MED23: a Mediator subunit's role in global gene transcription, regulation of craniofacial development and WNT signaling.

BY

Shachi Bhatt

Submitted to the graduate degree program in Anatomy and Cell Biology and to the graduate faculty of University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Co-chair	Paul Trainor, Ph.D.
Co-chair	Douglas Wright, Ph.D.
	Joan Conaway, Ph.D.
	Timothy Fields, M.D., Ph.D.
	Robb Krumlauf, Ph.D.

Date Defended: August 23rd, 2013

The Defense committee for **Shachi Bhatt** certifies that this is the approved version
of the following dissertation:

**MED23: a Mediator subunit's role in global gene transcription, regulation of
craniofacial development and WNT signaling**

Co-chair	Paul Trainor, Ph.D.

Co-chair	Douglas Wright, Ph.D.

Date approved: September 9th, 2013

Abstract

Expression of genes at the right time and place is crucial during adult homeostasis as well as embryonic development. Multicellular organisms regulate this spatiotemporal expression of genes by employing tissue specific transcription factors which bind to enhancer and repressor elements distant from the gene transcription start site. In embryonic development, most of the research understanding cell differentiation has focused on identifying such tissue specific transcription factors. What we do not understand clearly, is how these transcription factors that bind so far away from the gene promoter site influence the decision of RNA Pol II to transcribe or not transcribe. A recent discovery of a mega-dalton protein complex, called Mediator, has begun to answer this question. Mediator complex physically interacts with RNA Pol II and general transcription factors on one side and with transcription factors bound to enhancer/repressor sites on the other side. Mediator has thus been shown to act as a bridge that relays information between the transcription factors and RNA Pol II machinery and thus regulate gene expression. The various subunits of Mediator complex have been shown to interact with distinct transcription factors to regulate expression of specific genes. In this work, I describe the role of one such Mediator subunit, MED23.

The role of MED23 during mammalian embryonic development was identified through a forward genetics screen done in the lab. Loss of *med23* leads to mid-gestational lethality in

mice embryos along with defects in craniofacial, neural and vascular development. We currently do not know the exact cause for embryonic lethality in *med23* mutant embryos, but my results indicate that MED23 is crucial for endothelial cell-cell junction formation at E9.5, defects in which have previously been shown to affect embryonic survival. Specifically the formation of adherens and tight junctions between endothelial cells is affected in *med23* mutant embryos. Analysis of neuronal defects in *med23* mutant embryos suggests that MED23 is required during various steps of cranial placode development and this is regulated by MED23-mediated regulation of canonical WNT signaling.

How and why loss of MED23 leads to defects in these specific tissues is currently unknown, but work with conditional mutant analysis as well as transcription factor-binding screens are underway to figure this out. My work thus highlights a unique link between general transcription co-factor, Mediator, and its subunit MED23 with development of neural, vascular and craniofacial tissues and a crucial signaling pathway, WNT signaling.

Acknowledgements

First and foremost, I would like to dedicate this dissertation to the four most important people in my life, my parents, my sister and Rushi. This accomplishment would have been incomplete without your presence in my life. Mummy, Papa - In spite of all our disagreements ;), you have been the two strong pillars of my life and the strength that I gained from you has held me upright before and during my PhD; and Didu – if there is one person I can count on who will always be on my side no matter what, I would say it's you. Thank you for all your love and support that have sustained me through the thick and thin of my past seven years in US. We might have been physically apart but you were always there. Rushi – words are not enough for the amount of gratitude I owe to you for being by my side during smallest victories and the biggest scares of grad school. There isn't anybody else who could understand and relate to the frustrations and the excitement that grad school offers and I feel blessed to have had you to share it with. You are the fairest individual I have known in my life and daily inspire me to challenge myself and make things better in everyday life and in science. It is said that graduate school is one of the most stressful times in the career of a scientist, but your presence made it fun.

Of course, my dissertation would be incomplete without thanking you, Paul. I have yet to come across a more well-rounded personality than yours in my life. It seems to me that you

have figured it out; you are fabulous scientist who never forgets the bigger picture in life, a great mentor and just an amazing individual. Thank you for everything that you have done for me and for everything that I learnt from you in the past for years. I hope that my growth as a scientific researcher is something that makes you proud. Lisa, Amanda, Daisuke and Kim, you guys taught me the basics in the lab and I will never forget your contribution to my achievement today and hereafter. Every member of Trainor lab has been a great friend and a great colleague. From our scientific discussions to our fun hangouts at Westport, you all have provided me a family away from home.

I would also like to thank my committee members, Robb, Joan, Doug, Tim and Jay, whose inputs have been valuable to shaping this body of work and in helping me grow as a researcher. I am also very grateful to the department of Anatomy and Cell Biology at KUMC and all the core facilities at Stowers Institute for Medical Research who have provided me support and facilitated my work.

Table of Contents

List of Figures and Tables

Introduction

Part 1: Craniofacial Development

One third of all congenital deformities have a craniofacial structure anomaly. In their severe forms, craniofacial defects affect the basic functioning of the head, ranging from jaw closure defects that affect the ability of patients to talk and eat, to debilitating defects in sensory organs affecting cognition. Even in their mildest forms where the functioning is not highly affected, defects in facial appearance affect well-being of patients. Prevention of occurrence of these craniofacial occurrences warrants a detailed understanding of how a normal head structure forms and an understanding of crucial genetic and environmental factors responsible for its normal development. While some genes and environmental factors required for proper craniofacial development have been identified, we clearly do not have a comprehensive understanding of head development and defects.

The high occurrence of craniofacial anomalies is not a surprise given the highly complex nature of craniofacial development. Head induction begins as soon as the anterio-posterior axis of the embryo is defined at gastrulation. All three germ layers, ectoderm, mesoderm and endoderm contribute to the developing craniofacial complex and their induction, differentiation, proliferation and patterning have to be highly orchestrated for proper head development. In head, ectoderm gives rise to the nervous system (central and peripheral) and majority of the craniofacial skeleton; mesoderm forms the cephalic

musculature and blood vessels as well as some of the skull bones while the endoderm forms the foregut lining, pharynx and trachea along with other organs. This section gives a general overview of head induction including formation of anterio-posterior axis and gastrulation followed by brief description (when and how) of differentiation of each germ layer into the various structures within craniofacial complex.

I) Gastrulation and Formation of Anterio-posterior axis

Mouse embryos begin as a single cell zygote which undergoes a series of cell divisions and a process of compaction eventually forming a fluid filled epithelial vesicle known as blastocyst. At late blastocyst stage, the outer epithelium cells are committed to the **trophectoderm** lineage which subsequently forms the placenta while the cells inside the vesicle, known as **inner cell mass (ICM)**, is composed of two cell types, **epiblast** which gives rise to all cell of the embryo proper and some extra-embryonic membranes and **primitive endoderm** which primarily forms the endoderm layer of the extraembryonic yolk sacs[1]. These early events in a mouse embryo describing formation of blastocyst and first asymmetry with ICM have been extensively described elsewhere and summarized in **Figure 1**[2].

One of the crucial aspects of embryonic development is formation of the three axes, Organisms like *Drosophila* and Zebrafish, rely on asymmetric distribution of maternal

3

transcripts and proteins to generate polarity in the embryo. In mammals, however, transition of transcription under exclusive zygotic control (maternal-to-zygotic transition) occurs earlier than specification of axes. Following implantation the mouse blastocyst forms an egg cylinder with a proximal (the pole with the trophectoderm derived ectoplacental cone) – distal (P/D) axis[1]. Recent lineage tracing experiments have identified a specific group of *lefty-1* expressing cells within the ICM prior to implantation[3]. These *lefty-1+* cells form the **distal visceral endoderm (DVE)** of the egg cylinder, which populates the distal pole of the P/D axis, suggesting a possibility that P/D axis is formed prior to implantation. DVE cells were originally believed to give rise to **anterior visceral endoderm (AVE)**, an extra-embryonic signaling center crucial for imparting anterior character to the embryonic region adjacent to it [2,4,5, 6]. Recent works show that AVE cells are specified *de novo* and DVE is not required for its induction[3,7-9]. DVE however has been shown to be required initiating AVE migration toward the prospective anterior pole[3]. Epiblast cells receiving signals from AVE acquire anterior fate and contribute to head and brain structures while the cells away from AVE acquire a posterior fate[1].

The process of **gastrulation** forms the mesoderm and endoderm lineages from epiblast cells at the posterior end of the mouse embryo. In mice embryos, gastrulation begins with the formation of **primitive streak**. Primitive streak is formed by the epiblast cells undergoing polonaise movement at the future posterior end of the embryo. The polonaise motion results in

4
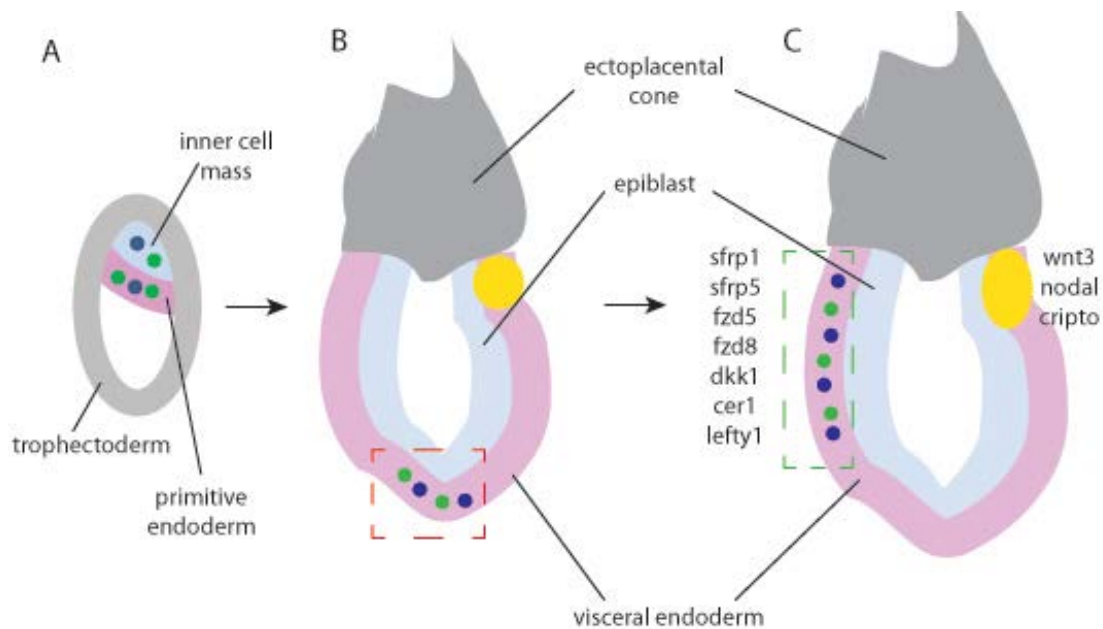
Figure 1: Emergence of asymmetry within a developing mouse embryo

A) At blastocyst stage, the embryo is already separated into three distinct cell types, ICM (light blue), Primitive endoderm (pink) and trophectoderm (grey). ICM cells already display asymmetric gene expression, and the *lefty1+* cells (dark blue and green) will eventually contribute to DVE (dark blue). AVE cells (green), also *lefty1+*, are specified later than DVE.

B) Implantation induced proximal (ectoplacental cone)–distal axis. Primitive endoderm eventually forms the visceral endoderm that surrounds the epiblast and extra-embryonic tissues. DVE and AVE cells migrate to the distal pole of the embryo (boxed area with red dotted lines). WNT signaling is activated (orange) at the future posterior end of the embryo.

C) Future posterior end expresses WNT and Nodal signaling ligands. Gastrulation begins. DVE and AVE cells migrate to the future anterior side of the embryo (boxed area with green dotted lines) and express WNT and Nodal signaling inhibitors.

epiblast cells moving in circles around a morphologically visible midline, which is the primitive streak. As time progresses the epiblast cells undergo epithelial-to-mesenchymal transition (EMT) and ingress through the streak into the embryo. These newly ingressed mesenchymal cells are a mixture of mesoderm and endoderm progenitors and signals emanating from the site of ingression help specify their eventual fate within the embryo. As gastrulation proceeds the embryo gets divided into three distinct germ layers: ectoderm which is the inside-most layer, endoderm which is the outside-most layer; and mesoderm, in between the two. The inside-outside positioning of the ectoderm and endoderm gets reversed when the mouse embryo undergoes turning at around E9.0.

Ia)   Molecular determinants and signaling pathways in gastrulation and A/P axis determination

The formation of the anterio-posterior (A/P) axis within the embryo requires two separate yet related processes; specification of the anterior end (involvement of AVE) and specification of the posterior end (marked by presence of primitive streak, site of gastrulation). Pre-gastrula embryos have been shown to display asymmetry in gene expression. For example, components of canonical WNT signaling, *wnt3*, *wnt2b* and activated β-catenin have a restricted pattern of expression at the future posterior end of the embryo[10-12]. Mouse knock-

out studies have also shown that embryos lacking functioning WNT signaling (*Wnt3* $^{-/-}$ [13] or

WNT co-receptors *Lrp5* $^{-/-}$ or *Lrp6* $^{-/-}$ [14]) fail to establish a primitive streak and thus lack

mesoderm and definitive endoderm lineages. All of this information together makes a strong

case for the involvement of **WNT signaling** in establishing **gastrulation site** and hence in

establishing **posterior end** of the embryo. At the same time it is crucial to block WNT signaling

for the specification of the anterior pole. This blockage of WNT signaling is achieved by

expression of WNT signaling inhibitors specifically at the anterior end. As mentioned earlier,

cells within AVE secrete signals that specify the anterior end of the embryo. One of these

secreted signals is a molecule called Dickkopf-1 (DKK-1), a well-known inhibitor of WNT

signaling[15]. The importance of blocking WNT signaling for anterior specification comes from

the fact that the complete knock-out of *Dkk-1* leads to expansion of WNT signaling and results

in complete absence of anterior head structures[16].

The seemingly straightforward role for WNT signaling does not hold true for the other

signaling pathways also involved A/P axis formation. **Nodal**, a member of TGFβ family of

secreted ligands has a more complex role in specifying anterior and posterior poles of the

embryo. Although it is well established that inhibition of Nodal signaling, achieved by secretion

of Nodal antagonist, LEFTY, from AVE cells is crucial for anterior specification[17], the

specification of DVE/AVE itself requires active Nodal signaling from the epiblast cells. DVE cells

are first specified within the ICM of a pre-implantation embryo at the future distal end. The

specification of proximal-distal axis at this stage (E3.5 – E4.0) requires active Nodal signaling.

Once the DVE cells are specified, active Nodal signaling is observed specifically at the

posterior–proximal end of the embryo and the newly specified DVE/AVE cells secrete Nodal

antagonists to restrict Nodal signaling to the posterior end of the embryo.

II)   Ectoderm

Following A/P axis specification and gastrulation, medial part of the ectoderm

undergoes neural induction forming a neural plate, which will eventually give rise to the entire

central nervous system (brain at the anterior end and spinal cord at the posterior end). Neural

induction is the first step of ectoderm differentiation and segregates the homogenous ectoderm

into neural ectoderm (forming the neural plate) and non-neural ectoderm or surface ectoderm

(everything else). The boundary between the two is called the **neural plate boundary (NPB)**

region. In the most anterior regions, NPB gives rise to two separate ectodermal derivatives,

neural crest and ectodermal placodes. The majority of the craniofacial skeleton is derived from

cranial neural crest cells, while the cranial peripheral nervous system has contributions from

both **cranial neural crest** and **ectodermal placodes.**

IIa)   Neural Crest Cells

First observed by William His in 1868 as a string of cells delaminating from in between the neural tube and the future epidermis of the spinal cord in a chicken embryo, neural crest cells were originally given the name "ganglionic crest"[18]. These cells are induced bilaterally at the border of neural and non-neural ectoderm throughout the A/P axis, undergo epithelial-to-mesenchymal transition and emigrate out in stereotypical trajectories to contribute to a variety of tissues within the body (**Figure 2**). Specifically, in the head region, crest cells follow a dorsolateral trajectory to form the craniofacial mesenchyme. The timing when neural crest emigration begins differs among various vertebrate species and in mice emigration begins before the neural plate forms the neural tube.

IIa.1)   Induction of Neural Crest cells

Induction of neural crest cells is a multi-step process that varies between the various vertebrate species (reviewed in details here[19]). Work in *Xenopus* embryos has shown that high levels of **BMP signaling** are required for epidermal differentiation while complete inhibition of BMP signaling is required for neural induction[20]. It has been proposed that chick neural crest cells, which arise from the territory in between the future epidermis and neural plate, require intermediate levels of BMP signaling for their induction, which are generated as a consequence

of the BMP gradient generated between BMP ligand expressing epidermal cells[21] and BMP antagonists (Cerberus, Noggin, Chordin, Follistatin etc) found in the underlying paraxial mesoderm[19,22,23]. In mice embryos, conclusive evidence depicting a crucial role for BMP gradient in neural crest induction is not established. However, other early aspects of neural crest development seem to require BMP signaling, for example, BMP5 and BMP7 have redundant roles in survival of post-migratory neural crest cells[24] and BMP2 has been shown to be important for formation of migratory neural crest cells[25]. It should be kept in mind that BMP signaling is in no way sufficient to induce neural crest cell marker expression even in *Xenopus* or chick embryos[23,26] suggesting that neural crest cell induction requires help from additional signals as well.

A role for **WNT signaling** in neural crest induction has been shown in all major vertebrate model systems[20,26-32]. WNT signaling ligands are expressed and secreted from the paraxial mesoderm and overlying surface ectoderm, both tissues that have been shown to be important for neural crest induction[33]. Activation of WNT signaling expands neural crest cell domain, while inhibition of WNT signaling perturbs neural crest induction in *Xenopus*, chick and fish embryos[20,26,27,29,34]. Interestingly, defects in neural crest induction caused by ablation of paraxial mesoderm in *Xenopus* embryos can be reversed by overexpression of WNT ligand, *wnt3a*[35]. In *Xenopus*, a two-step mechanism for induction of neural crest involving both WNT

and BMP signaling has been proposed[36]. In this model WNT and BMP signaling work antagonistically (WNT signaling activates and BMP signaling inhibits) in the first step for induction of crest cells, but once induced, neural crest cell maintenance requires tandem activation of both WNT and BMP signaling. This two-step model is supported by chick explant studies[37]; however, there is a lack of *in vivo* evidence for it. *Wnt1/Wnt3a* double knock-out mice show reduced numbers of neural crest cells and minor defects in craniofacial skeleton formation[30]. Elimination of β-catenin, the intracellular effector of canonical WNT signaling, exclusively within neural crest cells affects formation of craniofacial skeleton and cranial ganglia due to increased apoptosis of neural crest cells, suggesting a role for WNT signaling in neural crest survival but not in the neural crest induction[28]. At later stages, WNT signaling has also been shown to regulate differentiation of neural crest cells into sensory and melanocyte lineages in mice embryos[38-40].

FGF ligands represent another set of signaling cues that are capable of inducing neural crest. The role for FGF signaling in neural crest induction is mostly depicted in non-mammalian species. Overexpression of FGF8 transiently induces neural crest cells in *Xenopus* embryos[41] albeit with the help of other signals as exogenous FGF8 alone is not sufficient to induce neural crest marker expression[42]. FGF2 mediated up-regulation of neural crest cell and epithelial-to-mesenchymal transition marker, *snail2* in *Xenopus* embryos, requires

11

concomitant attenuation of BMP signaling[43, 44]. Recent evidence in *Xenopus* embryos suggests that FGF signaling induces neural crest cells indirectly through activation of *wnt8* in lateral mesoderm[45]. In chick embryos, however, FGF/MAPK signaling has been implicated in specification of the neural plate border during gastrulation[46]. Similar to what is observed with WNT signaling, mouse embryos lacking FGF ligand, FGF8, FGF10, FGF18 or FGF receptors, FGFR1 or FGFR2 display defects in craniofacial skeleton; however, neural crest cell induction is not abolished in these mutants (reviewed in details here[47]).

Recently, in a new theory, Garcia-Castro et al. propose that neural crest induction begins earlier than previously assumed, at gastrulation stage, and is independent of neural or mesodermal tissues[48]. Using *in situ* hybridization and immuno-staining, the researchers depict *pax7+* cells in two bilaterally symmetric oblique bands lateral to the Hensen's node in gastrulating chick embryos, which can *in vitro* form melanocytes and neurons. In gastrulating embryos, these *pax7+* cells do not overlap with neural plate, epidermis, ectodermal placode or neural plate border markers. Lack of marker expression however does not rule out the possibility that the *pax7+* cells might belong of any of those lineages. Interestingly, lineage tracing of this domain by DiI labeling showed that these (*pax7+*) cells eventually incorporate into the dorsal neural folds, arguing against their neural crest specific lineage and suggesting that the *pax7+* domain at gastrulation might represent a unique domain contributing the
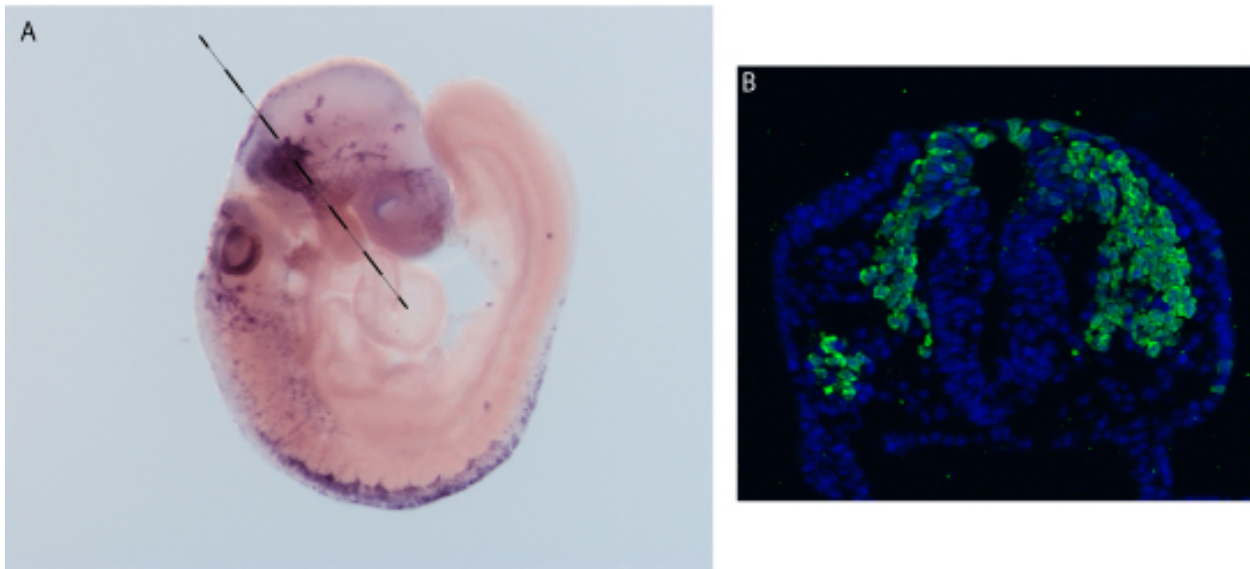
Figure 2: Mammalian neural crest cells

A) *Sox10* labeling shows dorsoventrally migrating neural crest cells throughout the anterio-posterior axis

B) A transverse section through a Wnt1-Cre; Rosa[eYFP] embryo immunostained for YFP (green) shows the neural crest cells arising at the dorsal end of the neural tube and migrating out. Figure A shows the approximate axial location of where the section would be.

neuroectoderm.[48] The involvement of *pax* genes in neural crest formation has been implicated

in both *Xenopus* and mouse as well; however, this early role of *pax* genes in neural crest

induction is not supported by current work done in either of these species. In *Xenopus*, loss of

pax3 or *pax7* leads to neural crest induction defects indirectly through defective development

of ectoderm and mesoderm respectively[49]. Both *pax3* and *pax7* mouse mutants individually

show defects in neural crest derivatives. Loss of *pax7* causes postnatal lethality with

malformations in facial structures involving maxilla and nose, while loss of *pax3* leads to

defects in the spinal cord, sensory ganglia and cardiac development[50, 51]. Normal neural crest

induction and formation in *pax3* and *pax7* mutant mice has been attributed to functional

redundancy between the two, a notion supported by the fact that knock-in of *pax7* into *pax3*

locus can rescue *pax3* mutant phenotype[52]. Interestingly, induction of avian *pax7+*

presumptive neural crest precursor population at gastrulation is affected by perturbation of

WNT and BMP signaling[48].

IIa.2) Delamination, Migration and Differentiation of Neural Crest cells

Neural crest cells are indistinguishable from the progenitor neuroepithelial pool before

the actual event of **delamination**. Delamination of neural crest cells from neuroepithelium

requires two events, epithelial-to-mesenchymal transition and a G1/S transition of progenitors,

as neural crest cells always delaminate in S-phase. Work in chicken embryos has shown that delamination of neural crest requires a transition of expression pattern from N-cadherin+ (non-neural crest) to Cadherin 6B+ (pre-migratory neural crest) to Cadherin7/11+ (migratory neural crest (reviewed in[53]). Down-regulation of Cadherin 6B has been shown to be directly downstream of a transcriptional repressor Snail2[54], whose expression in trunk (but not cephalic) neural crest cells have been shown to be under the control of WNT/BMP signaling[55-58]. A lot of our understanding of molecular control of neural crest delamination comes from work done on avian species and specifically in the trunk neural crest cells. Our understanding of delamination of cephalic neural crest cells, specifically, that of mouse cephalic neural crest cells is very murky. Even though the role for Snail proteins in inducing neural crest delamination is well understood in *Xenopus* and chick[54,59], mouse knock-out of both *Snail* homologues, *Snail1* and *Snail2*, individually or in combination does not result in neural crest defects[60,61]. There has been only one gene reported to have defects in mouse neural crest delamination, *Zfhx1b* (*Zeb2* or *Sip1*: smad interacting protein 1). *Zfhx1b* has also been shown to be important for G1/S cell cycle transition in EMT[62]. Mutations in *Zfhx1b* in mice result in complete lack of post-otic vagal neural crest cells and affect delamination of anterior cephalic neural crest cells[63]; however, analysis of neural crest-specific deletion of *Zfhx1b* in mice embryos suggests that this defect observed in complete null embryos is not neural crest cell-

autonomous[64]. No analysis of the role for *Zfhx1b* in neural crest delamination is done in other species.

Cranial neural crest cells delaminate from the neuroepithelium as a continuous wave of cells that then swiftly segregates into distinct stereotypical streams along the anterio/posterior axis. A sub-population of these cells halt at a relatively dorsal location and contribute to the peripheral nervous system of the head (Cranial sensory nervous system), while the rest migrate to ventral locations to make the craniofacial skeleton. The mechanisms responsible for segregation of this continuous wave along the A/P axis are similar among cephalic neural crest from different species. **Cranial neural crest migration** is regulated by both positive and negative interactions that occur between migrating crest cells and mesoderm surrounding them. Repulsive signaling, non-cell autonomously mediated by receptor tyrosine kinase Erbb4[65] and cell-autonomously mediated by Neuropilin/Semaphorin signaling[66,67] (also reviewed in[68]) have been shown to be required to maintain the neural crest streams entering branchial arches 1 and 2 separate. Similarly, repulsive interactions between Eph receptors and Ephrin ligands is required for segregation of neural crest streams entering pharyngeal arches 2, 3 and 4[69,70]. On the other hand, FGF signaling has been suggested to be an attractive signal that creates a permissive environment for neural crest cells to enter pharyngeal arch 2[71].

Cranial neural crest cells differentiate into a broad array of cell types within the head. There are two possible explanations as to how neural crest cells differentiate into these different cell types. Crest cells could be thought of as a multi-potent population of cells that subsequently differentiate into different cell types. Signals from surrounding tissues would be the major source of instructive cues to the multi-potent neural crest cells, suggesting extrinsic regulation of neural crest differentiation. Conversely, neural crest cells could comprise a heterogeneous mixture of progenitor cells, each of which can give rise to a separate cell type within the body. This would require pre-specification of neural crest cells as they migrate out of the neural tube, implying an intrinsic regulation of neural crest differentiation. This question of extrinsic versus intrinsic specification of neural crest has plagued researchers for more than a century, and our current understanding is that both these modes are utilized for neural crest differentiation and are in certain cases interdependent on each other. For instance, changes in a cell intrinsic factor like Sox10 influences how the crest cell interprets its external environment[72]. The detailed mechanisms of differentiation of cranial neural crest into specific neuroglial or skeletal cell types will be discussed later in specific chapters pertaining to those topics (Chapter 3 and 5 respectively).

IIb)   Ectodermal placodes

Ectodermal or cranial placodes are a unique derivative of cranial ectoderm originally defined by the thickened morphology within the cranial epithelium. Cranial placodes contribute to the developing paired sense organs and sensory ganglia and differentiate into various cell types based on the sense organ or sensory ganglia they are contributing to. In mice, the cranial placodes include adenohyphyseal, olfactory, lens, trigeminal, otic and epibranchial placodes amongst which the lens and adenohypophyseal placodes are non-neurogenic. The lens placode forms the lenses of the paired eyes is while the adenohypophyseal placode eventually forms the anterior and intermediate lobes of the pituitary gland. Fish and amphibia also possess lateral line placodes which give rise to the lateral line system which detects motion currents and aids in determining schooling behavior and prey detection. The lens, olfactory, otic and lateral line placodal cells form the paired sensory organs, while the trigeminal and epibranchial placodal cells differentiate into sensory neurons of the cranial sensory nervous system[73].

IIb.1) Induction of Cranial placodes

Formation of cranial placodes is thought to follow a hierarchal pattern of gene expression that correlates with the status of placodal differentiation. Generally, a pre-placodal region (PPR) is specified first from the cranial ectoderm, which is then segregated into multiple
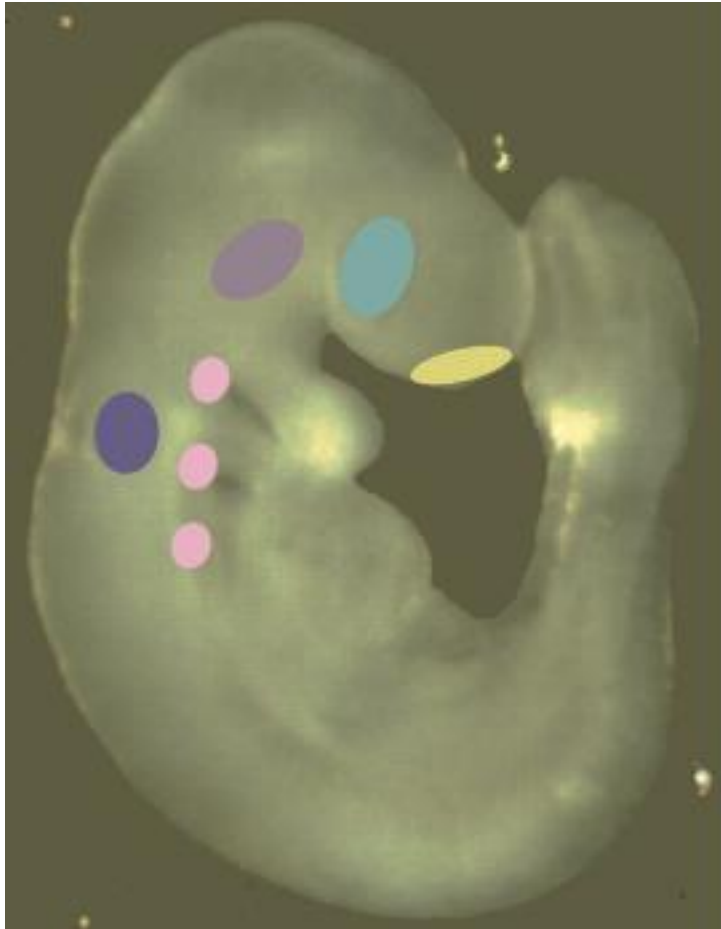
Anterior - to- posterior
Olfactory placode
Lens placode
Trigeminal placode
Epibranchial placodes
      Geniculate placode
      Petrosal placode
      Nodose placode
Otic placode

Figure 3: Ectodermal placodes of a developing mouse embryo

cranial placodes based on extrinsic and intrinsic signals. Each of these placodes then differentiates into a number of cell types specific to the particular placode.

Most of the work trying to understand the origin of cranial placodes comes from non-mammalian species. All cranial placodes have been suggested to come from a common ectodermal territory called the **Pre-placodal region (PPR)**, which is specified at neurulation[73]. Similar to neural crest induction, the importance of underlying head mesoderm and pharyngeal endoderm in formation of placodes has been shown in chick embryos[74]. Activation of FGF signaling and inhibition of WNT & BMP signaling together have been shown to be necessary and sufficient for PPR induction[75, 76]. Fate map analysis in Zebrafish, *Xenopus*, and chick and mouse embryos indicates that around the time of gastrulation the precursors for different placodes are widely intermingled with future neural, epidermal and neural crest cells[77].

IIb.2) Molecular determinants of cranial placodes

Differentiation of PPR into the various cranial placodes has been covered in depth in reviews by Gerard Schlosser, Andrea Streit and Sally Moody[73, 78, 79]. Around the time of neurulation, *six* and *eya* family of transcription factors *(six1/4, eya1/2)* are expressed in a horseshoe shaped region surrounding the rostral neural plate (forebrain to hindbrain levels)[77]

and eventually this *six1⁺/eya⁺* domain forms the pre-placodal region (PPR). Studies in Xenopus

have shown that s*ix1* is required for cells to acquire a PPR character and mis-expression of

six1 leads to up-regulation of genes specific to placodal precursors at the expense of neural

crest and epidermis markers[80]. Recent evidence in chick suggests that *six1* and *eya2* act

synergistically to promote PPR gene expression, suppressing the neural and neural crest fates.

These are, however, not sufficient to impart placodal competence to non-placodal cells, hence

do not induce ectopic placodes[81], suggesting that additional signals must be required to specify

PPR from ectoderm. Convincing visual evidence showing the presence of pre-placodal territory

in mice is not available. Hence, analysis of whether *six* and *eya* genes are responsible for its

formation is not possible, But whether a pre-placodal territory exists in mouse embryos or not,

the importance for *six* and *eya* gene families in overall formation of cranial placodes is

demonstrated through loss of function mutations in mice as well as humans. Mice

heterozygous for *eya1* display a phenotype similar to the Branchio-oto-renal (BOR) syndrome

in humans[82], in which the development of neck tissues, ear and kidneys is affected.

Homozygous *eya1* mutant mice have severe defects in inner ear formation. In addition,

trigeminal ganglia of these mice are reduced in size while the epibranchial placode derived

ganglia are completely missing or severely affected[83]. Human *six1* mutations have also been

associated with BOR syndrome. Null mutation in *six1* in mice also causes similar phenotype

like *eya1*: smaller otic vescicles, lack of vestibulo-cochlear ganglion, loss or reduction in the number of trigeminal and epibranchial placode derived neurons[84]. Information about *eya2* loss of function is very sparse, while *Six4* mutant mice have not been yet shown to have a placode phenotype[85]. However, *six1* and *six4* double mutant mice show a more severe phenotype than *six1* mutants alone [86] suggesting that Six1 might compensate for loss of *six4*.

Sox2 and *Sox3* of the *SoxB1* subfamily of HMG box containing transcription factors, as well as, homeobox transcription factors *irx1* and *irx6* have also been shown to be important for cranial placode development. These genes show widespread expression pattern in the PPR but do not cover it entirely. In Zebrafish, *irx* genes are required for placodal neurogenesis in the profundal/trigeminal placode[87] while *sox3* appears to be important for neurogenesis in the epibranchial placodes[88]. Cranial placode specific expression of *sox3* is not observed in mice embryos, highlighting the differences in the mechanism of placode specification between species. Both *irx* and *soxB1* family genes are down-regulated as soon as the placodal derived neurons migrate away and express neuronal determination and differentiation genes[88,89], suggesting their role in maintenance of a neural progenitor state in the placodes, similar to their function in the neural plate.

Members of the *pax2/5/8, pax 3/7* and *pax6* subfamilies but not *pax1/9* subfamily also play a role in specifying the different cranial placodes from the more general PPR. *Pax* genes interact with the regulatory network of *eya-six-dach* genes [90] and these genes are expressed in PPR. But the fact that *pax* genes are expressed in a more regionally restricted manner in the forming placodes has led to a hypothesis that *pax* genes are involved in conferring placode specific identity to the preplacodal cells[91-93]. *Pax6* is a marker of the olfactory and lens placodes while *pax3/7* markers of the ophthalmic trigeminal placode. *Pax3* and *Pax7* are expressed in the lateral neural plate (later dorsal neural tube) and neural crest as well as in the trigeminal placode. *pax3* mutants display severe hypoplasty in various cranial ganglia including the profundal ganglia (ophthalmic division of the trigeminal ganglion in amniotes), however whether these defects are of placodal origin or neural crest is not known [94]. *Pax 2/5/8* mark the otic placode and *pax2/8* expression is also observed in the developing epibranchial placodes[73, 92]. *Pax2* has been suggested to control epibranchial neural identity and *Pax2* and *pax8* have crucial but redundant functions in otic placode development[91]. *Pax2* mice mutants exhibit severe defects in sensory organ formation and neurogenesis from the inner ear [95]. Each *pax* gene is still expressed in multiple placodes and hence specification of placodal identity should be a result of cooperation between *pax* family of genes and some other transcription

factors. Detailed description of neuronal differentiation of some of these cranial placodes expressing *pax* gene subfamily members will be provided in later chapters (Chapter 3).

IIc)   Interactions between Neural crest and cranial placodes

In vertebrate head, neurogenic cranial placodes and neural crest cells work together to form the cranial sensory nervous system. Although most neurons of the cranial sensory nervous system are derived from the neurogenic placodes, proper migration and functioning of neural crest cells is required for the differentiation of placodal cells into neurons. Neural crest cells have been shown to be important for proper guidance of placode derived epibranchial neurons as they extend their axons towards hindbrain[96]. Improper migration of cranial neural crest cells, following loss of Semaphorin/Neuropilin signaling has also been shown to affect axon guidance of placode derived sensory neurons[97]. Neural crest cells have also been shown to influence the formation of cell-cell junctions between placodal cells thus helping in organization of trigeminal and epibranchial placodes which then affects their neuronal differentiation[98]. On the other hand, disruption of sensory neurons derived from epibranchial placodes has been shown to affect directional outgrowth of hindbrain motor neurons and the formation of neural crest derived proximal epibranchial ganglia[99]. Proper formation of cranial

PNS is thus highly dependent on proper formation, migration and differentiation of both neural

crest and cranial placodes; processes that have been shown to be interdependent.

III)   Mesoderm

Mesoderm, the middle germ layer formed during gastrulation, is an exclusive feature of

triploblastic organisms. Head mesoderm precursors undergo gastrulation through the primitive

streak prior to precursors of trunk mesoderm[100]. Within the head, mesoderm gives rise to most

of the musculature of the head and neck, some bones of the neurocranium as well as the

endothelial and supporting cells forming the cranial vasculature[101]. Differentiation and

patterning of head mesoderm into cranial musculature and blood vessels is highly

interdependent and proper patterning of these mesoderm derived tissues require normal

formation of craniofacial skeleton (neural crest derived) and pharyngeal endoderm.

IIIa)   Head Musculature

Head musculature includes about 60 distinct skeletal muscles that can be divided into 2

groups, 1) Six **extra-ocular muscles (EOM)** that move and rotate the eye and 2) **pharyngeal**

muscles (aka branchial arch muscles) which control jaw mastication, facial expression,

breathing as well pharyngeal and laryngeal function. Both EOM and pharyngeal muscles are

derived from head mesoderm. It is well established that pharyngeal muscles are derived from the subset of head mesoderm known as pharyngeal mesoderm (PM) (or paraxial mesoderm) which is located within the core of branchial arches and surrounding the developing pharynx[102]. EOM are largely derived from another subset of head mesoderm called prechordal mesoderm; however, whether there is any contribution from pharyngeal mesoderm is not clear[103]. Some of the muscles of the neck are derived from the mesoderm cells that originate in the anterior-most somites and migrate into the branchial arches[103].

IIIa.1) Regulation of Head musculature development

Unlike trunk mesoderm, which is segmented into metameric units called somites, head mesoderm is unsegmented. Head mesoderm patterning is heavily dependent on signals from pharyngeal endoderm and cranial neural crest cells migrating into the pharyngeal arches and is regulated by gene regulatory networks distinct from that governing trunk mesoderm development. For example, paired homeodomain transcription factor, PAX3, which is considered to be the master regulator of trunk myogenesis, is not expressed in head mesoderm and mutations in *pax3* do not affect head musculature formation cell-autonomously[104]. MYF5 and MYOD are key regulators of myogenesis in general. Combined loss of *Myod* and *Myf5* affects craniofacial muscle development much more drastically than trunk muscle

development, as presence of another transcription factor MRF4 is able to rescue trunk muscle differentiation[105]. Four transcription factors have been shown to be crucial for pharyngeal mesoderm differentiation. CAPSULIN (TCF21) and MYOR are transcriptional repressors, required for MYF5 activation in first branchial arch[106]. PITX2, a homeobox transcription factor is crucial for EOM formation from prechordal mesoderm as well as differentiation and survival of first arch derived muscles[107,108] while TBX1 is required for robust expression of MYF5 and MYOD throughout the pharyngeal mesoderm[109-111]. MYF5 activates SIX1, which has been shown to be important for maintenance of pharyngeal myogenesis[111,112] and together with a related family member SIX4, is required for formation of certain pharyngeal muscles[113].

Interestingly, even though both *pitx2* and *tbx1* are eventually expressed throughout the head mesoderm, their expression begins in a relatively regionalized manner. Early on, *pitx2* is expressed in the anterior head mesoderm (from di- to metencephalic levels) while *tbx1* is expressed in more posterior head mesoderm (adjacent to hindbrain). Given that loss of PITX2 affects EOM development[107,108] but loss of TBX1 does not[114], and anterior and posterior head mesoderm has distinct cell fates (EOM vs. pharyngeal muscles respectively), it is possible that early *pitx2* and *tbx1* expression boundaries represent a regionalization within head mesoderm[115]. These restricted patterns of *pitx2* and *tbx1* are achieved by a network of signaling pathways regulating each other. Recent work from Dietrich lab suggests that head

mesoderm patterning begins much earlier than thought. Regionalization within the head

mesoderm is observed immediately following gastrulation (chicken embryo stage HH5–6). An

initial inhibition of Retinoic acid signaling in the anterior head mesoderm combined with

activation of FGF signaling in the posterior head mesoderm, at this time, is required for

regionalizing *pitx2* and *tbx1* expression in anterior and posterior head mesoderm respectively.

Anterior regionalization is refined by active BMP signaling, which activates expression of *Myor*

and *alx4* in the *pitx2* expressing region, while posterior regionalization is refined by increasing

levels of FGF signaling. Subsequently anteriorly spreading FGF signals spread *tbx1* expression

throughout the pharynx region eventually leading to a final pattern of expression where

anterior *pitx2* expression labels precursors of EOM while posterior *tbx1* expression labels

precursors of pharyngeal muscles[116]. Analysis of mandibular muscle specification in Zebrafish

shows that at much later stages (20 hours post fertilization ~ equivalent to a HH22 stage

chicken embryo) BMP, Hedgehog and WNT signaling are dispensable for patterning of

mandibular muscles, while active FGF signaling still seems to be important for its patterning[117].

IIIb)   Endothelial Cells

Organogenesis is critically dependent on blood supply. Failure to form proper blood

vessel network has been shown to lead to major defects during development and has been

shown to play a key role in many diseases (reviewed in[118]). Blood vessels comprise of an endothelial tube, which is surrounded by a covering of support cells, comprising pericytes, vascular smooth muscle cells, and connective tissue. Almost all of the blood vessels in the entire embryo and extra-embryonic tissues are derived from mesoderm, except for the vascular smooth muscle cells and pericytes surrounding some of the cranial arteries (great arteries, branchial arch arteries) which are derived from cranial neural crest.

Formation of blood vessel network in amniotes is usually believed to begin in extra-embryonic tissues around the time of gastrulation. Mesoderm cells in yolk sac first differentiate into angioblasts, which are precursors to endothelial cells. The locally differentiated endothelial cells coalesce into the first tubular network, through a process called **vasculogenesis**. This initial vessel network, known as the primary capillary plexus, then expands and remodels by **angiogenesis**. Angiogenesis involves several morphogenetic events that can expand the primary plexus through endothelial cell proliferation as well as remodel the primary network through branching, directional migration and breaking and making of cell-cell junctions. The result of angiogenesis is formation of a mature blood vessel network. Although separated in time, both vasculogenesis and angiogenesis utilize similar mechanisms and signaling pathways for regulation of blood vessel formation.

One of the major signaling pathways crucial for differentiation of endothelial cells from mesoderm is Vascular Endothelial Growth Factor (**VEGF**) signaling. Expression of ligand *Vegf* is observed throughout the newly formed endoderm of a gastrulation stage embryo. At the same time, its receptors, VEGFR1 and VEGFR2 are expressed in the newly formed yolk sac mesoderm. VEGF signaling has a dose dependent requirement during blood vessel development. The importance of VEGF signaling is highlighted by the fact that loss of *vegfr2* leads to a complete absence of endothelial and blood cell formation[119]. Loss of one or both copies of *Vegf* ligand also leads to defects in blood vessel development resulting in embryonic lethality in mice[120]. On the other hand too much VEGF signaling has also been shown to be detrimental for blood vessel formation. Loss of VEGFR1, which is a negative regulator of embryonic angiogenesis, or up-regulation of VEGF levels have both been shown to cause mid-gestational embryonic lethality in mice[121, 122].

During angiogenic sprouting, VEGF ligand has been shown to act a chemoattractive cue[123]. Interestingly, it was shown that not all endothelial cells react to VEGF chemoattractive cue equally. During angiogenic sprouting, endothelial cells could be of two types, tip cells or stalk cells, depending on their location within the forming sprout; tip cells are at the tip of the sprout, first ones to experience the external cues while stalk cells as the name suggests are

behind the tip cells. Recent study has found that the tip versus stalk cell fate of endothelial cells is highly dynamic and depends on cell–cell inhibition by active **Notch signaling**[124].

IV)   Endoderm

The contribution of endoderm to the developing head is very poorly understood. Endoderm contributes to the pharynx and foregut lining in the head region and is required for segregating the developing pharyngeal arches and preventing mixing of the neural crest- and mesoderm- derived mesenchyme between the pharyngeal arches. The endoderm that is derived from gastrulation is known as **Definitive Endoderm**. Prior to gastrulation, the inner cell mass (ICM) of the embryo differentiates into two cell types: Epiblast, which contributes only to the embryo proper, and **Primitive Endoderm,** which was originally thought to contribute to the extra-embryonic tissues. Recently, live imaging has shown that some cells from the primitive endoderm get incorporated in the early gut tube[125]. The eventual fate of these primitive endoderm derived cells within the gut of mammalian embryos is currently unknown. Similar to mesoderm, induction of endoderm during gastrulation requires active WNT and Nodal signaling. Post specification formation of the gut tube lining from endodermal cells requires a cohort of transcription factors, which include members of GATA, Forkhead factor, and Sox families (reviewed here[126]).

Part 2: Mediator and Transcriptional Control of Gene Expression

Expression of genes at the right time and place is crucial for proper functioning of all biological processes. This is especially true during embryonic development, where gene expression mistakes or delays can result in congenital deformities. This control of gene expression is achieved through many mechanisms, one of the most prominent being regulation of messenger RNA (mRNA) synthesis by RNA polymerase II (Pol II). This regulation is achieved by use of transcription factors that bind to regulatory elements upstream of a gene and either activate or repress gene expression. The activation or repression of gene expression is achieved through recruitment of large co-activator or co-repressor complexes. About two decades ago, researchers identified a multi-subunit mega-dalton protein complex in yeast that acts as a transcriptional co-activator. This complex, called Mediator, which has now been shown to be conserved from yeast to humans, also plays an important role in gene expression regulation, through its various subunits and their interactions with other intracellular proteins and protein complexes.

Mediator complex

Mediator complex is required for transcription of most (if not all) protein-coding genes, housekeeping and activated. Binding studies have shown that Mediator complex, through its

various subunits, conveys information from the gene specific transcription factors bound upstream to the Pre-initiation complex present at the transcription initiation site[127-129 130]. When first identified in yeast, Mediator complex was shown to be composed of 25 subunits. Five more metazoan specific mediator subunits have been identified since then. Electron microscopy and biochemical studies have shown that the different subunits of Mediator complex reside within four modules, Head, Middle, Tail and Kinase **(Figure 9)**, and this modular architecture is conserved from yeast to humans.

Regulation of transcription by Mediator

Mediator has been associated with both active and inactive genes[131, 132]. Both yeast and human studies show Mediator binds to and recruits of RNA Pol II to the gene promoter site[133-136] and thus enhances basal and activated transcription by facilitating pre-initiation complex (PIC) formation[137,138]. Apart from being required to assemble the PIC, Mediator has also been shown to aid in post-recruitment processes. The interaction between Mediator and transcription factor, hepatocyte nuclear factor 4 (HNF4) is required for activation by HNF4 but not for recruitment of RNA Pol II[139]. Similarly, interaction of Mediator tail subunit, MED23 with transcription factor ELK1 has been shown to affect downstream gene expression at a much higher fold level than its effect in RNA Pol II binding at the gene promoter site[140]. CDK7,
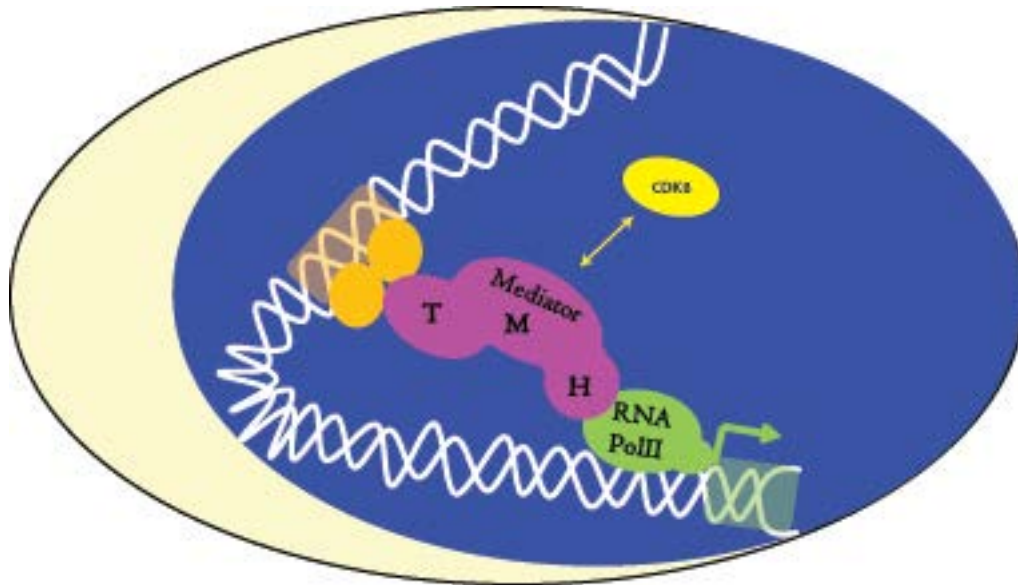
Figure 4: Composition of the Mediator Complex

Mediator complex is divided into 4 modules, Head (H), middle (M), and tail (T) and kinase (CDK8). Subunits of the

Head module interact with the pre-initiation complex (PIC) machinery (green) sitting at the gene promoters while

the tail subunits interact with the transcription factors (orange) at the distal regulatory sites.

the kinase component of TFIIH, a general transcription co-factor subunit of PIC, is important for phosphorylating Serines 5 and 7 of RNA Pol II C-terminal domain (CTD) and allowing the transition of transcription initiation to transcription elongation[141]. Notably, it has recently been shown that the phosphorylation of Serine 7 by CDK7 is regulated by Mediator, thus suggesting a role for Mediator beyond PIC assembly[142]. Evidence showing a role for Mediator in transcription elongation comes from recent work showing interactions between Mediator subunit MED26 and elongation factors and a role for this interaction in releasing paused polymerase[143].

The recruitment of RNA Pol II and other general transcription factors to pre-initiation complex is facilitated by the conformation changes caused in Mediator complex by its association with transcription factors. For example, binding of p53 Activator Domain to Mediator forms a large pocket domain in Mediator and transitions the pre-initiation complex from a stalled state to an elongation state[144], suggesting pre- and post- recruitment roles for Mediator. In a very elegant study, it was recently shown that Mediator facilitates gene expression by assembling enhancer/core promoter DNA loop complex[145]. In this study, Chip-Sequence analysis showed Mediator occupancy at both enhancers and core promoters in actively transcribed genes in mouse ES cells along with Cohesin (protein required for chromatid attachments) and Cohesin loading factor Nipb1. Chromosome Conformation

Capture (3C) experiments had previously shown the proximity of enhancers and promoter regions during gene activation[146],[147]. All these observations combined suggest a formation of Mediator – Cohesin – Nipb1 complex, which allows for enhancer/core promoter DNA looping[145].

Head Module: Interactions of Mediator with Pre-Initiation complex (PIC)

The subunits of the Head module directly bind to components of the PIC (RNA Pol II + general transcription co-factors). Rbp3 subunit of RNA Pol II has been shown to interact with Med17, Med18 and Med22 subunits of the Head module[148,149]. Cryo-EM techniques have also shown that the interaction of Mediator head module with Rbp4-7 subunits of RNA Pol II triggers a conformational change in RNA Pol II structure so as to improve the accessibility of enzyme binding site to DNA sequence of the promoter[150]. This data provides an explanation for findings that showed that Head module alone was able to influence basal transcription[151],[152]. Med1 and Med8 subunits of Head module interact with general transcription factors TFIIH and TBP[153,154]. Mediator interactions with the general transcription co-factors of pre-initiation complex help strengthen Mediator – RNA Pol II interactions and help tether RNA Pol II at the site of gene promoter[155].

Table 1: Mediator subunits and transcriptional activators.
Table adapted from *"Interactions between subunits of the Mediator complex with gene-specific transcription factors" by Borgreffe and Yue, Seminars in Cell and Developmental Biology, 2011*

Mediator subunits	Transcriptional activators
MED1	TRα/β, PPARα/gamma, RARα, RXRα; VDR; ERα/β; RORα; GR; FXR; AHR; HNF4; GATA-1; PGC-1α ; BRCA1; GABPα ; Pit-1; GATA2 ; C/EBPβ ; p53
MED12	SOX9; RTA; Gli3; β-catenin; G9a; AICD
MED14	GR; HNF4; STAT2; SREBP-1_; PPARgamma
MED15	Smad2/3/4; SREBP-1α
MED16	DIF
MED17	VP16; p53; HSF; DIF; RXR; STAT2; p65
MED19	REST
MED21	TRα/β
MED23	E1A-CR3; Elk1; DIF and HSF; ESX; C/EBPβ
MED25	VP16; DIF and HSF; RARα; HNF4; SOX9
MED26	REST
MED28	Merlin and Grb2
MED29	DSXF
CDK8	c-myc

Tail Module: Interactions of Mediator with Transcription regulators

At the other end, **Tail module** subunits interact with gene specific transcription regulators. Interestingly, it has been shown that even though diverse in sequence composition, all the binding domains within transcription factors responsible for their interactions with Mediator Tail subunits, form similar 3-dimensional arrangements upon binding to Mediator tail subunits. These structural transitions in the binding domains of transcription factors are dynamic, thus generating a situation where the same binding domain is flexible to form varied conformations, allowing it to interact with different Mediator subunit interfaces[156]. Transcription regulator – Mediator Tail subunit interaction recruits Mediator complex to the site of gene expression. It also leads to a conformation change in the Mediator complex that allows formation of a binding pocket for interaction with Pol II and other transcriptional co-factors thus regulating gene expression[144, 157-159].

Mediator interactions with transcription factors: Involvement in Cell Differentiation

Transcription factor specific binding of Mediator subunits has been reported in all organisms possessing Mediator complex **(Table 1)**. In yeast, MED10 has been shown to specifically interact with GAL10, and mutations in both these proteins lead to severe defects in transcription of genes involved in carbon metabolism and amino acid synthesis, leaving the

transcription of all other genes intact[160]. The *C. elegans* homologue of MED23 subunit of Mediator complex has been shown to directly interact with WNT and Ras signaling pathways and regulate vulval development[161], while MDT-15, *C.elegans* homologue for MED15 has been shown to be important for expression of fatty acid metabolism genes[162]. RNAi screen analysis showed distinct requirements for *Drosophila* Mediator subunits, MED16 and MED23 in transcription of specific genes *attacin A* and *hsp26[163]*.

MED1, which is a subunit of the Middle module of Mediator complex and has been shown to be a part of only about 20% Mediator complexes[164], is one of the most studied subunits of Mediator in terms of transcription factor binding. Loss of MED1 does not affect the overall integrity of Mediator complex. However, its loss affects Mediator interactions with specific transcription factors like various nuclear hormone receptors (Vitamin D receptor, glucocorticoid receptor, estrogen receptor, retinoic acid receptors and others (Table 2)), GATA1 and GATA2, p53, C/EBPβ and many others[165-171]. MED1 is also important for adipogenesis through its interaction with PPARgamma[172]. MED1 was the only Mediator subunit shown to interact with nuclear receptors until recently, when MED14 was shown to be important for adipogenesis as well through its interaction with PPARgamma[173]. MED14 has also been shown to interact with glucocorticoid receptor and can act in an independent manner as well as in

co-operation with MED1 to enhance glucocorticoid receptor transactivation[174]. A null mutation for MED14 has not been reported yet.

Similar to MED1 and MED14, another subunit of Tail Mediator complex, **MED23** has also been shown to be important for adipogenesis but through a different mechanism. Under the influence of active Insulin signaling, MED23 has been shown to interact with transcription factor ELK1, and this interaction is crucial for activation of *egr2 (krox20)*, which is crucial for adipogenic differentiation[175]. MED23 was originally identified as a co-regulator of adenovirus E1A and has been shown to be important for expression of *egr1 (aka krox24)* through its binding with ELK1[140 ,176]. Both active and inactive forms of C/EBPβ interact with Mediator through MED23[177].

MED15 is another Mediator Tail subunit, which has been shown to mediate gene expression downstream of TGFβ/Activin signaling through its interaction with intracellular TGFβ signaling effector complex Smad 2/3/4[178]. Like *C.elegans*, a lipid homeostasis role for MED15 in higher eukaryotes has not been reported. However, given the sequence similarity such a role would not be a surprise[162]. **MED25** has also been shown to regulate lipid metabolism through its interaction with transcription factor HNF4[179] and regulate mouse embryonic development through its interaction with an E3 ubiquitin ligase WWP2[180]. **MED19** and **MED26,** though not a part of the Mediator Tail complex, have been shown to interact with

RE-1 silencing transcription factor (REST) and regulating epigenetic silencing of neuronal gene expression[181].

Similar to Mediator Tail subunits, subunits of CDK8 module have been shown to directly interact with specific transcription factors. Work done in HeLa cells has shown that the kinase module subunit, **MED12** can bind to β-catenin and this interaction is crucial for transcription of WNT signaling responsive genes[182]. CDK8 itself has been shown to bind to MYC, a protein highly up-regulated in cancer cells. Whether this binding has any role to play in cancer progression is unknown, but the recruitment of CDK8 by MYC has been shown to be sufficient for activation of a synthetic promoter construct[183].

Mediator Kinase module (Activator or Repressor?)

From the four modules making up Mediator complex, Kinase (CDK8) module is variably associated with the Mediator and its interaction with Mediator has been usually associated with rendering the complex transcriptionally inactive and unable to RNA Pol II C-terminal domain[158]. The CDK8 module of Mediator consists of four subunits, MED12, MED13, cyclin C and CDK8. Reconstitution of human CDK8 module has shown that MED12, but not MED13, is required for its kinase activity[184] while MED13 is required for its interaction with the rest of the Mediator[185]. In spite of being members of cyclin and cyclin dependent kinase families,

41

Cyclin C and CDK8 (respectively) have not been shown to have any role in cell cycle regulation[186]. A paralog of CDK8, with high amino acid sequence conservation, has been observed in vertebrates, named CDK19 (earlier known as CDK11), which has also been shown to associate with Mediator complex[135]. CDK8 knockout leads to embryonic lethality in mice and in spite of high sequence similarity, presence of CDK19 is unable to compensate for CDK8 loss, suggesting that the two kinases are not functionally redundant[187]. In fact, work done on viral activator VP16-dependent transcription suggests that the two kinases, CDK8 and CDK19 have opposite roles[188, 189]. Similar to CDK8, MED12 and MED13 also have vertebrate paralogs, MED12L and MED13L[135], and whether they have a preferential binding for CDK8 containing or CDK19 containing kinase modules is currently unknown[186].

The first indication for the role of CDK8 kinase module in mediating transcription repression came from electron microscopy studies done in yeast, *S. pombe*. CDK8 module was shown to be bound to the same cleft in Mediator where RNA Pol II was observed to be bound earlier, suggesting a steric hindrance to Mediator – RNA Pol II interaction[190]. Repression of activated transcription by human CDK8 module was independent of its kinase activity but was dependent on availability of its subunits MED12 and MED13[185]. Two *in vivo* studies have highlighted the role for CDK8 association with inactive Mediator. C/EBPβ is an intrinsic repressor whose conversion into activator form requires active RAS signaling. The Leutz group

has shown that the transition from repressor C/EBPβ to activated C/EBPβ under influence of active RAS signaling is associated with an exchange of Mediator containing CDK8 module with Mediator devoid of CDK8 module[177]. In another study it was shown that active Retinoic Acid (RA) requires DNA binding protein PARP-1 for dissociation of CDK8 containing Mediator complex for target gene expression[191].

Recently, an activator role for CDK8 module has come to light. These studies highlight a context dependent role for CDK8 module, which might act as an activator or a repressor in a gene-specific manner. CDK8 module and its kinase activity have been shown to be important for RNA Pol II recruitment to and activation of a gene called *dio1*[192] and for transcriptional activation of p53 target gene *p21*[193]. CDK8 mediated gene activation has also been shown down-stream of signaling pathways like TGFβ and Notch signaling[194, 195]. Colon cancer cell lines show a copy number gain in CDK8 and a requirement for CDK8 in cancer cell proliferation and β-catenin mediated gene transcription[196]. The transactivator domain of β-catenin binds to MED12 subunit of CDK8 module and recruits Mediator to WNT responsive genes *in vivo*. Null mutations in MED12 or specific mutations in MED12 affecting β-catenin binding, block β-catenin mediated gene activation[182]. CDK8 has also been shown to influence β-catenin mediated gene activation indirectly by phosphorylating and inhibiting transcription regulator, E2F1, known to cause protein degradation of β-catenin[197]. RNA Pol II CTD

phosphorylation and assembly of elongation complexes requires active CDK8 module, suggesting that CDK8 module might be important for post-recruitment activation and transcription elongation[198].

Mediator and Embryonic Development

Above section showcases how Mediator proteins interact with various transcription factors to influence specific cellular differentiation cascades. Some of these known Mediator-Transcription factor interactions have been shown to be important in embryonic development and highlighted in the section above. Mutant analyses in drosophila, zebrafish, mice and human cell lines have shined light on Mediator subunits required for specific developmental processes, and for some of these no Mediator-Transcription factor interactions are currently known. **Table 2** summarizes the mutant analyses for various Mediator subunits that appear to be involved in embryonic development.

Although known as a general transcription co-factor, only one Mediator subunit has yet been shown to be involved in general transcription. ES cells homozygous for *med21* deletion cannot be generated and *med21-/-* mouse embryos die at blastocyst stage, a time point correlated with onset of zygotic transcription[199]. Similar to MED21, MED6 has been shown to regulate most, but not all, housekeeping and cell homeostasis genes in drosophila, and loss of

med6 affects drosophila larval development[200]. *C.elegans* homologues of MED6, MED7 and MED10 (ceMed6, ceMed7 and ceMed10) have been shown to be required for transcription of developmental genes but not housekeeping genes. RNAi mediated inhibition of ceMed6, ceMed7 and ceMed10, individually showed defects *C.elegans* embryonic development post 300cell stage but not prior. Low doses of RNAi caused defects in germ cell development and generated sterile adults (Kwon PNAS 1999). As mentioned above, MED1 has been shown to interact with GATA transcription factors (amongst many others). This interaction between MED1 and GATA transcription factors seems to be most pertinent in terms of early embryonic development as *med1-/-* mouse mutants display embryonic lethality at 11.5dpc associated with impaired placental and cardiovascular development, processes for which its interaction with GATA proteins seems to be important[201-203].

Both MED12 and MED13 have been shown to be important for formation of eye and wing imaginal disc in drosophila[204-206]. Hedgehog and WNT signaling pathways have been shown to be disrupted in eye and wing imaginal discs of *med12* and *med13* mutant flies[204,206,207]. MED12 and MED13 have also been shown to be involved in mediating GATA/RUNX regulated embryonic crystal cell formation in Drosophila hematopoiesis[208,209]. The interaction of MED12 and WNT signaling in *C.elegans* is shown to be opposite of that observed in

Table 2 – Mediator subunits and vertebrate embryonic development

Mediator gene	Organism	Mutant phenotype
Med1	Mouse	Cardiac, vascular and growth defects
Med12	Zebrafish	Neural crest and cartilage development, hindbrain patterning and cardiovascular defects
Med12	Mouse	Neural tube, somitogenesis and cardiovascular defects
Med14	Zebrafish	Retina and rod cell development
Med21	Mouse	Arrest at blastocyst stage
Med23	Mouse	Systemic circulatory failure
Med24	Zebrafish	Enteric neuron differentiation defect
Med24	Mouse	Cardiovascular, neural tube and cell growth defects
Med25	Zebrafish,	Mouse Palatal malformations
Med27	Zebrafish	Retina development
Med31	Mouse	Cell proliferation defect and developmental delay

Drosophila. In *Drosophila*, MED12 is required for WNT target gene activation, while in *C.elegans;* MED12 is shown to be an inhibitor of WNT signaling during neurogenesis[210]. MED12 has also been shown to be important for *C.elegans* vulval development; however, this role of MED12 is independent of WNT signaling, but dependent on active RAS signaling[211]. Vertebrate requirement for MED12 is very widespread. In zebrafish, multiple *med12* mutations have been discovered from mutagenesis screens, suggesting a role for MED12 in brain development, endoderm development, neural crest development and kidney development[212-214]. MED12 has been shown to be a co-activator for activation of SOX9 target genes involved in neural crest, cartilage and ear development in zebrafish; however, an involvement of WNT signaling has not been reported[215]. In mice too, **MED12** has been shown to interact with canonical and non-canonical WNT signaling. *med12* mouse mutants display defects in neural tube development, axis elongation, somitogenesis and heart development through its interaction with canonical and non-canonical WNT signaling[216]. MED12 and MED13 have also been shown to be important for pattern formation in Arabidopsis, highlighting the evolutionary conservation of the subunits and their importance in embryonic development[217].

Loss of *med23*, a metazoan specific Mediator Tail subunit has been shown to cause embryonic lethality in mice at 10.5dpc possibly through a systemic circulation failure[218]. Although a few transcription factor interactions have been reported for MED23, none can

provide an explanation for causing mid-gestational embryonic lethality. In absence of MED23, its two Tail module interacting partners MED16 and MED24 also do not incorporate into the Mediator complex[176]. Loss of MED24 also leads to mid-gestational embryonic lethality along with defects in cardiovascular development, central nervous system development and placenta formation[219]. MED23, MED16 and MED24 form a small subunit that is missing in both *med23-/-* and *med24-/-* mutants. Loss of MED24 has also been analyzed in zebrafish. Zebrafish *med24* mutants display defects specifically in neural crest derived components like craniofacial structure and enteric nervous system[220]. Differentiation of neural crest cells in mouse *med24* mutants has not been undertaken owing to early embryonic lethality of mutant embryos. Hence, the defects observed in each of these mutants could be a due to a compound loss of both, a possibility that has not been explored in details yet. Loss of another metazoan specific subunit, MED25 also causes neural crest and craniofacial development defects in zebrafish through an indirect effect on SOX9[180]. Most of the subunits show somewhat similar defects between species. Loss of MED31 however, seems to have different roles in drosophila and mouse development. In drosophila, MED31 seems to be important for anterior-posterior axis formation[221], while loss of MED31 in mouse embryos does not affect early patterning; however, general developmental delay and proliferation defects are observed post-midgestation[222].

Summary

The past two decades of work has shown us very clearly that Mediator and its subunits are crucial for general and activated gene transcription, a process especially crucial for embryonic development. While the interaction of head module subunits with RNA Pol II and general transcription co-factors anchors the pre-initiation complex at the polymerase start site, the selective interactions of the rest of the Mediator subunits with specific transcription factors is what gives Mediator a role in regulating gene transcription. The fact that loss of specific Mediator subunits themselves or specific Mediator subunit-transcription factor interactions affects transcription of only a subset of genes, calls for the presence of a "Mediator Code", which regulates transcription of a specific group of genes. Evidence for presence of such multiple forms of Mediator complexes has been observed *in vivo*, but we are currently far away from understanding the finite details of how each of these different complexes function. This current work is compilation of studies done to understand the role of one of these Mediator subunits, MED23 in transcription of a specific set of genes involved in specific differentiation processes during mammalian embryonic development.

Chapter 1

MED23 is required for mammalian embryonic development

Summary

Mammalian embryonic development is a complex process that requires orchestration of multiple differentiation events happening at the same time. Precise transcriptional control is one of the mechanisms by which the embryo ensures the expression of genes at the right time and place. Through an unbiased 3-generation forward genetics screen, our lab identified ten mutants that show defects in normal embryonic development, specifically craniofacial development[223]. Here, I describe one of these mutants, *snouty*, which leads to embryonic lethality, craniofacial, neural and vascular development defects. Dr. Lisa Sandell, a previous post-doctoral fellow in our lab, identified *snouty* to be a point mutant in *med23*, which encodes a subunit of the Mediator complex. Our *snouty* phenotype phenocopies published *med23*$^{-/-}$ phenotype, suggesting that the *snouty* allele is a null allele of *med23*. We think *snouty* is a great tool to understand the role of ubiquitously expressed transcription co-factors in mammalian embryonic development and have generated additional null and conditional alleles of *med23* for a comprehensive study of its role in mammalian embryonic development.

Introduction

Craniofacial anomalies are a part of one-third of all congenital anomalies. A lot of craniofacial anomalies have been found to be associated with defects in neural crest-derived craniofacial skeleton and could arise from defects in neural crest formation (Treacher Collins syndrome)[224,225], migration (Bardet-Biedl syndrome)[226] or differentiation (Craniosynostosis)[227]. Bone and cartilage derivatives of neural crest cells serve as important scaffold for formation of mesoderm derived muscles, tendons and vasculature. At the same time, the migration of neural crest cells through the surrounding mesoderm requires proper formation and patterning of the mesoderm itself. Signals from ectoderm and endoderm are also required for proper neural crest survival, patterning and growth. Thus, overall, craniofacial disorders, though usually manifested as defects in bone and cartilage formation, may arise also due to causes extrinsic to neural crest cell development. Defects in proper development and patterning of any of the germ layers, ectoderm, mesoderm and endoderm can lead to defects in craniofacial development.

Formation of the craniofacial complex uses common molecular pathways like WNT, FGF, SHH, BMP and RA (Retinoic acid). We have some understanding of how these signaling molecules and the factors involved in and affected by their intracellular cascades regulate the different aspects of craniofacial development, but not all the details are known. Most of our

understanding of these molecules in mammalian systems comes from gene knock-out strategies, which focus of loss-of-function phenotypes or very rarely occurring spontaneous mutations in the laboratory mouse. Completion of mouse genome sequence, however, now allows us to utilize the tool of forward genetics screen even with a small focus. Forward genetics screen, using alkylating agent N-ethyl-N-nitrosourea (commonly also known as ENU) has been successfully used in many model organisms, including laboratory mouse, to explore the genetic basis of many biological processes. ENU is injected into healthy adult males, where it potently induces point mutations in premeiotic male germ line. The point mutations are the most unique feature of ENU mediated mutagenesis as functionally they lead to all kinds of mutations, null, hypomorphic and hypermorphic. Also, point mutations can provide us an insight into the structural and functional details of the various domains within a protein and the single point mutations generated by ENU are unbiased.

With all this in mind, our lab designed a simple three-generation forward genetics screen to identify novel genes that might be involved in craniofacial development[223]. The overall scheme for the screen involved breeding ENU-injected C57Bl/6 male mice to generate founder males (F1) heterozygous for the ENU-induced mutation. The founder males were outcrossed to FVB females and the daughter offspring were then backcrossed to each founder male to generate lines that reproducibly produced autosomal recessive mutant phenotypes. The

time point of observation was E10.5 and the embryos were analyzed for morphological defects in craniofacial development. Using this screen, ten recessive mutant lines were identified, each of which was analyzed morphologically for defects in embryo size, frontonasal prominence and branchial arch patterning, development of sensory organs (eye, ear, olfactory), formation of neural tube, neural crest development and neurogenesis and vasculogenesis. The phenotypes of all the mutants identified from the screen ranges from exencephaly to frontonasal and branchial arch hypoplasty to defects in vascular development as well as remodeling. Almost all of the mutants identified from this screen are embryonically lethal at mid-gestation, suggesting the crucial role that the genes involved play during mammalian embryonic development. The embryos also typically display a smaller size and have mild-to-severe defects in neural crest cell formation, migration or differentiation.

The goal for the screen was to identify novel genes involved in craniofacial development. Each mutant line was generated essentially from a cross between C57Bl/6; 128Sv mixed background mutagenized male and an FVB background female. Using a panel of microsatellite markers polymorphic between C57Bl/6; 128Sv genetic background from males and FVB genetic background from females, we have been able to identify the genomic interval associated with each phenotype. Once the genomic interval is identified various approaches, from RT-PCR to exon sequencing to whole chromosome sequencing have been used for the

different mutants to conclusively identify the one single gene whose mutation is responsible for the phenotype. We have been able to identify known and previously unknown roles of multiple genes during craniofacial development using this approach ([205] & personal observation, *data not published*).

Here, I characterize the phenotype of one such mutant, *snouty*, identified from the above-mentioned screen. *snouty* is an autosomal recessive allele, and its homozygosity causes defects in embryonic survival beyond E10.5. As described below in details, *snouty* mutant embryos display defects in frontonasal process and branchial arch-1 formation along with defects in neurogenesis and vascular remodeling. Dr. Sandell, a previous member of our lab identified *snouty* as a single point mutation in *med23*, a gene encoding for a universally expressed transcription co-factor complex subunit, MED23.

Results

I.   *snouty* mutation leads to craniofacial development defects and embryonic lethality

snouty mutant embryos have a variable phenotype with the most severe embryos being much smaller and are quite developmentally delayed (up to a day) than their wild-type littermates. Most of the severe-phenotype *snouty* embryos display an open neural tube and lack of axial turning. *snouty* embryos with mild phenotypes also display a reduced size in

comparison to the wild-type counterparts, however they are bigger in size than the most severe

mutant embryos, and these embryos do complete most of the neural tube closure. Some mild-

phenotype *snouty* embryos do display lack of neural tube closure in the most anterior regions

that include the forebrain and the midbrain. The mild *snouty*-phenotype is the predominant

phenotype in these mutants; hence, most of my analysis from here on focuses on the mild-

phenotype embryos.

The smaller size of the mild-phenotype mutant embryos, observed at E9.5, does not

seem to be due to developmental delay, as these *snouty* embryos are only 4-6 hours behind in

development as determined by their somite number. By E9.5, *snouty* embryos display a smaller

and malformed frontonasal prominence (FNP) and first branchial arch (BA) (**Figure 5A and B**),

the regions of the embryo which will give rise to the face (snout) of the adult mouse; a

characteristic which lead to the mutant phenotype being christened *snouty*. *snouty* embryos

also display defects in eye and ear development, as observed by the smaller optic and otic

capsules in the mutants (**arrows, figure 5A and B**). The optic capsule of the *snouty* embryos

seems to be ventrally shifted in comparison to their wild-type littermates; this could however

be an artifact due to lack of FNP structures that are normally formed ventral to the optic

capsule. The forming heart (*h,* **Figure 5A and B**) should have already undergone cardiac

looping by E9.5, a process which is delayed in *snouty* embryos. The representative mild-
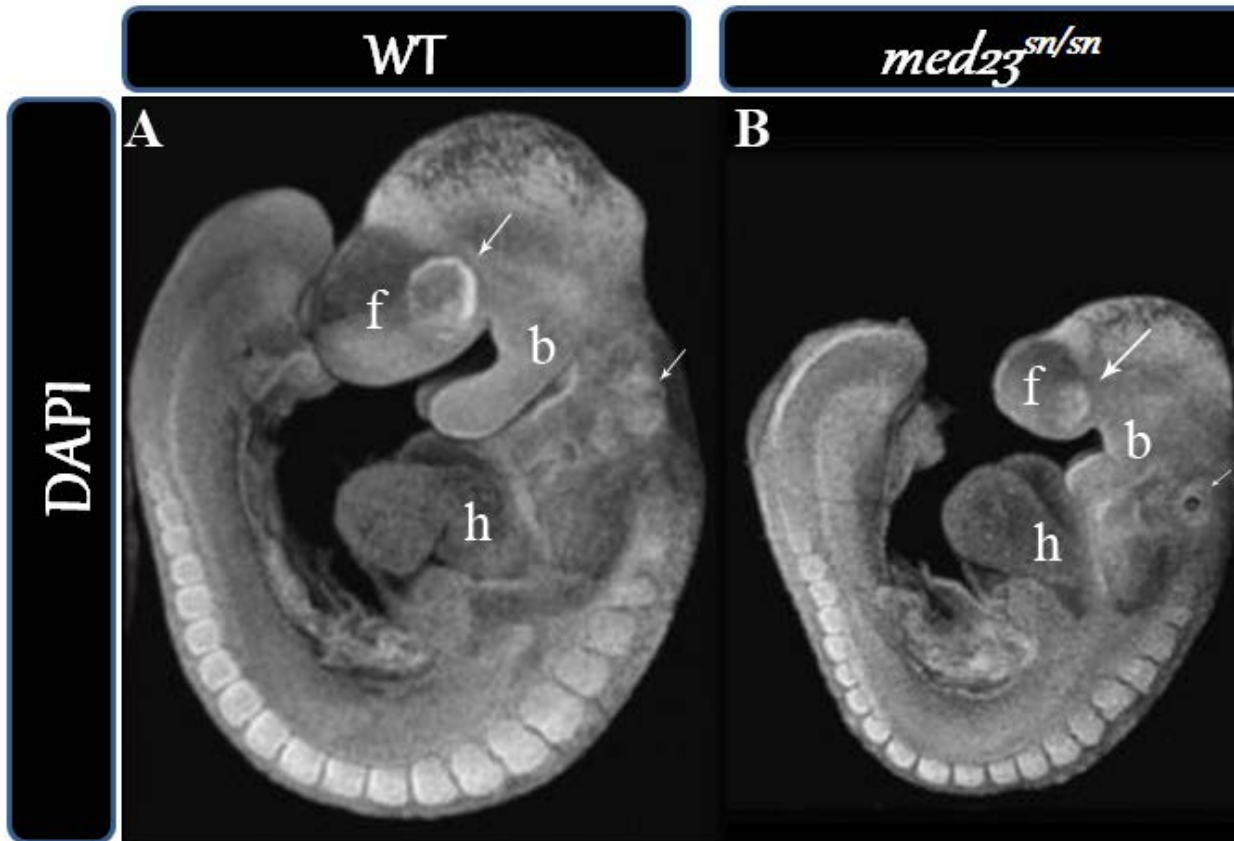
Figure 5: *snouty* embryos display a smaller size and craniofacial defects at E9.5

Wild-type (A) and *snouty* (B) E9.5 mouse embryo littermates were stained with DAPI and imaged with confocal microscope. *Arrows- optic and otic capsules, h- heart, f - frontonasal process, b- branchial arch 1.*

phenotype *snouty* embryo is about 2/3rd size as that of its wild-type embryo (Figure 7A). By E10.5, *snouty* embryos, even though alive as assessed by live beating heart, have heavily stunted growth leading to a very sick looking mutant embryo. Considering this, we selected E9.5 – E10.0 as our time-point for analysis to make sure that we are able to differentiate between the direct consequences of *snouty* mutation versus the indirect defects in the mutant embryos owing to their unhealthy state at E10.5.

snouty* mutation causes mid-gestation embryonic lethality and the mutant embryos die by E10.5. Majority of organ systems of the mouse embryo are established post-E10.5. Hence, the embryonic lethality phenotype of *snouty*, although proving a crucial role for the mutated gene in embryogenesis, prevents us from understanding its role in organ system formation. However, two systems, nervous system and cardio-vascular system, both extremely crucial for embryonic development, have already begun forming and differentiating by this time point. Defects in both nervous system development as well as embryonic and extra-embryonic vascular development have been associated with mid-gestational embryonic lethality. Considering this, we analyzed the formation of neurons and blood vessels in *snouty* embryos at E10.0.

II   *snouty* mutant embryos have defects in neural and vascular development

Neural Development

Development of neurons was analyzed by immuno-staining with an antibody against neuronal cell specific cytoskeletal marker β-tubulin III. At E10.0, β-tubulin III labels both the cell bodies and axons of the developing neurons of the central and peripheral nervous systems (CNS and PNS). In **Figure 6A**, we can see that β-tubulin III (green fluorescence) labels the developing neuronal network of the midbrain (*mb*) which is a part of the CNS. Here, β-tubulin III is also labeling the cell bodies and axons of the developing neurons of the cranial sensory nervous system (CSNS), which includes trigeminal, facial/vestibuloacoustic, glossopharyngeal and vagal ganglia, as well as the developing trunk sensory neurons whose cell bodies coalesce to form dorsal root ganglia (DRG). *snouty* mutant embryos are able to form the CNS neurons as can be seen by the presence of β-tubulin III immuno-stained neuronal cells in the midbrain neuronal network (*mb*, **Figure 6B**), however, they lack the neuronal differentiation in the developing CSNS, with only few cells showing positive β-tubulin III immuno-staining in the forming trigeminal ganglion (TG) and no cells showing β-tubulin III immuno-staining in the epibranchial ganglia (EG). *snouty* embryos also show a reduced neuronal differentiation in the DRGs; however the extent of this defect is variable amongst the mutants. The neurons of the CSNS are derived from a mixture of two cell populations, neural crest and cranial placodes.
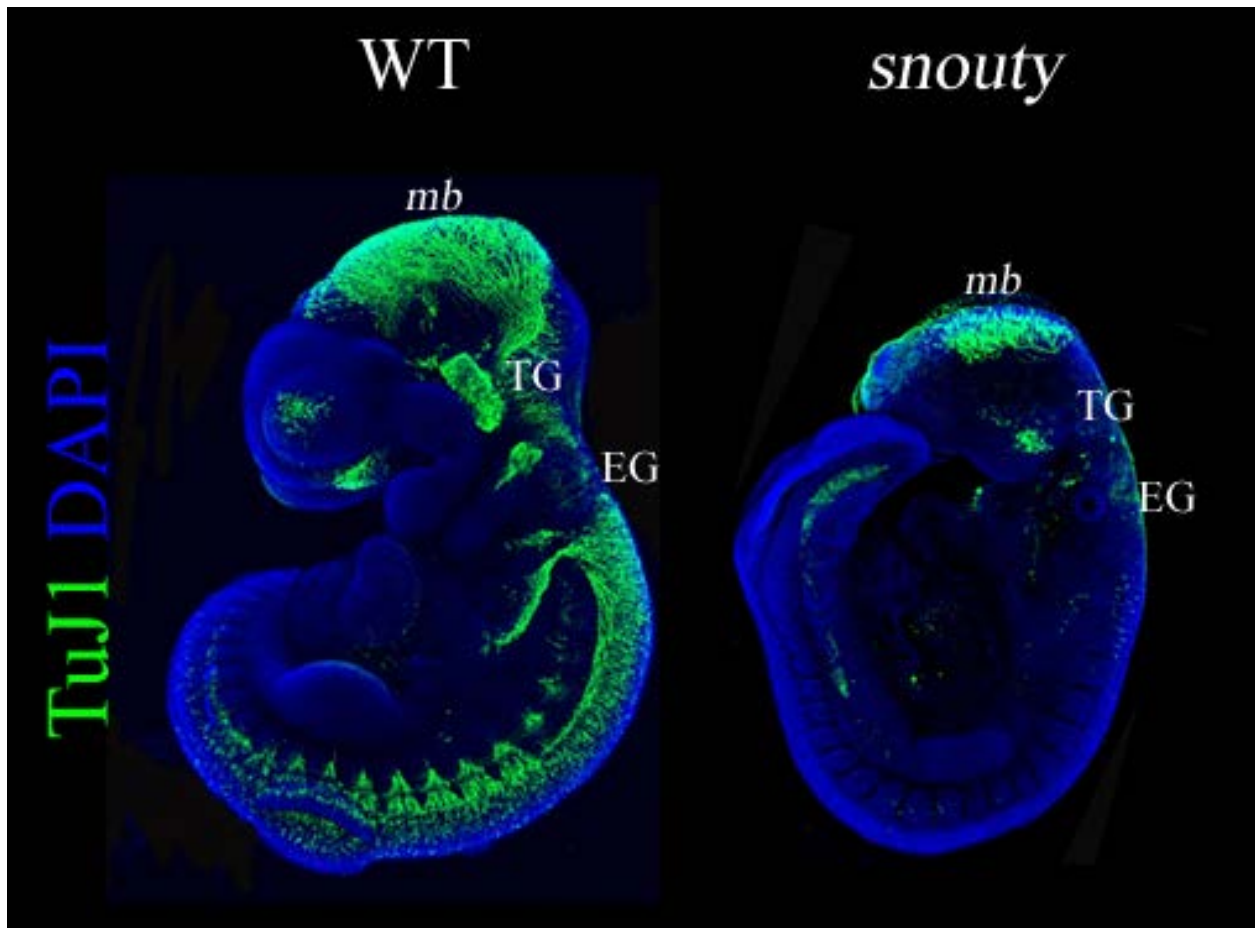
Figure 6: *snouty* embryos have defects in peripheral nervous system formation.

Wild-type and *snouty* E9.5 littermate embryos were immuno-stained for antibody against TuJ1 (neuron marker). *snouty* embryos display disrupted cranial sensory nervous system formation (very few TuJ1+ cells in TG – trigeminal ganglion and no TuJ1+ cells in EG – epibranchial ganglia) but relatively normal neurogenesis in midbrain (mb).

Subsequent chapters will analyze which of the two cell populations are affected in *snouty* embryos leading to the defect in CSNS differentiation. The question of whether this defect is a differentiation defect or a cell survival defect will also be addressed.

Vascular defect

Having established that *snouty* embryos have a defect in formation of PNS, we next analyzed the formation of the embryonic and extra-embryonic blood vasculature in these embryos. Platelet endothelial cell adhesion marker (Pecam-1) aka CD31 is a protein expressed at the junctions of embryonic and extra-embryonic endothelial cells as they are forming blood vessels. We analyzed *snouty* embryos for the blood vessel development by immuno-staining the embryos using an antibody against Pecam-1. We know that *snouty* embryos are smaller in size than their wild-type littermates at E9.5. This reduction in size could be a consequence of reduced nutrient and oxygen supply to the developing embryo owing to defects in blood vessel formation. Hence, we decided to first look at the development and organization of the blood vasculature in wild-type and *snouty* embryos and yolk sacs at E9.5 **(Figure 7)**. By E9.5, wild-type embryos form a very nicely organized vascular tree in the head and epibranchial regions of the embryo **(asterisk, Figure 7E)**. *snouty* embryos display a mis-organization of the vascular
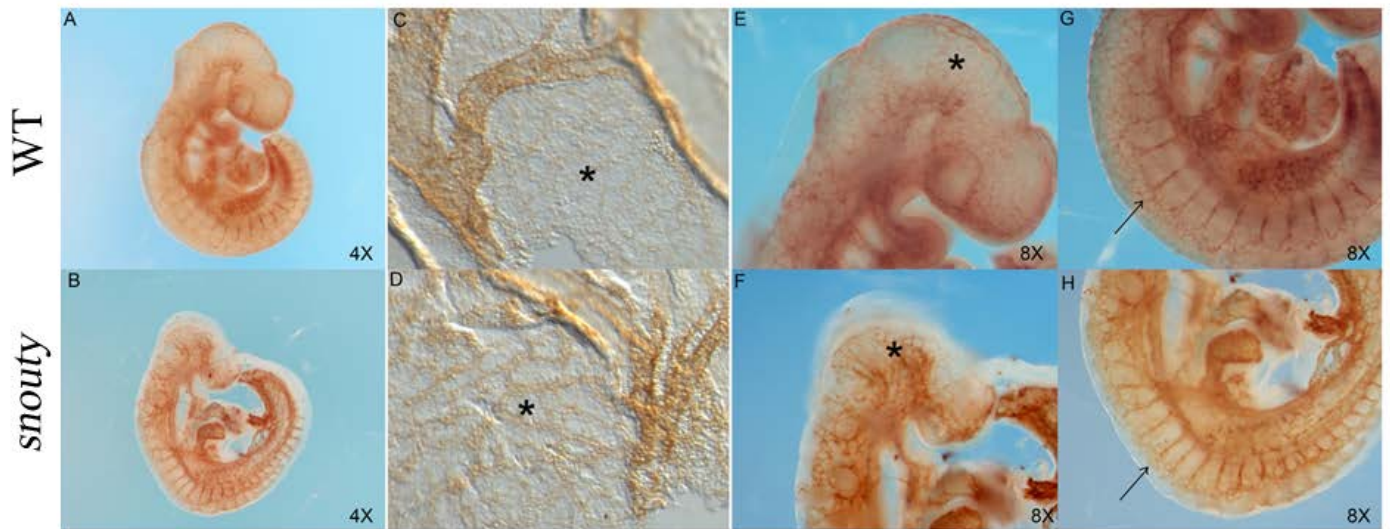
Figure 7: *snouty* embryos have defects in vascular remodeling

Pecam-1 immuno-staining of Wildtype (A, C, E, F) and *snouty* (B, D, F, H) E9.5 embryos. Mis-

regulation of vascular tree like network is visible in yolk sacs (C, D) and in the head (*) and

epibranchial regions (E, F). Intersomitic vessel formation (arrow, G, H) is not affected.

tree in the head and epibranchial regions (asterisk, Figure 7F). The inter-somitic vessels (ISM,

Figure 7G and H) develop normally in the *snouty* embryos. The vessels infiltrating the heart

(Figure 7A, B) also seem to be dilated, but that could be a consequence of delayed and defective

cardiac development in *snouty* embryos. The yolk sac vasculature (Figure 7C, D) of *snouty*

embryos displays a similar phenotype to embryonic vasculature. The blood vessels of the yolk

sacs in *snouty* embryos are also mis-organized, thicker and more dilated. The characteristic

vascular tree formation in the embryos and yolk sac is achieved by remodeling of the newly

formed blood vessels during angiogenesis. Given that the blood vessels are formed in *snouty*

embryos but the refined vascular tree network is not formed, it seems that *snouty* embryos have

a defect in the vascular remodeling.

III   *snouty* mutation was identified as a single nucleotide change in *med23*, a gene encoding for Mediator complex subunit, MED23

The mapping for *snouty* mutation was done in our lab by a previous post-doctoral

candidate, Dr. Lisa Sandell. Using SNP and microsatellite markers exclusive to C57BL/6 or FVB

strain the exact point of mutation in *snouty* was determined to be a single base pair change in

exon 22 of a gene *med23*, located on chromosome 10 of mice (Figure 8). The single base pair

change leads to a single amino acid change from Valine to Aspartic acid (V to D) in the
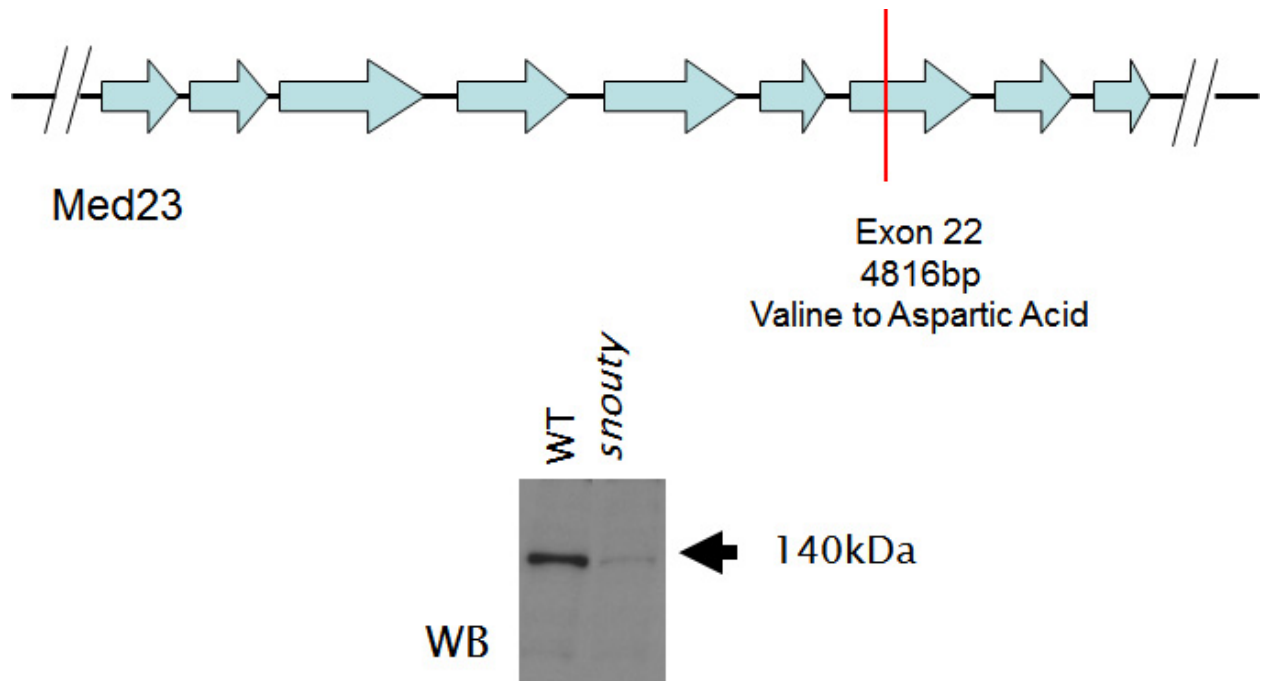
Figure 8: *snouty* is a mutant in *med23*

snouty mutation was identified to be a single base pair mutation on Exon 22 of *med23* gene leading to a single amino acid change, valine to aspartic acid, in the protein. Western blot against N-terminal of MED23 shows drastic reduction of MED23 protein levels in *snouty* mutant embryos

transcribed protein. Putative structure analysis suggests that MED23 protein comprises of multiple alpha helices joined together by linker motifs V-to-D mutation in $med23^{sn/sn}$ $(sn=snouty)$ falls in one for the linkers connecting the alpha helices. We speculated that the mutation might lead to instability in the transcribed protein. Western blot against N-terminal domain of MED23 showed a that MED23 protein in $med23^{sn/sn}$ embryo was reduced by ~85% in comparison to wild-type littermate embryos (**Figure 8**).

IV Generation of knock-out *med23* mouse strain

To additionally confirm that *snouty* mutant carries a mutation in *med23* and that $med23^{sn}$ allele is a functionally null allele of *med23*, we procured ES cells carrying a gene-trap mutation in *med23* from Knock-out mouse consortium project (KOMP). The schematic of the gene-trap mouse allele is shown in **Figure 9A**. The KOMP-*med23* allele contains a (splice acceptor) SA-lacZ-neo cassette, known as β-geo from now on, introduced within the intron between exons 12 and 13. Because of the presence of SA site 5' of β-geo cassette and a polyA tail 3' of β-geo cassette, this KOMP-*med23* allele (known as $med23^{bgeo}$ from hereon) transcribes into a truncated RNA product consisting of exons 1-12 and a fused β-geo (**Figure 9B**). The translated protein is hence truncated at exon 12 and fused with LACZ. Formation of such a protein product gives us two advantages. First, the truncated protein from $med23^{bgeo}$

65

(A) *Med23*βgeo

(B) spliced mRNA product of (A)
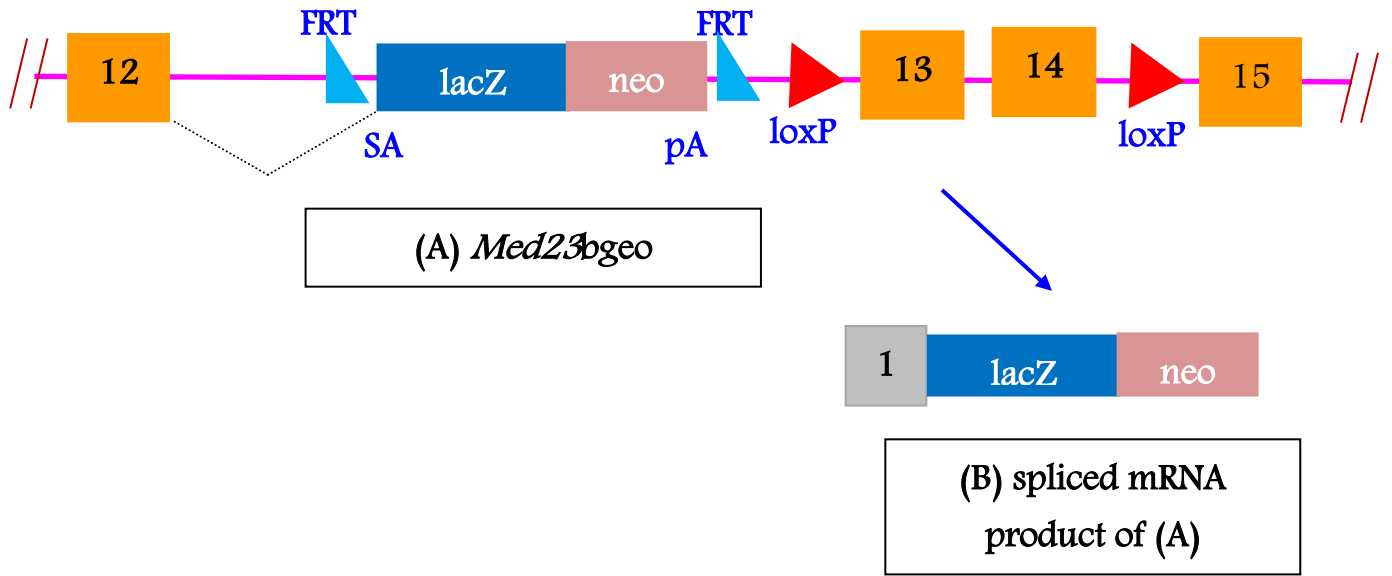
C
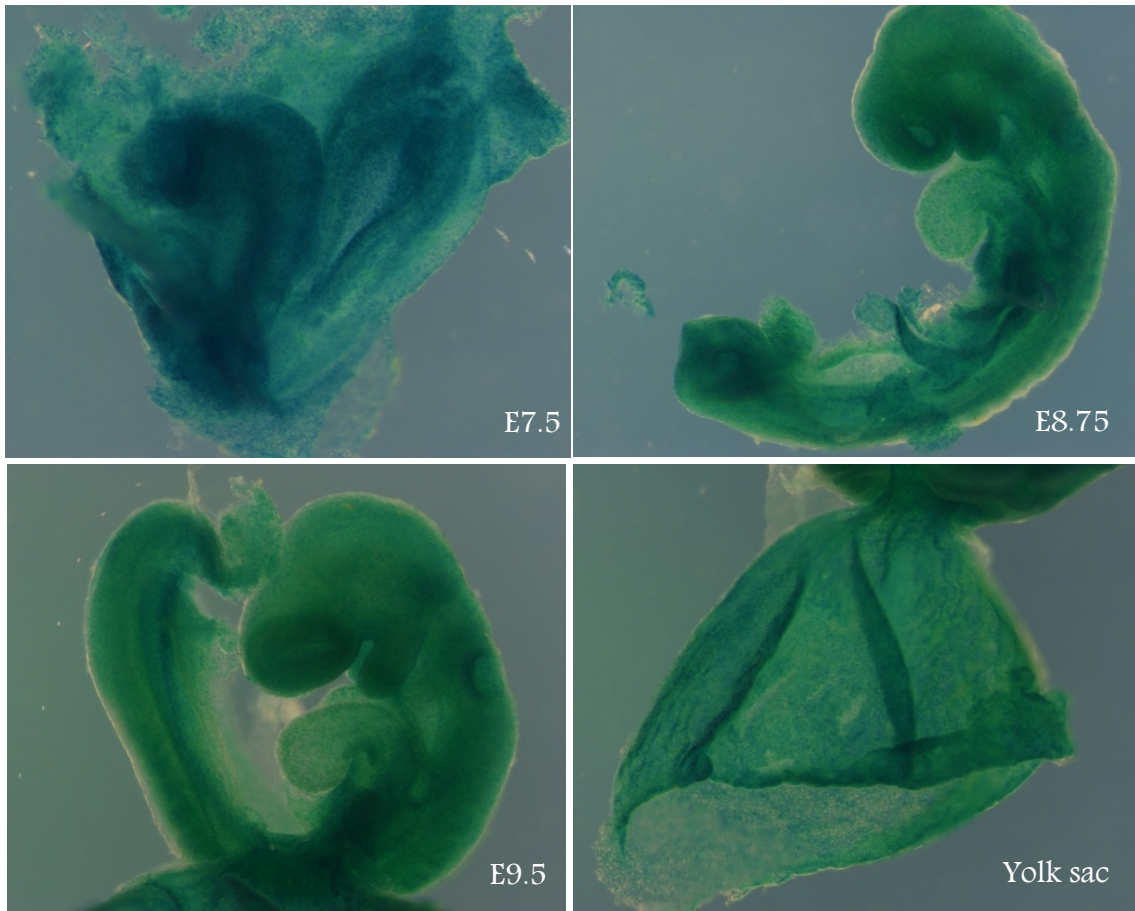
Figure 9:

A) Schematic representation of *med23^bgeo* allele

B) Formation of LacZ-fused truncated *med23* RNA product from *med23^bgeo* allele

C) Ubiquitous expression of LacZ (representative of *med23* expression) throughout the embryonic and extraembryonic yolk sac from E7.5 to E9.5.

(MED23*) cannot perform same functions of MED23, and hence $med23^{bgeo}$ allele serves as a functionally null mutation. Secondly, the MED23*-LACZ fusion protein can be utilized to analyze the expression pattern of $med23$ within the developing embryo. ES cells containing $med23^{bgeo}$ allele were on C57BL/6 background. To generate $med23^{bgeo/+}$ mouse strain these ES cells were cultured and injected into the C57BL/6 blastocysts generating chimeras which were then injected into a pseudo-pregnant C57Bl/6 females. Germ-line transmission was confirmed through tail-genotyping of the first generation and second generation offspring. $med23^{bgeo/+}$ animals were intercrossed and embryos were obtained at E9.5.

$med23$ is ubiquitously expressed in all cells of the developing embryo

We made use of the MED23*-lacZ fusion protein formed in $med23^{bgeo/+}$ embryos to analyze the expression pattern of $med23$ during embryogenesis. $med23^{bgeo/+}$ males were mated with C57BL/6 females to harvest embryo litters from E7.5 to E9.5 (**Figure 9C**). At each stage the embryos were subjected to X-gal staining, in which regional distribution of LACZ enzyme is determined using its property to cleave X-gal molecule and generate a blue colored product. The presence of blue staining indicates the presence of LACZ, which in this case indicates the site of $med23$ expression. As can be seen from Figure 9C, $med23$ is expressed ubiquitously in all embryonic and extra-embryonic tissues.
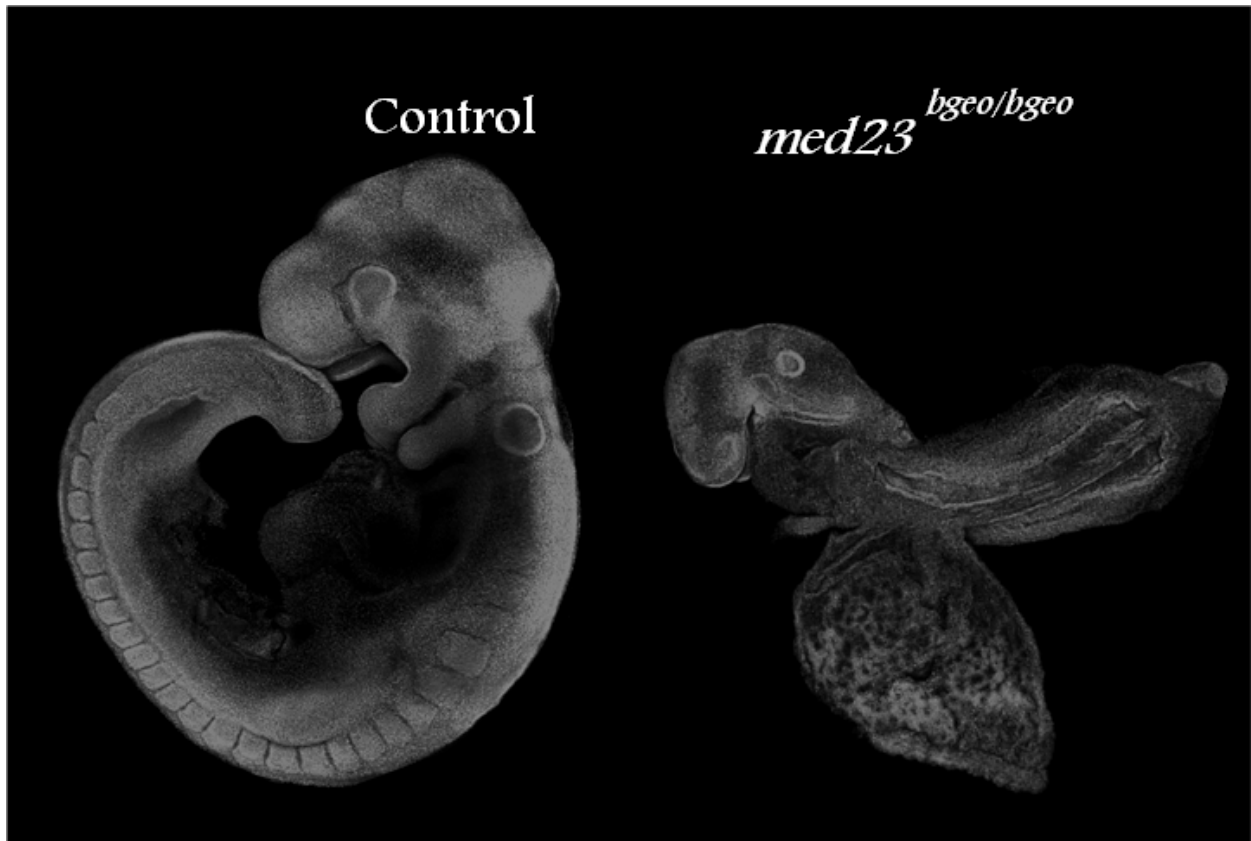
Figure 10: *med23^bgeo/bgeo* embryos halt their development at E8.75

DAPI immuno-stained littermate control and *med23^bgeo/bgeo* embryos at E9.5 show the developmental delay in the latter. *med23^bgeo/bgeo* embryos fail to undergo axial turning, display edematous heart, and are developmentally halted at E8.75.

V $med23^{bgeo}$ homozygosity is embryonically lethal and leads to phenotype more severe than $med23^{sn/sn}$ embryos

$med23^{bgeo}$ strain is on C57BL/6 mouse background, which is different from the FVB background of $med23^{sn}$ strain. Hence, we analyzed the morphology of $med23^{bgeo/bgeo}$ embryos to compare to $med23^{sn/sn}$ embryos and determine whether there is any strain dependence of $med23$ loss-of-function (LOF) phenotype. $med23^{bgeo/bgeo}$ embryos do not survive beyond E10.5, a phenotype similar to $med23^{sn/sn}$ embryos. The difference between $med23^{sn/sn}$ and $med23^{bgeo/bgeo}$ embryos is that $med23^{bgeo/bgeo}$ embryos halt their development at E8.75. At E9.5, even though $med23^{bgeo/bgeo}$ embryos are alive with a beating heart, the mutant hearts are edematous (Figure 10A and B), they have not undergone axial turning and their FNP and 1st BA (future craniofacial structures) have not developed equivalent to the littermate E9.5 wild-type embryos.

.

VI Complementation test to confirm *snouty* mutation as a functionally null mutation in $med23$

The severity of $med23^{bgeo/bgeo}$ phenotype as compared to $med23^{sn/sn}$ embryos is most likely due to the strain differences between the backgrounds of the mouse lines. However, there remains a faint possibility that $med23^{sn/sn}$ is a hypomorph and $med23^{bgeo/bgeo}$ represents the

$med23^{bgeo/+}$ **X** $med23^{sn/+}$

Litters at E9.5

$med23^{bgeo/sn}$

| similar to $med23^{sn/sn}$
snouty is null allele | | severe than $med23^{sn/sn}$
snouty is hypomorph allele |

Milder than $med23^{sn/sn}$
$Med23^{bgeo}$ is a hypomorph allele
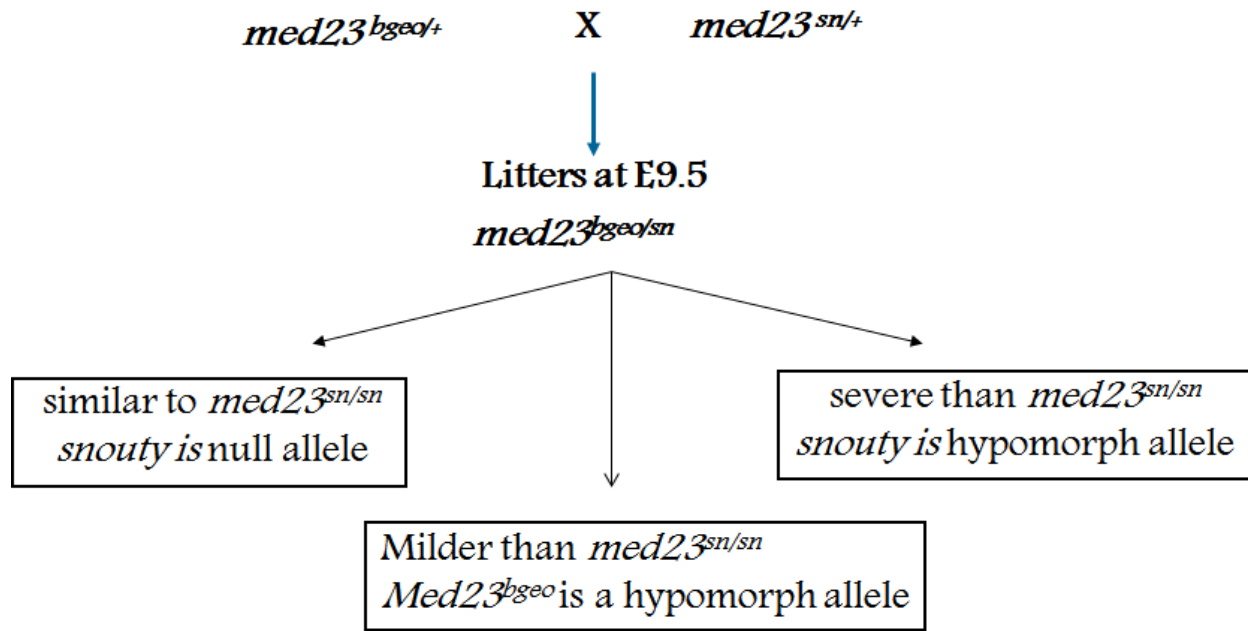
Figure 11: Schematic of Complementation test

complete null phenotype for *med23* LOF. To test for this possibility we crossed *med23$^{bgeo/+}$* and

med23$^{sn/+}$ adult animals and harvested the double mutant embryos at E9.5. The scheme for

mating and expected outcomes is described in **Figure 11**. The analyses are performed by

examining the phenotype of *med23$^{bgeo/sn}$* (double mutant) embryos and compare it to

med23$^{sn/sn}$ and *med23$^{bgeo/bgeo}$* embryos. If *med23^{sn}* allele is indeed a hypomorphic allele, then

combining one *snouty* – hypomorphic allele with a *bgeo* – null allele should still lead to

formation of some functional MED23 protein (at levels lower than *med23$^{sn/sn}$* embryos), and

the phenotype of the double mutant *med23$^{bgeo/sn}$* embryos should be more severe than

med23$^{sn/sn}$ embryos. In another case if *med23^{sn}* allele is a functionally null allele, then the

phenotype of the combined knock–out *med23$^{bgeo/sn}$* embryos should be similar to that of

med23$^{sn/sn}$ embryos. There is also a third possibility where the phenotype of double mutant

med23$^{bgeo/sn}$ embryos is milder than *med23$^{sn/sn}$* embryos; this would suggest that the *med23^{bgeo}*

allele is a hypomorph, something that has been observed in *gene-trap* alleles containing the *β–*

geo cassette. Given the highly severe phenotype for *med23$^{bgeo/bgeo}$* embryos, the third possibility

is highly unlikely in our case.

We know that *med23$^{sn/sn}$* embryos display defects in anterior morphology, neural

differentiation and blood vessel organization at E9.5. Hence, we analyzed the combined mutant

med23$^{bgeo/sn}$ embryos for the same three processes at E9.5. Embryonic morphology was

Control   *med23*[bgeo/sn]
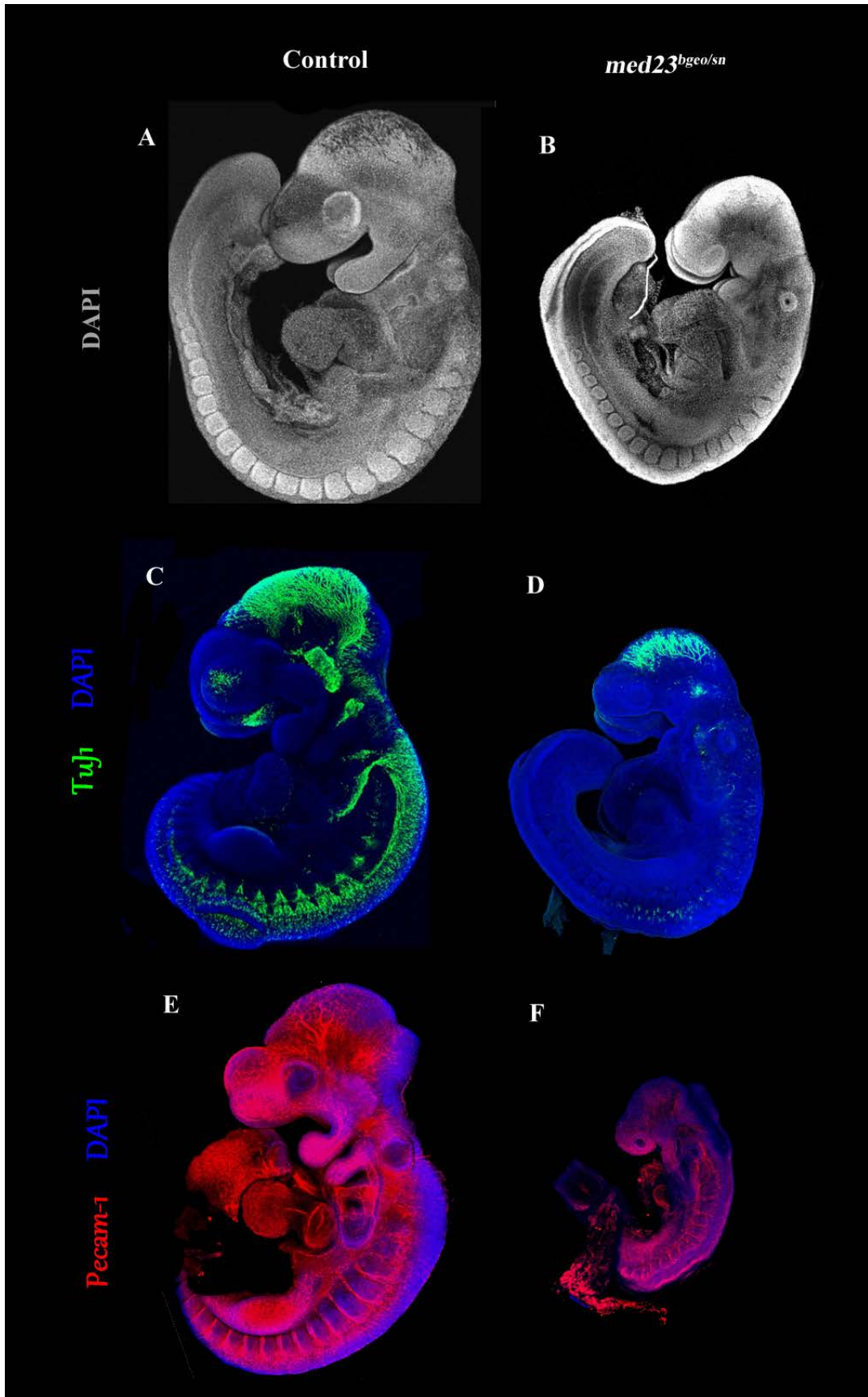
Figure 12: *snouty* is a functionally null allele

Wildtype (A, C, E) and $med23^{bgeo/sn}$ (B, D, F) littermate embryos were immunostained for DAPI

(A, B), TuJ1 (C, D) and Pecam-1 (E, F). Morphological, neural and vascular defects in

$med23^{bgeo/sn}$ embryos are similar to $med23^{sn/sn}$ embryos suggesting that $med23^{sn}$ is a null allele.

determined by pseudo-SEM DAPI staining **(Figure 12A and B)**, neural differentiation was determined by β-tubulin III immuno-staining **(Figure 12C and D)** and blood vessel formation was determined by Pecam-1 immuno-staining of endothelial cells **(Figure 12E and F)**. In all the three processes, the defects observed in $med23^{bgeo/sn}$ embryos were similar (neither milder nor more severe) to $med23^{sn/sn}$ embryos. At E9.5, $med23^{bgeo/sn}$ embryos are smaller in size than their wild-type littermates, their FNP and 1st BA **(Figure 12B)** are smaller in size and mis-formed and the heart is under-developed **(Figure 12B)**. The forming nervous system of $med23^{bgeo/sn}$ embryos displays similar defects as that of $med23^{sn/sn}$ embryos. $med23^{bgeo/sn}$ embryos also lack β-tubulin III immuno-stained differentiating neurons in the forming cranial sensory nervous system and have fewer and mis-organized β-tubulin III immuno-stained differentiating neurons in the forming trunk sensory nervous system **(Figure 12D)**. Moreover, Pecam-1 immuno-staining shows that the defects in vascular organization of $med23^{bgeo/sn}$ embryos **(Figure 12F)** are also similar to that of $med23^{sn/sn}$ embryos. $med23^{bgeo/sn}$ embryos do not survive beyond E10.5 similar to $med23^{sn/sn}$ embryos. These results from the complementation test suggest that *snouty* is indeed a functionally null mutation of *med23* and the phenotypic differences between $med23^{sn/sn}$ and $med23^{bgeo/bgeo}$ embryos are due to the background strain differences.

VII Generation of *med23* conditional knock-out mice

To overcome the early embryonic lethality of *med23^{bgeo/bgeo}* embryos and to analyze the role for MED23 during later gestation and postnatally, we decided to generate conditional mutant mouse lines for *med23*. *med23^{bgeo}* allele obtained from KOMP (Figure) has been designed such that the genetrap cassette within intron 12 is flanked by FRT sites. To make use of the Flp/FRT system, we crossed the *med23^{bgeo}* mice to a C57BL6 Flpper mouse strain. The flippase enzyme excises out the genetrap sequence flanked between FRT sites leaving two loxP sites flanking exons 13 and 14 of *med23* (Figure). This mouse line, *med23^{floxed}* will be used to mate with various *cre* lines to generate tissue specific and time specific conditional mice. The scheme of matings is depicted in **Figure 13**.

Unfortunately, much after the Flpper and *med23^{bgeo}* mating was underway we realized that the Flpper mouse is not very efficient. In the *med23^{bgeo}* allele, the FRT sites flank the complete '*bgeo*' cassette, which includes the genome sequence encoding for lacZ as well. Using lacZ staining in the developing gonads of embryos obtained from *med23^{bgeo}* x *flpper* mice, we observed that majority of the embryos that are carry both *flp* and *med23^{bgeo}* alleles are still unable to excise out '*bgeo*' cassette and express *lacZ* and stain blue upon X-gal staining (**Figure 14**). The FLP/FRT mediated excision in this particular system is mosaic and as a result we ended up with animals of mixed genotypes containing *med23^{bgeo}*, *med23^{floxed}* and *flp* alleles all
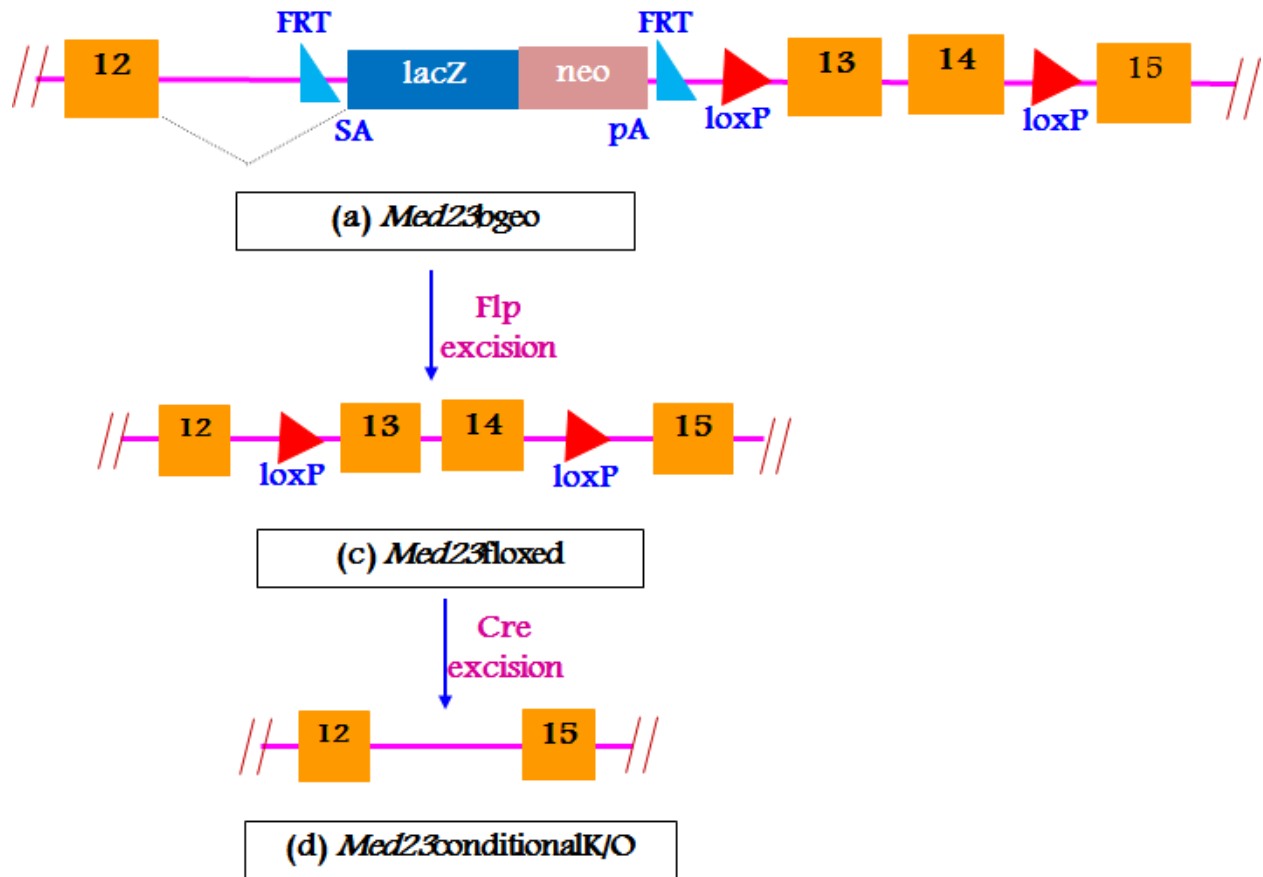
Figure 13: Mating scheme to generate conditional mice from *med23*^{bgeo} animals
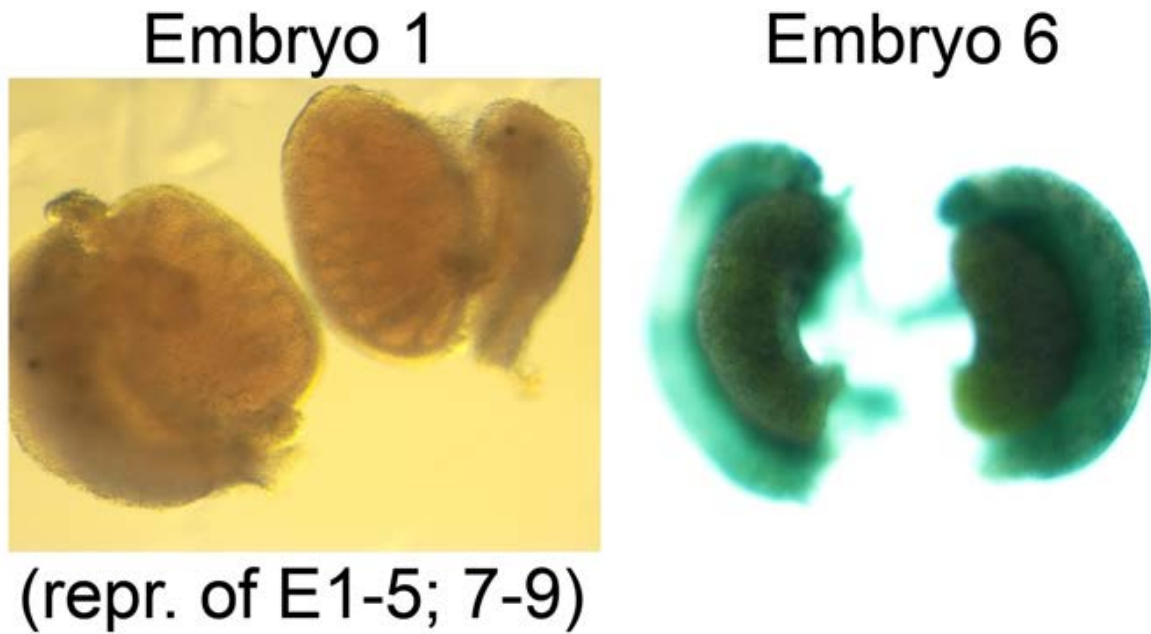
Figure 14: Mosaic activity of Flpper mice

C57Bl/6 Flpper mice were crossed with $med23^{bgeo/+}$ animals and embryos were harvested at E14.5 to analyze their genital ridges for complete excision. LacZ staining from $bgeo$ genetrap allele was used to determine if the excision occurred or not. Excision of $bgeo$ genetrap by Flpper would result in absence of lacZ staining, however 1 out of 9 (embryo # 6) embryos from C57B/6 Flpper x $med23^{bgeo}$ cross still expressed lacZ (in spite of having the Flp allele genotypically) showing that Flpper excision is not complete.

together. We did not want to generate our conditional mice with these mixed genotypes and getting around this ended up extending our timeline beyond previous expectations. In order to avoid leakiness of *med23^{bgeo}* allele into the conditional animals (*med23^{floxed}*), we have screened all the offspring of Flpper x *med23^{bgeo}* mating by genotyping not just for the presence of the newly '*floxed*' allele but also for absence of '*lacZ*' or '*bgeo*' allele and generated our *med23^{floxed}* founder males and females (*med23^{floxed}* F2 generation) only from these newly identified *floxed+; lacZ–* animals (*med23^{floxed}* F1 generation). Since our genotyping is done with tail clips, we had to bear in mind that the mosaicism caused by inefficient Flppase enzyme activity could result in a situation where the all the tail cells end up with perfectly excised *med23^{floxed}* alleles (also *lacZ–*) but the germ cells could still carry some residual *med23^{bgeo}* alleles. This indeed did happen as some of F2 animals were still genotyped as *lacZ+*; our solution to this problem was to discard the *lacZ+* F2 animals and maintain only the *floxed+; lacZ–* ones. This solution has been successful yet quite slow in generating the *med23^{floxed}* mouse line.

In spite of these hurdles and time delays, we have now been able to cross the *med23^{floxed}* line to various *cre* lines (*Wnt1^{cre}, Tek^{cre}*, ERT2^{cre} and *ZP3^{cre}*). *Wnt1^{cre}; med23^{floxed}* line will help us excise *med23* specifically out of neural crest cells, while *Tek^{cre}* (*Tie2^{cre}*) will excise *med23* out of all endothelial cells in yolk sac and embryo beginning E9.5[228]. ERT^{cre} is a mouse line which expresses *cre* in a tamoxifen-dependent manner, hence ERT2^{cre}; *med23^{floxed}* mouse line
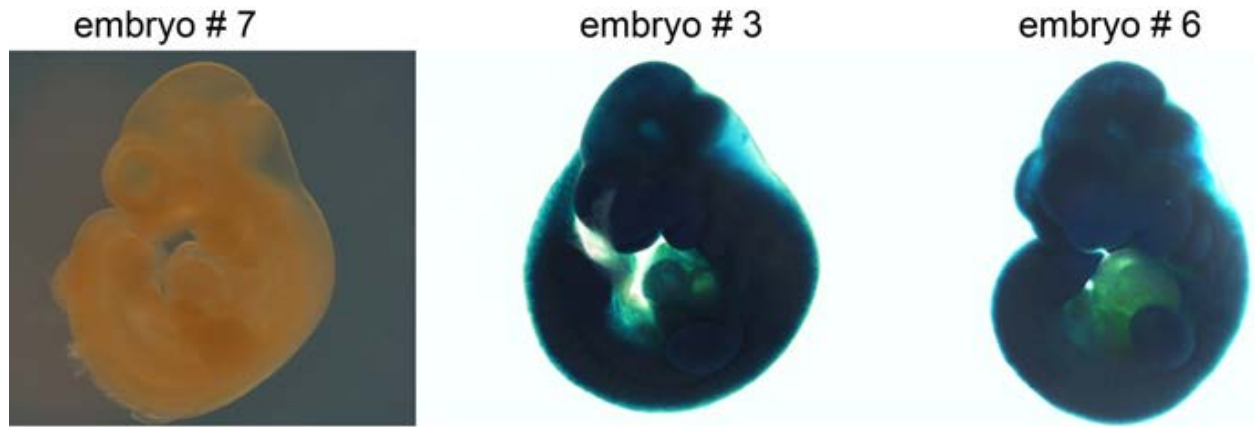
Figure 15: Tamoxifen inducible ERT2^{Cre} is able to excise loxP floxed alleles at 5mg/kg dosage

ERT2^{Cre} mouse line was crossed with R26RlacZ mouse line, where "stop" codon upstream of lacZ gene is flanked by loxP sites. Pregnant mothers were treated with oral gavage of 5mg/kg tamoxifen (+1mg/kg Progesterone) at E9.5 and embryos were analyzed for excision at E10.5. As shown above Embryo # 3 and Embryo # 5, carrying *cre* alleles show ubiquitous lacZ staining suggesting ubiquitous excision by ERT2^{cre} allele at this dose.

will be used to surpass the early embryonic lethality of $med23^{sn/sn}$ embryos and excise $med23$ out during later stages of embryonic development. Our preliminary studies have identified 5mg/kg dosage of Tamoxifen as optimum for complete excision of *floxed* allele by $ERT2^{cre}$ (Figure 15). At this dose, Tamoxifen requires 24hours post-oral gavage to completely excise the *floxed* allele. The purpose of $ZP3^{Cre}$; $med23^{floxed}$ mouse line would be to excise $med23$ in the egg cells and generate another model for $med23$ null mutation to compare to $med23^{bgeo/bgeo}$ and $med23^{sn/sn}$ embryos.

Discussion

The mega-dalton protein complex, Mediator, is well conserved from yeast to humans. When first discovered, Mediator complex was assumed to serve as a tether between the regulating transcription factors and the PIC (Polymerase Initiation Complex) assembly. However, subsequent work done by many other labs has shown that Mediator is not a passive player in transcription. Mediator subunits themselves are arranged in three separate modules, Head, Middle and Tail. These subunits, through their interaction with transcription factors, intracellular signaling components, and polymerase machinery subunits create a system which provides an added layer of transcription initiation regulation. Mediator complex comes in two forms; the activator form of Mediator is associated with subunit MED26 while the repressor

form is associated with the CDK8 module. Both MED26 and CDK8 bind to the middle module of Mediator. Recent work has also shown that MED26 subunit containing Mediator complex plays a role in regulation of transcript elongation. MED23 subunit (mutated in *snouty* and *med23^bgeo*) has been shown to be associated with both activator and repressor forms of Mediator[177].

It is intriguing to see that loss of a single subunit of transcription co-factor complex, Mediator, results in such specific embryonic defects. Our data shows that *med23* is ubiquitously expressed. Intuitively, we would expect a RNA Polymerase II co-factor to be crucial for all transcription within the embryo which would mean that losing a subunit of this co-factor complex would have severe defects within the homozygous embryo beginning as soon as the start of zygotic transcription, which happens really early in mammalian embryos. However, our data points to the contrary. How a mutation in a ubiquitously expressed subunit of a transcription co-factor complex can lead to defects specific to craniofacial, peripheral neurons and cardio-vascular tissue development, while sparing the other organ systems (central nervous system, muscle progenitor somatic tissue) is perplexing but not unprecedented. Some other Mediator subunits, like MED12, have shown roles in specific organ system development and specific signaling pathway regulation[216]. One possible explanation for such tissue specific role for MED23 could be that loss of MED23 subunit does not affect the

assembly of the rest of the Mediator[176]. We also know that MED23 is a subunit of the tail sub-

complex of Mediator, whose function is to bind and interact with specific transcription factors.

All of this suggests a system in which the different subunits of Tail sub-complex interact with

specific transcription factors and these specific interactions have roles in regulation of specific

processes only, thus making MED23 the tail subunit important for formation of frontonasal

process, peripheral nervous system and cardiovascular system within the mammalian embryo.

The *med23^{sn/sn}* mutant embryos die before we can analyze late gestational or post natal role for

MED23. We have now generated conditional *med23* mice that will help us overcome this mid-

gestational lethality.

snouty is a point mutant of *med23*; which means that *snouty* could be a null or a

hypomorphic allele of *med23*. Our work, which also supports published reports[176,218] argues

against *snouty* being a hypomorph. The phenotype of *snouty* embryos matches perfectly with

the published *med23^{-/-}* embryo phenotype, in spite of differences in the mouse strains carrying

the two mutations. We also see a loss of about 85% of MED23 protein product in *snouty*

mutant embryos presumably due to instability of the mutant MED23 protein. We have

generated a truncated allele of *med23* (*med23^{bgeo}*), which in homozygosity also leads to mid-

gestational lethality, but slightly earlier than *snouty*. Our complementation test tells us that

snouty and *med23^{bgeo}* allele are both functionally null, and the only reason for increased

severity of $med23^{bgeo/bgeo}$ embryos is the background strain differences between *snouty* and

$med23^{bgeo}$. $med23^{bgeo}$ allele still forms a truncated RNA product consisting of first 12 exons.

The expectation is that this truncated mRNA would undergo Nonsense Mediated Decay (NMD)

however, some transcript is still observed in the mutant embryos. No information is currently

available from our or other labs to suggest that the truncated mRNA product is still active,

however, the possibility cannot be completely ruled out. The evidence that *snouty* and

$med23^{bgeo}$ are indeed functionally null alleles also comes from a comparison with a published

phenotype of a *med23* null allele. Berk lab has generated a mutant allele of *med23* in which

they have knocked out exons 2 and 3 of the gene ($med23^{-/-}$)[176]. Analysis of this $med23^{-/-}$

phenotype revealed that the mutant embryos die between E9.5 to E10.5 as observed our *snouty*

embryos[218]. Berk lab $med23^{-/-}$ embryos also display a growth defect at E9.5 along with defects

in formation of the PNS and organization of blood vasculature. In spite of being on different

mouse strain backgrounds and having different sites of mutation, *snouty* embryos (FVB strain;

point mutation exon 22) and Berk lab $med23^{-/-}$ embryos (F1 strain; deletion exon 2 and 3)

display similar defects in embryogenesis. This suggests to us that even though $med23^{sn}$ allele

translates about 15% of the mutant MED23 protein; the translated product is functionally

inactive. Our $med23^{bgeo/bgeo}$ embryos phenotype is still more severe that the Berk lab $med23^{-/-}$

and $med23^{sn/sn}$ embryo phenotype, suggesting that these differences might be more due to the

underlying strain differences. Comparing the Berk lab *med23-/-* embryo phenotype with

med23$^{sn/sn}$ and *med23$^{sn/bgo}$* (complementation) embryo, it seems that *med23^{sn}* is a null allele.

Chapter 2

MED23 is required for mammalian vascular development

Summary

Proper blood vasculature development ensures proper blood flow to all organs and cell types within the body. Availability of oxygenated blood and nutrition is just as important, if not more, during embryonic development as it is in maintaining body homeostasis. As described earlier, *med23^{sn/sn}* embryos seem to display a defect in formation for proper vascular network within the yolk sac and embryo. Our analysis shows that MED23 is dispensable for vasculogenesis (the process of formation of vasculature *de novo*) but seems to be important for remodeling of the blood vessels after the primary plexus has been established. It seems that loss of MED23 disrupts endothelial cell-cell junction formation. Later roles for *med23* in differentiation of vascular smooth muscle cells has been shown in zebrafish embryos, however, *med23^{sn/sn}* mice embryos die much before those analyses could be performed. Early on, preliminary vascular smooth muscle development seems unaffected in these embryos. We have also observed an increase in *Vegfa* transcript levels following loss of *med23*, which has been reported to cause embryonic lethality in mice embryos due to cardiovascular development defects. Whether the combination of all these vascular defects in *med23^{sn/sn}* embryos is severe enough to cause the mid-gestational lethality is currently debatable. We hope that our analysis with endothelial cell-specific deletions of *med23* will give us better understanding of how MED23 regulates blood vasculature development.

Introduction

Development and functioning of all organs depends on a properly functioning vascular network. Blood vessels consist of an endothelial tube surrounded by **mural or support cells** (**vascular smooth muscle cells** for arteries and veins or **pericytes** for arterioles, venules and capillaries), which regulate vessel diameter and blood flow. **Endothelial cells** that line the entire blood vessel network are thought to derive *de novo* from mesoderm that first forms a primary vascular plexus within the extra-embryonic tissue and share their lineage with hematopoietic cells through a common progenitor, called hemangioblast, which has been described in drosophila, zebrafish, chick and mice embryos[229-234].

Blood vessel formation is typically divided into two stages, **vasculogenesis (***de novo* formation of endothelial tubes from angioblasts) and **angiogenesis** (remodeling of existing blood vessels into new ones).

Vasculogenesis

Vasculogenesis begins in the yolk sac during gastrulation and involves differentiation of mesodermal cells into angioblasts. Angioblasts are endothelial cell precursors that come together and form the primary vascular plexus within the yolk sac. This differentiation of primary endothelial cells has been shown to require signals similar to the ones required for

mesoderm specification during gastrulation, BMPs and FGFs. Loss of BMP signaling effector proteins, SMAD4 or SMAD5, result in yolk sac vasculature defects leading to embryonic lethality[235-237]. In mouse embryonic stem cells, BMP signaling has been shown to initiate a FGF2-dependent signaling program that regulates specification of angioblasts[238, 239]. The hierarchy of BMP and FGF signaling cascades is reversed in human embryonic stem cells where FGF signaling has been shown to be required for stem cell survival and proliferation, while BMP signaling is required to promote endothelial cell differentiation[240]. BMP signaling in this case is under regulation of Hedgehog (IHH) pathway whose components are expressed in the yolk sac visceral endoderm and mesoderm at the time of vasculogenesis and their loss affects endothelial cell differentiation[241].

Angiogenesis

Angiogenesis or vascular remodeling involves proliferation, migration and sprouting of endothelial cells to form new vessels from pre-existing ones and also involves differentiation of endothelial cells into arterial, venous and blood-forming hemogenic phenotypes. One of the signaling pathways crucial for angiogenesis is **Notch signaling**[242, 243]. Loss of ligands, *notch1* and *notch4* together, causes severe defects in embryonic and extra-embryonic vessel remodeling leading to embryonic lethality[244] while mice lacking a copy of Notch receptor, *dll4,*

89

exhibit smaller dorsal aorta and remodeling defects in yolk sac vessels[245]. HEY1 and HEY2 are downstream effectors of Notch signaling and have been shown to be crucial for vascular development. Loss of both *hey1* and *hey2* together leads to embryonic lethality in mice at E9.5, specifically due to extra-embryonic (yolk sac and placenta) vessel remodeling defects[246, 247].

The process of **Angiogenic sprouting** involves loss of cell-cell junctions between endothelial cells, degradation of basement membrane by proteolysis followed by migration of 'tip' endothelial cells in response to angiogenic stimuli. The other endothelial cells that trail the earliest migrating tip endothelial cells are known as 'stalk' cells and these stalk cells are responsible for proliferating and expanding the newly forming endothelial sprouts. The specification of **'tip' versus 'stalk' cells** is regulated by VEGF and Notch signaling[248]. Tip cells are characterized by their expression of Notch ligand, *dll4*[249]. VEGF signaling regulates this expression of *dll4* in tip cells[250], which then reduces the expression of *flk1* in adjacent endothelial cells through activation of Notch signaling in those cells. Reduction in *flk1* expression reduces the ability of the adjacent cells to respond to VEGF stimuli thus preventing them from acquiring tip cell fate and conferring them stalk cell identity[251-253]. Loss of *dll4* and inhibition of Notch signaling results in increased angiogenic sprouting and hyper – proliferative endothelial cells[251, 254]. To make matters more complex, the specification of tip-

versus-stalk identity within the sprouting endothelial cells itself has been shown to be a dynamic process. Through live imaging, it was shown that the sprouting endothelial cells are continuously in struggle to become the tip cells and at each point of time whichever cell expresses the *dll4* ligand acquires the tip cell identity for that time period[255].

Apart from angiogenic sprouting, VEGF and Notch signaling cross-talk is also crucial for **arterial and venous differentiation of endothelial cells.** Mice lacking a copy of Notch receptor, *dll4*, exhibit smaller dorsal aorta and remodeling defects in yolk sac vessels and have complete loss of arterial identity[256,257]. On the other hand, overexpression of *dll4* causes enhanced arterialization also leading to vascular defects[245]. Dorsal aorta development was also affected in *hey1; hey2* double mutant embryos[246][247]. Loss of *Rbpj*, encoding a transcription factor downstream of Notch signaling, also results in defects in arterial endothelial cell development[257], suggesting that Notch signaling pathway regulates arterial differentiation during endothelial cell development via RBPJ/HEY1-HEY2. VEGF signaling has also been suggested to have a role in arterio-venous differentiation of endothelial cells. *In vitro* VEGF induces expression of arterial markers in endothelial cells while morpholino knockdown of *vegf* in zebrafish embryos leads to sustenance of venous cell markers at the cost of arterial makers[258,259]. Specification of venous cell identity, on the other hand, is under the control of an orphan nuclear receptor, COUPTFII, which is expressed in endothelial cells specific to veins

and lymphatic vasculature beginning E8.5[260]. Endothelial cell specific deletion of *CouptfII* (using *Tie2[re]*) leads to embryonic lethality and loss of venous identity. Overexpression of *CouptfII* in endothelial cells on the other hand also leads to aberrant vessel development due to fusion of artery-vein structures, a phenotype similar to *notch*- deficient embryos[261, 262]. COUPTFII, thus seems to be required for suppressing Notch activity (which is regulates arterial endothelial cell differentiation) and thereby regulate venous cell identity. This hypothesis is supported by the fact that double knock-out for *CouptfII* and *Rbpj* (downstream target for Notch signaling) partially restores venous identity for endothelial cells[263].

VEGF signaling in Vasculature development

VEGF signaling has been shown to be important in regulating various aspects of endothelial cell function, including survival, proliferation and vessel permeability[264, 265]. VEGFA is the most extensively studied VEGF family member and is expressed its expression in the extra-embryonic visceral endoderm at E7.5 in mice correlates perfectly with blood island formation in yolk sac[266]. Loss of one or both copies of *Vegfa* leads to cardiovascular defects and embryonic lethality in mice[267, 268]. Over-expression of *Vegfa* also impairs cardiac development and results in mid-gestational embryonic lethality suggesting a dose-dependent crucial role for VEGF in heart and vascular development[121]. VEGFA signals mainly through two receptors,

VEGFR1 and VEGFR2 (or FLT1 and FLK1 respectively) but it has also been shown to interact with Neuropilin receptors 1 and 2 (NPN1 and NPN2). VEGFR1 (FLT1) has a higher affinity for VEGFA than VEGFR2 (FLK1), however VEGFR1 lacks the tyrosine kinase ability to signal downstream post- VEGFA binding event and hence has been considered to be a negative regulator (decoy receptor) of VEGFA[269]. In support of this, loss of *flt1* results in increased number of hemangioblasts leading to an overgrowth of endothelial cells and vascular channels and eventually causes mid-gestational embryonic lethality in mice[122]. Role of VEGFR2 or FLK-1 in endothelial cell generation and blood vessel development is very well established. Loss of *flk1* does not affect formation of angioblasts but still results in complete lack of yolk sac blood islands and embryonic and extra-embryonic blood vessels leading to embryonic lethality[119]. Analysis of chimeric embryos generated from wild-type and *flk1-null* cells has shown that *flk1* is cell autonomously required for endothelial cell differentiation and suggests that the main role for FLK1 is to ensure that the mesodermal precursors of blood islands end up at the correct locations within the yolk sac[270]. This suggests that VEGFA signaling probably regulates survival and proliferation of endothelial cells and their precursors but not necessarily the differentiation of angioblasts from mesoderm.

Angiopoeitin signaling in vascular development

Angiopoetin/Tie signaling pathway is unique such that it is almost exclusively endothelial cell specific and is crucial not for *de novo* endothelial cell differentiation (or vasculaogenesis) but for blood vessel remodeling and maturation (angiogenesis) during later stages of embryonic and postnatal development. Angiopoetins (ANG1, ANG2 and ANG4) are ligands that bind to tyrosine kinase receptor Tie2 (also known as Tek) and regulate endothelial cell survival and cell-cell junction stabilization. Deletion of *tie2* in mice embryos leads to mid-gestational embryonic lethality due to impaired cardiac development and reduced numbers of endothelial cells[271], while loss of *angiopoeitin-1* (ANG1) leads to vascular remodeling defects and a disruption of endothelial cell – extracellular matrix interaction[272, 273]. Tie1, a homolog of Tie2, on the other hand, does not directly bind to any Angiopoeitin ligands, but its loss still affects blood vasculature development, specifically angiogenic capillary growth and endothelial cell survival, leading to hemorrhage and death of mutant embryos[274,275].

Formation and Regulation of Endothelial cell-cell junctions

Endothelial cell-cell junctions are critical to regulate vascular permeability. During development, regulation of junction formation is crucial for forming endothelial cell tubes (blood vessels) and regulated disruption of the junctions is required during angiogenic sprouting. Homotypic junctions between endothelial cells come in multiple flavors, two of the

well-studied ones are **tight and adherens junctions (Figure 16)**. Adhesion via adherens junctions is mediated via VE-Cadherin, an endothelial specific member of the Cadherin family. Inside the cells, VE-Cadherin is linked to a large number of intracellular partners, one of them being the actin cytoskeleton, which can control the dynamic opening and closing of the cell-cell junctions. Adhesion in tight junctions is mediated by the Claudin family of membrane proteins. Multiple claudin family members are expressed in tight junctions of all cell types, such as occluding, nectin and others; Claudin-5 amongst these is specific to endothelial cells[276]. Disrupting **VE-Cadherin** in mouse embryonic stem cells does not affect differentiation of endothelial cells but affects their organization into a vessel like pattern[277]. Loss of VE-Cadherin in embryos affects formation of capillary plexus within mouse allantois and leads to mid-gestational embryonic lethality following vascular defects[278]. VE-Cadherin association with another junctional protein β-catenin seems to be crucial for its vascular remodeling function[279]. Recently, it has been shown that VE-Cadherin is not required for formation of nascent blood vessels but is required for maintaining their vessel-like assembly by maintaining the endothelial cell-cell junctions[280]. Another cadherin, **N-cadherin**, has also been shown to be expressed in endothelial cells and its ablation within the endothelial cell lineage leads to loss of *ve-cadherin* expression, vascular development defects and mid-gestational embryonic lethality[281]. However, *in vitro,* embryonic stem cells deficient for N-cadherin were able to form
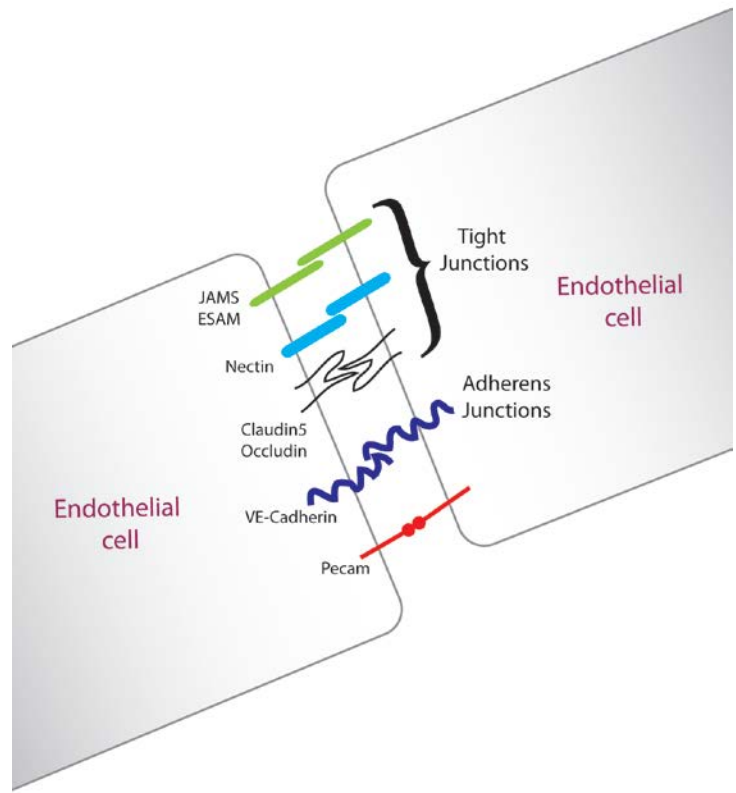
Figure 16: Endothelial cell–cell junctions

vessel like structures but showed defects in endothelial cell-support cell junctions (pericytes) suggesting a role for N-cadherin in maintaining heterotypic endothelial cell connections[282]. Loss of **Claudin-5**, which is an endothelial cell specific claudin, surprisingly does not disrupt overall morphology of blood vasculature and tight junction structure; however, it does affect the formation of blood brain barrier which leads to perinatal embryonic lethality in mutants[283, 284]. Apart from adherens and tight junctions, endothelial cells also form homo- and heterotypic junctions via proteins like **PECAM-1** (Platelet and Endothelial Cell Adhesion Molecule-1) and **VCAM-1** (Vascular Cell Adhesion Molecule-1), which are expressed preferentially in endothelial cells and hence are usually used as markers of vasculature but are not indispensable for blood vessel development[285, 286].

Loss of MED23 results in mid-gestational embryonic lethality. Our initial analysis showed that *med23^sn/sn* embryos display disruption in embryonic and extra-embryonic blood vascular network at E9.5. Here we are trying to characterize this blood vessel formation defect in more details. We see defects in expression of endothelial cell-cell junction markers in embryonic vasculature at E9.5 suggesting a disruption in vascular remodeling due to loss of *med23*. Whether these defects are sufficient to cause embryonic lethality has currently not been tested, but is in process with conditional deletion of *med23* from endothelial cells.

Results

I. *flk1* expression is similar in wild-type and *med23^{sn/sn}* littermate embryos

PECAM-1 immuno-staining depicted the presence of disorganized blood vessel network in *med23^{sn/sn}* embryos and yolk sac at E9.5. A microarray done on E9.5 wild-type and *med23^{sn/sn}* embryos showed a 1.6fold increase in *Vegfa* transcript levels. Increasing *Vegfa* levels by 2-3 folds in mice embryos result in disruption of cardiovascular development and mid-gestational lethality[121]. It has been hypothesized that in some mutants, lack of proper vasculature results in hypoxia-like conditions, which trigger an over-expression of *Vegfa* to compensate. Thus, the increase in *Vegfa* levels in *med23^{sn/sn}* embryos could be the cause for or result of abnormal vascular development. To determine if this mis-regulation of *Vegfa* transcription is real, we analyzed the expression pattern of VEGFA receptor, FLK1. *flk1* is known to be expressed in all endothelial cells from the time that they first differentiate from mesoderm and by E9.5; it should be expressed throughout the vascular network. Wild-type embryos in *med23^{sn/sn}* embryos also, *flk1+* cells are detected throughout the embryo (**Figure 17**). This is not surprising as we do see a vascular network being formed in *med23^{sn/sn}* embryos by PECAM-1 immuno-staining and not a complete lack of vasculature, which would be expected had we lost *flk1* expression.
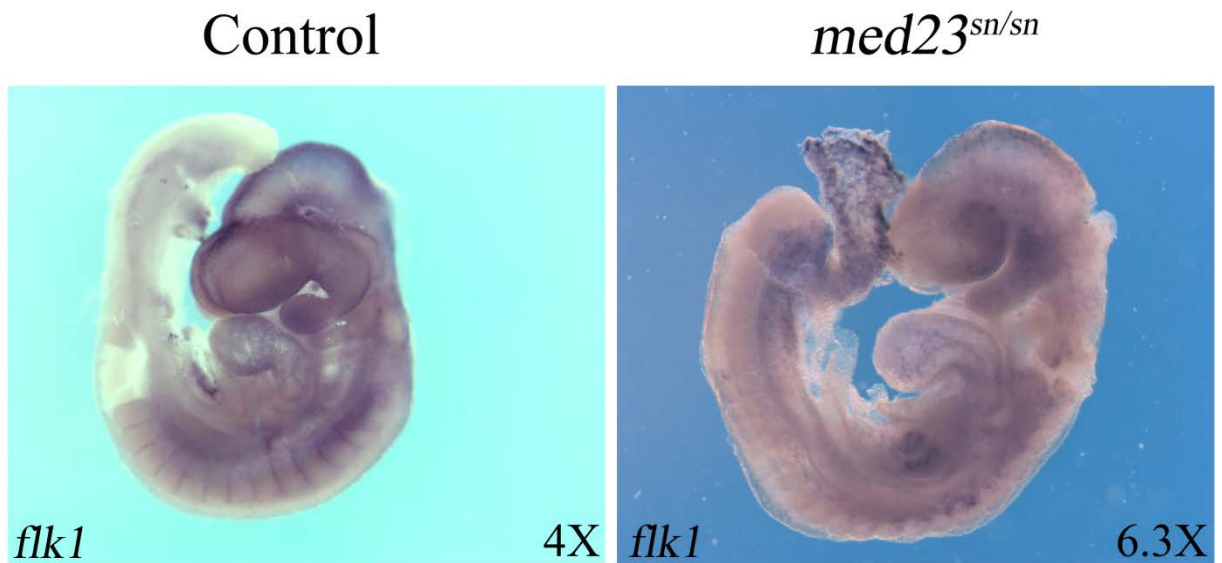
Figure 17: *flk1* expression in wildtype and *med23^{sn/sn}* embryos.

flk1 expression in the *med23^{sn/sn}* embryo at late day E9.5 marks the blood vessel network. f*lk1+* cells are observed in the allantois, heart, intersomitic vessels and head and epibranchial regions of the mutant embryo similar to control littermate.

II.   Formation of endothelial cell–cell junctions is affected in *med23*^{sn/sn} embryos

In our effort to characterize the defects in blood vessel development in *med23*$^{sn/sn}$ embryos we decided to analyze the expression of endothelial cell–cell junction markers in the mutant embryos. The two main Homotypic junctions formed between endothelial cells are adherens junctions (marked by VE–Cadherin) and tight junctions (marked by Claudin–5). Both VE–Cadherin and Claudin–5 are junction proteins exclusive to endothelial cells only.

Adherens Junction

When analyzed at E9.0, a time point when *med23*$^{sn/sn}$ phenotype is first visible, expression of *ve-cadherin* seems to be normal in wild–type and mutant embryos alike (**Figure 18A, B**). However, at E9.5, two out of three embryos analyzed displayed a mild disruption in *ve-cadherin* expression pattern throughout the embryo (representative image, **Figure 18 C, D**). Instead of the beautiful network of blood vessels observed in control embryos, mutant embryos display dispersed *ve-cadherin+* cells lacking a tree–like vascular network. One of the three mutant embryos analyzed had a severe defect, where the *ve-cadherin+* endothelial cells were dispersed and fewer in number, especially in the head and epibranchial region (**Figure 18 E, F**). Specifically the network around the otic vescicle in the epibranchial region is severely disrupted in both mutant embryos (**Figure 18D, F**). Since, *ve- cadherin+* cells are present

Control   *med23*^{sn/sn}

E9.0
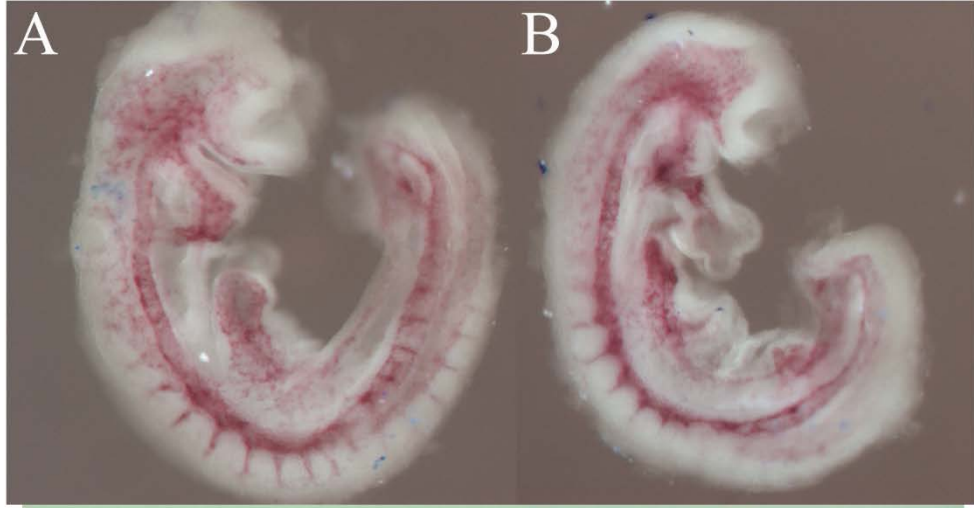
E9.5
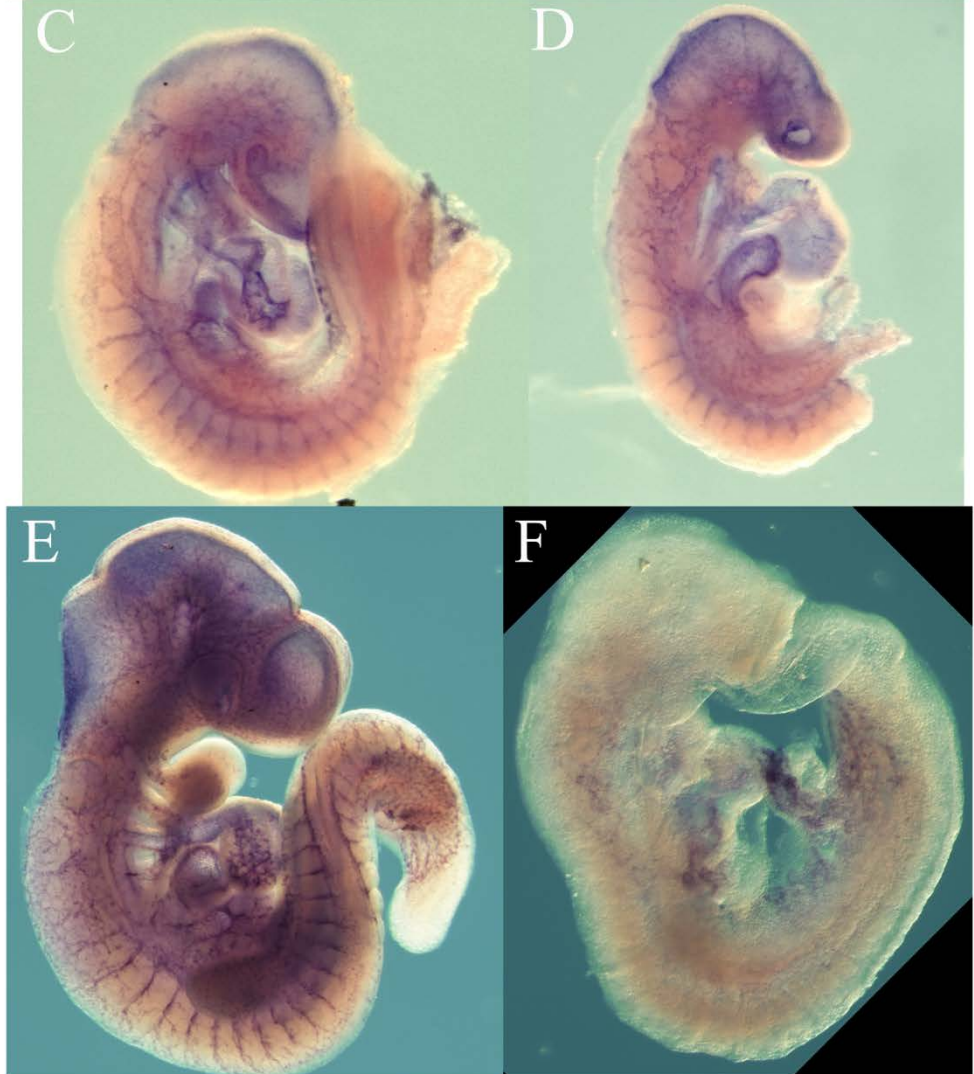
Figure 18: Adherens junction marker; *ve-cadherin* expression is reduced in *med23$^{sn/sn}$* embryos at E9.5 but not at E9.0

A, B *ve-cadherin* ISH at E9.0 shows similar expression between wild-type and mutant embryos.

C, D At E9.5, mild phenotype *ve-cadherin* ISH shows abnormal vascular network in *med23$^{sn/sn}$* embryos reminding of Pecam-1 immunostaining phenotype.

E, F In a severe phenotype, very few *ve-cadherin+* cells are observed in the *med23$^{sn/sn}$* embryos and vascular pattern of expression is lacking.

in that region half a day earlier (Figure 17B), and PECAM-1 immuno-staining suggests the presence of a vascular network at E9.5, it seems that the expression of adherens junction marker is specifically affected at that stage and that MED23 might be required for its maintenance.

Tight Junctions

Since we saw a disruption of adherens junction marker expression in E9.5 $med23^{sn/sn}$ embryos, we also decided to analyze the expression of tight junction marker in the mutant embryos. *claudin-5* is a tight junction marker exclusive to endothelial cells. *claudin-5* is expressed in all endothelial cells however its role is specific to blood brain barrier formation. RNA *in situ* hybridization of wild-type and $med23^{sn/sn}$ embryos at E9.5, display a defect in *claudin5* expression in mutant embryos (**Figure 19 A, B**). Similar to *ve-cadherin*, at E9.5, $med23^{sn/sn}$ embryos display a disrupted *claudin-5* expression pattern in the epibranchial region (**Figure 19C, D**). Given that PECAM-1 immuno-staining shows the presence of a vascular network in the epibranchial region, it seems that tight junction marker *claudin-5* expression is specifically disrupted following loss of MED23.

Control   *med23*$^{sn/sn}$

claudin5

A B

C D

E F
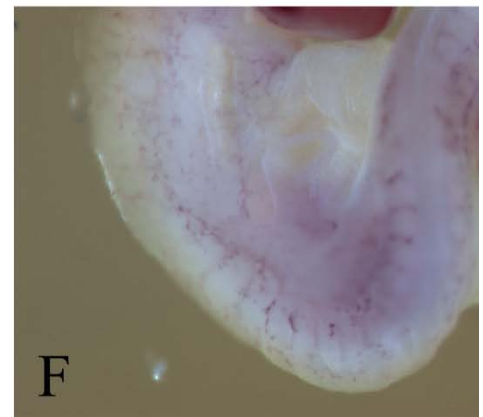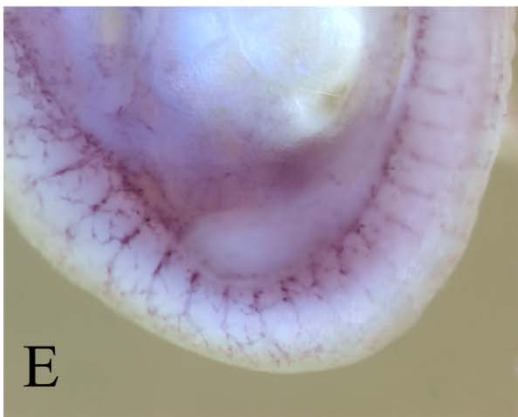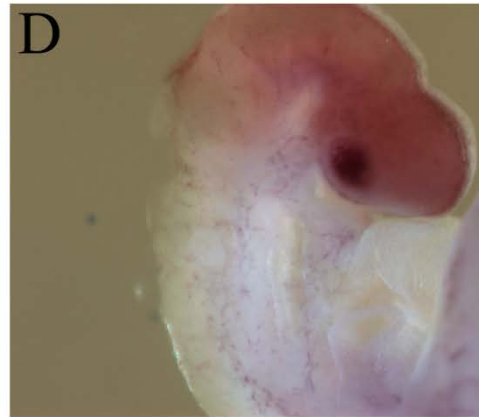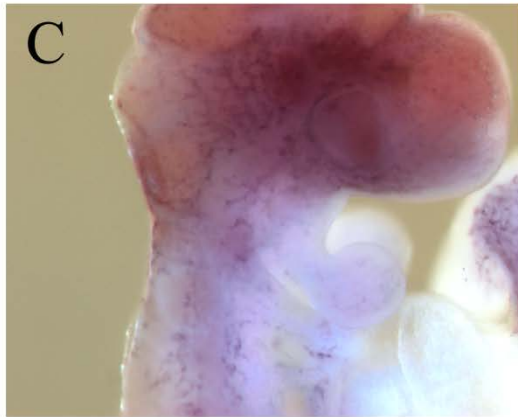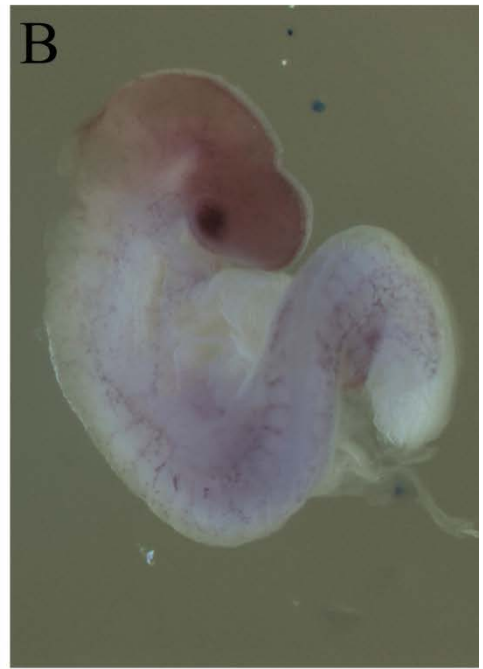
Figure 19: Expression of tight junction marker, *claudin5* is reduced in *med23*^{sn/sn} embryos at

E9.5

Analysis of *claudin5* expression in control and *med23*^{sn/sn} embryos at E9.5 shows reduced

claudin5+ cells in the epibranchial and head region of mutant embryos (A – D) and in the

intersomitic vessels.

III.   Early development of vascular smooth muscle cells is unaffected in *med23*^{sn/sn} embryos

The vascular defect observed in *med23*^{sn/sn} embryos could be endothelial cell-autonomous or non-autonomous. The loss of cell-cell junction markers in mutant embryos could also result from lack of support from mural cells. By E9.5, vascular smooth muscle cells have already begun to be recruited in the developing cardiac tissue and the developing aorta to surround and support the endothelial cell tubes. We utilized an antibody against SM22α (Smooth muscle 22 alpha), one of the earliest markers of vascular smooth muscle cells to analyze early stages of smooth muscle differentiation in mutant embryos. At E9.0, wild-type embryos express SM22α throughout out the developing heart as well as in yolk sac. SM22α expression in E9.0 *med23*^{sn/sn} embryos is also detected in the heart and yolk sac at levels similar to its wild-type littermate **(Figure 20A, B)** suggesting that early differentiation of vascular smooth muscle cells does not require MED23.

Discussion

Loss of MED23 results in disruption of vascular network and also causes mid-gestational embryonic lethality. We sought to figure out whether the embryonic and extra-embryonic vascular defects of the mutant embryos are related to its lethality by analyzing the various aspects of vascular development in these embryos. Our analysis suggests that MED23 is
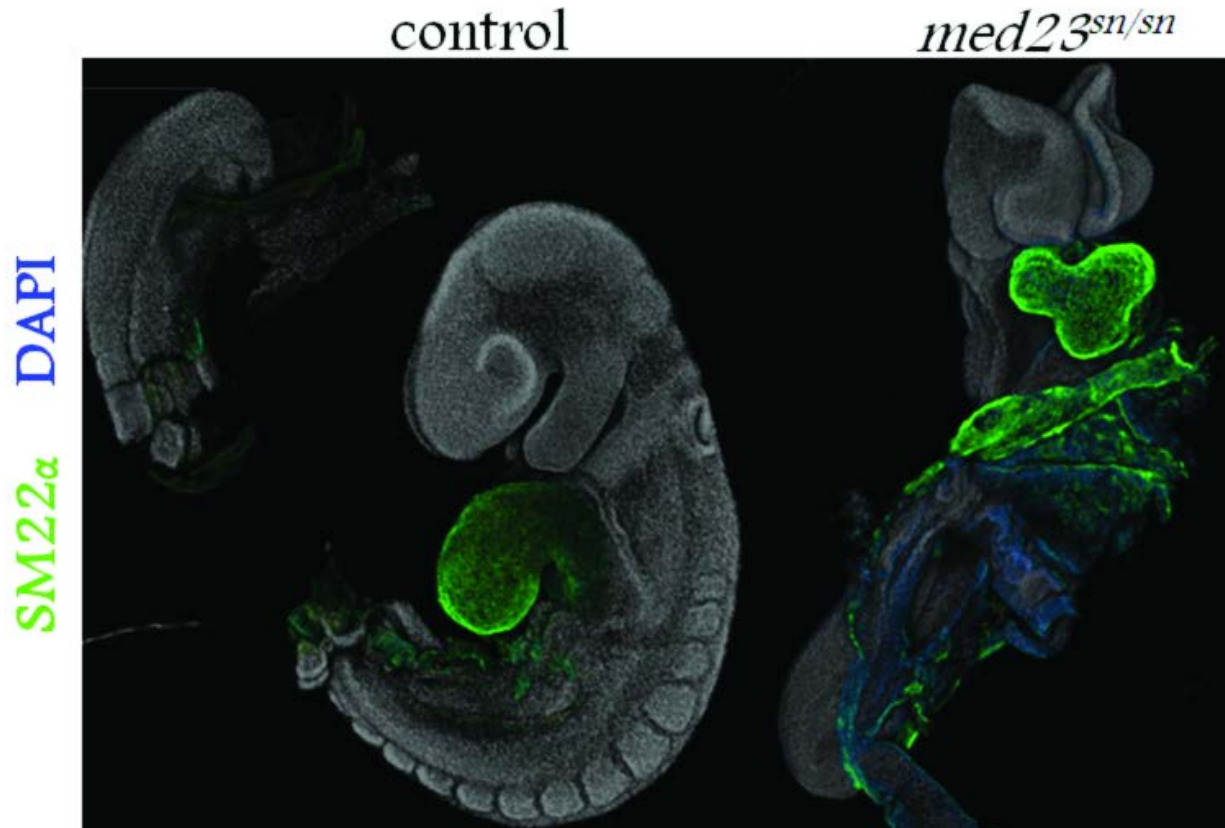
Figure 20: Early formation of vascular smooth muscle cells is not affected in control and

med23^sn/sn littermate embryos at E9.0.

Analysis of Control and *med23^sn/sn* embryos were immuno-stained for SM22α, a marker of

vascular smooth muscles, showed similar staining in the developing heart of mutant and wild-

type embryos.

required for vascular remodeling and maintenance of endothelial cell-cell junction markers, *ve-cadherin* and *claudin-5*.

In spite of an increase in transcript levels of *Vegfa* in *med23*[sn/sn] embryos, we do not see over-expression of VEGFA receptor *flk1* in mutant endothelial cells, which matches with our PECAM-1 immuno-staining, where we do not see hyper-vascularization of the embryo. *In vitro,* the ability of VEGF to increase microvasculature permeability and cause vasodilation has been well known[287, 288, 289]. VEGF signaling through its receptor VEGFR2 (or FLK1) induces phosphorylation of cell junction proteins (like ZO-1, Occludin and VE-Cadherin) leading to their destabilization resulting in increase in permeability[290,291,292,293,294]. Ultrastructural analysis has shown that VEGF signaling activation leads to a very rapid disruption of endothelial cell-cell junctions, including loss of both tight and adherens junctions[295]. What has not been investigated is if VEGF signaling has any role in regulating the formation of these endothelial cell-cell junctions via regulation of junctional protein transcription. Interestingly, in our system we see a reduction in expression of endothelial cell-cell adherens and tight junction markers (loss of *ve-cadherin* and *claudin-5* transcripts) as well as an increase in *Vegfa* transcript levels. It would be interesting to look at the protein distribution of VE-Cadherin and Claudin-5 within the endothelial cells and see if their protein levels are reduced by loss of MED23. That would indicate that MED23 is required (directly or indirectly) to

108

suppress *Vegfa* transcript levels, mis-regulation of which affects endothelial cell junctional protein stability in *med23^{sn/sn}* embryos. On the other hand, MED23 might just be directly required for maintenance of *ve-cadherin* and/or *claudin-5* expression. VE-Cadherin itself has been recently shown to regulate the formation of tight junctions[296] suggesting that loss of *claudin-5* in E9.5 *med23^{sn/sn}* embryos might also be an indirect effect of loss of MED23-mediated VE-Cadherin expression. In my opinion, the loss of *ve-cadherin* and *claudin-5* in *med23^{sn/sn}* embryos is less of a direct effect of MED23 loss on their expression and more of an effect in disruption of endothelial cell-cell junction maintenance. Specific loss of *claudin-5* expression within the head region of *med23^{sn/sn}* embryos suggests that if allowed to develop till birth, loss of MED23 may affect the formation of blood brain barrier in perinatal and postnatal animals, similar to what is observed after loss of *claudin-5*[284].

Recently, a study in zebrafish suggested that MED23 is required for regulation of adipogenesis and smooth muscle cell formation. Morpholinos against *med23* resulted in zebrafish embryos with increased vascular smooth muscle cells along with an increased vasculature at the cost of adipocytes; specifically the expressions of vascular smooth muscle markers, like SM22α and α-SMA (smooth muscle actin) were both significantly increased in the *med23* morpholino treated embryos in comparison to control embryos[297]. Our analysis with one of the vascular smooth muscle markers, SM22α, shows a completely opposite result.

Loss of MED23 in mice embryos does not affect early smooth muscle cell differentiation. The major difference between the two studies is the time point of analysis. The increase in vascular smooth muscle cells, post-*med23* morpholino treatment, was observed in fish at 48 hours-post-fertilization (hpf) is way beyond the time point E10.5 in mice embryos when *med23*$^{sn/sn}$ embryos die. Thus, it might be that if somehow allowed to live longer, loss of MED23 might result in over-differentiation of vascular smooth muscle cells, a defect currently not observed in mutant embryos.

Currently, we are focused on understanding whether the specification of arterial versus venous fate of endothelial cells is in any way affected by loss of MED23. Formation of endothelial cell-cell junctions is crucial to stabilize the proliferation of endothelial cells and thus stabilize the newly formed or sprouted endothelial tubes[298]. It would be interesting to see if such endothelial cell specific hyper-proliferation would be observed in *med23*$^{sn/sn}$ embryos due to loss of cell-cell junction markers. We are also working on understanding the endothelial cell specific role for MED23 in regulation of vascular development using *Tie2*^{cre}*; med23*^{floxed} mice, which will excise out *med23* specifically from endothelial cells beginning E9.5. Together all of this information should provide us a better understanding into how MED23 regulates vascular remodeling, endothelial cell-cell junction marker expression and thus endothelial cell-cell junction formation.

Chapter 3

MED23 is required for Peripheral Nervous system formation

Parts of "Introduction" in this chapter (Trunk Sensory Nervous system and Autonomous Nervous system) have

been adapted from a book chapter, *Bhatt S., Diaz R., Trainor P.* (2013) Signals and Switches in Mammalian Neural

crest differentiation, published by Cold Spring Harbor Perspectives in Biology

Summary

In our initial analysis, we observed a defect in peripheral nervous system formation in *med23^{sn/sn}* embryos, specifically the cranial sensory nervous system. In amniotes, the cranial sensory nervous system is comprised of the three paired sense organs, eyes, nose and ear and four neurogenic cranial ganglia, trigeminal, petrosal, nodose and glossopharyngeal as well as all the cranial nerves (sensory and/or motor) associated with each of the paired sensory organs and cranial ganglia. DAPI immuno-staining showed the defects in the morphology of the paired sensory organs while TuJ1 (neuron specific β-tubulin) marker immuno-staining showed defects in formation of the cranial nerves and associated cranial ganglia. RNA expression analysis determined that the major defect underlying the abnormal development of neurogenic cranial ganglia in *med23^{sn/sn}* embryos is in formation of cranial placodes. Cranial placodes form from the ectoderm at gastrulation as a single pre-placodal region (PPR) that then goes to segregate and differentiate into multiple placodes, which contribute to the various sensory organs and ganglia. Here, we show that in *med23^{sn/sn}* embryos, cranial placode formation is affected at multiple steps. Although very early specification of PPR is relatively unaffected, *med23* seems to be required for proper organization and neurogenic differentiation of the neurogenic cranial placodes.

Introduction

In multicellular organisms, the central nervous system (CNS) needs a way to sense and react to external stimuli. This function is carried out by the peripheral nervous system (PNS) of the body. PNS comprises of sensory and motor neurons that relay information back and forth from the viscera, smooth muscles, skin and the exocrine glands of the body to the brain and spinal cord of the central nervous system. In keeping with this function, PNS consists of nerves and ganglia located outside the brain and spinal cord which are grouped into two subsets, 1) Sensory nervous system: cranial and trunk sensory nervous system and 2) Autonomous nervous system (ANS): sympathetic, parasympathetic and enteric nervous system. The sensory nervous system, as the name suggests, is responsible for sensing external stimuli and consists of afferent fibers that transmit information from the "somatic" (skin, skeletal muscles, tendons and joints) and "visceral" (smooth and cardiac muscle and glands) structures to the CNS; while ANS consists of efferent motor neuron fibers which are responsible for transmitting information from CNS to the somatic and visceral structures.

Sensory Nervous system

Based on the location within the body, the sensory nervous system is divided into cranial and trunk sensory nervous systems. Cranial and trunk sensory neurons are pseudo-

unipolar; their cell bodies lie within the peripheral ganglia, cranial ganglia (for cranial sensory nervous system) and spinal or dorsal root ganglia (for trunk sensory nervous system) and their axons are split into two branches; one extending to the periphery while the other extending towards the CNS (brain for cranial and spinal cord for trunk sensory nervous system).

Cranial Sensory Nervous System

Cranial sensory neurons work in conjunction with cranial motor component to regulate the functions of paired sensory organs, eyes, ears and nose as well as movement of the craniofacial muscles including the muscles involved in facial expression and mastication (through the cranial ganglia) **(Table 3)**. The sensory nerves are derived from two separate ectodermal-origin cell populations, neural crest and cranial placodes and associate with two anatomically distinct set of cranial sensory ganglia which have differential contributions from these two cell types **(Figure 21)**. The proximal subset of cranial ganglia (dorso-medial region of trigeminal ganglion, a small portion of proximal geniculate ganglion, acoustic ganglion, superior ganglion and jugular ganglion associated with cranial nerves V (trigeminal), VII (facial), VIII (vestibulo-cochlear) and proximal portions of IX (glossopharyngeal) and X (vagal) respectively) is anatomically located closer to the central nervous system, and sensory neurons associated with these ganglia are derived from cranial neural crest, and trigeminal and otic

114

Table 3: Innervation of cranial nerves (human)

Cranial Nerve Name	Number	Site of innervation
Olfactory	I	Nasal cavity
Optic	II	Retina
Oculomotor	III	Eye muscles
Trochlear	IV	Eye muscles
Trigeminal	V	Skin of the face and muscles of mastication
Abducens	VI	Muscle of the eye orbit
Facial	VII	Muscles of facial expression, anterior $2/3^{rd}$ of the tongue
Vestibulocochlear	VIII	Inner ear
Glossopharyngeal	IX	Posterior $1/3^{rd}$ of the tongue
Vagal	X	Laryngeal and pharyngeal muscles, soft palate
Spinal accessory	XI	Sensory component joins Vagal nerve, motor component innervates muscles of the neck
Hypoglossal	XII	Muscles of the tongue
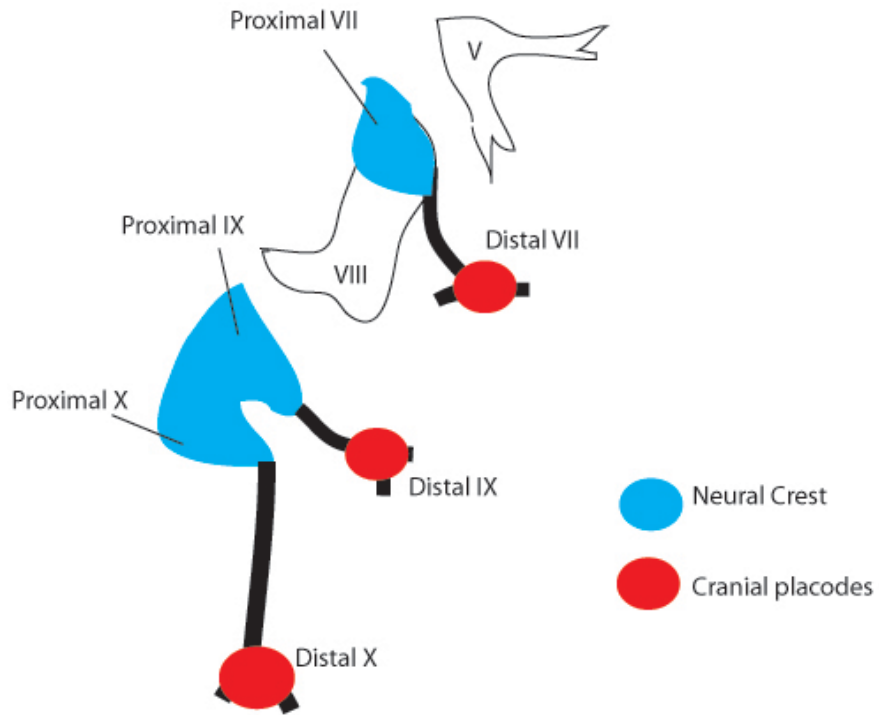
Figure 21: Contribution of neural crest and cranial placodes to the cranial sensory ganglia in vertebrates

placodes. The distal subset of cranial ganglia (distal portions of geniculate (facial or cranial nerve VII), petrosal (glossopharyngeal or cranial nerve IX) and nodose (vagal or cranial nerve X) ganglia) is located further away from the central nervous system and neurons associated with these ganglia are derived from epibranchial placodes.

Differentiation of cranial placodes into cranial sensory neurons

The early development of cranial placodes and neural crest into cranial sensory neurons is independent of each other[299,300] however once specified the placodal derived sensory neurons require input from neural crest cells to form proper axonal projections[96, 300]. Cranial placodes are first specified as a single pre-placodal region (PPR). This PPR region then segregates into separate cranial placodes (Introduction – pages 23–32). Four sets of genes are expressed in each placode during initial steps of differentiation in the following temporal manner, 1) *six* and *eya*, 2)*sox*, 3) *pax* and 4) bHLH (proneural determination and differentiation basic helix–loop–helix) genes **(Figure 22)**. The temporal expression of genes in the developing cranial placodes has been reviewed in great details elsewhere[301]. Knock–outs of some of these genes in mice have suggested an important role in mammalian placode development; however mechanisms by which they regulate mammalian placodal development are understudied.
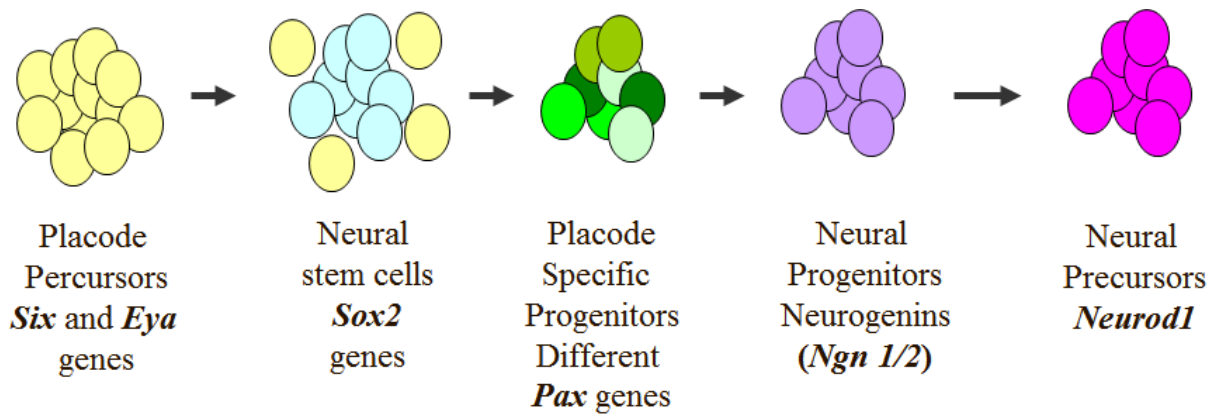
Figure 22: Hierarchy of genes expressed in placodal development from PPR to neuronal differentiation.

six and *eya* family genes are expressed during initial placodal development and continue to be expressed throughout the various stages of cranial placode differentiation, albeit with a slight variation between various placodes (reviewed in[73]). Their continued expression suggests a role in maintenance placodal identity and initiation of placodal differentiation. It has been suggested that *six1* is involved in specification of PPR and maintenance of "stem-like" properties in placodal cells[78] as timing of placodal differentiation correlates with down-regulation of *six1* expression[302, 303] and its overexpression post-PPR establishment, diminishes expression of later placodal genes (reviewed by S*ally Moody, Principles of Developmental Genetics, Book chapter, 2007*). On the other hand, expression of other *six* family genes (*six2, six4)* and *eya* family genes (*eya1* and *eya2*) persists into later stages of placodal development suggesting later roles in differentiation[304, 305]. Work in non-mammalian model organisms has shown a cranial placode specific expression of *sox* family genes, *sox2* and *sox3*. Both *sox2* and *sox3* are known to be expressed in the developing neural plate and seem to have a similar role in maintenance of neural progenitor pool in cranial placodes as well[306]. However, the fact that *sox2* and *sox3* are also expressed in non-neurogenic lens placode and not expressed in neurogenic trigeminal placodes suggests that they may have non-neural roles as well[306, 307]. In mice embryos, expression of *sox2* is specific to the cranial placodes giving rise to the paired sensory organs (eye and ear) while *sox3* expression is not placode specific.

Segregation of PPR into specific cranial placodes is visualized by expression of placodal specifier genes of *pax* family (reviewed in[73]). *pax3* is expressed in the ophthalmic branch of trigeminal placode, *pax6* is expressed in olfactory and lens placodes and *pax2/5/8* are expressed in the otic placode. Pre-placodal region (PPR) first segregates into anterior and posterior pre-placodal area (APA and PPA) and PPA then goes to form the otic and epibranchial placodes. *pax2* has been shown to be expressed in the PPA and required for its maintenance[308]. Work in chick embryos suggests a role for *pax2* in regulation of epibranchial neuron identity[91], while in zebrafish *pax2* acts synergistically and probably redundantly with *pax8* in specifying the otic placode[309, 310]. Mice knock-outs of *pax2* and *pax8* individually, both show defects in formation of the inner ear and associated ganglion[95, 311, 312]. Loss of *pax3* in mouse embryos leads to hypoplasty of all cranial ganglia including the trigeminal ganglion[313, 94]. However, given the important role played by *pax3* in neural crest development and the contribution of neural crest to sensory neurons and glia within the cranial ganglia, it is difficult to conclusively pinpoint these defects as cranial placode formation defects and claim a role for *pax3* in cranial placode development. These restricted patterns of *pax* genes right before the cranial placodes begin to separate[92, 306]and their observed requirement in maintenance of the various cranial placodes in which they are expressed, suggest existence of a **"pax code"** that provides the identity to each cranial placode.

Once specified, each cranial placode (except for the lens placode) differentiates into cranial sensory neurons. Mouse knock-out studies have shown that neuronal differentiation of proximal subset of cranial ganglia, which derive from both neural crest and some cranial placodes require expression of a basic helix-loop-helix (bHLH) transcription factor, *neurogenin1*[314], while neuronal differentiation of distal subset of cranial ganglia that derive exclusively from cranial placodes, require expression of another bHLH transcription factor, *neurogenin2*[315]. The importance of bHLH transcription factors in neurogenesis was first identified in *Drosophila* (reviewed in[316]). In *Xenopus, neurogenin (ngn1)* expression is observed in prospective ganglion cells and is lost as the coalescing ganglion cells differentiate into neurons and glia. Both *ngn1* and *ngn2* directly regulate the expression of another bHLH transcription factor, **neuroD** whose expression correlates well with the acquisition of neuronal and glial characteristics. Loss of *neuroD1* in mice affects formation of otic placode derived inner ear hair cells and in humans has been associated with Branchio-oto-renal disorder.

Trunk Sensory Nervous system

Trunk sensory nervous system comprises of a metameric string of Dorsal Root Ganglia (DRG) which consist of variety of sensory neuron cell bodies, axons of which innervate various parts of the body and enable us to sense touch, temperature, and pain and limb movements.

Each of these functions is attributed to a specific type of sensory neuron, all of which are derived from trunk NC cells. Each functional type of sensory neuron is characterized by its own unique set of ion channels and forms through interactions of unique sets of transcription factors. The different types of neurons within each DRG rely on separate neurotrophic factors for their survival and growth and hence express distinct neurotrophic receptors. The small diameter pain and thermal sensory neurons require NGF (Neurotrophic Growth factor) and express NGF receptor *TrkA* and Ret[317, 318, 319] while the subpopulation of touch neurons require BDNF (Brain derived neurotrophic factor) and express BDNF receptor *TrkB*[320, 321, 322]. The larger diameter movement sensing neurons innervate skeletal muscles and depend on Neurotrophin-3 (NT-3) for survival and express NT-3 receptor *TrkC*[319, 323].

Differentiation of neural crest into trunk sensory neurons

Similar to cranial sensory neurons, trunk sensory neurogenesis also requires expression of bHLH transcription factors, *neurogenin1* (*ngn1*) and *neurogenin2* (*ngn2*). Trunk sensory neurogenesis occurs in two waves. The first wave of neurogenesis, controlled by *ngn2*, occurs in the migrating neural crest cells and forms the large diameter propioceptive and mechanoreceptive neurons while the second wave, controlled by *ngn1*, occurs after DRG coalescence and produces both small nociceptive and large mechanoreceptor and

proprioceptor neurons. Similar to cranial neurons, *neurogenin* expression in trunk controls

expression of another family of bHLH transcription factors (*neuroD*) which serve as neuronal

differentiation factors. Combined loss of both *ngn1* and *ngn2* leads to complete loss of *neuroD*

expression and lack of DRGs[324, 325]. Analyses of *ngn1* and *ngn2* null mutations suggests a

predominant role for *ngn1* in formation of small diameter pain *TrkA+* neurons and a lesser role

in the formation of large diameter *TrkB+* and *TrkC+* neurons, and a transient role for *ngn2* in

formation of large diameter *TrkB+* and *TrkC+* neurons. DRG formation in *ngn2* mutant

embryos is normal due to functional compensation by *ngn1*. *ngn2+* cells also contribute to a

small but significant fraction of TrkA+ nociceptive neurons[326]. A number of transcription

factors, like POU domain transcription factor, POU4F1, Runt related transcription factors,

RUNX1 and RUNX3, and homeobox transcription factor, SHOX2, have all been shown to

regulate the differentiation of DRG sensory neurons into various subtypes.

Two major reports highlight the role of WNTs released from dorsal neural tube in

trunk sensory neurogenesis[39, 38]. Activation of canonical WNT signaling causes stabilization of

cytoplasmic protein, β-catenin, which then translocates into the nucleus and activates WNT

responsive genes with the help of Lef/TCF factors. Genetic ablation of β-catenin in neural crest

leads to a complete absence of dorsal root ganglia and loss of both waves of sensory

neurogenesis[39]. Lack of active WNT signaling at the sites of post-migratory neural crest cells

and at DRG sites suggests that WNT signaling is not directly involved in the induction of the secondary wave of DRG neurogenesis[327]. Constitutive activation of canonical WNT signaling in the neural crest progenitor cells in the dorsal neural tube promotes sensory neuron differentiation *in vivo*, at the expense of other neural crest progeny. In vitro clonal analysis proved that this effect of canonical WNT signaling in sensory cell differentiation is not caused by WNT mediated "sensory progenitor" cell proliferation and expansion but is a true cell fate specification[38].

Autonomic Nervous system

ANS is a neural crest derivative that provides a way for the central nervous system (CNS) to send commands to the rest of the body and regulate the involuntary functions of the visceral organs. Functionally, ANS is divided into three major components, sympathetic nervous system (SyNS) and parasympathetic nervous system (PSNS) which function together to maintain body homeostasis and the enteric nervous system (ENS) which controls the gut motility amongst other things. Axons from pre-ganglionic neurons of the CNS (brainstem and spinal cord in this case) project and synapse with the post-ganglionic cell bodies located paravertebrally (for SyNS) or inside the target organs (in case of PSNS); the post-ganglionic axons innervate the target site (organs and tissues throughout the body). In the gut, the nor-

adrenergic and cholinergic ganglia form the enteric nervous system (ENS) which exclusively innervates the gastro-intestinal (GI) tract.

Differentiation of autonomic neurons

Within the ANS, formation of sympathetic neurons has been studied in most details. Anatomically, sympathetic nervous system is hallmarked by a ventrally located string of sympathetic ganglia (SG) aligned parallel to the vertebrae on both sides of the embryo. Formation of SGs requires coalescence of sympathetic precursor neural crest cells which is achieved by combinatorial action of Eph/ Ephrin, Semaphorin/Neuropilin and N-cadherin signaling[328, 329, 330, 331].

BMP signals emanating from the dorsal aorta have been shown to be the regulators of sympathetic neuron differentiation[332]. Over-expression of *Bmp4* and *Bmp7* in migrating neural crest cells leads to an increase in the size of sympathetic ganglia as well as formation of ectopic ganglia in chick embryos while blocking BMP signaling *in vivo* leads to prevention of sympathetic neuron generation hallmarked by the absence of sympathetic neuron marker gene expression. *Bmp4* and *7*[332] and *Bmp2* and *4*[333] are expressed at dorsal aortae, the site of primary SG chain formation of chick and mice respectively while neural crest cells express BMP receptors *Bmpr1a* and *Bmpr1b*[334].

BMP signaling activates expression of a multitude of transcription factors, MASH-1, Phox2a/2b, Hand2, and Gata2/3, which co-regulate each other and form a network key for sympathetic differentiation. Together, they regulate expression of Noradrenaline synthesis enzymes like TH (tyrosine hydroxylase) and DBH (Dopamine beta hydroxylase) in sympathetic neurons. Phox2 genes are highly conserved paired-type homeodomain transcription factor, expressed in sympathetic, parasympathetic as well as enteric ganglia of the developing peripheral autonomic nervous system and are necessary and sufficient for specification of sympathetic neurons from trunk neural crest cells[335, 336]. PHOX2B is required to maintain the expression of MASH-1, another transcription factor involved in sympathetic differentiation which in turn regulates expression of another paired-type homeodomain transcription factor, *phox2a* [335,337,338,339,340,341]. PHOX2A and PHOX2B can both, independently, induce transcription of adrenergic enzymes, TH and DBH[342,343,344]. The helix-loop-helix transcription factor HAND2 (also called dHAND) works synergistically with PHOX2A to induce *dbh* transcription[345,346] and can also interact with MASH-1[347]. Over-expression of HAND2 in chick peripheral nerves leads to induction of pan-neuronal (SCG10 & NF160) and adrenergic marker (*th* and *dbh*) expression. Activation of PHOX2A/B, MASH-1 and HAND2 under active BMP signaling, is followed by activation of transcription of *gata* factors. GATA2 in chick and

GATA3 in mice embryos have been shown to be required for expression of *th* and *dbh* in sympathetic precursors[348, 349].

Cholinergic vs. Nor-adrenergic neuronal differentiation

Trunk NC cells generate both noradrenergic and cholinergic neurons (in limited numbers) while mesencephalic crest predominantly form cholinergic parasympathetic neurons (of the ciliary ganglia). BMPs synthesized in the local microenvironment are essential for the differentiation of both noradrenergic and cholinergic neurons (reviewed in[350, 351, 352]. In the mesencephalic crest, *phox2* but not *hand2* genes are expressed under the influence of BMP signaling[353] suggesting that HAND2 may be required for *th* and *dbh* expression[353, 354]. Recently, it has also been shown that trophic factors NGF and NT3 have differential requirements in cholinergic and adrenergic neuron differentiation respectively[355].

As described in Chapter 1, loss of MED23 results in a defect in peripheral nervous system development. We have determined that MED23 is required for cranial placode development, loss of which leads to cranial sensory nervous system formation defects. Fewer neuro-gliogenic neural crest cells are observed in *med23^{sn/sn}* embryos but whether this leads to

a defect in formation of trunk sensory ganglia or the autonomic nervous system will be determined from conditional mutant analysis only.

Results

I.   *med23^{sn/sn}* embryos do not have drastic defects in neural crest development

Majority of the peripheral nervous system in vertebrates is derived from neural crest cells. Expression of sox10 is observed in all migrating neural crest cells at early stages but later on by E9.5 gets restricted to neuro-gliogenic fate neural crest only. We decided to make use of this specific expression pattern of *sox10* at E9.5, to analyze the formation and migration of neuro-gliogenic neural crest. As can be seen in **Figure 23A**, in wild-type embryos, *sox10* expression is observed in the neural crest cells populating the cranial ganglia. *med23^{sn/sn}* littermate embryos also display the presence of *sox10⁺* neuro-gliogenic fate neural crest cells in the developing cranial ganglia; however the number of cells seems to be reduced **(Figure 23B)**. Neural crest cells majorly contribute to the forming glia in the cranial ganglia. Given the presence of *sox10⁺* cells in cranial, it seems neural crest cells are able to populate sites of cranial and trunk sensory ganglia in *med23^{sn/sn}* embryos, their numbers are however reduced.

II *med23^{sn/sn}* **embryos have drastic defects in cranial placode formation**
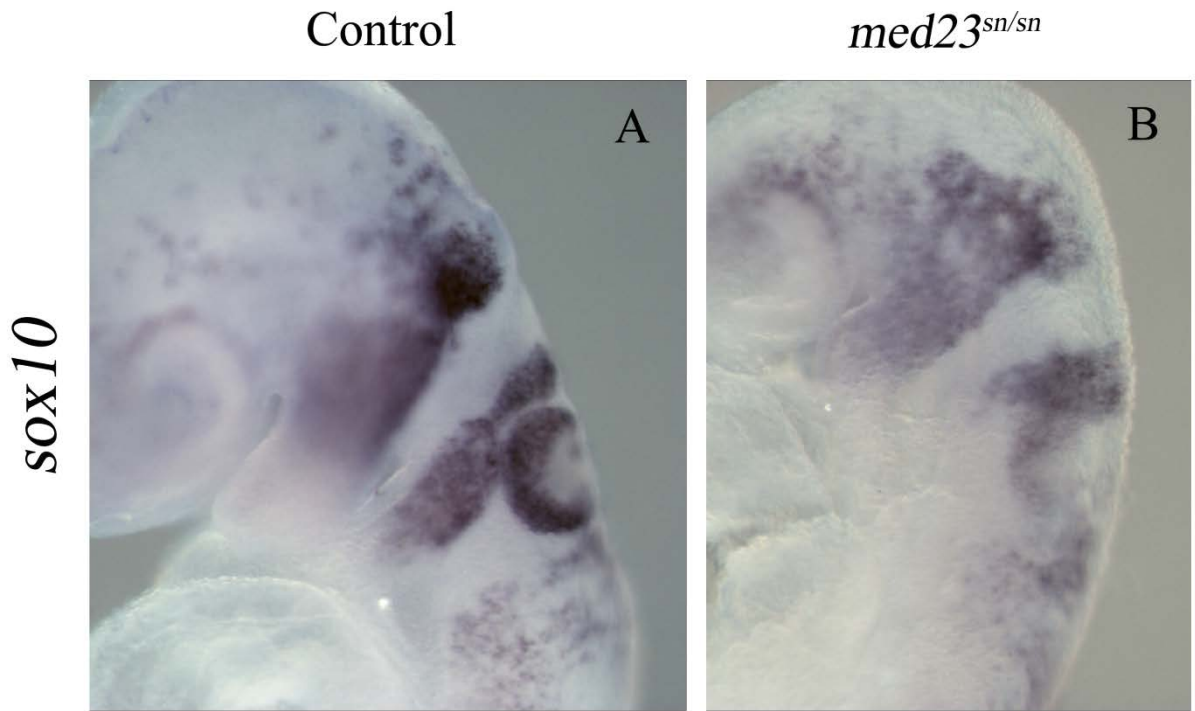
Control $med23^{sn/sn}$

Figure 23: *sox10+* cells are reduced in *med23$^{sn/sn}$* embryos

Sox10 ISH of control (A) and *med23$^{sn/sn}$* (B) embryos at E9.5 shows reduced *sox10+* cells entering the cranial ganglia region near the 1st branchial arch and around the otic vesicle.

As described earlier, the major defect in the peripheral nervous system formation in

med23$^{sn/sn}$ embryos seems to be in the formation of the cranial sensory nervous system. Cranial

ganglia (ganglia of the cranial sensory nervous system) are derived from two separate

populations of cells. All the neurons of epibranchial ganglia and a subset of the neurons of

trigeminal ganglia come from cranial placodes. Since we did not see a drastic defect in

formation and migration of cranial neural crest cells in *med23$^{sn/sn}$* embryos, we decided to

analyze the formation of the cranial placodes in these embryos.

IIA Neuronal differentiation of cranial placodes is affected in *med23$^{sn/sn}$* embryos

Our first hint that neuronal differentiation could be affected in *med23$^{sn/sn}$* embryos

came from analysis of microarray done on the wild-type and mutant littermate embryos at

E9.5. *neurod1*, a gene involved in peripheral and central neuron differentiation was down-

regulated by 1.6fold on the microarray. In the peripheral neurons, *neurod1* is expressed

specifically during cranial sensory neuron differentiation. We confirmed the microarray data

using RNA *in situ* hybridization. As expected, *neurod1* transcripts are observed in the

neurogenic trigeminal and epibranchial ganglia at E9.5 **(Figure 24A)**. On the contrary,

littermate *med23$^{sn/sn}$* embryos display an almost complete loss of *neurod1* expression in the
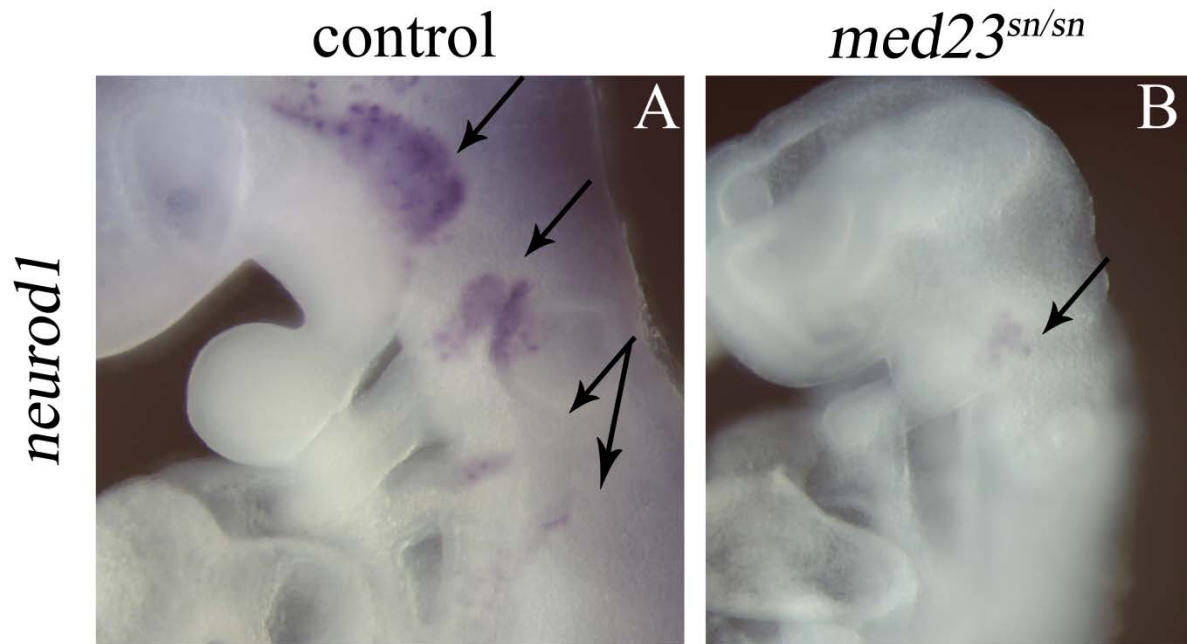
Figure 24: *neurod1+* cells are drastically reduced in the developing cranial ganglia of

med23^sn/sn embryos at E9.5

Control (A) and *med23^sn/sn* embryos were analyzed for *neurod1* expression at E9.5. Trigeminal

and epibranchial ganglia in control embryos (arrows) display *neurod1+* cells while *med23^sn/sn*

embryos very few positive *neurod1+* cells in the trigeminal ganglion and no *neurod1+* cells in

the epibranchial ganglia.

developing cranial ganglia (**Figure 24B**). Very few cells in the trigeminal ganglion still express

neurod1. These few *neurod1*⁺ cells in the trigeminal ganglion co-relate with the few TuJ1⁺ cells

observed in the trigeminal ganglion by immuno-staining, suggesting that lack of *neurod1*

expression might be the cause for lack of TuJ1 marker expression.

As explained earlier in this chapter, cranial placode development follows a

hierarchical pattern of gene expression. Thus, from beginning from specification of pre-

placodal territory to differentiation of cranial placodal cells, each stage of cranial placode

development can be delineated using stage-specific gene expression as the marker. The lack of

neurod1⁺ cells in the forming cranial ganglia region in *med23*$^{sn/sn}$ embryos could be a result of

loss of *neurod1* or loss of cranial placodal cells. In order to figure that out, we analyzed cranial

placode formation by analyzing the hierarchical gene expression. Neurogenins are

transcription factors which during cranial ganglia development have been shown to be

upstream of *neurod1* expression. *ngn1 (neurogenin1)* in trigeminal ganglion and *ngn2*

(neurogenin2) in epibranchial ganglia have been shown to be directly upstream of *neurod1*

and are crucial to the neuronal differentiation of trigeminal ganglion and epibranchial ganglia

respectively. We analyzed the expression of both *ngn1* and *ngn2* in wild-type and *med23*$^{sn/sn}$

littermate embryos. At E9.5, we observe *ngn1* expression mainly in the developing trigeminal

ganglion and faintly in the developing epibranchial ganglia (**Figure 25A**). When analyzed in
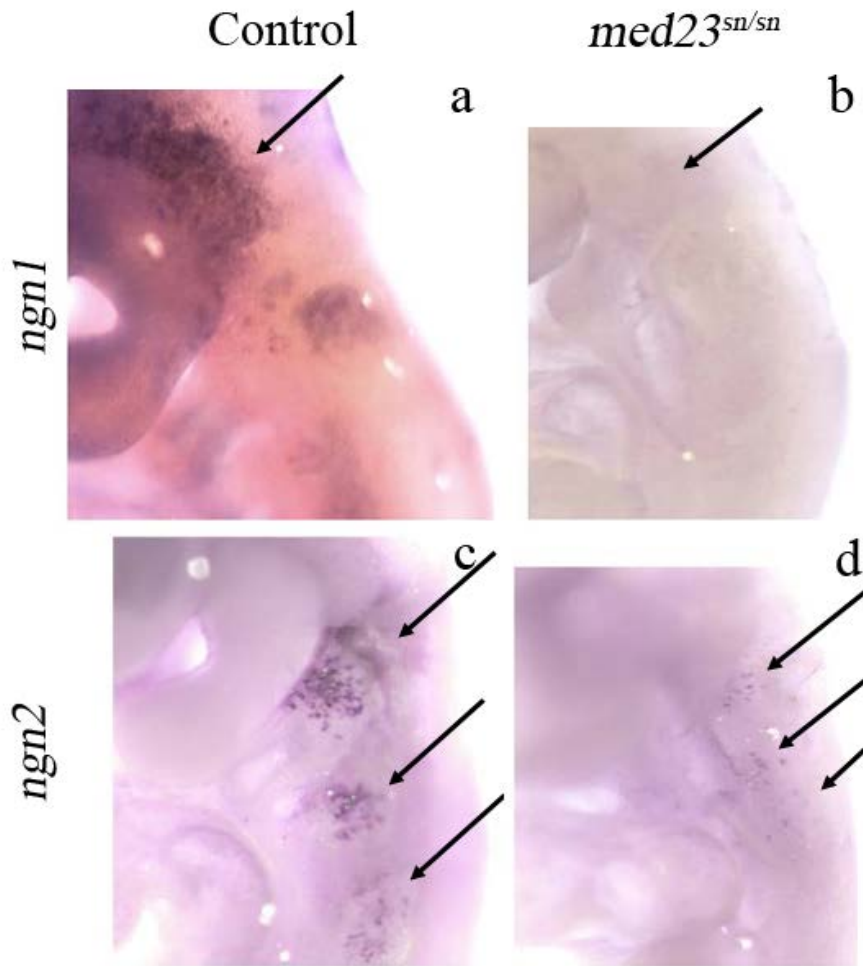
Figure 25: *neurogenin expression* is affected in the cranial ganglia of *med23^{sn/sn}* embryos

A, B *ngn1* is expressed in the trigeminal (arrow) and few epibranchial cells in control embryos

(A) *ngn1* expression in the trigeminal (arrow) and epibranchial regions is lost in *med23^{sn/sn}* embryos.

C, D *ngn2+* cells are observed in the epibranchial ganglia of control embryos (arrows, C). *ngn2+* cell number is drastically reduced in the epibranchial ganglia of *med23^{sn/sn}* embryos (arrows, D).

med23$^{sn/sn}$ littermate embryos, very few cells in the developing trigeminal ganglion display presence of *ngn1* transcript. No cells in the epibranchial ganglia/placode region are *ngn1$^+$* (**Figure 25B**). Similar results were observed with *ngn2*, *ngn2* displays a temporally dynamic expression pattern. Although crucial for epibranchial ganglia development, *ngn2* also has a brief time window during which it is expressed in the developing trigeminal ganglion. At E9.5, we observe *ngn2$^+$* cells present only in the epibranchial placodal cells in wild-type embryos (**Figure 25C**). The number of *ngn2$^+$* cells in the developing epibranchial placodes is drastically reduced in *med23$^{sn/sn}$* embryos (**Figure 25D**). Interestingly, these few *ngn2$^+$* epibranchial placodal cells fail to turn on the expression of *neurod1* and differentiate into TuJ1$^+$ epibranchial neurons.

IIB Cranial placodes are properly specified but loosely organized in *med23$^{sn/sn}$* embryos

In our quest to understand what steps in cranial placode development require MED23, we further analyzed if cranial placodes are appropriately specified in *med23$^{sn/sn}$* embryos. *Pax* gene expression is an indication of newly specified cranial placodes. Specifically, *pax2* and *pax8* have been shown to be expressed in the newly specified otic and epibranchial placodes.
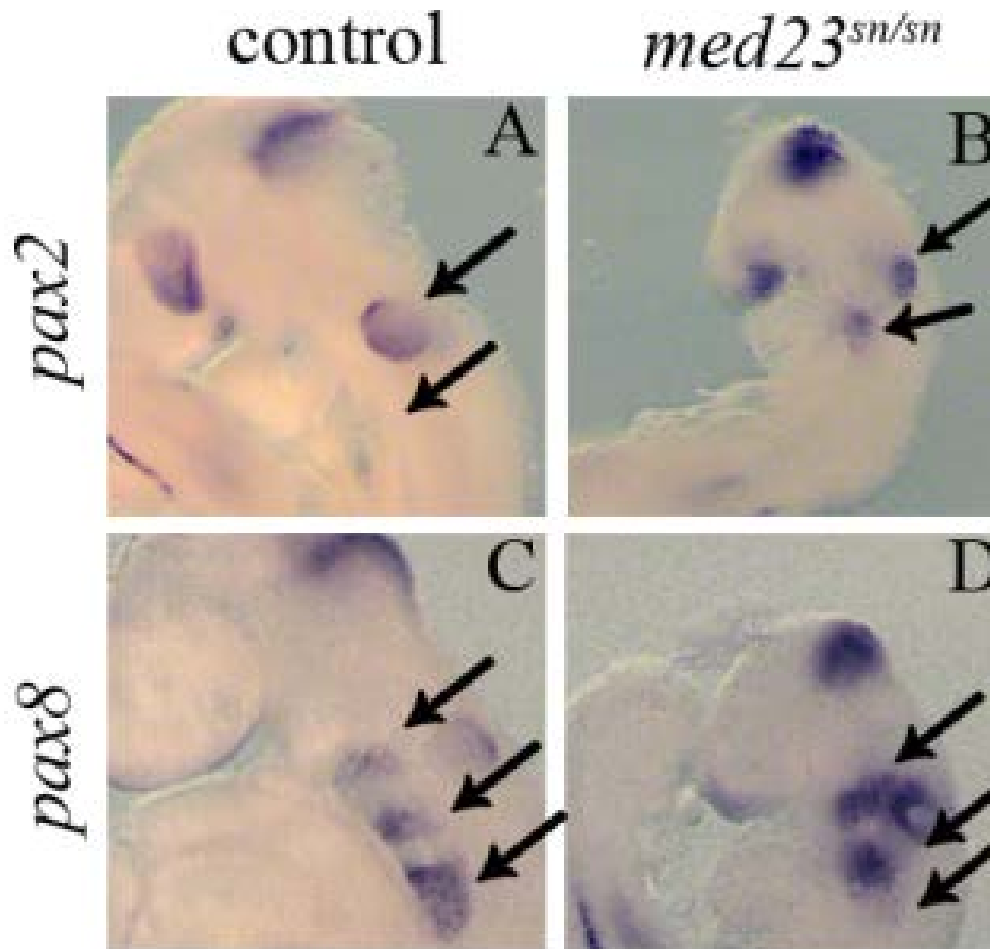
Figure 26: Coalescence but not specification of cranial placodes is affected in *med23^{sn/sn}*
embryos

A, B *pax2+* cells are present in the optic, otic and isthmus of both control and *med23^{sn/sn}*
embryos

C, D *pax8+* cells are present but loosely organized in the epibranchial region, otic placode and
isthmus of *med23^{sn/sn}* embryos (arrows, D) in comparison to control embryos (arrows, C).

At E9.5, *pax2* expression is observed in the eye, nasal and otic placodes, in the epibranchial region and isthmus (**Figure 26A**). This expression pattern of *pax2* persists in littermate *med23^{sn/sn}* embryos (**Figure 26B**), however, the delineation between the optic and nasal placode is difficult in mutant embryos due to abnormal frontonasal morphology. The expression of *pax2* in the epibranchial region seems to be expanded in *med23^{sn/sn}* embryos, implying either an expansion in the *pax2+* territory or a persistence of *pax2* expression. *pax8* expression is more specific to the epibranchial placodes and the gene is known to have roles in regulating the segregation of otic and epibranchial placodes from the posterior pre-placodal ectoderm (PPA). Wild-type embryos show expression of *pax8* in the isthmus as well as the otic and epibranchial placodes (**Figure 26C**). Similar to *pax2*, this expression of *pax8* is also maintained in the littermate *med23^{sn/sn}* embryos, however the *pax8+* cells in the developing epibranchial placodes do not seem to be as well coalesced in the mutants as in the wild-type embryos (**Figure 26D**). This lack of coalescence within the epibranchial placodes may be the cause for neuronal differentiation defect in the *med23* mutant embryos.

IIC *med23^{sn/sn}* embryos have defects in maintenance PPR markers

Cranial placodes begin as a single ectodermal-cell population called Pre-placodal region (PPR), which then segregates into anterior and posterior pre-placodal regions that
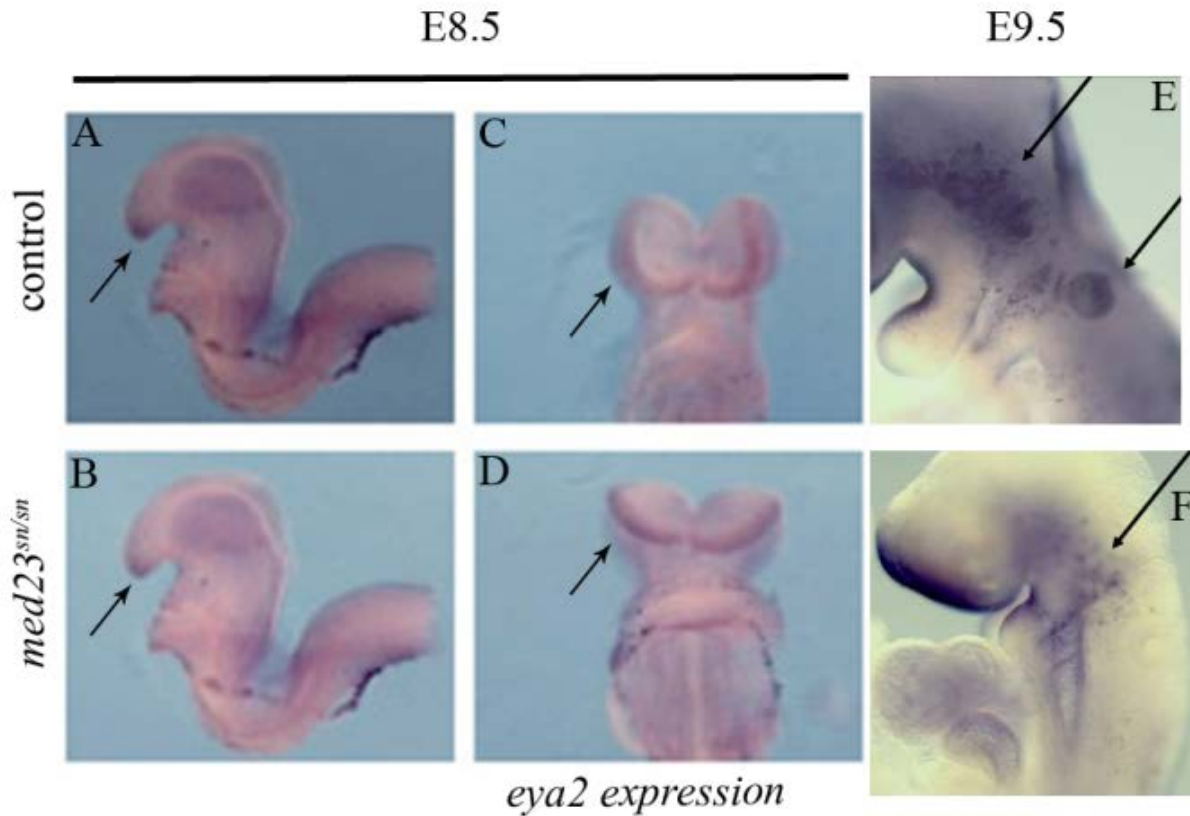
Figure 27: *med23^sn/sn* embryos do not maintain the expression of PPR markers in cranial sensory ganglia

A – D *eya2* expression in control (A, C) and *med23^sn/sn* (B, D) embryos shows proper formation of anterior pre-placodal area (arrows, A – D) in mutant embryos

E, F control embryos at E9.5 (E) express *eya2* in trigeminal placode and few epibranchail placode cells (arrows, E). Very few *eya2+* cells are observed in the trigeminal and epibranchial regions of *med23^sn/sn* embryos (arrow, F).

separate into specific placodes. *six* and *eya* family members, specifically *six1* and *4* and *eya1* and *2,* are expressed at the time of PPR specification and their expression is maintained as the PPR segregates into specific placodes and differentiates into various cell types. We analyzed the expression of one of these PPR genes, *eya2,* at two different stages of cranial placode segregation from PPR. At E8.5 (**Figure 27A**; *lateral view* & **27C**; *ventral view*), *eya2* expression is observed in the anterior pre-placodal region (that later separates into olfactory, optic and pituitary placodes) in both wild-type and mutant embryos. At E8.5 the expression of *eya2* does not seem to be altered in the *med23$^{sn/sn}$* embryos (**Figure 27B, D**) suggesting that the formation of anterior pre-placodal region is not affected by loss of *med23.* On the other hand, the expression of *eya2* is more pan-placodal at E9.5 (**Figure 27E**). Cells in trigeminal and epibranchial placodes are positive for *eya2* in wild-type embryos at E9.5 but *med23$^{sn/sn}$* embryos display a reduction in the number of *eya2+* cells in the developing trigeminal placode and no *eya2^{+}* cells are observed in the forming epibranchial placodes in the mutant embryos (**Figure 27F**). Thus, MED23 seems to be required for maintenance of placodal marker expression, suggesting that MED23 has a role in maintenance of placodal identity, loss of which may contribute to the neuronal differentiation phenotype observed in *med23* mutants.

Discussion

Our initial analysis showed to us that loss of MED23 leads to fewer TuJ1+ neurons in the cranial sensory nervous system. *med23^{sn/sn}* embryos display only a few differentiated TuJ1+ neurons in the trigeminal ganglion and no differentiated neurons in any of the epibranchial ganglia. We sought out to determine the cause for this neurogenesis defect in *med23^{sn/sn}* embryos. Our analysis has shown to us that **there is a role for MED23 in development of neural crest and cranial placodes,** the two ectoderm derived precursor of cranial sensory neurons. The extent of MED23 requirement, however, varies between the two.

Despite almost complete loss of neurogenesis, *med23^{sn/sn}* embryos do display proper formation and migration of neural crest cells as observed by *sox10* expression analysis, suggesting that MED23 is not required in the early steps of neural crest formation or in determining neural crest migratory pathways. By E9.5, expression of *sox10* becomes restricted to neuro-gliogenic fated neural crest cells. In *med23^{sn/sn}* embryos, the total number of *sox10+* neural crest cells is reduced, which could mean a role for MED23 in neural crest survival or proliferation. Reduction in the number of *sox10+* neural crest cells was observed both in cranial and trunk regions. Trunk neural crest cells differentiate into sensory (DRG) and autonomic (sympathetic, parasympathetic and enteric) neurons. Whether this reduction in the trunk neural crest cell numbers leads to defects in sensory and autonomic neurogenesis within the trunk of *med23^{sn/sn}* embryos is not known. The timing of differentiation of trunk neural

crest cells into sensory and autonomic neurons is much later than that of cranial sensory neurons, and *med23$^{sn/sn}$* embryos die before a comprehensive analysis of trunk sensory and autonomic neuron differentiation can be done. Future understanding of the role for MED23 in differentiation of trunk neural crest cells requires analysis of conditional mutant *med23* alleles that will surpass the early embryonic lethality of complete *med23* knock-out. In our lab, we also study another mouse model, *tcof1$^{+/-}$*, which shows a reduction in neural crest numbers leading to defects in peripheral nervous system development as well. Similar to *med23, tcof1* is also expressed in all cell types within the embryo. In *tcof1$^{+/-}$* mouse model, the cause for reduced number of neural crest cells is reduced survival and increased cell death of crest precursors, neural plate cells. Such a possibility cannot be ruled out right now in *med23$^{sn/sn}$* embryos. An analysis of the cell survival and proliferation within the distinct tissues of *med23$^{sn/sn}$* embryos should be able to answer this question.

TuJ1 (β-tubulin III) is a neural specific cytoskeletal marker, whose expression coincides with acquisition of neural character. All differentiating neurons (central and peripheral) express TuJ1 as one of the final steps of neuronal differentiation. Work from various species supports a theory that cranial placodes originate as a single ectodermal cell population known as pre-placodal region (PPR) which segregates into different placodes that express neuronal determination genes and differentiate into neurons. Expression of TuJ1

happens during this final step of neuronal differentiation. In order to figure out which exact step(s) during cranial placodal differentiation require MED23, we analyzed the formation of cranial placodes all the way from pan-placodal region marker expression to neuronal differentiation.

Our analysis of early pre-placodal region marker, *eya2* suggests that formation of the anterior pre-placodal area (at E8.5) does not require MED23. Anterior pre-placodal area is fated to segregate and differentiate into optic, olfactory placodes and adenohypophysis of the pituitary gland. This fits in perfectly with our previous observation that a day later, *med23$^{sn/sn}$* embryos have smaller and more ventrally located but still morphologically normal eye development. On the other hand, it is known that even though a marker of pre-placodal region, *eya2* is expressed throughout cranial placode differentiation. This persistent expression of *eya2* is required for maintenance of placodal identity and maybe even for differentiation of cranial placodes. At later stages, *med23$^{sn/sn}$* embryos lose *eya2* expression the developing otic and epibranchial ganglia as well as the number of *eya2+* cells in the trigeminal ganglion of mutant embryos is reduced, thus suggesting in absence of MED23, the neurogenic cranial placodes are unable to maintain their placodal identity. MED23 however does not seem to be the ultimate authority for regulation of cranial placodal identity. PAX2 and PAX8 are both considered to be markers of otic and epibranchial placodal identity and their expression

usually follow that of *eya* genes. *med23^sn/sn* embryos have *pax2+* and *pax8+* cells in the developing otic and epibranchial ganglia, suggesting that cranial placodal identities are established even in absence of MED23. The *pax2+* or *pax8+* cells however, do not coalesce well within the epibranchial placodes. Lack of coalescence due to loss of Slit-Robo and N-cadherin signaling has been shown to be responsible for defects in trigeminal ganglion[98]. It would not be surprising if similar compact three-dimensional arrangement is a requirement for differentiation of epibranchial placodes as well. It is possible that loss of MED23 affects intra-placodal cell-cell adherence, lack of which affects the maintenance of pan-placodal marker (*eya2*) which may cause the loss of neuronal differentiation in epibranchial ganglia of mutant embryos. It would be interesting to see if coalescence of newly segregated *pax3+* trigeminal placodal cells is also affected by loss of MED23.

The distal subset of epibranchial ganglia, geniculate, petrosal and nodose associate with the VIIth, IXth and Xth cranial nerves and are derived exclusively from epibranchial placodes. Neuronal differentiation of these distal epibranchial ganglia requires expression of neuronal determination gene, *ngn2* which then turns on *neurod1* and TuJ1 expression in these cells. Even though these placodal cells are specified for epibranchial fate in mutant embryos, loss of *med23* leads to a complete loss of TuJ1+ and *neurod1+* cells in the epibranchial region. Few cells expressing *ngn2* are still observed in these distal epibranchial placode regions; this

reduction in *ngn2+* cell number could be a result of a requirement for MED23 in maintenance of *ngn2* expression, survival of *ngn2+* cells or induction of *ngn2* expression. Conversely, formation of a coalesced epibranchial placode could be required for neuronal differentiation of those cells and lack of such coalescence in *med23^{sn/sn}* embryos, as observed by *pax2* and *pax8* expression, could result in lack of *ngn2* expression leading to defect in neuronal differentiation. Current data suggests that any or all of these possibilities could be true; however, one thing is for sure, that those few *ngn2+* cells are unable to turn on the expression of *neurod1* and TuJ1 in the developing distal epibranchial placodes of *med23^{sn/sn}* embryos, suggesting that MED23 is required (directly or indirectly) for acquisition of ultimate neuronal fate in epibranchial placode cells.

Neurons in proximal set of epibranchial ganglia and trigeminal ganglion have mixed origins, from neural crest and placodes. Developing trigeminal and proximal epibranchial cells express another bHLH transcription factor, *ngn1* and rely on it for their neuronal differentiation. At E9.5, apart from the site of developing trigeminal ganglion *ngn1+* cells are also observed at the sites of vestibulocochlear (cranial nerve VIII) and proximal geniculate (cranial nerve VII) ganglia. Interestingly, amongst all the cranial sensory ganglia analyzed at E9.5 in *med23^{sn/sn}* embryos trigeminal ganglion is the only one that still shows some extent of neuronal differentiation, as observed by a few TuJ1+ cells. When we analyzed the expression of

neuronal determination genes in the cells (crest or placodal) destined for trigeminal fate, we observed that both *ngn1* and its downstream gene *neurod1* are expressed in the developing trigeminal neurons in *med23^{sn/sn}* embryos. The number of *neurod1+* or *ngn1+* cells in the putative trigeminal neurons in mutant embryos is fewer than that in wild-type littermates similar to the fewer number of TuJ1+ neurons observed in mutant embryos. Unlike trigeminal ganglion, no *ngn1+* cells are observed at the sites of putative otic and proximal geniculate ganglia in *med23^{sn/sn}* embryos. Combining this with the results from distal epibranchial ganglia a theme emerges, where MED23 seems to be required for the neuronal determination of epibranchial ganglia in general (distal and proximal). The few TuJ1+, *neurod1+* and *ngn1+* cells in the trigeminal ganglion could be of neural crest origin or trigeminal placode origin. On the other hand there is a complete lack of TuJ1+, *ngn1+* and *neurd1+* cells in the crest- and/or placode- derived epibranchial ganglia. Given the normal formation and migration of neural crest cells we are tempted to hypothesize that in trigeminal ganglion the neuronal differentiation of cranial neural crest cells is unaffected but that of trigeminal placodal cells is affected in *med23^{sn/sn}* embryos. Conversely, MED23 might have a small role in neuronal differentiation of neural crest cells at proximal epibranchial ganglia. Neural crest specific *med23* knock-out studies are currently underway to determine whether MED23 has a role for neuronal differentiation of cranial neural crest cells in trigeminal and proximal epibranchial

144

ganglia. These studies will also help us understand if MED23 is required for the sensory and autonomic differentiation of trunk neural crest cells.

Thus, overall, this analysis unequivocally states a role for MED23 in various stages of cranial placode development, including placodal identity maintenance, organization of newly segregated cranial placodes and neuronal differentiation of neurogenic placodes, all together leading to a defect in formation and differentiation of cranial sensory nervous system.

Chapter 4

MED23 is important for the anterior development in

mammalian embryos

Summary

Our initial analysis showed a defect in frontonasal process and branchial arch-1 development in mice embryos following loss of MED23. Here, we are trying to understand what tissues and signaling pathways within the craniofacial apparatus are specifically affected by loss of MED23. Craniofacial defects can be identified in *med23^{sn/sn}* embryos as early as E9.0. Craniofacial skeleton develops majorly from neural crest cells, and its patterning depends on a complex interaction network between FGF, WNT, BMP and SHH signaling. Here we show that *med23^{sn/sn}* embryos display a reduction in the number of neural crest cells populating the frontonasal and branchial arch-1 mesenchyme; however this reduction is not due to reduced cell proliferation or increased cell apoptosis. Forebrain development (as assessed by expression of forebrain markers, *otx2* and *six3*) and formation of zone of frontonasal ectoderm (FEZ) (as assessed by *fgf8* expression and active SHH signaling) seems to be unaffected by loss of MED23. On the other hand, WNT signaling activity is up-regulated in the developing frontonasal process of *med23^{sn/sn}* embryos, and this could be the underlying cause for craniofacial defects in embryos lacking MED23.

Introduction

All vertebrates share a common blueprint for facial development. A series of small buds of tissue called the facial prominences (or primordia) form around the primitive mouth (stomodeum) and are the basis for all structures of an adult face. These five prominences, single unpaired **frontonasal prominence (FNP)** and **paired maxillary and mandibular prominences** form the upper, lateral and lower borders of the stomodeum respectively. The paired maxillary and mandibular prominences are formed from the first pair of branchial arches on either side of the developing foregut and give rise to the cheek and sides of nose (maxillary) and lower lip and lower jaw structures (mandibular) of an adult face. All of these five prominences are filled with mesenchyme derived mostly from neural crest cells migrating from the fore-, mid- and hind- brain. As neural crest cells populate these prominences, they encounter signals from surrounding brain structures and covering surface ectoderm and differentiate into craniofacial skeleton. The mesenchymal core of each branchial arches also consists of some mesoderm derived cells which form the musculature of the head and is lined with ectoderm on the outside and endoderm on the inside.

Formation of the final craniofacial structure requires fusion of these various prominences. Before any fusion event occurs, the frontonasal prominence develops two sets of bilateral swellings, optic and nasal (olfactory) placodes (that respectively contribute to the

148

paired sensory organs, eyes and nose – *see Chapter 3*). Following this, the two (bilaterally located) mandibular prominences fuse at the midline forming a contiguous base underneath the mouth opening, stomodeum. The maxillary prominences on each side then fuse with the frontonasal prominence, and the two lines of fusion form the **nasolacrimal groove**. At the same time, nasal placodes invaginate to form nasal pits which are surrounded by horse shoe shaped eminences. The lateral portion of these eminences is called **lateral nasal processes** and it lies exactly next to the nasolacrimal groove (also known as the naso-optic furrow). Later on as development proceeds, the lateral nasal processes fuse with the maxillary processes on both sides obliterating the nasolacrimal grooves. The medial portion of these eminences is called **medial nasal processes**. Growth of the maxillary processes pushes the two medial nasal processes towards each other where they fuse with each other in the center and with the maxillary processes on the sides extending the mouth cavity. The fusion of the medial nasal processes at midline forms the intermaxillary process which contributes to the primary palate. In mice embryogenesis, this fusion occurs by 12.5dpc. Failure of this fusion leads to cleft lip, a congenital defect observed in 1 in 1000 live births. Along with this, the frontonasal prominence and medial nasal processes also contribute to forehead and nasal ridge in midface. The sides of the nose and cheek have contributions from both lateral nasal processes and maxillary prominences while the lower jaw comes from the fused mandibular prominences.

Later in development the outgrowths from maxillary prominences fuse to form the secondary palate, failure of which leads to a human congenital defect, cleft palate.

Cranial Neural Crest contribution to the developing Face

The "face" is largely the product of cranial neural crest cells. Dorsolateral migration of neural crest cells mainly from the forebrain and anterior midbrain contribute to the frontonasal ectomesenchyme and form the cartilage, bone and connective tissue of the face; while the ventromedially migrating neural crest cells from midbrain proper and anterior hindbrain axial levels populate the branchial arches and contribute to the cranial ganglia and branchial arch derived skeleton. Branchial arches made up of mesenchymal cores consisting of ectodermally derived cranial neural crest and some mesodermally derived cells, sandwiched between an outer ectodermal layer and the inner endodermal layer. Both the ectodermal and endodermal layers signal to the crest and mesoderm derived mesenchyme to regulate their differentiation into skeletal and muscular structures.

Patterning the face

Face is unique as in it does not possess a single "facial organizer", but instead multiple "organizing centers" have been identified that regulate development of distinct facial

structures. Facial morphogenesis is achieved by controlled outgrowth of the above-mentioned facial prominences which is regulated by specialized signaling centers that express known important signaling molecules, like, SHH, BMPs, FGFs and WNTs. Release of these signals first regulates the specification and growth of the craniofacial tissues and later their differentiation and skeletogenesis.

Inhibition of WNT signaling is crucial for anterior specification in vertebrates (Introduction); however at later stages, activation of **WNT signaling** is required for proper cell proliferation, differentiation, patterning and survival of the cells forming the various facial structures. The indication that WNT signaling is important for craniofacial development comes from the fact that various WNT ligands are expressed within the facial prominences[356, 357]. Canonical WNT signaling stabilizes and enhances the function of β-catenin as a transcriptional co-activator and majorly all canonical WNT signaling target genes contain a β-catenin binding site in their promoter. Taking advantage of this, multiple WNT signaling reporter transgenes, *TOPgal* and *BATgal* have been constructed and their expression in facial prominences and their derivatives has been reported; further suggesting a role for WNT/β-catenin signaling in facial morphogenesis[358, 359, 360, 361]. WNT signaling has been shown to coordinate multiple epithelial signals during the formation of upper and lower jaw elements. Increasing β-catenin levels specifically in the branchial arch-1 epithelium supports lower jaw development and induces

molecular reprogramming of mesenchymal cells from maxillary to mandibular-like fate while deletion of β-catenin specifically in the branchial arch-1 epithelium causes severe truncation or absence of mandibular skeletal elements[362]. On the other hand, manipulation of canonical WNT signaling in frontonasal ectoderm[363] or both frontonasal and branchial arch-1 ectoderm[364] suggests a role for WNT signaling in formation of both maxillary and mandibular derived structures. Ablation of WNT signaling co-receptor, *lrp6*, causes hypoplasia of facial processes and reduced cell proliferation in the nasal processes leading to a Cleft lip like phenotype[365]. Consistent with this, homozygous mutations in WNT ligands, *wnt3* in both mice and humans and *wnt9b* in mice have been shown to cause cleft lip phenotype[366, 367].

Changing levels of canonical WNT signaling, through its regulation of cell proliferation, has been implicated to be the basis of facial morphogenesis variety in nature[360, 368] For example, the increased cell proliferation in the maxillary processes while no cell proliferation in the frontonasal process in mice embryos correlates with presence of active WNT signaling in the maxillary processes whilst lack of active WNT signaling in the frontonasal process; resulting in a midline furrow that forms a snout or a muzzle in mice. On the other hand, in avian embryos which eventually form a beak, the frontonasal process undergoes higher cell proliferation than the maxillary process which is reflected by increasing WNT signaling responsiveness in the frontonasal process (vs. maxillary processes) in chick embryos[360].

Hedgehog signaling from both foregut endoderm and overlaying ectoderm has been shown to regulate development of the upper face[369, 370, 371, 372, 373, 374]. The most anterior pharyngeal endoderm is necessary for formation of premaxilla (front-most bone of the upper jaw) as well as mesethmoid (the ventral midline bar in nasal capsule) in chicken embryos as surgical removal of this SHH releasing "endoderm zone" prevents mesethmoid development while grafting of supernumerary "endoderm zone" leads to ectopic formation of the mesethmoid cartilage[371]. The cause for disrupted mesethmoid cartilage development in ablation studies seems to be a massive apoptosis of the neural crest cells that populate the frontonasal mesenchyme and the 1st branchial arch which could be somewhat restored by exogenous supplementation of SHH[369], suggesting a neural crest cell survival role for foregut endoderm and SHH signals released from it. Interfering with SHH signaling with a function-blocking antibody inhibits facial primordia fusion while SHH signaling removal causes failure of frontonasal growth[375]. Hedgehog signaling is also required for proper patterning of stomodeum ectoderm which regulates neural crest condensation and formation of the anterior neurocranium (ANC)[374].

The role for **FGF signaling** in the development of craniofacial mesenchyme has long been established. Among 22 FGF genes, at least seven (*fgf3, fgf8, fgf9, fgf10, fgf15, fgf17* and *fgf18*) are expressed in the ectoderm of facial processes between 9.5dpc to 10.5dpc in mice

153

embryos[376]. Mutations in FGF ligands, *fgf3, fgf7, fgf10, fgf18* and FGF receptor, *fgfr1* were recently shown to contribute to non-syndromic cleft lip and palate in humans[377]. During craniofacial development, *fgf8* expression is first observed in the head mesenchyme, as early as 8dpc. The frontonasal process and branchial arch-1 surface ectoderm specific expression is observed half a day later, 8.5dpc, which persists until later (10.5dpc) in the maxillary and mandibular ectoderm as well as ectoderm of nasopharynx and nasal pit ectoderm , in infundibulum, telencephalon, diencephalon and mesencephalon[378, 379]. Branchial arch-1 ectoderm specific deletion of *fgf8* reveals its dual role: promoting survival and differentiation of BA-1 mesenchyme during craniofacial morphogenesis[380]. Even reduction in FGF8 levels within the branchial arch-1 ectoderm leads to neural crest cell apoptosis[381]. On the other hand, too much FGF8 in cranial neural crest cell cultures increases cell proliferation and induces chondrogenesis[382]. Another FGF ligand, FGF18 has been shown to be important survival and osteogenic and chondrogenic differentiation craniofacial mesenchyme, but its requirement seems to be later in development[383].

Within the facial ectoderm, the expression domains of *shh* and *fgf8* are adjacent to each other. This molecular boundary in the frontonasal process ectoderm, known as the **"Zone of Frontonasal Ectoderm (FEZ)"** has now been shown to be a site that regulates dorso-ventral and proximo-distal patterning of the frontonasal mass. Studies done by ectopically transplanting

the FEZ within avian species and between avian and mammalian species (mice–chick chimera) suggest that the regulatory role for FEZ in proliferation of neural crest mesenchyme into maxillary and mandibular structures is conserved through evolution[384, 385]. Inhibition of FEZ formation leads to truncations in the developing upper jaw[386, 387]. FEZ does not repattern the mesenchyme, as the tissues retain their identity (as frontonasal or maxillary); however it does determine the dorso-ventral patterning of the forming upper jaw[384]. Formation of FEZ itself is not very clearly understood currently. The FEZ is established just prior to the outgrowth of the frontonasal prominence and regulates the three-dimensional expansion and orientation of the upper jaw. Expression of *fgf8* has been shown to be required for initial FEZ activity, but its *fgf8* down–regulation is required later for normal development of FEZ[388]. Expression of *shh*, on the other hand, seems to be required for FNP outgrowth and ectopic expression of SHH leads to expansion of the FNP[386, 387, 375]. *Shh* signaling from the forebrain is important for the proper positioning of the FEZ[385]. Apart from *fgf8* and *shh*, several genes encoding bone morphogenic proteins (*bmp2, bmp4 and bmp7*) are also expressed in the FEZ[389, 390, 384, 387] and regulation of BMP expression in FEZ seems to be important for development of neural crest mesenchyme[391]. BMP signaling within the head is required for maxillary outgrowth[389] and disruption of BMP signaling has been shown to affect formation of FEZ itself[390]. *Fgf8* and *shh* signals themselves are not capable of replacing the FEZ in transplant experiments, suggesting that other factors

like the BMPs might be crucial for FEZ function. On the other hand, disruptions in WNT/β-catenin signaling leads to defects in FEZ formation suggesting an upstream role for canonical WNT signaling in FEZ formation and craniofacial development.

Taking together all the work done on various vertebrate species, a picture emerges where cross-regulation between the crucial signaling pathways, like BMP, WNT, FGF and SHH and physical and molecular interactions between the various facial prominences and cell types is required for proper craniofacial development. As described in Chapter 1, loss of MED23 leads to morphological defects in frontonasal prominence and branchial arch-1 development. Early embryonic lethality of $med23^{sn/sn}$ embryos prevents us from analyzing whether these defects translate into defects in craniofacial mesenchyme differentiation or not. In this chapter, I have tried to understand the underlying cause for the morphologically evident craniofacial defects in $med23^{sn/sn}$ embryos and have probed whether the development of the FEZ, neural crest cells and forebrain is affected by loss of MED23.

Results

I. Cranial neural crest cells are marginally reduced in $med23^{sn/sn}$ embryos

The mesenchyme of the frontonasal prominence and branchial arch is majorly composed of cranial neural crest cells. Since we see a size difference in the frontonasal
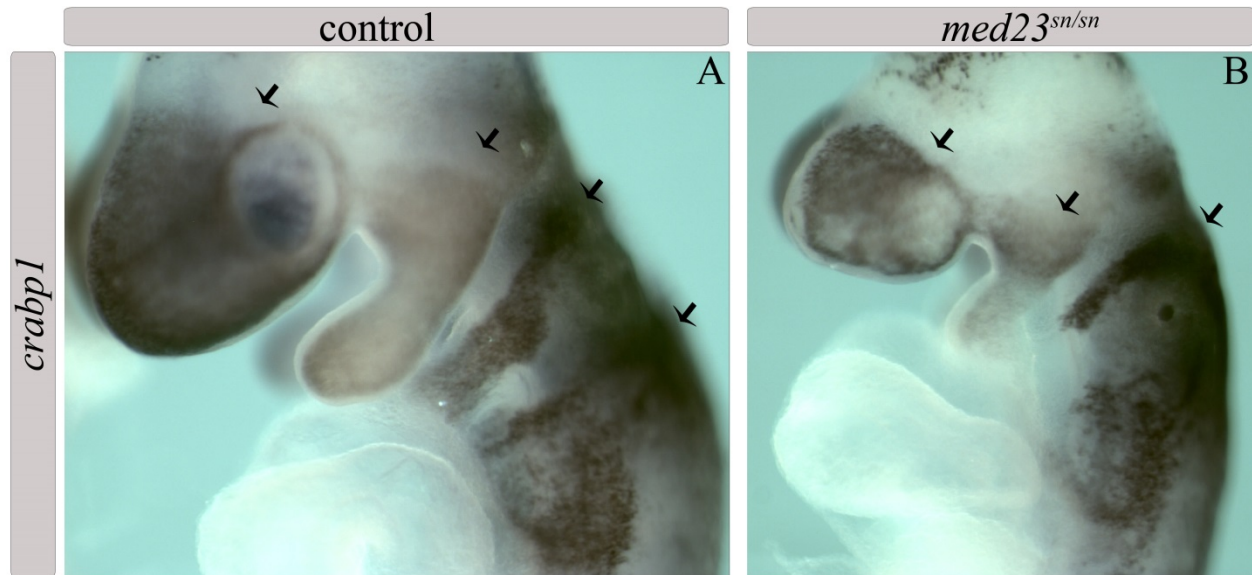
Figure 28: Reduced contribution of *crabp1+* neural crest cells to the craniofacial regions in

med23^{sn/sn} embryos.

Control (A) and *med23^{sn/sn}* embryos were analyzed for *crabp1* expression. *Med23^{sn/sn}* embryos display a reduced contribution of *crabp1+* cells to the frontonasal process and 1st branchial arch (arrows). Migration of *crabp1+* neural crest cells in the second arch stream seems to have halted before it can enter the arch (B).

prominence of *med23^{sn/sn}* embryos at E9.5, we decided to analyze the contribution of neural crest cells to the developing FNP. *crabp1* is an intracellular retinoic acid binding protein has been well utilized to characterize neural crest cell development[392]. Using RNA *in situ* hybridization technique, we analyzed the expression of *crabp1* in the neural crest cells of wild-type and *med23^{sn/sn}* littermate embryos at E9.5 (**Figure 28**). Wild-type E9.5 embryos show beautiful *crabp1^+* dorso-ventral neural crest streams entering into the frontonasal process, branchial arches (**Figure 28A**). Littermate *med23^{sn/sn}* embryos also display presence of *crabp1^+* neural crest cells following similar dorso-ventral trajectories and entering frontonasal process and branchial arches (**Figure 28B**). However, the number of *crabp1+* crest cells is reduced in the mutant frontonasal process and 1st branchial arch, specifically the distal part which gives rise to the mandibular process, suggesting that this reduction in the number of neural crest cells could be the cause for smaller FNP and first branchial arch in *med23^{sn/sn}* embryos.

II.   **Neural crest cell reduction in frontonasal process of *med23^{sn/sn}* embryos is not due to lack of cell proliferation or increased cell apoptosis**

There could be multiple causes for the reduction in *crabp1+* neural crest cells entering the frontonasal process and branchial arch-1in *med23^{sn/sn}* embryos. Since the crest cell streams were observed entering the frontonasal process and branchial arch-1 in *med23^{sn/sn}* embryos

WT   *med23^{sn/sn}*   *med23^{sn/sn}*

PH3 ● TUNEL ● DAPI
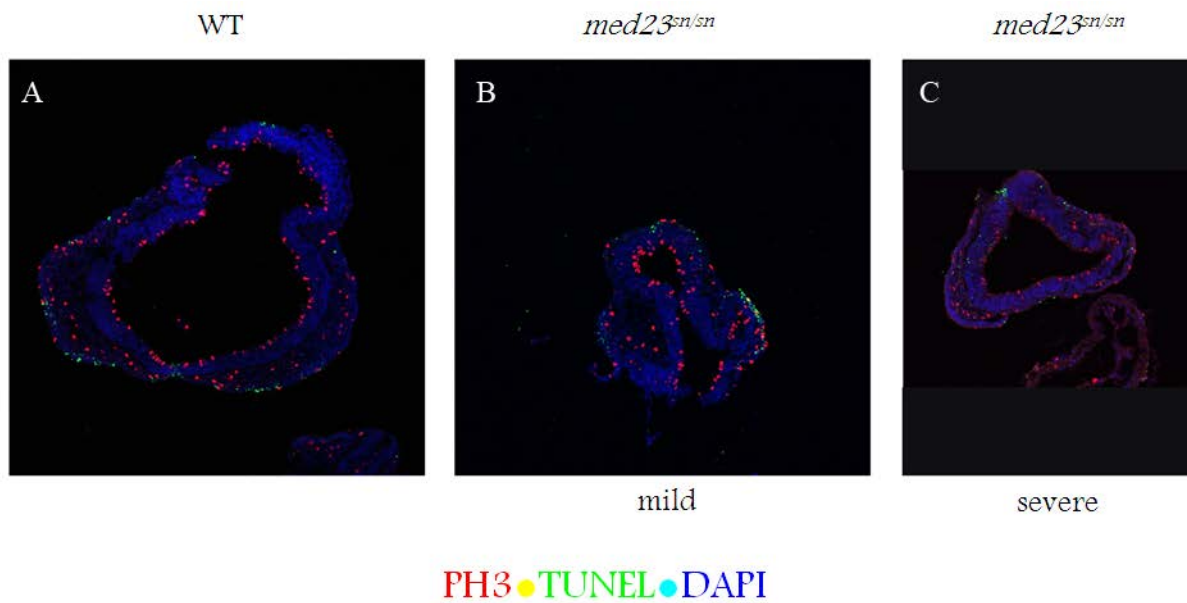
**Figure 29: Cell proliferation and cell death are not altered in the frontonasal process of *med23^{sn/sn}* embryos at E9.5**

Phosphohistone H3 immunostaining (red) and TUNEL (green) labeling show no difference in number of mitotic and apoptotic cells (respectively) in cryosections through the frontonasal process of wild-type (A), mild *med23^{sn/sn}* (B) and severe *med23^{sn/sn}* (C) phenotype embryos.

we wanted to know what happens to these cells once they populate the craniofacial region. We

used phospho-Histone H3 (pHH3) and TUNEL to analyze for cell proliferation and cell death

respectively in the frontonasal prominence (**Figure 29**). pHH3 marks for cells in their mitotic

phase of cell cycle. Increased pHH3 immuno-staining would indicate either an increase in cell

proliferation or a persistence of mitotic phase. TUNEL immuno-staining labels for nicks in DNA

which occur following cell apoptosis or necrosis. Frontal cryosections through the frontonasal

prominences of E9.5 wild-type and both severe and mild phenotype *med23$^{sn/sn}$* embryos were

stained using antibodies against pHH3 and TUNEL and analyzed by confocal microscopy.

Interestingly, no changes in cell proliferation or cell death were observed in frontonasal

ectoderm or frontonasal mesenchyme between *med23$^{sn/sn}$* embryos and its wild-type

littermates at E9.5 (**Figure 29A–C**).

III.   Formation of FEZ seems to be unaffected in *med23$^{sn/sn}$* embryos

In order to understand the cause behind frontonasal process defect in *med23$^{sn/sn}$*

embryos, we also analyzed the formation of the signaling centers within the facial primordia.

As explained above, the juxtaposition of *shh* and *fgf8* expression and signaling within the facial

ectoderm is crucial for proper dorsoventral expansion of frontonasal prominence, and this

zone of juxtaposition is known as the "zone of frontonasal ectoderm" (FEZ). We looked at the

expression of *fgf8* in wild-type and *med23^{sn/sn}* embryos, at E9.5. At E9.5, *fgf8* expression within

the facial ectoderm is specific to the anterior frontonasal ectoderm (also known as anterior

neural ridge) and branchial arch-1 ectoderm (**Figure 30A**). *fgf8* transcripts in the developing

midbrain-hindbrain junction (or isthmus) are also required for survival of cells in the

developing midbrain and hindbrain). In *med23^{sn/sn}* littermates at E9.5, *fgf8* expression in the

anterior neural ridge, branchial arch-1 ectoderm and midbrain-hindbrain junction is

unaffected (**Figure 30B**). The role of FGF8 released from the facial ectoderm is to support cell

survival and proliferation within the craniofacial mesenchyme. Similar levels of *fgf8*

transcripts in *med23^{sn/sn}* and wild-type littermate embryos, supports of our data where we do

not see any changes in reduced cell proliferation or increased cell death within the *med23^{sn/sn}*

craniofacial mesenchyme as compared to littermate wild type embryos.

The other half of FEZ is composed of *shh* expression. SHH signaling functions by

binding to its membrane receptor PATCHED-1, hence *patched-1* expressing cells are

considered SHH signaling recipient cells. SHH signaling itself is known to regulate the

expression of *patched-1*. We utilized a reporter mouse, *patched-1^{lacZ}* in which one of the

alleles for *patched-1* has been replaced with *lacZ* sequence, thus the expression and activity of

LacZ (as determined by the blue colored product formation post-X-gal staining) depicts the site

of *patched-1* expression and thus the site of active SHH signaling. We crossed *med23^{sn}* mouse

line to *patched-1^{lacZ}* mouse line, and analyzed the LacZ pattern in wild-type; *patched^{lacZ}* and littermate *med23^{sn/sn}; patched^{lacZ}* embryos obtained from their offsprings. In both wild-type and mutant embryos at E9.5, lacZ expression and activity was observed to be the same in the endoderm and notochord region in the face and body respectively (**Figure 30C, D**), suggesting that SHH signaling activity is also unaffected in *med23^{sn/sn}* embryos.

IV.   Forebrain development seems to be unaffected in *med23^{sn/sn}* embryos

Apart from FEZ, signals from the forebrain are also crucial for frontonasal process development. In our effort to understand the defects in anterior development in *med23^{sn/sn}* embryos we analyzed if the forebrain itself is developed well in absence of MED23. One of the genes crucial for forebrain and midbrain development in mouse embryos is a homeodomain transcription factor, *otx2*. Loss of *otx2* leads to disruption and complete absence of forebrain and midbrain structures in mice embryos[393, 394]. Another homeobox gene, whose expression in the craniofacial apparatus is restricted to forebrain, is *six3*[395]. Six3 has been shown to be important to regulate expression of *shh* within the forebrain which in turn is important for *shh* expression in FEZ and loss of *six3* causes holoprosencephaly in mice embryos, due to mis-regulation of SHH signaling[396, 397]. We analyzed the expression of both *otx2* and *six3* in the forebrain of wild-type and littermate *med23^{sn/sn}* embryos at E9.5 and found no differences

162

between the two (**Figure 31A–D**), suggesting that patterning of forebrain is not affected in *med23^{sn/sn}* embryos.

V.   WNT signaling is disrupted in the developing frontonasal process of *med23^{sn/sn}* embryos

One of the other crucial signaling pathways regulating cell growth, proliferation and patterning within the craniofacial mesenchyme is canonical WNT signaling. As explained above, WNT signaling has been proposed to correlate with zones of cell growth and proliferation in the developing embryos in avian and mammalian species. BATgal is a canonical WNT signaling transgenic reporter line that expresses LacZ under the regulation of β–catenin responsive sites. We crossed BATgal line with our *med23^{sn}* line to generate a *med23^{sn}*; BATgal strain, animals from which were intercrossed to get wild-type and *med23^{sn/sn}* offspring that contained BATgal allele. In wild-type embryos, canonical WNT signaling has very regionally restrictive zones of activity. Within the craniofacial region of wild-type; BATgal embryos, at E9.5, strong WNT signaling activity was observed in the developing midbrain, hindbrain, and midline of frontonasal process, otic vesicle and 1st branchial arch (**Figure 32A**). Interestingly, left and right lateral zones of the frontonasal process were devoid of WNT signaling activity, and this has been suggested to be crucial for the proper snout formation in mice. LacZ staining pattern in *med23^{sn/sn}* embryos at E9.5 had two key differences (**Figure 32B**).
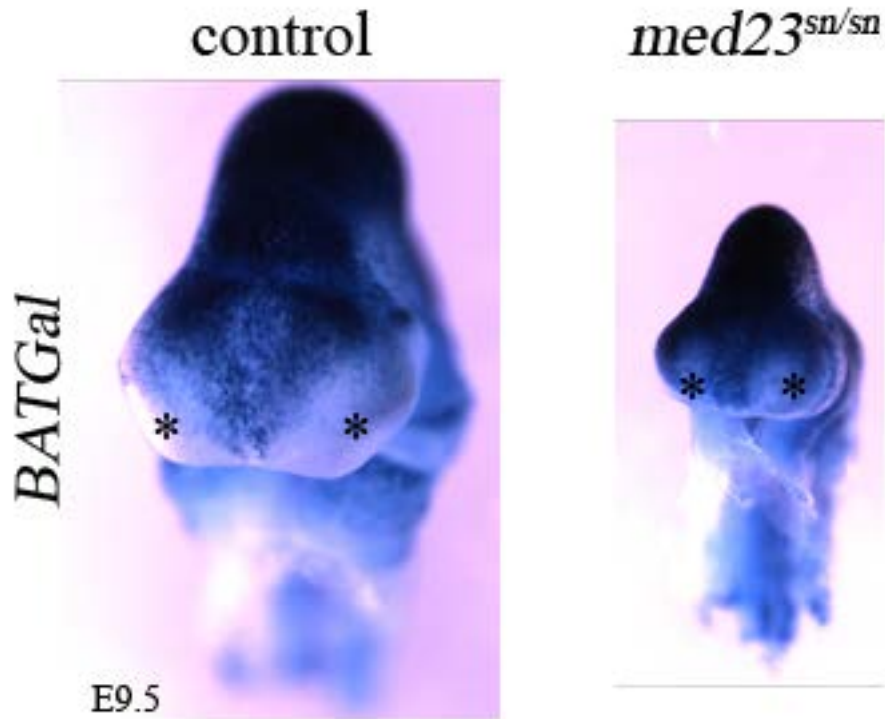
Figure 32: WNT signaling is upregulated and ectopically expressed in the lateral nasal processes of *med23^sn/sn* embryos at E9.5.

Frontal images of control; BATgal and *med23^sn/sn*; BATGal embryos stained for lacZ show an increase in WNT signaling in the frontonasal processes. Lateral nasal processes which are usually devoid of active WNT signaling (*, asterisk, control) show positive lacZ staining (WNT activity) in *med23^sn/sn* embryos (*, asterisk, *med23^sn/sn*).

Overall, the intensity of LacZ staining was highly up-regulated in the mutant embryos. And within the frontonasal process, we did not observe any zones (lateral) that were devoid of WNT signaling (**Figure 32B**). This result is supports an earlier observation made in the lab, where the levels of *dkk-1* transcript, encoding for a WNT signaling inhibitor, were down-regulated in *med23^{sn/sn}* embryos (observed by microarray and qPCR). Increased WNT signaling can affect frontonasal process development, and complete loss of *dkk-1* has been shown to lead to complete absence of anterior structures in mice. Thus, this increased WNT signaling might underlie the frontonasal process defects in *med23^{sn/sn}* embryos.

(Disclaimer: The original *med23^{sn}*; BATGal matings and analysis were done before I joined the lab, by Dr. Lisa Sandell).

VI. Craniofacial defects in *med23^{sn/sn}* embryos are evident at E9.0

In whole mount, *med23^{sn/sn}* embryos are indistinguishable from the wild type littermates before E9.0. At E9.0, most *med23^{sn/sn}* embryos are of similar size in comparison to the wild-type littermates (**Figure 33**), however they have smaller first branchial arch (arrow, and their maxillary-mandibular furrow is reduced in depth. The frontonasal prominence of the control and *med23^{sn/sn}* embryos is also slightly deformed at this time point.

Figure 33: Craniofacial defects in *med23^sn/sn* embryos are evident at E9.0.

DAPI immunostaining of Control and *med23^sn/sn* embryos at E9.0. *med23^sn/sn* embryos are not

smaller in size than their wild-type littermates at E9.0, but do display smaller and abnormal

branchial arch-1 (arrow) at this stage.

Discussion

Loss of MED23 leads to defects specific to the formation of frontonasal process and branchial arch 1 suggesting a specific role for MED23 in anterior development. Here, we tried to characterize the craniofacial defects in *med23*[sn/sn] embryos. Homozygosity of the ENU allele for *med23* leads to mid-gestational embryonic lethality which precludes us from understanding the role for MED23 in skeletogenesis and musculogenesis during craniofacial development, but by E9.5 the frontonasal process and the first branchial arch are already filled with cranial neural crest cells and the blueprint for dorsoventral and proximo-distal patterning is already in place.

We first analyzed if neural crest cells populate the developing frontonasal process and 1st branchial arch in *med23*[sn/sn] embryos. Neural crest cells migrate into the frontonasal process and the 1st branchial arch as early as E8.5[398]. In our observation, when analyzed by neural crest marker expression, by E9.5, wild-type neural crest cells populate the frontonasal process, and 1st and 2nd branchial arches as well as the DRGs in trunk (*data not shown*). In absence of MED23, fewer neural crest cells are observed in the developing frontonasal prominence and branchial arch-1, but as we showed this reduction in neural crest numbers are not due to increased cell death or reduced cell proliferation. We currently do not know how early the neural crest cell defects in *med23*[sn/sn] embryos begin. The defects could be due to

a reduction in the number of crest cells emigrating out of the dorsal neural tube or could be caused due to a lack of survival of crest cells within the frontonasal prominence and branchial arch-1 at earlier stages. The first morphological phenotype of *med23* loss is a very apparent smaller and deformed branchial arch-1 and a very slightly deformed frontonasal process at E9.0. Given this, we suspect that if analyzed we would already be able to see a reduction in crest cell numbers in the frontonasal process and 1st branchial arch of *med23$^{sn/sn}$* embryos at this early stage as well. One thing to bear in mind is that expression analysis can never substitute for lineage tracing. Even though less likely in this case, it is always possible that the crest cells populating the frontonasal process and 1st branchial arch in *med23$^{sn/sn}$* embryos are present but have turned down *crabp1* expression. Smaller size of the frontonasal process and 1st arch in mutant embryos argues against this possibility. Also, using RNA *in situ* hybridization for another neural crest cell marker, we observed a reduced number of *sox10+* neural crest cells migrating out of the neural tube (Chapter 3), suggesting that loss of MED23 might truly affect the formation or migration of neural crest cells into the craniofacial regions. Lineage tracing of neural crest cells (using *Wnt1^{cre}* or *Pax3^{cre}* mouse lines) in presence or absence of *med23* would be able to confirm this.

Genetic manipulation leading to loss of neural crest cell potential to respond to SHH stimuli leads to formation of smaller frontonasal process and branchial arch-1 in mutants[399];

the occurrence of this phenotype however is later in time in comparison to $med23^{sn/sn}$ embryos. Similarly, loss of $fgf8$ from the facial ectoderm has been shown to lead to increased mesenchymal apoptosis in $med23^{sn/sn}$ embryos, which we do not observe at E9.5 and later stages. $fgf8$ expression in the facial ectoderm begins as early as E8.5, and neural crest cells have already migrated into the frontonasal process and 1st branchial arch by then, thus we need to analyze the expression of cell survival factor, $fgf8$ as well as cell proliferation and cell death markers, pHH3 and TUNEL at earlier time points to see if an early hit on neural crest cell population within the craniofacial mesenchyme would lead to smaller frontonasal and arch size in $med23^{sn/sn}$ embryos. Out of the four signaling pathways crucial for craniofacial development, we show that canonical WNT signaling pathway is mis-regulated in $med23$ mutant embryos. Canonical WNT signaling affects species specific facial morphogenesis by specifically activating cell proliferation in regionally restricted fashion. In $med23^{sn/sn}$ embryos, this regional restriction of WNT signaling activity is lost, specifically at E9.5 but not really at half a day earlier, E9.0 when the defect is first observed (see data in Chapter 5). The activity of WNT signaling during craniofacial development is very dynamic[358, 400] WNT signaling up-regulation following homozygous loss of dkk-1 is however also associated with some forebrain patterning defects (assessed by $six3$ expression)[400], a feature that we do not observe in $med23^{sn/sn}$ embryos. However, it does seem that MED23 regulates WNT signaling in a unique

way that does not affect forebrain or facial ectoderm patterning, but still affects frontonasal prominence and branchial arch-1 development. Stabilizing β-catenin specifically in the facial ectoderm, leads to increased BATgal activity in the lateral nasal processes and maxillary and mandibular processes at E9.5, similar to our observation, however it also leads to defects in specification of FEZ and shows no overt morphological defects in frontonasal and 1st arch formation at this early stage[364]. This is contrary to what we observe; however, it has to be kept in mind that we currently have no evidence to conclude that WNT signaling is the only pathway affected during frontonasal and branchial arch-1 development in *med23$^{sn/sn}$* embryos, as we have not yet analyzed downstream targets of FGF8 and BMP pathways in *med23$^{sn/sn}$* mutants. Also, in our mouse line, *med23* is lost in both facial ectoderm and mesenchyme, which would probably affect WNT signaling in both these populations, leading to a different phenotype than what is observed by stabilizing β-catenin in facial ectoderm only.

Chapter 5

MED23 regulates WNT signaling during cranial ganglia

development

Summary

To understand the underlying mechanisms by which MED23 regulates craniofacial, vascular and neural development during mouse embryogenesis, our lab had performed a microarray on wild-type and *med23sn/sn* littermate embryos at E9.5. The microarray was already performed before I joined the lab and insights from *med23sn/sn* transcriptome data were crucial to my understanding of MED23 role. One of the crucial signaling pathways that seemed to be mis-regulated in mutant embryos was WNT signaling. WNT signaling has been shown to be crucial for anterior-posterior axis specification and head development, induction of neural crest cells, formation and differentiation of cranial sensory ganglia and endothelial cell remodeling and differentiation, some of which are disturbed by loss of MED23. The up-regulation of WNT signaling observed in *med23sn/sn* embryos seems to be responsible for cranial sensory differentiation defects as manipulating the levels back to approximate normal partially restores neuronal differentiation in cranial ganglia. This, however, does not diminish craniofacial development or vascular remodeling defects in the mutants, suggesting that they might be WNT signaling-independent.

Introduction

WNT proteins are a family of highly conserved secreted proteins that were first identified for their role in carcinogenesis and fly embryonic development[401, 402]. Mice boast 19 secreted WNT ligands which bind to anyone of its 15 transmembrane protein receptors and co-receptors and activate three types of intra-cellular cascades, that have been historically classified as β-catenin dependent and β-catenin independent (**Figure 34**). Through these intracellular cascades, WNT signaling regulates a multitude of embryonic developmental processes, including specification of embryonic axis formation, body patterning as well as stem cell development[403, 404]. The complexity of WNT signaling is amplified by the presence of various antagonists, like dickkopf related protein 1 (Dkk1), secreted frizzled related proteins (sFRPs), WNT inhibitory factor (WIF), Sclerostin, other agonists like R-spondin family members and Norrin and the presence of WNT signaling modulators (context dependent activators or inhibitors) like Wise, all of which can bind to the various WNT signaling receptors and co-receptors and influence WNT signaling. The various WNT ligands activate β-catenin dependent or β-catenin independent pathways depending upon their cellular and molecular context. Much work has been done over the past two decades to define a WNT ligand–receptor "combinatorial code" that can predict the intra-cellular cascade the ligand-receptor binding activates. Barring certain exceptional circumstances, it is generally believed that WNT ligands,

173
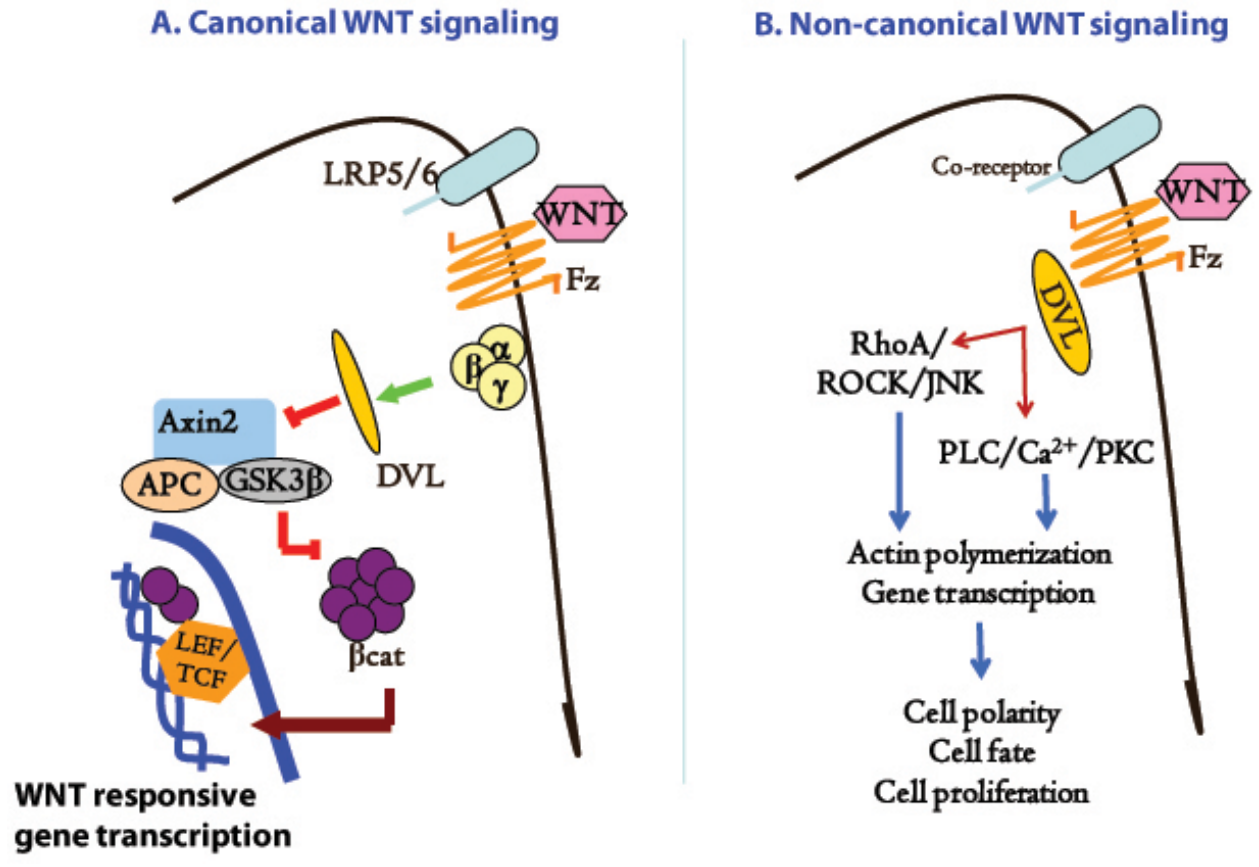
Figure 34: WNT signaling pathways

WNT1, WNT3A and WNT8 activate a β-catenin dependent signaling while ligands, WNT5A and WNT11 typically activate a β-catenin independent pathway.

β-catenin dependent or Canonical WNT signaling

The canonical WNT signaling or WNT/β-catenin pathway is one of the most studied pathways during mammalian embryonic development and is described in **Figure 34A**. β-catenin is a multifactorial protein that interacts with cadherins to maintain apical junctions between cells[405]. Current understanding of subcellular localization of β-catenin suggests that cells have two pools of β-catenin molecules: one that is required for maintenance of cell-cell junctions while another that responds to canonical WNT signaling and translocates to the nucleus. Cross-talk between these two β-catenin pools has been observed but is not completely understood. In absence of WNT signaling ligands, the intracellular pool of β-catenin molecules gets targeted for proteasomal degradation by the 'destruction complex'. The destruction complex comprises adenomatosis polyposis coli (APC), Axin2, casein kinase 1α and (β-catenin phosphorylating enzyme) GSK3β. Interaction between WNT ligands and its transmembrane receptor, Frizzled, along with WNT co-receptors, LRP5 and/or LRP6, leads to inhibition of GSK3β thereby preventing the degradation of β-catenin. Cytoplasmic β-catenin, hence, accumulates and can now translocate into the nucleus, where it serves as a transcriptional co-

activator for transcriptional factors TCF (T-cell factor) and LEF (lymphoid enhancer binding factor) to regulate transcription of WNT signaling target genes[406].

β-catenin independent or Non-canonical WNT signaling

Non-canonical WNT signaling encompasses all those pathways that do not use β-catenin as their transcriptional effector **(Figure 34B)**. Based on the intracellular cascades involved, non-canonical WNT signaling pathways are classified as WNT/JNK pathway (closely related to drosophila planar cell polarity (PCP) signaling), WNT/Ca++ pathway, WNT/ROR2 pathway, WNT/PKA pathway, WNT/mTOR pathway, WNT/Rap1 pathway, WNT/GSK3MT pathway and WNT/αPKC pathway[407]. Signaling through non-canonical WNT pathways may or may not affect the transcriptome of the receiving cells. **WNT/PCP signaling** is one of the best characterized non-canonical WNT signaling pathways and involves activation of small GTPases of Rho family (including RACK, CDC42, RHO, JNK and RHO Kinase). Activation of WNT/PCP signaling is required for establishing cellular polarity and asymmetric distribution of polarized proteins within the cell. During embryogenesis, WNT/PCP signaling components are important during all processes that require polarized cell movements, for example, gastrulation, neural crest migration and cardiac outflow tract development. **WNT/Ca++ signaling**, on the other hand, has also been shown to be important during development for

establishment of dorsoventral polarity, convergent extension during gastrulation and organogenesis (liver, heart and kidney formation)[408].

Role of canonical WNT signaling during embryonic development

The role of WNT/β-catenin signaling during **anterior-posterior axis specification** in mice embryos has been described in earlier sections (Introduction-1). Briefly, WNT/β-catenin signaling is crucial for specification of mesendoderm in a gastrulating mouse embryo and inhibition of canonical WNT signaling is absolutely essential for anterior specification. Later on, however, WNT signals coming from dorsal neural tube influence survival and delamination of the newly formed neural crest cells as they migrate into the embryo to differentiate into craniofacial skeleton, peripheral nervous system derivatives and pigment cells. During **neural crest cell differentiation**, canonical WNT signaling is absolutely essential at two points, one during differentiation of trunk neural crest cells into sensory neurons and later during differentiation of dorsolaterally migrating neural crest cells into melanocytes.

As described earlier, **cranial sensory ganglia** develop from a mixed population of cranial placodes and cranial neural crest cells. The role for WNT signaling in regulation of cranial placode specification and development is multifaceted. Inhibition of WNT signaling early on is required for specification of the pre-placodal region (PPR) – a precursor tissue to all cranial

placodes[79]. Within the neural plate border (NPB) region, the level of WNT signaling determines if the NPB cell will adopt a neural crest fate or a PPR fate and sustained activation of canonical WNT signaling prevents expression of PPR specific gene markers[76]. On the other hand, WNT/β-catenin signaling which has been shown to be active in the ophthalmic branch of trigeminal placode is required by those cells to maintain their placodal identity and differentiate into trigeminal neurons[409]. Canonical WNT signaling has also shown to be required for determining otic placode versus epidermis fate as well as determine otic placode size and regulate otic placode cell survival[410, 411, 412]. Inhibiting canonical WNT signaling in *pax2+* otic placodal cells, reverts their fate back to epidermis while activation of canonical WNT signaling increases the size of the otic placode. On the contrary formation of epibranchial placode is inhibited by persistent WNT/β-catenin signaling, suggesting that the segregation of PPA (posterior pre-placodal area) into otic and epibranchial placodal fates is WNT signaling dependent[79]. Canonical WNT signaling is also later required for both proliferation and differentiation of inner hair cells within the cochlea, which is a derivative of the otic placode[413].

BATGal is a transgenic canonical WNT signaling reporter mouse line, that expresses *lacZ* under the regulation of β-catenin responsive sites. LacZ staining in BATGal mice embryos has shown that canonical WNT signaling is active in **endothelial cells**[359]. Targeted mutations of

many canonical WNT signaling ligands, *wnt2, wnt4, wnt7b* and a canonical WNT signaling receptor, *fzd5* show variable degrees of defects in vascular development[414, 415, 416, 417, 418]. Specifically loss of *wnt2* and *fzd5* leads to embryonic lethality due to yolk sac and placental angiogenesis defects[414, 418]. Overexpression and inhibition of WNT signaling in chick paraxial mesoderm increases vascular density or inhibits angiogenesis respectively[419, 420]. Inhibition of WNT signaling also leads to decreased *flk1+* cells[421] and affects mesoderm specification while forced expression of stabilized β-catenin does not induce mesoderm marker expression, including expression of *flk1*[422], suggesting that canonical WNT signaling is necessary but not sufficient for endothelial cell differentiation. Inactivating β-catenin specifically in the endothelial cell lineage (using *Tie2^cre*) causes defects in vascular remodeling and cardiovascular cushion and valve development leading to embryonic lethality while sustained expression of β-catenin in endothelial cells leads to defects in arterio-venous fate specification through regulation of Notch signaling[423, 424]. WNT signaling is specifically important during CNS angiogenesis. Endothelial cells in the central nervous system form an elaborate network of tight junctions forming the blood-brain-barrier (BBB), which is essential for neural protection[425]. Canonical WNT signaling has been shown to be necessary and sufficient for inducing BBB-characteristics in endothelial cells[426]. Inactivation of β-catenin in *flk1+* cells (*flk1^cre; β-catenin^floxed*) induced defective angiogenesis in central nervous system with

phenotypically normal development in cardiac and hemovascular systems[427] suggesting a crucial role for WNT/β–catenin signaling in maintenance of BBB.

Here, we describe up–regulation of WNT/β–catenin signaling in *med23^{sn/sn}* embryos. We questioned whether this up–regulation of canonical WNT signaling is the underlying cause for the various craniofacial, neural and vascular defects observed in *med23^{sn/sn}* embryos by genetically suppressing WNT signaling in the mutant embryos and observing the phenotype of combined mutants. Interestingly, reducing canonical WNT signaling by loss of a copy of WNT–co–receptor *lrp6* or WNT signaling modulator, *wise*, resulted in a partial restoration of epibranchial neuron differentiation, but not in craniofacial development or embryonic lethality. This suggests to us that MED23 is crucial for regulating canonical WNT signaling during cranial ganglia development, loss of which affects cranial ganglia differentiation in *med23^{sn/sn}* embryos.

Results

I.   Canonical WNT signaling is up–regulated in *med23^{sn/sn}* embryos at E9.5

Microarray analysis of *med23^{sn/sn}* embryos suggested a reduction in *dkk–1* (canonical WNT signaling inhibitor) transcript levels at E9.5. Dr. Sandell, a previous post–doctoral fellow in our lab, confirmed these results by quantitative PCR analysis **(Figure 35)**.
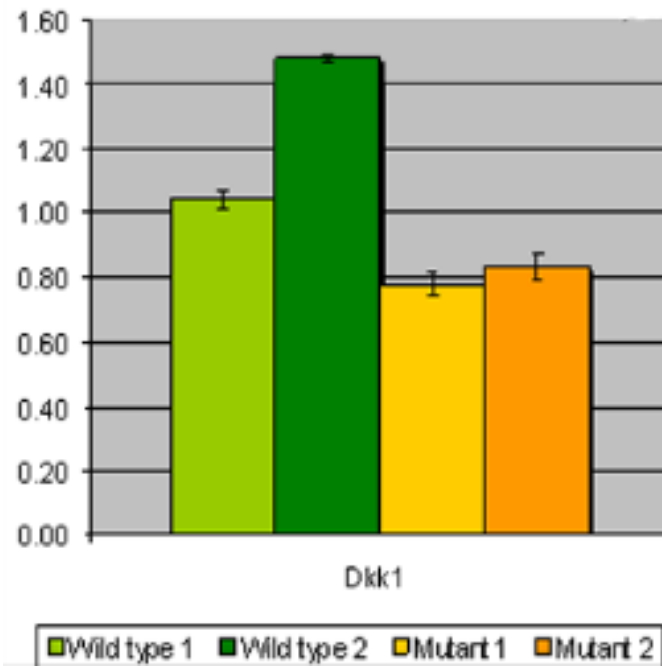
Figure 35 Expression of Dkk-1 (WNT inhibitor) is reduced in *med23^{sn/sn}* embryos.

qPCR analysis on cDNA obtained from E9.5 wild-type (greens) and *med23^{sn/sn}* (oranges) littermate embryos shows a reduction in levels of *dkk-1*.

To investigate the effect of loss of *dkk-1* on WNT signaling *in vivo* in *med23^{sn/sn}* embryos, we took advantage of the BATGal reporter mouse line that expresses *lacZ* under regulation of β-catenin responsive sites. *med23^{sn}; BATGal* mouse line was generated and embryos from their intercrosses were analyzed for LacZ activity from E8.5 to E9.5 (**Figure 36A–H**). As depicted in Chapter 4, lateral frontonasal processes display up-regulated and ectopic canonical WNT signaling in *med23^{sn/sn}* embryos at E9.5. We also observe an overall up-regulation of WNT signaling levels at E9.5 (**Figure 36 G, H**). At E8.5 however, the anterior regions of control and *med23^{sn/sn}* embryos display similar pattern and levels of LacZ staining, suggesting that a normal pattern for canonical WNT signaling in *med23^{sn/sn}* embryos at this stage. Lateral view also shows similar levels of WNT signaling overall in the embryo (**Figure 36A, B**). Anterio-posterior patterning of WNT signaling however is affected by loss of *med23* at this stage (**Figure 36C, D**). In order to find out how early the frontonasal process of *med23^{sn/sn}* embryos shows aberrant WNT signaling, we analyzed *med23^{sn/sn}; BATGal* embryos at E9.0. Interestingly, lacZ staining levels in the frontonasal process and the rest of the *med23^{sn/sn}* embryo were comparable to their wild-type littermates at this stage (**Figure 36 E, F**).

II.   **Genetic modulation of WNT signaling in *med23^{sn/sn}* embryos results in partial restoration of cranial sensory neuron differentiation**

control $med23^{sn/sn}$

E8.5

E9.0

E9.5

A   B

C   D

E   F

G   H
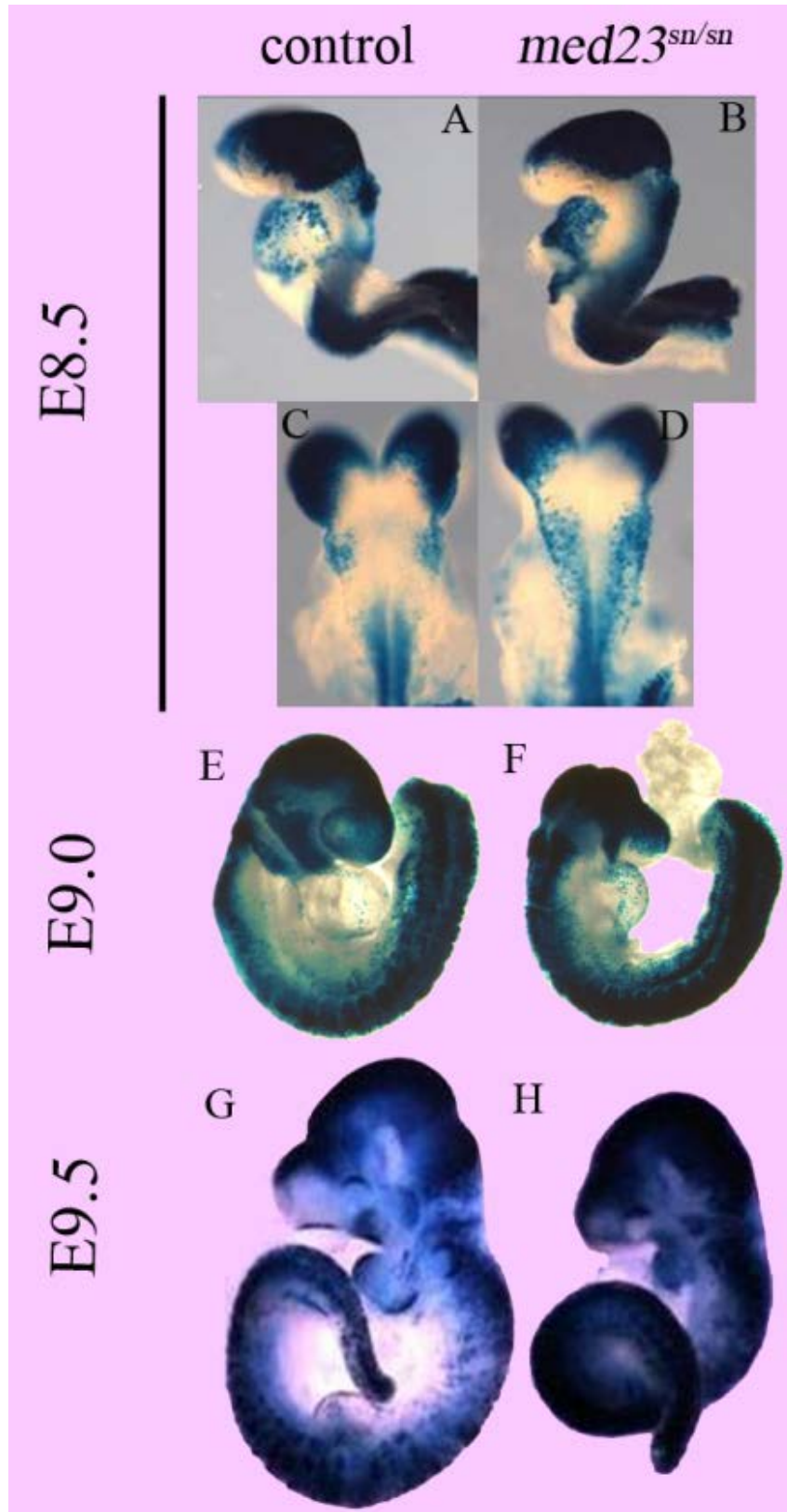
Figure 36: WNT signaling is up-regulated throughout the embryo specifically at E9.5.

A – D, X-gal staining of E8.5 wild-type; BATGal (A, C) and *med23$^{sn/sn}$;* BATGal (B, D) shows

ectopic WNT signaling in the hindbrain regions of mutant embryos (D)

E, F X-Gal staining of E9.0 wild-type; BATGal (E) and *med23$^{sn/sn}$;* BATGal (F) embryos shows

similar levels of WNT signaling between the two embryos

G, H WNT signaling is overall up-regulated in *med23$^{sn/sn}$*; BATGal embryos (H) at E9.5 in

comparison to wild-type; BATGal embryos (G)

Proper dosage of canonical WNT signaling is crucial for many aspects of mammalian embryonic development. We hypothesized that up-regulation of canonical WNT signaling underlies the defects observed in *med23*$^{sn/sn}$ embryos. To test for this, we crossed *med23*^{sn} mouse line to mutants of WNT signaling pathway genes, WNT co-receptor, *lrp6* and WNT signaling modulator, *wise*. *med23*^{sn}*; lrp6*$^{+/-}$ and *med23*^{sn}*; wise*$^{+/-}$ mouse lines were generated and intercrossed to get *med23*$^{sn/sn}$ embryos that had lost one or both copies of *lrp6* and/or *wise* and the double or triple knock-out embryos were then analyzed for neuronal differentiation using antibodies against neuron specific β-tubulin III or TuJ1.

We first analyzed the *lrp6*$^{+/-}$ and *wise*$^{+/-}$ embryos themselves to confirm that loss of a copy of *lrp6* or *wise* does not affect neuronal differentiation. As can be seen in **Figure 37**, embryos that have lost a copy of *lrp6* or a copy of *wise* allele were able to generate and pattern TuJ1+ neurons similar to their wild-type littermates. We then analyzed the TuJ1 expression pattern in the double mutant embryos. **Figure 38A – D** shows the epibranchial region in wild-type, *med23*$^{sn/sn}$, *med23*$^{sn/sn}$*; lrp6*$^{+/-}$ and *med23*$^{sn/sn}$*; wise*$^{+/-}$ embryos at E9.5 respectively. As you can see in the boxed area, mutant embryos that have lost of a single copy of *lrp6* or a single copy of *wise* show some TuJ1+ cells within the epibranchial region (while no
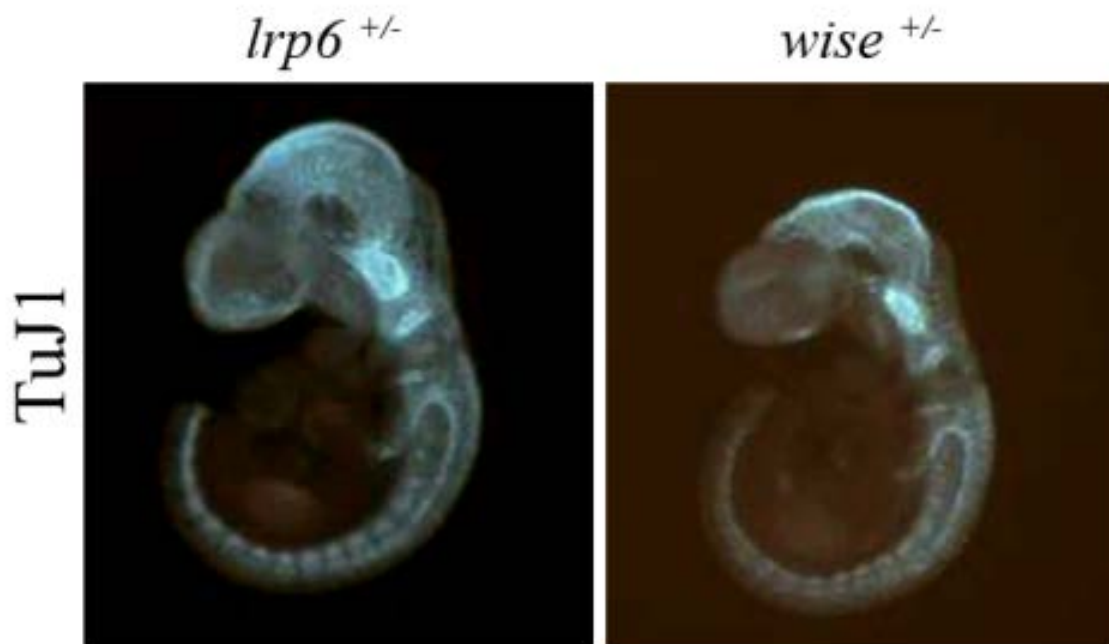
Figure 37: Formation of nervous system is not affected by losing a copy of *lrp6* or a copy of *wise*

TuJ1 immuno-staining of *lrp6*$^{+/-}$ and *wise*$^{+/-}$ embryos at late day E9.5 shows beautiful formation of trigeminal, epibranchial and trunk peripheral nervous system as well as midbrain neuron differentiation.

TuJ1+ is observed in epibranchial region of exclusive $med23^{sn/sn}$ embryos) (**Figure 38B–D**). Interestingly, the morphology of the epibranchial neuron ganglia is slightly different between $med23^{sn/sn}$; $lrp6^{+/-}$ and $med23^{sn/sn}$; $wise^{+/-}$ embryos (**Figure 38C, D**). Mutant embryos that have lost a copy of $lrp6$ seem to coalesce the TuJ1+ cells in a more ganglion like form while the restored TuJ1+ neurons in mutants that have lost a copy of $wise$ are much more dispersedly organized, suggesting that LRP6 and Wise regulate WNT signaling and cranial ganglia differentiation with slightly different mechanisms and might be important for separate aspects of cranial ganglia development.

Since WISE has been shown to be a canonical WNT signaling modulator, such that it can act both as a WNT signaling inhibitor or an activator depending on the cellular and molecular context, similar phenotype of loss of $lrp6$ and loss of $wise$ in $med23^{sn/sn}$ embryos was intriguing and suggestive of a WNT signaling activator role in cranial neuron differentiation context. To confirm this, we analyzed the neuronal differentiation in triple mutants carrying $med23^{sn/sn}$; $lrp6^{+/-}$; $wise^{+/-}$ or $med23^{sn/sn}$; $lrp6^{+/-}$; $wise^{-/-}$ genotype (**Figure 38A–D**). Interestingly, the restoration of TuJ1 immuno-staining and cranial neuron differentiation in the triple mutants is similar to that of both double mutants indicating that in cranial neuron differentiation both WISE and LRP6 are required for positive canonical WNT signaling.
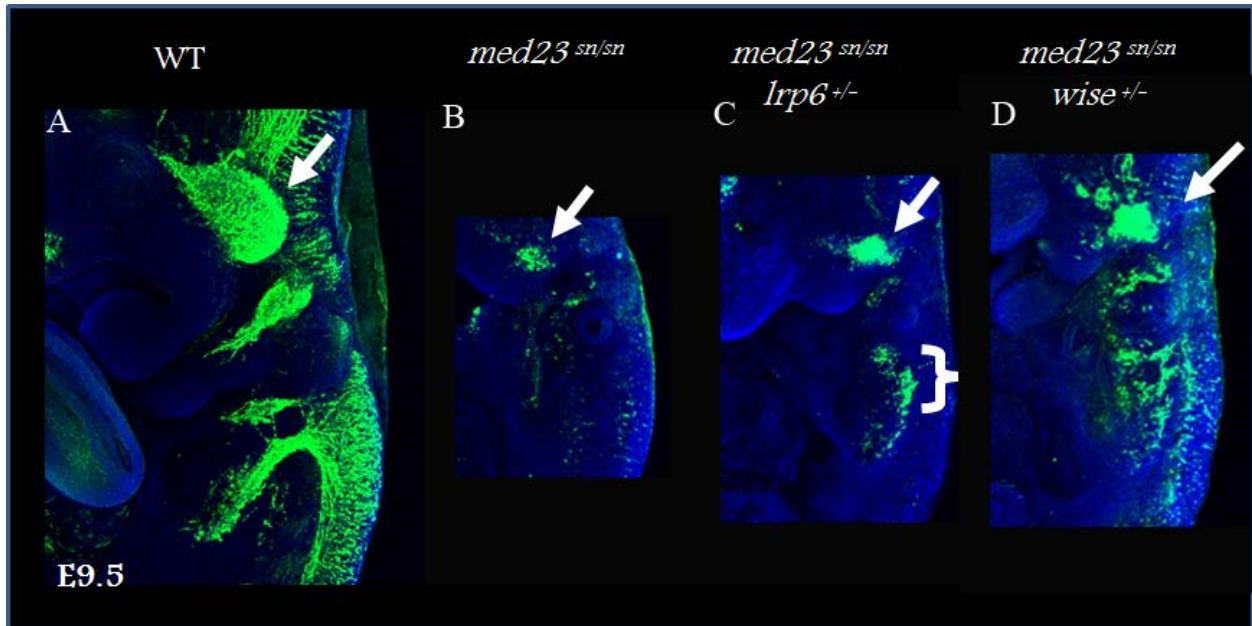
Figure 38: Modulation of canonical WNT signaling partially restores epibranchial ganglia development in *med23*^{sn/sn} embryos.

A)Wild-type E9.5 embryo, B) *med23*^{sn/sn} E9.5 embryo, C) *med23*^{sn/sn} embryo which has lost a copy of *lrp6*, and D) *med23*^{sn/sn} embryo that has lost a copy of *wise*, were all immuno-stained for TuJ1. Losing a copy of *lrp6 or* wise restores neuronal differentiation in epibranchial region of *med23*^{sn/sn} embryo (C, D).
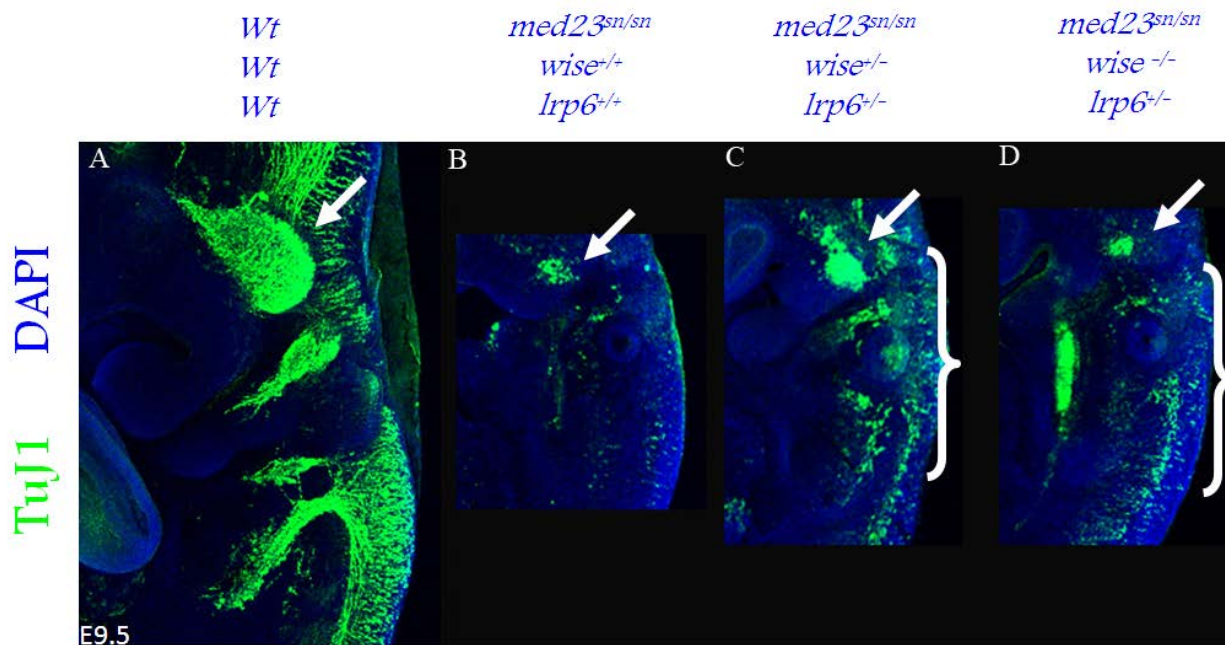
Figure 39: Wise and Lrp6 activate WNT signaling during cranial ganglia formation.

Combined loss of *lrp6* and *wise* (C, D) gives similar phenotype to loss of *lrp6* only (Figure 37, C) suggesting that in cranial ganglia formation Wise activates WNT signaling.

III.   Partially restored cranial neurons in *med23^{sn/sn}; wise^{+/-}* embryos express *neurod1*

Since loss of a copy of *wise* gave us much more widespread restoration of cranial neuron differentiation, we decided to do our further analysis with *med23^{sn/sn}; wise^{+/-}* double mutant embryos only. It was important for us to know whether manipulation of canonical WNT signaling by losing a copy of WISE (or LRP6) restored TuJ1 immuno-staining through restoration of neuronal differentiation of cranial placodal cells. To that effect we analyzed the expression of *neurod1*, neuronal differentiation marker of cranial placodal cells, in control and *med23^{sn/sn}; wise^{+/-}* embryos at E9.5. As expected, similar to TuJ1 immuno-staining, double knock-out embryos, display *neurod1+* cells in the epibranchial region at E9.5 (**Figure 40**), while no such cells were observed in this region in just *med23^{sn/sn}* embryos (Figure 24). Also similar to TuJ1immuno-staining, the pattern of *neurod1+* cells in the epibranchial region of the double knock-out embryos is very dispersed and not in a coalesced ganglion form.

IV.   Early placodal marker expression is still not maintained in *med23^{sn/sn}; wise^{+/-}* embryos

Partial restoration of neuronal differentiation and *neurod1* expression in *med23^{sn/sn}; wise^{+/-}* embryos suggests that WNT signaling regulation is crucial for cranial neuron differentiation. Our expression marker analysis has shown to us that MED23 is required at multiple steps during cranial placode development. However, the time-point during cranial placode
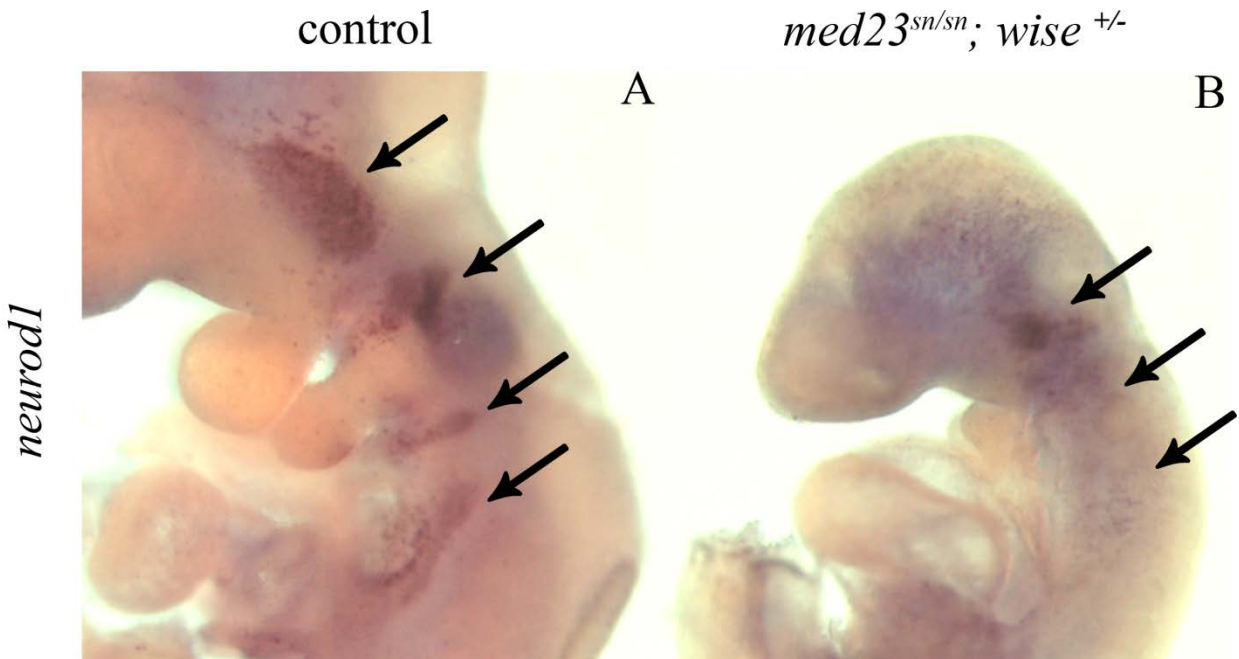
Figure 40: Epibranchial neurons restored by modulating of WNT signaling express *neurod1*

Losing a copy of *wise* in *med23^{sn/sn}* embryos restores *neurod1* expression in the epibranchial region (Compare with complete lack of *neurod1+* cells *med23^{sn/sn}* embryos in Figure 24)
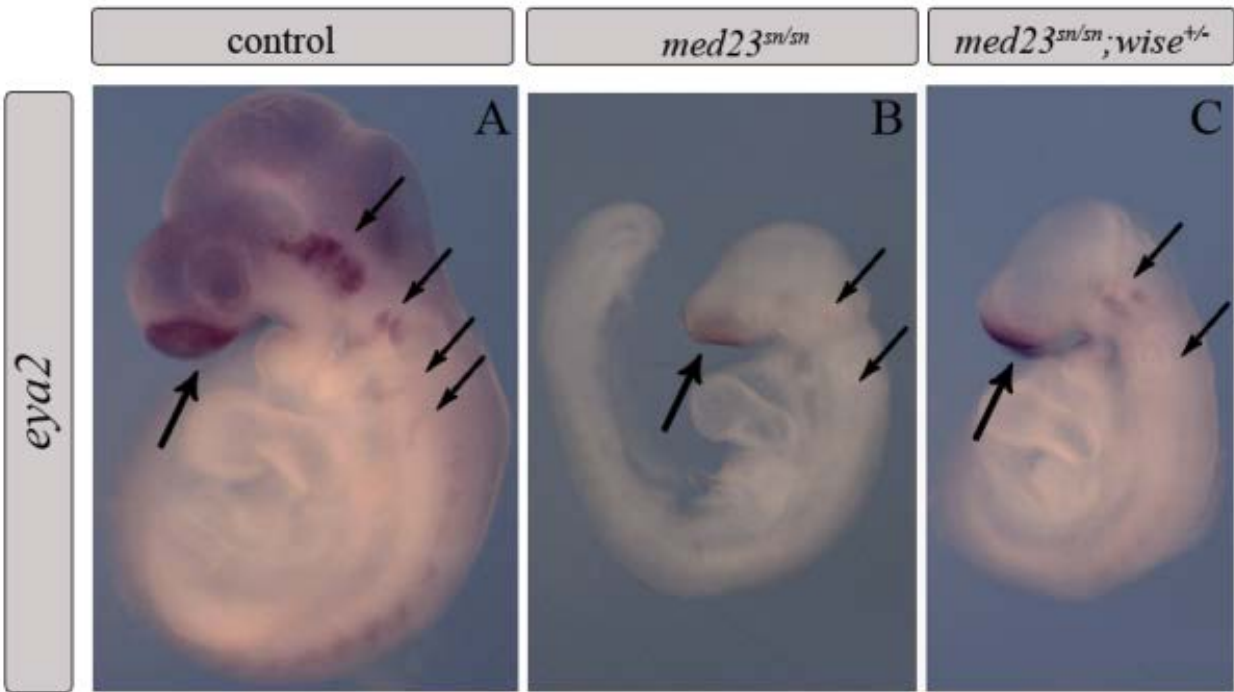
Figure 41: Defects in maintenance of early PPR gene expression of *med23^sn/sn* embryos are not restored by modulating the WNT signaling levels.

Wild-type (A), *med23^sn/sn* (B) and *med23^sn/sn; wise^+/-* embryos were analyzed for expression of PPR marker *eya2*. Fewer *eya2+* cells are present in the trigeminal and epibranchial placodes of both *med23^sn/sn* and *med23^sn/sn; wise^+/-* embryos when compared to wild-type *eya2* expression.

development, when loss of a *wise* copy restores neuronal differentiation, was still unknown. To that effect, we decided to analyze the earliest marker of cranial placode development. As showed earlier, *eya2* expression in cranial placode development begins as soon as pre-placodal region (PPR) is specified and is maintained and required throughout cranial placode development. At E9.5, *eya2* expression can still be observed in the cells in olfactory, trigeminal and some epibranchial placodal cells of wild-type embryos (**Figure 41A**) while in *med23*[sn/sn] embryos only a few cells within the trigeminal placode are *eya2+* (**Figure 41B**). Similar to *med23*[sn/sn] embryos, even the *med23*[sn/sn]; *wise*^{+/-} embryos display a reduced number of *eya2+* in the trigeminal placode and absence of *eya2+* cells in the epibranchial regions (**Figure 41C**), suggesting that even though the double mutants partially restore neurogenesis, loss of a single copy of *wise* does not completely restore cranial placode development in *med23*[sn/sn] embryos.

V.   *wise* expression itself is unaffected in *med23*[sn/sn] embryos

Wise modulates WNT signaling by binding to WNT co-receptors, Lrp5 and Lrp6[428, 429]. *In vitro*, Wise has been shown to bind to WNT antagonist Lrp4[430], transcript levels of which are reduced in *med23*[sn/sn] embryos as observed by the microarray done on wild-type and *med23*[sn/sn] E9.5 littermates. We wanted to analyze if similar to *lrp4*, Wise expression was

VI.
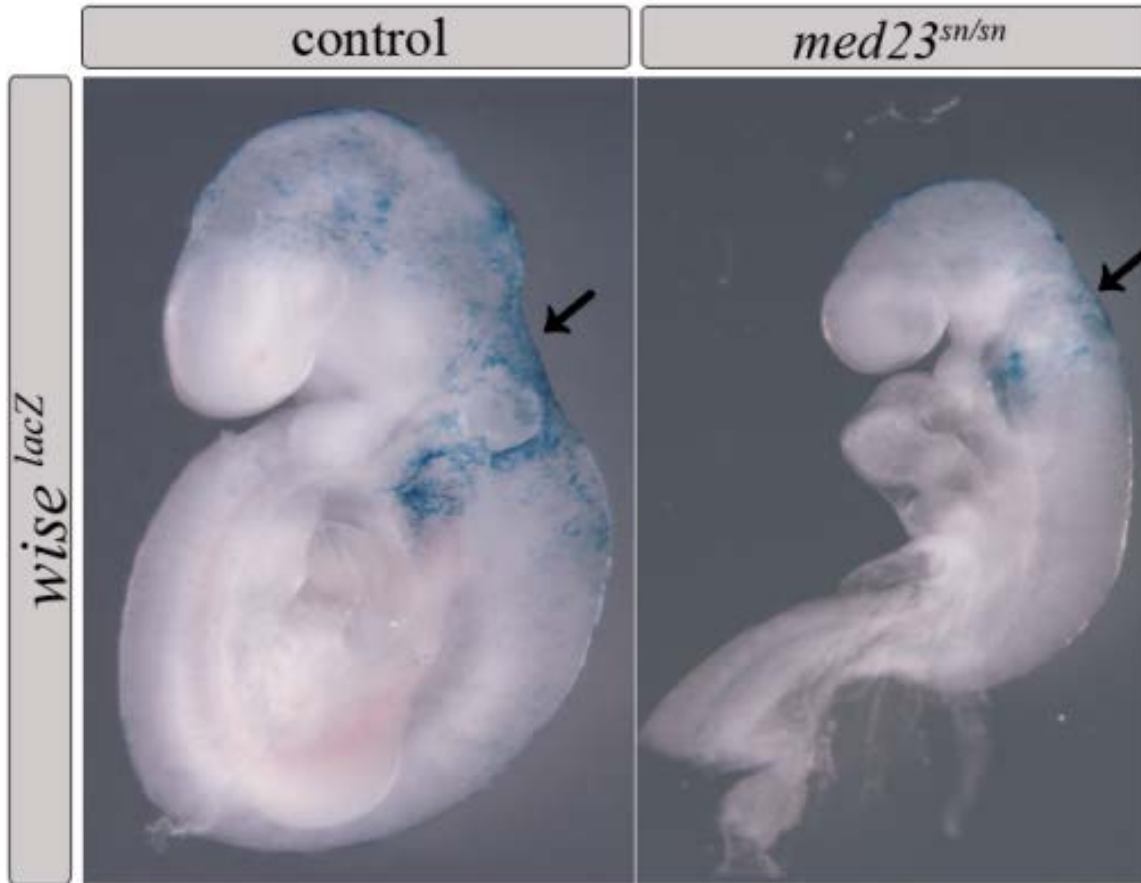
Figure 42: Loss of *med23* does not affect *wise* expression

Using transgenic *wise^{lacZ}* mouse line, we determined that wild-type and *med23^{sn/sn}* embryos

display similar *wise* expression pattern.

affected in *med23^{sn/sn}* embryos. Dr. Youngwook in Krumlauf lab generously shared the *Wise^{lacZ}*

transgenic mouse line with us which we then used to mate with *med23^{sn}* to generate *med23^{sn}*;

wise^{lacZ} line. Animals from this line were intercrossed and embryos were analyzed for LacZ

activity (indicating *wise* expression) in wild-type and *med23^{sn/sn}* embryos at E9.5. Earliest

expression of *Wise*, as detected by lacZ staining, was observed at E8.5 in the trunk region (*data

not shown*). At E9.5, wild-type embryos display lacZ activity specifically within the

epibranchial region of the developing embryo. This epibranchial region specific expression of

wise is maintained even in *med23^{sn/sn}* embryos (**Figure 42 A, B**) suggesting that loss of MED23

does not affect *wise* expression.

VII. Manipulation of canonical WNT signaling does not restore Frontonasal process formation and embryonic lethality in *med23^{sn/sn}* embryos

Loss of *dkk1* affects anterior development in mice embryos due to aberrant increases in

canonical WNT signaling[16]. Hence, our hypothesis was that the craniofacial defects observed in

med23^{sn/sn} embryos were due to reduced *dkk1* transcript and aberrant WNT signaling in the

frontonasal prominence. Interestingly, manipulating the levels of WNT signaling in the mutant

embryos, by loss of a copy of *lrp6* or a copy of *wise* did not restore frontonasal prominence

defects observed in *med23^{sn/sn}* embryos (**Figure 43**). Knock-out embryo phenotypes for *lrp6*

and *wise* are both milder than what is observed in *med23^{sn/sn}* embryos. Mouse embryos null for

lrp6 have a smaller skull with a crooked nose while complete loss of *wise* affects tooth

development and results in palatal defects[431, 432]. Aberrant canonical WNT signaling might still

be the cause for frontonasal prominence and first arch defects in *med23^{sn/sn}* embryos; however

these results suggest that aberrant WNT signaling in frontonasal process of *med23^{sn/sn}* embryos

is not mediated through LRP6 or Wise. Loss of a copy of *lrp6* or a copy of *wise* is also unable to

restore the embryonic lethal phenotype of caused by loss of MED23 as the double knock-out

embryos die at around E10.5 as well.

Discussion

Mis-regulation of canonical WNT signaling seems to be at the heart of cranial ganglia

defects in *med23^{sn/sn}* embryos. Even though both loss and gain of canonical WNT signaling

have been shown to affect cranial ganglia development and differentiation, the detailed role for

WNT signaling during each step of cranial placode development and cranial sensory neuron

differentiation is not clearly understood in mice or other model organisms. Here we show that

MED23 is important for regulation of WNT signaling levels during mouse embryogenesis, and

this dosage dependent regulation of WNT signaling is crucial for appropriate cranial ganglia

development. WNT signaling activation is temporally regulated during mouse development,

and loss of MED23 is specifically crucial at E9.5. Currently, we do not know if there is a tissue specific up-regulation of WNT signaling within the *med23^{sn/sn}* embryos in comparison to their wild-type littermates and whether MED23 is cell-autonomously required for regulation of WNT signaling. Analysis of conditional mutants should answer some of these questions. Regulation of WNT signaling by Mediator subunit is not unprecedented. Another Mediator subunit, MED12 has also been shown to regulate WNT signaling during mouse embryogenesis[216]. MED12 has been shown to bind to β-catenin *in vivo*, but whether this binding event is crucial to its regulation of WNT signaling is not known[182]. Such a binding has not been described for MED23, and right now we think that MED23-mediated regulation of WNT signaling might be through an indirect mechanism.

Wise was identified as a conserved WNT modulator that interacts with WNT signaling co-receptors LRP5 and LRP6[433, 428, 429]. Animal cap experiments in *Xenopus* have shown that Wise can also act as a WNT signaling activator, where ectopic expression of Wise lead to an altered A/P patterning phenotype that was similar to the one observed by over-expression of canonical WNT signaling ligand, *wnt8a*[433]. On the other hand, the interaction between Wise and LRP5 and 6 has been shown to inhibit WNT signaling during tooth patterning[434] and bone development[428, 429]. In our context, loss of *lrp6* or *wise* or both together, in embryos already carrying a mutation for *med23*, lead to cranial neuron differentiation suggesting that they

might be acting in the same pathway. Loss of a *wise* allele seems to suppress WNT signaling levels similar to loss of a *lrp6* allele. In mice embryos, this is the first indication of Wise acting as a WNT signaling activator *in vivo*. The morphology of the cranial ganglia in *med23$^{sn/sn}$; lrp6$^{+/-}$* and *med23$^{sn/sn}$; wise$^{+/-}$* is slightly different. WNT signaling modulation through loss of *lrp6* seems to confer a more ganglion like morphology in *med23$^{sn/sn}$* embryos. Work in chick embryos has shown a role for *wise* in trigeminal ganglion coalescence[435] suggesting that this loss of ganglion like morphology in *med23$^{sn/sn}$; wise$^{+/-}$* embryos could be a compound effect of aberrant WNT signaling and loss of *wise*. Overall, this suggests a possibility that LRP6 and Wise-mediated canonical WNT signaling might be required for coalescence of epibranchial ganglia.

There are multiple possibilities as to why double knock-out embryos display a restoration of TuJ1+ and *neurod1+* cells but not that of *eya2+* cells in the epibranchial region. One possibility is that MED23 regulates the various steps of cranial placode development and differentiation via separate mechanisms and mis-regulation of canonical WNT signaling specifically affects only the neuronal differentiation of the cranial ganglia. On the other hand, this disparity in restoration might be more due to the specific pattern and timing of expression of *wise* itself; *wise* expression in the epibranchial region of mouse embryos does not begin until E9.5, a time point by which the various cranial placodes have already been specified. Thus loss

of *wise* in *med23^{sn/sn}* embryos does not alter WNT signaling levels until after cranial placode specification and hence, the early defects in cranial placode development (like loss of *eya2* expression) can no longer be restored. Unlike *wise, lrp6* is expression in the developing mouse embryo has been observed as early as E7.5[14]. It would be interesting to see the expression pattern of *eya2* in *med23^{sn/sn}; lrp6^{+/-}* embryos and compare it to that of *med23^{sn/sn}; wise^{+/-}* embryos. A lack of normal cranial placode *eya2* expression pattern in *med23^{sn/sn}; lrp6^{+/-}* embryos would suggest that LRP6 mediated canonical WNT signaling does not regulate early steps of cranial placode development.

Lack of frontonasal process defect or embryonic lethality rescue by the double mutants suggests that disruption of LRP6 and Wise mediated WNT signaling is not the cause for these defects in *med23^{sn/sn}* embryos, but it does not rule out the involvement of other WNT signaling co-receptors and modulators. Overall, this work suggests that MED23 mediated regulation of canonical WNT signaling is required for multiple steps of cranial ganglia development.

Discussion

Mediator has been called an integrative hub of transcriptional regulation. The evidence for role of Mediator complex in embryonic development comes from mutation analysis. Specific Mediator subunits have been shown to regulate transcription of specific set of genes and control cell and tissue differentiation. This work describes the role for one such Mediator subunit, MED23, which seems to be required for regulation of craniofacial, vascular and neural development specifically.

MED23 is a context dependent regulator of genes

The human MED23 subunit was identified as a factor required for adenovirus E1A mediated transcription[436]. Over the past decade a role for MED23 in regulation of Insulin- and Ras- signaling mediated gene transcription has emerged[175, 177, 297]. Insulin signaling induces adipogenesis in wild-type mouse embryonic fibroblasts. This *in vitro* differentiation of adipocytes from MEFs was reduced in absence of *med23* as MED23-deficient Mediator complex is unable to induce transcription of a crucial adipogenesis gene, *egr2*. Interestingly, *egr2* gene activation requires binding of MED23 to phosphorylated form of ELK1, phosphorylation of which is regulated by Insulin signaling[175]. Alternatively, MAPK signaling-mediated ELK1 phosphorylation and MED23-pELK1 interaction induces expression *egr1*[140, 176]. Epigenetic marks for active gene transcription as well as Pol II and general transcription factor

binding at *egr1* locus in MAPK-induced *med23*^{-/-} cells was equivalent or only slightly reduced

in comparison to the MAPK induced wild-type cells; however the transcription of *egr1* gene

itself was drastically reduced in cells devoid of MED23[140]. Recently, it has been shown that

MED23 regulated transcription of *egr1* is also regulated at the transcript elongation step.

med23^{-/-} cells are unable to recruit CDK-9 and through it, the elongation factor, P-TEFb, and

thus *egr1* expression is inhibited[437]. Ras-signaling mediated MED23-pELK1 binding is crucial

for proliferation and tumorigenicity of lung cancer cells[438]. Another transcription factor,

C/EBPβ has also been shown to interact with MED23 and this interaction is capable of

transcription activation, however the role for this interaction *in vivo* has not been

established[177].

One important thing to bear in mind is that all the above studies have been performed

in vitro and whether these interactions and gene transcription specific roles of MED23 hold

true within a live animal or a developing embryo have not been analyzed. In our system,

microarray analysis shows that *med23*[sn/sn] embryos display a down-regulation of *egr-1*

transcript levels by 9-fold compared to wild-type. This provides us a good validation that our

med23[sn] mutation causes similar defects to the existent *med23*[-] allele. Along with many other

developmental defects, *egr1* mutation in mice also causes a defect in growth hormone levels,

which might explain the smaller size of *med23*[sn/sn] embryos as compared to wild-type
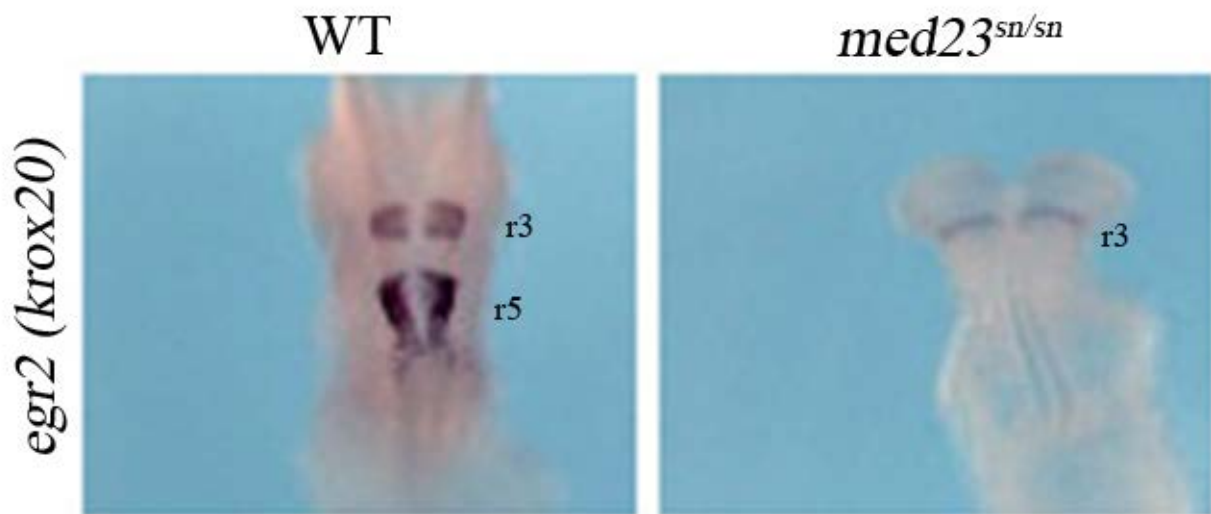
Figure 44: *egr2 (krox20)* is expressed in the hindbrain of *med23^{sn/sn}* embryos

Wild-type and *med23^{sn/sn}* embryos display hindbrain specific expression of *egr2*. Lack of *egr2* expression in r5 is due to the developmental delay in *med23^{sn/sn}* embryos. Analysis of *med23^{sn/sn}* embryos at a later stage displays *egr2* expression in both r3 and r5 (*data not shown*).

littermates. However, transcript levels of *egr2*, whose transcription has also been shown to be regulated by MED23 *in vitro*, do not seem to be altered in mutant embryos, by our microarray analysis. During early mouse development, *egr2 (krox20)* is an important marker of hindbrain patterning and has been shown to be crucial for maintaining the rhomobomere 3 and 5 identities. Our analysis of *egr2* transcription, suggests that during rhombomeres patterning, *med23^{sn/sn}* embryos express *egr2* in the right domains at E8.5 (**Figure 44A, B**; Note: *med23^{sn/sn}* embryo in B is developmentally delayed than the wild-type embryo in A, hence the r5 specific expression of *egr2* is not observed here, but I have observed it in late-stage *med23^{sn/sn}* embryos (*data not shown*)). This suggests to us that the requirement of MED23 for *egr2* expression is very specific to the developmental context; it is required during adipogenesis (as published) but does not seem to be required during hindbrain patterning. How this context specific role for MED23 is achieved can right now only be speculated. Since MED23 is ubiquitously present in all cells at all points of time, this context specificity could be achieved either by a spatial and/or temporal specific expression of a co-factor or by presence or absence of different signaling pathways at different contexts. It would be interesting to find out if ELK1 is expressed in the hindbrain tissues at the time of hindbrain patterning and see if MED23-ELK1 interaction is present at that time point.

This context dependent regulation of gene expression by MED23 is also evident in our analysis of vascular smooth muscle cells and could be species or time dependent as well. Morpholino injections inhibiting *med23* displayed an amplification of vascular smooth muscle differentiation at the cost of adipogenesis in zebrafish embryos[297]. Mechanistically, MED23 was shown to bind to ELK1-SRF complex at the smooth muscle cell differentiation genes (*acta2, sm22alpha*) and repress their expression directly and loss of *med23* abrogated this repression. In contrast, my analysis on *med23^{sn/sn}* embryos at E9.0 suggests that the expression of SM22alpha, in the developing vascular smooth muscle cells is unaffected in *med23^{sn/sn}* embryos as compared to the wild-type littermates. If there were a way to extend the viability of *med23^{sn/sn}* embryos beyond E10.5, then we might be able to analyze whether this smooth muscle cell versus adipocyte role of MED23 is conserved between zebrafish and mice embryos. Mis-regulation of anterio-posterior pattern of WNT signaling in *med23^{sn/sn}* embryos at E8.5 but not at E9.5 or up-regulation of WNT signaling levels in the frontonasal process of *med23^{sn/sn}* embryos at E9.5 but not at E8.5 or E9.0 is yet another example of how the context influences the MED23 requirement.

Summary of Results and Future experiments: MED23 is required for craniofacial, vascular and cranial placode development

This work highlights the role of MED23 containing Mediator complex in various aspects of embryonic development. MED23 is specifically required in development of frontonasal process and branchial arches, vascular remodeling and peripheral nervous system. Through comprehensive analysis **frontonasal process development**, I have shown that the frontonasal process and branchial arch development defects observed in *med23^{sn/sn}* embryos do not reflect any defects in forebrain patterning or SHH signaling or reduction in cell proliferation or increased cell apoptosis. There is however a reduction in the number of neural crest cells entering the frontonasal process and first branchial arch in mutant embryos in comparison to wild-type, which might be the cause for the smaller FNP and 1st arch in mutants. This lack of neural crest cells could be due a cell autonomous or a non-cell autonomous requirement of *med23*. Future experiments designed to excise *med23* specifically out of neural crest cell lineage, using Wnt1-Cre line should be able to tell us more about the cell-autonomous role of MED23 in neural crest cells and craniofacial development.

During **vascular development**, MED23 seems to be required for vascular remodeling and not for *de novo* vascular development. Specifically there is a defect in the expression of genes encoding endothelial cell-cell junction markers. Knock-down of VE-Cadherin in human umbilical cord vein cells (HUVEC) leads to disruption in cell-cell junction formation, increased responsiveness to VEGF and failure to halt angiogenic sprouting[439]. Over-sprouting of

endothelial tubes is associated with increased vascular permeability and failure to suppress sprouting may lead to vascular remodeling defects. Electron microscopy has shown that redistribution of VE-Cadherin away from the endothelial cell-cell junctions of already formed blood vessels causes blebbing of endothelial cells and formation of gaps within the endothelial layer[440]. *med23^{sn/sn}* embryos show normal expression of endothelial adherens junction marker, *ve-cadherin* at E9.0, but this expression is reduced compared to wild-type embryos at E9.5. In future, I would like to analyze the endothelial cell-cell junctions in *med23^{sn/sn}* embryos using electron microscopy to see if the reduced expression of junctional proteins at E9.5 results in disrupted endothelial cell-cell junctions. The initial the formation of vascular smooth muscle cells does not seem to be affected in mutant embryos. It is difficult to say right now whether the endothelial cell defects in *med23^{sn/sn}* embryos underlie their embryonic lethal phenotype and to analyze for that I plan to use conditional deletion of *med23* specifically within the endothelial cell lineage (by Tie2-Cre line). Endothelial cell specific stabilization of β-catenin leads to defects in vascular remodeling, elongation of intersomitic vessels and loss of venous identity[424]. Mutations in other canonical WNT signaling components (Frizzled-5, Norrin and Lrp) show a role for WNT signaling in vascular growth and endothelial cell-mural (support) cell interaction. Ectopic activation of Frizzled/Lrp signaling leads to defects in embryonic angiogenesis and mid-gestational embryonic lethality[441,442]. Keeping these in mind, I expect

Tie2-Cre; *med23^{floxed/floxed}* embryos to have increased angiogenic sprouting, defects in cell–cell junction and disorganized vasculature which can be investigated by endothelial junction marker expression and ink injection.

MED23 is important for multiple aspects of **cranial ganglia development**, from maintenance of early cranial placode markers to coalescence of segregated cranial placodal cells to neuronal differentiation. MED23-mediated regulation of WNT signaling seems to be crucial for the neuronal differentiation of cranial ganglia. We do not know whether this requirement for MED23 in cranial placode development is cell autonomous. Excising *med23* specifically out of pre-placodal ectoderm or *ex vivo* culture and differentiation of pre-placodal region from *med23^{sn/sn}* embryos into cranial sensory neurons would be the best way to figure out the role for MED23 in cranial placode development. However, no such genetic tool or cell culture protocol is currently available for use. The Wnt1-Cre; *med23^{floxed/floxed}* mice analysis can tell us if the cranial ganglia defect observed in *med23^{sn/sn}* embryos are due to lack of support cells (glia) which differentiate from neural crest. Lack of cranial ganglia defect in Wnt1-Cre; *med23^{floxed/floxed}* embryos would suggest (but not prove) a placode specific role for MED23. Reduction in WNT signaling levels in *med23^{sn/sn}* embryos using genetic mutants can partially restore cranial neuron differentiation in *med23^{sn/sn}* embryos; however, in future it would be interesting to see the actual extent of WNT signaling in the double mutant mice.

Crossing BATGal mouse line into *med23*$^{sn/+}$*; wise*$^{+/-}$ or *med23*$^{sn/+}$*; lrp6*$^{+/-}$ mouse line would display the spatiotemporal pattern of WNT signaling in the double mutants. In spite partial restoration of cranial neuron differentiation, the double mutants do not show any rescue of frontonasal process defect. Combining this with our knowledge that *wise* expression in the epibranchial placodes is seen only after E9.0, I expect WNT signaling levels in *med23*$^{sn/sn}$*; wise*$^{+/-}$*; BATgal* embryos to be more close to wild-type; BATgal embryos in the epibranchial region but still aberrant in the frontonasal process at E9.5. Alternatively, ubiquitous expression of *lrp6* during mouse embryogenesis has been reported[443], but *med23*$^{sn/sn}$*; lrp6*$^{+/-}$embryos still do not display a restoration of frontonasal process development. *med23*$^{sn/sn}$*; lrp6*$^{+/-}$ embryos might reduce WNT signaling at levels lower than *med23*$^{sn/sn}$ embryos, but not enough to restore frontonasal process development. Involvement of Lrp6 and Wise with pathways other than canonical WNT signaling has been suggested[444]. A rare possibility is that the double mutants restore cranial ganglia via modulation of signaling pathways other than canonical WNT signaling. In such a scenario no restoration of WNT signaling levels in triple mutants (*med23*$^{sn/sn}$*; lrp6*$^{+/-}$*; BATgal* or *med23*$^{sn/sn}$*; wise*$^{+/-}$*; BATgal*) would be observed.

MED23 has a crucial requirement during the 12-hour E9.0 – E9.5 time window during mouse embryogenesis

One of the salient features of the mutant embryos is their smaller size at E9.5, which could be result of reduced nutritional support indicating vascular defects. Lack of nutrition could also be indicative of lack of proper placental development, analysis of which in the *med23^{sn/sn}* embryos would be really informative. Just half a day earlier, however, the size of mutant and wild-type littermate embryos is comparable, indicating that MED23 has a crucial requirement during that time window. This time window from **E9.0 to E9.5** seems to be crucial not just for size determination and overall growth, but also for maintenance of cell-cell junctions in blood vessels. Immuno-staining for Pecam-1, marker of endothelial cell-cell junctions, suggests formation of abnormal but nevertheless present vascular network, however, the lack of other endothelial cell-cell junction marker gene expression (*ve-cadherin* and *claudin5*) suggests that this vascular network might not be robust. Interestingly, when analyzed half a day earlier, the expression of endothelial cell-cell junction marker, *ve-cadherin* was not reduced in mutant embryos compared to wild-type littermates. Whether this normal expression pattern of *ve-cadherin* at E9.0 is actually translated into normal adherens junction formation between endothelial cells remains to be analyzed. It can be speculated that loss of endothelial cell-cell junctions would result in leaky blood vessel formation which would not function properly to transport nutrients and oxygen to the developing tissues. Ink injection into the blood vessel network of wild-type and *med23^{sn/sn}* embryos would be a simple yet

informative experiment to test their leakiness. This crucial requirement of MED23 from E9.0 to E9.5 is also observed in our analysis of WNT signaling activity. Our experiments suggest that MED23 might be specifically required for regulation of WNT signaling dosage between E9.0 to E9.5. Reporter activity experiments indicate that the levels of WNT signaling are up-regulated in $med23^{sn/sn}$ embryos at E9.5. However, the overall LacZ activity (indicative of active WNT signaling) at E9.0 does not seem to be up-regulated in mutant embryos in comparison to wild-type littermates. This mis-regulation of WNT signaling leads to defects in cranial ganglia differentiation, which can be restored in $med23^{sn/sn}$ embryos by genetic inhibition of other WNT signaling pathway genes. Canonical WNT signaling has been shown to be important for cell adhesion while β-catenin (canonical WNT signaling effector) also has roles in cell adhesion and has been shown to interact with adherens junction proteins, suggesting that the up-regulation of WNT signaling at E9.5 and the aberrant expression of endothelial cell-cell junctions also observed at E9.5 could be related.

It would be great if we could analyze the transcriptome of $med23^{sn/sn}$ embryos at E9.0 and E9.5 and compare them to each other. One other way to understand this time critical role for MED23 during embryogenesis would be temporal excision of $med23$ at E9.0. We have already generated the ERT2-Cre; $med23^{floxed}$ mouse line, which will induce excision of $med23$ following Tamoxifen oral gavage. It would be interesting to see the phenotype of ERT2-Cre;

med23^{floxed/floxed} embryos and analyze if their frontonasal process, vascular development and WNT signaling levels are affected similar to *med23^{sn/sn}* embryos.

Mediator and WNT signaling

This work shows a requirement for MED23 in regulation of WNT signaling in mice embryos, a process that is critical for cranial ganglia development. Another Mediator subunit, MED12 has also been shown to regulate WNT signaling during embryonic development, which leads to severe defects in axis elongation, neural tube closure, somitogenesis and cardiac development[216]. Interestingly, in *med12* loss of function mutants, activation of canonical WNT signaling pathway target genes is down-regulated suggesting that MED12 might be required downstream of WNT signaling to transcribe its target genes. This is supported by the fact that MED12 has been shown to bind to β-catenin, canonical WNT signaling effector protein[182] and this MED12-β-catenin interaction might be required for recruitment of PIC to the WNT signaling target genes. The requirement of MED23 in WNT signaling regulation on the other hand seems to be quite different. MED23 might be required upstream of WNT signaling to regulate transcription of WNT ligands or receptors or inhibit transcription of WNT antagonists, or might be required downstream of WNT signaling to stabilize the β-catenin – Mediator binding, thereby increasing WNT signaling response. Such an interaction between β-catenin

and MED23 has not been reported yet, but it would be highly interesting to find out. The perfect experiment would be to screen through all WNT/β-catenin signaling effector proteins to check for their binding affinity to MED23 and the ability for the interaction complex to activate downstream WNT signaling target genes. It is well established that the various WNT signaling pathways interact with each other, and it is known that some components of WNT/PCP signaling pathway are important for cranial ganglia coalescence; however we currently have no information to say whether PCP signaling is affected in our mutant embryos or not.

MED23 interaction partners and downstream target genes

I would like to understand how a Mediator complex subunit, like MED23 which is ubiquitously expressed in all cell types, regulates formation of specific organs and tissues during development. Since MED23 is ubiquitously expressed in all the cell types within the embryo, my expectation is that its diverse binding partners are specifically expressed in the cells at specific times and that their transcription is regulated by the various signaling pathways required for tissue-specific differentiation. In the past decade, two main binding partners of MED23 have emerged, ELK1 and C/EBPβ and their interaction with MED23 has been shown to be regulated by Insulin and Ras signaling. Complete loss of ELK1 and C/EBPβ

213

during mouse embryogenesis however does not lead to drastic development defects and embryonic lethality as seen in *med23*$^{sn/sn}$ embryos, suggesting to us that other transcription factors might be interacting with MED23 and those interactions would be critical for the formation of frontonasal process, cranial ganglia and blood vasculature during development. In order to understand, what these factors might be, I used the help of screening facility at SIMR to design a mammalian two hybrid screen that would screen 1500 mouse and human transcription factors for their ability to bind MED23. Literature searches will help refine the list of identified candidates for further analysis. To understand what transcription factors bind to MED23 *in vivo* during embryonic development, we could also undertake MUDPIT analysis of MED23 interacting complexes isolated from wild-type embryos at different time points and from specific different tissues. Stoichiometrically, most of the interacting partners identified from MUDPIT would be other Mediator complex proteins. A lot of work done over the past decade argues in favor of the presence of a dynamic Mediator (See Introduction) whose composition varies based on the genes being activated and the tissue types undergoing differentiation. The MUDPIT data could be used to analyze Mediator complex composition in different cell types and at different times. Alternatively, a variant bioinformatics approach could be taken for the MUDPIT data analysis to identify the transcription factor binding partners of MED23. To understand expression of what genes is regulated by MED23

containing Mediator complex, the ideal experiment to do would be MED23 ChIP-sequence analysis. Using a comprehensive comparison of the MED23 ChIP-Sequence data between different time points and different tissue types, we would then be able to come up with a scheme of MED23 activation during mouse embryonic development. However, MED23 not being a direct DNA binding protein makes this a difficult task.

Concluding Remarks

In this work, I have attempted to understand the role of a Mediator complex subunit, MED23 during mouse embryonic development. Mediator complex, when originally identified was believed to a general transcription co-factor for RNA Polymerase II, however, work done over more than two decades has now drawn a picture in which the various subunits of Mediator are in constant conversation with all the other factors present in the nucleus. This crosstalk between cellular proteins and Mediator subunits creates a regulatory network which is essential for maintenance of proper gene expression. Mechanistically, the binding of Mediator subunits to the cellular proteins generates a structural change within the Mediator complex which allows or inhibits it from interacting with RNA Pol II machinery and thus regulate gene transcription. This, however, seems to be just one of the mechanisms of Mediator-mediated regulation of gene expression, as two recent reports show that Mediator

can regulate gene transcription through regulation of transcript elongation as well. Mediator itself has now been found to be a dynamic complex; multiple versions of it exist that consist of variable numbers of subunits. This has been termed as a "Mediator Code" where a Mediator of a specific composition is required for the expression of a specific set of genes only.

In this project, I have analyzed embryonic developmental defects in a mutant for *med23*, a gene that transcribes for a tail subunit of Mediator complex. Loss of *med23* results in embryonic lethality and causes defects in craniofacial, vascular and cranial ganglia differentiation. MED23 is definitely required for expression of cranial neural crest markers in the frontonasal process and cell-cell junction markers in the endothelial cells. I show that MED23 is specifically required for regulation of WNT signaling during cranial ganglia development and restoration of WNT signaling levels to somewhat normalcy partially restores the cranial neuron differentiation in the *med23* mutant embryos. The role for WNT signaling during formation of cranial ganglia is not very clear, neither is our understanding of how the Mediator subunits regulate tissue specific gene expression; hopefully this body of work, which highlights the role of Mediator subunit MED23 in various aspects of embryonic development, including the regulation of WNT signaling during cranial ganglia formation, will shed some light in both those areas.

Materials and Methods

Embryos used

Briefly, the male and female animals were time-mated at a suitable age (42d or older) and checked for plugs. The day when the female was plugged was considered to be E0.5 at noon (considering that the animals mated at midnight). 9 days after the plug date, at E9.5 noon, the pregnant females were sacrificed by Carbon dioxide (CO_2) asphyxiation followed by cervical dislocation; both procedures are in compliance with the Stowers Institute of Medical Research (SIMR) IACUC protocol. The embryos were harvested and the yolk sacs are saved for genotyping. The yolk sacs were sent to Transnetyx, which already has the probes to detect for the multiple *med23* alleles.

Whole Mount Immunostaining

Briefly, the embryos were harvested as described above. Following harvest, the embryos were immediately transferred to 4%PFA/PBS and fixed at 4`C overnight. The embryos were dehydrated through an increasing series of Methanol washes and stored in Methanol at (-)20`C till use. To perform whole mount immunostaining, the embryos were bleached in Dent's bleach (4:1:1 Methanol: DMSO: 30% Hydrogen Peroxide) for two hours at room temperature. The embryos were then rehydrated through a decreasing series of Methanol to PBS and blocked in 3%BSA/PBS for two hours at room temperature. The embryos were then incubated the

218

primary antibody (Mouse α βtubulin III (TuJ1) at 1:1000 dilution, *Covance Research products, Catalog # MMS-435P*) diluted in blocking solution for 12-16 hours (overnight) at 4`C. The following day, the embryos were washed 4-5 times for 1 hour each with PBS at room temperature, followed by overnight incubation at 4`C in secondary antibody (1:300 dilutions) diluted in blocking solution. On the third day, the embryos were washed thrice in PBS for 10 minutes each and counterstained with DAPI (1:1000 dilutions in PBS for 20 minutes). The embryos were cleared in series of glycerol till 50%Glycerol/PBS and transferred to Vectashield. The embryos were mounted in Vectashield and imaged using an upright confocal microscope.

Section Immunostaining

The embryos were harvested as described above. Following harvest the embryos were immediately fixed in 4%PFA/PBS for two hours at room temperature. Following fixation the embryos were equilibrated with an increasing series of sucrose solutions over the course of next 3 days (7.5% Sucrose/PBS to 15%Sucrose/PBS to 30%Sucrose/PBS to 1:1 OCT: 30%Sucrose/PBS). For each equilibration step the embryos were left in the solution for overnight at 4`C. On the fifth day, the embryos were embedded in OCT for cryosectioning. The blocks were saved in (-)80`C until use. The embryo blocks were sectioned on a cryostat at 10μm size. The sections were dried on a heat block for 90 minutes. Following drying, the slides

were saved in (–)80`C until use. For immunostaining, the slides were thawed to room temperature, followed by blocking in 10% Sheep serum/PBS for 20 minutes at room temperature. The sections were then incubated in primary antibody (Rabbit α pH3 at 1:500 dilution, *Upstate Biotechnology, Catalog # 06570* ; diluted in the blocking solution for two hours at room temperature. The sections were then washed thrice in PBS followed by incubation in secondary antibody (1:300 dilutions, *Molecular Probes*) diluted in the blocking solution. The sections were then washed thrice in PBS and mounted in Vectashield with DAPI. The sections were imaged immediately on an upright confocal microscope.

TUNEL assay

TUNEL labeling on cryosections was done in conjunct with immunostaining. Post-secondary antibody wash, the slides were incubated in permeabilization solution (freshly prepared 0.1% Triton X–100, 0.1% Sodium citrate), and then incubated in TUNEL reaction mix (10% Enzyme solution + 90% labeling mix) (*In situ cell death detection kit, Fluorescein, Roche Applied* labeling solution *Science, Cat # 11684795910)* and incubated for 1 hour at 37`C. Post TUNEL labeling, the slides were rinsed in PBS and mount with Vectashield with DAPI and imaged on upright confocal as quickly as possible.

RNA *In Situ* Hybridization

The embryos were harvested as above. The embryos were fixed in 4%PFA/depcPBS overnight at 4`C. The next day the embryos were dehydrated through an increasing series of Methanol and stored in 100% MeOH at (–) 20`C until use. For RNA ISH, Digoxigenin labeled antisense mRNA probes were synthesized in the lab using a standard protocol. Constructs containing the selected genes were linearized and utilized in riboprobe synthesis reaction with the appropriate RNA polymerase. The following protocol is abridged for the sake of brevity. The protocol was performed over the course of 4 days and began with rehydrating the embryos, Proteinase K digestion, post-fixation and hybridization of the embryos with appropriate Digoxigenin labeled antisense mRNA probe overnight at 68`C on Day 1. On Day 2 the embryos were subjected to post-hybridization washes and incubated with Alkaline phosphatase labeled anti-Digoxigenin antibody overnight at 4`C. Day3 consisted of post antibody washes, followed by color development of the Alkaline phosphatase activity by substrates NBT/BCIP (Nitroblue tetrazolium chloride / 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) on Day 4.

X-Gal staining

For whole mount X-Gal staining, the embryos were dissected in Tyrode's solution and immediately fixed on ice in 2%PFA/0.2%Glutaraldehyde in PBS for time periods corresponding

to the embryonic stage (E8.5 – 30 min fix, E9.5 – 45min fix). Following fixation, the embryos were washed in Tissue solution A *(Millipore corporation, Catalog # BG-6-B)* for 30 minutes at room temperature. The embryos were then transferred to Tissue solution B *(Millipore corporation, Catalog # BG-7-B)* for 15 minutes at 37`C. The embryos were then incubated in 1:40 dilution of X-Gal (40mg/ml X-Gal in DMF) in pre-warmed Tissue Stain Base solution *(Millipore corporation, Catalog # BG-8-C)* in dark at 37`C till complete staining was observed. Once stained, the embryos were post-fixed in 4%PFA for 2 hours at room temperature and then transferred to PBST (PBS + 0.1% Triton X-100) and imaged on brightfield microscope.

Bibliography

1. Stephenson, R.O., Rossant, J. & Tam, P.P. Intercellular interactions, position, and polarity in establishing blastocyst cell lineages and embryonic axes. *Cold Spring Harb Perspect Biol* **4**(2012).

2. Rossant, J. & Tam, P.P. Blastocyst lineage formation, early embryonic asymmetries and axis patterning in the mouse. *Development* **136**, 701-13 (2009).

3. Takaoka, K., Yamamoto, M. & Hamada, H. Origin and role of distal visceral endoderm, a group of cells that determines anterior-posterior polarity of the mouse embryo. *Nat Cell Biol* **13**, 743-52 (2011).

4. Beddington, R.S. & Robertson, E.J. Anterior patterning in mouse. *Trends Genet* **14**, 277-84 (1998).

5. Beddington, R.S. & Robertson, E.J. Axis development and early asymmetry in mammals. *Cell* **96**, 195-209 (1999).

6. Madabhushi, M. & Lacy, E. Anterior visceral endoderm directs ventral morphogenesis and placement of head and heart via BMP2 expression. *Dev Cell* **21**, 907-19 (2011).

7. Takaoka, K. et al. The mouse embryo autonomously acquires anterior-posterior polarity at implantation. *Dev Cell* **10**, 451-9 (2006).

8. Chazaud, C. & Rossant, J. Disruption of early proximodistal patterning and AVE formation in Apc mutants. *Development* **133**, 3379-87 (2006).

9. Torres-Padilla, M.E. et al. The anterior visceral endoderm of the mouse embryo is established from both preimplantation precursor cells and by de novo gene expression after implantation. *Dev Biol* **309**, 97-112 (2007).

10. Rivera-Perez, J.A. & Magnuson, T. Primitive streak formation in mice is preceded by localized activation of Brachyury and Wnt3. *Dev Biol* **288**, 363-71 (2005).

11. Kemp, C., Willems, E., Abdo, S., Lambiv, L. & Leyns, L. Expression of all Wnt genes and their secreted antagonists during mouse blastocyst and postimplantation development. *Dev Dyn* **233**, 1064-75 (2005).

12. Mohamed, O.A., Clarke, H.J. & Dufort, D. Beta-catenin signaling marks the prospective site of primitive streak formation in the mouse embryo. *Dev Dyn* **231**, 416-24 (2004).

13. Liu, P. et al. Requirement for Wnt3 in vertebrate axis formation. *Nat Genet* **22**, 361-5 (1999).

14.   Kelly, O.G., Pinson, K.I. & Skarnes, W.C. The Wnt co-receptors Lrp5 and Lrp6 are essential for gastrulation in mice. *Development* **131**, 2803-15 (2004).

15.   Kimura-Yoshida, C. et al. Canonical Wnt signaling and its antagonist regulate anterior-posterior axis polarization by guiding cell migration in mouse visceral endoderm. *Dev Cell* **9**, 639-50 (2005).

16.   Mukhopadhyay, M. et al. Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. *Dev Cell* **1**, 423-34 (2001).

17.   Perea-Gomez, A. et al. Nodal antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks. *Dev Cell* **3**, 745-56 (2002).

18.   Hall, B.K. The neural crest as a fourth germ layer and vertebrates as quadroblastic not triploblastic. *Evol Dev* **2**, 3-5 (2000).

19.   Sauka-Spengler, T. & Bronner-Fraser, M. A gene regulatory network orchestrates neural crest formation. *Nat Rev Mol Cell Biol* **9**, 557-68 (2008).

20.   LaBonne, C. & Bronner-Fraser, M. Neural crest induction in Xenopus: evidence for a two-signal model. *Development* **125**, 2403-14 (1998).

21.   Liem, K.F., Jr., Tremml, G., Roelink, H. & Jessell, T.M. Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-79 (1995).

22.   Tribulo, C., Aybar, M.J., Nguyen, V.H., Mullins, M.C. & Mayor, R. Regulation of Msx genes by a Bmp gradient is essential for neural crest specification. *Development* **130**, 6441-52 (2003).

23.   Wilson, P.A., Lagna, G., Suzuki, A. & Hemmati-Brivanlou, A. Concentration-dependent patterning of the Xenopus ectoderm by BMP4 and its signal transducer Smad1. *Development* **124**, 3177-84 (1997).

24.   Solloway, M.J. & Robertson, E.J. Early embryonic lethality in Bmp5;Bmp7 double mutant mice suggests functional redundancy within the 60A subgroup. *Development* **126**, 1753-68 (1999).

25.   Correia, A.C. et al. Bmp2 is required for migration but not for induction of neural crest cells in the mouse. *Dev Dyn* **236**, 2493-501 (2007).

26.   Garcia-Castro, M.I., Marcelle, C. & Bronner-Fraser, M. Ectodermal Wnt function as a neural crest inducer. *Science* **297**, 848-51 (2002).

27.   Lewis, J.L. et al. Reiterated Wnt signaling during zebrafish neural crest development. *Development* **131**, 1299-308 (2004).

28.   Brault, V. et al. Inactivation of the beta-catenin gene by Wnt1-Cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. *Development* **128**, 1253-64 (2001).

29.   Saint-Jeannet, J.P., He, X., Varmus, H.E. & Dawid, I.B. Regulation of dorsal fate in the neuraxis by Wnt-1 and Wnt-3a. *Proc Natl Acad Sci U S A* **94**, 13713-8 (1997).

30.   Ikeya, M., Lee, S.M., Johnson, J.E., McMahon, A.P. & Takada, S. Wnt signalling required for expansion of neural crest and CNS progenitors. *Nature* **389**, 966-70 (1997).

31.   Dorsky, R.I., Moon, R.T. & Raible, D.W. Control of neural crest cell fate by the Wnt signalling pathway. *Nature* **396**, 370-3 (1998).

32.   Chang, C. & Hemmati-Brivanlou, A. Neural crest induction by Xwnt7B in Xenopus. *Dev Biol* **194**, 129-34 (1998).

33.   Knecht, A.K. & Bronner-Fraser, M. Induction of the neural crest: a multigene process. *Nat Rev Genet* **3**, 453-61 (2002).

34.   Tamai, K. et al. LDL-receptor-related proteins in Wnt signal transduction. *Nature* **407**, 530-5 (2000).

35.   Elkouby, Y.M. et al. Mesodermal Wnt signaling organizes the neural plate via Meis3. *Development* **137**, 1531-41 (2010).

36.   Steventon, B., Araya, C., Linker, C., Kuriyama, S. & Mayor, R. Differential requirements of BMP and Wnt signalling during gastrulation and neurulation define two steps in neural crest induction. *Development* **136**, 771-9 (2009).

37.   Patthey, C., Edlund, T. & Gunhaga, L. Wnt-regulated temporal control of BMP exposure directs the choice between neural plate border and epidermal fate. *Development* **136**, 73-83 (2009).

38.   Lee, H.Y. et al. Instructive role of Wnt/beta-catenin in sensory fate specification in neural crest stem cells. *Science* **303**, 1020-3 (2004).

39.   Hari, L. et al. Lineage-specific requirements of beta-catenin in neural crest development. *J Cell Biol* **159**, 867-80 (2002).

40.   Hari, L. et al. Temporal control of neural crest lineage generation by Wnt/beta-catenin signaling. *Development* **139**, 2107-17 (2012).

41.   Monsoro-Burq, A.H., Fletcher, R.B. & Harland, R.M. Neural crest induction by paraxial mesoderm in Xenopus embryos requires FGF signals. *Development* **130**, 3111-24 (2003).

42. Noden, D.M. & Trainor, P.A. Relations and interactions between cranial mesoderm and neural crest populations. *J Anat* **207**, 575-601 (2005).

43. Mayor, R., Guerrero, N. & Martinez, C. Role of FGF and noggin in neural crest induction. *Dev Biol* **189**, 1-12 (1997).

44. Villanueva, S., Glavic, A., Ruiz, P. & Mayor, R. Posteriorization by FGF, Wnt, and retinoic acid is required for neural crest induction. *Dev Biol* **241**, 289-301 (2002).

45. Hong, C.S., Park, B.Y. & Saint-Jeannet, J.P. Fgf8a induces neural crest indirectly through the activation of Wnt8 in the paraxial mesoderm. *Development* **135**, 3903-10 (2008).

46. Stuhlmiller, T.J. & Garcia-Castro, M.I. FGF/MAPK signaling is required in the gastrula epiblast for avian neural crest induction. *Development* **139**, 289-300 (2012).

47. Stanier, P. & Pauws, E. Development of the lip and palate: FGF signalling. *Front Oral Biol* **16**, 71-80 (2012).

48. Basch, M.L., Bronner-Fraser, M. & Garcia-Castro, M.I. Specification of the neural crest occurs during gastrulation and requires Pax7. *Nature* **441**, 218-22 (2006).

49. Maczkowiak, F. et al. The Pax3 and Pax7 paralogs cooperate in neural and neural crest patterning using distinct molecular mechanisms, in Xenopus laevis embryos. *Dev Biol* **340**, 381-96 (2010).

50. Franz, T. & Kothary, R. Characterization of the neural crest defect in Splotch (Sp1H) mutant mice using a lacZ transgene. *Brain Res Dev Brain Res* **72**, 99-105 (1993).

51. Mansouri, A., Stoykova, A., Torres, M. & Gruss, P. Dysgenesis of cephalic neural crest derivatives in Pax7-/- mutant mice. *Development* **122**, 831-8 (1996).

52. Relaix, F., Rocancourt, D., Mansouri, A. & Buckingham, M. Divergent functions of murine Pax3 and Pax7 in limb muscle development. *Genes Dev* **18**, 1088-105 (2004).

53. Theveneau, E. & Mayor, R. Neural crest delamination and migration: from epithelium-to-mesenchyme transition to collective cell migration. *Dev Biol* **366**, 34-54 (2012).

54. Taneyhill, L.A., Coles, E.G. & Bronner-Fraser, M. Snail2 directly represses cadherin6B during epithelial-to-mesenchymal transitions of the neural crest. *Development* **134**, 1481-90 (2007).

55. Chalpe, A.J., Prasad, M., Henke, A.J. & Paulson, A.F. Regulation of cadherin expression in the chicken neural crest by the Wnt/beta-catenin signaling pathway. *Cell Adh Migr* **4**, 431-8 (2010).

56. Burstyn-Cohen, T., Stanleigh, J., Sela-Donenfeld, D. & Kalcheim, C. Canonical Wnt activity regulates trunk neural crest delamination linking BMP/noggin signaling with G1/S transition. *Development* **131**, 5327-39 (2004).

57. Graham, A., Francis-West, P., Brickell, P. & Lumsden, A. The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. *Nature* **372**, 684-6 (1994).

58. Tzahor, E. et al. Antagonists of Wnt and BMP signaling promote the formation of vertebrate head muscle. *Genes Dev* **17**, 3087-99 (2003).

59. Carl, T.F., Dufton, C., Hanken, J. & Klymkowsky, M.W. Inhibition of neural crest migration in Xenopus using antisense slug RNA. *Dev Biol* **213**, 101-15 (1999).

60. Jiang, R., Lan, Y., Norton, C.R., Sundberg, J.P. & Gridley, T. The Slug gene is not essential for mesoderm or neural crest development in mice. *Dev Biol* **198**, 277-85 (1998).

61. Murray, S.A. & Gridley, T. Snail family genes are required for left-right asymmetry determination, but not neural crest formation, in mice. *Proc Natl Acad Sci U S A* **103**, 10300-4 (2006).

62. Mejlvang, J. et al. Direct repression of cyclin D1 by SIP1 attenuates cell cycle progression in cells undergoing an epithelial mesenchymal transition. *Mol Biol Cell* **18**, 4615-24 (2007).

63. Van de Putte, T. et al. Mice lacking ZFHX1B, the gene that codes for Smad-interacting protein-1, reveal a role for multiple neural crest cell defects in the etiology of Hirschsprung disease-mental retardation syndrome. *Am J Hum Genet* **72**, 465-70 (2003).

64. Van de Putte, T., Francis, A., Nelles, L., van Grunsven, L.A. & Huylebroeck, D. Neural crest-specific removal of Zfhx1b in mouse leads to a wide range of neurocristopathies reminiscent of Mowat-Wilson syndrome. *Hum Mol Genet* **16**, 1423-36 (2007).

65. Golding, J.P., Trainor, P., Krumlauf, R. & Gassmann, M. Defects in pathfinding by cranial neural crest cells in mice lacking the neuregulin receptor ErbB4. *Nat Cell Biol* **2**, 103-9 (2000).

66. Gammill, L.S., Gonzalez, C. & Bronner-Fraser, M. Neuropilin 2/semaphorin 3F signaling is essential for cranial neural crest migration and trigeminal ganglion condensation. *Dev Neurobiol* **67**, 47-56 (2007).

67. Schwarz, Q., Vieira, J.M., Howard, B., Eickholt, B.J. & Ruhrberg, C. Neuropilin 1 and 2 control cranial gangliogenesis and axon guidance through neural crest cells. *Development* **135**, 1605-13 (2008).

68.   Minoux, M. & Rijli, F.M. Molecular mechanisms of cranial neural crest cell migration and patterning in craniofacial development. *Development* **137**, 2605-21 (2010).

69.   Davy, A., Aubin, J. & Soriano, P. Ephrin-B1 forward and reverse signaling are required during mouse development. *Genes Dev* **18**, 572-83 (2004).

70.   Adams, R.H. et al. The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. *Cell* **104**, 57-69 (2001).

71.   Trokovic, N., Trokovic, R., Mai, P. & Partanen, J. Fgfr1 regulates patterning of the pharyngeal region. *Genes Dev* **17**, 141-53 (2003).

72.   Paratore, C., Goerich, D.E., Suter, U., Wegner, M. & Sommer, L. Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. *Development* **128**, 3949-61 (2001).

73.   Schlosser, G. Induction and specification of cranial placodes. *Dev Biol* **294**, 303-51 (2006).

74.   Begbie, J., Brunet, J.F., Rubenstein, J.L. & Graham, A. Induction of the epibranchial placodes. *Development* **126**, 895-902 (1999).

75.   Glavic, A. et al. Role of BMP signaling and the homeoprotein Iroquois in the specification of the cranial placodal field. *Dev Biol* **272**, 89-103 (2004).

76.   Litsiou, A., Hanson, S. & Streit, A. A balance of FGF, BMP and WNT signalling positions the future placode territory in the head. *Development* **132**, 4051-62 (2005).

77.   Streit, A. The preplacodal region: an ectodermal domain with multipotential progenitors that contribute to sense organs and cranial sensory ganglia. *Int J Dev Biol* **51**, 447-61 (2007).

78.   Brugmann, S.A. & Moody, S.A. Induction and specification of the vertebrate ectodermal placodes: precursors of the cranial sensory organs. *Biol Cell* **97**, 303-19 (2005).

79.   Streit, A. The cranial sensory nervous system: specification of sensory progenitors and placodes. in *StemBook* (Cambridge (MA), 2008).

80.   Brugmann, S.A., Pandur, P.D., Kenyon, K.L., Pignoni, F. & Moody, S.A. Six1 promotes a placodal fate within the lateral neurogenic ectoderm by functioning as both a transcriptional activator and repressor. *Development* **131**, 5871-81 (2004).

81.   Christophorou, N.A., Bailey, A.P., Hanson, S. & Streit, A. Activation of Six1 target genes is required for sensory placode formation. *Dev Biol* **336**, 327-36 (2009).

82.   Abdelhak, S. et al. A human homologue of the Drosophila eyes absent gene underlies branchio-oto-renal (BOR) syndrome and identifies a novel gene family. *Nat Genet* **15**, 157-64 (1997).

83.   Xu, P.X. et al. Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nat Genet* **23**, 113-7 (1999).

84.   Ozaki, H. et al. Six1 controls patterning of the mouse otic vesicle. *Development* **131**, 551-62 (2004).

85.   Ozaki, H. et al. Six4, a putative myogenin gene regulator, is not essential for mouse embryonal development. *Mol Cell Biol* **21**, 3343-50 (2001).

86.   Kobayashi, H., Kawakami, K., Asashima, M. & Nishinakamura, R. Six1 and Six4 are essential for Gdnf expression in the metanephric mesenchyme and ureteric bud formation, while Six1 deficiency alone causes mesonephric-tubule defects. *Mech Dev* **124**, 290-303 (2007).

87.   Itoh, M., Kudoh, T., Dedekian, M., Kim, C.H. & Chitnis, A.B. A role for iro1 and iro7 in the establishment of an anteroposterior compartment of the ectoderm adjacent to the midbrain-hindbrain boundary. *Development* **129**, 2317-27 (2002).

88.   Abu-Elmagd, M. et al. cSox3 expression and neurogenesis in the epibranchial placodes. *Dev Biol* **237**, 258-69 (2001).

89.   Ahrens, K. & Schlosser, G. Tissues and signals involved in the induction of placodal Six1 expression in Xenopus laevis. *Dev Biol* **288**, 40-59 (2005).

90.   Li, X. et al. Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. *Nature* **426**, 247-54 (2003).

91.   Baker, C.V. & Bronner-Fraser, M. Establishing neuronal identity in vertebrate neurogenic placodes. *Development* **127**, 3045-56 (2000).

92.   Baker, C.V. & Bronner-Fraser, M. Vertebrate cranial placodes I. Embryonic induction. *Dev Biol* **232**, 1-61 (2001).

93.   Begbie, J., Ballivet, M. & Graham, A. Early steps in the production of sensory neurons by the neurogenic placodes. *Mol Cell Neurosci* **21**, 502-11 (2002).

94.   Tremblay, P., Kessel, M. & Gruss, P. A transgenic neuroanatomical marker identifies cranial neural crest deficiencies associated with the Pax3 mutant Splotch. *Dev Biol* **171**, 317-29 (1995).

95.   Burton, Q., Cole, L.K., Mulheisen, M., Chang, W. & Wu, D.K. The role of Pax2 in mouse inner ear development. *Dev Biol* **272**, 161-75 (2004).

96. Begbie, J. & Graham, A. Integration between the epibranchial placodes and the hindbrain. *Science* **294**, 595-8 (2001).

97. Schwarz, Q. et al. Plexin A3 and plexin A4 convey semaphorin signals during facial nerve development. *Dev Biol* **324**, 1-9 (2008).

98. Shiau, C.E. & Bronner-Fraser, M. N-cadherin acts in concert with Slit1-Robo2 signaling in regulating aggregation of placode-derived cranial sensory neurons. *Development* **136**, 4155-64 (2009).

99. Coppola, E. et al. Epibranchial ganglia orchestrate the development of the cranial neurogenic crest. *Proc Natl Acad Sci U S A* **107**, 2066-71 (2010).

100. Kinder, S.J. et al. The orderly allocation of mesodermal cells to the extraembryonic structures and the anteroposterior axis during gastrulation of the mouse embryo. *Development* **126**, 4691-701 (1999).

101. Evans, D.J. & Noden, D.M. Spatial relations between avian craniofacial neural crest and paraxial mesoderm cells. *Dev Dyn* **235**, 1310-25 (2006).

102. Nathan, E. et al. The contribution of Islet1-expressing splanchnic mesoderm cells to distinct branchiomeric muscles reveals significant heterogeneity in head muscle development. *Development* **135**, 647-57 (2008).

103. Noden, D.M. & Francis-West, P. The differentiation and morphogenesis of craniofacial muscles. *Dev Dyn* **235**, 1194-218 (2006).

104. Hacker, A. & Guthrie, S. A distinct developmental programme for the cranial paraxial mesoderm in the chick embryo. *Development* **125**, 3461-72 (1998).

105. Kassar-Duchossoy, L. et al. Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. *Nature* **431**, 466-71 (2004).

106. Lu, J.R. et al. Control of facial muscle development by MyoR and capsulin. *Science* **298**, 2378-81 (2002).

107. Shih, H.P., Gross, M.K. & Kioussi, C. Cranial muscle defects of Pitx2 mutants result from specification defects in the first branchial arch. *Proc Natl Acad Sci U S A* **104**, 5907-12 (2007).

108. Dong, F. et al. Pitx2 promotes development of splanchnic mesoderm-derived branchiomeric muscle. *Development* **133**, 4891-9 (2006).

109. Kelly, R.G., Jerome-Majewska, L.A. & Papaioannou, V.E. The del22q11.2 candidate gene Tbx1 regulates branchiomeric myogenesis. *Hum Mol Genet* **13**, 2829-40 (2004).

110. Sambasivan, R. et al. Distinct regulatory cascades govern extraocular and pharyngeal arch muscle progenitor cell fates. *Dev Cell* **16**, 810-21 (2009).

111. Lin, C.Y. et al. The transcription factor Six1a plays an essential role in the craniofacial myogenesis of zebrafish. *Dev Biol* **331**, 152-66 (2009).

112. Lin, C.Y. et al. Myogenic regulatory factors Myf5 and Myod function distinctly during craniofacial myogenesis of zebrafish. *Dev Biol* **299**, 594-608 (2006).

113. Grifone, R. et al. Six1 and Six4 homeoproteins are required for Pax3 and Mrf expression during myogenesis in the mouse embryo. *Development* **132**, 2235-49 (2005).

114. Grifone, R. et al. Properties of branchiomeric and somite-derived muscle development in Tbx1 mutant embryos. *Dev Dyn* **237**, 3071-8 (2008).

115. Bothe, I. & Dietrich, S. The molecular setup of the avian head mesoderm and its implication for craniofacial myogenesis. *Dev Dyn* **235**, 2845-60 (2006).

116. Bothe, I., Tenin, G., Oseni, A. & Dietrich, S. Dynamic control of head mesoderm patterning. *Development* **138**, 2807-21 (2011).

117. Knight, R.D., Mebus, K. & Roehl, H.H. Mandibular arch muscle identity is regulated by a conserved molecular process during vertebrate development. *J Exp Zool B Mol Dev Evol* **310**, 355-69 (2008).

118. Carmeliet, P. Angiogenesis in health and disease. *Nat Med* **9**, 653-60 (2003).

119. Shalaby, F. et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376**, 62-6 (1995).

120. Carmeliet, P. et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380**, 435-9 (1996).

121. Miquerol, L., Langille, B.L. & Nagy, A. Embryonic development is disrupted by modest increases in vascular endothelial growth factor gene expression. *Development* **127**, 3941-6 (2000).

122. Fong, G.H., Rossant, J., Gertsenstein, M. & Breitman, M.L. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376**, 66-70 (1995).

123. Blanco, R. & Gerhardt, H. VEGF and Notch in tip and stalk cell selection. *Cold Spring Harb Perspect Med* **3**, a006569 (2013).

124.   Gale, N.W. et al. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc Natl Acad Sci U S A* **101**, 15949-54 (2004).

125.   Kwon, G.S., Viotti, M. & Hadjantonakis, A.K. The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. *Dev Cell* **15**, 509-20 (2008).

126.   Grapin-Botton, A. Endoderm specification. in *StemBook* (Cambridge (MA), 2008).

127.   Baek, H.J., Malik, S., Qin, J. & Roeder, R.G. Requirement of TRAP/mediator for both activator-independent and activator-dependent transcription in conjunction with TFIID-associated TAF(II)s. *Mol Cell Biol* **22**, 2842-52 (2002).

128.   Flanagan, T., Taylor, L., Poulter, L., Viveros, O.H. & Diliberto, E.J., Jr. A novel 1745-dalton pyroglutamyl peptide derived from chromogranin B is in the bovine adrenomedullary chromaffin vesicle. *Cell Mol Neurobiol* **10**, 507-23 (1990).

129.   Rani, P.G., Ranish, J.A. & Hahn, S. RNA polymerase II (Pol II)-TFIIF and Pol II-mediator complexes: the major stable Pol II complexes and their activity in transcription initiation and reinitiation. *Mol Cell Biol* **24**, 1709-20 (2004).

130.   Kornberg, R.D. & Lorch, Y. Chromatin-modifying and -remodeling complexes. *Curr Opin Genet Dev* **9**, 148-51 (1999).

131.   Andrau, J.C. et al. Genome-wide location of the coactivator mediator: Binding without activation and transient Cdk8 interaction on DNA. *Mol Cell* **22**, 179-92 (2006).

132.   Zhu, X. et al. Genome-wide occupancy profile of mediator and the Srb8-11 module reveals interactions with coding regions. *Mol Cell* **22**, 169-78 (2006).

133.   Kim, Y.J., Bjorklund, S., Li, Y., Sayre, M.H. & Kornberg, R.D. A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. *Cell* **77**, 599-608 (1994).

134.   Hengartner, C.J. et al. Association of an activator with an RNA polymerase II holoenzyme. *Genes Dev* **9**, 897-910 (1995).

135.   Sato, S. et al. A set of consensus mammalian mediator subunits identified by multidimensional protein identification technology. *Mol Cell* **14**, 685-91 (2004).

136.   Malik, S., Baek, H.J., Wu, W. & Roeder, R.G. Structural and functional characterization of PC2 and RNA polymerase II-associated subpopulations of metazoan Mediator. *Mol Cell Biol* **25**, 2117-29 (2005).

137. Cantin, G.T., Stevens, J.L. & Berk, A.J. Activation domain-mediator interactions promote transcription preinitiation complex assembly on promoter DNA. *Proc Natl Acad Sci U S A* **100**, 12003-8 (2003).

138. Baek, H.J., Kang, Y.K. & Roeder, R.G. Human Mediator enhances basal transcription by facilitating recruitment of transcription factor IIB during preinitiation complex assembly. *J Biol Chem* **281**, 15172-81 (2006).

139. Malik, S., Wallberg, A.E., Kang, Y.K. & Roeder, R.G. TRAP/SMCC/mediator-dependent transcriptional activation from DNA and chromatin templates by orphan nuclear receptor hepatocyte nuclear factor 4. *Mol Cell Biol* **22**, 5626-37 (2002).

140. Wang, G. et al. Mediator requirement for both recruitment and postrecruitment steps in transcription initiation. *Mol Cell* **17**, 683-94 (2005).

141. Akhtar, M.S. et al. TFIIH kinase places bivalent marks on the carboxy-terminal domain of RNA polymerase II. *Mol Cell* **34**, 387-93 (2009).

142. Boeing, S., Rigault, C., Heidemann, M., Eick, D. & Meisterernst, M. RNA polymerase II C-terminal heptarepeat domain Ser-7 phosphorylation is established in a mediator-dependent fashion. *J Biol Chem* **285**, 188-96 (2010).

143. Takahashi, H. et al. Human mediator subunit MED26 functions as a docking site for transcription elongation factors. *Cell* **146**, 92-104 (2011).

144. Meyer, K.D., Lin, S.C., Bernecky, C., Gao, Y. & Taatjes, D.J. p53 activates transcription by directing structural shifts in Mediator. *Nat Struct Mol Biol* **17**, 753-60 (2010).

145. Kagey, M.H. et al. Mediator and cohesin connect gene expression and chromatin architecture. *Nature* **467**, 430-5 (2010).

146. Jiang, H. & Peterlin, B.M. Differential chromatin looping regulates CD4 expression in immature thymocytes. *Mol Cell Biol* **28**, 907-12 (2008).

147. Miele, A. & Dekker, J. Long-range chromosomal interactions and gene regulation. *Mol Biosyst* **4**, 1046-57 (2008).

148. Soutourina, J., Wydau, S., Ambroise, Y., Boschiero, C. & Werner, M. Direct interaction of RNA polymerase II and mediator required for transcription in vivo. *Science* **331**, 1451-4 (2011).

149. Imasaki, T. et al. Architecture of the Mediator head module. *Nature* **475**, 240-3 (2011).

150. Cai, G. et al. Interaction of the mediator head module with RNA polymerase II. *Structure* **20**, 899-910 (2012).

151.   Takagi, Y. et al. Head module control of mediator interactions. *Mol Cell* **23**, 355-64 (2006).

152.   Takagi, Y. & Kornberg, R.D. Mediator as a general transcription factor. *J Biol Chem* **281**, 80-9 (2006).

153.   Esnault, C. et al. Mediator-dependent recruitment of TFIIH modules in preinitiation complex. *Mol Cell* **31**, 337-46 (2008).

154.   Lariviere, L. et al. Structure and TBP binding of the Mediator head subcomplex Med8-Med18-Med20. *Nat Struct Mol Biol* **13**, 895-901 (2006).

155.   Bernecky, C., Grob, P., Ebmeier, C.C., Nogales, E. & Taatjes, D.J. Molecular architecture of the human Mediator-RNA polymerase II-TFIIF assembly. *PLoS Biol* **9**, e1000603 (2011).

156.   Lariviere, L. et al. Structure of the Mediator head module. *Nature* **492**, 448-51 (2012).

157.   Ebmeier, C.C. & Taatjes, D.J. Activator-Mediator binding regulates Mediator-cofactor interactions. *Proc Natl Acad Sci U S A* **107**, 11283-8 (2010).

158.   Taatjes, D.J., Naar, A.M., Andel, F., 3rd, Nogales, E. & Tjian, R. Structure, function, and activator-induced conformations of the CRSP coactivator. *Science* **295**, 1058-62 (2002).

159.   Taatjes, D.J., Schneider-Poetsch, T. & Tjian, R. Distinct conformational states of nuclear receptor-bound CRSP-Med complexes. *Nat Struct Mol Biol* **11**, 664-71 (2004).

160.   Han, S.J. et al. Activator-specific requirement of yeast mediator proteins for RNA polymerase II transcriptional activation. *Mol Cell Biol* **19**, 979-88 (1999).

161.   Howard, R.M. & Sundaram, M.V. C. elegans EOR-1/PLZF and EOR-2 positively regulate Ras and Wnt signaling and function redundantly with LIN-25 and the SUR-2 Mediator component. *Genes Dev* **16**, 1815-27 (2002).

162.   Taubert, S., Van Gilst, M.R., Hansen, M. & Yamamoto, K.R. A Mediator subunit, MDT-15, integrates regulation of fatty acid metabolism by NHR-49-dependent and -independent pathways in C. elegans. *Genes Dev* **20**, 1137-49 (2006).

163.   Kim, T.W. et al. MED16 and MED23 of Mediator are coactivators of lipopolysaccharide- and heat-shock-induced transcriptional activators. *Proc Natl Acad Sci U S A* **101**, 12153-8 (2004).

164.   Zhang, X. et al. MED1/TRAP220 exists predominantly in a TRAP/ Mediator subpopulation enriched in RNA polymerase II and is required for ER-mediated transcription. *Mol Cell* **19**, 89-100 (2005).

165. Taatjes, D.J. & Tjian, R. Structure and function of CRSP/Med2; a promoter-selective transcriptional coactivator complex. *Mol Cell* **14**, 675-83 (2004).

166. Yuan, C.X., Ito, M., Fondell, J.D., Fu, Z.Y. & Roeder, R.G. The TRAP220 component of a thyroid hormone receptor- associated protein (TRAP) coactivator complex interacts directly with nuclear receptors in a ligand-dependent fashion. *Proc Natl Acad Sci U S A* **95**, 7939-44 (1998).

167. Zhu, Y., Qi, C., Jain, S., Rao, M.S. & Reddy, J.K. Isolation and characterization of PBP, a protein that interacts with peroxisome proliferator-activated receptor. *J Biol Chem* **272**, 25500-6 (1997).

168. Zhu, Y. et al. Amplification and overexpression of peroxisome proliferator-activated receptor binding protein (PBP/PPARBP) gene in breast cancer. *Proc Natl Acad Sci U S A* **96**, 10848-53 (1999).

169. Kang, Y.K., Guermah, M., Yuan, C.X. & Roeder, R.G. The TRAP/Mediator coactivator complex interacts directly with estrogen receptors alpha and beta through the TRAP220 subunit and directly enhances estrogen receptor function in vitro. *Proc Natl Acad Sci U S A* **99**, 2642-7 (2002).

170. Warnmark, A., Almlof, T., Leers, J., Gustafsson, J.A. & Treuter, E. Differential recruitment of the mammalian mediator subunit TRAP220 by estrogen receptors ERalpha and ERbeta. *J Biol Chem* **276**, 23397-404 (2001).

171. Burakov, D., Wong, C.W., Rachez, C., Cheskis, B.J. & Freedman, L.P. Functional interactions between the estrogen receptor and DRIP205, a subunit of the heteromeric DRIP coactivator complex. *J Biol Chem* **275**, 20928-34 (2000).

172. Ge, K. et al. Transcription coactivator TRAP220 is required for PPAR gamma 2-stimulated adipogenesis. *Nature* **417**, 563-7 (2002).

173. Grontved, L., Madsen, M.S., Boergesen, M., Roeder, R.G. & Mandrup, S. MED14 tethers mediator to the N-terminal domain of peroxisome proliferator-activated receptor gamma and is required for full transcriptional activity and adipogenesis. *Mol Cell Biol* **30**, 2155-69 (2010).

174. Chen, W., Rogatsky, I. & Garabedian, M.J. MED14 and MED1 differentially regulate target-specific gene activation by the glucocorticoid receptor. *Mol Endocrinol* **20**, 560-72 (2006).

175. Wang, W. et al. Mediator MED23 links insulin signaling to the adipogenesis transcription cascade. *Dev Cell* **16**, 764-71 (2009).

176. Stevens, J.L. et al. Transcription control by E1A and MAP kinase pathway via Sur2 mediator subunit. *Science* **296**, 755-8 (2002).

177. Mo, X., Kowenz-Leutz, E., Xu, H. & Leutz, A. Ras induces mediator complex exchange on C/EBP beta. *Mol Cell* **13**, 241-50 (2004).

178. Kato, Y., Habas, R., Katsuyama, Y., Naar, A.M. & He, X. A component of the ARC/Mediator complex required for TGF beta/Nodal signalling. *Nature* **418**, 641-6 (2002).

179. Rana, R., Surapureddi, S., Kam, W., Ferguson, S. & Goldstein, J.A. Med25 is required for RNA polymerase II recruitment to specific promoters, thus regulating xenobiotic and lipid metabolism in human liver. *Mol Cell Biol* **31**, 466-81 (2011).

180. Nakamura, Y. et al. Wwp2 is essential for palatogenesis mediated by the interaction between Sox9 and mediator subunit 25. *Nat Commun* **2**, 251 (2011).

181. Ding, N. et al. MED19 and MED26 are synergistic functional targets of the RE1 silencing transcription factor in epigenetic silencing of neuronal gene expression. *J Biol Chem* **284**, 2648-56 (2009).

182. Kim, S., Xu, X., Hecht, A. & Boyer, T.G. Mediator is a transducer of Wnt/beta-catenin signaling. *J Biol Chem* **281**, 14066-75 (2006).

183. Eberhardy, S.R. & Farnham, P.J. Myc recruits P-TEFb to mediate the final step in the transcriptional activation of the cad promoter. *J Biol Chem* **277**, 40156-62 (2002).

184. Knuesel, M.T., Meyer, K.D., Donner, A.J., Espinosa, J.M. & Taatjes, D.J. The human CDK8 subcomplex is a histone kinase that requires Med12 for activity and can function independently of mediator. *Mol Cell Biol* **29**, 650-61 (2009).

185. Knuesel, M.T., Meyer, K.D., Bernecky, C. & Taatjes, D.J. The human CDK8 subcomplex is a molecular switch that controls Mediator coactivator function. *Genes Dev* **23**, 439-51 (2009).

186. Galbraith, M.D., Donner, A.J. & Espinosa, J.M. CDK8: a positive regulator of transcription. *Transcription* **1**, 4-12 (2010).

187. Westerling, T., Kuuluvainen, E. & Makela, T.P. Cdk8 is essential for preimplantation mouse development. *Mol Cell Biol* **27**, 6177-82 (2007).

188. Tsutsui, T. et al. Human mediator kinase subunit CDK11 plays a negative role in viral activator VP16-dependent transcriptional regulation. *Genes Cells* **13**, 817-26 (2008).

189. Furumoto, T. et al. A kinase subunit of the human mediator complex, CDK8, positively regulates transcriptional activation. *Genes Cells* **12**, 119-32 (2007).

190. Elmlund, H. et al. The cyclin-dependent kinase 8 module sterically blocks Mediator interactions with RNA polymerase II. *Proc Natl Acad Sci U S A* **103**, 15788-93 (2006).

191. Pavri, R. et al. PARP-1 determines specificity in a retinoid signaling pathway via direct modulation of mediator. *Mol Cell* **18**, 83-96 (2005).

192. Belakavadi, M. & Fondell, J.D. Cyclin-dependent kinase 8 positively cooperates with Mediator to promote thyroid hormone receptor-dependent transcriptional activation. *Mol Cell Biol* **30**, 2437-48 (2010).

193. Donner, A.J., Szostek, S., Hoover, J.M. & Espinosa, J.M. CDK8 is a stimulus-specific positive coregulator of p53 target genes. *Mol Cell* **27**, 121-33 (2007).

194. Alarcon, C. et al. Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. *Cell* **139**, 757-69 (2009).

195. Fryer, C.J., White, J.B. & Jones, K.A. Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. *Mol Cell* **16**, 509-20 (2004).

196. Firestein, R. et al. CDK8 is a colorectal cancer oncogene that regulates beta-catenin activity. *Nature* **455**, 547-51 (2008).

197. Morris, E.J. et al. E2F1 represses beta-catenin transcription and is antagonized by both pRB and CDK8. *Nature* **455**, 552-6 (2008).

198. Donner, A.J., Ebmeier, C.C., Taatjes, D.J. & Espinosa, J.M. CDK8 is a positive regulator of transcriptional elongation within the serum response network. *Nat Struct Mol Biol* **17**, 194-201 (2010).

199. Tudor, M., Murray, P.J., Onufryk, C., Jaenisch, R. & Young, R.A. Ubiquitous expression and embryonic requirement for RNA polymerase II coactivator subunit Srb7 in mice. *Genes Dev* **13**, 2365-8 (1999).

200. Gim, B.S., Park, J.M., Yoon, J.H., Kang, C. & Kim, Y.J. Drosophila Med6 is required for elevated expression of a large but distinct set of developmentally regulated genes. *Mol Cell Biol* **21**, 5242-55 (2001).

201. Ito, M., Yuan, C.X., Okano, H.J., Darnell, R.B. & Roeder, R.G. Involvement of the TRAP220 component of the TRAP/SMCC coactivator complex in embryonic development and thyroid hormone action. *Mol Cell* **5**, 683-93 (2000).

202. Zhu, Y. et al. Deletion of PBP/PPARBP, the gene for nuclear receptor coactivator peroxisome proliferator-activated receptor-binding protein, results in embryonic lethality. *J Biol Chem* **275**, 14779-82 (2000).

203. Crawford, S.E. et al. Defects of the heart, eye, and megakaryocytes in peroxisome proliferator activator receptor-binding protein (PBP) null embryos implicate GATA family of transcription factors. *J Biol Chem* **277**, 3585-92 (2002).

204. Treisman, J. Drosophila homologues of the transcriptional coactivation complex subunits TRAP240 and TRAP230 are required for identical processes in eye-antennal disc development. *Development* **128**, 603-15 (2001).

205. Lim, J., Lee, O.K., Hsu, Y.C., Singh, A. & Choi, K.W. Drosophila TRAP230/240 are essential coactivators for Atonal in retinal neurogenesis. *Dev Biol* **308**, 322-30 (2007).

206. Janody, F., Martirosyan, Z., Benlali, A. & Treisman, J.E. Two subunits of the Drosophila mediator complex act together to control cell affinity. *Development* **130**, 3691-701 (2003).

207. Carrera, I., Janody, F., Leeds, N., Duveau, F. & Treisman, J.E. Pygopus activates Wingless target gene transcription through the mediator complex subunits Med12 and Med13. *Proc Natl Acad Sci U S A* **105**, 6644-9 (2008).

208. Milchanowski, A.B., Henkenius, A.L., Narayanan, M., Hartenstein, V. & Banerjee, U. Identification and characterization of genes involved in embryonic crystal cell formation during Drosophila hematopoiesis. *Genetics* **168**, 325-39 (2004).

209. Gobert, V. et al. A genome-wide RNA interference screen identifies a differential role of the mediator CDK8 module subunits for GATA/ RUNX-activated transcription in Drosophila. *Mol Cell Biol* **30**, 2837-48 (2010).

210. Zhang, H. & Emmons, S.W. A C. elegans mediator protein confers regulatory selectivity on lineage-specific expression of a transcription factor gene. *Genes Dev* **14**, 2161-72 (2000).

211. Moghal, N. & Sternberg, P.W. A component of the transcriptional mediator complex inhibits RAS-dependent vulval fate specification in C. elegans. *Development* **130**, 57-69 (2003).

212. Hong, S.K. et al. The zebrafish kohtalo/trap230 gene is required for the development of the brain, neural crest, and pronephric kidney. *Proc Natl Acad Sci U S A* **102**, 18473-8 (2005).

213. Shin, C.H. et al. Multiple roles for Med12 in vertebrate endoderm development. *Dev Biol* **317**, 467-79 (2008).

214. Hong, S.K. & Dawid, I.B. The transcriptional mediator component Med12 is required for hindbrain boundary formation. *PLoS One* **6**, e19076 (2011).

215. Rau, M.J., Fischer, S. & Neumann, C.J. Zebrafish Trap230/Med12 is required as a coactivator for Sox9-dependent neural crest, cartilage and ear development. *Dev Biol* **296**, 83-93 (2006).

216. Rocha, P.P., Scholze, M., Bleiss, W. & Schrewe, H. Med12 is essential for early mouse development and for canonical Wnt and Wnt/PCP signaling. *Development* **137**, 2723-31 (2010).

217. Gillmor, C.S. et al. The MED12-MED13 module of Mediator regulates the timing of embryo patterning in Arabidopsis. *Development* **137**, 113-22 (2010).

218. Balamotis, M.A. et al. Complexity in transcription control at the activation domain-mediator interface. *Sci Signal* **2**, ra20 (2009).

219. Ito, M., Okano, H.J., Darnell, R.B. & Roeder, R.G. The TRAP100 component of the TRAP/Mediator complex is essential in broad transcriptional events and development. *EMBO J* **21**, 3464-75 (2002).

220. Pietsch, J. et al. lessen encodes a zebrafish trap100 required for enteric nervous system development. *Development* **133**, 395-406 (2006).

221. Bosveld, F., van Hoek, S. & Sibon, O.C. Establishment of cell fate during early Drosophila embryogenesis requires transcriptional Mediator subunit dMED31. *Dev Biol* **313**, 802-13 (2008).

222. Risley, M.D., Clowes, C., Yu, M., Mitchell, K. & Hentges, K.E. The Mediator complex protein Med31 is required for embryonic growth and cell proliferation during mammalian development. *Dev Biol* **342**, 146-56 (2010).

223. Sandell, L.L. et al. A phenotype-driven ENU mutagenesis screen identifies novel alleles with functional roles in early mouse craniofacial development. *Genesis* **49**, 342-59 (2011).

224. Dixon, J. et al. Tcof1/Treacle is required for neural crest cell formation and proliferation deficiencies that cause craniofacial abnormalities. *Proc Natl Acad Sci U S A* **103**, 13403-8 (2006).

225. Jones, N.C. et al. Prevention of the neurocristopathy Treacher Collins syndrome through inhibition of p53 function. *Nat Med* **14**, 125-33 (2008).

226. Tobin, J.L. et al. Inhibition of neural crest migration underlies craniofacial dysmorphology and Hirschsprung's disease in Bardet-Biedl syndrome. *Proc Natl Acad Sci U S A* **105**, 6714-9 (2008).

227. Morriss-Kay, G.M. & Wilkie, A.O. Growth of the normal skull vault and its alteration in craniosynostosis: insights from human genetics and experimental studies. *J Anat* **207**, 637-53 (2005).

228. Kisanuki, Y.Y. et al. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Dev Biol* **230**, 230-42 (2001).

229.   Choi, K. The hemangioblast: a common progenitor of hematopoietic and endothelial cells. *J Hematother Stem Cell Res* **11**, 91-101 (2002).

230.   Liao, W. et al. The zebrafish gene cloche acts upstream of a flk-1 homologue to regulate endothelial cell differentiation. *Development* **124**, 381-9 (1997).

231.   Liao, E.C. et al. SCL/Tal-1 transcription factor acts downstream of cloche to specify hematopoietic and vascular progenitors in zebrafish. *Genes Dev* **12**, 621-6 (1998).

232.   Xiong, J.W., Yu, Q., Zhang, J. & Mably, J.D. An acyltransferase controls the generation of hematopoietic and endothelial lineages in zebrafish. *Circ Res* **102**, 1057-64 (2008).

233.   Chung, Y.S. et al. Lineage analysis of the hemangioblast as defined by FLK1 and SCL expression. *Development* **129**, 5511-20 (2002).

234.   Huber, T.L., Kouskoff, V., Fehling, H.J., Palis, J. & Keller, G. Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* **432**, 625-30 (2004).

235.   Chang, H. et al. Smad5 knockout mice die at mid-gestation due to multiple embryonic and extraembryonic defects. *Development* **126**, 1631-42 (1999).

236.   Sirard, C. et al. The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev* **12**, 107-19 (1998).

237.   Yang, X., Li, C., Xu, X. & Deng, C. The tumor suppressor SMAD4/DPC4 is essential for epiblast proliferation and mesoderm induction in mice. *Proc Natl Acad Sci U S A* **95**, 3667-72 (1998).

238.   Pearson, S., Sroczynska, P., Lacaud, G. & Kouskoff, V. The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF. *Development* **135**, 1525-35 (2008).

239.   Huber, T.L., Zhou, Y., Mead, P.E. & Zon, L.I. Cooperative effects of growth factors involved in the induction of hematopoietic mesoderm. *Blood* **92**, 4128-37 (1998).

240.   Kelly, M.A. & Hirschi, K.K. Signaling hierarchy regulating human endothelial cell development. *Arterioscler Thromb Vasc Biol* **29**, 718-24 (2009).

241.   Byrd, N. et al. Hedgehog is required for murine yolk sac angiogenesis. *Development* **129**, 361-72 (2002).

242.   Harvey, N.L. & Oliver, G. Choose your fate: artery, vein or lymphatic vessel? *Curr Opin Genet Dev* **14**, 499-505 (2004).

243. Swift, M.R. & Weinstein, B.M. Arterial-venous specification during development. *Circ Res* **104**, 576-88 (2009).

244. Krebs, L.T. et al. Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev* **14**, 1343-52 (2000).

245. Trindade, A. et al. Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos. *Blood* **112**, 1720-9 (2008).

246. Fischer, A., Schumacher, N., Maier, M., Sendtner, M. & Gessler, M. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes Dev* **18**, 901-11 (2004).

247. Kokubo, H., Miyagawa-Tomita, S., Nakazawa, M., Saga, Y. & Johnson, R.L. Mouse hesr1 and hesr2 genes are redundantly required to mediate Notch signaling in the developing cardiovascular system. *Dev Biol* **278**, 301-9 (2005).

248. Carmeliet, P. & Jain, R.K. Molecular mechanisms and clinical applications of angiogenesis. *Nature* **473**, 298-307 (2011).

249. Ridgway, J. et al. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature* **444**, 1083-7 (2006).

250. Lobov, I.B. et al. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc Natl Acad Sci U S A* **104**, 3219-24 (2007).

251. Suchting, S. et al. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc Natl Acad Sci U S A* **104**, 3225-30 (2007).

252. Siekmann, A.F. & Lawson, N.D. Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature* **445**, 781-4 (2007).

253. Sainson, R.C. et al. Cell-autonomous notch signaling regulates endothelial cell branching and proliferation during vascular tubulogenesis. *FASEB J* **19**, 1027-9 (2005).

254. Hellstrom, M. et al. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* **445**, 776-80 (2007).

255. Jakobsson, L. et al. Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. *Nat Cell Biol* **12**, 943-53 (2010).

256. Duarte, A. et al. Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev* **18**, 2474-8 (2004).

257. Krebs, L.T. et al. Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. *Genes Dev* **18**, 2469-73 (2004).

258. Lawson, N.D., Vogel, A.M. & Weinstein, B.M. sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Dev Cell* **3**, 127-36 (2002).

259. Mukouyama, Y.S., Gerber, H.P., Ferrara, N., Gu, C. & Anderson, D.J. Peripheral nerve-derived VEGF promotes arterial differentiation via neuropilin 1-mediated positive feedback. *Development* **132**, 941-52 (2005).

260. Pereira, F.A., Qiu, Y., Zhou, G., Tsai, M.J. & Tsai, S.Y. The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. *Genes Dev* **13**, 1037-49 (1999).

261. Huppert, S.S. et al. Embryonic lethality in mice homozygous for a processing-deficient allele of Notch1. *Nature* **405**, 966-70 (2000).

262. You, L.R. et al. Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature* **435**, 98-104 (2005).

263. Srinivasan, R.S. et al. The nuclear hormone receptor Coup-TFII is required for the initiation and early maintenance of Prox1 expression in lymphatic endothelial cells. *Genes Dev* **24**, 696-707 (2010).

264. Lohela, M., Bry, M., Tammela, T. & Alitalo, K. VEGFs and receptors involved in angiogenesis versus lymphangiogenesis. *Curr Opin Cell Biol* **21**, 154-65 (2009).

265. Bates, D.O. Vascular endothelial growth factors and vascular permeability. *Cardiovasc Res* **87**, 262-71 (2010).

266. Breier, G., Clauss, M. & Risau, W. Coordinate expression of vascular endothelial growth factor receptor-1 (flt-1) and its ligand suggests a paracrine regulation of murine vascular development. *Dev Dyn* **204**, 228-39 (1995).

267. Carmeliet, P. et al. Role of tissue factor in embryonic blood vessel development. *Nature* **383**, 73-5 (1996).

268. Ferrara, N. et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380**, 439-42 (1996).

269. Hiratsuka, S. et al. Membrane fixation of vascular endothelial growth factor receptor 1 ligand-binding domain is important for vasculogenesis and angiogenesis in mice. *Mol Cell Biol* **25**, 346-54 (2005).

270. Shalaby, F. et al. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell* **89**, 981-90 (1997).

271.   Dumont, D.J. et al. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev* **8**, 1897-909 (1994).

272.   Suri, C. et al. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* **87**, 1171-80 (1996).

273.   Jeansson, M. et al. Angiopoietin-1 is essential in mouse vasculature during development and in response to injury. *J Clin Invest* **121**, 2278-89 (2011).

274.   Partanen, J. et al. Cell autonomous functions of the receptor tyrosine kinase TIE in a late phase of angiogenic capillary growth and endothelial cell survival during murine development. *Development* **122**, 3013-21 (1996).

275.   Puri, M.C., Rossant, J., Alitalo, K., Bernstein, A. & Partanen, J. The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO J* **14**, 5884-91 (1995).

276.   Dejana, E. & Giampietro, C. Vascular endothelial-cadherin and vascular stability. *Curr Opin Hematol* **19**, 218-23 (2012).

277.   Vittet, D., Buchou, T., Schweitzer, A., Dejana, E. & Huber, P. Targeted null-mutation in the vascular endothelial-cadherin gene impairs the organization of vascular-like structures in embryoid bodies. *Proc Natl Acad Sci U S A* **94**, 6273-8 (1997).

278.   Gory-Faure, S. et al. Role of vascular endothelial-cadherin in vascular morphogenesis. *Development* **126**, 2093-102 (1999).

279.   Carmeliet, P. et al. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* **98**, 147-57 (1999).

280.   Crosby, C.V. et al. VE-cadherin is not required for the formation of nascent blood vessels but acts to prevent their disassembly. *Blood* **105**, 2771-6 (2005).

281.   Luo, Y. & Radice, G.L. N-cadherin acts upstream of VE-cadherin in controlling vascular morphogenesis. *J Cell Biol* **169**, 29-34 (2005).

282.   Tillet, E. et al. N-cadherin deficiency impairs pericyte recruitment, and not endothelial differentiation or sprouting, in embryonic stem cell-derived angiogenesis. *Exp Cell Res* **310**, 392-400 (2005).

283.   Morita, K., Sasaki, H., Furuse, M. & Tsukita, S. Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. *J Cell Biol* **147**, 185-94 (1999).

284. Nitta, T. et al. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J Cell Biol* **161**, 653-60 (2003).

285. Duncan, G.S. et al. Genetic evidence for functional redundancy of Platelet/Endothelial cell adhesion molecule-1 (PECAM-1): CD31-deficient mice reveal PECAM-1-dependent and PECAM-1-independent functions. *J Immunol* **162**, 3022-30 (1999).

286. Preiss, D.J. & Sattar, N. Vascular cell adhesion molecule-1: a viable therapeutic target for atherosclerosis? *Int J Clin Pract* **61**, 697-701 (2007).

287. Senger, D.R. et al. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* **219**, 983-5 (1983).

288. Glass, C.A., Harper, S.J. & Bates, D.O. The anti-angiogenic VEGF isoform VEGF165b transiently increases hydraulic conductivity, probably through VEGF receptor 1 in vivo. *J Physiol* **572**, 243-57 (2006).

289. Ku, D.D., Zaleski, J.K., Liu, S. & Brock, T.A. Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. *Am J Physiol* **265**, H586-92 (1993).

290. Antonetti, D.A., Barber, A.J., Hollinger, L.A., Wolpert, E.B. & Gardner, T.W. Vascular endothelial growth factor induces rapid phosphorylation of tight junction proteins occludin and zonula occluden 1. A potential mechanism for vascular permeability in diabetic retinopathy and tumors. *J Biol Chem* **274**, 23463-7 (1999).

291. Kevil, C.G., Payne, D.K., Mire, E. & Alexander, J.S. Vascular permeability factor/vascular endothelial cell growth factor-mediated permeability occurs through disorganization of endothelial junctional proteins. *J Biol Chem* **273**, 15099-103 (1998).

292. Esser, S. et al. Vascular endothelial growth factor induces endothelial fenestrations in vitro. *J Cell Biol* **140**, 947-59 (1998).

293. Dejana, E., Spagnuolo, R. & Bazzoni, G. Interendothelial junctions and their role in the control of angiogenesis, vascular permeability and leukocyte transmigration. *Thromb Haemost* **86**, 308-15 (2001).

294. Wang, W., Merrill, M.J. & Borchardt, R.T. Vascular endothelial growth factor affects permeability of brain microvessel endothelial cells in vitro. *Am J Physiol* **271**, C1973-80 (1996).

295. Michel, C.C. & Neal, C.R. Openings through endothelial cells associated with increased microvascular permeability. *Microcirculation* **6**, 45-54 (1999).

296. Taddei, A. et al. Endothelial adherens junctions control tight junctions by VE-cadherin-mediated upregulation of claudin-5. *Nat Cell Biol* **10**, 923-34 (2008).

297. Yin, J.W. et al. Mediator MED23 plays opposing roles in directing smooth muscle cell and adipocyte differentiation. *Genes Dev* **26**, 2192-205 (2012).

298. Caveda, L. et al. Inhibition of cultured cell growth by vascular endothelial cadherin (cadherin-5/VE-cadherin). *J Clin Invest* **98**, 886-93 (1996).

299. Barlow, L.A. Cranial nerve development: placodal neurons ride the crest. *Curr Biol* **12**, R171-3 (2002).

300. Baker, C.V., Bronner-Fraser, M., Le Douarin, N.M. & Teillet, M.A. Early- and late-migrating cranial neural crest cell populations have equivalent developmental potential in vivo. *Development* **124**, 3077-87 (1997).

301. Park, B.Y. & Saint-Jeannet, J.P. in *Induction and Segregation of the Vertebrate Cranial Placodes* (San Rafael (CA), 2010).

302. Pandur, P.D. & Moody, S.A. Xenopus Six1 gene is expressed in neurogenic cranial placodes and maintained in the differentiating lateral lines. *Mech Dev* **96**, 253-7 (2000).

303. Schlosser, G. & Northcutt, R.G. Development of neurogenic placodes in Xenopus laevis. *J Comp Neurol* **418**, 121-46 (2000).

304. Niiya, A., Ohto, H., Kawakami, K. & Araki, M. Localization of Six4/AREC3 in the developing mouse retina; implications in mammalian retinal development. *Exp Eye Res* **67**, 699-707 (1998).

305. Ohto, H. et al. Tissue and developmental distribution of Six family gene products. *Int J Dev Biol* **42**, 141-8 (1998).

306. Schlosser, G. & Ahrens, K. Molecular anatomy of placode development in Xenopus laevis. *Dev Biol* **271**, 439-66 (2004).

307. Kamachi, Y., Uchikawa, M., Collignon, J., Lovell-Badge, R. & Kondoh, H. Involvement of Sox1, 2 and 3 in the early and subsequent molecular events of lens induction. *Development* **125**, 2521-32 (1998).

308. Freter, S. et al. Pax2 modulates proliferation during specification of the otic and epibranchial placodes. *Dev Dyn* **241**, 1716-28 (2012).

309. Hans, S., Liu, D. & Westerfield, M. Pax8 and Pax2a function synergistically in otic specification, downstream of the Foxi1 and Dlx3b transcription factors. *Development* **131**, 5091-102 (2004).

310. Mackereth, M.D., Kwak, S.J., Fritz, A. & Riley, B.B. Zebrafish pax8 is required for otic placode induction and plays a redundant role with Pax2 genes in the maintenance of the otic placode. *Development* **132**, 371-82 (2005).

311. Mansouri, A. The role of Pax3 and Pax7 in development and cancer. *Crit Rev Oncog* **9**, 141-9 (1998).

312. Torres, M., Gomez-Pardo, E. & Gruss, P. Pax2 contributes to inner ear patterning and optic nerve trajectory. *Development* **122**, 3381-91 (1996).

313. Epstein, D.J., Vekemans, M. & Gros, P. Splotch (Sp2H), a mutation affecting development of the mouse neural tube, shows a deletion within the paired homeodomain of Pax-3. *Cell* **67**, 767-74 (1991).

314. Ma, Q., Chen, Z., del Barco Barrantes, I., de la Pompa, J.L. & Anderson, D.J. neurogenin1 is essential for the determination of neuronal precursors for proximal cranial sensory ganglia. *Neuron* **20**, 469-82 (1998).

315. Fode, C. et al. The bHLH protein NEUROGENIN 2 is a determination factor for epibranchial placode-derived sensory neurons. *Neuron* **20**, 483-94 (1998).

316. Guillemot, F. Vertebrate bHLH genes and the determination of neuronal fates. *Exp Cell Res* **253**, 357-64 (1999).

317. Chen, C.L. et al. Runx1 determines nociceptive sensory neuron phenotype and is required for thermal and neuropathic pain. *Neuron* **49**, 365-77 (2006).

318. Luo, W. et al. A hierarchical NGF signaling cascade controls Ret-dependent and Ret-independent events during development of nonpeptidergic DRG neurons. *Neuron* **54**, 739-54 (2007).

319. Snider, W.D. & Wright, D.E. Neurotrophins cause a new sensation. *Neuron* **16**, 229-32 (1996).

320. Gonzalez-Martinez, T. et al. Absence of Meissner corpuscles in the digital pads of mice lacking functional TrkB. *Brain Res* **1002**, 120-8 (2004).

321. Shimizu, S., Ichikawa, H., Nakagawa, H., Kiyomiya, K. & Matsuo, S. Effect of BDNF depletion on the formation of Ruffini endings in vibrissa follicles and the survival of their mechanoreceptive neurons in trigeminal ganglion. *Brain Res* **1154**, 95-104 (2007).

322. Perez-Pinera, P. et al. Characterization of sensory deficits in TrkB knockout mice. *Neurosci Lett* **433**, 43-7 (2008).

323. Hasegawa, H. & Wang, F. Visualizing mechanosensory endings of TrkC-expressing neurons in HS3ST-2-hPLAP mice. *J Comp Neurol* **511**, 543-56 (2008).

324. Ma, Q., Fode, C., Guillemot, F. & Anderson, D.J. Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia. *Genes Dev* **13**, 1717-28 (1999).

325. Marmigere, F. & Ernfors, P. Specification and connectivity of neuronal subtypes in the sensory lineage. *Nat Rev Neurosci* **8**, 114-27 (2007).

326. Zirlinger, M., Lo, L., McMahon, J., McMahon, A.P. & Anderson, D.J. Transient expression of the bHLH factor neurogenin-2 marks a subpopulation of neural crest cells biased for a sensory but not a neuronal fate. *Proc Natl Acad Sci U S A* **99**, 8084-9 (2002).

327. Kleber, M. et al. Neural crest stem cell maintenance by combinatorial Wnt and BMP signaling. *J Cell Biol* **169**, 309-20 (2005).

328. Eickholt, B.J., Mackenzie, S.L., Graham, A., Walsh, F.S. & Doherty, P. Evidence for collapsin-1 functioning in the control of neural crest migration in both trunk and hindbrain regions. *Development* **126**, 2181-9 (1999).

329. Kawasaki, T. et al. Requirement of neuropilin 1-mediated Sema3A signals in patterning of the sympathetic nervous system. *Development* **129**, 671-80 (2002).

330. Bron, R., Eickholt, B.J., Vermeren, M., Fragale, N. & Cohen, J. Functional knockdown of neuropilin-1 in the developing chick nervous system by siRNA hairpins phenocopies genetic ablation in the mouse. *Dev Dyn* **230**, 299-308 (2004).

331. Kasemeier-Kulesa, J.C., Bradley, R., Pasquale, E.B., Lefcort, F. & Kulesa, P.M. Eph/ephrins and N-cadherin coordinate to control the pattern of sympathetic ganglia. *Development* **133**, 4839-47 (2006).

332. Reissmann, E. et al. Involvement of bone morphogenetic protein-4 and bone morphogenetic protein-7 in the differentiation of the adrenergic phenotype in developing sympathetic neurons. *Development* **122**, 2079-88 (1996).

333. Shah, N.M., Groves, A.K. & Anderson, D.J. Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. *Cell* **85**, 331-43 (1996).

334. McPherson, C.E., Varley, J.E. & Maxwell, G.D. Expression and regulation of type I BMP receptors during early avian sympathetic ganglion development. *Dev Biol* **221**, 220-32 (2000).

335. Pattyn, A., Morin, X., Cremer, H., Goridis, C. & Brunet, J.F. The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. *Nature* **399**, 366-70 (1999).

336. Stanke, M. et al. The Phox2 homeodomain proteins are sufficient to promote the development of sympathetic neurons. *Development* **126**, 4087-94 (1999).

337. Schneider, C., Wicht, H., Enderich, J., Wegner, M. & Rohrer, H. Bone morphogenetic proteins are required in vivo for the generation of sympathetic neurons. *Neuron* **24**, 861-70 (1999).

338. Pattyn, A., Goridis, C. & Brunet, J.F. Specification of the central noradrenergic phenotype by the homeobox gene Phox2b. *Mol Cell Neurosci* **15**, 235-43 (2000).

339. Brunet, J.F. & Pattyn, A. Phox2 genes - from patterning to connectivity. *Curr Opin Genet Dev* **12**, 435-40 (2002).

340. Hirsch, M.R., Tiveron, M.C., Guillemot, F., Brunet, J.F. & Goridis, C. Control of noradrenergic differentiation and Phox2a expression by MASH1 in the central and peripheral nervous system. *Development* **125**, 599-608 (1998).

341. Lo, L., Tiveron, M.C. & Anderson, D.J. MASH1 activates expression of the paired homeodomain transcription factor Phox2a, and couples pan-neuronal and subtype-specific components of autonomic neuronal identity. *Development* **125**, 609-20 (1998).

342. Zellmer, E. et al. A homeodomain protein selectively expressed in noradrenergic tissue regulates transcription of neurotransmitter biosynthetic genes. *J Neurosci* **15**, 8109-20 (1995).

343. Yang, C. et al. Paired-like homeodomain proteins, Phox2a and Phox2b, are responsible for noradrenergic cell-specific transcription of the dopamine beta-hydroxylase gene. *J Neurochem* **71**, 1813-26 (1998).

344. Lo, L., Morin, X., Brunet, J.F. & Anderson, D.J. Specification of neurotransmitter identity by Phox2 proteins in neural crest stem cells. *Neuron* **22**, 693-705 (1999).

345. Xu, H., Firulli, A.B., Zhang, X. & Howard, M.J. HAND2 synergistically enhances transcription of dopamine-beta-hydroxylase in the presence of Phox2a. *Dev Biol* **262**, 183-93 (2003).

346. Rychlik, J.L., Gerbasi, V. & Lewis, E.J. The interaction between dHAND and Arix at the dopamine beta-hydroxylase promoter region is independent of direct dHAND binding to DNA. *J Biol Chem* **278**, 49652-60 (2003).

347. Firulli, A.B. A HANDful of questions: the molecular biology of the heart and neural crest derivatives (HAND)-subclass of basic helix-loop-helix transcription factors. *Gene* **312**, 27-40 (2003).

348. Lim, K.C. et al. Gata3 loss leads to embryonic lethality due to noradrenaline deficiency of the sympathetic nervous system. *Nat Genet* **25**, 209-12 (2000).

349. Tsarovina, K. et al. Essential role of Gata transcription factors in sympathetic neuron development. *Development* **131**, 4775-86 (2004).

350. Goridis, C. & Rohrer, H. Specification of catecholaminergic and serotonergic neurons. *Nat Rev Neurosci* **3**, 531-41 (2002).

351. Rohrer, H. The role of bone morphogenetic proteins in sympathetic neuron development. *Drug News Perspect* **16**, 589-96 (2003).

352. Howard, M.J. Mechanisms and perspectives on differentiation of autonomic neurons. *Dev Biol* **277**, 271-86 (2005).

353. Muller, F. & Rohrer, H. Molecular control of ciliary neuron development: BMPs and downstream transcriptional control in the parasympathetic lineage. *Development* **129**, 5707-17 (2002).

354. Lee, V.M., Bronner-Fraser, M. & Baker, C.V. Restricted response of mesencephalic neural crest to sympathetic differentiation signals in the trunk. *Dev Biol* **278**, 175-92 (2005).

355. Andres, R., Herraez-Baranda, L.A., Thompson, J., Wyatt, S. & Davies, A.M. Regulation of sympathetic neuron differentiation by endogenous nerve growth factor and neurotrophin-3. *Neurosci Lett* **431**, 241-6 (2008).

356. Summerhurst, K., Stark, M., Sharpe, J., Davidson, D. & Murphy, P. 3D representation of Wnt and Frizzled gene expression patterns in the mouse embryo at embryonic day 11.5 (Ts19). *Gene Expr Patterns* **8**, 331-48 (2008).

357. Geetha-Loganathan, P. et al. Expression of WNT signalling pathway genes during chicken craniofacial development. *Dev Dyn* **238**, 1150-65 (2009).

358. Mani, P., Jarrell, A., Myers, J. & Atit, R. Visualizing canonical Wnt signaling during mouse craniofacial development. *Dev Dyn* **239**, 354-63 (2010).

359. Maretto, S. et al. Mapping Wnt/beta-catenin signaling during mouse development and in colorectal tumors. *Proc Natl Acad Sci U S A* **100**, 3299-304 (2003).

360. Brugmann, S.A. et al. Wnt signaling mediates regional specification in the vertebrate face. *Development* **134**, 3283-95 (2007).

361. Al Alam, D. et al. Contrasting expression of canonical Wnt signaling reporters TOPGAL, BATGAL and Axin2(LacZ) during murine lung development and repair. *PLoS One* **6**, e23139 (2011).

362. Sun, Y. et al. Asymmetric requirement of surface epithelial beta-catenin during the upper and lower jaw development. *Dev Dyn* **241**, 663-74 (2012).

363. Wang, Y., Song, L. & Zhou, C.J. The canonical Wnt/beta-catenin signaling pathway regulates Fgf signaling for early facial development. *Dev Biol* **349**, 250-60 (2011).

364. Reid, B.S., Yang, H., Melvin, V.S., Taketo, M.M. & Williams, T. Ectodermal Wnt/beta-catenin signaling shapes the mouse face. *Dev Biol* **349**, 261-9 (2011).

365. Song, L. et al. Lrp6-mediated canonical Wnt signaling is required for lip formation and fusion. *Development* **136**, 3161-71 (2009).

366. Niemann, S. et al. Homozygous WNT3 mutation causes tetra-amelia in a large consanguineous family. *Am J Hum Genet* **74**, 558-63 (2004).

367. Juriloff, D.M., Harris, M.J., McMahon, A.P., Carroll, T.J. & Lidral, A.C. Wnt9b is the mutated gene involved in multifactorial nonsyndromic cleft lip with or without cleft palate in A/WySn mice, as confirmed by a genetic complementation test. *Birth Defects Res A Clin Mol Teratol* **76**, 574-9 (2006).

368. Mallarino, R. et al. Two developmental modules establish 3D beak-shape variation in Darwin's finches. *Proc Natl Acad Sci U S A* **108**, 4057-62 (2011).

369. Brito, J.M., Teillet, M.A. & Le Douarin, N.M. An early role for sonic hedgehog from foregut endoderm in jaw development: ensuring neural crest cell survival. *Proc Natl Acad Sci U S A* **103**, 11607-12 (2006).

370. Couly, G., Creuzet, S., Bennaceur, S., Vincent, C. & Le Douarin, N.M. Interactions between Hox-negative cephalic neural crest cells and the foregut endoderm in patterning the facial skeleton in the vertebrate head. *Development* **129**, 1061-73 (2002).

371. Benouaiche, L., Gitton, Y., Vincent, C., Couly, G. & Levi, G. Sonic hedgehog signalling from foregut endoderm patterns the avian nasal capsule. *Development* **135**, 2221-5 (2008).

372. Ruhin, B. et al. Patterning of the hyoid cartilage depends upon signals arising from the ventral foregut endoderm. *Dev Dyn* **228**, 239-46 (2003).

373. Tyler, M.S. & Hall, B.K. Epithelial influences on skeletogenesis in the mandible of the embryonic chick. *Anat Rec* **188**, 229-39 (1977).

374. Eberhart, J.K., Swartz, M.E., Crump, J.G. & Kimmel, C.B. Early Hedgehog signaling from neural to oral epithelium organizes anterior craniofacial development. *Development* **133**, 1069-77 (2006).

375. Hu, D. & Helms, J.A. The role of sonic hedgehog in normal and abnormal craniofacial morphogenesis. *Development* **126**, 4873-84 (1999).

376. Bachler, M. & Neubuser, A. Expression of members of the Fgf family and their receptors during midfacial development. *Mech Dev* **100**, 313-6 (2001).

377. Riley, B.M. et al. Impaired FGF signaling contributes to cleft lip and palate. *Proc Natl Acad Sci U S A* **104**, 4512-7 (2007).

378. Crossley, P.H. & Martin, G.R. The mouse Fgf8 gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-51 (1995).

379. Heikinheimo, M., Lawshe, A., Shackleford, G.M., Wilson, D.B. & MacArthur, C.A. Fgf-8 expression in the post-gastrulation mouse suggests roles in the development of the face, limbs and central nervous system. *Mech Dev* **48**, 129-38 (1994).

380. Trumpp, A., Depew, M.J., Rubenstein, J.L., Bishop, J.M. & Martin, G.R. Cre-mediated gene inactivation demonstrates that FGF8 is required for cell survival and patterning of the first branchial arch. *Genes Dev* **13**, 3136-48 (1999).

381. Abu-Issa, R., Smyth, G., Smoak, I., Yamamura, K. & Meyers, E.N. Fgf8 is required for pharyngeal arch and cardiovascular development in the mouse. *Development* **129**, 4613-25 (2002).

382. Abzhanov, A., Tzahor, E., Lassar, A.B. & Tabin, C.J. Dissimilar regulation of cell differentiation in mesencephalic (cranial) and sacral (trunk) neural crest cells in vitro. *Development* **130**, 4567-79 (2003).

383. Ohbayashi, N. et al. FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. *Genes Dev* **16**, 870-9 (2002).

384. Hu, D., Marcucio, R.S. & Helms, J.A. A zone of frontonasal ectoderm regulates patterning and growth in the face. *Development* **130**, 1749-58 (2003).

385. Hu, D. & Marcucio, R.S. Unique organization of the frontonasal ectodermal zone in birds and mammals. *Dev Biol* **325**, 200-10 (2009).

386. Cordero, D. et al. Temporal perturbations in sonic hedgehog signaling elicit the spectrum of holoprosencephaly phenotypes. *J Clin Invest* **114**, 485-94 (2004).

387. Marcucio, R.S., Cordero, D.R., Hu, D. & Helms, J.A. Molecular interactions coordinating the development of the forebrain and face. *Dev Biol* **284**, 48-61 (2005).

388. MacDonald, M.E., Abbott, U.K. & Richman, J.M. Upper beak truncation in chicken embryos with the cleft primary palate mutation is due to an epithelial defect in the frontonasal mass. *Dev Dyn* **230**, 335-49 (2004).

389. Abzhanov, A. & Tabin, C.J. Shh and Fgf8 act synergistically to drive cartilage outgrowth during cranial development. *Dev Biol* **273**, 134-48 (2004).

390. Foppiano, S., Hu, D. & Marcucio, R.S. Signaling by bone morphogenetic proteins directs formation of an ectodermal signaling center that regulates craniofacial development. *Dev Biol* **312**, 103-14 (2007).

391. Hu, D., Colnot, C. & Marcucio, R.S. Effect of bone morphogenetic protein signaling on development of the jaw skeleton. *Dev Dyn* **237**, 3727-37 (2008).

392. Maden, M. et al. Domains of cellular retinoic acid-binding protein I (CRABP I) expression in the hindbrain and neural crest of the mouse embryo. *Mech Dev* **37**, 13-23 (1992).

393. Acampora, D. et al. Forebrain and midbrain regions are deleted in Otx2-/- mutants due to a defective anterior neuroectoderm specification during gastrulation. *Development* **121**, 3279-90 (1995).

394. Boyl, P.P. et al. Forebrain and midbrain development requires epiblast-restricted Otx2 translational control mediated by its 3' UTR. *Development* **128**, 2989-3000 (2001).

395. Kobayashi, M., Toyama, R., Takeda, H., Dawid, I.B. & Kawakami, K. Overexpression of the forebrain-specific homeobox gene six3 induces rostral forebrain enlargement in zebrafish. *Development* **125**, 2973-82 (1998).

396. Jeong, Y. et al. Regulation of a remote Shh forebrain enhancer by the Six3 homeoprotein. *Nat Genet* **40**, 1348-53 (2008).

397. Geng, X. et al. Haploinsufficiency of Six3 fails to activate Sonic hedgehog expression in the ventral forebrain and causes holoprosencephaly. *Dev Cell* **15**, 236-47 (2008).

398. Achilleos, A. & Trainor, P.A. Neural crest stem cells: discovery, properties and potential for therapy. *Cell Res* **22**, 288-304 (2012).

399. Jeong, J., Mao, J., Tenzen, T., Kottmann, A.H. & McMahon, A.P. Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. *Genes Dev* **18**, 937-51 (2004).

400. Lewis, S.L. et al. Dkk1 and Wnt3 interact to control head morphogenesis in the mouse. *Development* **135**, 1791-801 (2008).

401. Nusse, R. & Varmus, H.E. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* **31**, 99-109 (1982).

402. Sharma, R.P. & Chopra, V.L. Effect of the Wingless (wg1) mutation on wing and haltere development in Drosophila melanogaster. *Dev Biol* **48**, 461-5 (1976).

403. Niehrs, C. On growth and form: a Cartesian coordinate system of Wnt and BMP signaling specifies bilaterian body axes. *Development* **137**, 845-57 (2010).

404. Wray, J. & Hartmann, C. WNTing embryonic stem cells. *Trends Cell Biol* **22**, 159-68 (2012).

405.   Bullions, L.C. & Levine, A.J. The role of beta-catenin in cell adhesion, signal transduction, and cancer. *Curr Opin Oncol* **10**, 81-7 (1998).

406.   Saito-Diaz, K. et al. The way Wnt works: components and mechanism. *Growth Factors* **31**, 1-31 (2013).

407.   Semenov, M.V., Habas, R., Macdonald, B.T. & He, X. SnapShot: Noncanonical Wnt Signaling Pathways. *Cell* **131**, 1378 (2007).

408.   De, A. Wnt/Ca2+ signaling pathway: a brief overview. *Acta Biochim Biophys Sin (Shanghai)* **43**, 745-56 (2011).

409.   Lassiter, R.N. et al. Canonical Wnt signaling is required for ophthalmic trigeminal placode cell fate determination and maintenance. *Dev Biol* **308**, 392-406 (2007).

410.   Freter, S., Muta, Y., Mak, S.S., Rinkwitz, S. & Ladher, R.K. Progressive restriction of otic fate: the role of FGF and Wnt in resolving inner ear potential. *Development* **135**, 3415-24 (2008).

411.   Jayasena, C.S., Ohyama, T., Segil, N. & Groves, A.K. Notch signaling augments the canonical Wnt pathway to specify the size of the otic placode. *Development* **135**, 2251-61 (2008).

412.   Ohyama, K., Das, R. & Placzek, M. Temporal progression of hypothalamic patterning by a dual action of BMP. *Development* **135**, 3325-31 (2008).

413.   Jacques, B.E. et al. A dual function for canonical Wnt/beta-catenin signaling in the developing mammalian cochlea. *Development* **139**, 4395-404 (2012).

414.   Monkley, S.J., Delaney, S.J., Pennisi, D.J., Christiansen, J.H. & Wainwright, B.J. Targeted disruption of the Wnt2 gene results in placentation defects. *Development* **122**, 3343-53 (1996).

415.   Jeays-Ward, K. et al. Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad. *Development* **130**, 3663-70 (2003).

416.   Shu, W., Jiang, Y.Q., Lu, M.M. & Morrisey, E.E. Wnt7b regulates mesenchymal proliferation and vascular development in the lung. *Development* **129**, 4831-42 (2002).

417.   Lobov, I.B. et al. WNT7b mediates macrophage-induced programmed cell death in patterning of the vasculature. *Nature* **437**, 417-21 (2005).

418.   Ishikawa, T. et al. Mouse Wnt receptor gene Fzd5 is essential for yolk sac and placental angiogenesis. *Development* **128**, 25-33 (2001).

419. Nimmagadda, S., Geetha-Loganathan, P., Scaal, M., Christ, B. & Huang, R. FGFs, Wnts and BMPs mediate induction of VEGFR-2 (Quek-1) expression during avian somite development. *Dev Biol* **305**, 421-9 (2007).

420. Aicher, A. et al. The Wnt antagonist Dickkopf-1 mobilizes vasculogenic progenitor cells via activation of the bone marrow endosteal stem cell niche. *Circ Res* **103**, 796-803 (2008).

421. Lengerke, C. et al. BMP and Wnt specify hematopoietic fate by activation of the Cdx-Hox pathway. *Cell Stem Cell* **2**, 72-82 (2008).

422. Lindsley, R.C., Gill, J.G., Kyba, M., Murphy, T.L. & Murphy, K.M. Canonical Wnt signaling is required for development of embryonic stem cell-derived mesoderm. *Development* **133**, 3787-96 (2006).

423. Cattelino, A. et al. The conditional inactivation of the beta-catenin gene in endothelial cells causes a defective vascular pattern and increased vascular fragility. *J Cell Biol* **162**, 1111-22 (2003).

424. Corada, M. et al. The Wnt/beta-catenin pathway modulates vascular remodeling and specification by upregulating Dll4/Notch signaling. *Dev Cell* **18**, 938-49 (2010).

425. Engelhardt, B. Development of the blood-brain barrier. *Cell Tissue Res* **314**, 119-29 (2003).

426. Daneman, R. et al. Wnt/beta-catenin signaling is required for CNS, but not non-CNS, angiogenesis. *Proc Natl Acad Sci U S A* **106**, 641-6 (2009).

427. Stenman, J.M. et al. Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature. *Science* **322**, 1247-50 (2008).

428. Li, X. et al. Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *J Biol Chem* **280**, 19883-7 (2005).

429. Semenov, M., Tamai, K. & He, X. SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor. *J Biol Chem* **280**, 26770-5 (2005).

430. Ohazama, A. et al. Lrp4 modulates extracellular integration of cell signaling pathways in development. *PLoS One* **3**, e4092 (2008).

431. Carter, M. et al. Crooked tail (Cd) model of human folate-responsive neural tube defects is mutated in Wnt coreceptor lipoprotein receptor-related protein 6. *Proc Natl Acad Sci U S A* **102**, 12843-8 (2005).

432. Kassai, Y. et al. Regulation of mammalian tooth cusp patterning by ectodin. *Science* **309**, 2067-70 (2005).

433.	Itasaki, N. et al. Wise, a context-dependent activator and inhibitor of Wnt signalling. *Development* **130**, 4295-305 (2003).

434.	Ahn, Y., Sanderson, B.W., Klein, O.D. & Krumlauf, R. Inhibition of Wnt signaling by Wise (Sostdc1) and negative feedback from Shh controls tooth number and patterning. *Development* **137**, 3221-31 (2010).

435.	Shigetani, Y. et al. Wise promotes coalescence of cells of neural crest and placode origins in the trigeminal region during head development. *Dev Biol* **319**, 346-58 (2008).

436.	Boyer, T.G., Martin, M.E., Lees, E., Ricciardi, R.P. & Berk, A.J. Mammalian Srb/Mediator complex is targeted by adenovirus E1A protein. *Nature* **399**, 276-9 (1999).

437.	Wang, W. et al. Mediator MED23 regulates basal transcription in vivo via an interaction with P-TEFb. *Transcription* **4**, 39-51 (2013).

438.	Yang, X. et al. Selective requirement for Mediator MED23 in Ras-active lung cancer. *Proc Natl Acad Sci U S A* **109**, E2813-22 (2012).

439.	Abraham, S. et al. VE-Cadherin-mediated cell-cell interaction suppresses sprouting via signaling to MLC2 phosphorylation. *Curr Biol* **19**, 668-74 (2009).

440.	Corada, M. et al. Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. *Proc Natl Acad Sci U S A* **96**, 9815-20 (1999).

441.	Ye, X. et al. Norrin, frizzled-4, and Lrp5 signaling in endothelial cells controls a genetic program for retinal vascularization. *Cell* **139**, 285-98 (2009).

442.	Dejana, E. The role of wnt signaling in physiological and pathological angiogenesis. *Circ Res* **107**, 943-52 (2010).

443.	Pinson, K.I., Brennan, J., Monkley, S., Avery, B.J. & Skarnes, W.C. An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* **407**, 535-8 (2000).

444.	Gray, J.D. et al. LRP6 exerts non-canonical effects on Wnt signaling during neural tube closure. *Hum Mol Genet* (2013).