 conditional double mutants that may reveal potentially earlier role of Meis factors during NCC development.

Even though we did not see a major difference in Sox9 expression in early embryos, anomalies in the craniofacial skeleton of Wnt1-Cre/Sox9 cKO [47] and our AP2α-Cre/Meis2 cKO are similar in the mandible, tongue, the otic capsule and the hyoid bone. This suggests that Sox9 and Meis2 cooperate in a similar differentiation process during chondrogenesis. It remains to

be elucidated what genes are direct targets of Meis factors. Altogether, our loss-of-function studies show that Meis2 transcription is an important player in the gene regulatory network determining differentiation of cardiac and cranial NCC.

Methods

Generation of Meis2 null mice

The LoxP recognition elements for the Cre recombinase were inserted in the introns 2 and 6 of the *Meis2* gene at the Gene Targeting & Transgenic Facility, University of Connecticut, USA. Transgenic mice termed Meis2 cKO were created by standard techniques using homologous recombination in mouse embryonic stem cells (129SvEv-Tac/C57BL/6 J F1) also at the Gene Targeting & Transgenic Facility. A neomycin selection cassette was removed using FLP-FRT recombination. Meis2 cKO were crossed to Hprt-Cre mice (strain 129S1/Sv-Hprt[tm1(cre)Mnn]/J, stock 004302, The Jackson Laboratory) with the zygotic activity of the Cre recombinase to obtain animals that were heterozygous for Meis2 (Meis2+/-) in the mixed genetic background. Primers for genotyping Meis2+/- alleles: Mrg1-lox-F (forward) GAGGGGACAGTGGGTAAACA, Mrg1-frt-R (reverse) TCAGACCCAGGAATTTGAGG, a PCR product of 256 bp. Wild-type allele: Mrg1-frt-F GCAAGGGTGCTGAGGTTAAA and Mrg1-frt-R TCA GACCCAGGAATTTGAGG, a PCR product of 235 bp. (Fig. 1a). Alternatively, Mrg1-lox-F GAGGGGACAGTG GGTAAACA, Mrg1-lox-R GCGTTGCAGCTCACAAGA AT, a PCR product of 142 bp.

All procedures involving experimental animals were approved by the Institutional Committee for Animal Care and Use (permission #PP-071/2011). This work did not include human subjects.

Immunohistochemistry

Embryos were fixed in 4 % paraformaldehyde overnight at 4 °C. 8-10 μm cryosections or 5-μm (paraffin-embedded) sections were permeabilized in 0.1 % Triton X-100 in PBS (PBT). After blocking sections were incubated overnight in a primary antibody (1 % BSA in PBT), washed with PBS and incubated with a fluorescent secondary for 1 h. Nuclei were visualized by DAPI (4,6-diamidino-2-phenylindol, 0.1 μg ml^{-1}, Roche). Primary antibodies: anti-Meis2 and anti-Meis1 (a gift from Dr. Buchberg), anti-Myl7 (1:250, Santa Cruz), smooth muscle actin (SMA) (1:1000, Sigma), Sox10, Twist1 (all Santa Cruz Biotech), 2H3 (neurofilaments), 3B5 (Tfap2a) and Pax3 (all DSHB), anti-alpha sarcomeric actin clone 5C5, anti-alpha smooth muscle actin clone 1A4 (both Sigma), anti-Myl7 (H60) (Santa Cruz Biotech), Mitf (a gift from Dr. H. Arnheiter), anti-Ter119 (BD Pharmingen). Secondary antibodies: anti-mouse (-rat, -rabbit) Alexa Fluor488 or 594 (Life Technologies). Biotinylated-anti-mouse, -anti-rabbit, -anti-rat (Vector Laboratories), Vectastain ABC Elite kit and ImmPACT DAB substrate (all Vector Laboratories). Images were acquired in Leica MZ APO stereomicroscope with DC200 camera or Olympus SZX9 with DP72 camera. Fluorescence images were acquired in Zeiss Axioskop 2 microscope with objectives Ph3 Plan-Neofluar 40x/1.3 oil or Ph1 Plan-Neofluar 10x/0.3 and confocal Leica SP5. Bright-field light images were acquired in Nikon Diaphot 300 with objectives 4x/0.1 and 10x/0.25.

Scale2 protocol

Dissected embryonic hearts were fixed overnight in 4 % PFA, washed in PBS and transferred into ScaleA2 reagent (4 M urea, 10 % glycerol, 0.1 % triton X-100) as described in [49]. After two hours at RT hearts were photographed.

Alcian blue/Alizarin red staining

Embryos at E16.5-17.5 were dissected and scalded in hot water (65-70 °C, 2 min). They were dehydrated in 95 % ethanol for 48-72 h, changing solution every 12 h. After Alcian blue (Sigma) staining for 12 h, they were rinsed twice in ethanol and kept overnight. After clearing in 1 % KOH for 2 h and they were stained with Alizarin red (Sigma) for 5 h. Further clearing in 2 % KOH was carried out overnight, then in Glycerol (25 %) and 2 % KOH (75 %) for 8 h and Glycerol (50 %) and 2 % KOH (50 %) for 48 h. Tissue sections were rehydrydated and stained in 0.04 % Alcian solution for 10 min. Pictures were obtained using binocular microscope Olympus SYX9 and camera Olympus DP72.

Whole-mount in situ hybridization

Riboprobes: Mouse Foxd3 was cloned into pGEM-T-easy vector (Promega) using primers F-GGACCGCAA GAGTTCGCGGA, R-TCCGGAGCTCCCGTGTCGTT and antisense mRNA was transcribed with T7 polymerase. Mouse Sox9 gene was cloned into pGEM-T-easy using primers F-GAGCACTCTGGGCAATCTCAG, R-CTCAGGGTCTGGTGAGCTGTG and antisense mRNA was transcribed with T7 polymerase. Whole-mount in situ hybridization was performed using standard protocols.

Conclusions

We present a pioneering functional description of Meis2, a member of TALE-class homeodomain transcription factors, which is strongly expressed in cranial neural crest cells. We generated a conditional allele of the Meis2 gene. Using systemic and neural crest-specific inactivation of Meis2, we provide evidence that Meis2 is an important player in the regulatory network controlling cranial and cardiac neural crest cells.

Additional file

Additional file 1: Figures S1-S4. Fetal liver in Meis2-/- at E13.5 contains less erythrocytes and loses cell viability. **Figure S2.** Meis2 is abundant in the mesenchyme of the aorta-gonadmesonephros (AGM). **Figure S3.** Meis2 is strongly expressed in migrating NCC and in mesenchymal cells at E10.5. **Figure S4.** Proliferation and cell death of migrating NCC appears normal in Meis2-/- mutants. (PDF 1406 kb)

Abbreviations
ao: aorta; cKO: conditional knock-out; DORV: double outlet right ventricle; MO: morpholino; NCC: neural crest cells; OFT: outflow tract; pa: Pulmonary artery; PTA: persistent truncus arteriosus; EMT: epithelial-mesenchymal transtition.

Competing interests
The authors declare no competing financial and non-financial interests.

Authors' contributions
OM designed and performed experiments, interpreted data and wrote the manuscript. JM commented on the manuscript and performed experiments. OM performed experiments. SK, ZK interpreted data and commented on the manuscript. All authors have read and approved the manuscript.

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liver WT E13

WT   E13.5   Meis2-/-

A Meis1   Meis2

B WT   E13.5   Meis2-/-

1 mm   1 mm

C cresyl violet

D H&E

E Ter119

F TUNEL

Fig. S1

A

Meis2

B

Meis2 CD31 DAPI   Meis2 CD31   Meis2   CD31

DA

Meis1 CD31 DAPI   Meis1 CD31   Meis1   CD31

DA

C

Meis2 Runx1 DAPI   Meis2 Runx1   Meis2   Runx1

DA

Fig. S2

Fig. S3

control   E10.5   Meis2-/-

A Sox9

B PCNA

C Sox9 PCNA

D PH3 DAPI
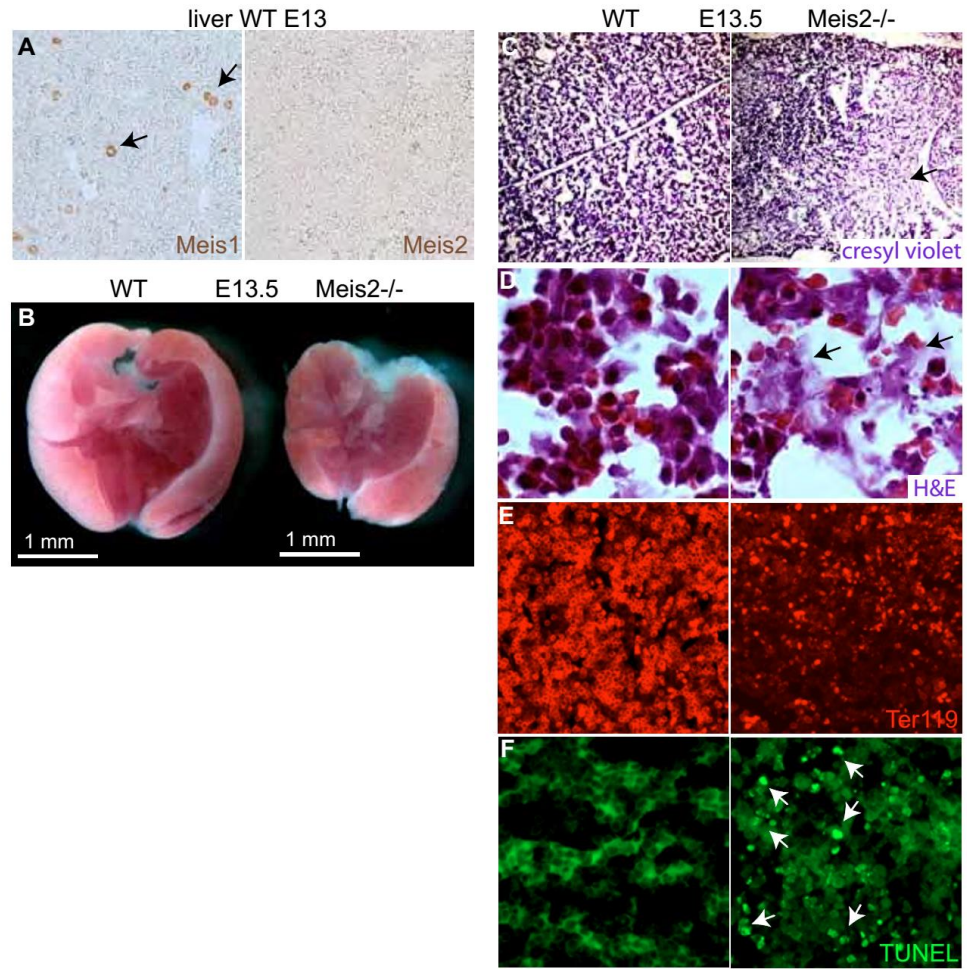
E Cas3
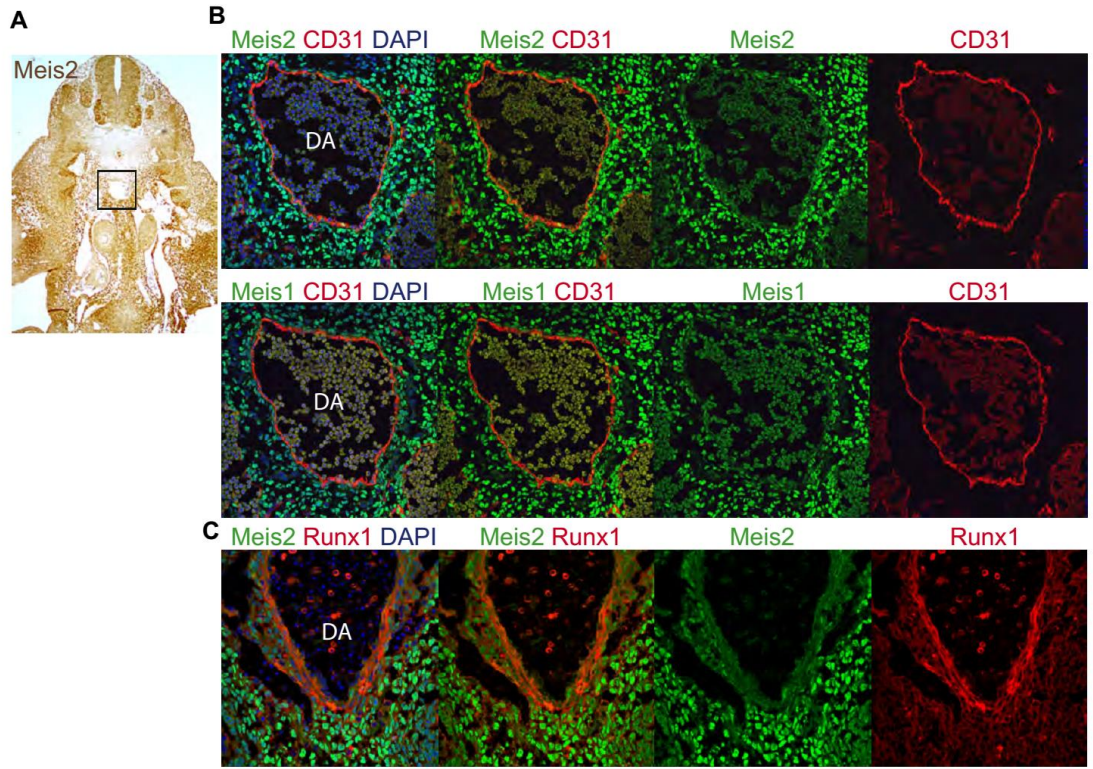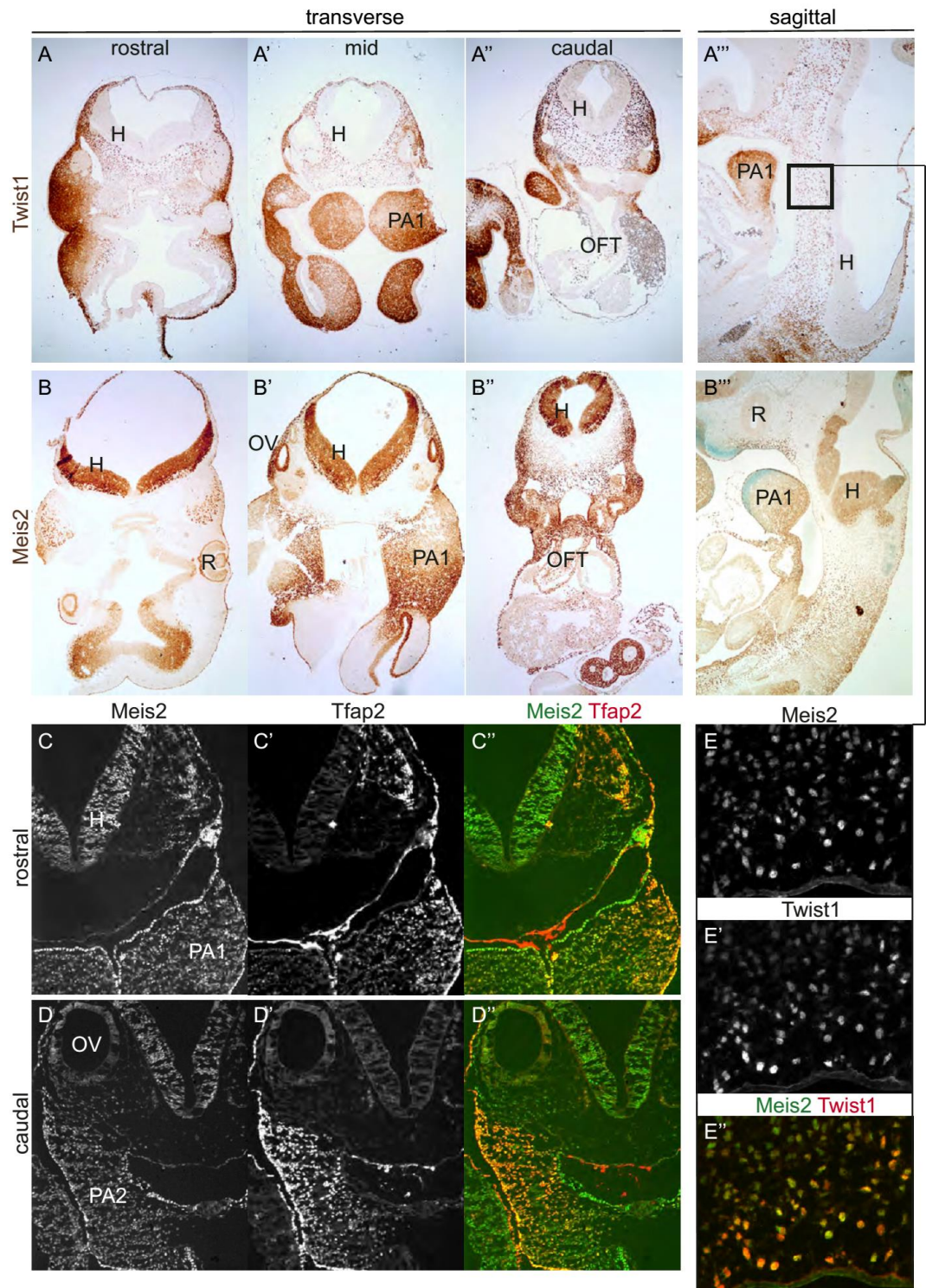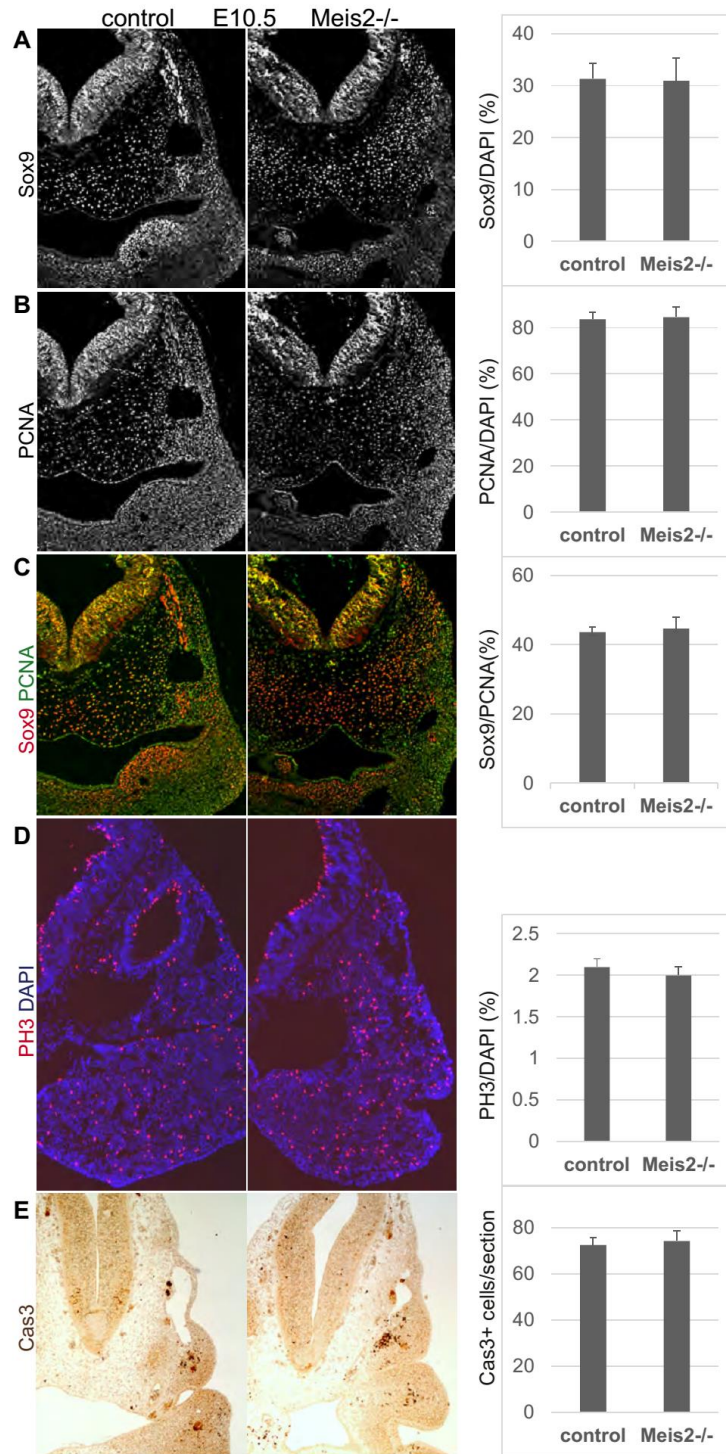
Fig. S4

Figure S1. Fetal liver in Meis2-/- at E13.5 contains less erythrocytes and loses cell

viability. (A) Meis2 is not expressed in the fetal liver in contrast to Meis1 that is

readily detected by immunohistochemistry. **(B)** Comparison of the liver size in

controls and Meis2-/-. **(C-D)** Some regions in the mutant liver show disrupted cellular

network and cell viability as revealed by cresyl violet and hematoxylin/eosin staining

at a higher magnification. **(E)** Erythrocyte immunofluorescence using anti-Ter119 on

liver sections at E13. **(F)** Apoptosis assay on liver sections using Cell Death Detection

kit (Roche).

Figure S2. Meis2 is abundant in the mesenchyme of the aorta-gonad-

mesonephros (AGM). (A) An overview of Meis2 immunohistochemistry in the trunk

of mouse embryo at E10.5. **(B)** Immunofluorescent double-labeling of Meis2 or

Meis1 and CD31 in AGM in which both proteins are absent in the endothelial wall

(CD31+) of the dorsal aorta (DA) but strongly expressed in the surrounding

mesenchyme. **(C)** Immunofluorescent double-labeling of Meis2 and Runx1 in the

dorsal aorta at the AGM region showing no expression of Meis2 in circulating

hemapoietic stem cells.

Figure S3. Meis2 is strongly expressed in migrating NCC and in mesenchymal

cells at E10.5. (A-A'') Rostral to caudal transverse sections at a low-power

magnification show the presence of Twist1 in mesenchymal cells around the otic

vesicle, in PA1-PA2 and in the aortic sac. (A''') Sagittal view on E10.5 embryo

stained for Twist1. **(B-B'')** Rostral to caudal transverse sections at a low-power

magnification show Meis2 expression at E10.5 in mesenchymal cells, neuroectoderm,

otic vesicle, PA1-PA2 and heart, (B''') sagittal view on Meis2 pattern. **(C-D)**

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Immunofluorescent double-labeling of Meis2 and Tfap2 in a rostral region with PA1

(C-C') and in a caudal region with otic vesicle (D-D'') demonstrating a high number

of NCC co-expressing both proteins. **(E-E'')** Immunofluorescent double-labeling of

Meis2 and Twist1 in the region depicted by rectangle in (A'''). F, forebrain; H,

hindbrain; OFT, outflow tract; OV, otic vesicle; PA1-2, pharyngeal arches; R, retina.

Figure S4. Proliferation and cell death of migrating NCC appears normal in

Meis2-/- mutants. (A-C) Sox9 and PCNA alone and double labeling (C) of

transverse sections in the region of the otic vesicle in Meis2-/- E10.5 embryos.

Corresponding quantifications (as the percentage relative to all DAPI cells on a

section) are shown to the right. **(D)** PH3 immunofluorescence and its counting with no

significant difference in proliferating cells in the mutants. **(E)** Quantification of

apoptosis assayed as the number of Cas3-positive cells after immunostaining of

embryonic sections at E10.5 (brown). Bars represent the average from three

experiment with standard deviations.

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1   **Tcf7l1 protects the anterior neural fold from adopting the neural crest fate**

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17   Keywords: Tcf/Lef; Wnt signaling; neural crest; forebrain

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24   **Summary statement**

25   Tcf7l1 mediated repression of the Wnt/β-catenin pathway at the anterior neural tissue,
26   protects the cell identity of the prospective prosencephalon from conversion to the neural
27   crest.

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Abstract

The neural crest (NC) is crucial for evolutionary diversification of vertebrates. NC cells are induced at the neural plate border (NPB) by a coordinated action of several signaling pathways including Wnt/β-catenin. NC cells are normally generated in posterior NPB, whereas the anterior neural fold is devoid of NC cells. Using the mouse model, we show here that active repression of Wnt/β-catenin signaling is required for maintenance of neuroepithelial identity in the anterior neural fold and for inhibition of NC induction. Conditional inactivation of Tcf7l1, a transcriptional repressor of Wnt target genes, leads to aberrant activation of Wnt/β-catenin signaling in the anterior neuroectoderm and its conversion into the NC. This reduces the developing prosencephalon without affecting the anterior-posterior neural character. Thus, Tcf7l1 defines the border between the NC and the prospective forebrain via restriction of the Wnt/β-catenin signaling gradient.

Introduction

Neural crest (NC) cells are vertebrate-specific, multipotent, highly migratory cells originating from the ectoderm of the neural plate border (NPB). NC cells possess the ability to differentiate into a wide range of cell types including the bone and cartilage of the skull, sensory neurons, outflow tract of the heart, enteric ganglia and pigment cells. During embryogenesis, a combination of gene expression driven by Wnt, FGF and BMP pathways determine the future characteristics of the head ectoderm, transforming it either into placodes, neural plate, epidermis or NC cells (Garnett et al., 2012; Groves and LaBonne, 2014; Litsiou et al., 2005; Simoes-Costa and Bronner, 2015; Steventon et al., 2009). Wnt/β-catenin signaling represents one of the most extensively studied signaling cascade. Binding of secreted Wnt proteins to their Frizzled receptors and their Lrp5/6 co-receptors initiates a complex cascade of intracellular events leading to the stabilization of β-catenin. Stabilized β-catenin accumulates in the cytoplasm and enters the nucleus where it binds to the Tcf/Lef family of transcription factors, thereby regulating the expression of Wnt target genes (Clevers and Nusse, 2012; http://web.stanford.edu/group/nusselab/cgi-bin/wnt/).

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56   Four Tcf/Lef genes have been identified in vertebrates; *Lef1* (alias *Lef1*) (Travis et

57   al., 1991), *Tcf7* (alias *Tcf1*) (van de Wetering et al., 1991), *Tcf7l1* and *Tcf7l2* (alias *Tcf3*

58   and *Tcf4*) (Castrop et al., 1992). Tcf/Lefs share several characteristic protein features,

59   such as the N-terminal β-catenin binding motif, GRG motifs for binding of Groucho/TLE

60   co-repressors, and the HMG box containing the DNA binding domain. Tcf7 and Tcf7l2

61   also contain a C-clamp, a second DNA binding motif that further modulates gene target

62   specificity (Cadigan and Waterman, 2012). Distinct expression patterns of individual

63   Tcf/Lefs and their unequal capability to activate/repress Wnt target genes, represent the

64   ultimate regulation step of the Wnt/β-catenin signaling pathway. Tcf7l1, expressed in the

65   anterior ectoderm at E7.5 and in the anterior neural fold (ANF) at E8.5 of the developing

66   mouse embryo (Korinek et al., 1998; Merrill et al., 2004), is the least potent activator but

67   the strongest repressor from the whole family (Cole et al., 2008; Liu et al., 2005; Ombrato

68   et al., 2012). Tcf7l1-deficient mice, that display duplicated axis and a severely reduced

69   anterior part of the embryo, die shortly after gastrulation (Merrill et al., 2004). Tcf7l1

70   inactivation appears to allow Wnt/β-catenin signaling to spread towards the anterior

71   neural fold in both zebrafish and mouse. This leads to a deficiency in expression of the

72   ANF specific genes accompanied with severe defects in head formation (Andoniadou et

73   al., 2011; Dorsky et al., 2003; Kim et al., 2000). All these data have been interpreted in a

74   manner consistent with the repression of Wnt signaling in the ANF being required for A-P

75   axis determination of the neural tube.

76   Experiments in *Xenopus*, zebrafish and chicken have shown a requirement for

77   Wnt/β-catenin signaling from the earliest steps of neural crest development - at first

78   during gastrulation, when Wnt/β-catenin signaling drives the expression of the NC

79   specifier genes *Pax3* (Bang et al., 1999; Garnett et al., 2012; Taneyhill and Bronner-

80   Fraser, 2005; Zhao et al., 2014), *FoxD3* (Janssens et al., 2013) and *Gbx2* (Li et al.,

81   2009), later during NC maintenance (Kleber et al., 2005; Steventon et al., 2009) and the

82   epithelial-to-mesenchymal transition (EMT), via triggering the *Slug* gene expression

83   (Burstyn-Cohen et al., 2004; Sakai et al., 2005; Vallin et al., 2001). Compared to non-

84   mammalian experimental models, a clear picture about the role of Wnt/β-catenin

85   signaling in early NC cell populations in mammals is absent. This is partly caused by a

86   lack of appropriate Cre drivers for the early pre-migratory NC lineage. The widely used

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87   Wnt1-Cre mouse targets the NC population after its induction and specification (Danielian
88   et al., 1998; Lewis et al., 2013). Depletion of β-catenin using Wnt1-Cre leads to
89   malformations of the craniofacial structures, accompanied with a deficiency in sensory
90   neurons (Brault et al., 2001; Gay et al., 2015). Similar phenotypic changes have been
91   observed in compound Wnt1/Wnt3a mutants that also show impaired formation of
92   sensory neurons, and defects in several bones and cartilage derived from the NC (Ikeya
93   et al., 1997). Ectopic Wnt1-Cre driven expression of stabilized β-catenin (β-cat$^{Ex3fl/+}$)
94   results in excessive differentiation of NC progenitors into NeuroD and Brn3a-positive
95   sensory neurons, thus limiting NC multipotency (Lee et al., 2004). Collectively, these data
96   confirm the requirement of Wnt/β-catenin signaling in NC maintenance and differentiation
97   however, they do not clarify the role of Wnts in NC induction and specification. The
98   apparent inconsistency may be explained in two ways; either the signaling pathway
99   network governing NC induction, and specification differs between mouse and other
100   species (Barriga et al., 2015) or, more probably, the Wnt1-Cre driver targets the already
101   specified NC, thus not allowing the investigation of the earlier events of NC development.

102   The aim of our work was to investigate the role of Wnt/β-catenin signaling in the
103   early development of the mammalian NC. We used a Cre-line derived from a NC inducer
104   gene, Tfap2α (Ap2α-Cre) (Macatee et al., 2003), in order to achieve conditional deletion
105   of Tcf7l1 in the earliest possible steps of the NC development. Conditional inactivation of
106   Tcf7l1 leads to trans- differentiation of ANF into NC cells accompanied with a loss of
107   anterior neural markers. Our data suggest that Tcf7l1, acting as a Wnt/β-catenin
108   repressor, is required to maintain the anterior neural character by restricting the
109   expansion of the Wnt-induced NC cells.

110   **RESULTS**

111   **AP2α-Cre mediated deletion of Tcf7l1 during mouse NC specification results in**
112   **exencephaly.**

113   Tcf7l1 is detected in the developing mouse ectoderm prior to gastrulation, and
114   later at E7.5, and it is expressed in the anterior ectoderm and mesoderm (Korinek et al.,
115   1998; Merrill et al., 2004). We characterized the expression of *Tcf7l1* during neurulation,

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116   using whole mount *in situ* hybridization. *Tcf7l1* mRNA was detected in the anterior neural

117   plate at the 1 somite pair (s) stage, being further restricted to the ANF region and along

118   the anterior NPB with the progress of neurulation 3-4s (Fig. 1A). We next wanted to relate

119   the expression pattern of *Tcf7l1* to that of known NC-specific genes and the area of

120   Wnt/β-catenin signaling. *Tfap2α* is one of the key genes involved in the NC induction,

121   while *FoxD3, Sox9, Sox10* have been linked with the NC specification (Groves and

122   LaBonne, 2014; Simoes-Costa and Bronner, 2015). During neurulation, the Tfap2α

123   protein is continuously expressed along the whole NPB (Fig. 1B). First *Sox9* and *FoxD3*

124   transcripts were detected in the neural plate as early as at the 1-2s stage, at 3-4s were

125   expressed robustly in two stripes along the anterior neural plate border (Figs 1C, S1A).

126   The onset of *Sox10* mRNA was detected later (4-5s) and slightly more caudally, in a very

127   similar pattern as the expression of *Sox9* (Fig. S1B). To map the activity of the Wnt/β-

128   catenin pathway, we used the BAT-gal reporter mice (Maretto et al., 2003). The BAT-gal

129   signal, detected by immunostaining, spread from the 2-3s stage across the prospective

130   mesencephalon and metencephalon, but not reaching the ANF at the 4-5s stage (Fig.

131   1D). In conclusion, *Tcf7l1* expression in the ANF is reciprocal to that of *Sox9*, *FoxD3*,

132   *Sox10* and BAT-gal, that resides in the caudal areas of the neural plate, but it overlaps

133   with Tfap2α, lining the whole NPB.

134   In order to study the function of Tcf7l1 during NC induction and specification, we

135   inactivated Tcf7l1 by crossing Tcf7l1 conditional knock-out mice (EUCOMM) with AP2α-

136   IRESCre (AP2α-Cre) transgenics (Macatee et al., 2003). Tracing of the AP2α-Cre-driven

137   recombination using the Rosa26$^{EYFPfl/+}$ reporter strain revealed the first EYFP-positive

138   cells scattered throughout the embryo at E7.0 (Fig. 1E). The EYFP signal became

139   focused along the entire NPB at the 4s stage with the highest levels detected rostrally, in

140   the neural- and non-neural ectoderm of the ANF at the 5s stage. AP2α-Cre most probably

141   mediates the recombination in the entire NC population as demonstrated by complete co-

142   localization of double immunofluorescence of EYFP-positive cells and Sox10-positive NC

143   cells in AP2α-Cre;Rosa26$^{EYFPfl/+}$ embryos at the 10s stage. At E10.5, the EYFP signal

144   was present in NC derivatives (brachial arches, dorsal root ganglia and sensory neurons),

145   the head ectoderm, lens placodes, and in the anterior telencephalon (Fig. 1E, Fig. S1C).

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146　　　　In AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants, *Tcf7l1* mRNA was completely lost, whereas control

147　　embryos showed high levels of transcripts along the anterior NPB including the ANF at

148　　the 5s stage (Fig. 1F-G). At this stage, phenotypical changes following *Tcf7l1* deletion

149　　became apparent; the shape of neural folds changed from rounded into more a narrow

150　　morphology and the neural plate expanded laterally at the prosencephalic-mesencephalic

151　　boundary (dashed lines in frontal views in Fig. 1F,G). At later stages (E10.5), we

152　　observed a variability in the penetrance of morphological changes among individual

153　　mutants. Therefore, we decided to classify the defects into two categories: (i) a strong

154　　phenotype, represented by a severe reduction of the telencephalic tissue and

155　　exencephaly (arrow Fig. 1H); and (ii) a mild phenotype, with reduced telencephalon and

156　　bilateral anopthalmia (arrowhead Fig. 1H). Quantification of phenotype incidence,

157　　between E10.5-E13.5, revealed that the strong phenotype was present in 60.5% and the

158　　mild phenotype in 39.5% of the analyzed embryos (n=38) (Fig. 1I). Mutant embryos with

159　　the strong phenotype did not survive beyond E13.5, exhibiting massive exencephaly and

160　　severe defects in craniofacial structures (Fig. S1D, E). These data demonstrate that

161　　Tcf7l1 function in the ANF is crucial for the development of the mouse forebrain.

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163   **Tcf7l1 is required for the expression of anterior neural markers**

164   Tcf7l1 has been implicated in A-P patterning of the developing brain in both

165   zebrafish and mouse since its loss-of-function mutations result in severe reductions of

166   anterior neural regions, accompanied with apparent expansion of midbrain and hindbrain

167   markers towards the anterior (Dorsky et al., 2003; Merrill et al., 2004). In contrast to

168   published data, we did not observed any shift in mRNA expression of the core regulators

169   of the midbrain-hindbrain boundary (MHB). *Fgf8*, *Gbx2* and *Wnt1* were expressed

170   anteriorly when comparing Tcf7l1 conditional mutant embryos and controls at E9.5

171   (arrowheads in Figs 2A-B´, S3C). Remarkably, we observed a progressive loss of the

172   posterior prosencephalon marker *Tcf7l2* (Tcf4) mRNA (Fig. 2C-C´´), illustrating the

173   gradual loss of anterior neural tissue in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants with mild (C´) or

174   strong (C´´) phenotype at E9.5. Furthermore, the expression of the ANF specific

175   transcripts, *Six3* (Lagutin et al., 2003), *FoxG1* (Hanashima et al., 2004) and *Fgf8* (Meyers

176   et al., 1998) was lost or strongly reduced in mutants at the 8s stage (Fig. 2D-F´). Next we

177   tested whether the ANF cells affected by Tcf7l1 deletion retained their neural character.

178   Reduced expression of the neural marker *Sox2,* revealed by RNA *in situ* hybridization,

179   uncovered an expansion of *Sox2* negative cells along the whole lateral border of the ANF

180   in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants (Fig. 2G,G´), suggesting that the cells had lost their neural

181   identity.

182   **Tcf7l1 deletion results in aberrant trans-differentiation of the ANF cells into the NC**

183   NC cells need to lose their neuroepithelial character in order to undergo EMT and

184   migrate towards their target destinations. We decided to investigate the expression of the

185   transcription factors FoxD3, Sox9 and Sox10, key regulators of the NC specification,

186   responsible for initiation of EMT in *Xenopus* and chicken (Cheung et al., 2005; Dottori et

187   al., 2001; Liu et al., 2013), in the absence Tcf7l1. As can be seen from the whole-mount

188   immunostaining, Sox9 positive NC cells expanded into the Sox1 positive ANF region in

189   AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants compared with controls at the 5s stage (Fig. 3A). Examination

190   under higher magnification showed decreased neural Sox1 staining towards the anterior

191   part of the mutant embryo. A frontal view on the same embryos clearly showed ectopic

192   Sox9-positive NC cells along the ANF in mutants (Fig. 3B). This was further confirmed by

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193   *in situ* hybridization of *Sox9* mRNA (arrowhead Fig. S2A). Consistently, the expression of

194   *Foxd3* mRNA also shifted anteriorly in mutant embryos compared to controls at the 10s

195   stage (Fig. 3C). Later at E9.0, aberrant *Foxd3* mRNA was detected in the rostral part of

196   the head in mutants (arrowhead Fig. S2B), while it was completely absent in controls.

197   Expression of *Sox10* mRNA was apparently delayed in the absence of Tcf7l1, being

198   barely detectable at the 6s stage (Fig. 3D). Two days later, at E9.5, however, RNA *in situ*

199   hybridization revealed robust ectopic expression of *Sox10* in the anterior part of the head

200   of mutant embryos (arrowhead Fig. 3D). Interestingly, we also observed a cell fate shift,

201   characterized by the change from Sox1 to Sox9, in caudal neural tissue. Coronal

202   sectioning of the developing hindbrain at E9.0 also uncovered aberrant Sox9-

203   positive/Sox1-negative cells in the mutants (arrowheads Fig. S2C). Taken together, the

204   expression of several genes responsible for the NC specification and EMT initiation is

205   expanded towards ANF in the AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants, suggesting that Tcf7l1

206   maintains the neuroepithelial character of the ANF and restricts spreading of NC within

207   the anterior head.

208   **Trans-differentiation of the ANF is driven by aberrant Wnt/β-catenin signaling**

209   　　Tcf7l1 is a very efficient repressor of Wnt/β-catenin signaling target genes, as has

210   been shown in both mouse embryonic stem cells and *in vivo* (Cole et al., 2008; Shy et al.,

211   2013; Wu et al., 2012). Gain- and loss-of-function experiments in non-mammalian

212   vertebrates have shown that the Wnt/β-catenin pathway is required for physiological, and

213   sufficient for ectopic, induction and expansion of the NC (Garcia-Castro et al., 2002;

214   Heeg-Truesdell and LaBonne, 2006; Steventon et al., 2009; Taneyhill and Bronner-

215   Fraser, 2005; Wu et al., 2005). In our transient reporter assays using the SuperTopFlash

216   reporter in HEK293 cells, Tcf7l1 was able to compete with other Tcf/Lefs, reducing Wnt

217   reporter signal significantly (Fig. S3A). Therefore we asked, whether the expansion of NC

218   cells in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants, was caused by upregulation of Wnt/β-catenin

219   signaling. We crossed BAT-Gal reporter mice with Tcf7l1 conditional mutants and stained

220   whole-mount embryos with anti-galactosidase and anti-Sox9 antibodies.

221   　　As expected, the Wnt reporter BAT-gal immunofluorescence expanded anteriorly

222   in the AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants (arrow, Fig. 4A). This was accompanied with a high

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223   number of ectopic Sox9-positive cells, as clearly visible from the frontal view of the 8s

224   stage embryos (white arrowheads, Fig. 4A). The coexpression of Sox9 and BAT-gal

225   positive cells does not seem to be aberrant, as cross-sections from the 8s old wild-type

226   embryos showed large overlaps between the Sox9 and BAT-gal immunofluorescence

227   (Fig. S3B), indicating that Sox9 may be induced by Wnts. Furthermore, expression of *Sp5*

228   mRNA, a known Wnt/β-catenin target gene, was also upregulated and expanded rostrally

229   in the mutant embryos at 6s stage (arrowheads, Fig. 4B), confirming the ectopic increase

230   of the Wnt/β-catenin signaling activity in the anterior neuroectoderm. Apart from its role in

231   MHB formation (McMahon and Bradley, 1990; Thomas and Capecchi, 1990) is Wnt1

232   required also during NC development (Ikeya et al., 1997; Saint-Jeannet et al., 1997).

233   Interestingly, we found aberrant *Wnt1* mRNA expression in the ANF of the Tcf7l1 mutants

234   at the 5-6s stage (Fig. 4C). The ectopic pattern of *Wnt1* mRNA expression along the NPB

235   of the prosencephalon was even more obvious later, at E9.0 (arrowheads, Fig. 4C). We

236   observed no difference in *Wnt1* mRNA expression in the MHB between controls and

237   Tcf7l1 mutants (arrowheads, Fig. S3C) at the same stage, suggesting that the aberrant

238   expression of *Wnt1* reflects the NC phenotype, rather than changes in A-P pattering.

239   To confirm that the phenotype observed in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants resulted

240   from an aberrant activation of the Wnt/β-catenin pathway, we activated the signaling

241   using β-cat$^{ΔEx3fl/fl}$ mice (Harada et al., 1999), in which a truncated, non-degradable form of

242   β-catenin is produced upon AP2α-Cre-mediated recombination. The AP2α-Cre;β-

243   cat$^{ΔEx3fl/+}$ embryos exhibited neural tube closure defects and did not survived beyond

244   E10.5. The crossing of the BAT-gal reporter into AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants revealed

245   activation of the reporter in the forebrain, which was even more pronounced in AP2α-

246   Cre;β-cat$^{ΔEx3fl/+}$ mutants that also showed a strong over-activation of the signal in most of

247   the head ectoderm when compared with controls (Fig. 4D-F). A lateral view of the Tcf7l1

248   and β-cat$^{ΔEx3fl/+}$ mutants clearly showed an aberrant population of BAT-gal-positive cells

249   migrating towards the branchial arches (arrowheads, Fig. 4D′-F′). Cross sections of these

250   embryos uncovered aberrant BAT-gal staining in the forebrain of both Tcf7l1 and β-

251   cat^{ΔEx3} mutants (Fig. 4D′′-F′′).

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252   Next we assayed the EMT using immunofluorescence of neuroepithelial cells
253   marked with N-cadherin and migrating NC cells labelled with Sox9 antibodies. In control
254   embryos, we observed only a thin layer of Sox9 positive cells between the N-cadherin
255   positive forebrain tissue and the epidermis. The hindbrain in controls labeled with N-
256   cadherin was found to be completely devoid of Sox9 positive cells at E9.5. (Fig. 4G). In
257   contrast, AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants clearly displayed aberrant Sox9-positive cells in the
258   hindbrain prior to EMT (arrowheads, Figs 4H, S2C). Concomitantly, N-cadherin
259   expression was weaker in the anterior forebrain and the dorsal hindbrain mutants with
260   "strong" phenotype (arrows Fig. 4H). Intriguingly, the same analysis of AP2α-Cre;β-
261   cat$^{Ex3fl/+}$ embryos revealed that almost the whole mutant forebrain converted into Sox9
262   positive NC. Similarly to Tcf7l1 conditional mutants, we also detected aberrant Sox9
263   expressing cells in the hindbrain, accompanied with a massive decrease in the levels of
264   N-cadherin (arrows, Fig. 4I). Further, protein levels of the Wnt/β-catenin transcriptional
265   activator and target gene Lef1 (Galceran et al., 1999; Kratochwil et al., 2002; Liu et al.,
266   2005), were increased in the forebrain of the Tcf7l1 mutants in comparison with controls
267   (Fig. 4J,K). β-cat^{Ex3} mutants displayed very strong upregulation of Lef1 in the
268   mesenchyme adjacent to the hindbrain as well as in the forebrain (Fig. 4L). Whole-mount
269   immunofluorescence of wild-type embryos revealed colocalization of Lef1 and Sox10-
270   positive cells at the 8s stage (arrowhead, Fig. S3C). It should be noted that in addition to
271   the effects on NC population, the strong activation of the Wnt/β-catenin pathway in the
272   AP2α-Cre;β-cat$^{Ex3fl/+}$ mutants disorganized both the A-P patterning of the neural tissue
273   and the non-neural ectoderm surrounding the NC. This was clearly visible from the
274   expansion of *Gbx2* and *Fgf8* mRNA at E9.5 (Fig. S3E,F). Taken together, deletion of
275   Tcf7l1 resulted in an ectopic activation of the Wnt/β-catenin pathway, suppression of
276   neuroepithelial character and premature initiation of NC cell fate. Similar observations
277   were made in conditional AP2α-Cre;β-cat$^{Ex3fl/+}$ mutants that yielded even more profound
278   changes in the expression of described cell markers and in larger areas than in Tcf7l1
279   mutants.

280   **The phenotype can be rescued with a truncated form of TCF4 but not with Tcf7l1**
281   **fused with β-catenin**

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282   Our data suggest that the loss of Tcf7l1-mediated repression of Wnt targets is the
283 primary cause of NC cell fate expansion. In order to verify this hypothesis, we performed
284 rescue experiments using two mouse strains, conditionally expressing either dominant
285 Tcf repressor or activator. As a dominant repressor we used a mouse strain
286 Rosa26$^{dnTCF4fl/+}$ containing the N-terminally truncated form of the human TCF7L2 targeted
287 into the ROSA26 locus 3′to the lox-STOP TdTomato (TdT) cassette (originally referred to
288 as Rosa26$^{+/tdTomato}$). This form cannot bind β-catenin and thus acts as dominant repressor
289 of Wnt/β-catenin targets (Janeckova et al., in press). Secondly, we generated the mouse
290 strain Rosa26$^{ctβcat-Tcf7l1fl/+}$, allowing conditional expression of full-length mouse Tcf7l1
291 fused with the transactivation domain of β-catenin (aa696-781), analogous to the
292 described Cat-Lef fusion (Hsu et al., 1998). This construct, that mimics the β-catenin
293 binding to Tcf7l1, was able to deliver activation of the Wnt reporter (Fig. S4A). Details
294 about the strain generation and characterization are present in the supplementary (Fig.
295 S4B-G). Following Cre recombination, the TdT cassette is excised, allowing production of
296 either dnTCF4 or ctβcat-Tcf7l1 mRNA (Fig. 5A).

297   We crossed the Tcf7l1$^{fl/fl}$;Rosa26$^{dnTCF4fl/fl}$ and AP2α-Cre;Tcf7l1$^{fl/+}$ strains and
298 evaluated the strength of the phenotype in AP2α-Cre;Tcf7l1$^{fl/fl}$;Rosa26$^{dnTCF4fl/+}$ compound
299 mutants at E10.5 stage. Intriguingly, overexpression of the dnTCF4 was able to reduce
300 the occurrence of the strong phenotype by more than half (27.3%), compared with the
301 60.5% incidence in the AP2α-Cre;Tcf7l1$^{fl/fl}$ mutant. The effect of the rescue on NC
302 population was further analyzed by *in situ* hybridization showing no or very little ectopic
303 *Sox10* mRNA in the AP2α-Cre;Tcf7l1$^{fl/fl}$;Rosa26$^{dnTCF4fl/+}$ 'rescued' mutants (Fig. 5B,D). On
304 the contrary, the βcat-Tcf7l1 fusion protein showed significantly (P=0.049, Fishers test)
305 decreased capability to rescue the phenotype than the dnTCF4, as demonstrated by
306 85.7% of analyzed embryos displaying the strong phenotype in the AP2α-Cre;Tcf7l1$^{fl/fl}$;
307 Rosa26$^{ctβcat-Tcf7l1fl/+}$ compound mutants. Expression of ctβcat-Tcf7l1 was also unable to
308 reduce the aberrant, *Sox10* mRNA positive, NC population (Fig. 5C,D). In summary,
309 using transgenic mice strains, we have genetically confirmed that Tcf7l1, acting as a
310 repressor of Wnt/β-catenin signaling, protects anterior neural tissue from conversion to a
311 NC cell fate.

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312 **Discussion**

313 **How does the progressive activation of Wnt/β-catenin pathway affect development**
314 **of the anterior neural tissue and neural crest?**

315   Spatio-temporal regulation of Wnt/β-catenin pathway activation, required for proper
316 development of the prosencephalon, is secured by expression of several ANF specific
317 negative regulators of the Wnt/β-catenin signaling. Our data (Figs 4,5,S3), and evidence
318 from others (Cole et al., 2008; Liu et al., 2005; Ombrato et al., 2012; Wu et al., 2012)
319 characterize Tcf7l1 as a Wnt/β-catenin target gene repressor, implicating aberrant
320 activation of the Wnt/β-catenin pathway as the cause of strong A-P patterning defects
321 that follow Tcf7l1 deficiency both in zebrafish (Dorsky et al., 2003; Kim et al., 2000) and
322 mouse (Merrill et al., 2004). In zebrafish, the Tcf7l1a loss-of-function in headless (*hdl*)
323 mutants, as well as MO-mediated knock-down of Tcf7l1a and Tcf7l1b variants, result in
324 the expansion of MHB markers, *Pax2.1* and *Gbx1* mRNA expression anteriorly,
325 accompanied by the loss of *Six3* and *Rx3* expression (Dorsky et al., 2003). The Tcf7l1
326 mutants in mouse also displayed the expansion of the midbrain mRNA marker *Engrailed,*
327 joined with the downregulation of *Hesx1* expression (Merrill et al., 2004). Similarly, the
328 loss of anterior neural tissue in mouse follows deletion of *Dkk1* (Mukhopadhyay et al.,
329 2001), *Six3* (Lagutin et al., 2003), *Hesx1* (Andoniadou et al., 2007) and *ICAT* (Satoh et
330 al., 2004) and has been interpreted as a defect in A-P patterning induced by aberrant
331 Wnt/β-catenin signaling. We contend that this is too generalized view and that, in the
332 context of the ANF phenotypes, is only partially true. In *Xenopus*, low levels of ectopic
333 Wnt/β-catenin activation enrich the NC population without affecting A-P patterning, while
334 high doses affect both (Wu et al., 2005). This might be also valid for mammals, as mice
335 carrying ENU-induced activating point-mutations in Lrp6 and β-catenin display a gradual
336 loss of the prospective prosencephalon, depending on the strength of the Wnt/β-catenin
337 pathway overactivation, but does not manifest a shift of posterior markers towards the
338 anterior part of the embryo (Fossat et al., 2011). Furthermore, compound deletion of one
339 *Hesx1* allele and both alleles of *Tcf7l1* in mouse, using prosencephalon-specific Hesx1-
340 Cre, results in the aberrant activation of Wnt/β-catenin signaling, accompanied with loss
341 of anterior neural tissue without affecting the A-P patterning (Andoniadou et al., 2011).

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342   Similarly, we did not detect any changes in the expression of the MHB markers *Fgf8* or

343   *Gbx2* in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants. Nevertheless, we do observe a gradual loss

344   (depending on the strength of the phenotype) of the posterior prosencephalon marker

345   *Tcf7l2* and the ANF markers *Six3*, *Fgf8* and *FoxG1* (Fig. 2). This was accompanied by the

346   expansion of *Sox9*, *FoxD3* and *Sox10*-positive NC populations along the NPB anteriorly

347   (Figs 3,S2). Therefore we conclude that the loss of Tcf7l1 mediated repression of Wnt

348   target genes in the AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants allows the gradient of Wnt ligands to

349   influence cell fate in a wider region of the ANF than in controls, without having an effect

350   on A-P patterning. This is not the case in AP2α-Cre;β-cat$^{Ex3fl/+}$ mutants, in which ectopic

351   stabilization of β-catenin facilitates strong over-activation of Wnt/β-catenin driven

352   transcription, affecting both NC (Fig. 4) and A-P patterning (Fig. S3). Notably, we

353   observed effects on the population of undifferentiated Sox9-positive NC cells in AP2α-

354   Cre;β-cat$^{Ex3fl/+}$ mutants at E9.5 and earlier (data not shown). In contrast, the imbalance in

355   NC differentiation observed in Wnt1-Cre/β-cat$^{Ex3fl/+}$ mutants occurred one day later at

356   E10.5 (Lee et al., 2004). This merely reflects the earlier onset of the AP2α-Cre (Macatee

357   et al., 2003) mediated Cre recombination (Fig. 1) than in the widely used Wnt1-Cre

358   (Danielian et al., 1998; Lewis et al., 2013), making AP2α-Cre a more suitable tool for

359   studying early events of NC development. Our data provide clear evidence for a notably

360   earlier role of the Wnt/β-catenin pathway in NC development in regard to previously

361   published findings from the mouse model showing the requirement of the Wnt/β-catenin

362   pathway in NC maintenance and differentiation (Brault et al., 2001; Gay et al., 2015; Lee

363   et al., 2004).

364   The effects of the Tcf7l1 deficiency in mice and zebrafish on NC development

365   were not, until now, characterized in detail. Analogously to the NC expansion in AP2α-

366   Cre;Tcf7l1$^{fl/fl}$ mutant mice (Figs 3,4,S2), we observed ectopic expression of *FoxD3*, *Pax3*

367   and *Sox9* mRNA along the entire anterior NPB upon Tcf7l1a MO-mediated knock-down

368   in zebrafish (Fig. S2D), implicating the conservation of Tcf7l1 function across vertebrate

369   species. In accord with this, the enrichment of the NC population, marked by the

370   expansion of Snail2 mRNA expression towards the anterior portion of the embryo, follows

371   the MO-mediated knock-down of Dkk1 in *Xenopus* (Carmona-Fontaine, 2007). In mouse,

372   Hesx1 mutants display expansion of Wnt target genes as well as the NC mRNA markers

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373   *Pax3* and *FoxD3* anteriorly (Andoniadou et al., 2007) and the loss of the Wnt inhibitor

374   Dkk1 leads to the expansion of the NC mRNA marker *Sox10* (Carmona-Fontaine et al.,

375   2007), completely in agreement with the interpretation of the data presented here.

376   Furthermore, mutual dispensability of these factors was demonstrated in zebrafish, where

377   injection of either *Six3* or *Hesx1* mRNA rescued the *hdl* phenotype (induced by Tcf7l1a

378   deletion) (Andoniadou et al., 2011; Lagutin et al., 2003).

379   In summary, collective evidence point towards a coordinated expression of several

380   Wnt/β-catenin pathway inhibitors in the anterior neuroectoderm that together define,

381   under physiological conditions, a spatial border to the expansion of the NC. Similarity in

382   the phenotypes following their deficiency signifies that the ANF region is very close to a

383   threshold of the NC inducing Wnt/β-catenin activation. Therefore, from the evolutionary

384   perspective, attenuation of the sensitivity to Wnt/β-catenin signaling in the ANF region by

385   fine-tuning the expression levels of Wnt signaling inhibitors may represent a very

386   sensitive tool that allows simultaneous modification of two prominent features of the

387   vertebrates, the neural crest and the forebrain.

388   **Key question remains: which Wnt/β-catenin target genes act as NC inducers?**

389   Our analysis of the AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants identify Tcf7l1 as a negative

390   regulator of the NC population, suggesting that other Tcf/Lefs are responsible for

391   activation of Wnt/β-catenin targets in the NC. It is likely that Lef1, Tcf7 and Tcf7l2 are

392   redundant in NC cells since whole body double knock-outs of Lef1/Tcf7 and Tcf7/Tcf7l2

393   in mouse do not display any obvious NC phenotype (Galceran et al., 1999; Gregorieff et

394   al., 2004). Ectopic Sox9-positive cells (Fig. 4K,L) in AP2α-Cre;β-cat$^{ΔEx3fl/+}$ mutants

395   spatially overlap with the elevation of the BAT-gal reporter (Fig. 4H′,I′), Lef1, Tcf7 but not

396   Tcf7l2 proteins (Fig. 4N,O and data not shown), suggesting that Lef1 and Tcf7 are

397   positively regulated by Wnt/β-catenin signaling and probably participate in the expression

398   of Wnt responsive NC specific genes. Sp5, another well-defined Wnt target gene and

399   transcriptional repressor (Fujimura et al., 2007) was strongly upregulated in AP2α-

400   Cre;Tcf7l1$^{fl/fl}$ mutants spreading its expression across the entire ANF (Fig. 4). Work in

401   *Xenopus* has identified Sp5 as a novel NC specific inducer (Park et al., 2013). MO-

402   mediated knock-down of *Sp5* leads to the upregulation of *Zic1*, making it a candidate

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403   target of Sp5 repression. Zic1 acts as a NC inducer only when expressed in appropriate

404   balance with Pax3, another NC inducer (Hong and Saint-Jeannet, 2007; Park et al.,

405   2013). Pax3 has been recently identified as a direct target of Tcf/Lefs (Zhao et al., 2014),

406   providing an additional link to the GRN driving NC induction. It is quite possible that

407   aberrant induction of Sp5 mediated repression of Zic1, in combination with the ectopic

408   expression of Wnt1 ligand (Fig. 4) enhancing the β-catenin/Tcf/Lef driven transcription of

409   Pax3, might serve as an artificial positive feedback loop, further potentiating the effects

410   on NC induction. Additionally, the aberrant Wnt/β-catenin signaling present in AP2α-

411   Cre;Tcf7l1$^{fl/fl}$ mutants might upregulate the expression of the transcription factor Axud1, a

412   novel Wnt dependent, positive regulator of NC specification (Simoes-Costa et al., 2015).

413   In summary, we demonstrated that Tcf7l1 mediated repression of β-catenin target

414   genes protects the anterior neuroectoderm from converting into NC and through that we,

415   (i) help to eliminate the ambiguity in the interpretation of NC development in mouse and

416   other vertebrate model organisms and, (ii) provide a model explaining the phenotypes

417   previously acquired in mouse after de-repression of the Wnt/β-catenin signaling pathway

418   during early brain development.

419

420   **Materials and methods**

421   **Mouse strains**

422   Housing of mice and *in vivo* experiments were performed in compliance with the

423   European Communities Council Directive of 24 November 1986 (86/609/EEC) and

424   national and institutional guidelines. All procedures involving experimental animals were

425   approved by the Institutional Committee for Animal Care and Use (permission PP-

426   071/2011, PP-281/2011). This work did not include human subjects. AP2α-IRESCre

427   (AP2α-Cre) was described previously (Macatee et al., 2003), Cre reporter lines

428   Rosa26$^{EYFPfl/+}$ (B6.129X1-*Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$*/J, stock No: 00614) and ROSA26

429   (B6.129S4-Gt(ROSA)26Sor^{tm1Sor}/J), stock No: 003474) were purchased from The

430   Jackson Laboratory. Tcf7l1$^{fl/+}$ strain (Tcf7l1$^{tm1a(EUCOMM)Wtsi}$) was purchased from the

431   EUCOMM. Transgenic mouse strain BAT-gal (Maretto et al., 2003) was kindly provided

15

432 by S. Piccolo. Mice with a conditional "floxed" β–catenin (β-cat$^{Ex3fl/+}$) were kindly provided

433 by Dr. M. M. Taketo (Harada et al., 1999). Generation of Rosa26$^{dnTCF4fl/fl}$ was described in

434 (Janeckova et al., in press). Briefly, human TCF7L2 coding sequence lacking N-terminal

435 domain (aa 1-31) was cloned into the ROSA26 targeting vector containing arms for

436 homologous recombination into ROSA26 locus in a same way as when generating the

437 Rosa26$^{ctβcat-Tcf7l1fl/+}$. Details about the Rosa26$^{ctβcat-Tcf7l1fl/+}$ mouse line generation are

438 present in Fig. S4. A C-terminal part of β-catenin (aa 696-781) was fused with the mouse

439 full length Tcf7l1 and inserted into ROSA26 locus using homologous recombination in ES

440 cells. The construct carries tdTomato coding sequence flanked with the LoxP sites

441 serving as a stop cassette for ctβcat-Tcf7l1. Excision of the tdTomato cassette via Cre-

442 mediated recombination, allows transcription of ctβcat-Tcf7l1 mRNA (Fig. 5A). PGK-Neo

443 cassette was removed using FLP-FRT recombination, F1 animals were crossed to the

444 Actb:FLPe strain (Rodriguez et al., 2000) and kept on a C57BL/6J background. Primers

445 for genotyping Rosa26$^{ctβcat-Tcf7l1fl/+}$ allele: JM21F GAGGAGCCATAACTGCAGAC, JM23R

446 CCTGGCTATCCTAGATAGAAC and JM27R GTACTGTTCCACGATGGT the expected

447 size of the PCR product is 965bp in the mutant and 300bp in the wild-type.

448 **MO knock-down in zebrafish**

449 Zebrafish wild-type, single-cell embryos were injected with combination of previously

450 described Tcf7l1a (5′ CTCCGTTTAACTGAGGCATGTTGGC 3′) and Tcf7l1b (5′

451 CGCCTCCGTTAAGCTGCGGCATGTT 3′) morpholinos (Dorsky et al., 2003), in 1:1 ratio

452 at a final concentration of 1.6ng/nl (1-2nl injected) (Gene Tools). Embryos were than

453 raised at 28°C and harvested at 12hpf.

454 **Immunohistochemistry and RNA in situ hybridization**

455 At least three embryos of each genotype and similar developmental stage were analyzed

456 per staining. All used primary antibodies are listed in Supplementary Table S1.

457 Secondary antibodies: anti-mouse (-chicken, -rabbit, -goat) Alexa Fluor488 or 594 (Life

458 Technologies), Biotinylated anti-rabbit (Vector Laboratories), Vectastain ABC Elite kit and

459 ImPACT DAB substrate (all Vector Laboratories). For wholemount immunohistochemistry

460 we used the protocol published by Sandell et al. (Sandell et al., 2014). Briefly, embryos

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461 were fixed overnight in 4% PFA, permeabilized using Dent's bleach and 100% methanol
462 and rehydrated. Blocking for 2 hours and antibody incubation overnight were performed
463 in 3% BSA in 0.1M Tris-Cl, pH 7.5, 0.15 M Nacl, 0.03% Triton X-100. After 5 times of 1-
464 hour washing in PBS, overnight incubation with secondary antibody in the blocking buffer
465 followed. The next day, embryos were stained for nuclei using Hoechst (Life
466 technologies), washed three times in PBS (20 minutes each) and stored in 2%
467 formaldehyde (Sigma). Whole-mount immunofluorescence was captured using binocular
468 microscope Apotome.2 Ax10 zoom.V16 (Zeiss). Fluorescence and bright-field light
469 images were acquired in Nikon Diaphot 300 with objectives 4x/0.1 and 10x/0.25 and
470 confocal Leica SP5.

471 Whole-mount *in situ* hybridization was performed using standard protocols. Riboprobes
472 were cloned into pGEM-T-easy vector (Promega) using primers specified in the
473 Supplementary Table S1.

474 **SuperTop Flash reporter assay.**

475 HEK293 cells (30 000 cells/well/96-well plate) were transiently transfected using
476 Lipofectamine 2000 (Invitrogen) with 30 ng of Super8X TopFlash and 1 ng of Renilla
477 luciferase constructs and indicated amount of the expression vector, filled with mock DNA
478 to final concentration of 200ng DNA per well. Luciferase assays were performed 48h later
479 with a Dual-Glo luciferase kit (Promega). The luciferase signal was normalized to a co-
480 transfected Renilla readout. Experiments were performed on at least 6 biological
481 replicates per condition.

482 **Acknowledgments**

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490   **Competing interest statement**

491   The authors declare no competing financial interests.

492   **Author contribution**

493   J.M., O.M., and Z.K. conceived and designed the experimental approach. V.K. and
494   M.M.T. provided new analytic tools. J.M. and O.M. performed the experiments and
495   analyzed the data. J.M., O.M. and Z.K. wrote the manuscript.

496   **Supplementary material**

497   Supplementary material for this article is available at

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669   **Fig. 1**

670   **Tcf7l1 is expressed in a mutually exclusive manner with markers of specified NC and**
671   **is required for proper forebrain development**

672   (A-D) Expression of *Tcf7l1* mRNA, early NC marker Tfap2α protein, marker of specified NC *Sox9*
673   mRNA and Wnt reporter BAT-gal in the NPB and the ANF (encircled) of developing mouse
674   embryo. Please note the overlap of *Tcf7l1* transript and Tfap2α protein, and of *Sox9* mRNA and
675   BAT-gal signal.

676   (E) Mapping of recombination delivered by AP2α-Cre using Rosa26$^{EYFPfl/+}$ reporter mouse at
677   various stages E7.0-E10.5. EYFP was detected either directly or using anti-GFP antibody. The
678   recombination occurred along the entire NPB (arrowheads, 10s stage). Co-staining of GFP with
679   anti-Sox10 antibody revealed recombination in the whole cranial NC population at 10s stage.

680   (F-G) *In situ* hybridization shows loss of *Tcf7l1* mRNA expression in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants.
681   ANF in mutant is wider at PM, but narrower towards anterior tip of the ANF (dashed line) than in
682   the control at 5s stage, scale 30μm. Control embryo (left) and typical examples of strong (middle)
683   and mild (right) AP2α-Cre;Tcf7l1$^{fl/fl}$ phenotype.

684   (I) Quantification of strong and mild phenotype incidence between E10.5-E13.5 ANF- anterior
685   neural fold, NPB-neural plate border, NC-neural crest, NNE-non-neural ectoderm, PM-
686   prosencephalic-mesencephalic boundary, s-somite pairs. All embryos are shown from a lateral
687   view if not stated otherwise. Nuclei were stained with Hoechst. See also Fig. S1.

688   **Fig. 2**

689   **Tcf7l1 deletion induces loss of ANF specifiers, without affecting more posterior brain**
690   **structures**

691   (A-B´) *In situ* hybridization shows unchanged expression of *Fgf8* and *Gbx2* mRNA in MHB
692   (arrowheads), all lateral view.

693   (C-C´´) *Tcf7l2* mRNA in the posterior prosencephalon of control and AP2α-Cre;Tcf7l1$^{fl/fl}$ embryos
694   displaying mild and strong phenotype at E9.5, all lateral view.

695   (D-G´) Tcf7l1 deletion resulted in marked reduction of endogenous mRNA expression of *Six3* and
696   *Sox2* at 7-8s stage, *FoxG1* at 11-12s stage and of *Fgf8* at E9.0 in the ANF of AP2α-Cre;Tcf7l1$^{fl/fl}$
697   embryos, all frontal view.

23

698   **Fig. 3**

699   **The NC population expands anteriorly in the absence of Tcf7l1**

700   (A) Whole mount immunohistochemical staining of Sox1 and Sox9 protein at 5s stage, lateral
701   view. Higher magnification reveales reduction of Sox1 staining in the ANF (dashed line) of the
702   AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants. Nuclei were stained with Hoechst.

703   (B) Same embryos as shown in (A), frontal view, higher magnification documents the expansion
704   (dashed line) of Sox9 positive cells along the ANF border in mutants.

705   (C) Lateral and frontal views revealed aberrant expression of the NC markers *FoxD3* along the
706   ANF in the mutants while it is absent in the control embryos at 10s stage.

707   (D) *Sox10* mRNA is less abundant in the Tcf7l1 mutants at 6s stage but becomes ectopically
708   expressed later in anterior head (arrowhead) of AP2α-Cre;Tcf7l1$^{fl/fl}$ embryos at E9.5. V-trigeminal
709   ganglion. See also Fig. S2.

710   **Fig. 4**

711   **The ectopic activation of Wnt/β-catenin pathway promotes NC formation in mouse**

712   (A) BAT-gal signal (detected with β-galactosidase antibody) and Sox9 staining expands anteriorly
713   in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants at the 8s stage.

714   (B, C) *In situ* hybridization shows upregulation of the Wnt target *Sp5* and Wnt ligand *Wnt1* along
715   the NPB of the ANF in Tcf7l1 mutants at 5s and E9.5 stage.

716   (D-F´) Frontal and lateral view on the whole mount BAT-gal staining of the control, AP2α-
717   Cre;Tcf7l1$^{fl/fl}$ and AP2α-Cre;β-cat$^{Ex3fl/+}$ embryos at E9.5.

718   (D´´-F´´) Coronal sections in the dashed line shown in (D-F), respectively. Different embryos were
719   used in F, F´ and F´´.

720   (G-I) Coronal sections immunostained with antibodies against N-Cadherin and Sox9 at E9.5.
721   Sox9$^+$ population is increased and N-Cadherin staining decreased in the AP2α-Cre;Tcf7l1$^{fl/fl}$ and
722   AP2α-Cre;β-cat$^{Ex3fl/+}$ mutants, but not in control embryos.

723   (J-L) Lef1 immunostaining of coronal sections of the control (M), AP2α-Cre;Tcf7l1$^{fl/fl}$ (N) and
724   AP2α-Cre;β-cat$^{Ex3fl/+}$ (O) embryos at E9.5. fb-forebrain, t-tail. See also Fig. S3.
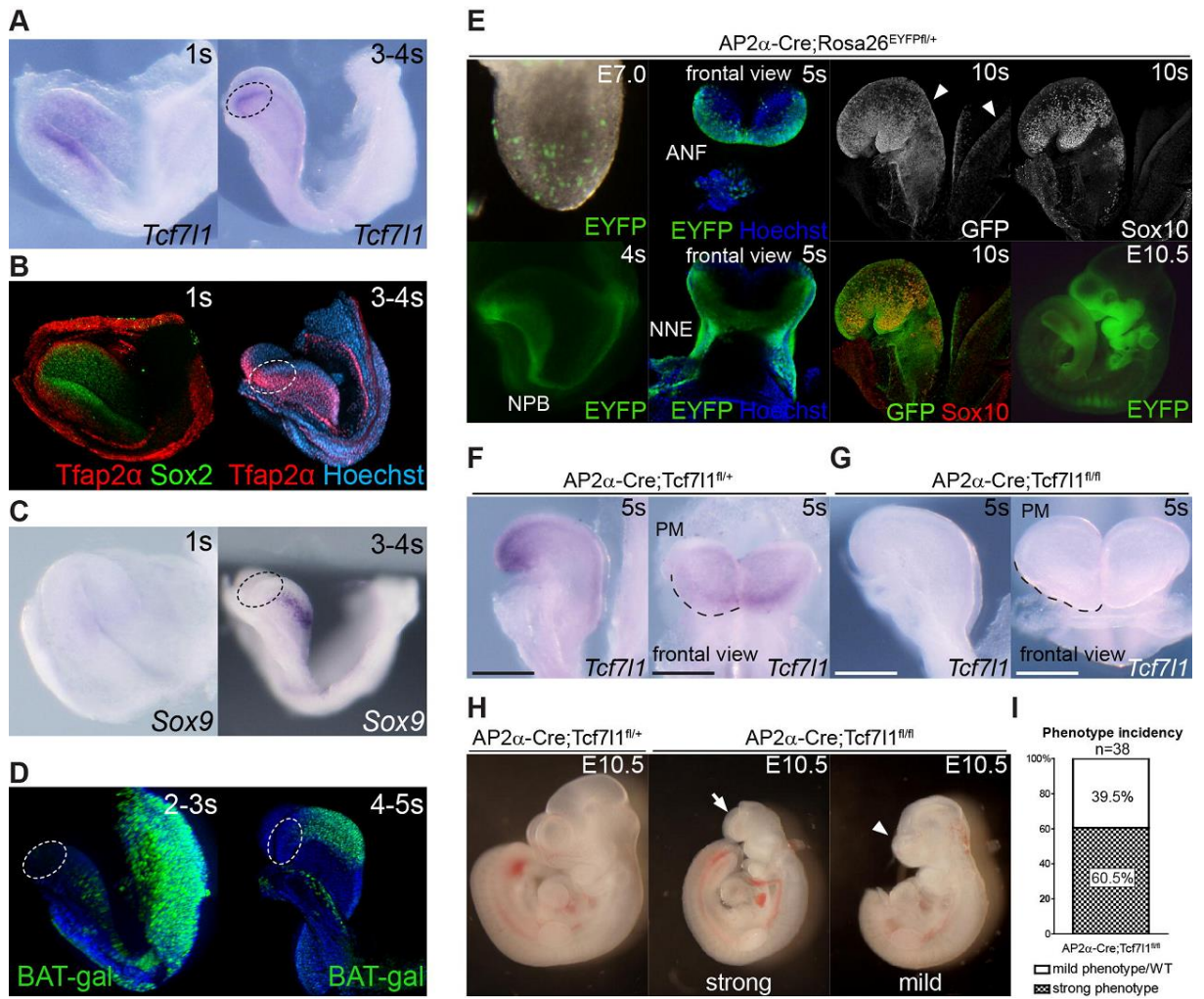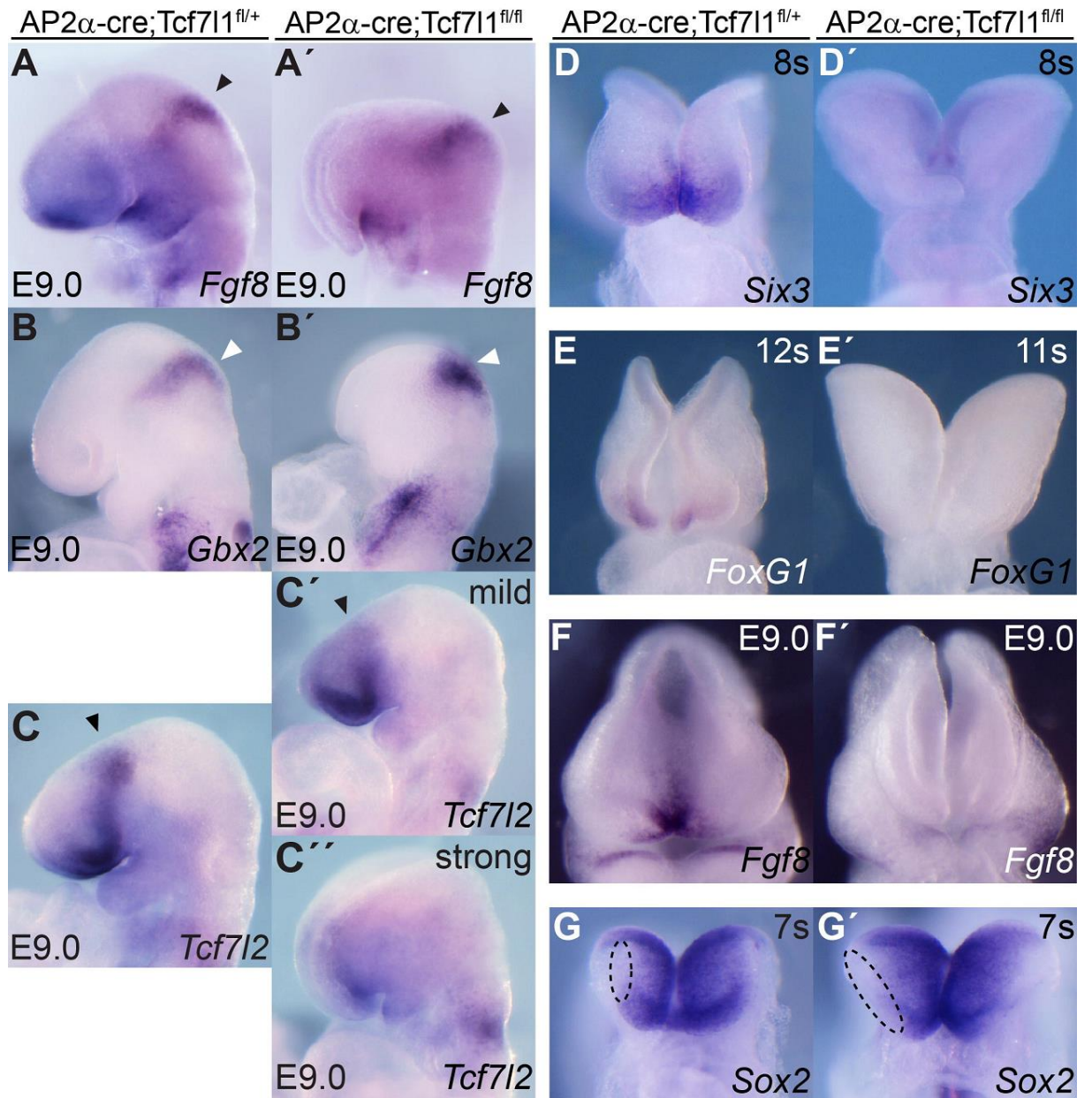
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725   **Fig. 5**

726   **A constitutively repressing form of Tcf is able to rescue ANF absence in AP2α-Cre;Tcf7l1$^{fl/fl}$**
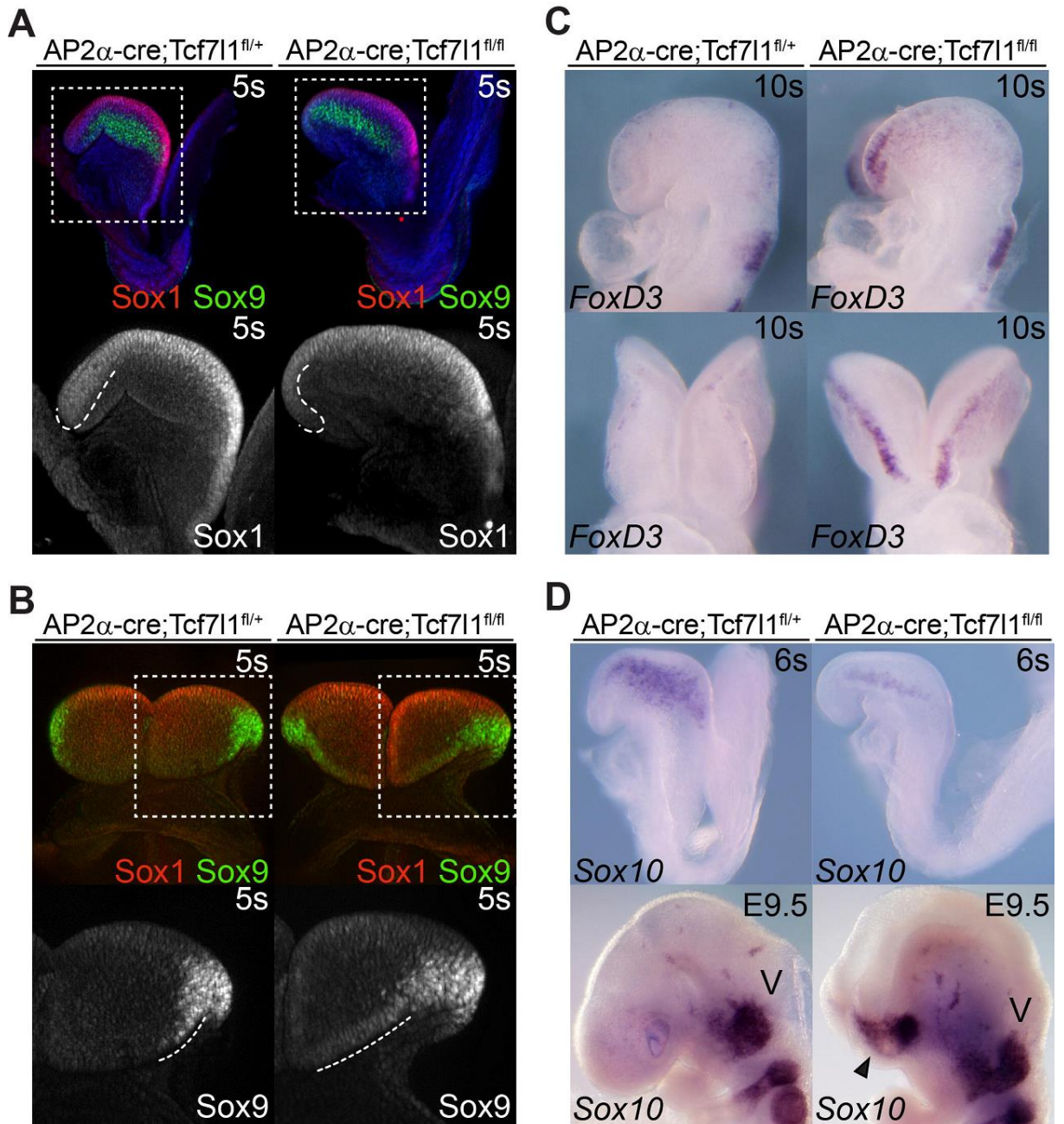727   **mutants**

728   (A) Diagram describing Rosa26$^{dnTCF4fl/+}$ and Rosa26$^{ctβcat-Tcf7l1fl/+}$ mouse strains used in the rescue
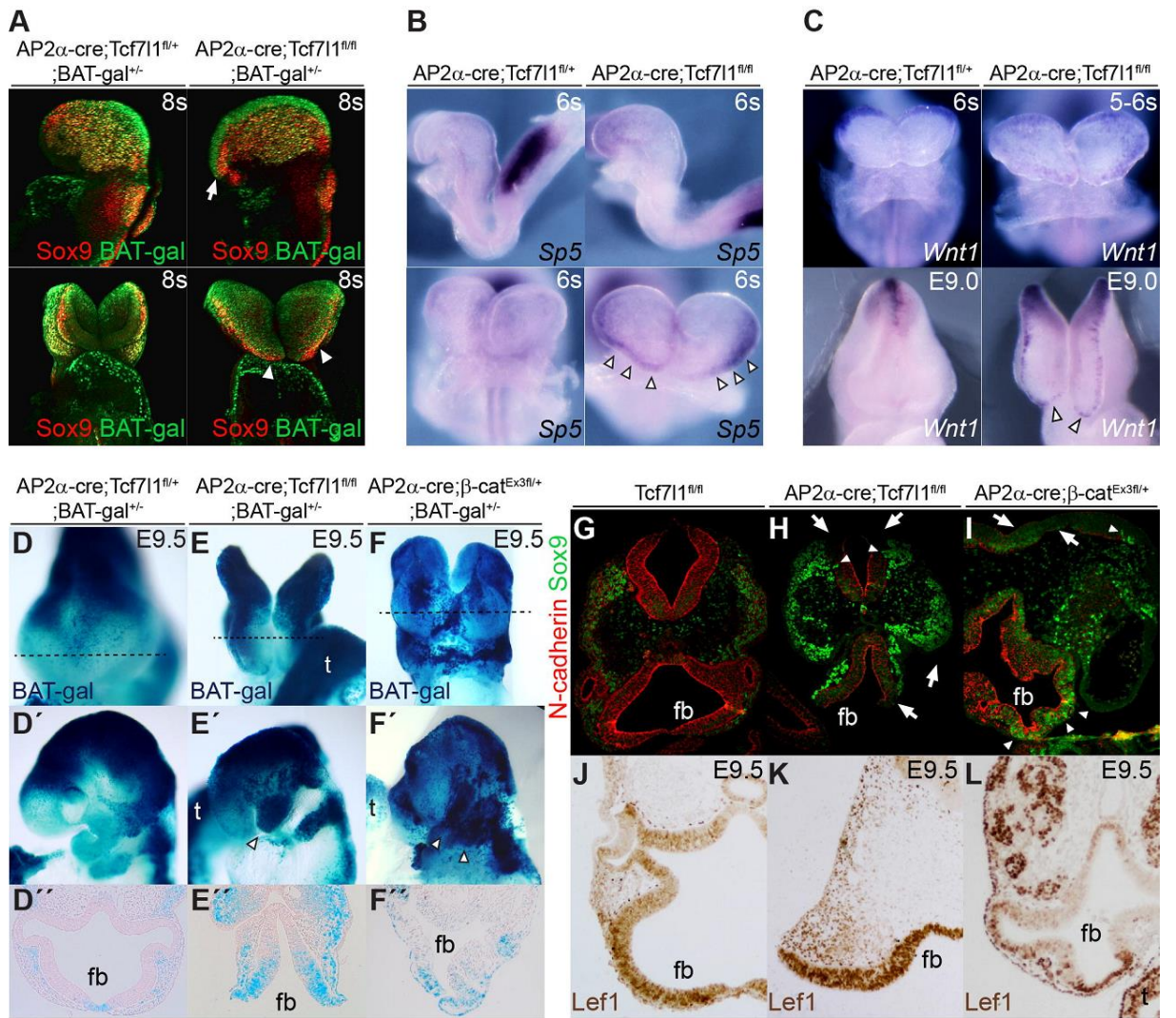729   experiments. SA-splice acceptor, pA-poly-adenylation signal.

730   (B,C) Expression of *Sox10* transripts was either unaffected or only mildly affected in the AP2α-
731   Cre;Tcf7l1$^{fl/fl}$;Rosa26$^{dnTCF4fl/+}$ compound mutants, confirming the rescue capability of dnTCF4.
732   AP2α-Cre;Tcf7l1$^{fl/fl}$; Rosa26$^{ctβcat-Tcf7l1fl/+}$ compound mutants, displayed no rescue or even stronger
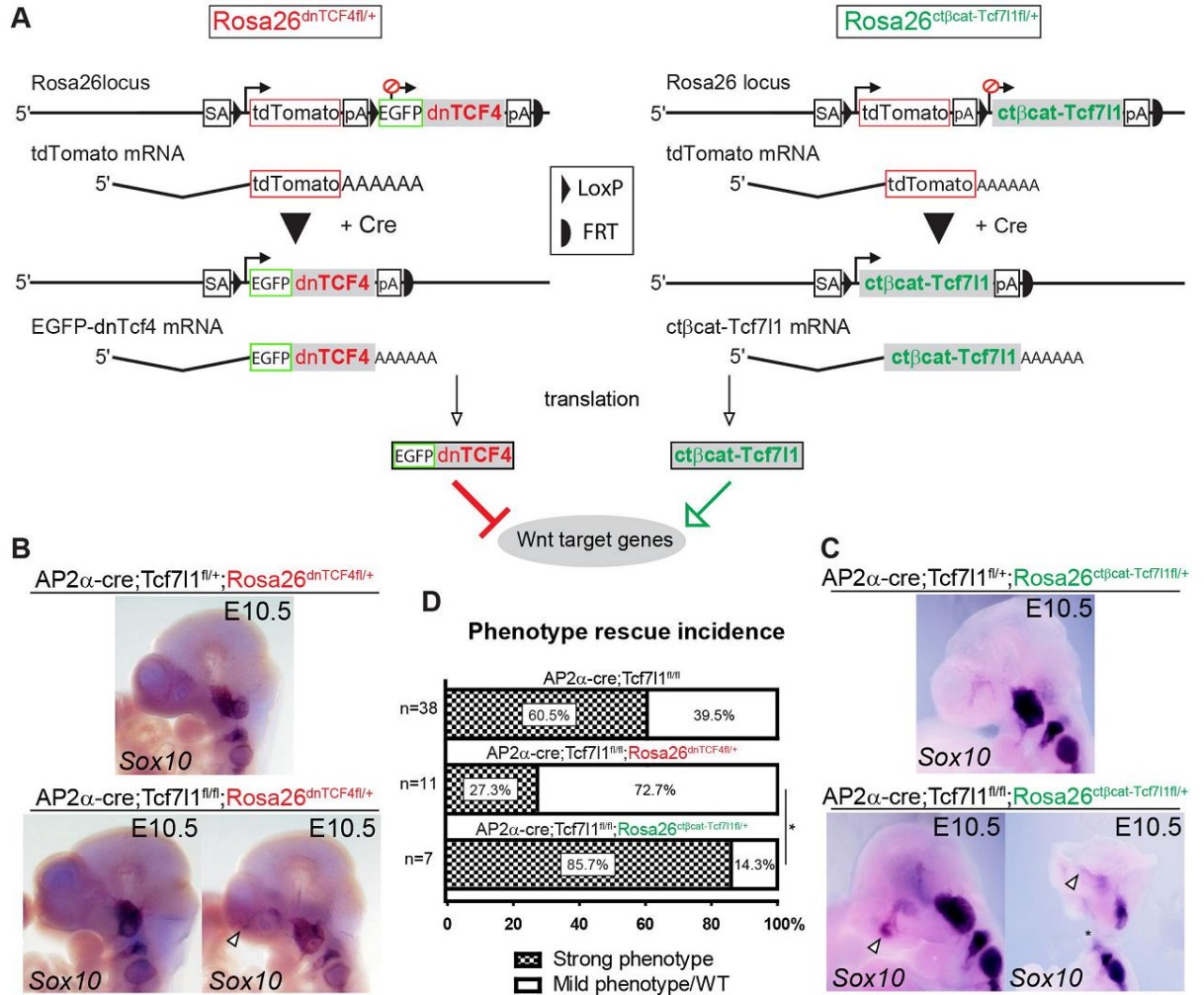733   phenotype. * indicates artifact caused during preparation.

734   (D) Quantification of the strong and mild phenotype incidence and rescue capability of dnTCF4
735   and ctβcat-Tcf7l1, scored at E10.5-13.5. *(P=0.049, Fishers test), n-number of embryos analyzed.
736   See also Fig. S4.

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A

B

C

D

E

F

G

H

I

A

AP2α-cre;Tcf7l1^{fl/+}   AP2α-cre;Tcf7l1^{fl/fl}

Sox1 Sox9   Sox1 Sox9

Sox1   Sox1

B

AP2α-cre;Tcf7l1^{fl/+}   AP2α-cre;Tcf7l1^{fl/fl}

Sox1 Sox9   Sox1 Sox9

Sox9   Sox9

C

AP2α-cre;Tcf7l1^{fl/+}   AP2α-cre;Tcf7l1^{fl/fl}

FoxD3   *FoxD3*

FoxD3   *FoxD3*

D

AP2α-cre;Tcf7l1^{fl/+}   AP2α-cre;Tcf7l1^{fl/fl}

Sox10   *Sox10*

Sox10   *Sox10*

A

Rosa26^dnTCF4fl/+

Rosa26^ctβcat-Tcf7l1fl/+

B

AP2α-cre;Tcf7l1^fl/+;Rosa26^dnTCF4fl/+

E10.5

Sox10

AP2α-cre;Tcf7l1^fl/fl;Rosa26^dnTCF4fl/+

E10.5   E10.5

Sox10   *Sox10*

D

Phenotype rescue incidence

AP2α-cre;Tcf7l1^fl/fl

n=38   60.5%   39.5%

AP2α-cre;Tcf7l1^fl/fl;Rosa26^dnTCF4fl/+

n=11   27.3%   72.7%

AP2α-cre;Tcf7l1^fl/fl;Rosa26^ctβcat-Tcf7l1fl/+

n=7   85.7%   14.3%

0   20   40   60   80   100%

▦ Strong phenotype
▢ Mild phenotype/WT

C

AP2α-cre;Tcf7l1^fl/+;Rosa26^ctβcat-Tcf7l1fl/+

E10.5

Sox10

AP2α-cre;Tcf7l1^fl/fl;Rosa26^ctβcat-Tcf7l1fl/+

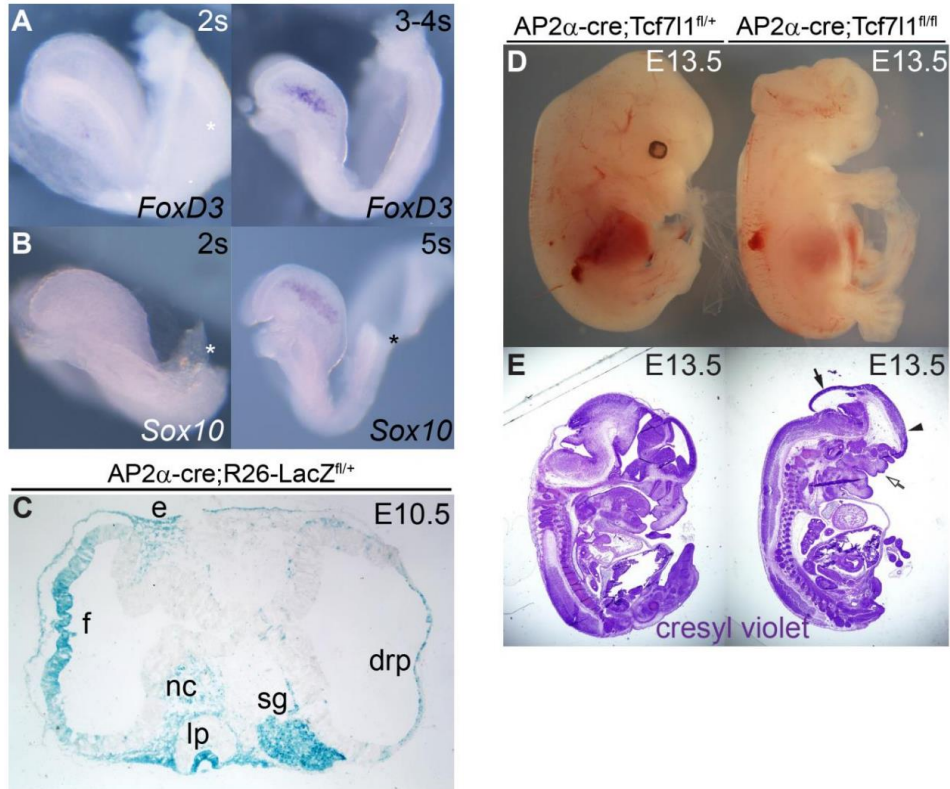E10.5   E10.5

Sox10   *Sox10*

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1   **Fig. S1, related to Fig. 1**

2

3   **Expression of NC genes during early mouse embryogenesis.**

4   **(A-B)** RNA *in situ* hybridisation of *FoxD3* at 2s and 3-4s stage, and *Sox10* transcripts at 2s and 5s
5   stage, respectively. **Mapping of AP2α-Cre delivered recombination (C)** Coronal section of AP2α-
6   Cre;Rosa26$^{LacZfl/+}$ at E10.5 stage. Recombination was detected enzymatically with β-galactosidase.
7   drp-dorsal roof plate, e-ectoderm,f-forebrain, lp-lens pit, nc-neural crest, sg-sensory ganglion.
8   **Tcf7l1 deletion results in exencephaly (D,E)** Histological analysis of saggittal sections from the
9   AP2α-Cre;Tcf7l1$^{fl/fl}$ 'strong' mutant at E13.5, sections stained with cresyl violet show severe defects
10   in the splanchnocranium (white arrow), forebrain (black arrowhead) and midbrain (black arrow) of
11   the mutant embryo.

12

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13   **Fig. S2, related to Fig. 3**
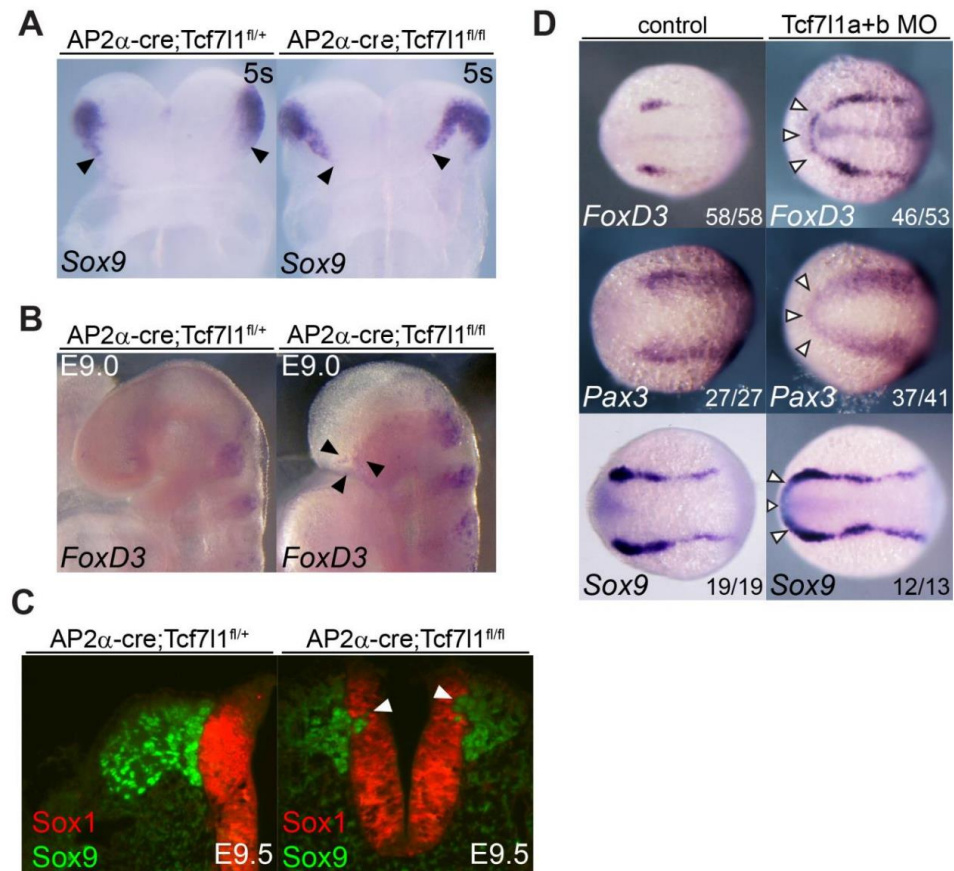
14

15   **Expansion of NC markers follows Tcf7l1 deletion in mouse and zebrafish**

16   **(A-C)** Expression of *Sox9* transcripts expanded rostrally in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutant at 5s stage.
17   Abberant *FoxD3* mRNA expressing cells are present in the Tcf7l1 mutant at E9.0.
18   Immunnohistological staining of coronal sections from the E9.0 hindbrain reveled ectopic Sox9-
19   positive/Sox1-negative cells in AP2α-Cre;Tcf7l1$^{fl/fl}$ mutants. **(D)** Expression of *FoxD3*, *Pax3* and
20   *Sox9* mRNA expands along the anterior NPB upon MO-mediated knock-down of both Tcf7l1a and
21   Tcf7l1b variants in zebrafish at 12h stage.

22

2

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23   **Fig. S3, related to Fig. 4**
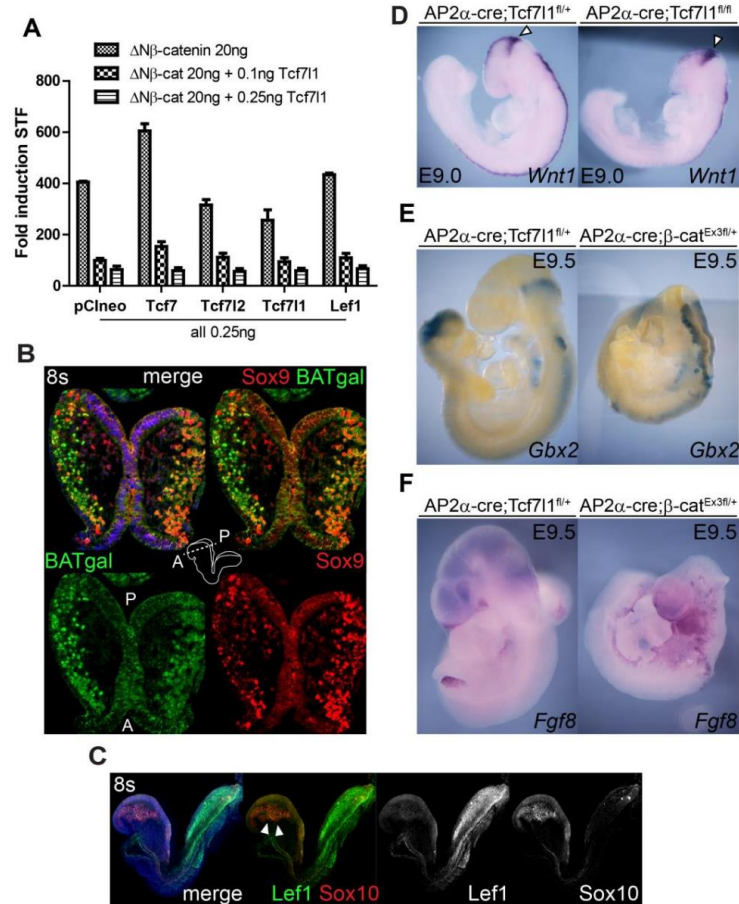
24

25   **Balance between Wnt/β-catenin pathway activation and Tcf7l1 delivered repression**
26   **regulates NC specification.**

27   **(A)** SuperTopFlash luciferase reporter assay in HEK293 cells documents Tcf7l1 ability to repress
28   Tcf/Lef driven gene expression. **(B)** Immunostaining of coronal sections from 8s stage wild-type
29   embryos, A-anterior, P-posterior. Please note a large overlap between the Sox9-positive and BAT-
30   gal-positive delaminating cells. Nuclei were stained with DAPI. **(C)** Whole mount
31   immunofluorecence revealed ovelrlap of Lef1 and Sox10-positive cells in wild-type embryo at 8s
32   stage. Nuclei were stained with Hoechst. **(D)** The expression of *Wnt1* transcripts is unchanged in
33   the MHB of AP2α-Cre;Tcf7l1^{fl/fl} mutants at E9.0. **Ectopic activation of the Wnt/β-catenin pathway**
34   **severely affects NPB development. (E-F)** RNA *in situ* hybridisation of the MHB markers *Gbx2* (E)
35   and *Fgf8* (E) in the AP2α-Cre;β-cat[Ex3fl/+] mutants at E9.5.

3

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36   **Fig. S4, related to Fig. 5**
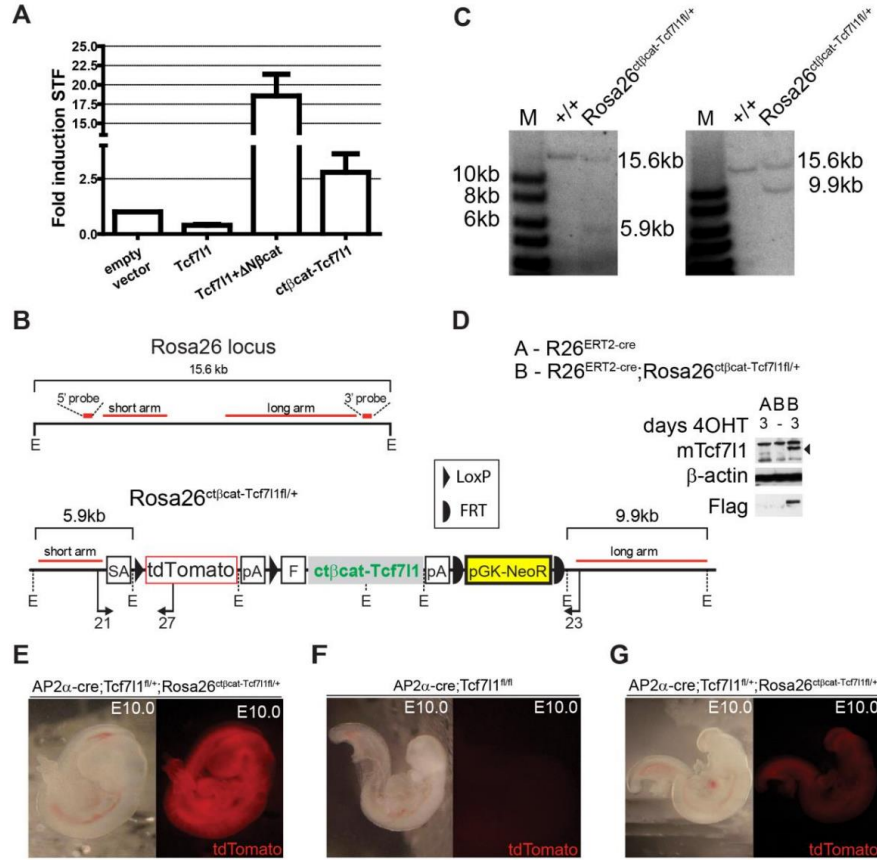
38   **Generation and validation of the mouse strain Rosa26$^{ct\beta cat-Tcf7l1fl/+}$.**

39   **(A)** SuperTopFlash assay in HEK293 cells showed ctβcat-Tcf7l1 mediated activation of the Tcf/Lef
40   driven luciferase reporter. **(B)** Scheme of the Rosa26 locus and the targeting vector, homology
41   arms are depicted in red. E-EcoRI cleavage site, SA-splice acceptor, pA-poly-adenylation signal,
42   pGK-NeoR–neomycin expressing cassette, F-Flag tag sequence, primers for genotyping 21-
43   JM21F, 23-JM23R and 27-JM27R. **(C)** Southern blotting analysis confirms correct homologous
44   recombination into mES cells, short arm is on the left. **(D)** Western blotting of lysates from mouse
45   embryonic fibroblasts (MEFs) isolated from the R26$^{ERT2-cre}$;Rosa26$^{ctbcat-Tcf7l1fl/+}$ embryos revealed
46   presence of the ctβcat-Tcf7l1 fusion protein 3 days after administration of 4-OHT (4-
47   *hydroxitamoxifen*). **(E-G)** Rosa26$^{ctbcat-Tcf7l1fl/+}$ mice ubiquitously express fluorescent protein
48   tdTomato, the expression is lost in the regions where recombination occurs, visible in the AP2α-
49   Cre;Tcf7l1$^{fl/fl}$; Rosa26$^{ct\beta cat-Tcf7l1fl/+}$ compound mutants, AP2α-Cre;Tcf7l1$^{fl/fl}$ serves as a negative
50   control.

4

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51   **Supplemental Table S1 (Related to Materials and methods)**

ISH probes		
Gene	forward	reverse
FoxD3	GGACCGCAAGAGTTCGCGGA	TCCGGAGCTCCCGTGTCGTT
Sox9	GAGCACTCTGGGCAATCTCAG	CTCAGGGTCTGGTGAGCTGTG
Gbx2	Gift from Peter Rathjen	Adelaide University
Sp5	CGTGAAGACGCACCAAAATA	TATTTTCACGCTGCCAACTG
Fgf8	CAGGTCCTGGCCAACAAG	GAGCTCCCGCTGGATTCCT
Sox10	Gift from Anthony Firulli	Indiana University, USA
Sox2	Open Biosystems	BC057574
FoxG1	Gift from Stefan Krauss	OUS, Oslo, Norway
Six3	Gift from Guillermo Oliver	St. Jude Hospital, Memphis, USA
Wnt1	Gift from Andy McMahon	USC, USA
Tcf7l1	Open Biosystems	BC128306
Tcf7l2	Open Biosystems	BC052022
Antibodies		
Gene	Company	Dilution
Sox1	RαD AF3369	1:1000
Tfap2α	Sanαta Cruz Biotech SC-184	1:1000
Sox10	Santa Cruz Biotech SC-17342	1:1000
Sox9	Millipore AB5535	1:1000
N-cadherin	BD Transduction Lab. 610920	1:2000
GFP	Life Technologies A11122	1:1000
β-galactosidase	Abcam ab9361-250	1:1000
Lef1	Cell Siganling C12A5	1:1000

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