CIS-REGULATORY ANALYSIS OF THE KEY DEVELOPMENTAL GENE, SOX10, IN NEURAL CREST AND EAR

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ABSTRACT

In vertebrates, Sox10 is a transcription factor essential for the formation of neural crest cells and their derivatives as well as placode-derived inner ear structures. At gastrula stages, both presumptive neural crest and placode cells reside at the neural plate border, between the non-neural ectoderm and the neural plate. Despite their common site of origin, these two cell populations have different characteristics. Neural crest cells are a multipotent, stem cell-like population that arises at all axial levels, migrates extensively in the embryo and forms a wide array of derivatives ranging from neurons to melanocytes and cartilage. On the other hand, placode cells are restricted to the cranial region, have limited migratory capacity and contribute only to sensory structures such as the eye, ear, olfactory epithelium and distal portions of the cranial sensory ganglia. Interestingly, I have identified *cis*-regulatory modules responsible for the regulation of *Sox10* in these two different embryonic regions and at different times. Among these modules, I found a downstream novel cis-regulatory region, Sox10E2,that mediates initial *Sox10* expression in cranial neural crest cells and otic placode cells. Through a combination of computational analysis, experimental perturbation of putative upstream transcription factors and their binding sites within the Sox10E2 regulatory module, plus chromatin immunoprecipitation, I revealed a set of direct inputs into Sox10E2 regulatory region. The results show that cMyb, Sox9 and Ets1 are responsible for the initial *Sox10* expression in delaminating cranial neural crest cells, whereas cMyb, Sox8 and Pea3 regulate *Sox10* expression in the otic placode. Analyzing *Sox10* regulation through the enhancer Sox10E2 has helped unravel gene regulatory inputs contributing to both neural crest formation and otic placode development.

The finding that paralogous factors activate the same regulatory module in these two populations suggests the intriguing possibility of an ancient cooption of regulatory function and/or a common ancestral crest-placode origin.

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Introduction: Gene Regulatory Interactions at the Neural Plate Border, Neural Crest and Presumptive Placodal Region

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Introduction

The neural crest, often referred to as the "fourth germ layer" (Hall 2000), is a multipotent stem cell-like population of highly migratory cells that contribute derivatives to a wide variety of tissues and organs of the vertebrate embryo. These include but are not limited to sensory and autonomic ganglia, adrenal and thyroid glands, smooth muscle of major blood vessels, cartilage and bone of the face, and pigmentation of the skin. As a defining feature of vertebrates, neural crest formation has been extensively studied using different vertebrate model organisms, ranging from lampreys and fish, to frogs, chicken and mouse.

Neural crest cells form over a long period of developmental time that starts at gastrulation and extends into late organogenesis. This process is initiated by a combination of inductive signals emanating from neighboring tissues, such as underlying mesoderm or adjacent neural and non-neural ectoderm, which set up the presumptive neural crest region. As a result, the territory between neural and non-neural ectoderm, termed the neural plate border, is competent to respond to signals specifying bona fide progenitors. The neural crest precursors subsequently undergo an epithelial to mesenchymal transition, delaminate from the neuroepithelium, and migrate along stereotypical pathways. After settling in various and sometimes distant sites in the embryo, they differentiate into a multitude of derivatives.

In addition to neural crest cells, the unique neural plate border territory also contains precursors for dorsal neural tube and cranial placode cells. Fate map analyses

demonstrate that the preplacodal territory and presumptive neural crest cells largely overlap at the neural plate border (Streit 2007). The preplacodal region contributes to the eye, ear and olfactory, epithelium sensory structures. Placodes are defined as thickened ectoderm that with the exception of the lens placode, can delaminate from the epithelium and migrate into the adjacent mesenchyme in a similar way to neural crest cells (Schlosser 2008). Some of the signals and transcription factors involved in the induction of the placodal territory will be discussed later in this chapter.

For over a century, the neural crest and ectodermal placodes have provided a productive paradigm for addressing essential questions regarding cell interactions that underlay induction, specification and differentiation events during development. In particular, the neural crest is the subject of a very extensive literature and descriptive database. In combination with recent genomic *cis*-regulatory and gene knockdown data, this provides a critical mass of information regarding the molecular underpinnings that guide neural crest formation. Such a compelling database calls for a systematic approach to integrate diverse information into a multi-step gene regulatory network that describes the process of neural crest formation. In contrast, there is comparatively less information known about placode development. However, the otic placode, which is the best studied of the ectodermal placodes, has a growing body of molecular information concerning its formation and differentiation.

Accruing molecular information relevant to neural crest induction, specification and migration has led to the formulation of a putative vertebrate gene regulatory network (GRN) that orchestrates neural crest formation (Meulemans and Bronner-Fraser 2004,

Sauka-Spengler and Bronner-Fraser 2008a, Steventon et al. 2005). Because of variation between species, the main challenge has been to incorporate the pertinent data, obtained from a number of vertebrate developmental models, into a single, pan-vertebrate network. In addition to discrepancies in the patterns of gene expression and differences in deployment of paralogous genes among various vertebrates (Meulemans and Bronner-Fraser 2004), there are also remarkable differences between populations of neural crest cells originating from different axial levels within a given species. These include differences in mechanisms of delamination and developmental potential, such as the ability to generate skeletal structures (Graham et al. 2004). For example, although both cranial and trunk crest cells can generate the full repertoire of neural crest cell derivatives (McGonnell and Graham 2002), the skeletogenic potential of trunk crest cells is suppressed during normal development (Graham et al. 2004). Thus, different neural crest populations may well be exposed to at least a subset of unique regulatory interactions.

Finally, only a limited number of *cis*-regulatory studies of neural crest genes has been reported thus far, making it difficult to discern direct regulatory interactions. The majority of known direct regulatory interactions have been elucidated in differentiating neural crest derivatives (Sauka-Spengler and Bronner-Fraser 2008a). Thus, the current formulation of the neural crest (NC) GRN is largely a consolidation of regulatory predictions. Nevertheless, many regulatory steps appear to be highly conserved even in basal vertebrate systems (Sauka-Spengler et al. 2007), suggesting that it should be possible to assemble a scaffold of regulatory interactions that may be common to all vertebrates and may function at all axial levels.

In this chapter, I will first introduce the formation of neural crest cells by generating an updated representation of the NC-GRN. For this purpose I will attempt to integrate the most current neural crest regulatory information and present possible circuit connections inferred largely from loss of function analysis together with direct regulatory interactions, thus far documented mostly at later stages of differentiation. I will also take into account separate spatial subpopulations of neural crest at different levels of the neural axis. As a starting point, I will focus on the regulatory state of cranial neural crest cells (Fig. 1, table 1), which are the first crest population to form and initiate migration in the vertebrate embryo. These cells contribute derivatives mainly to the facial skeleton, peripheral nervous system and pigmentation in the head.

To present this updated NC-GRN, I used a generic drawing software Biotapestry (http://www.biotapestry.org/), which employs symbolic representation of genes to describe their regulatory interactions and integrate experimentally-derived network features (Longabaugh et al. 2009).

Finally, for the otic placode, given that there is limited information regarding gene regulatory interactions occurring during placode formation and differentiation, I will focus on the current state of knowledge of otic placode development as introduction to understanding the gene regulatory connections controlling formation of this ectodermal placode.

Initial signaling inputs into the neural crest gene regulatory network: BMP, Wnt, FGF and Notch pathways in induction and specification

The classical view suggested that neural crest cell induction occurred during the process of neurulation, as the neural folds elevated. This was thought to occur as a consequence of interactions resulting from the juxtaposition of epidermis on elevating neural plate (Mancilla and Mayor 1996, Selleck and Bronner-Fraser 1995). However, recent findings in frog (Monsoro-Burq et al. 2005) and chicken demonstrate that neural crest induction is underway much earlier, during gastrulation (Basch et al. 2006). In chicken, for instance, the transcription factor Pax7 is expressed in the neural plate border domain where neural crest cells originate, in the mid-gastrula as early as stage HH4+. When tissue explants from this Pax7-positive domain of the gastrula were cultured in the absence of exogenous inductive signals, they were able to generate neural crest cells (Basch et al. 2006), despite the lack of added factors or other tissue interactions. Recent fate maps studies show that the neural plate border region is wider and overlaps partially with BMP4-expressing domain at gastrula stages (Ezin et al. 2009), consistent with the possibility that signaling cues are already at play at this place and time.

Evidence of early specification of neural plate border in frog and chicken has also been substantiated by studies in lamprey, where these events are conserved, but happen at a much slower rate, making lamprey a suitable system for studying signaling inputs and neural plate border specifier readout with much better temporal resolution and therefore in much higher detail (Nikitina N. et al. 2008). Interestingly, the induction program and resulting expression of transcription factors specifying neural plate border is shared by non-

vertebrate chordates that do not possess neural crest (Meulemans and Bronner-Fraser 2004, Sauka-Spengler and Bronner-Fraser 2008b). Thus, all the evidence suggests that the neural crest cell induction in vertebrate embryos occurs during gastrulation. However, the early inductive events remain unexplored in some species, such as the mouse, highlighting the importance of performing comparative analysis in numerous vertebrates.

The induction of prospective neural crest within the neural plate border is thought to occur in response to signaling molecules emanating from adjacent tissues. The "response" that sets future neural crest cells apart from other border cells requires the activation of a battery of transcription factors that imbues them with multipotency, characteristics of proliferating cell, and the competence to respond to later neural crest–specifying signals. Identifying the signaling inputs that initiate neural crest induction has been challenging since information obtained from different vertebrate systems is sometimes contradictory. Fate map studies suggest that presumptive neural crest cells are in proximity to three different regions: presumptive epidermis, neural plate and mesoderm. These tissues are thought to secrete signaling ligands, including BMPs, Wnts and FGFs, that have all been demonstrated as essential for the early induction, maintenance and differentiation of neural crest cells (Knecht and Bronner-Fraser 2002). Although there are differences between neural crest populations at various levels of the neural axis, the inductive signals appear similar regardless of axial level.

Bone morphogenetic proteins (BMPs). In *Xenopus* embryos, high levels of BMP have been shown to be necessary for acquisition of epidermal fate, while inhibition of BMPs is required for neural induction (LaBonne and Bronner-Fraser 1998). The neural

plate border territory that lies between non-neural ectoderm (future epidermis) and neural ectoderm contains neural crest precursors, preplacodal ectoderm, dorsal neural tube and epidermis, all of which are exposed to BMP signals. In chicken explant culture experiments, juxtaposition of non-neural ectoderm and intermediate neural plate tissue, which normally forms only neural tube, can generate neural crest cells. Addition of BMP4 and BMP7, endogenously expressed in the non-neural ectoderm, is able to substitute for non-neural ectoderm such that neural crest cells are induced from intermediate neural plate explants (Liem et al. 1995). It has been proposed that intermediate levels of BMP, obtained as a result of diffusion of secreted BMP molecules throughout the ectoderm (BMP gradient) are responsible for the induction of neural crest cells. In support of the gradient model, zebrafish BMP pathway mutants show either expansion or reduction of the neural crest cell domain depending on the alteration of BMP levels (Knecht and Bronner-Fraser 2002, Nguyen et al. 1998). Alternatively, a gradient that would create the intermediate levels of BMP required for neural crest induction may be established by antagonistic interactions with cerberus, noggin, chordin, follistatins ligands, secreted by the forming neural plate cells, (Sauka-Spengler and Bronner-Fraser 2008a, Tribulo et al. 2003, Wilson et al. 1997). Regardless of the way a BMP gradient is established, intermediate levels of BMP alone are not sufficient to induce expression of neural crest cell markers in *Xenopus* or any other vertebrate model organisms (Garcia-Castro et al. 2002, LaBonne and Bronner-Fraser 1998, Wilson et al. 1997). BMP signaling is therefore an important initial step but additional signals are required for induction of the neural crest.

Fibroblast growth factors (FGFs). The FGF family of growth factors represents

another set of signaling cues implicated in neural crest induction. In *Xenopus* animal cap assays, FGF2 ligand, together with attenuated BMP signaling, up-regulates expression of an early neural crest cell marker, Snail2, whereas over-expression of dominant negative FGF receptor blocks Snail2 without affecting neural plate markers (Mayor et al. 1997, Villanueva et al. 2002). In *Xenopus*, over-expression of FGF8, normally expressed in the paraxial mesoderm, transiently induces neural crest cells (Monsoro-Burq et al. 2003). However, exogenous FGF8 alone is not sufficient to induce the full range of neural crest markers (Noden and Trainor 2005). Furthermore the requirement for FGF signaling may vary between species, making it difficult to make definitive conclusions about its universality. For example, mouse null mutant embryos lacking either FGF or FGFR have no obvious defects in neural crest formation (Jones and Trainor 2005). This could be explained by functional redundancy of FGF signaling factors. Similarly, in zebrafish, neural crest cells develop normally in the absence of mesoderm (Jones and Trainor 2005), and mutant embryos carrying mutations in FGF signaling components show no neural crest defects.

Wnt signaling pathway (Wnts). Wnt family members are involved in many aspects of neural crest development. Numerous family members, e.g. Wnt6, Wnt7b, Wnt3a, Wnt1 and Wnt8, are expressed in the right tissue and at the proper time to play a role in induction (Knecht and Bronner-Fraser 2002). Wnts are present in the paraxial mesoderm in frog (Christian et al. 1991, Knecht and Bronner-Fraser 2002) and in the non-neural ectoderm adjacent to the neural folds in chicken embryos (Garcia-Castro et al. 2002). Gain and loss of function experiments in frog, chicken and fish have shown that the activation of the Wnt

pathway is essential for neural crest cell induction and specification (Garcia-Castro et al. 2002, LaBonne and Bronner-Fraser 1998, Lewis et al. 2004). For instance, in zebrafish, an inducible Wnt inhibitor activated during early neurulation specifically interferes with neural crest cell formation without altering the formation of neurons from the central nervous system (Lewis et al. 2004). In chicken, Wnt6 is expressed by ectodermal cells at the time of neural crest cell induction, and exposing neural plate explants to Wnt6 induces the formation of neural crest cells in culture (Garcia-Castro et al. 2002, Schubert et al. 2002). However, the role of Wnt signaling in induction of the neural crest during gastrulation has yet to be examined in the mouse embryo. While *Wnt1/Wnt3a* double mutants exhibit defects in a wide range of neural crest derivatives (cranial skeleton, cranial and even dorsal root ganglia as well as melanocytes), it is not yet clear if this results from early induction defects, as the analysis of mutant phenotype in the neural plate border has yet to be performed (Ikeya et al. 1997). All other gene knock-out experiments used as evidence to suggest a role for Wnt signaling in mouse are confined to the lineage specification and neural crest cell differentiation rather than early induction. These have been performed by targeting the Wnt signaling pathway components in the dorsal neural tube (Jones and Trainor 2005), representing a relatively late time point, by which *bone fide* neural crest progenitors reside within the dorsal aspects of neural folds/tube. Thus, it is too late to address the role of Wnt signaling in induction events, which have normally taken place during gastrulation. In addition, due to gene duplications, and the particularly large number of Wnt ligands in mouse genome, it is possible that Wnts act redundantly during neural crest cell development in mouse. Their early inductive role may have been missed in single Wnt knock-outs, whereas the effects of simultaneous inactivation of several Wnts

have not been examined to date (Jones and Trainor 2005). Thus, it remains unclear if Wnt signaling pathways play an inductive role at early stages in the mouse embryo.

Local cell-cell signals such as Notch/Delta are also found in the vicinity of and/or on developing neural crest cells (Endo et al. 2002, Glavic et al. 2004, Williams et al. 1995). In chick, Notch is confined to the neural folds together with *Hairy2*, its direct downstream effector, whereas Delta is expressed in the presumptive epidermis (Endo et al. 2002). It has been reported that Notch-Delta signaling acts upstream of BMP4 in chick and *Xenopus* embryos and can affect expression of *Snail* and other neural crest specifier genes (Endo et al. 2002, Glavic et al. 2004). However, the function and requirement for Notch during neural crest cell development may vary among different vertebrates. In mouse, *Delta1* null mutants have no apparent early neural crest defects, even though cranial neural crest cells express several *Notch* genes (De Bellard et al. 2002, Williams et al. 1995). It is possible that Notch signalling in those cells may be activated by a different ligand. In zebrafish, mutants in components of the Notch pathway appear to affect trunk but not cranial neural crest (Cornell and Eisen 2005), consistent with the possibility that this signaling pathway plays more of a role in trunk than cranial crest, where there may be functional redundancy with other signaling pathways.

Despite some species specific differences, it is generally agreed that a combination of inductive signals activates a battery of immediate downstream genes in the neural plate border that give the cells the capacity to become neural crest cells. For instance, the combination of low levels of BMP plus Wnt family members can induce expression of *Snail2* and other neural crest genes in *Xenopus* explants (LaBonne and Bronner-Fraser

1998)

Neural Plate Border Specifiers

Signaling inputs into the neural plate border territory activate a battery of transcription factors whose collective expression sets presumptive neural crest cells apart from other border progenitors by conferring to them the competence to respond to neural crest specifying signals. These genes, termed neural plate border specifiers, appear early during neurulation and include homeobox transcription factors Mxs1/2, Dlx5, Pax3/7 and Gbx2, as well as zinc finger-containing Zic proteins. Although little is known about direct inputs that regulate their expression or about regulatory interactions that occur among them, gain- and loss-of-function experiments suggest possible hierarchical interrelationships. Understanding their regulatory interrelationships helps expand links within the gene regulatory network (GRN), adds a number of testable hypotheses, and can serve as an experimental guide.

In *Xenopus*, integration of inputs from BMP, FGF, Wnt and Notch signaling pathways activates expression of *Msx1* (Monsoro-Burq et al. 2005, Tribulo et al. 2003). *Zic1* and *Pax3* are also downstream of Wnt, BMP and FGF signals (Sato et al. 2005), while FGF8 can experimentally induce *Zic5* expression, but is not required to do so endogenously (Monsoro-Burq et al. 2003). While BMP and FGF signals can regulate individual expression of *Zic1* and *Pax3*, both transcription factors need to be simultaneously activated to achieve neural crest specification. In *Xenopus* embryos, high levels of either transcription factor alone (Pax3 or Zic1) promotes alternative neural plate border fates

(hatching gland or pre-placodal progenitors, respectively) (Hong and Saint-Jeannet 2007). Furthermore, FGF8 and Wnt signals act in parallel at the neural plate border and seem to independently converge onto Pax3 (Monsoro-Burq et al. 2005). Hairy2, a direct downstream effector of Delta/Notch input into the neural plate border territory, also participates in regulation of neural crest specifier genes (Glavic et al. 2004). *Dlx5*, regulated by attenuated levels of BMP (Luo et al. 2001), expands *Msx1* expression domain upon ectopic activity (Woda et al. 2003).

Since neural plate border specifiers are the first transcription factors to appear at the border, it is not surprising that they may be directly activated by simultaneous input of multiple signaling pathways. Although evidence for direct interactions is sparse, Brugger and colleagues show direct conversion of the intermediate levels of BMP signal onto the *Msx2* promoter (Brugger et al. 2004). Recently, Li and colleagues found that *Gbx2*, a gene essential for the anteroposterior partitioning of the neural folds, is expressed in an ectodermal region that includes the future neural plate border from which crest cells will arise (Li et al. 2009). The authors demonstrated that *Gbx2* is an immediate direct downstream target of Wnt signaling. Furthermore, epistatic rescue experiments reveal that Gbx2 is positioned upstream of the earliest previously reported neural plate border specifiers, *Msx1* and *Pax3*. These results suggest Gbx2 as a candidate for mediating the earliest Wnt inductive signaling input into NC GRN.

Studying the hierarchical interrelationships between newly activated neural plate border specifiers is challenging due to the inaccessibility and/or rapidity of the induction and border specification processes in most vertebrate models. Due to their slow

development, however, lamprey embryos allow unprecedented temporal resolution of neural plate border specification. This has enabled chronological ordering of the onset of gene expression amongst neural plate border specifiers, as well as gene perturbation assays to establish their hierarchical relationships. The study by Nikitina and colleagues establishes Msx, but also the neural crest specifier AP-2a, at the top of the neural plate border cascade, with many of the factors present at the border (both known border specifiers such as Msx, Pax3/7 or Zic, as well as early crest specifiers, such as AP-2a, n-Myc or Id) feeding back and regulating each other's expression (Nikitina N. et al. 2008). It will be interesting to further investigate direct regulatory relationships at the border as well as test similar interactions in higher vertebrates, such as chick embryo, which also have good temporal resolution of neural plate border specification.

Neural crest specifier genes

The regulatory state during neural crest specification is defined by the cumulative expression of a set of genes, termed neural crest specifiers, in the premigratory and early migrating *bona fide* neural crest progenitors. Some neural crest specifiers persist in the migrating and differentiating neural crest (such as Sox10), while others such as Snail2 are only present at the onset of the specification process and epithelial to mesenchymal transition prior to their emigration. Some neural crest specifiers have a biphasic expression pattern, first being present in neural crest progenitors and again later in differentiating derivatives (e.g. Sox9). A subgroup of transcription factors such as AP-2a, Snail1/2, Id, c-Myc and Twist are expressed even before neural crest progenitors become apparent, though

the timing of their onset and presence within the neural plate border varies among different vertebrates. In a basal vertebrate, the lamprey, expression of this subgroup of early-expressing neural crest specifiers begins at the early neurula stage, preceding expression of canonical neural crest markers such as Sox10 and FoxD3 (Nikitina N. V. and Bronner-Fraser 2009, Sauka-Spengler et al. 2007). This raises the intriguing possibility these genes may function as a key regulatory link between establishing competence in presumptive crest at the neural plate border and specification of *bona fide* neural crest cells. During specification, neural crest specifier genes are either directly or indirectly regulated by neural plate border genes. They also receive signaling pathways inputs, and undergo intricate cross-regulatory activity with other neural crest specifiers.

The regulatory control of *Snail2* exemplifies how signaling pathways and regulatory factors merge to direct the expression of a key gene involved in the epithelial to mesenchymal transition of neural crest cells. *Cis*-regulatory analysis shows that *Snail2* is directly regulated by intermediate levels of BMP and modulated by Wnt pathway input. Accordingly, the *Snail2* regulatory region contains binding motifs for Smad1, a transcription factor that mediates BMP signaling input (Sakai et al. 2005) and Tcf/Lef1, mediating b-catenin-dependent Wnt signal (Vallin et al. 2001). Furthermore, in *Xenopus* animal cap explants, a combination of the BMP inhibitor chordin and Wnt8 is sufficient to induce the expression of *Snail2* as well as *Id3*, helix-loop-helix transcriptional regulator involved in specification of neural crest (Kee and Bronner-Fraser 2005). Over-expression of Hairy2, a direct downstream effector gene of Notch signaling causes an expansion of *Snail2* expression in *Xenopus* (Glavic et al. 2004) and has been proposed as a direct input

into the *Snail2* regulatory region. Finally, it has been demonstrated that the neural plate border specifiers, Zic1, Msx1 and Pax3/7, are independently necessary and sufficient for the expression of a group of neural crest cell specifiers including *Snail2* (Meulemans and Bronner-Fraser 2004, Sato et al. 2005, Tribulo et al. 2003). This suggests that regulatory signaling inputs activating *Snail* may be mediated by neural plate border specifiers, such as Zic1, Mxs1 and Pax3/7. Conversely, signaling inputs can act in parallel with upstream border specifiers to control neural crest specifiers expression. For instance, in *Xenopus* embryos, b-catenin-dependent canonical Wnt signals cooperate with Zic1 and Pax3/7 to activate *Snail2* expression (Sato et al. 2005).

Far less is known about the regulation of other neural crest specifiers. *Twist*, for instance, is ectopically activated upon Snail2 and FoxD3 misexpression in *Xenopus* embryos and ectodermal explants, perhaps indirectly via Zic (Meulemans and Bronner-Fraser 2004, Sasai et al. 2001). In contrast, expression of a constitutively activated truncated version of a Notch receptor in *Xenopus* embryos down-regulates *Twist* expression, simultaneously causing the neural plate to expand and the epidermis to regress. Thus, it is not clear if the loss of *Twist* expression is a result of regulatory changes caused by a shift in signaling or a secondary effect due to neural plate expansion at the expense of neural crest (Coffman et al. 1993, Cornell and Eisen 2005). Although it is intriguing to speculate that Notch-Twist regulation is mediated by Zic1, there is currently no data either supporting or refuting this possibility. Some early neural crest cell specifiers, such as Id and cMyc, appear to function within the neural crest gene regulatory network to maintain the neural crest cells in a multipotent state, mediating critical cell cycle and/or cell fate

decisions (Bellmeyer et al. 2003, Kee and Bronner-Fraser 2005, Light et al. 2005). Id is a transcriptional repressor that possesses a helix-loop-helix (HLH) domain for dimerization, but lacks a basic domain for DNA binding. Id proteins interfere with gene expression by binding to transcriptional activators from bHLH families and preventing them from activating their direct targets. In lamprey, initial expression of *Id* at the neural plate border precedes that of *cMyc* (Nikitina N. V. and Bronner-Fraser 2009). However, in *Xenopus* embryos cMyc can directly regulate *Id* expression (Light et al. 2005) indicating that other factors, such as AP-2a or Zic1, may be responsible for the initial expression of *Id* (Nikitina N. et al. 2008). Therefore, cMyc functions directly upstream of *Id*, via the identified cis-regulatory region, to maintain its expression in the premigratory neural crest cells.

By the time premigratory and delaminating neural crest cells express transcription factors such as FoxD3, Sox9, Snail2 or Sox10, they are specified to a neural crest fate. The winged-helix transcription factor FoxD3 appears to pay a role in maintaining neural crest multipotency by preventing early differentiation (Lister et al. 2006). Direct regulatory inputs responsible for *FoxD3* activation and maintenance in the pre-migratory and later migrating neural crest cells have yet to be described. Similar to *Snail2* activation, there is evidence from studies in *Xenopus* embryos suggesting that a Hairy2 mediated Notch signal regulates *FoxD3* (Wettstein et al. 1997). In addition, the collective activity of Zic1 and Pax3/7 complemented with Wnt input induces *FoxD3* expression (Sato et al. 2005). Gain and loss of function experiments in *Xenopus* have also shown that Msx1 regulates the expression of *FoxD3* (Tribulo et al. 2003).

The SoxE family of transcription factors, most notably Sox9 and Sox10, have well-

established roles in neural crest development. In *Xenopus*, *Sox9* expression has been shown to be dependent on activity of AP-2a (Lee H. Y. et al. 2004a, Luo et al. 2003, Saint-Germain et al. 2004). Moreover, AP-2a binding motifs have been identified within early-acting *Sox9 cis*-regulatory region in mouse, using *in silico* database searches (Bagheri-Fam et al. 2006). In *Xenopus* Gbx2 together with Zic1 can induce the expression of neural crest specifiers genes including *Sox9* and *Snail2* while inhibiting pre-placodal fate (Li et al. 2009). However, the direct regulatory inputs into *Sox9* have yet to be experimentally demonstrated. In addition to the neural crest, SoxE genes are also expressed in the developing ear. However, far less is known about their roles during otic placode development.

Using comparative bioinformatics and in ovo electroporation of reporter plasmid constructs carrying candidate *Sox10* regulatory fragments, I identified the earliest acting Sox10 *cis*-regulatory region, referred to as Sox10E2, which is downstream of the coding region. Recently through extensive characterization of this initial Sox10-activating *cis*-regulatory element in chicken embryo (Betancur et al. 2010), I demonstrated that the synergistic activity of cMyb, Sox9 and Ets1 directly and specifically regulate the onset of Sox10 in the cranial neural crest via this *Sox10* enhancer, while another combination of factors, including cMyb, Sox8 and Pea3 regulates *Sox10* expression through this same enhancer in the otic placode. Since cMyb participates in the regulation of *Sox10* in both placode and neural crest cells, it appears to have a conserved regulatory role on the initiation of *Sox10* expression. The possible role of the proto-oncogene cMyb in neural crest cells development was first suggested in migrating trunk neural crest cells, where the

knockdown of cMyb reduced *Snail2* expression (Karafiat et al. 2005). However cMyb expression in chicken begins much earlier, at gastrula stage. It becomes confined to the neural folds as the neural plate begins to invaginate and later continues to be expressed in migrating crest cells (Betancur et al. 2010). By morpholino, I demonstrated that the knock down of cMyb in the cranial neural crest causes a diminution of *Sox10* expression, confirming that this factor acts upstream of Sox10. Ets1 expression is specific to the cranial crest population, and first appears in neural crest progenitors in chicken embryos as the neural folds are closing (Theveneau et al. 2007). Trunk neural crest cells, which normally do not express Ets1, arrest in the G1 phase of the cell cycle prior to separating from neuroepithelium and synchronously enter the S phase upon delamination. Interfering with G1/S transition prevents the delamination process (Burstyn-Cohen and Kalcheim 2002). Ectopic expression of Ets1 in the trunk region promotes massive emigration independent of cell cycle (Sauka-Spengler and Bronner-Fraser 2008a, Theveneau et al. 2007), more like migration in the cranial region. All this data together with the finding that Ets1 directly regulates *Sox10* specifically in cranial crest cells, raises the intriguing possibility that Ets1 may have a unique function in the cranial neural crest of establishing a regulatory state that activates cranial crest specific effector genes responsible for the pre-migratory to migratory transition. The differential expression of Ets1 and its regulatory relationship to other neural crest genes highlights interesting differences between neural crest populations at different levels of the neural axis.

Neural crest cell specifiers, in general, represent a “node point” onto which inductive inputs mediated by or acting in parallel with neural plate border specifiers

converge. Those specifying transcription factors in turn control the expression of effector genes that will give neural crest cells their unique migratory and multipotent characteristics. Therefore, in the life cycle of a neural crest cell, it is critical to keep the specifier genes running as a unit in the network. For this purpose in frog, there seems to exist high interdependence among neural crest cell specifiers. Gain and loss of function experiments suggest that Snail2 regulates *FoxD3, Twist* and *Sox10* expression, probably in an indirect fashion (Aoki et al. 2003, Aybar et al. 2003). Ectopic expression of AP-2a in the neural plate activates the ectopic expression of *Snail2* (Spokony et al. 2002), while Sox10 feeds back to maintain *Snail2*, *Sox9* and *FoxD3* expression (Honore et al. 2003). However, in mouse and zebrafish, there is less tight cross-regulation among neural crest cell specifiers since knockouts of *Snail1* and *Snail2, Sox10* and *AP-2a* have effects later, during differentiation in selective neural crest derivatives rather than at this state of specification (Meulemans and Bronner-Fraser 2004). Perhaps in other organisms, neural crest specifier genes have a more redundant function during specification, and their function becomes more restricted as the neural crest advance to the differentiating state. Conversely, this discrepancy may be due to the higher rate of gene duplication and functional compensation by redundant paralogs (Lister et al. 1999, Luo et al. 2001, Yan et al. 2005). Only through characterization of *cis*-regulatory modules will we be able to understand the degree of importance of these cross-regulatory events of neural crest cell specifiers.

Genes regulating neural crest emigration and migration

To initiate migration, premigratory neural crest cells must delaminate from the

neuroepithelium. Thus, transcription factors acting on the neural crest precursor pool must not only maintain them in a multipotent and proliferating state, but also activate or repress effector genes involved in their epithelial to mesenchymal transition (EMT). To allow cells to become less compact and acquire motility, EMT induces changes at the cellular level that include switches in cell junctions and adhesion properties and major cytoskeletal rearrangements. One characteristic of the EMT process includes a switch in cadherin expression, with up-regulation of type II cadherins, that allow for less adhesiveness and concomitant down-regulation of type I cadherins and other factors characteristic of epithelial cell types. In trunk neural crest cells in the chick, forced expression of FoxD3 down-regulates N-cadherin (Type I cadherin) while concomitantly up-regulating expression of Cad-7, a type II cadherin, and b1 integrin (Cheung et al. 2005). Since FoxD3 is a repressor, the latter effect is likely to be indirect. Confirming a role of FoxD3 during delamination, mis-expression of FoxD3 along the entire dorsoventral axis of chicken neural tube caused an increase in expression of neural crest cell markers, including Cad-7, and promoted delamination and migration from more ventral regions of the neural tube while simultaneously repressing interneuron differentiation (Dottori et al. 2001). Normally, Cad-7 is only expressed in migrating crest cells and excluded from neural tube (Nakagawa and Takeichi 1995). Similar to FoxD3, Sox10 over-expression induces b1 integrin expression while inhibiting N-Cad expression (Cheung et al. 2005). Although it is difficult to ascribe direct gene regulatory interactions, it is clear that both FoxD3 and Sox10 affect expression of EMT effector genes, such as cadherins, whose orchestrated regulation is crucial for EMT to occur.

Snail1 and *Snail2* genes have a clear role in controlling cell adhesiveness and many other aspects of epithelial to mesenchymal transitions in embryonic and metastatic cells (Thiery and Sleeman 2006). Snail1 is directly responsible for the negative regulation of *E-cadherin*, a cell adhesion molecule characteristic of epithelial cells (Cano et al. 2000). Similarly, Snail2 acts directly to negatively regulate the expression of *Cadherin 6B*, a molecule that characterizes cell-cell adhesion amongst dorsal neural tube cells, most of which are premigratory neural crest progenitors (Taneyhill et al. 2007). Sox5, a member of the SoxD family, is another transcription factor proposed to have a regulative role during neural crest cell delamination. Sox5 mis-expression causes an increase in the number of cranial neural crest cells generated. Sox5 up-regulates *Snail2, FoxD3*, and *Sox10* transcription factors in migrating crest cells and RhoB, a member of the Rho family of small GTPases that controls a variety of signal transduction pathways (Perez-Alcala et al. 2004). RhoB is a well-known regulator of events that change cell morphology, such as the actin cytoskeleton rearrangements, the formation of focal adhesions and stress fibers (Liu J. P. and Jessell 1998). All these cellular changes are necessary for neural crest delamination (Nobes and Hall 1995). The function of RhoB in cranial crest cells appears to be distinct from that in the trunk where it acts as a negative modulator, down-regulating N-cadherin and preparing cells for delamination (Groysman et al. 2008). Again, *cis*-regulatory profiling will confirm if the sub-circuit initiated by Sox5 consists of direct feeding onto *Snail*, *FoxD3, Sox10* and *RhoB* regulatory modules in delaminating cranial crest cells. In other studies it has been demonstrated that Sox5 can bind to *cis*-regulatory modules via known motifs, previously identified as *Sox9* and *Sox10* binding sites, and can modulate expression of downstream target genes by recruiting specific co-factors during neural crest

cell differentiation (Hattori et al. 2008, Stolt et al. 2008). It is likely that the same regulatory mechanism is used during cranial crest delamination. Since Sox5 appears early in the premigratory neural crest, it may be also be involved in the regulatory interactions that take place during neural crest specification. However, this possibility remains to be explored.

Most of the transcription factors that are involved in neural crest cell specification continue to be expressed in neural crest cells as they migrate. However, other unidentified upstream inputs, different from those that initiate expression of the neural crest specifiers in the pre-migratory state, may be responsible for maintaining their expression during migration. Moreover, different upstream regulators may be characteristic of neural crest cells with various differentiation potential, correlated with their future fate. For example, inactivation of Wnt signaling input sites within the *Sox9* enhancer decreased reporter expression exclusively in neural crest cells migrating into the first but not second or third branchial arches (Bagheri-Fam et al. 2006). *Cis*-regulatory analysis in mouse has shown that during neural crest migration, *Sox10* is directly regulated by Pax3, AP-2 and Sox9, but also receives Wnt signaling input (Werner et al. 2007). Analysis in zebrafish confirms that a Wnt signal feeds directly onto the Sox10 regulatory element during migration, but also strongly suggests SoxE, NFkB as well as Notch signals as potential direct *Sox10* regulatory inputs (Dutton J. R. et al. 2008). These studies also demonstrated that there is no direct regulatory interaction between FoxD3 and *Sox10*, despite the presence of FoxD3 binding motifs in *Sox10 cis*-regulatory regions. However, FoxD3 has been reported as a negative regulator of *Sox10* (Pohl and Knochel 2001, Sasai et al. 2001). It is plausible that the

negative feed of FoxD3 repressor on *Sox10* regulatory module may have been missed because assays employed to identify direct regulators are more targeted to isolation of positive regulatory influences (activators). Conversely, FoxD3 may function as regulator of *Sox10* activity via yet unidentified enhancers. Alternatively, the loss of *Sox10* expression after FoxD3 inactivation may suggest that their functional interactions are not direct and perhaps involves an intermediary inhibitor.

Prior to and during neural crest migration, cells acquire signaling receptors that allow them to interact with their environment and help guide them along specific pathways. Such molecules include Neuropilin-1/2, Robo-1/2 and Ephrin receptors among others in the cranial region (Sauka-Spengler and Bronner-Fraser 2008a). However, the transcription factors that regulate expression of these signaling molecules remain elusive. Similarly, there is not much information regarding the upstream regulators of genes that are involved in cell cycle decisions prior to cranial neural crest cell delamination; only a few regulatory interactions that prevent cells from undergoing apoptosis have been described. In *Sox9* null mice, neural crest cells undergo massive apoptosis (Cheung et al. 2005). Similarly, zebrafish neural crest cells lacking Sox9 within the branchial arches show a predominant cell death phenotype (Yan et al. 2005). Gain and loss of function experiments in *Xenopus* suggest a direct regulatory connection between Sox9 and another anti-apoptotic factor, Snail1 (Aoki et al. 2003).

In summary, the combined regulatory function of neural crest specifier genes and their downstream effectors endows neural crest cells with the characteristics rendering them mesenchymal, proliferative and motile. However, out of a very large number of

neural crest downstream effector genes, there is only a scarce number whose direct regulatory inputs and links to upstream neural crest specifiers is known, making it difficult to ascribe their precise positions within the neural crest gene regulatory network.

The transition from migration to differentiation

How neural crest cells lose their migratory and multipotent characteristics as they prepare to differentiate remains an open question. It is logical to postulate that a separate set of gene batteries is deployed in each neural crest lineage. *Cis*-regulatory analysis combined with functional and binding affinity assays, have revealed several sub-circuits of direct gene regulatory interactions for each lineage. After neural crest cells have migrated and reached their final destinations, typically expression of most early neural crest cell specifiers, including *Snail/Snail2, FoxD3, Id and AP-2,* is down-regulated, although the direct regulatory interactions triggering this down-regulation are elusive (Meulemans and Bronner-Fraser 2004). Nevertheless, there is some evidence to suggest that FoxD3 participates in repression of *Snail1b* (previously Snail2) in zebrafish. Its absence causes prolonged expression of *Snail1b* when it would normally be turned off (Lister et al. 2006). Exogenous expression of FoxD3 in *Xenopus* causes repression of endogenous *FoxD3* indicating that FoxD3 can directly down-regulate its own expression in a negative autoregulatory loop (Pohl and Knochel 2001). Down-regulation of FoxD3 in migrating cells prior to differentiation does not take place in all neural crest derived lineages. While absent from melanoblasts, FoxD3 expression persists in neural/glial precursors, where it prevents Pax3 from binding to the promoter of Microphtalmia-associated transcription

factor (MITF) and thus protects sensory precursors from assuming a pigment cell fate (Thomas and Erickson 2009). These data demonstrate the importance of negative regulation in acquisition of cell fate in cell types with multiple developmental potentials, like the neural crest. It will be essential to study differential upstream inputs that confine FoxD3 or other possible repressive circuits that act as regulatory switches between different lineages.

Notable exceptions are SoxE transcription factor family members, Sox9 and Sox10, which persist in specific subpopulations of neural crest cell derivatives and appear to be master regulators of terminal differentiation in the majority of neural crest derivatives (Kelsh 2006, Sauka-Spengler and Bronner-Fraser 2008b). The necessity of different SoxE genes for specification of distinct neural crest sublineages has recently been demonstrated in zebrafish (Arduini et al. 2009). Sox9 and Sox10 are maintained in cartilage and neuron/glial/melanocyte lineages, respectively, such that Sox10 persists in melanoblasts and elements of peripheral nervous system, while Sox9 is characteristic of neural crest-derived chondrocytes. Experiments in *Xenopus* suggest that the HLH transcriptional repressor Id prevents premature neural crest cell differentiation during neural crest migration. Constitutive expression of Id family members in migrating neural crest cells populating the pharyngeal arches, most of which would normally adopt a cartilage fate, extends *Sox10* expression which is normally downregulated in this population when they enter the arches (Light et al. 2005).Furthermore, over-expression of Id3 in melanocytes or neural crest-derived cartilage cells, independently affects differentiation of these two cell lineages (Light et al. 2005). Thus, downregulation of Id is necessary for occurrence of the

initial steps of neural crest cell differentiation. It is plausible that endogenous down-regulation of Id indirectly releases inhibitors that feed into the neural crest specifier module, while the maintainance of Sox9 in cartilage and Sox10 expression in melanocyte derivatives, may be independent of the Id regulatory cascade. Alternatively, there is strong evidence indicating that Id helps establish the time window during which cells respond to differentiating signals (Light et al. 2005). At the proper time, it may release activator genes involved in differentiation and maintenance of Sox9 and Sox10 expression in their respective differentiated lineages.

Another possibility is that the inhibitory activity of Id maintains *Sox10* and perhaps *Sox9* expression at low levels. It has been suggested that low concentrations of Sox10 sustain the multipotency of neural crest cells and at higher levels inhibit neuronal differentiation and promotes glia and melanoblast formation (Kim et al. 2003, Paratore et al. 2001). Resolving regulatory interactions to the detail that would allow unraveling of these complex events remains a challenge. The battery of genes involved in maintaining neural crest cells may change such that new regulatory interactions emerge, some involving redeployment of transcription factors involved in early neural crest cell specification to perform a later function in cell differentiation. For example, the way that Sox9 and Sox10 acquire new instructive role for directing the fate of certain neural crest derivatives may involve acquisition of new cofactors.

Differentiation of the cranial neural crest

Neural crest cells give rise to a widely varied of derivatives ranging from

melanocytes, glia, neurons, to skeletal components of the head. In general, the type of derivative depends upon the axial level from which the neural crest originate and the time of their emigration from the neuroepithelium. For example, midbrain and rhombomere (r) 1 and r2 neural crest contribute to neurons and glia of the trigeminal ganglion as well as the skeleton of the upper and lower jaw. Neural crest cells from r4 give rise to neurons of the proximal facial ganglion and the hyoid bone. Neurons of the proximal and jugular ganglia and skeletal components of the post-pharyngeal arches are derived from post otic neural crest streams r6 and r7 (Graham et al. 2004, Lumsden et al. 1991, Schilling and Kimmel 1994). Vagal neural crest forms enteric nervous system as well as cardiac and aortic arch components. Trunk neural crest forms sensory and autonomic ganglia and the adrenal medulla.

The time of migration also influences the types of derivatives that neural crest cells form. Early migrating cranial neural crest cells populate the pharyngeal arches to generate bone, cartilage and connective tissue (skeletal structures) while the later wave stays close to central nervous system and generates neurons and glia of the cranial ganglia (Graham et al. 2004). Melanocytes are derived from neural crest from all axial levels. In mouse, a subpopulation of neural crest cells within a dorsomedial domain of the neural tube at the midbrain-hindbrain junction migrate exclusively into the developing dermis and express melanocyte lineage markers (Trainor 2005).

Of all the cranial neural crest derivatives, melanocytes and chondrocytes are the two lineages in which the most *cis*-regulatory work has been performed, allowing predictions regarding regulatory subcircuits. In melanocytes, Sox10, in synergy with Pax3,

directly regulates *Mitf* by binding to a proximal region of its promoter (Bondurand et al. 2001, Verastegui et al. 2000). Then, in collaboration with Mitf, Sox10 directly regulates expression of an enzyme necessary for melanin synthesis, dopachrome tautomerase (Dct/TRP2) (Ludwig et al. 2004).

Sox5 also plays a direct modulatory role in melanocyte differentiation. Sox5 and members of the SoxD family of transcription factors are characterized by their lack of a transactivation domain (Lefebvre et al. 1998). It has been speculated that they regulate transcription by recruiting other co-activators or co-repressors to regulatory regions. In melanocytes, Sox5 binds to the Mitf and Dct/TRP2 promoter regions through Sox10 identified binding elements. It recruits the co-repressors CtBP2 and HDAC to compete with Sox10 for binding of these regulatory regions, therefore modulating Sox10 inducing activity (Stolt et al. 2008). During chondrocyte development, on the other hand, Sox9 directly regulates expression of important cartilage markers such as *Col2a1* (Lefebvre et al. 1997), *Col11a2* (Bridgewater et al. 1998), and CD RAP (Xie et al. 1999) by binding to sites in identified enhancer regions. Interestingly, Sox5 null mice have skeletal defects and particularly craniofacial defects (Smits et al. 2001). This suggests another role for Sox5, in chondrocyte development. Consistent with this possibility, it was recently found that Sox5 cooperates with Sox9 and other co-factor in chondrocytes to regulate expression of *Col2a1* by binding to Sox9 target sites (Hattori et al. 2008). These inputs at the effector level of the neural crest gene regulatory network are a few notable examples how precise gene regulatory subcircuits can guide neural crest subpopulation to differentiate into specific derivatives.

For specification and differentiation of cranial neural crest cells into glia and neurons, there is little known about direct regulatory interactions. Most of knowledge about direct regulatory interactions in neurogenic neural crest derivatives comes from experiments performed in the trunk neural crest cells. These studies show that differentiation into neural crest-derived neurons and glia requires re-deployment of factors utilized earlier, during neural crest induction and specification. As an example, Notch and Delta proteins are expressed in neural crest cells that populate the presumptive trigeminal ganglion region, where they undergo gliogenesis and neurogenesis. The Notch signaling pathway promotes gliogenic differentiation while inhibiting neuronal differentiation (Nakamura et al. 2000, Ohtsuka et al. 1999). Furthermore, the cell fate decision between gliogenic and skeletogenic differentiation appears to be controlled in part by the use of different mediators of Notch signaling. While the Deltex-mediated Notch pathway controls gliogenesis, simultaneous activation of the RBP-J and the Deltex-dependent Notch pathways leads to chondrogenic specification (Ijuin et al. 2008), mediated by previously characterized Notch downstream effectors, Hes1 and Hes5 (Jarriault et al. 1995). The downstream readouts used to differentiate the gliogenic and chondrogenic lineages were glial fibrillary acidic protein (GFAP) and Collagen type II a (Col2aı), respectively. Thus, the above-mentioned studies place these specific markers as potential effectors genes, acting directly downstream of Notch signaling inputs mediated by Hes1 and Hes5 factors (Ijuin et al. 2008).

In addition to its role in melanocyte differentiation, Sox10 also controls specification of glial and neuronal fates in neural crest derivatives specification. Sox10

appears to further participate in the differentiation of glia, as its expression within this lineage persists into terminal differentiation stages (Kelsh 2006). During glial differentiation, Sox10 directly regulates the expression of protein zero (P0) (Peirano et al. 2000), myelin basic protein (MBP), peripheral myelin protein 22 (PMP22) and the gap junction protein connexin 32 (Cx32), thus affecting all major components of the myelination process (Bondurand et al. 2001). Finally, evidence concerning the direct regulatory role of Sox10 during differentiation of neural crest-derived neurons comes from studies of sensory and autonomic lineages in the trunk. In mouse neural crest cultures, Sox10 regulates the expression of mouse achaete-scute homologue 1 (MASH1) and the paired homeodomain (Phox2b), transcription factors that are essential for autonomic neurogenesis (Kim et al. 2003). Sensory neurons derived from the dorsal root ganglia transiently express Sox10, shown to regulate the expression of proneural gene neurogenin 1 in zebrafish (Carney et al. 2006). It is possible that similar interactions, involving direct Sox10 regulatory inputs and expression of the sensory neuronal marker Neurogenin1, take places during cranial neurogenesis.

Finally, it is important to stress the role of negative regulation during steps of terminal differentiation into neural crest derivatives. The recent study by Sun and colleagues, shows that LIM-homeodomain factor Islet1 specifically regulates subprograms within different sensory neuron lineages (Sun et al. 2008). Islet1 is specifically required to repress/terminate gene expression at the end of the neurogenic phase of development, such as Neurogenin1 or NeuroD family members. Interestingly, Islet1 is also required to repress a number of transcription factors not normally expressed in the sensory ganglia, but found

in the spinal cord and hindbrain, such as LIM-homeobox factors Lhx1 and Lhx2 and oligodendrocyte markers Olig1 and Olig2. This suggests that Islet1 inhibition also serves as a control switch, keeping the cells within the sensory lineage (Sun et al. 2008).

Ectodermal placodes also arise at the neural plate border

For otic placode development, regulatory information and particularly *cis*-regulatory analysis are limited and a GRN has yet to be assembled. Nevertheless, a compilation of data suggests that ectodermal cells at the neural plate border first acquire a "preplacodal state" (Schlosser 2008, Streit 2007), characterized by expression of a set of transcription factors in a common preplacodal domain from which all placode precursors emerge. These are subsequently maintained in all placodes and lost from non-placodal ectoderm. Among the specific placodal transcription factors identified so far, are members of the Six and Eya families. These factors have been implicated in multiple processes during placode formation and the requirement of these genes for normal placode derivatives development has been extensively studied. For instance, mice heterozygous in *Eya1*, display a phenotype similar to an inherited form of deafness in humans, the branchio-otorenal (BOR) syndrome (Abdelhak et al. 1997, Xu et al. 1999). *Eya1* null mice have severe inner ear defects characterized by the arrest of otic formation at early stages. Mutations in *Six1* also cause similar defects to those observed in the human syndrome BOR. Furthermore, in the otic vesicle loss of Eya1 and Six1 lead to reduced proliferation (Bonini et al. 1993). Thus, the importance of Six and Eya family members together with their specific early placodal expression have made these factors good candidates for

defining the placode territory at early developmental stages (Streit 2007).

However, these factors are not the only presumptive preplacodal factors. There are a number of factors that are found in a wide domain which encompasses neighboring preplacodal territories such as the non-neural ectoderm, neural plate and presumptive neural crest. Furthermore, signaling molecules involve in the induction of these neighboring territories also are responsible for the induction of the preplacodal domain. Importantly, the requirement for these signaling pathways is different for the individual induction of the aforementioned territories. For instance, the BMP and Wnt pathways are required for the establishment of the neural plate border territory while preplacodal regionalization requires the inhibition of Wnt and BMP most likely achieved through the antagonistic activity of the BMP inhibitor DAN and the Wnt antagonist Cerberus (Streit 2007). As in neural crest cell induction, FGF8 is also important for the preplacodal domain, as well as the otic placode induction. Early over-expression of FGF8 promotes the expression of the preplacodal marker *eya2* (Brugmann et al. 2004), while missexpression of FGF8 leads to an enlarged otic vesicle only within the field where both otic placode markers, *Foxi* and *Dlx3b* are co-expressed; in addition, FGF8 is required for the induction of these two genes (Hans et al. 2004, Hans et al. 2007). Interestingly, as placode begin to segregate into different subpopulations, a trio of SoxE family factors, Sox8, Sox9 and Sox10, among other transcription factors, become concomitantly expressed in the otic placode. However, their order of expression in the placode is different than in the neural crest, with Sox8 expressed first followed by Sox10 in chick. It has been suggested that the role of the SoxE genes in the otic placode is similar to their role during neural crest cell specification. However, there is not much information regarding this function in the otic.

Since the previously described Sox10E2 enhancer is active in both cranial neural crest cells and otic placode, I used this *Sox10* regulatory region to begin to estabish regulatory connections on *Sox10* in the otic region. By mutational analysis of the *Sox10* regulatory fragment and morpholino knock down of upstream putative activators, I located three *Sox10* activators cMyb, Sox8 and Pea3. It is also intriguingly that the same binding motifs within the Sox10E2 regulatory region respond to the input of different sets of transactivators to activate initial *Sox10* expression in each neural crest and otic placode cells. This suggests that *Sox10 cis*-regulatory machinery has been highly conserved and that the differences in deployment of upstream activators may have been the result of duplication and divergence during evolutionary changes.

Conclusion and future prospects

In this chapter, I first presented an overview of a gene regulatory network orchestrating formation of neural crest cells, focusing on the cranial level. Formulation of this network relies on information largely inferred from studies of molecular mechanisms underlying neural crest formation in several vertebrate model organisms. I attempted to include all known *cis*-regulatory information obtained to date, providing evidence for direct regulatory interactions and architectural circuitry between the molecular factors involved. I briefly introduced signaling pathways and factors involved in the induction and specification of

the common preplacodal domain in general and the otic placode in particular. However, there is little information available at the level of regulatory interactions, let alone regarding direct interactions. Finally, I presented how the direct activation of the early neural crest *Sox10* enhancer by cMyb, Sox9 and Ets1 fits into the neural crest cell gene regulatory network. The characterization of the Sox10E2 enhancer also helps to expand knowledge of direct regulatory interactions in the otic placode by placing Sox8 upstream of Sox10 and also implicating two new *Sox10* regulators, Pea3 and cMyb, in this region. The finding that the same enhancer functions in both neural crest and otic placode, while being activated by paralogous transcription factors, poses the intriguing possibility of a close evolutionary relationship between these two cell types at the regulatory level.

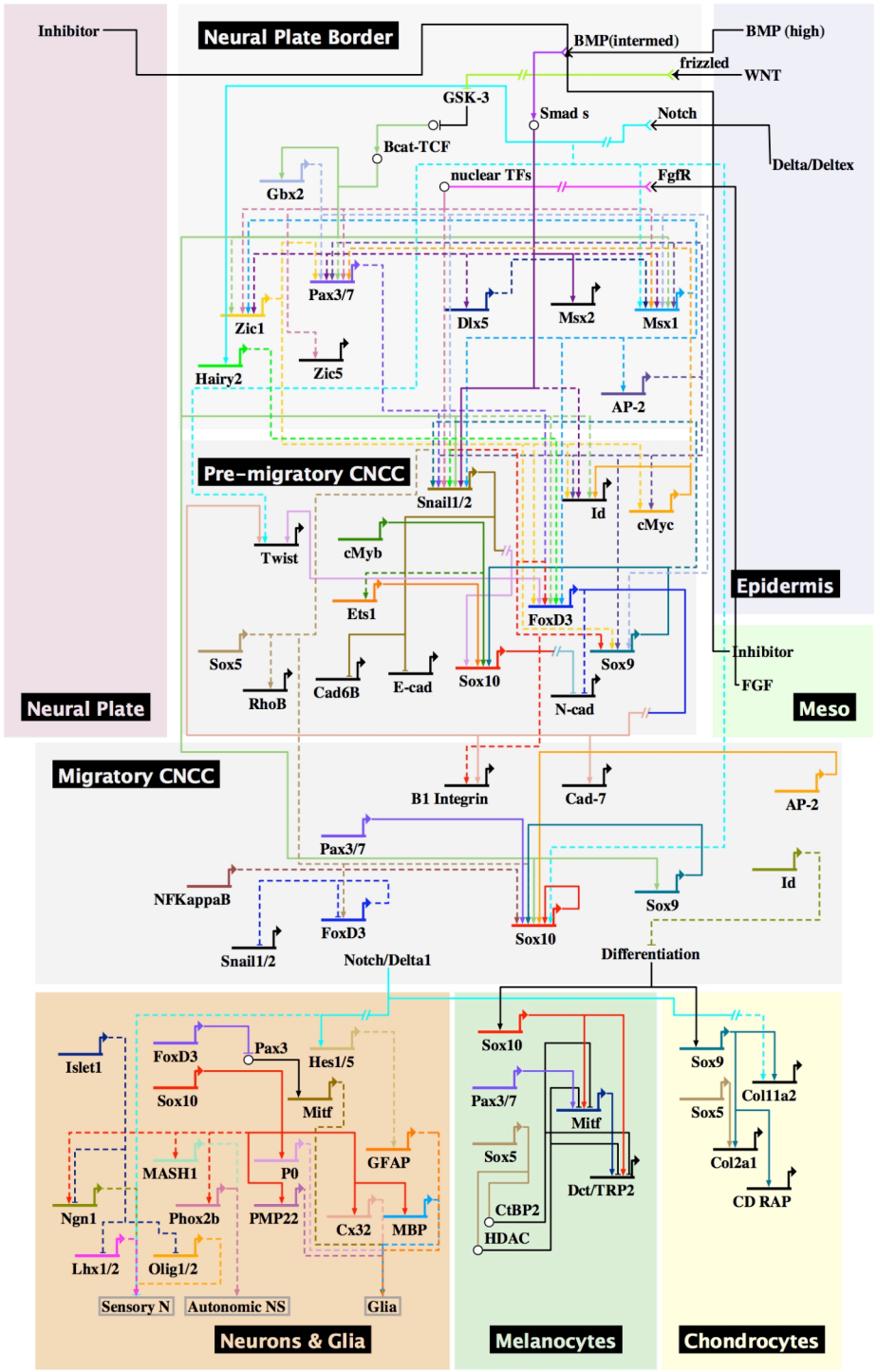
Inhibitor
Neural Plate Border
BMP(intermed)
BMP (high)
frizzled
WNT
GSK-3
Smad s
Notch
Bcat-TCF
Delta/Deltex
nuclear TFs
FgfR
Gbx2
Pax3/7
Zic1
Dlx5
Msx2
Msx1
Hairy2
Zic5
AP-2
Pre-migratory CNCC
Snail1/2
Id
cMyc
Twist
cMyb
Epidermis
Ets1
FoxD3
Sox5
Sox9
Inhibitor
RhoB
Cad6B
E-cad
Sox10
FGF
N-cad
Neural Plate
Meso
Migratory CNCC
B1 Integrin
Cad-7
AP-2
Pax3/7
Id
NFKappaB
FoxD3
Sox9
Sox10
Snail1/2
Notch/Delta1
Differentiation
Islet1
FoxD3
Pax3
Hes1/5
Sox10
Mitf
MASH1
P0
GFAP
Ngn1
Phox2b
PMP22
Cx32
MBP
Lhx1/2
Olig1/2
Sensory N
Autonomic NS
Glia
Neurons & Glia
Sox10
Pax3/7
Mitf
Sox5
Dct/TRP2
CtBP2
HDAC
Melanocytes
Sox9
Col11a2
Sox5
Col2a1
CD RAP
Chondrocytes

Figure 1. Gene regulatory network model (a view from all nuclei) mapping vertebrate hierarchical gene regulatory interactions during cranial neural crest cell (CNCC) development. The model is built using BioTapestry software (Longabaugh et al 2009). The network is partitioned into sub-networks that regroup regulatory interactions during induction and specification at the neural plate border, in pre-migratory and migrating neural crest cells and in differentiating neural crest derivatives. Most of the linkages in the network model are inferred from available gene perturbation data from *Xenopus*, chicken, mouse, zebrafish and lamprey. Direct regulatory interactions, based on promoter and *cis*-regulatory analysis, are indicated with solid connector lines. Dash connectors show potential direct regulatory interaction inferred from gene perturbation studies. Broken lines are used to represent potential interactions that are indirect. Bubble nodes indicate protein-protein interaction. Transcriptional orientation was not taken into consideration in this case since it varies among different vertebrate models.

TABLE 1. Evidence for gene regulatory interactions in cranial neural crest cells

Source	Interaction	Target	System	Evidence
BMP	Promotes *	Msx2	Mouse	Brugger, 2004
BMP	Promotes	Dlx5	Xenopus	Luo, 2001
BMP, FGF, Wnt, Notch, Gbx2, Dlx5, AP-2, Myc	Promotes	Msx1	Xenopus, lamprey	Monsoro-Burq,2005; Tribulo, 2003; Li, 2009; Woda, 2003; Nikitina, 2008
BMP, Wnt, FGF, Gbx2, AP-2, Zic, Myc	Promotes	Pax3/7	Xenopus, lamprey	Sato, 2003; Hong, 2007; Monsoro-Burq, 2005; Li, 2009; Nikitina, 2008
BMP, Wnt, FGF, Msx	Promotes	Zic1	Xenopus, lamprey	Sato, 2003; Hong, 2007; Nikitina, 2008
FGF	Promotes	Zic5	Xenopus	Monsoro-Burq, 2003
Wnt	Promotes*	Gbx2	Xenopus	Li, 2009
Notch	Promotes*	Hairy2	Xenopus	Glavic, 2004
Msx	Promotes	AP-2	Lamprey	Nikitina, 2008
FGF, Hairy2, Zic1, Msx1, Pax3/7, Gbx2, AP-2, Sox9, Sox10, Sox5	Promotes	Snail1/2	Xenopus, chicken	Mayor, 1997; Villanueva, 2002; Glavic, 2004; Sato, 2005; Tribuolo, 2003; Maulemans, 2004; Li, 2009; Spokony, 2002; Aoki, 2003; Honore, 2003; Perez-Alcala, 2004
BMP, Wnt,	Promotes*	Snail1/2	Mouse, xenopus	Sakai, 2005; Vallin, 2001
FoxD3	Represses	Snail1/2	Zebrafish	Lister, 2006
Notch	Promotes	Twist	Xenopus	Coffman, 1993; Cornell, 2005
Snail1/2, FoxD3	Promotes (Ind)	Twist	Xenopus	Aoki, 2003; Aybar, 2003; Sasai, 2001; Maulemans, 2004
cMyc	Promotes*	Id	Xenopus	Light, 2005
BMP, Wnt, Zic, AP-2	Promotes	Id	Xenopus, lamprey	Kee, 2005; Nikitina, 2008
Zic, AP-2	Promotes	Myc	Lamprey	Nikitina, 2008
Hairy2, Zic1, Pax3/7, Msx1, Sox10, Sox5	Promotes	FoxD3	Xenopus, chicken	Wettstein, 1997; Sato, 2005; Tribulo, 2003; Honore, 2003; Perez-Alcala, 2004
Snail1/2	Promotes (Ind)	FoxD3	Xenopus	Aoki, 2003; Aybar, 2003

FoxD3	Represses	FoxD3	Xenopus	Pohl, 2001
cMyb	Promotes	Ets1	Chicken	Betancur, unpublished data
Wnt	Promotes*	Sox9	Mouse	Bagueri-Fam, 2006
Ap-2, Gbx2, Zic1, Sox10	Promotes	Sox9	Xenopus, mouse	Lee, 2004; Luo, 2003; Saint-Germain, 2004; Bagheri-Fam, 2006; Li, 2009; Honore, 2003
Id	Represses	Sox9	Xenopus	Light, 2005
cMyb, Ets1, Sox9, Wnt, Pax3/7, AP-2	Promotes*	Sox10	Chicken, mouse, zebrafish	Betancur, 2010; Werner, 2007; Dutton, 2008
Sox5, Notch, NFKappaB	Promotes	Sox10	Chicken	Perez-Alcala, 2004; Dutton, 2008
Snail1/2	Promotes (Ind)	Sox10	Xenopus	Aoki, 2003; Aybar, 2003
Id	Represses	Sox10	Xenopus	Light, 2005
Snail1/2	Represses*	Cad6B, Ecad	Chicken, mouse & human cell lines	Taneyhill, 2007; Cano, 2000
Sox10	Represses (Ind)	Ncad	Chicken, mouse	Cheung, 2005
FoxD3	Represses	Ncad	Chicken, mouse	Cheung, 2005
RhoB	Modulates	Ncad		Groysman, 2008
FoxD3	Promotes (Ind)	Cad7	Chicken, mouse	Cheung, 2005
FoxD3	Promotes (Ind)	B1 Integrin	Chicken, mouse	Cheung, 2005
Sox10	Promotes	B1 integrin	Chicken, mouse	Cheung, 2005
Sox5	Promotes	RhoB	Chicken	Perez-Alcala, 2004
Notch	Promotes*	Hes1/5	HeLa cell line	Jarriault, 1995
Hes1/5	Promotes	GFAP, Col2a1	Mouse	Ijuin, 2008
Sox10, Pax3	Promotes*	Mitf	Cell lines	Verastegui, 2000; Bondurand, 2001
Sox5	Modulates*	Mitf	Mouse	Stolt, 2008
FoxD3	Represses (Ind)	Mitf	Quail	Thomas and Erickson, 2009
Sox10, Mitf	Promotes*	Dct/TRP2	Cell lines	Ludwig, 2004
Sox5	Modulates*	Dct/TRP2	Mouse	Stolt, 2008

Sox10	Promotes *	P0, Cx32, MBP, PMP22	Mouse, cell lines	Peirano, 2000; Bondurad, 2001
Sox10	Promotes	Phox2B, MASH1, Ngn1	Rat cell culture, zebrafish	Kim, 2003; Carney, 2006
Sox9, Sox5	Promotes*	Col2a1	Cell lines, mouse	Lefebvre, 1997; Hattori, 2008
Sox9	Promotes*	Col11a2, CD RAP	Cell lines, mouse	Bridgewater, 1998; Xi, 1999
Islet1	Represses	Ngn1, Lhx1/2, Olig1/2	Mouse	Sun, 2008

* Direct regulatory interaction (data available)
(Ind) Possible indirect interaction

C h a p t e r 2

Genomic Code for Sox10 Activation Reveals a Key Regulatory Enhancer for Cranial Neural Crest

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Abstract

The neural crest is a multipotent, stem cell-like population that migrates extensively in the embryo and forms a wide array of derivatives ranging from neurons to melanocytes and cartilage. Analyses of the gene regulatory network (GRN) driving neural crest development revealed Sox10 as one of the earliest neural crest specifying genes, cell-autonomously driving delamination and directly regulating numerous downstream effectors and differentiation gene batteries. In search of direct inputs to the neural crest specifier module, I dissected the chick *Sox10* genomic region and isolated two downstream regulatory regions with distinct spatiotemporal activity. A novel element, Sox10E2 represents the earliest-acting neural crest *cis*-regulatory element, critical for initiating *Sox10* expression in newly formed cranial, but not vagal and trunk neural crest. A second element, Sox10E1, acts in later migrating vagal and trunk crest cells. Deep characterization of Sox10E2 reveals Sox9, Ets1 and cMyb as direct inputs mediating enhancer activity. Chromatin immunoprecipitation, DNA-pulldown and gel shift assays demonstrate their direct binding to the Sox10E2 enhancer *in vivo*, whereas mutation of their corresponding binding sites, or inactivation of the three upstream regulators, abolishes both reporter and endogenous *Sox10* expression. Using *cis*-regulatory analysis as a tool, this study makes critical connections within the neural crest GRN, thus establishing the first direct link of upstream effectors to a key neural crest specifier.

Introduction

Because of its stem cell properties and numerous derivatives, the vertebrate neural crest (NC) represents an excellent system for examining questions of cell specification and differentiation during development. Along the neural axis, neural crest cells are subdivided into several subpopulations, cranial, vagal, trunk and sacral, distinct in their migratory pathways and derivatives. Although the molecular underpinnings of these regional differences are unknown, an intriguing possibility is that these may be due to differential regulation of neural crest marker genes. Consistent with this, some transcription factors, like *Id2* and *Ets1*, are selectively expressed at cranial but not vagal or trunk levels (Martinsen and Bronner-Fraser 1998, Tahtakran and Selleck 2003).

It has been proposed that a gene regulatory network (GRN) defines the regulatory state of neural crest cells (Meulemans and Bronner-Fraser 2004), such that modules of transcription factors function sequentially to first specify the neural plate border and then the nascent neural crest. The intricate regulatory interactions within the NC-GRN start with a group of transcription factors comprising an evolutionarily "inflexible" neural plate border regulatory unit, whose essential upstream function is to establish identity of the progenitor territory (Nikitina N. et al. 2008). Although neural plate border genes are thought to regulate genes of the neural crest specification circuit, virtually nothing is known about direct regulatory connections between these border and specifier modules. Activation of transcription factors in a temporally and spatially controlled fashion assures not only that NC cells acquire migratory properties, but also that they differentiate into numerous derivatives appropriate for their axial level of origin. Identification of region-specific

regulatory elements promises to provide an important tool for understanding how neural crest cells are regionally specified and how this relates to the global NC-GRN.

The present state of knowledge of the NC-GRN has been largely derived from transperturbation experiments using morpholino-mediated knock-down in frog, zebrafish and chick, generally focused on the cranial neural crest (CNC). Due to the nature of the analysis, understanding hierarchical relationships within the NC-GRN has been indirect. As a consequence, information directly connecting hypothetical upstream neural plate border regulators to neural crest specifiers is sorely lacking. The evolutionary addition of the crest specifier link to a neural plate border module, already present in non-vertebrate chordates, was a critical step for invention of migratory and multipotent NC cells in the vertebrate lineage (Sauka-Spengler and Bronner-Fraser 2006). To fill this void and connect currently distant portions of the NC-GRN, *cis*-regulatory analysis of neural crest specifier genes is required. However, this has been classically problematic due to the difficulty of performing high throughput regulatory analysis and the paucity of genomic information in those vertebrates most amenable to experimental manipulation.

Previous studies have identified Sox10 as a key regulator of numerous effector genes in the NC-GRN. It is critical not only for neural crest delamination/migration but also for specification of multiple neural crest lineages (autonomic neurons, glia, melanocytes) by directly regulating genes involved in differentiation (Kelsh 2006, Sauka-Spengler and Bronner-Fraser 2008a). Thus, Sox10 is a linch pin for understanding the process of neural crest specification. Although *Sox10* enhancer elements controlling expression in neural crest derivatives and late migrating cells have been noted in other species (Antonellis et al. 2006, Antonellis et al. 2008, Dutton J. R. et al. 2008, Werner et al.

2007), no regulatory element(s) controlling initial activation of any neural crest specifier, let alone *Sox10*, has been uncovered to date.

Here, I provide the necessary *cis*-regulatory analysis that links activation of *Sox10* in newly formed cranial neural crest cells within the NC-GRN. By dissecting the *cis*-regulatory regions of this essential neural crest specifier, I have isolated two enhancers with distinct regulatory activities. Mutational analysis, reveal previously unknown *cis*-regulatory inputs active in nascent cranial neural crest cells. Three transcription factors, Sox9, Ets1 and cMyb, acting via the newly identified enhancer Sox10E2, are required for direct initial activation of endogenous *Sox10* expression and the specification of delaminating/migrating cranial neural crest. This study adds additional, previously uncharacterized players to the early phase of the NC-GRN. By establishing direct regulatory connections to *Sox10* activation within the cranial neural crest, the data add important information for decoding and understanding the NC-GRN as a whole.

Results and Discussion

Identification of Sox10 genomic fragments with regulatory activity in newly formed neural crest

To guide experimental tests of regulatory activity, comparative genomic analysis was employed to identify conserved elements. Genomic sequences surrounding the *Sox10* coding region from chicken, zebrafish, *Xenopus*, opossum, mouse, rat and human were

compared *in silico* (Fig1. A), employing the ECR Browser program (http://ecrbrowser.dcode.org/). Using *Sox10* BAC clone, genomic fragments of ~3-5kb, containing one or more conserved regions (≥70% homology), were cloned into EGFP reporter vector upstream of thymidine kinase (tk) basal promoter (Uchikawa et al. 2003) and functionally tested *in vivo* for ability to recapitulate *Sox10* expression during early neural crest formation. Using an *ex ovo* and *in ovo* electroporation techniques (Sauka-Spengler and Barembaum 2008), the entire epiblast of stage 4 chick embryos, according to Hamburger and Hamilton (HH), or dorsal neural tube of stage HH8-12 embryos were transfected with reporter construct (green), together with a pCI-H2B-RFP (red) ubiquitous tracer to assess transfection extent and efficiency. Embryos were collected after 8-48 hrs (HH8+-18), fixed and analyzed for EGFP expression.

The results reveal a 3.5kb fragment, ~1kb downstream of the *Sox10* coding region, that activates EGFP reporter expression (Figs. 1B-F) in a manner that recapitulates endogenous Sox10 transcription (Figs. 1G-J), as the neural crest delaminates and migrates from the neural tube. EGFP transcripts were detected in cranial neural crest cells as early as HH8+ (Fig. 1B), in embryos with 6 somites, when *Sox10* is first distinguishable by *in situ* hybridization (Fig. 1G). Both the EGFP reporter and endogenous *Sox10* were maintained on actively migrating cranial neural crest (Figs. 1D, F, I) as expression initiates progressively caudally (Figs. 1I, J; (Cheng et al. 2000)). However, while endogenous *Sox10* is down-regulated as crest cells enter the branchial arches (Fig. 1J), expression of the EGFP reporter was maintained in branchial arches (similar to SFig. 2b). Both *Sox10* and EGFP were also expressed in otic placode cells by stage HH10 (Figs. 1C, H) and later, more

caudally, in actively migrating, but not early delaminating vagal and trunk neural crest (Figs. 1D, E, I, J).

Thus, this 3.5kb *Sox10* genomic fragment (denoted Sox10E) contains regulatory modules that mediate initial Sox10 activation during early neural crest delamination at the cranial but not more caudal levels. Of six other fragments upstream of the coding region, five lacked functional activity at the time points of interest. Another 5kb fragment, denoted Sox10L8 (Fig. 1A), exhibited weak EGFP activity in neural crest and otic cells by HH13 (6/6), but not in emigrating neural crest and therefore was not pursued further.

Two highly conserved regions within Sox10E genomic fragment activate distinct spatiotemporal reporter expression

I used the ECR browser program to search for highly conserved sequences, potentially representing minimal essential core regulatory elements. By screening for 70% conservation across 100bp windows within multiple aligned genomic regions between *Sox10* and *POLR2F*, the first downstream neighboring gene, the program revealed two clusters of ~160bp and ~267bp within the 3.5kb Sox10E fragment (Fig. 2A). As no recognizable sequence homology was observed with either zebrafish or *Xenopus* sequences, these species were excluded. While there are no studies addressing Sox10 regulation in *Xenopus*, in zebrafish a 4.9 kb region upstream of Sox10 can recapitulate endogenous *Sox10* expression in sox10:GFP transgenics (Carney et al. 2006). Interestingly, despite the lack of obvious sequence conservation between their corresponding genomic

regions murine Sox10 regulatory elements drive reporter expression in transgenic zebrafish in similar spatial, but not completely overlapping temporal patterns, to those observed in transgenic reporter mice (Antonellis et al. 2008). Rather than using conventional sequence conservation approach, the search for conserved smaller motifs in a constrained arrangement has led to identification of a zebrafish Sox10 enhancer, further confirming that regulatory factors controlling Sox10 expression across vertebrate appears conserved (Dutton J. R. et al. 2008). Assaying two smaller fragments, each containing one identified conserved region, revealed that they activated EGFP expression in spatially distinct populations and in temporally distinct manners. A 600bp Sox10E1 fragment lacked activity in emigrating or migrating cranial crest (SFigs. 2a, d). It was first active in migrating vagal crest at HH15 (Fig. 2C) and in trunk crest, otic vesicle and condensed trigeminal ganglia (Fig2. D, Table1 on Fig. 2E), but did not drive EGFP expression in delaminating vagal or trunk neural crest.

Systematic deletions within the Sox10E region revealed a second active region--a 264bp minimal enhancer fragment, Sox10E2, comprised of an essential highly conserved 160bp core and supporting elements within 59bp upstream thereof (SFig. 1). In contrast to the late activating Sox10E1, Sox10E2 displayed enhancer activity as early as HH8+ in the first cranial crest emigrating from the neural tube, mimicking Sox10E activity (Fig. 1B) that intensified through HH9 (Fig. 2B). At HH12-15, Sox10E2 reporter expression was maintained in periocular crest, rostral hindbrain streams and otic vesicle (OV: SFigs. 2b, e), but absent from caudal hindbrain or trunk levels (SFig. 2b, c, e, f). Just as Sox10E displays regulatory activity within the branchial arches, Sox10E2 drives EGFP expression in rostral

hindbrain crest populating the first two arches (SFigs. 2b, e), and Sox10E1 is active in vagally-derived (r6-8) crest of posterior branchial arches (BA3-5; Fig. 2D). In contrast, endogenous *Sox10* is downregulated upon entering the arches (Fig. 1J). This ectopic expression suggests loss of a repressor element from the Sox10E fragments. The results show that both *cis*-regulatory fragments (Sox10E1/E2) can regulate *Sox10* expression in neural crest and otic regions, but in spatially and temporally distinct patterns. Interestingly, each reflects a portion of endogenous *Sox10* expression, which initiates in a rostrocaudal temporal sequence (Table1 on Fig. 2E). Fragment Sox10E1 is conserved and exhibited similar expression to a previously identified mouse D6 enhancer (Fig. 2A) (Werner et al. 2007). In contrast, no conserved region homologous to Sox10E2 has been reported.

Binding motifs for SoxE, Ets and Myb are necessary for Sox10E2 regulatory activity

To identify putative transcription factor binding sites within the 264bp Sox10E2 regulatory fragment, the corresponding sequences from human, mouse, rat, opossum and *Xenopus* genomic regions were aligned to chicken and screened for conserved motifs. Concomitantly, sequences were analyzed for known transcription factor consensus sites using Transfac 7.0, rVista and Jaspar programs. This revealed three highly conserved binding motifs (100% across amniotes), two for SoxE proteins and one for Ets factors. Conservation of other putative binding motifs ranged from 50-80% (Fig. 3A). Computationally identified binding motifs within Sox10E2 were tested for function via mutation/deletion analyses. Mutated versions of Sox10E2-EGFP constructs were generated

for individual putative binding motifs, electroporated into chicken embryos, and analyzed after 10-12 hrs (HH10-12).

Mutation of a putative Ets binding motif, within the enhancer core (M9;Fig3A), completely abolished Sox10E2 expression (Fig. 3C; 8/8). Similarly, reporter activity in cranial neural crest (SFig. 3b) was eliminated upon mutation of either SoxE binding site within the essential core region (M8, M11; Fig. 3A), indicating both were required for its activity (SFig. 3b; 13/13). Interestingly, there are two putative binding motifs for Myb factors in Sox10E2, one within the core and the other in the upstream adjacent supporting region (M2, M12; Fig. 3A), each contributing to regulatory activity. When both were replaced with random sequences, this double mutation completely abolished reporter expression (SFig. 3c; 7/7). Individual mutation of other computationally identified motifs only reduced enhancer activity. Perturbations of SoxD (M13; 10/10), Elk/Ets (M4; 7/7) and single Myb (M2,M12; 6/6) sites diminished EGFP signal intensity (Fig. 3A, D; SFig. 3d) suggesting they enhance regulatory function. In contrast, several mutations had no effect; e.g. simultaneous mutation of 4 putative Pax binding sites (M1, M3, M5, M7; Fig. 3A, E; 7/7), deletion of 45bp within the core region (Fig. 3A faded portion; 11/11), or mutation of either NFkB binding site (M6, M10; SFig. 3e; 6/6). Taken together, these results show that SoxE, Ets and Myb binding motifs are each necessary for Sox10E2 regulatory function. In addition to neural crest expression, these mutations also affected expression of the Sox10E2 reporter in the otic placode.

I tested whether SoxE, Ets and Myb binding sites, within the 264bp Sox10E2 fragment, are essential for regulatory activity of a larger construct from the Sox10 locus. To this end, I mutated these same sites (M2, M8, M9, M11, M12; Fig. 3A) within a much larger genomic fragment (~3.5kb) to test whether other genomic regions surrounding these enhancers could compensate for the loss of activity. Whereas the full length, non-mutated construct gave robust GFP staining that recapitulated endogenous *Sox10* expression, reporter activity in delaminating neural crest was completely eliminated in the same construct bearing mutations in SoxE, Ets and Myb binding sites within Sox10E2 (Fig. 3F; 6/6). These results strongly suggest that 264bp Sox10E2 fragment represents an essential regulatory module, and that binding sites for SoxE, Ets and Myb proteins are absolutely required for early *Sox10* expression within the context of the *Sox10* locus.

Knockdown of Ets1, cMyb or Sox9 diminishes Sox10E2 regulatory activity

To test if Ets1, cMyb and Sox9 transcription factors are required for exogenous Sox10E2 regulatory activity in delaminating neural crest, I co-electroporated either Ets1, cMyb, or Sox9 morpholino with the Sox10E2 reporter construct. The right side of each embryo received morpholino plus Sox10E2 reporter, whereas the left side received reporter plasmid alone. When the reporter construct was co-electroporated with control morpholino, the reporter signal on the right side was unaffected and comparable to the contralateral side (Figs. 4A-C; 10/10). Conversely, in the presence of cMyb (Figs. 4D-F; 11/15), Ets1 (Figs. 4G-I; 13/15), or Sox9 morpholino (SFig. 4;15/15) expression was greatly decreased or

abolished. These results show that Ets1, cMyb and Sox9 are independently required for the normal Sox10E2 regulatory activity, therefore making them good candidate factors responsible for the initial regulation of Sox10 through the identified Ets, Myb and SoxE functional binding motifs within Sox10E2.

Knockdown of Ets1, cMyb or Sox9 diminishes endogenous Sox10 expression

Although *cMyb* transcripts have been detected in early embryogenesis (Karafiat et al. 2005), their distribution was unknown and has not been described within the context of the neural crest gene regulatory network. Results obtained by *in situ* hybridization show that *cMyb* is expressed at stage HH6 in the neural plate border (Fig. 4J), and that transcripts accumulate in the neural folds by HH8, with strongest expression at the dorsal margins containing neural crest precursors (Figs. 4K, K'). At HH10, transcripts are seen in neural crest cells delaminating and emigrating from the cranial neural tube (Figs. 4L, L'). Thus, *cMyb*, like *Sox9 (*Fig. 4M) and *Ets1 (*Fig. 4N), is expressed in presumptive cranial neural crest prior to *Sox10*. The presence of *cMyb* at the neural plate border and premigratory neural crest illuminates a new role, at the onset of *Sox10* expression, in neural crest cell specification. Its initial expression coincides with that of early neural crest specifiers such as *AP-2*, *c-Myc* or *Snail2*. Furthermore, overexpression of cMyb upregulates *Msx1* and *Snail2*, and may participate in BMP4 input into the epithelial-mesenchymal transition of trunk neural crest (Karafiat et al. 2005).

I performed *in vivo* validation that endogenous Ets1, Sox9 or cMyb proteins are required as upstream regulators of *Sox10* in delaminating crest *in vivo* by examining the effects of cMyb, Ets1 or Sox9 morpholinos on endogenous *Sox10* expression at HH8+-9. The results reveal a dose-dependent effect on *Sox10* expression on the electroporated

versus contralateral side. I observed a mild diminution when individual morpholinos were electroporated at 1mM (Sox9 3/3; cMyb 9/10; Ets1 7/10), but a marked decrease at 3mM (Sox9 n=5, cMyb and Ets1 n=6, p<0.02; Fig. 5A-C, E-G). The effect of cMyb knockdown was less strong than either Ets1 or Sox9 inactivation (phenotypes ranging from 50-75% loss in Sox10 transcript). In contrast, electroporation of a control morpholino had no effect (10/10;Figs. 5L, M) and co-electroporation of morpholinos with the corresponding mRNAs mutated within the morpholino target region successfully rescued the loss-of-function phenotype (Sox9,n=6, p<0.03; cMyb n=5, $p\leq$0.04; Ets1,n=5, p<0.03; Figs. 5I-K). No statistically significant differences were noted in phosphohistone H3 or TUNEL staining between electroporated and control sides of embryos receiving either individual or all three morpholinos (~3mM). Thus, changes in cell proliferation or cell death cannot account for loss of Sox10 transcript (SFig. 5). The cumulative results suggest that Sox9, cMyb and Ets1 are each required for expression of endogenous *Sox10*. Importantly, the combined electroporation of all three morpholinos virtually eliminated transcript expression on the electroporated side (n=6, $p\leq$0.01; Fig. 5D, H). Our data confirm that Sox9, cMyb and Ets1 together are necessary for initial activation of *Sox10*.

Sox9, Ets1 and cMyb ectopically activate and are required for Sox10E2 reporter expression

All three SoxE genes, *Sox8*, *Sox9* and *Sox10,* are expressed by neural crest progenitors (Haldin and Labonne 2009). Because these genes can act redundantly (Finzsch

et al. 2008, Stolt et al. 2004, Taylor and Labonne 2005), theoretically any could activate the Sox10E2 reporter construct within the endogenous context. In all vertebrates examined, however, Sox9 expression precedes Sox10 (Antonellis et al. 2006, Dutton J. R. et al. 2008, Hong and Saint-Jeannet 2005, Werner et al. 2007); e.g. chick Sox9 is expressed in dorsal neural folds as early as HH8, before either Sox10 or Sox8 (Cheung and Briscoe 2003). This narrow (4-6 hr) time delay and the Sox9 morpholino knock down results presented in this study, suggest that Sox9 directly regulates *Sox10* onset and that this SoxE protein is responsible for initiating *Sox10* expression. To test if Sox9 can regulate the identified Sox10E2 regulatory element, Sox9 protein was ectopically expressed using ubiquitous H2B-RFP expression vector. Whereas no ectopic reporter expression was seen when Sox10E2 reporter was co-electroporated with control plasmid (Figs. 6A, F; 9/9), co-electroporation with *Sox9* plasmid caused ectopic reporter activity in extraembryonic region (Figs. 6B, G; 6/6). Similar results were obtained when cMyb was ectopically expressed (Figs. 6D, I; 3/3). Because Sox9 is expressed only transiently in migrating neural crest cells, it is likely that Sox10 and/or Sox8 later act to maintain *Sox10* expression.

Interestingly, co-electroporation of *Ets1* plasmid with Sox10E2 reporter resulted in ectopic reporter activation not only in extraembryonic regions but also in the trunk neural tube, which normally does not express Ets1 (Tahtakran and Selleck 2003) (arrowheads;Figs. 6C, H; 12/12). In the embryo, Ets1 plays a role in cranial neural crest delamination and appears to mitigate the requirement for S phase synchronization to promote crest emigration in a cluster-like fashion. Moreover, ectopic expression of Ets1 in the trunk results in excess, cluster-like emigration of *Sox10*-expressing cells

(Theveneau et al. 2007). Since expression of both *Ets1* and the Sox10E2-driven reporter is cranial-specific, it is intriguing to speculate that this newly identified element may act as one of the switches distinguishing head and trunk crest populations. Since *Sox9* and *cMyb*, but not *Ets1*, are normally expressed in the trunk neural tube (Cheung and Briscoe 2003, Karafiat et al. 2005, Tahtakran and Selleck 2003), ectopic Ets1 in this location may cooperate with these other factors to induce reporter expression. In support of this, combined overexpression of *Sox9*, *Ets1* and *cMyb* has a broader effect and induces strong ectopic Sox10E2 expression not only extraembryonically, but also along the neural tube, and in the ectoderm (SFig6a,d;5/5).

Sox9, *Ets1* or *cMyb* are each sufficient to trigger ectopic Sox10E2 enhancer activity. However, mutation of individual binding motifs or knock down of individual factors in the endogenous context shows that all three factors are necessary for normal Sox10E2 regulatory activity. Since ectopic reporter activity driven by overexpression of individual transcription factors occurs mainly in the extraembryonic region, we speculate that these naïve cells may already contain regulatory factors characteristic of multipotent tissue and are, thus, competent to switch on a neural crest-like transcriptional program in response to the proper single inputs.

To test if regulatory activity is mediated via the corresponding binding motifs of Sox9, Ets1 and Myb within Sox10E2 enhancer, I assayed their ability to ectopically activate mutated reporter constructs. Either Sox9-H2B RFP (6/6) or cEts1-H2B RFP (6/6) were co-electroporated with Sox10E2 construct with corresponding binding motif mutations. In all cases, electroporated embryos lacked ectopic reporter expression (Figs. 6E, J). However, ectopic reporter expression was also affected when overexpressing either

Ets1, cMyb or Sox9 with other Sox10E2 versions containing mutations within non-cognate biding sites. For example, when Sox9 and cMyb were overexpressed and combined with a Sox10E2 reporter carrying a mutation within the Ets motif (M9), ectopic reporter expression in the extraembryonic region was not observed (SFigs. 6b, e; 6/6). If Sox9 and Ets1 were over expressed together with a Sox10E2 carrying a single mutated Myb site (M12), ectopic reporter expression was weak (SFigs. 6c, f; 3/3). This shows that for the Sox10E2 enhancer to have ectopic regulatory activity all binding sites need to be functional, and suggests a cluster-like conformation of the motifs and synergistic action of the corresponding upstream regulators.

Sox9, Ets1 and cMyb directly bind to the Sox10E2 element

To test if Sox9, Ets1 and cMyb can bind directly to the corresponding motifs within the Sox10E2 element, EMSA assays were performed, using biotinylated double stranded oligonucleotides containing the corresponding Sox10E2 sub-fragments (underlined,Fig3A). A clear electrophoretic shift was observed in samples incubated with nuclear extracts from chicken embryonic fibroblasts overexpressing Sox9, Ets1 or cMyb, but not from the cells transfected with control plasmid (white arrowheads;Fig6K). This binding was out-competed by adding 200-fold excess of the corresponding non-labeled (cold) fragment to the binding reaction showing specificity. Next, to confirm the identity of the transcription factors directly binding to Sox10E2 subfragments, a streptavidin-biotin DNA pulldown approach was used followed by Western blot with specific antibodies. Using biotinylated target and scrambled control fragments as bait, it was show that specific subfragments pull down corresponding binding proteins (Sox9, Ets1 and cMyb) from the embryonic nuclear

extracts. Conversely non-coated streptavidin-conjugated magnetic beads or beads coated with scrambled control fragments display no specific protein binding (Fig6L).

Finally, the direct binding of these transcription factors to the Sox10E2 enhancer *in vivo* using quantitative ChIP (qChIP) was tested. Crosslinked chromatin isolated from cranial regions of HH8-12 somite embryos was immunoprecipitated using Sox9, Ets1 and cMyb antibodies and ChIP-enriched DNA was used in site-specific QPCR, with primers designed to amplify fragments within the Sox10E2 region. Results show significant (4-8X) enrichment over non-specific antibody indicating that Sox10 locus and, in particular, Sox10E2 regulatory element, was occupied by endogenous Sox9, Ets1 and cMyb proteins in the cranial region of HH8-10 chicken embryos (Fig6M).

Conclusion

In summary, I have isolated and dissected a novel regulatory module, representing the first known element responsible for driving gene expression in newly emigrating cranial neural crest cells. Of the neural crest specifiers, Sox10 is a key regulator for specification of numerous genes important for neural crest migration and differentiation (Kelsh 2006, Sauka-Spengler and Bronner-Fraser 2008a). Although *Sox10* enhancer elements controlling expression in neural crest derivatives and late migrating cells have been noted in other species (Carney et al. 2006, Deal et al. 2006, Werner et al. 2007), this is the first report of a regulatory element(s) controlling onset of any neural crest specifier. I find that a cluster of transcription factors, Ets1, Sox9 or cMyb, directly converge onto this

cis-regulatory element to regulate the reporter and onset of endogenous *Sox10* expression in cranial neural crest cells. This introduces and establishes the role of two new neural crest specifier genes, *cMyb* and *Ets1*, in the NC-GRN and confirms Sox9 as an essential input into *Sox10* specific activation at the cranial level. Such in depth analysis combining perturbations of regulatory sequences and their candidate upstream regulators, substantiated with EMSA, DNA pulldown and chromatin immunoprecipitation assays, provides powerful tools for identifying direct binding interactions and testing regulatory outcomes *in vivo*. By connecting upstream regulators of the NC-GRN directly to *Sox10* via the cranial crest enhancer Sox10E2, new elements to the NC-GRN can be added to expand our knowledge of its architecture.

Materials and Methods

Ex ovo/in ovo electroporations

Chicken embryos were electroporated at stages HH4-8 to target the cranial neural crest cell population and at stages HH10-12 to target vagal and/or trunk neural crest cells following previously described electroporation procedures (Sauka-Spengler and Barembaum 2008). In *ex-ovo* experiments, the DNA plasmid constructs (enhancer driven reporter with ubiquitously-expressing tracer) were introduced in the entire epiblast of the early chicken embryo, while in *in-ovo* electroporations only one half of the neural tube received the DNA. Injected DNA plasmid concentrations were as follows: 2μg/μl of ptk-

EGFP or ptk-Cherry reporter construct, containing each of Sox10 putative *cis*-regulatory regions or the Sox10E2 mutated versions, combined with 1μg/μl of either tracer (pCI H2B-RFP) or expression constructs (Sox9-pCI H2B-RFP or Ets1-pCI H2B-RFP).

Morpholino-mediated knock-down experiments were performed by injecting the translation-blocking, FITC-labelled morpholino antisense oligonucleotides in one half of the epiblast (right to the primitive streak) or, in some cases, by double electroporations to differentially transfect each half of the embryo *ex-ovo*. Double electroporations were performed by introducing each morpholino combined with the Sox10E2-Cherry plasmid on the right side and the Sox10E2-Cherry reporter only on the left side of the embryo. Morpholinos used in this study were obtained from Gene Tools (Philomath, OR) and their sequences are as follows:

Ets1_5'-GCTTCAGGTCCACCGCCGCCTTCAT-3';

cMyb_5'-ATGGCCGCGAGCTCCGCGTGCAGAT-3';

Sox9_5'-GGGTCTAGGAGATTCATGCGAGAAA-3';

Control_5'-ATGGCCTCGGAGCTGGAGAGCCTCA-3'.

Microscopy and inmunohistochemistry

The electroporated embryos were collected at different stages, fixed in 4% paraformaldehyde O/N and then washed three times in PBS at room temperature. A Zeiss Axioskop2 Plus fluorescence microscope equipped with the AxioVision software was employed to image the embryos. Images were processed using Adobe Photoshop CS2. After imaging, embryos were cryo-protected in two steps: 15% sucrose/PBS and 7.5%

gelatin/15% sucrose/PBS, equilibrated and mounted in 20% gelatin/PBS and frozen in liquid nitrogen. 12μm cryosections were collected on Super Frost Plus slides (Fischer Scientific, Pittsburgh, PA) and de-gelatinized for 2x10 minutes at 42°C in PBS. To intensify EGFP signal, the sections were washed 4x in PBS for 5 minutes, blocked for 1 hour in 10% donkey serum / PBTW (PBS/0.1% Tween-20) and stained with 1:1000 anti-GFP primary antibody (Abcam Inc., Cambridge, MA) followed by 1:2000 donkey anti-goat Alexa-Fluor 488-conjugated secondary antibody (Molecular Probes). Sections were subsequently washed, cover-slipped and imaged using the same imaging procedure described for the whole-mounts.

Isolation of regulatory regions and cloning

Sox10 genomic regions were amplified with Expand High Fidelity Plus PCR System (Roche Applied Science, Indianapolis, IN), using BAC DNA clones as the template (Chicken BAC library Chori 26, BACPAC Resources, Oakland, CA). Each fragment, ranging from ~3kb-5kb in size, was cloned into the SmaI-linearized ptk-EGFP vector. The ptk-EGFP reporter vector has the Herpes simplex virus thymidine kinase basic promoter upstream of enhanced GFP and was a kind gift of Dr. Hisato Kondoh. The clones with the appropriate orientation were identified by colony PCR and sequenced. The plasmid DNA of the correct clones was prepared and purified using the Endo-free maxi kit (Qiagen) and eluted in EDTA-free buffer.

ptk-Cherry and pCI H2B-RFP plasmids were generated for use in this study. ptk-Cherry reporter vector was made by swapping EGFP with Cherry fluorescent protein in the

ptk-EGFP reporter vector (Uchikawa et al. 2003). pCI H2B-RFP, a tracer construct that yields ubiquitous expression under the control of chicken beta actin promoter is a bicistronic vector allowing for exogenous expression of proteins of interest and of a fusion protein of human histone 2B and monomeric RFP protein, translated from the IRES. The pCI H2B-RFP construct was made by replacing the 3xNLS-EGFP sequence within the pCI-GFP vector (Megason and McMahon 2002) with the H2B-mRFP1 sequence.

Dissection of Sox10 downstream putative regulatory region and mutation of candidate binding sites

The 3.5kb genomic region downstream *Sox10* coding (Sox10E) was divided into smaller regions, dissected and mutated fragments amplified using Expand High Fidelity Plus PCR System (Roche Applied Science, Indianapolis, IN). For the initial dissection of the Sox10E fragment, the following primers were used:

Sox10E1_ 5', 5'-ATTAGGTACCTCTGATACAGATGCAAGGCTG-3' and Sox10E1_ 3', 5'-TAATCTCGAGAATTTGCAGCACTGTGGCCTT-3';

Sox10E2_5', 5'-AATTGGTACCGGCAAGAGTGGCAATTTAACC-3' and Sox10E2_3', 5'-ATTACTCGAGATTGCTTCCCCCTAGACAGTT-3';

Sox10E3_5', 5'-TTTTGGTACCTAACCAGGGAGGAGTTGTGG-3' and Sox10E3_3', 5'-AATTCTCGAGAAGGCCCACAGCAGAGTG-3'.

To perturb candidate binding sites within the Sox10E2 fragment, we used single or fusion PCR (Heckman and Pease 2007, Szewczyk et al. 2006). Only the primers bearing the mutations are listed here. Mutated regions are underlined, the mutated nucleotides are shown in bold and fusion primer sequences are italicized:

First 2 Pax sites clusters

(M1,M3)_5',5'-

AATTGGTACCGGCAA**AGCCCATG**ATTTAACCTACAACTGCTGAGCTTGTAGG

A **AGCCCATG**GGCGACTGTGCTTCCGGCT-3';

Myb(M2)_5',5'-

ATTAGGTACCTGGCAAGAGTGGCAA**GGGATGGACTGGTA**GATG**GAAG**TGTA

GGACTGTGACTGGCGA-3';

Second 2 Pax sites clusters

(M5,M7)_3',5'-

TCCCTGCTCCTGCTGCTTATCA**TGGGCT**GGGATCCCCTTTCA**TGGGCT**CTGCCC

CAGCCGGAAGCACAGT-3';

Ets/Elk(M4)_5',5'-

ATTAGGTACCTGGCAAGAGTGGCAATTTAACCTACAACTGCTGAGCTTGTAGG

ACTGTGACTGGCGACTGT**ATGGTTAAT**TGGGGCAGTGCCACTGAAA-3';

NFKB1(M6)_3',5'-

TGCTGCTTATCAGTGATG**AGCCCATGGTCT**CAGTGGCACTGCCCCAG3';

Lef/Tcf/SoxE(M8)_3',5'-

TCTCATCAAATCACCT**CCATCTA**CCCTGCTCCTGCTGCTTATCAGT-3';

Ets(M9)_3',5'-

AATTCTCGAGATTGCTTCCCCCTAGACAGTTGGGCCTTTGTGCCCTGAGCAGGT

TGCTGTGGAAACCCCCAAT**GGG**CTCTCTGGCCAGAGCTGGCT-3';

NFKB1/Lef/Tcf/Ets1_3',5'-

AATTCTCGAGTTGCTTCCCCCTAGACAGTTGGGCCTTTGTGCCCTGAGCAGGTT

GCTGTGG**AGCCCATGGTC**TTCCTCTCTGGCCAGAGC-3';

SoxE/Lef/Tcf(M10)_3',5'-

ATTACTCGAGATTGCTTCCCCCTAGACAGTTGGGC**G**T**A**TG**C**GCCCTGAGCAGG

TTGCTGTGGAAA-3';

Myb(M11)_3',5'-

ATTACTCGAGATTGCTTCCCCCTA**CTCCA**T**AA**GGCCTTTGTGCCCTGAGCA-3';

SoxD (M12)_3', 5'-ATTACTCGAG**GCCAA**TTCCCCCTAGACAGTTGGGC-3';

Δ1_3',5'-*AATTTCCTCTCTGGCCAGA*AAATCACCTATTGTTTCCCT-3';

R1_3',5'-

AATTTCCTCTCTGGCCAG**CCTC**GG**GGTACA**TCC**G**CTC**GGAGGAGGCCTCCCA**

GCCCATGGTCTAAATCACCTATTGTTTCCCT-3'.

After PCR amplification, each fragment was purified using PCR product purification kit (Qiagen) and cloned into KpnI/XhoI digested ptk-EGFP or ptk-Cherry reporter vectors.

Over expression constructs

Open reading frames of chick *Sox9, cMyb* and *Ets1* genes were amplified from full length cDNA clones or chicken cDNA (Sox9 cDNA clone was a kind gift from Yi-Chuan Cheng, Ets1 clone was isolated from the stage 8-12 somites chicken macroarrayed cDNA library constructed by Laura Gammill (Gammill and Bronner-Fraser 2002)) and cloned into

XhoI/EcoRV or XhoI/ClaI digested pCI H2B-RFP expression vector. The Sox9 and Ets1 rescue constructs were generated from expression constructs by PCR, using primers carrying mutations within the morpholino target sites that do not alter the amino acid sequence of the recombinant proteins. The following primers were used:

Sox9_5', 5'-AATTCTCGAGGCCACCTGCTCAAGGGCTACGACTGG-3' and Sox9_3', 5'-ATTAGATATCTTTAAGGCCGGGTGAGCTGC-3';

Ets1_5', 5'-AATACTCGAGGGCCTCAACCATGAAGGCGGCGGTGGA-3' and Ets1_3', 5'-ATTAGATATCTCACTCATCAGCATCTGGCTTG-3';

cMyb_5',5'-ATTACTCGAGgccaccATGGCCCGGAGAC;

cMyb_3',5'-ATTAATCGATTCACATCACCAGAGTCC;

Sox9mut_5',5'-ATTACTCGAGgccaccATGAActTgtTgGAtCCCTTCATGAAAATGAC;

Ets1mut_5',5'-

ATTACTCGAGTCAACCATGAAaGCtGCcGTcGAttTaAAaCCCACCCTGACCATCA.

Comparative genomic analyses

To identify highly conserved genomic regions, the **ECR Browser** software (http://ecrbrowser.dcode.org/) was employed. Chicken, zebrafish, *Xenopus*, opossum, mouse, chimpanzee, dog, rat and human genomic sequences were downloaded using UCSC genome browser (http://genome.ucsc.edu/) Following instructions available on the ECR website, these sequences were computationally compared from between all the species, with conservation parameters set to 70-80%. The "zoom in" feature, built into the program, was used to closely analyze the sequence conservation by increasing the threshold up to 90% and using different window sizes ranging from 20bp to 50bp.

To search for putative binding motifs, we used the jaspar_core database from **Jaspar** (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl) and the P-Match program available through the **Transfac** database (http://www.gene-regulation.com/pub/programs.html). Briefly, the 264bp-long sequence of Sox10E2 genomic fragment was uploaded into these programs using desired parameters and the programs returned potential binding motifs based on the position weight matrix (PWM) score. Simultaneously, using **rVista 2.0** (http://rvista.dcode.org/), Sox10E2 sequence was aligned and compared to the corresponding mouse sequence to screen the latter sequence for conserved putative binding motifs identified in chicken by either Jaspar, Transfac or both search engines. We used the position weight matrix (PWM) score, a value given to a site based on the distribution frequency of each base at each position (Hollenhorst et al. 2007) to determine the probability of binding score. This was then used to guide mutational analysis, since, as with any informatics approach, there are several caveats: 1) this only predicts motifs for factors with known consensus sites; 2) not all functional sites have high PWM scores since they can differ greatly from consensus (Bajard et al. 2006); and 3) not all sites with high PWM are functional.

In situ hybridization

Whole-mount *in situ* hybridizations were performed using a procedure previously described (Wilkinson 1992). Fluorescent *in situ* procedure using GFP probe was adapted from (Acloque et al. 2008). With the exception of the Sox9 and Sox10 probes, which were prepared using full length cDNA constructs (a gift from Yi-Chuan Cheng) as a template, all other digoxigenin-labeled antisense RNA probes were prepared from chicken EST clones

obtained from ARK Genomics and MRC Geneservice. Sox10 template was digested with HindIII, while all EST clones were linearized using NotI restriction enzyme. All antisense RNA probes was synthesized using T3 RNA polymerase, according to standard protocols.

Chromatin immunoprecipitation with Sox9, Ets1 and cMyb antibodies

For each preparation of nuclei, cranial regions from 20 stage HH8-12 somite embryos were dissected in Ringer's solution and transferred to 1ml isotonic buffer (0.5% Triton X-100, 10mM Tris-HCl, pH 7.5, 3mM $CaCl_2$, 0.25 M sucrose, protease inhibitor tablet (Complete Protease Inhibitor EDTA-free, Roche), 1 mM DTT, and 0.2 mM PMSF) on ice (Buchholz et al. 2006). Tissue was homogenized using Dounce homogenizer and cells cross-linked by adding formaldehyde to a final concentration of 1% and nutated for 10' at room temperature. Glycine (final concentration of 125mM) was added to stop the cross-linking reaction and the solution was incubated by nutation for 5' at RT. The cross-linked cells were washed 3 times and cell pellets were snap frozen in liquid nitrogen and kept at -80°C until the ChIP procedure. Preparations were kept up to a month without altering the quality of results. The pellets were re-suspended in isotonic buffer and nuclei isolated using Dounce homogenizer washed and lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) for 10'-1h. The lysate was then diluted three-fold with ChIP dilution buffer (0.01% SDS, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1 mM DTT, 0.4 mM PMSF, and protease inhibitors) and ½ of chromatin (420 µl) was sonicated using Misonix 4000 sonicator at following settings: Amp 3, 10 consecutive cycles of 30'' sonication each with 1' pause in between. Triton X was added to the sonicated material to a final concentration of ~1%, chromatin was cleared by

centrifugation, diluted 3-4 times with ChIP Dilution Buffer w/1.1% Triton X-100 and was distributed between 2-3 antibody/bead complexes (400µl each) and incubated ON at 4°C. 50µl of the chromatin preparation was conserved at -80°C as the input fraction. Antibody/magnetic beads were prepared as per Young protocol (http://openwetware.org/wiki/ChIP). Post-immunoprecipitation washes were performed using RIPA wash buffer (50 mM Hepes-KOH, pH 7.6, 500 mM LiCl, 1mM EDTA, 1% NP-40, 0.7% Na-Deoxycholate). The complexes were then washed with Tris-EDTA/NaCl (10 mM Tris-HCl, 1 mM EDTA pH 8.0, 50 mM NaCl) for 5' and transferred to a new chilled tube, prior to last separation (Dahl and Collas 2008). The chromatin was eluted in elution buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0) and cross-link reversed overnight by incubation at 65°C. The samples were consecutively treated with RNase A (0.2mg/ml) and then Proteinase K (0.2 mg/ml), extracted with phenol/chloroform/isoamyl alcohol, precipitated and resuspended in 50µl of 10mM Tris pH 8.0. Real-time PCR reactions were performed in a 96-well plate ABI7000 QPCR machine. Reactions were set up using SybrGreen (Biorad), 450µM of each forward and reverse primer and 1µl of each ChIP reaction or 1:100-200 dilution of Input fraction. The $\Delta\Delta C_t$ method was used for quantification and calculations performed according to ChIP-qPCR Data Analysis instructions (SupperArray, Bioscience Corporation). In order to select suitable negative control primers, large regions (potentially corresponding to genomic deserts) of chromosome 1 were surveyed. Because Ets1 binding sites are present in large proportion of the chicken genome. non-specific binding was a concern; therefore, 8-10 different sets of primers were tested. The primers

presented in Fig. 6M corresponding to Sox10E2 fragment were: Sox10E2_1 for 5'-TGCTCCTGCTGCTTATCA-3'; Sox10E2_1 rev 5'-ATCAGCTCCACTGCACAT-3'; Sox10E2_2 for 5'-TGATAAGCAGCAGGAGCA-3'; Sox10E2_2 rev 5'-TGAGCAGGTTGCTGTGGAAA-3'. Control primer sets that amplify negative control region situated on the same chromosome as Sox10 locus were as follow: negcont_1 for 5'-TCGGATTTTAATGGGCTCAG-3'; negcont_1 rev 5'-CCGCAGATAGTTCTGCATCA-3' negcont_2 for 5'-GGTTGGATTTCCAGTCTCCA-3'; negcont_2 rev 5'-TGTTTTGCTGGACAATCTGC-3'.

EMSA and pull-down assays

Five Sox10E2 subfragments (M2, M4, M8, M9, M11/M12) and three control fragments were obtained by annealing synthetic oligonucleotides with or without 5' biotin modification (IDT Biotechnology). Double stranded fragments used in EMSA assay either had biotin tags on both ends or were not labeled (cold probes).

The sequences of Sox10E2 subfragments used in these approaches were:

M2,GCAATTTAACCTACAACTGCTGAGCTTGTA;

M4,GGCGACTGTGCTTCCGGCTGGGGCAGTG;

M8,GGAGCAGGGAAACAATAGGTGATTT;

M9,TGGCCAGAGAGGAAATTGGGGGTTT;

M11/12,CAGGGCACAAAGGCCCAACTG.

The sequences of scrambled controls were selected based on the absence of binding sites with homology of greater than 70%, according to an exhaustive survey of Jaspar and Transfac databases. They were as follows:

MybCo,TCTTCAAGTCCGCCATGCCCGAAGG;

Sox9Co,TACGGCAAGCTGTTCATCTGCACCA;

Ets1 Co, ATGTCTACGTCGAGCGCGACGGCGA.

EMSA. Nuclear extracts from chicken embryonic transfected with corresponding expression plasmids (Sox9- Ets1-, cMyb- or control-pCI H2BRFP) were obtained using standard hypotonic buffer (10mM HEPES pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 0.5 mM DTT, 1X Complete EDTA-Free Protease Inhibitors, 0.2 mM PMSF) to isolate the nuclei and extraction buffer (20 mM HEPES pH7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 1mM DTT, 1X Complete EDTA-Free Protease Inhibitors) to obtain nuclear extracts. Binding reactions and gel shift detection was performed using LightShift Chemiluminescent EMSA Kit (Thermo Fisher Scientific, Rockford, IL) following manufacturer's instructions.

PULL-DOWN ASSAYS. DNA fragments used in streptavidin-biotin pull-down assays carried a single biotin tag. Dynal streptavidin beads (Invitrogen) were coated according to manufacturer's instructions, using equimolar quantities of the fragment labeled on either 5' or 3' end. 400-500 µg of embryonic nuclear proteins (extracted from cephalic regions of chicken embryos at stage HH9-HH12) in final volume of ~800µl of binding buffer (12%glycerol, 12 mM HEPES pH 7.9, 4 mM Tris pH 7.9, 150 mM KCl, 1 mM EDTA, 1 mM DTE, and 0.1µg/µl poly(dI-dC). These were pre-exhausted using ~1.5mg of streptavidin Dynal beads and distributed among 4 Dynal bead preps: 2 coated with specific DNA fragment, one with scrambled control and one without DNA. Binding reactions were allowed to proceed for 30' at RT, and were subsequently washed 4 times with the binding buffer (only 1st wash contained poly(dI-dC)). After the 4th wash, the beads

were transferred to a new tube, bound proteins eluted in 30 µl of 50mM Tris pH 7.5, 100mM NaCl, 5mM EDTA, 1X Protease Inhibitors, 0.1% SDS and analyzed by Western blot using Sox9, Ets1 and cMyb antibodies.

Cell death and proliferation assays

HISTOLOGY. Embryos electroporated unilaterally with single or triple morpholino (@3mM concentration) were fixed in 4% paraformaldehyde and dehydrated to 100% methanol. After re-hydration, embryos were cryoprotected in 15% sucrose, equilibrated in 15% sucrose/7.5% gelatin, embedded in 20% gelatin and sectioned at 10 µm using Micron cryostat.

TUNEL REACTION. The slides were first washed twice in PBS at 42^{o}C for 10' to remove gelatin, followed by 3-4 10' washes in PBST (PBS+ 0.5% Triton X-100) at RT. After 10' incubation in Permeabilization solution (0.1% sodium Citrate, 0.5% Triton in PBS) and two PBST rinses, the slides were incubated in Tunel reaction mix (In Situ Cell Death Detection Kit, TMR red, Roche). Labeling solution was first diluted 10 times with Tunel buffer (30 mM Tris pH 7.2, 140 mM Sodium cacodylate, 1 mM $CoCl_2$) and then combined with enzyme mix as per manufacturer's instructions (1 part enzyme + 9 parts label). A positive control slide, pre-treated with DNAse I (2µl of 10U/µl stock in 100µl of DNase buffer: 10mM $CaCl_2$, 40mM Tris Cl pH7.4, 10mM $MgCl_2$, 10mM NaCl, for 1h at RT) was prepared in advance, rinsed with 2mM EDTA in PBST to quench DNase activity, washed twice in PBST and stained with Tunel reaction mix as well. Negative control slide

was incubated in tunel labeling mix, without TdT enzyme. The labeling was performed in the dark, for 4 h in a humidified chamber at 37°C. The slides were then washed 3x PBST for at least 15 min each time, the positive control slide was rinsed in a separate container.

IMMUNOSTAINING. The slides were incubated in blocking buffer (10% donkey serum in PBST) for 1 hour followed by primary antibodies diluted in blocking buffer overnight at 4^{o}C, (1:1000 rabbit antiPh3 (Phospho-Histone 3) and 1:800 Alexa 488 goat anti FITC). Slides were then rinsed 3-4 times in PBST, 30 min each wash. Secondary antibody was diluted in PBST or blocking buffer and applied for 1 hour at RT. As the TUNEL staining is red (TMR red), we used 1:1000 Alexa 350 goat anti-Rabbit (blue) to detect antiPh3 and FITC (morpholino) was labeled in green. All consecutive sections from the cranial region were counted and number of Ph3- (and Tunel-) positive cells within the neural fold was compared between morpholinoated and control sides for individual, triple and control morpholinos. We present the mean value of electroporated/control side ratio for triple and control morpholinos. The statistical values were calculated using unpaired student's t-test.

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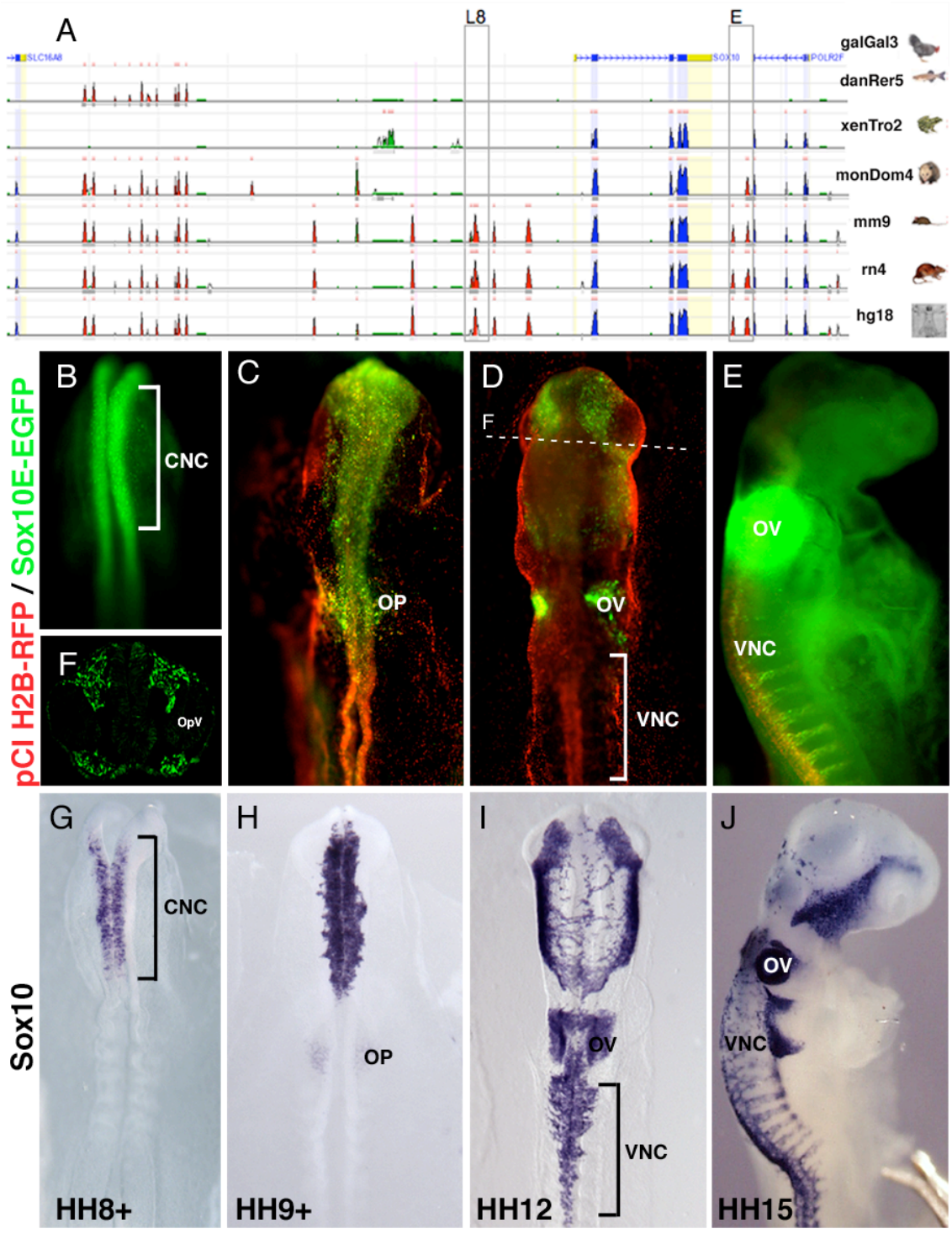
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L8
E
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HH12
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HH15

Figure 1. *Sox10 cis*-regulatory analysis. (A) Schematic diagram showing comparative genomic analysis using ECR browser. Chicken, zebrafish, *Xenopus*, opossum, mouse, rat and human genomic sequences were compared between *Sox10* and neighboring genes, *Slc16A8* and *PolR2F*. Red peaks = highly conserved elements; blue = coding exons; green = transposable elements and simple repeats. Boxed *Sox10* putative regulatory regions L8(L = late) and E(E = early) show activity in neural crest. UTRs shaded in yellow. (B) At HH8+, GFP transcripts are detected by fluorescent *in situ* hybridization in cranial neural crest (CNC) similar to endogenous *Sox10* expression(G). Distribution of EGFP transcripts (C, D, E; HH9+, 12, 15) is similar to endogenous *Sox10* in H-J, respectively. (D) EGFP expression at HH12 in rhombomere5 stream surrounding otic vesicle (OV) resembles endogenous *Sox10* (I), but is missing in vagal neural crest (VNC). (F) Cross section of embryo in D shows specific Sox10E regulatory activity in CNC around optic vesicle (OpV). (G-J) Endogenous *Sox10* expression at HH8+-15. OP, otic placode.

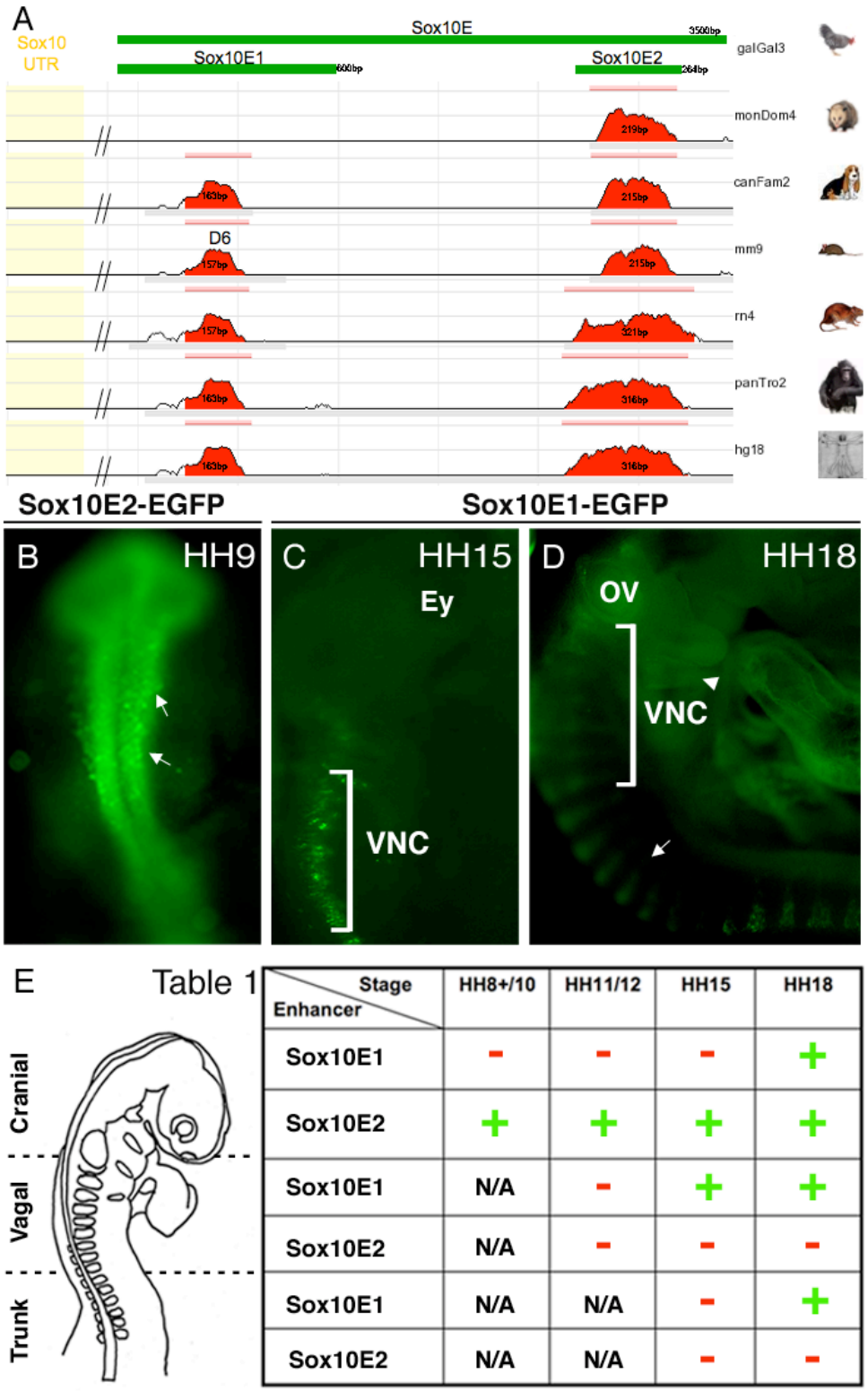
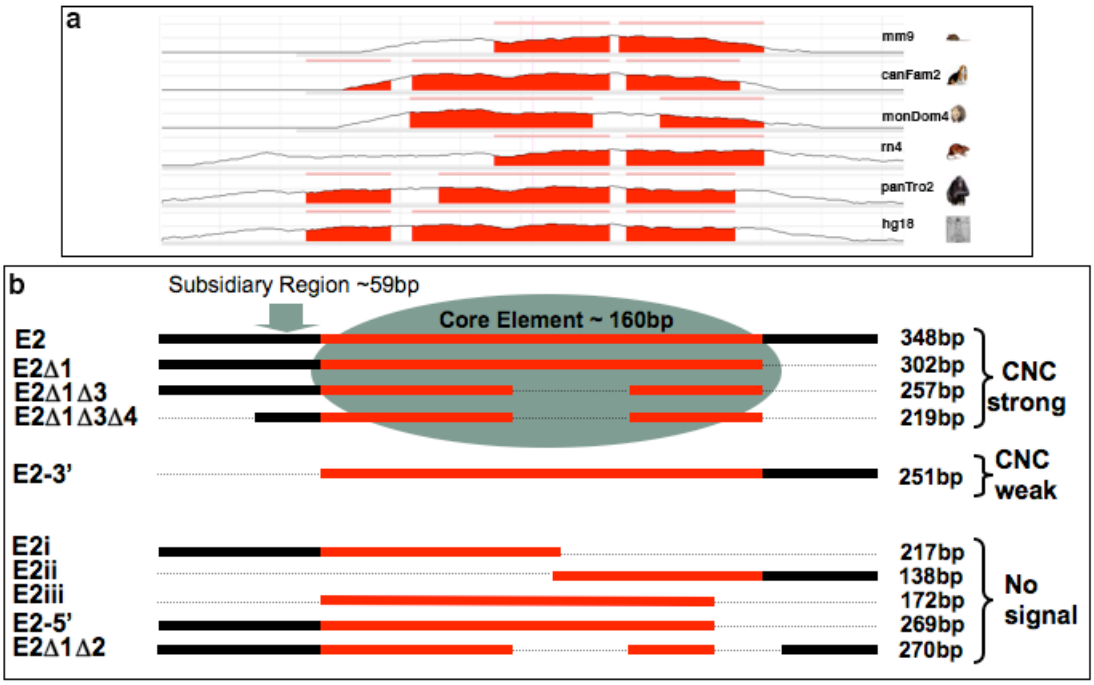


Table 1

Enhancer \ Stage	HH8+/10	HH11/12	HH15	HH18
Sox10E1	-	-	-	+
Sox10E2	+	+	+	+
Sox10E1	N/A	-	+	+
Sox10E2	N/A	-	-	-
Sox10E1	N/A	N/A	-	+
Sox10E2	N/A	N/A	-	-

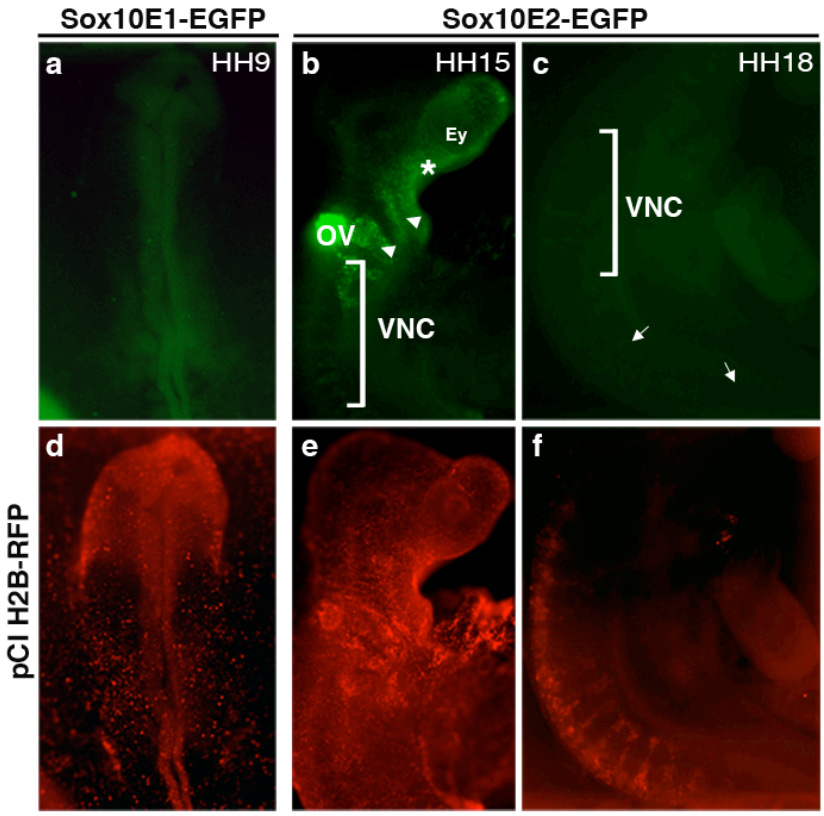
Figure 2. Sox10E contains distinct spatiotemporal regulatory elements: Sox10E2 in delaminating CNC and Sox10E1 in later migrating vagal (VNC) and trunk neural crest. (A) Schematic diagram representing dissection of Sox10E fragment, located ~1kb downstream of *Sox10* locus (UTR in yellow). Two smaller active regulatory fragments embedded within Sox10E (Sox10E1&E2) each contain a conserved region (red bar) with 70% sequence homology between amniotes. (B) Sox10E2 drives expression in delaminating CNC (arrows) at HH8+; Sox10E1 is first active in migrating VNC at HH15 (C). (D) Sox10E1 activity persists in migrating VNC, trunk neural crest (arrow), and branchial arches 3-5 (arrowhead). (E) Table1 summarizes distinct temporal (HH9-18) and spatial (cranial/vagal/trunk) regulatory activity of Sox10E1, E2. Red - = no expression; green + = EGFP reporter expression.



Supplementary Figure 1. Dissection of the Sox10E2 fragment reveals an essential core and auxiliary region important for optimal enhancer activity. (a) "Zoomed in" image showing the region Sox10E2 from the genomic comparative analysis illustrated in Fig. 2A. Each uncolored peak represents the conserved region equivalent to region Sox10E2 in chicken for each corresponding species. Inside the conserved regions, the red patches represent highly conserve portions (85%-90% each 30bp). The order of species from top to bottom: mouse, dog, opossum, rat, chimpanzee and human.

(b) Schematic diagram representing the different successive deletions (dotted lines) that were performed, guided by bioinformatics, to identify the main core element responsible for the regulatory activity of the Sox10E2 fragment. The horizontal black lines represent different fragments. Red portion of the lines denote a 160bp region (referred to as essential core element), highly conserved between dog, chimpanzee and human, capable of

producing weak tissue-specific regulatory activity in delaminating cranial neural crest cells. The gray arrow points to a non-conserved 59bp-long auxiliary region, necessary for achieving strong regulatory activity of the enhancer. CNC, cranial neural crest.



Supplementary Figure 2. Dissection of Sox10E reveals two regulatory regions that function in a specific spatiotemporal manner: Sox10E2 is activated as cranial neural crest cells delaminate whereas Sox10E1 is activated in later migrating vagal and trunk neural crest. (a) Sox10E1 displays no activity in the delaminating cranial neural crest. (b) Sox10E2 activity at HH15 persists in the periocular crest and otic vesicle, but also within the first two branchial arches, which lack endogenous Sox10; Sox10E2 is not expressed in vagal neural crest at this stage or later, at HH18 (c) in either the vagal or trunk regions. (d-f) Panels corresponding to a-c, respectively, show expression of the co-electroporated tracer pCI H2B-RFP to locate cells that received both tracer and reporter EGFP plasmid DNA.

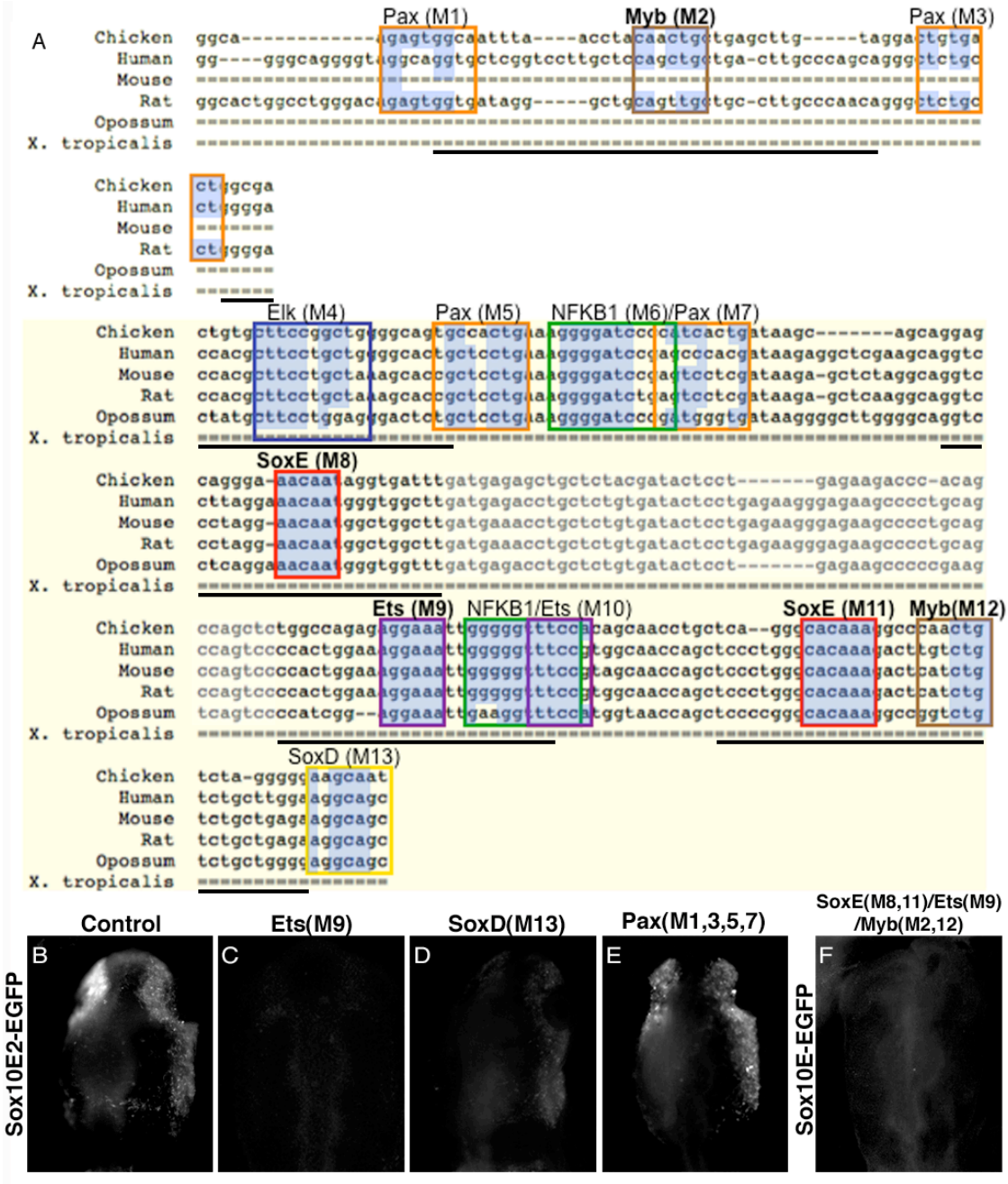
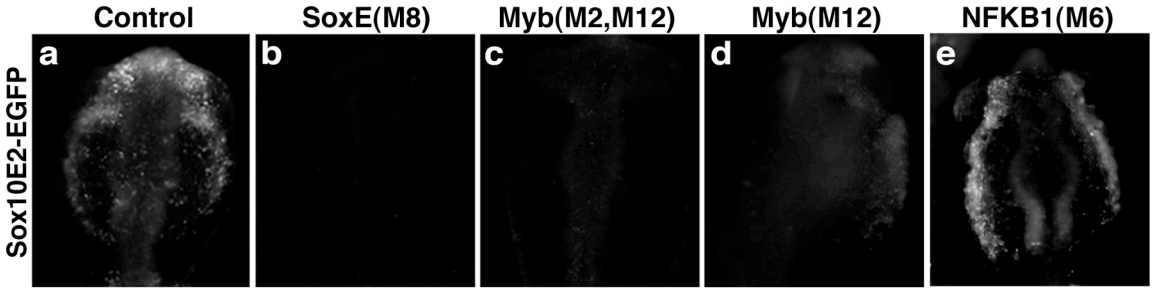


Figure 3. Transcriptional inputs into the Sox10E2 regulatory region. (A) Schematic diagram showing sequence alignment of 264bp Sox10E2 regulatory region; essential core

region shaded in yellow. Colored frames indicate computationally identified putative transcription factors binding motifs. Mutations M1-M13 were replaced by random sequences. Faded sequence shows a 45bp region deleted or replaced by mCherry coding sequence. Highlighted in blue are conserved nucleotides within putative binding motifs. Single dashed lines indicate no bases in aligned sequence. Thick dashed lines indicate nonalignable sequences. Thick solid underlines delineate Sox10E2 subfragments used in EMSA and pulldown assays. Sox10E2-driven EGFP expression in CNC (B) is abolished upon mutation of an Ets1 binding motif (C), but only decreased after mutation of putative SoxD motif (D), and not affected by simultaneous mutation of four putative Pax sites (E). (F) Simultaneous inactivation of SoxE, Ets and Myb binding sites (M2, M8, M9, M11, M12) within a much larger genomic region abolishes reporter expression in delaminating CNC.



Supplementary Figure 3. Transcriptional inputs into the Sox10E2 regulatory region. (a) EGFP pattern of expression in neural crest cells driven by the intact Sox10E2 regulatory region (b, c). EGFP expression (b, green) is abolished in cranial neural crest when binding motifs for putative upstream regulators, SoxE (b) and a pair of Myb (c), are mutated (mutations M8, M2/M12 respectively). (d) A mutation of a single Myb binding site mildly decreases EGFP reporter expression while a NFkB1 mutation does not affect reporter signal (e).

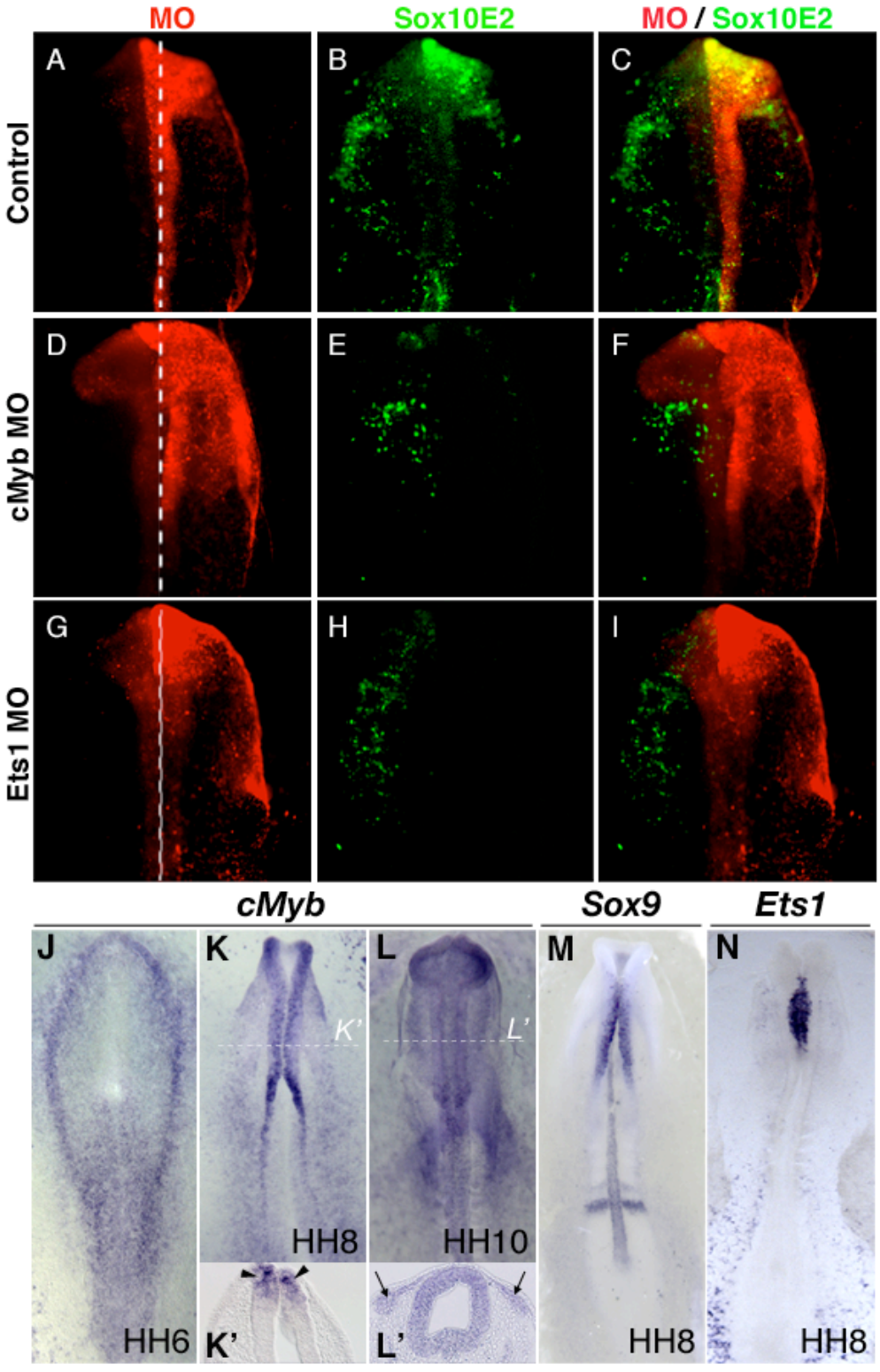
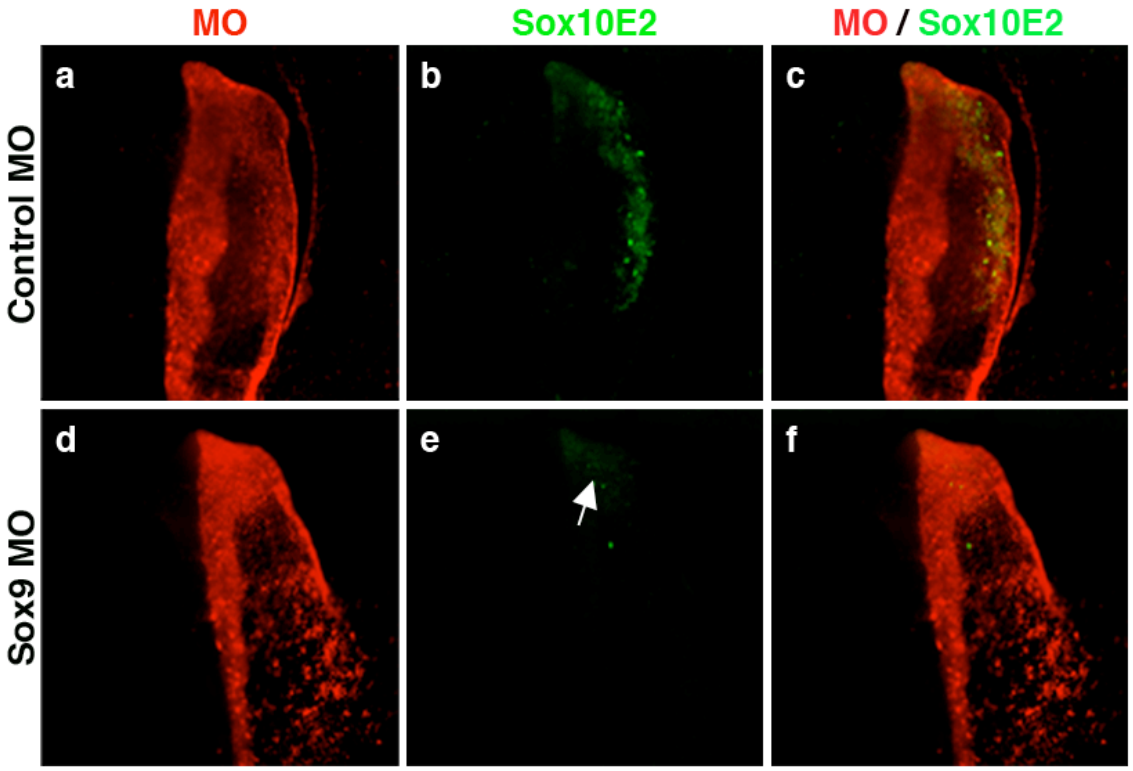

MO
Sox10E2
MO / Sox10E2
Control
cMyb MO
Ets1 MO
A
B
C
D
E
F
G
H
I
cMyb
Sox9
Ets1
J
K
L
M
N
K'
L'
HH6
HH8
HH10
HH8
HH8
K'
L'

Figure 4. Ets1 and cMyb transcription factors are necessary for activation of Sox10E2 regulatory element. (A) Control morpholino(right; red) has not effect on Sox10E2-driven Cherry (B; green) compared to non-electroporated (left) side. cMyb MO (D) significantly reduces, whereas Ets1 MO (G) abolishes Sox10E2-driven Cherry expression, (E,H, respectively; C, F, I are merged images of A/B, D/E and G/H, respectively). White dotted lines = midline. Green/red channels inverted for consistency. (J-L) Endogenous *cMyb, Sox9* and *Ets1* expression precedes that of *Sox10*, consistent with being upstream regulators. At HH6, *cMyb* is expressed within the neural plate border (J) and confined to dorsal neural folds containing CNC by HH8 (K;K'; arrowheads). At HH10, *cMyb* is observed in migrating neural crest (L and section at dotted line, L' arrows). At HH8, prior to Sox10 onset, Sox9 (M) and Ets1 (N) are expressed by presumptive cranial neural crest in the dorsal neural tube.



Supplementary Figure 4. Morpholino-mediated Sox9 knock-down significantly reduces Sox10E2 regulatory activity. (a) FITC-labeled control morpholino (in red) does not affect Sox10E2 driven Cherry expression (in green) (b). (c) Merged image of a and b shows overlap of control morpholino (in red) with Sox10E2 driving expression of Cherry (in green). (d) Sox9 FITC-labeled morpholino (in red) strongly reduces Sox10E2 driven Cherry expression (in green, white arrow) (e). (f) Merged image of d and e shows the effect of Sox9 morpholino (in red) on reporter expression of Cherry driven by Sox10E2 regulatory region (green). Embryos were electroporated on the right side only. The images were pseudo-colored using Photoshop, with green and red channels inverted for consistency, indicative of reporter expression.

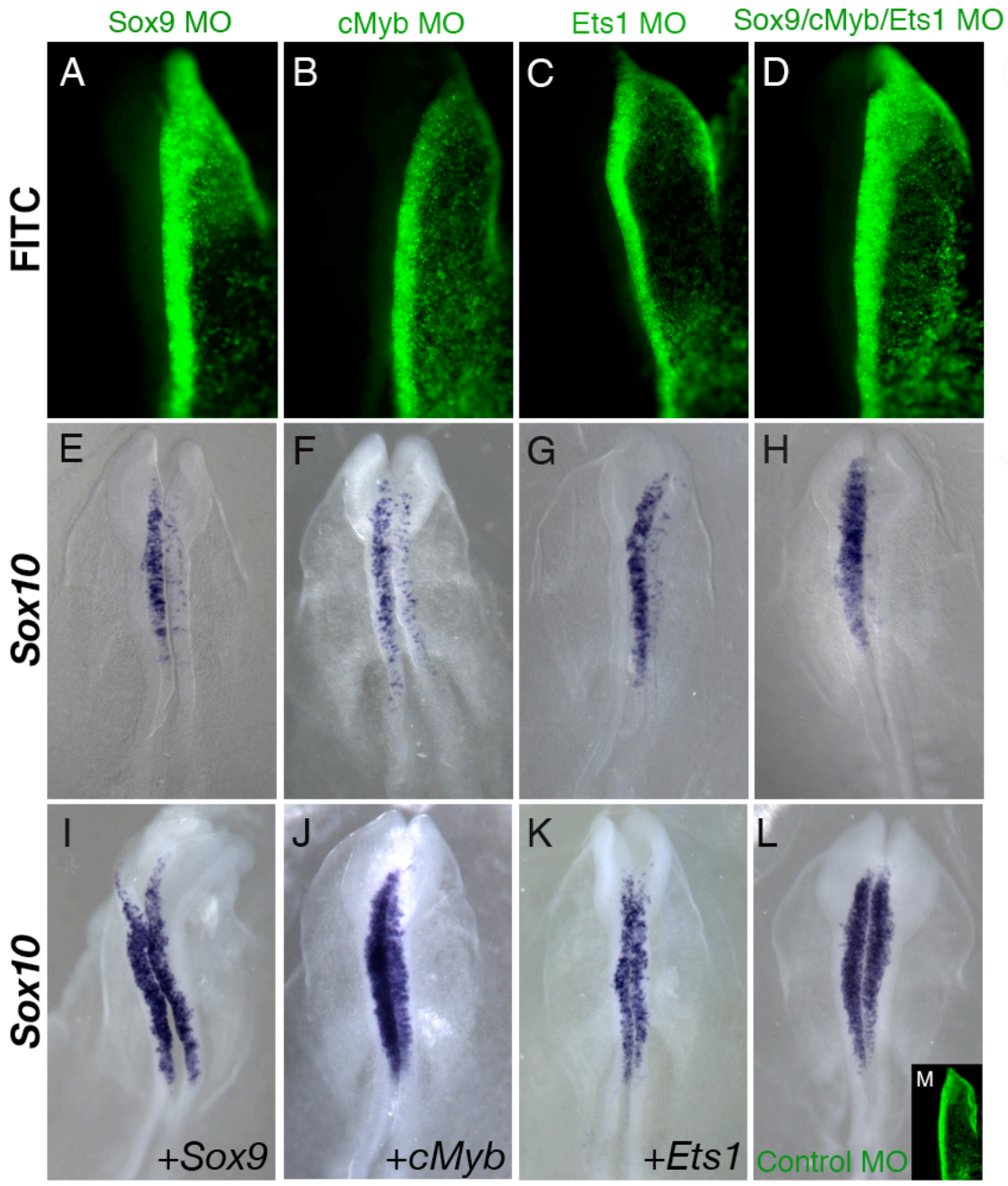
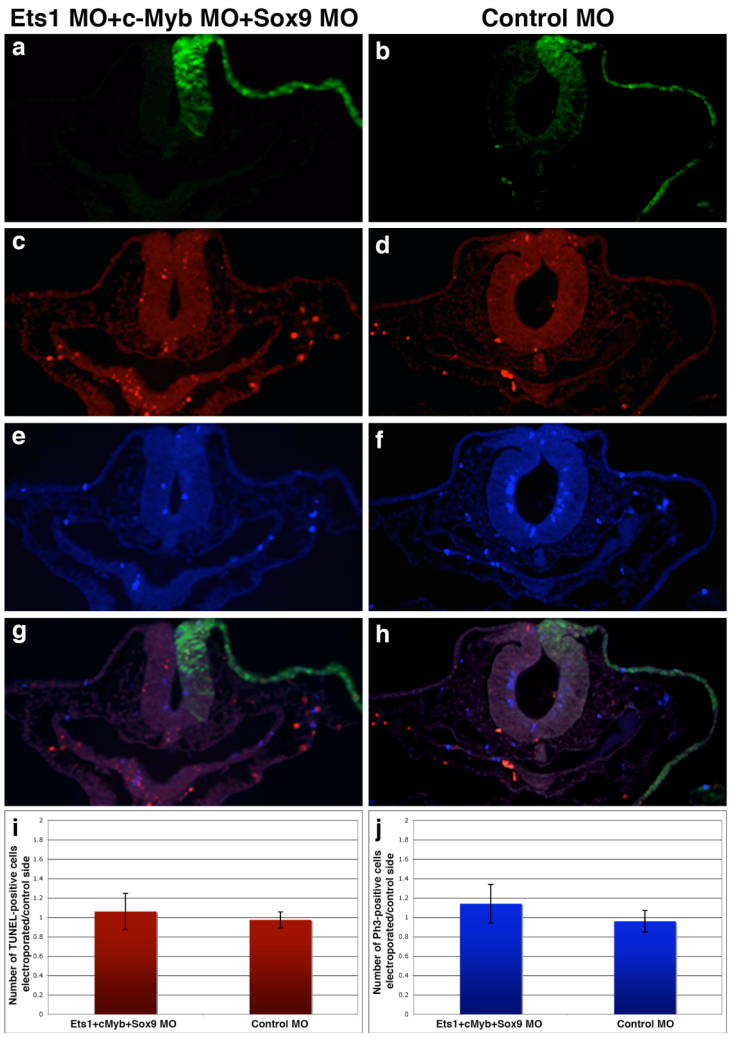


Figure 5. Sox9, cMyb and Ets1 are required for endogenous Sox10 expression in delaminating neural crest cells. HH8+ embryos with unilateral electroporation of Sox9

(A, E), cMyb (B, F) and Ets1 (C, G) morpholinos (MO; green) show significant decrease in endogenous *Sox10* expression in delaminating CNC compared to non-electroporated side, whereas control MO (L, M) has no effect. Co-electroporation of Sox9,cMyb,Ets1 MOs completely abolishes endogenous *Sox10* expression (D, H). Showing specificity, the effect is rescued by co-electroporation with corresponding expression construct: Sox9 MO+*Sox9* DNA (I), cMyb MO+*cMyb* DNA (J) or Ets1 MO+*Ets1* DNA (K). Statistical relevance by chi-squared test of MOs on *Sox10* expression was $p<0.02$; of rescues was $p<0.03$(Sox9;Ets1) and $p\leq0.04$(cMyb).



Supplementary Figure 5. Electroporation of Ets1, cMyb, Sox9 and control morpholinos does not affect cell proliferation and does not induce apoptotic cell death. Embryos electroporated with FITC-labeled triple (Ets1, cMyb and Sox9) or control morpholinos (a, b) were sectioned and with TUNEL staining (c, d) or anti-Phospho-Histone H3 (Ph3) antibody staining (e, f) was performed on sections. (g, h) Overlay of FITC, TUNEL and Ph3 staining presented in (a, c, e) and (b, d, f), respectively. All consecutive sections from the cranial region were counted and number of Ph3- (and Tunel-) positive cells within the neural fold was compared between morpholino-ed and control sides. Mean

value and standard deviation of electroporated/control side ratio from 4 independent embryos are presented (i, TUNEL, green bars; j, Ph3, blue bars). The statistical calculations performed using unpaired student's t-test show no statistically significant differences in cell death or proliferation counts between electroporated and control sides of embryos receiving either three specific morpholinos or control(@3mM).

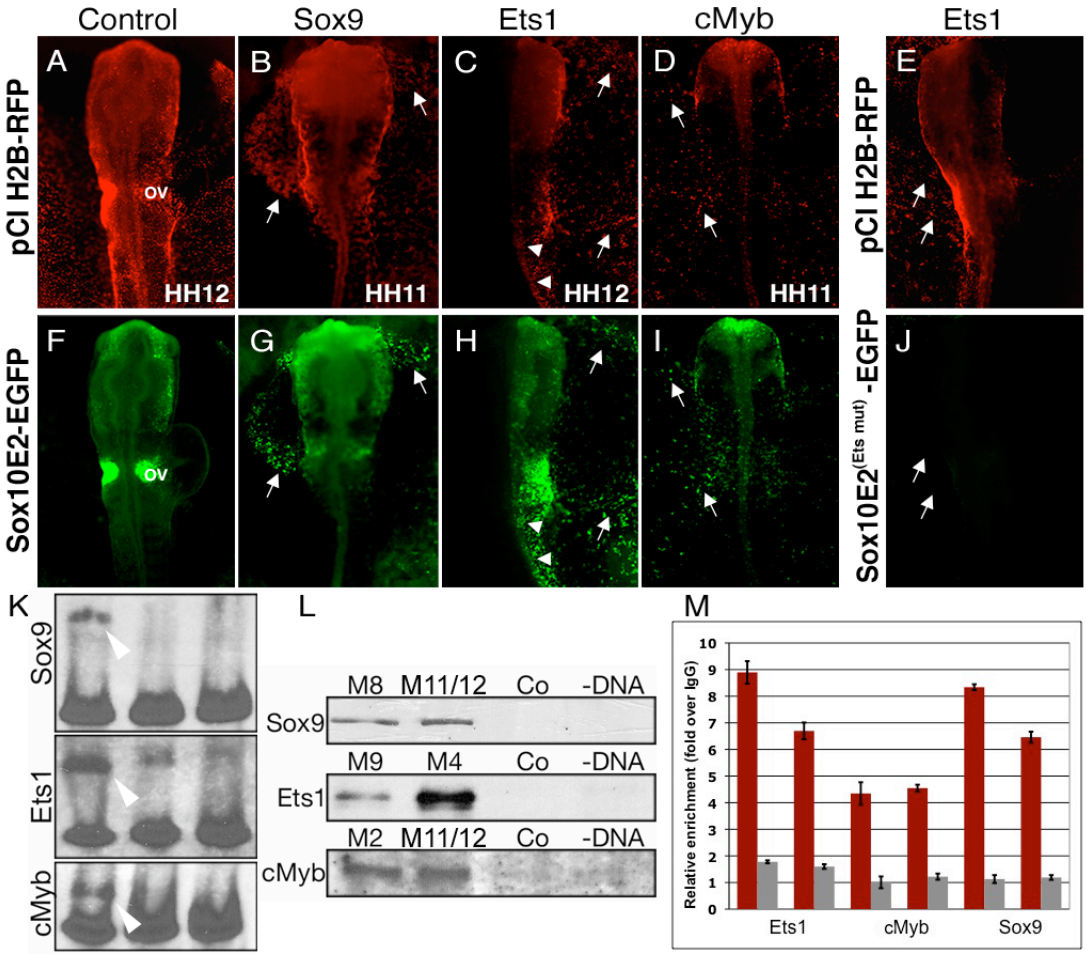
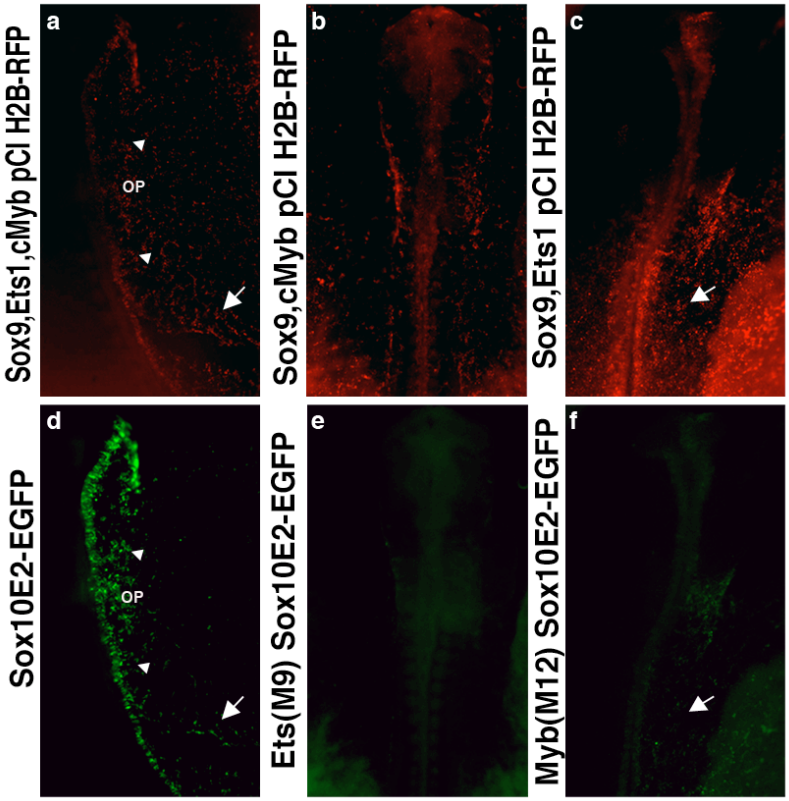


Figure 6. Sox9, cMyb and Ets1 overexpression ectopically induces Sox10E2 regulatory activity. EGFP is observed in migrating crest and otic vesicle (OV) when Sox10E2-EGFP is co-electroporated with control plasmid, pCI H2B-RFP. (A, F) overexpression of either Sox9 (B, G), Ets1 (C, H) or cMyb (D, I) ectopically activates Sox10E2-driven EGFP expression in extraembryonic ectoderm (white arrows). In (H) arrowheads show EGFP expression in posterior neural tube. Misexpression of Ets1 (E) fails to activate ectopic EGFP expression (J, arrows) in mutated Sox10E2 construct lacking an Ets binding motif (M9). (K) EMSA shows a clear band shift (white arrowhead) when nuclear extracts containing overexpressed Sox9, Ets1 or cMyb proteins are combined with

Sox10E2 subfragments, M11, M9 and M2, respectively (1st lane). This binding is outcompeted when excess non-labeled probe is added (2nd lane) and absent from nuclear extracts from control plasmid-transfected cells (3rd lane). (L) Biotinylated Sox10E2 subfragments (M8, M11-Sox9, M4, M9-Ets1 and M2, M12-cMyb), as well as scrambled control fragments and non-coated Dynal streptavidin beads, used as bait in a DNA pulldown assay show specific transcription factor binding as analyzed on a Western blot. (M) Direct binding of Ets1, cMyb and Sox9 to the Sox10E2 enhancer element *in vivo* as assessed by qChIP. Binding to Sox10E2 (red bars) or control region (grey bars) was assessed with two primer sets for each region and expressed as relative enrichment of target over control antibody; graph reflects mean $\pm$SD from a representative experiment. qChIP was performed 3-4 times for each factor.



Supplementary Figure 6. Binding motifs for SoxE, Ets and Myb within Sox10E2 enhancer need to be functional in order for ectopic reporter expression to occur when misexpressing Sox9, cMyb and/or Ets1, individually or in combination.

(a, d) Ectopic EGFP expression is observed in the extraembryonic region, ectoderm cells (arrowheads) and along the neural tube (d), when Sox9-pCI H2B-RFP, Ets1-pCI H2B-RFP and cMyb-pCI H2B-RFP (red) are simultaneously over-expressed (a). Combined Sox9 and cMyb misexpression (b) fail to activate ectopic EGFP expression through a mutated Sox10E2 regulatory region (M9) that lacks an Ets binding motif (e), whereas, overexpressing Sox9 and Ets1 simultaneously can activate weak reporter expression (arrows) through a mutated Sox10E2 lacking one Myb (M12) binding motif.

Chapter 3

cMyb, Sox8 and Pea3 Regulate the Onset of *Sox10* Expression by Converging on Enhancer Sox10E2 in the Otic Placode

Abstract

The otic placode, a specialized region of embryonic ectoderm, gives rise to the components of the inner ear, including semicircular canals, cochlea, an endolymphatic duct, as well as supporting and hair cells that form the sensory epithelium. In the gastrula stage embryo, placode and neural crest precursors are found at the neural plate border between the future epidermis and neural plate. Later, presumptive neural crest cells occupy the dorsal margins of the closing neural tube whereas placode cells come to reside outside of the neural plate in the patches of thickened ectoderm that comprise olfactory, trigeminal, otic and epibranchial placodes. The enhancer Sox10E2 has regulatory activity in both cranial neural crest and otic placode cells. Here, I demonstrate that three transcription factors mediate Sox10E2 expression in the otic placode region. cMyb, Sox8 and Pea3 are all necessary for the initial otic *Sox10* expression and mutating each of the corresponding binding motifs within the enhancer, greatly reduces enhancer activity in the ear. These data establish new regulatory connections and uncover a new function for cMyb in the development of the otic placode.

Introduction

Placodes are defined as thickened regions of cranial ectoderm that delaminate or invaginate from the surface ectoderm and migrate into the adjacent mesenchyme (Schlosser 2008). Placodal cells contribute to the formation of the lens of the eye, sensory structures in

the ear and olfactory epithelium as well as cranial ganglia. The best studied cranial placode is the otic placode, which thickens from the ectoderm adjacent to rhombomeres 5 and 6 (Hogan and Wright 1992). In chicken embryos, otic placode is first visible at stages HH9-10, then invaginates and separates from the surface ectoderm to form the otic vesicle, and later undergoes complex morphogenetic rearrangements to generate components of the inner ear (Dutton K. et al. 2009). Like neural crest cells, the otic placode expresses members of the SoxE family of transcription factors, Sox8, Sox9 and Sox10. As described in Chapter 1, the transcription factors Sox9 and Sox10 play important roles during neural crest cell specification, migration and differentiation. In particular, Sox10 plays a critical role in many aspects of neural crest development, from migration to differentiation. In contrast, not much is known about the function of *SoxE* genes in the otic placode. SoxE mutations such as those observed in Wardenburg syndrome type IV lead to defects in the ear. These defects, were previously attributed to a reduction in the number of melanocytes contributing to the ear (Bondurand et al. 2007) or cells that form glial cells of the spiral and vestibular ganglia (gVIII) (Evans and Noden 2006), both neural crest cell derivatives characterized by the expression of Sox10. However, recently it was noted that Sox8, Sox9 and Sox10 also are expressed in the otic epithelium, suggesting that SoxE genes have a more direct role in the development of the inner ear (Chiang et al. 2001, Dutton K. A. et al. 2001, O'Donnell et al. 2006).

In *Xenopus*, Sox9 has been reported to be involved in the invagination of the otic, based on the observation that Sox9 morphant placodes do not attach to the neural tube, fail to invaginate and undergo apoptosis (Barrionuevo et al. 2008). In zebrafish, *Sox9b* deletion mutants show a slightly small ear while double mutants for *Sox9a* and *Sox9b* lack or have

vestigial otic vesicles, due to a failure in the otic placode induction (Liu D. et al. 2003, Yan et al. 2005). The differences in Sox9 loss-of-function effects observed in zebrafish versus *Xenopus* may be due to differences in otic formation between the two different species. In zebrafish, rather than invagination, the formation of the otic cup involves cavitation. Similar to Sox9 mutants, zebrafish *sox10* mutants display subtle vesicle shape defects. At later stages, the size of the vesicle is smaller than normal, due partially to cell death (Dutton K. et al. 2009). A role for Sox9 and Sox10 in cell survival and maintenance of multipotency in the otic placode is not surprising given its similar previously demonstrated functions in neural crest cells (see Chapter 1). Just as in the neural crest, there appear to be cross-regulatory interrelationships among *SoxE* genes during ear development. For instance, de-repression of *Sox9* is observed in zebrafish *Sox10* mutants, while maintenance of *Sox10* expression throughout otic epithelia development depends upon SoxE function. Mutating *Sox10* and simultaneously knocking down sox9a and b is sufficient to greatly disrupt *Sox10* expression (Dutton K. et al. 2009). Nevertheless, these gene perturbations do not completely eliminate *Sox10* expression, suggesting that other factors, such as Sox8, also may be involved in the regulation of *Sox10*.

As opposed to maintenance of expression, less is known about what regulates onset of any of the *SoxE* genes. To tackle this question, I took advantage of the previously identified Sox10E2 enhancer and its regulatory activity in the otic, to probe for the upstream factors that regulate initial *Sox10* expression in this region. Interestingly, mutational analysis revealed that the same binding motifs within Sox10E2 that function in neural crest cells, are also required for the initial activity of the enhancer in the otic placode. However, a different set of activators mediate the enhancing activity of Sox10E2,

most likely through the interaction with these essential binding sites. These results show that cMyb together with two other factors the Ets family member, Pea3, and the SoxE family member, Sox8, are necessary for the initial endogenous expression of *Sox10*. Thus these data suggest a dual conserved role for the enhancer Sox10E2 in initial regulation of *Sox10* expression both in cranial neural crest cells and otic placode cells.

Results

Three core Sox10 enhancers: Sox10L8, Sox10E1 and Sox10E2 have regulatory activity in the forming otic placode and vesicle

The identification, isolation and cloning of the three Sox10 regulatory regions Sox10L8, Sox10E1 and Sox10E2, have been previously described (see Chapter 2). Each genomic fragment was tested for regulatory activity by electroporating the whole epiblast of chicken embryos at stage HH4. Embryos electroporated with the plasmid containing the enhancer Sox10E2 (2.1kb downstream Sox10) first exhibited EGFP reporter expression at stage HH9+ coincident with the onset of *Sox10* endogenous expression in the forming otic placode (Chapter 2, fig. 1C-E, H-J). On the other hand, weak EGFP reporter activity was observed later, at the otic vesicle stage, in the case of the two other Sox10 regulatory regions Sox10L8 (6.1Kb upstream) and Sox10E1 (1.1kb downstream) (Chapter 5, Table1). Thus Sox10E2 is the only identified enhancer that mediates reporter expression with the first appearance of endogenous *Sox10* expression in the otic placode.

Binding motifs for SoxE, Myb and Ets transcription factors are required for strong enhancing activity of Sox10E2 in the developing otic placode

Previously I demonstrated that a 264 core regulatory region, Sox10E2, was fundamental for the initial activation of *Sox10* expression in the cranial neural crest (Chapter 2, fig. 2A & B, Table1 on Fig2E). To test its function in the otic placode, careful dissection of the 264 bp fragment was performed by cutting around and through a highly conserve region (HCR) of ~160bp in length. Regulatory activity of each subfragment was tested by electroporating each plasmid version at stage HH4. Initial dissections showed that a 138bp fragment containing a portion of the 160bp HCR plus some sequence at the 3' end was sufficient to activate weak reporter expression. The 160bp HCR by itself was also able to drive weak EGFP expression while the 5' portion of Sox10E2 flanking a portion of the 160bp HCR failed to promote regulatory activity (Fig. 1). These results suggest that the minimal binding motifs required for basal activity of the enhancer in the otic placode were contained in the 3' portion of Sox10E2. Furthermore, Sox10E2 appears to contain many dispersed functional binding motifs, some of which are located in the 5' end, and are required for the strong enhancing activity. This experimental information was coupled with bioinformatics to recognize potential transcription factor binding motifs in order to locate possible inputs important for otic placode formation. Detailed mutational analysis showed that mutating two Myb, either SoxE, or one Ets sites significantly decreased EGFP reporter activity in the otic while the mutation of other sites such as Pax, NFKB, Elk/Ets or SoxD sites had no effect, when compared to embryos that received the construct carrying an intact Sox10E2 (Fig. 1 and Figs. 2A-H). Furthermore, a fragment containing an Ets site alone was not sufficient to regulate weak reporter expression (Fig. 1), suggesting that a

single Ets factor was not sufficient to account for initial Sox10 expression. Dissection and mutational results demonstrated that a minimal fragment containing a single SoxE and one Ets is sufficient to activate weak expression of EGFP (Fig. 1). The addition of a second SoxE and a single Myb functional site leads to recovery of strong expression of EGFP (Fig. 1). Interestingly, the simultaneous mutation of the five Myb, SoxE and Ets functional binding motifs (Fig. 1) in a larger genomic context that included enhancer SoxE1 eliminated reporter expression at early stages HH9+ through HH13 (Figs. 3A, B). There was only residual and scattered EGFP expression beginning to appear around stage HH13 (Fig. 3B) and weak reporter activity observed at stage HH15 (Fig. 3C), resembling the activity produced by enhancer Sox10E1 only after the otic vesicle has formed. This demonstrates that these five binding motifs are essential for the early regulation of *Sox10* in the otic placode and suggests that the role of Sox10E1 in the otic placode is that of maintaining *Sox10* expression.

cMyb, Pea3 or Sox8 but not Ets2 or Sox9 reduce EGFP reporter expression by acting on Sox10E2

Previously it has been shown that the transcription factors Pea3, a member of the Ets1 family, and Sox8 are present in the otic placode preceeding *Sox10* expression, whereas *Sox9* expression follows that of *Sox10* (Bagheri-Fam et al. 2006, Lunn et al. 2007, McKeown et al. 2005) (Geisha ID# pgm2n.pk009.c2 for Sox8). In addition, I confirmed by *in situ* hybridization that *cMyb* is present at stage HH9, just as *Sox10* transcripts begin to appear around the otic placode (Fig. 4A). Similarly, *Ets2* is present in the forming otic placode just prior to *Sox10* and continues to be expressed at stage HH9+ (Figs. 4B, C &

C'). These expression studies are consistent with the possibility that cMyb, Pea3, Ets2, Sox8 and/or Sox9, are candidates for mediating *Sox10* expression in the otic plaocode via Sox10E2. To test this possibility, I designed morpholino antisense oligonucleotides to specifically knock down each factor and examined the subsequent effects on the Sox10E2 regulatory activity. Since *Sox10* is expressed prior *Sox9* in otic placode, my prediction was that Sox9 would not affect Sox10 expression in early stages of otic placode development, whereas expression of the other transcription factors preceeds that of *Sox10*. After morpholino electroporations, embryos treated with cMyb, Pea3 or Sox8 morpholinos had a significant reduction of reporter signal driven by Sox10E2 enhancer (Figs. 5J-R) when compared to embryos treated with control (Figs. 5A-C). In contrast, little or no effect was noted with morpholino against either Sox9 or Ets2 (Figs. 5D-I). The results are consistent with the possibility that cMyb, Pea3 or Sox8 regulate *Sox10* expression through their corresponding functional SoxE, Ets and Myb binding motifs within the Sox10E2 regulatory region.

Endogenous Sox10 expression is reduced in the otic placode when either cMyb, Pea3 or Sox8 are knock down

Since reporter signal under the control of Sox10E2 enhancer was reduced in the presence of morpholinos against cMyb, Sox8 or Pea3, I next investigated if these perturbations have an effect on endogenous *Sox10* expression. Knocking down cMyb (Figs. 6C, D), Pea3 (Figs. 6E, F) or Sox8 (Figs. 6O, P) significantly reduced *Sox10* expression in embryos analyzed at stage HH11 and later at stage HH13 (Figs. 6J-M, S, T). In contrast, embryos electroporated with control morpholino (Figs. 6A, B) or Ets2 (Fig.

6G) showed no effect on *Sox10* expression (Figs. 6H, I & N). To check if the effect caused by morpholino electroporations was a specific reduction in *Sox10* transcripts, I used a preplacodal marker, Pax2 to immunostain embryos that were treated with Pea3 morpholino. There was no significant change in Pax2 expression on the morpholino electroporate compared with contralateral side (Figs. 6d-f) and embryos treated with control morpholino (Figs. 6a-c), confirming that the observed reduction was due to reduction in *Sox10* transcripts rather than secondary effects.

While individual knock down of each candidate factor reduced early *Sox10* expression, it was not abolished. However, simultaneous knock-down by electroporating morpholinos against all three (Figs. 6Q, R) completely eliminated *Sox10* expression at stage HH11 (Fig. 6U) and resulted in reduced expression at stage 13 (Fig. 6V). These results agree with the results obtained when mutating the binding sites for Myb Ets and SoxE in a larger genomic fragment containing Sox10E2 and Sox10E1 enhancers. The later recovery of endogenous *Sox10* expression may be due to the regulatory activity of other enhancers found in *Sox10* genomic locus including, perhaps, Sox10E1.

Discussion

A 264bp downstream Sox10 enhancer is sufficient to regulate gene expression in the forming otic placode

In the early chick embryo, I have identified three Sox10 genomic fragments, Sox10L8, Sox10E1 and Sox10E2, which had regulatory function in the developing otic

region. This study shows that the onset of regulatory activity of one of the identified fragments, Sox10E2, correlates with the onset of endogenous *Sox10* expression in the forming otic placode, while the activity of the other two regulatory regions appeared approximately 10 hrs later. This suggests that the Sox10E2 enhancer is responsible for initiation of *Sox10* expression in the otic region, whereas Sox10L8 and Sox10E1 and probably other unidentified regulatory regions are recruited to maintain *Sox10* expression in later ear development. Because reporter signal was much brighter for Sox10E2 than the other two regulatory fragments, These results suggest that Sox10E2 may play a primary role in the regulation of Sox10 in the ear and the other fragments may have supporting roles. For example, Sox10L8 and Sox10E1 might function synergistically as supporting elements, since they are both active and exhibit weak expression at later stages (~stg 13), when the otic vesicle is beginning to take shape.

I previously demonstrated that 264bp enhancer Sox10E2 is necessary for initial Sox10 expression in the nascent cranial neural crest (Betancur et al. 2010), albeit with different, paralogous inputs. That the same Sox10 regulatory region functions in two different cell populations--the cranial crest cells and placode cells—points to either common evolutionary ancestry or functional cooption between the two populations.

Characterization of Sox10E2 reveals critical binding motifs including two Myb, two SoxE and one Ets site required for strong activitation in the otic placode

Systematic deletion of portions within the 264bp enhancer Sox10E2 allowed identification of regions necessary for the strong enhancing activity. An HCR within the Sox10E2 sequence together with a ~33bp to the 3’ end of the HCR were sufficient to

activate strong reporter expression in the otic. In contrast, activity was weakened if the 33bp tail to the 3'end of the HCR was deleted regardless the presence of 38bp tail upstream of the HCR. Thus, it could be conclude that essential motifs responsible for the strong activity of the enhancer, are distributed along the highly conserved region and the non-conserved 3' end tail. Mutational analysis revealed that SoxE, Ets and either one of two Myb binding motifs, which I previously found to be necessary for the expression of Sox10E2 reporter in neural crest cells, are also employed for the strong activation of Sox10E2 in the otic placode. However, analysis of reporter expression in these cells revealed that each individual mutated motif, including a double myb mutation, decreased reporter signal intensity rather than completely abolishing the regulatory activity of Sox10E2 as was the case for neural crest cells. Therefore, I concluded that in the placodal region, each binding motif contributes to the enhancing activity of the regulatory region, albeit none is individually necessary. Rather, it is the cluster formed by the two SoxE, the Ets and either one Myb site that is sufficient for the strong activity of the enhancer during early *Sox10* expression in the otic placode. I also observed that the simultaneous mutation of all sites within the Sox10E2 region contained in a 3.5kb genomic fragment that also includes enhancer Sox10E1, delayed reporter expression until stage HH13, demonstrating that this cluster of binding motifs is necessary for initiation of Sox10E2 enhancer regulatory activity in the placode cells, whereas Sox10E1, regulates gene expression at later stages.

As opposed to neural crest cells, Sox10E2 in placodal cells seems to have basal regulatory activity that is not affected by individual mutation of Ets or SoxE sites or by the double mutation of Myb sites. In only one case, a fragment containing one Myb and one

SoxE but lacking the Ets and 3' end SoxE sites had no regulatory activity. Basal activity is restored when the fragment contains the functional Ets coupled with either SoxE functional sites. However, a small fragment containing only the Ets site fails to activate reporter activity (Fig. 1, orange fragment), confirming that the presence of other motifs, is required for basal activity of the regulatory region. Taken together, the results suggest that as long as one SoxE and the Ets site are present, Sox10E2 can produce basal regulatory activity that is enhanced when either Myb site is present.

cMyb, Pea3 or Sox8 control Sox10 expression through its identified regulatory region Sox10E2

In this study, I show that *cMyb* expression precedes that of *Sox10* in the otic placode, making it a good candidate to mediate the onset of *Sox10* expression. Morpholino perturbation of cMyb revealed that it is necessary for strong activity of Sox10E2. In a previous study, I found that cMyb binds directly to the identified Myb binding motifs within Sox10E2 (Betancur et al. 2010) in cranial neural crest cells. Thus, it is likely that cMyb can directly bind to the same sites of enhancer Sox10E2 in the otic placode cells. Similarly, in the same study, it was demonstrated that Sox9 or Ets1 factors in cranial neural crest cells bind and activate *Sox10* early expression through SoxE and Ets binding sites, respectively, within Sox10E2. Interestingly, I find that paralogous factors, Sox8, Pea3 and Ets2, are expressed in the otic placode, making them potential candidates for directly activating Sox10E2 in the placode. Moreover, they are expressed prior to *Sox10* in the otic placode cells. Both Ets2 and Pea3 are able to bind to and activate similar consensus sites (Fisher et al. 1991, Kopp et al. 2004). However, the morpholino studies performed here

demonstrate that Sox8 and Pea3 but not Ets2 knock-down reduce reporter expression driven by Sox10E2 enhancer in the otic placode. This suggests that Sox8 and Pea3 can regulate *Sox10* expression through Sox10E2 regulatory region. In addition, loss-of-function of either cMyb, Sox8 or Pea3 reduces early expression of endogenous *Sox10* and simultaneous knock-down of all three factors causes a complete loss of endogenous *Sox10*. This suggests that these three factors converge on the Sox10E2 regulatory region and all are necessary to initiate *Sox10* expression in the placode cells.

In summary, I have established that the Sox10E2 regulatory region is a dynamic enhancer, in which similar binding motifs are employed for regulation of Sox10 in two different populations of cells—the cranial neural crest and otic placode. Although SoxE, Ets or both Myb binding motifs enhance the activity of Sox10E2 in placodal cells, each motif is not completely necessary for some basal regulatory activity to occur. Importantly, I show that the combined action of transcription factors cMyb, Sox8 and Pea3 is required to initiate *Sox10* gene expression, thereby uncovering a novel regulatory function for these factors in otic placode development. Intriguingly, different family members of the SoxE and Ets family mediate initiation of *Sox10* expression in the neural crest by binding to the same motifs within Sox10E2. Thus, the function of a regulatory region has been conserved in two different cell populations whereas the upstream gene activators have changed. This may be due to a combination of gene duplication/divergence and changes in the *cis*-regulatory machinery that control the timing and spatial expression of these upstream factors, creating a different activating code for the *Sox10* enhancer unique to each cell population.

Materials and methods

Ex ovo/in ovo electroporations

Chicken embryos were electroporated at HH4-8 to target the cranial neural crest and at stages HH10-12 to target vagal and/or trunk neural crest following previously described procedures (Sauka-Spengler and Barembaum 2008). In morpholino-mediated knockdown experiments, only *ex-ovo* electroporations were performed. Morpholinos used in this study were obtained from Gene Tools (Philomath, OR).

Comparative genomic analyses and cloning of putative Sox10 regulatory regions

Highly conserved genomic regions were identified using ECR browser (http://rvista.dcode.org/). Binding motifs were predicted using **Jaspar** database (http://jaspar.genereg.net/cgi-bin/jaspar_db.pl) and P-Match program from **Transfac** database (http://www.gene-regulation.com/pub/programs.html). Putative regulatory regions were amplified with Expand High Fidelity Plus (Roche, Indianapolis, IN), from chicken BAC DNA (BACPAC, Oakland, CA) and cloned into the ptk-EGFP vector (Uchikawa et al. 2003). ptk-Cherry and pCI H2B-RFP constructs were generated for this study. For details regarding dissection and mutations of regulatory elements see Chapter 2, Materials and Methods.

Morpholinos

Morpholino-mediated knock-down experiments were performed by injecting the translation-blocking, FITC-labelled morpholino antisense oligonucleotides in one half of the epiblast (right to the primitive streak) embryo *ex-ovo*. Morpholinos used in this study were obtained from Gene Tools (Philomath, OR) and their sequences are as follows:

Control_5'-ATGGCCTCGGAGCTGGAGAGCCTCA-3';

cMyb_5'-ATGGCCGCGAGCTCCGCGTGCAGAT-3';

Pea3_5′-CTGCTGGTCCACGTACCCCTTCATC-3′;

Sox8_5'-CTCCTCGGTCATGTTGAGCATTTGG-3';

Ets2_5'-GTTTCTGATCGCAAATTCACTCATC-3';

Sox9_5'-GGGTCTAGGAGATTCATGCGAGAAA-3'.

In situ hybridization

Whole-mount *in situ* hybridization was performed using a procedure previously described (Wilkinson 1992). Fluorescent *in situ* procedure using GFP probe was adapted from (Acloque et al. 2008).

Microscopy and inmunohistochemistry

The electroporated embryos were collected at stages HH9-15, fixed in 4% paraformaldehyde overnight and then washed three times in PBS at room temperature. A Zeiss Axioskop2 Plus fluorescence microscope equipped with the AxioVision software

was employed to image the embryos. Images were processed using Adobe Photoshop CS2. After imaging, embryos were cryo-protected in two steps: 15%sucrose/PBS and 7.5%gelatin/15% sucrose/PBS, equilibrated and mounted in 20% gelatin/PBS and frozen in liquid nitrogen. 12mm cryosections were collected on Super Frost Plus slides (Fischer Scientific, Pittsburgh, PA) and de-gelatinized for 2 x 10 minutes at 42°C in PBS. Sections were subsequently washed, cover-slipped and imaged using the same imaging procedure described for the whole-mounts.

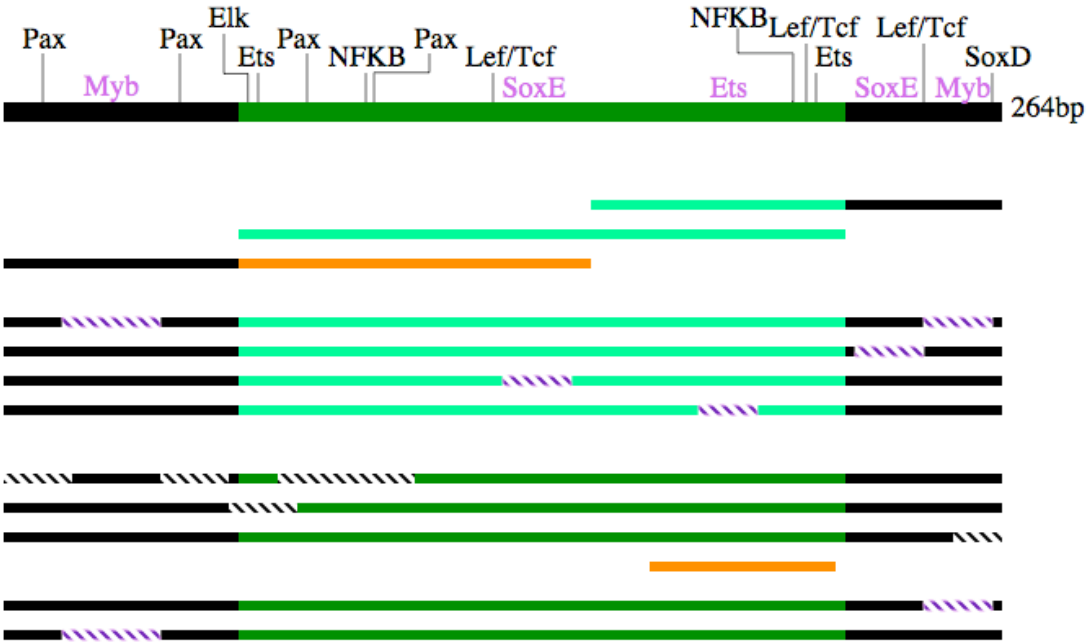


Figure 1. Analysis of reporter expression driven by different versions of enhancer Sox10E2 in the otic placode. Different versions of the regulatory region Sox10E2, were created by dissecting the 264bp fragment into smaller fragments, cutting through or around the 160bp highly conserved region (dark green line), or by mutating computationally located binding motifs (blocks of hatched lines). Fragment versions showing strong regulatory activity in the otic placode are depicted in dark green, light green represents weak activity whereas no activity is shown in orange. Identified functional binding motifs are shown in lavender.

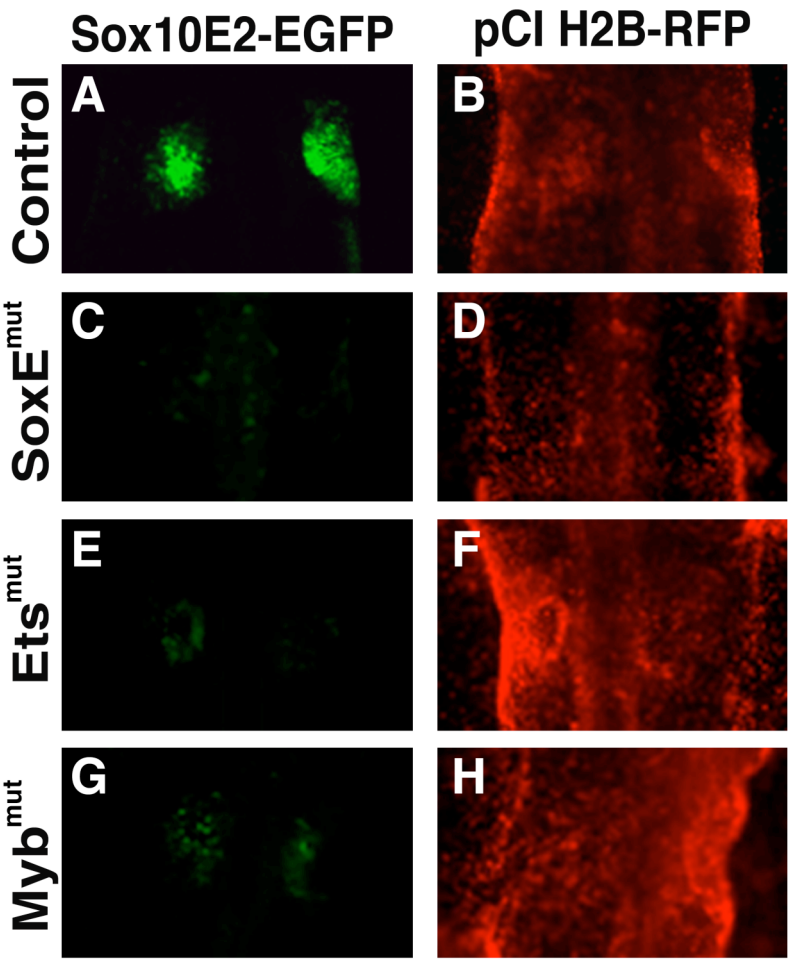


Figure 2. SoxE, Ets and Myb binding motifs are required for the enhancing activity of module Sox10E2 in the otic placode. EGFP reporter expression is greatly decreased in the otic placode of HH11-12 chicken embryos when mutating either SoxE (C), Ets (E) or both Myb (G) binding motifs within enhancer Sox10E2 (A). Panels B, D, F, H show expression of the co-electroporated tracer pCI H2B-RFP to locate cells that received both tracer and reporter EGFP plasmid DNA. Figure B corresponds to A, D to C, F to E and H to G, respectively.

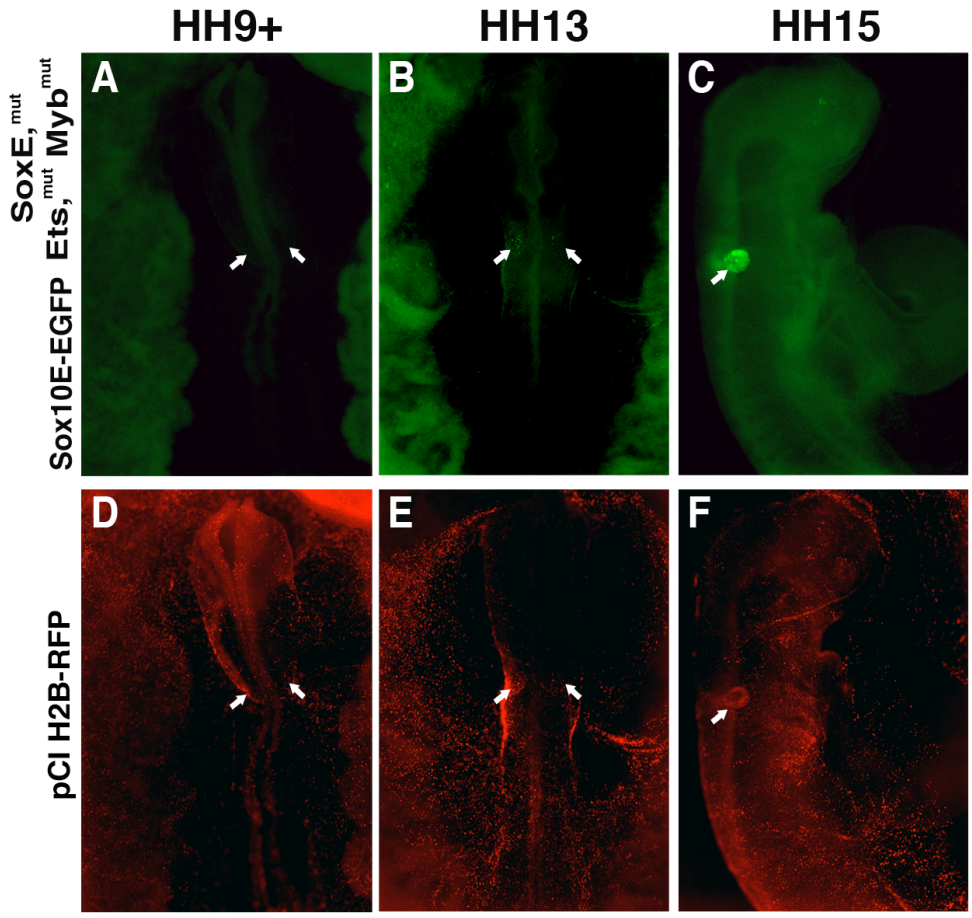


Figure 3. Simultaneous mutation of two SoxE, one Ets and two Myb identified sites within a much larger *Sox10* genomic region delays gene expression in the forming otic region. A 3.5 kb Sox10 genomic fragment containing the intact Sox10E1 and the Sox10E2 enhancer carrying mutations of the functional SoxE, Ets and Myb biding motifs, completely abolishes EGFP expression at HH9+ when endogenous *Sox10* expression is first observed in the forming otic placode (A, arrows). Weak and scattered EGFP activity reappears around HH13 (B, arrows), when the otic vesicle begins to shape and is back to normal levels around HH15 (C, arrows). Panels D-F correspond to figures A-C respectively and show expression of the co-electroporated tracer pCI H2B-RFP.

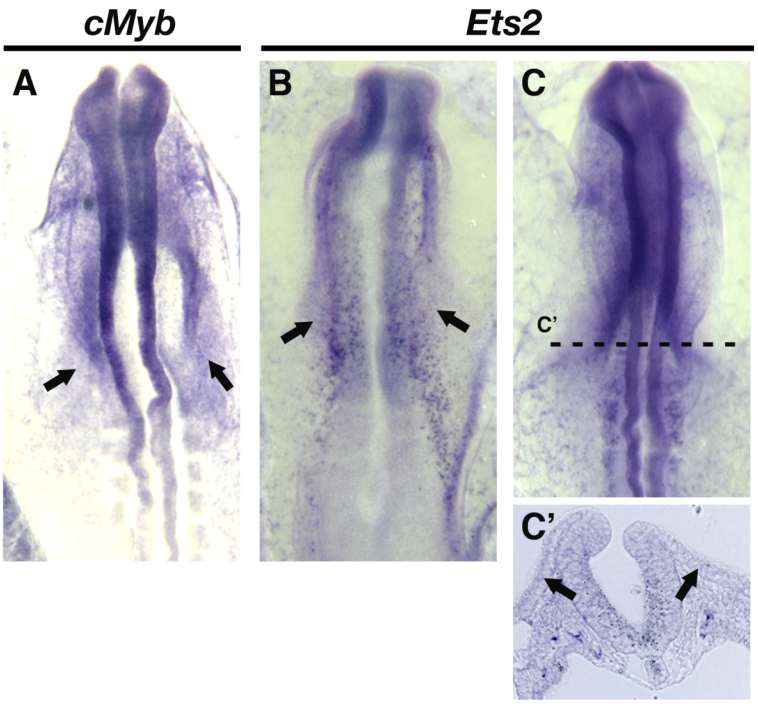
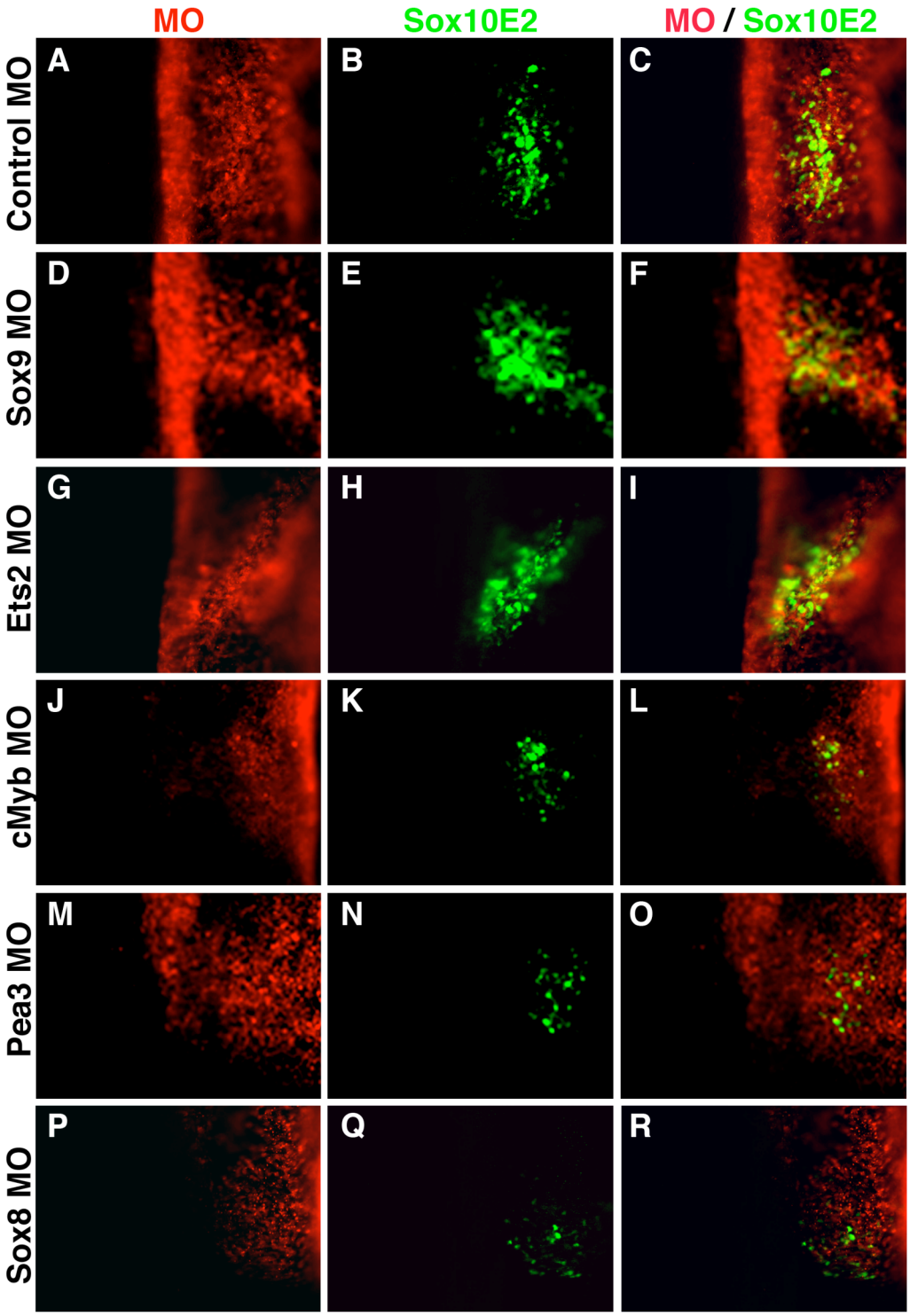


Figure 4. *cMyb* and *Ets2* are expressed in the presumptive otic area prior placode formation and *Sox10* expression. Endogenous *cMyb* is expressed at HH9 in the presumptive otic placode region (A, arrows). By HH7 *Ets2* is observed outside the neural plate (B, arrows) and otic placode region at HH9+ (C, C').


MO
Sox10E2
MO / Sox10E2
Control MO
Sox9 MO
Ets2 MO
cMyb MO
Pea3 MO
Sox8 MO
A
B
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I
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K
L
M
N
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Q
R

Figure 5. cMyb, Pea3 or Sox8 morpholino mediated knock-down dramatically reduces Sox10E2 regulatory activity in the otic placode. (A) FITC-labeled morpholino control (in red) does not affect Sox10E2 driven Cherry reporter expression (in green) in the otic placode of HH11-12 chicken embryos (B). Similarly Sox9 (D) or Ets2 (G) morpholino does not affect Sox10E2 regulatory activity (E, H) respectively. In contrast, morpholinos against either cMyb (J), Pea3 (M) or Sox8 (P), greatly reduce Cherry expression driven by the Sox10E2 enhancer (K, N, Q) respectively. Figures C, F, I, L, O and R are corresponding merged images of A/B, D/E, G/H, J/K, M/N and P/Q respectively. Embryos were electroporated on the right side only. The images were pseudo-colored using Photoshop. Green and red channels were inverted for consistency, indicative of reporter expression.

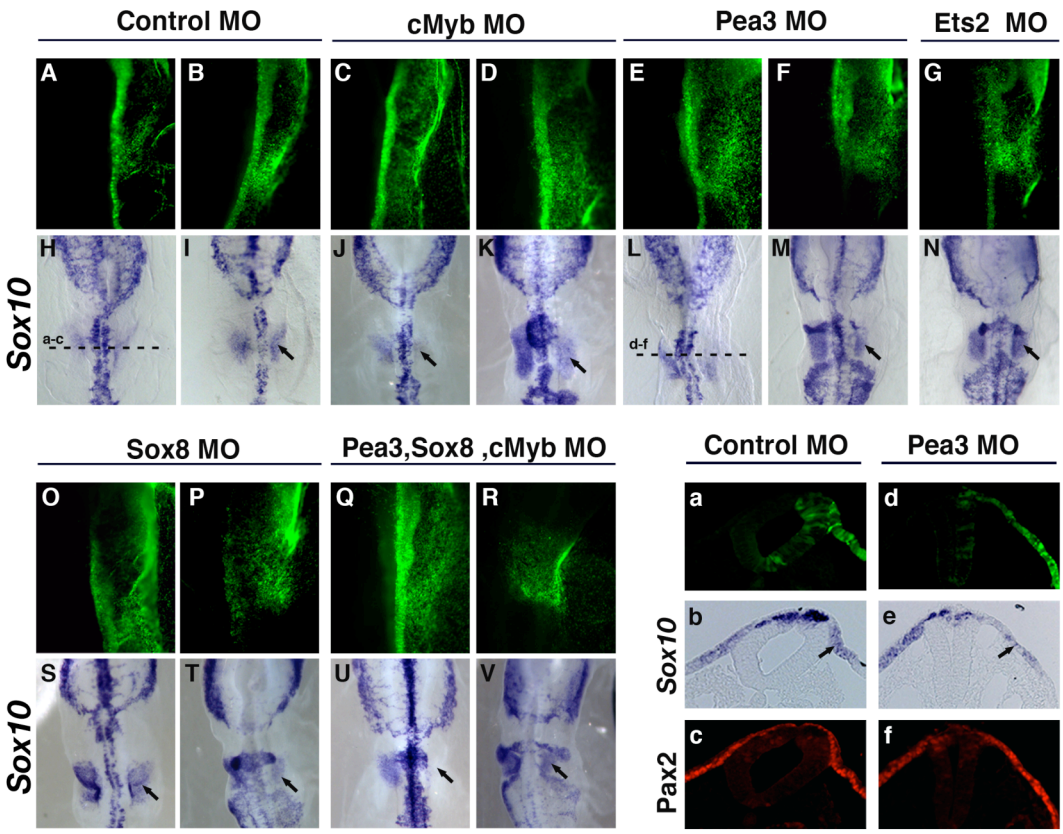

Figure 6. cMyb, Pea3 and Sox8 regulates the onset of *Sox10* expression in the otic placode. FITC-labeled morpholino control (A, B) does not affect *Sox10* expression in the otic placode at HH11 (H, b, arrow) and HH12 (I, arrow). Knocking down cMyb (C, D) strongly decreases *Sox10* expression at HH11 (J, arrow) more than at a later HH12 (K, arrow) stage. Pea3 morpholino (E, F) strongly decreases *Sox10* expression at both HH11 (L, e, arrow) and HH12 (M, arrow) stages, whereas Ets2 morpholino (G) has no effect on *Sox10* expression, even at stage HH12 (N, arrow). Similar to cMyb and Pea3 morpholino perturbations, knocking down Sox8 (O, P) reduces *Sox10* expression at HH12 (S, arrow) and HH13 (T, arrow) in the otic region. Depletion of *Sox10* expression in the otic placode (U, arrow) is observed when cMyb, Pea3 and Sox8 are knock-down simultaneously (Q).

However, after simultaneous knock-down of cMyb, Pea3 and Sox8 (R), *Sox10* expression recovers at HH13 (V, arrow). Pax2 antibody staining (c, f) of cross-section from embryos treated with control (a) or Pea3 (d) morpholino respectively, demonstrate that Pea3 morpholino does not affect the expression of Pax2 compared to Sox10 (b, e) and Pax2 immunostained contralateral untreated side. Morpholinos were electroporated unilaterally on the right side.

Chapter 4

CMyb Regulates the Expression of the Neural Crest Specifier *Ets1*

Abstract

Although the transcription factor *cMyb* has well known functions in hematopoiesis, little has been know about its distribution or function in the early embryo. Here, I show that *cMyb* transcripts are present in the early embryo during neurulation, as the neural folds are elevating, suggesting it may be involved in neural crest formation. In avian embryos, transcript expression initiates in the neural plate border and resolves to the neural folds by stage HH7-8. Expression is maintained in early migrating neural crest cells. Morpholino-mediated knock-down of *cMyb* has differential and specific effects on neural crest specifier genes. Most notably, loss of *cMyb* completely eliminates *Ets1* expression. Lesser effects were noted on *Snail2* and *Sox10* expression, with little or not effect on *FoxD3* or *Sox9*. These results show that *cMyb* is an early neural crest specifier gene that acts upstream of *Ets*1.

Introduction

In avian embryos, formation of the neural crest begins at the neural plate border during gastrulation stages (Basch et al., 2006), and appears to involve a sequential series of gene regulatory interactions. These begin by inductive signals like BMPs and Wnts that set up a domain at the neural plate border that expresses markers like *Msx* genes, *Pax7* and *Zic1*, that render the border distinct from neural plate and non-neural ectoderm.

Some time later, neural crest cells first become identifiable as a discrete population of premigratory cells within the dorsal neural tube, as they express a combination of neural crest specifier genes such as *Slug/Snail2*, *FoxD3*, and SoxE genes. However, little is known about events that occur between establishment of the neural plate border and the appearance of *bona fide* neural crest cells.

Neural crest cells share many transcription factors utilized in development of other stem cell populations. *cMyb* has important functions in a variety of developmental systems. Its role has been best studied during hematopoeisis, where it maintains T cells and other progenitors in a proliferating and immature state (Allen et al. 1999). Over-expression of cMyb in the trunk neural tube upregulates *Msx1* and *Snail2*, interpreted as evidence that cMyb may participate in BMP4 signaling input into the epithelial-mesenchymal transition of trunk neural crest (Karafiat et al. 2005). Interestingly, cMyb has been shown to have later effects on differentiation of neural crest derivatives. For example, it appears to influence melanocyte fate by regulating *c-kit* (Karafiat et al. 2007). Because many transcription factors are used reiteratively during development, we sought to examine the early expression and function of cMyb, using the early chick embryo as a model.

Results and Discussion

Isolation of full-length chick cMyb

As a first step in understanding the possible early functions of this gene in

embryonic patterning, the chick homologue of *cMyb* was isolated and its expression from gastrulation through early stages of nervous system development was examined in the chick. The full-length chick homologue of *cMyb* was obtained by a degenerate RT-PCR approach.

Expression pattern of cMyb in the early chick embryo during neural crest formation

The spatiotemporal expression pattern of chick *cMyb* was studied by whole mount *in situ* hybridization as the neural plate transforms and closes to form the central nervous system. The chick develops in an anterior to posterior progression, such that the neural folds first elevate and close in the presumptive head region and then closure moves progressively tailward. Using *in situ* hybridization, I find that *cMyb* is first expressed at HH6 in the neural plate border. As the cranial neural folds begin elevating at HH7 (Fig. 1A), highest *cMyb* expression is observed in the neural folds. As the cranial neural tube closes at HH8, *cMyb* expression remains in the presumptive neural crest region (Fig. 1B). Highest expression is observed at the dorsal margins of the closed neural tube, containing neural crest precursors, as seen in transverse section (Fig. 1E). At HH10, transcripts are seen in neural crest cells delaminating and emigrating from the cranial neural tube (Fig. 1C, F). At the caudal end of the embryo in the trunk region, the neural plate is still open; high expression is seen in the neural plate border and elevating neural folds, similar to expression in the trunk. Thus, *cMyb* is expressed early at the neural plate border and in presumptive as well as newly migrating neural crest cells.

Effects of cMyb knock-down on neural crest specifier gene expression

Since *cMyb* is expressed in the neural plate border and forming neural folds, I tested the effects of cMyb loss-of-function. To this end, I perturbed cMyb protein production by unilaterally electroporating FITC-tagged antisense morpholino oligonucleotide into one side of HH4 embryo. Embryos were allowed to develop until HH8 and the subsequent effect on expression of the neural crest markers *Sox9, FoxD3, Snail2/Slug* and *Ets1* was assayed.

The results show that knock-down of cMyb (Fig. 2D) completely abolished expression of *Ets1* (Fig. 2H; 5/5). Effects on *Snail2* expression were less severe (Figs. 2C, G; 3/3), with expression levels about half that seen on the contralateral side. In contrast, no changes were observed in expression of either *Sox9* (Figs2A,E; 3/3) or *FoxD3* (Figs. 2B, F; 3/3). Control morpholino injected embryos also had no changes between the electroporated and non-electroporated side.

This data shows that knock-down of cMyb causes selective down-regulation of other neural crest specifier genes. Most notably, there was complete elimination of *Ets1* expression within the neural crest territory. An intriguing possibility is that the main role of cMyb may be to first induce and then maintain *Ets1* expression levels, which in turn may act cooperatively to influence later neural crest specifier genes.

Material and Methods

In situ hybridization

Sox9 probes were prepared using full length cDNA constructs (a gift from Yi-

Chuan Cheng) as a template. Other digoxigenin-labeled antisense RNA probes, were prepared from chicken EST clones obtained from (ARK Genomics and MRC geneservice). All antisense RNA probes was synthesized using T3 RNA polymerase, according to standard protocols. Whole-mount *in situ* hybridizations were performed using a procedure previously described (Wilkinson 1992).

Embryo culture and electroporation

Fertilized chicken eggs were obtained from AA Laboratories, Westminster, CA and Chino Valley Ranchers, Arcadia, CA and incubated at 38^{o}C for approximately 24 hours to reach stage HH8-9. The embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton 1992).

Chicken embryos were electroporated at stages HH4 to target the cranial neural crest cell population. Morpholinos were introduced unilaterally to cover one side of the epiblast of the early chicken embryo (right to the primitive streak). The final molar concentration of each morpholino oligonucleotide used was 3 mM.

Morpholinos

To target the translation initiation site, FITC-labelled morpholino antisense oligonucleotides or controls (Gene Tools, Philomath, OR, USA) were designed following general manufacturer's instructions as follows:

cMyb_5'-ATGGCCGCGAGCTCCGCGTGCAGAT-3';

Control_5'-ATGGCCTCGGAGCTGGAGAGCCTCA-3'.

Microscopy and inmunohistochemistry

The electroporated embryos were collected at stages HH8-9, fixed in 4% paraformaldehyde overnight and then washed three times in PBS at room temperature. A Zeiss Axioskop2 Plus fluorescence microscope equipped with the AxioVision software was employed to image the embryos. Images were processed using Adobe Photoshop CS2. After imaging, embryos were cryo-protected in two steps: 15% sucrose/PBS and 7.5% gelatin/15% sucrose/PBS, equilibrated and mounted in 20% gelatin/PBS and frozen in liquid nitrogen. 12μm cryosections were collected on Super Frost Plus slides (Fischer Scientific, Pittsburgh, PA) and de-gelatinized for 2 x 10 minutes at 42°C in PBS. Sections were subsequently washed, cover slipped and imaged using the same imaging procedure described for the whole-mounts.

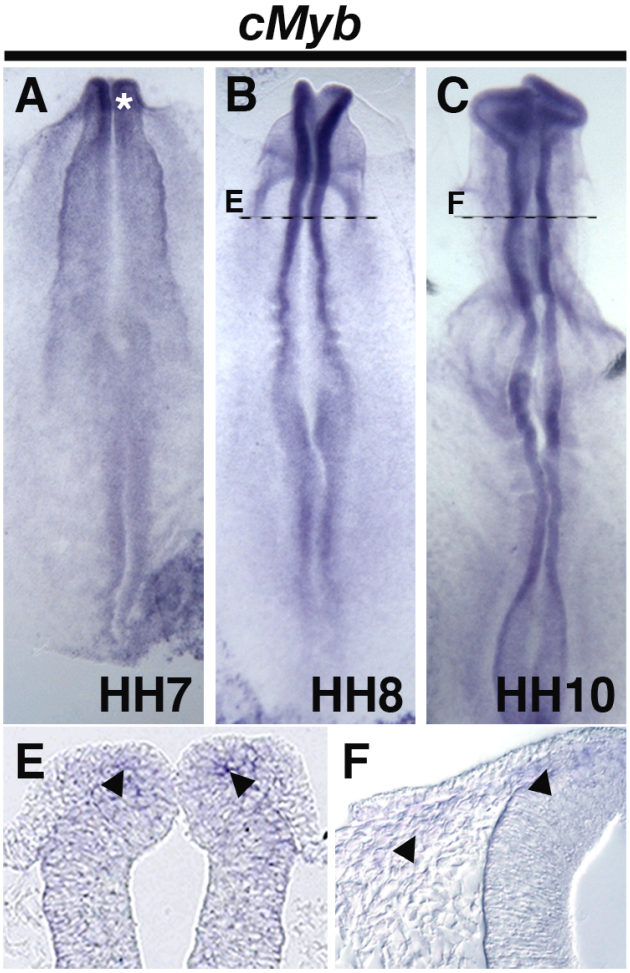

Figure 1. *cMyb* pattern of expression in the presumptive neural crest territory. At HH7, *cMyb* is expressed within the raising neural plate folds (A) and confined to dorsal neural folds containing CNC by HH8 (B, section at dotted line E, arrowheads). At HH10, *cMyb* is observed in migrating neural crest (C, section at dotted line F, arrowheads).

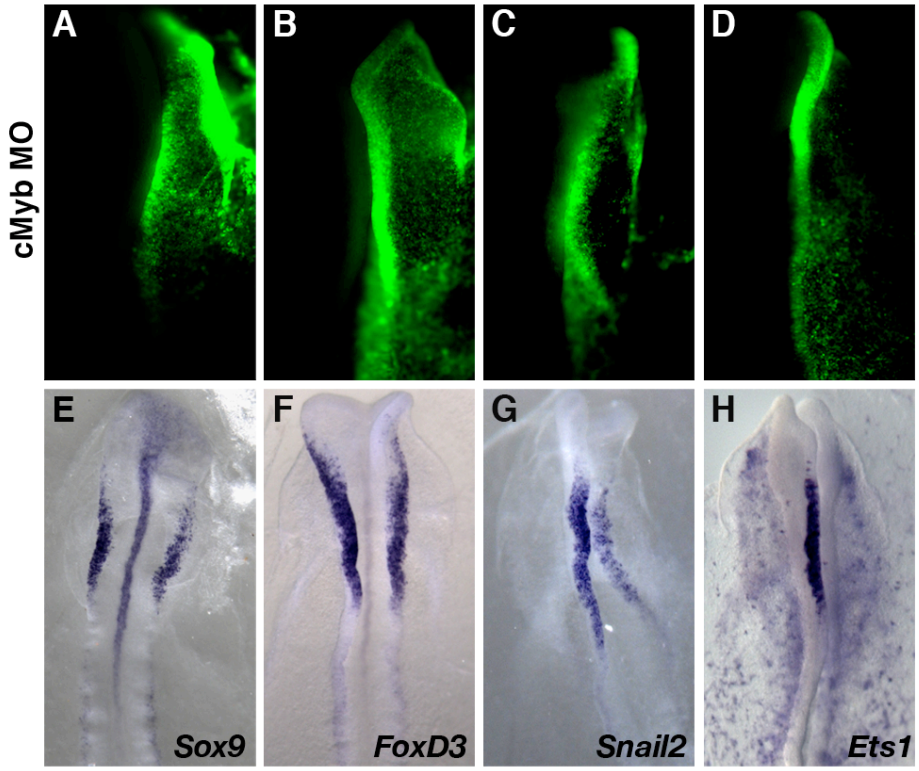

Figure 2. Effects of cMyb mediated knock-down morpholino on neural crest specifier genes at HH8 chicken embryonic stage. FITC- labeled morpholino cMyb (A, B) does not affect the expression of *Sox9* (E) or *FoxD3* (F). In contrast, knocking down cMyb (C, D) slightly reduces *Snail2/Slug* (G) and completely abolishes *Ets1* (H) expression. Morpholinos were electroporated unilaterally on the right side.

Chapter 5

CONCLUSIONS

My thesis project has involved the interrogation and expansion of the neural crest gene regulatory network by dissecting Sox10E2, the earliest acting *cis*-regulatory region of a well-known neural crest specifier gene, *Sox10*. The goal was to identify direct inputs that initially activate its expression. This analysis has provided a framework for identifying new regulators and direct transcriptional linkages mediating neural crest formation. Interestingly, the same regulatory region also mediates initial activation of *Sox10* in otic placode cells. Using this enhancer, I identified three *Sox10* key activators, cMyb, Sox8 and Pea3, that appear to mediate otic expression, thus establishing new regulatory connections to *Sox10* during ear development.

A downstream Sox10 cis-regulatory module activates gene expression specifically in early migrating cranial neural crest and otic placode cells

Comparative genomic analysis among seven species allowed the identification of seven conserved putative regulatory regions. Of those, Sox10L8 and Sox10E elements both regulated expression in neural crest and the otic region, though the former only in later, migrating crest cell populations and the forming otic vesicle. These elements controlling expression in late migrating neural crest, corresponding derivatives and the otic vesicle were similar to previously identified enhancers in mouse (Werner et al. 2007) (see Tables 1 and 2). Five other putative regulatory regions showed no regulatory activity at the stages tested in vivo, but may function at later times, different cell types or may contain isolated negative regulatory motifs that repress *Sox10* expression.

Dissection of Sox10E revealed a 264bp downstream regulatory region, Sox10E2, sufficient to regulate expression of *Sox10* at early phases of neural crest cell emigration and during early otic placode formation, thus mimicking the onset of *Sox10* expression. Further reduction of this fragment eliminated or largely diminished reporter expression in neural crest as well as otic placode cells, suggesting that Sox10E2 contains the minimal elements required for *Sox10* regulation in both populations. This minimal enhancer consists of an essential core element controlling specific spatiotemporal reporter expression and additional auxiliary regions that enhance activity. Thus, Sox10E2 represents the first early neural crest and otic placode *cis*-regulatory module responsible for the onset of *Sox10* expression in vertebrates. Recently, a conserved intronic regulatory region, that has no aparent homology to Sox10E2, has been shown to drive *Sox10* expression in neural crest and crest derivatives in zebrafish, but not in delaminating cranial crest (Dutton J. R. et al. 2008).

SoxE, Ets, and Myb binding motifs are necessary for Sox10E2 regulatory function

Putative binding motifs inside the Sox10E2 regulatory region were identified based on their position weight matrix (Hollenhorst et al. 2007) to determine the probability of binding score. *In silico* analysis was combined with *in vivo* perturbations by mutating predicted binding motifs, as well as other conserved sequences, to test their functional relevance. Through these combined approaches, I identified two functional SoxE sites and one Ets site in the essential core portion, individual mutation of which completely

eliminated reporter expression in neural crest cells while strongly reducing enhancer activity in otic placode cells. Simultaneous perturbation of two Myb binding sites also eliminated Sox10E2 reporter expression in neural crest and dramatically reduced reporter expression in the otic placode. Interestingly, while the function of each SoxE, Ets and both Myb binding motifs is required for the strong activity of the enhancer Sox10E2 in neural crest, the combination of all five binding motifs is necessary for the initial gene expression in placode cells. Thus, regulation of Sox10E2 is somewhat different for mediating *Sox10* expression in each cell population.

Intriguingly, it appears that the role of the Myb motifs and corresponding binding factors may vary from species to species. Whereas in *Xenopus* cMyb is expressed by neural crest cell progenitors (Amaravadi and King 1994), similar expression has not been reported in the mouse (Sitzmann et al. 1995) and *cMyb* mutant mice have no apparent neural crest defects (Mucenski et al. 1991). Far less is known about cMyb expression in the otic placode region of other species. Comparative analysis between chicken and mammals show that while SoxE and Ets binding motifs within Sox10E2 share 100% conservation, one of the Myb motifs does not align and the other is only 75% similar to corresponding sequences in the mouse. Thus, a new functional Myb site in chick Sox10E2 or evolutionary loss of one Myb motif from the partially homologous mouse regulatory region may account for observed species differences (Werner et al. 2007). Alternatively, the lack of apparent neural crest defects in the *cMyb* mutant mice may be due to other compensating regulatory factors.

cMyb, Sox9 and Ets1 are upstream of Sox10 during neural crest specification

My results show that *cMyb* is present at the neural plate border at stage HH6 and persists in premigratory crest in the dorsal neural folds, therefore preceding *Sox10*. The finding that mutation of two Myb sites in the Sox10E2 element abolishes reporter expression together with the cMyb loss-of-function experiments show that it is required for *Sox10* expression. Thus, illuminating a new role for cMyb in the gene regulatory network controlling neural crest cell specification. Recently, cMyb was shown to specify melanocyte fate by regulating *c-kit* (Karafiat et al. 2007), suggesting it acts within the neural crest specifier module. Its presence at the neural plate border suggests early involvement in the NC-GRN, concomitant with early neural crest specifiers such as *AP-2*, *c-Myc* or *Snail2*. Furthermore, overexpression of cMyb upregulates *Msx1* and *Snail2*, interpreted as evidence that cMyb may participate in BMP4 signaling input into the epithelial-mesenchymal transition of trunk neural crest (Karafiat et al. 2005).

Although several lines of experiments suggest that Sox9 can regulate *Sox10* expression, it is not clear wether Sox9 directly regulates the onset or maintenance of *Sox10*. *Sox9* expression precedes that of *Sox10* in all vertebrates examined (Hong and Saint-Jeannet 2005). Knock-down of Sox9 in *Xenopus* neural crest precursors depletes *Sox10* expression (Lee Y. H. et al. 2004b), whereas chick Sox9 misexpression induces ectopic *Sox10* in the trunk neural tube (Cheung and Briscoe 2003). In *Sox9* mutant mice, *Sox10* persists in early migrating trunk neural crest cells but is absent in neural crest derivatives (Cheung et al. 2005). Recently EMSA assays demonstrate that Sox9 and Sox10 proteins can bind to SoxE binding motifs within different mouse *Sox10* regulatory regions *in vitro* (Werner et al. 2007).

Finally, expression of a third putative upstream regulator, *Ets1,* precedes that of *Sox10* at cranial levels, but is absent in trunk neural crest progenitors (Tahtakran and Selleck 2003). Ets1 plays a role in cranial neural crest delamination and appears to mitigate the requirement for S phase synchronization to promote neural crest emigration in a cluster-like fashion (Theveneau et al. 2007). In contrast, trunk neural crest cells undergo cell-cycle arrest prior to delamination, dividing only once out of the neural tube (Burstyn-Cohen and Kalcheim 2002). Accordingly, ectopic expression of Ets1 in the trunk results in excess, cluster-like emigration of Sox10-expressing cells (Theveneau et al. 2007). Interestingly, ectopic expression of the Sox10E2 reporter in the trunk neural tube was observed when Ets1 is overexpressed (effect not observed when over expressiong either cMyb or Sox9, which are normally present in this location) (Cheung and Briscoe 2003, McKeown et al. 2005, Tahtakran and Selleck 2003, Theveneau et al. 2007). Thus addition of Ets1 may be sufficient to activate Sox10E2 reporter expression where it is normally lacking, raising the intriguing possibility that the newly identified Sox10E2 regulatory element may act as a switch between head and trunk neural crest populations, accounting for the morphological differences in delaminating cranial (clustered) versus trunk (individual cell-cycle arrested) crest.

Furthermore, co-expression of Sox10E2 reporter construct with Sox9, Ets1 or cMyb results in ectopic enhancer activation in the extraembryonic region. Extraembryonic regions retain regulatory factors characteristic of multipotent tissue, and often are considered “naïve” (Streit and Stern 2008, Streit et al. 1997). Thus, these cells retain competence to respond to multiple inducing signals and may possess intrinsic signals that, together with an added specific factor, ectopically activate the Sox10E2

enhancer. One possibility is that this occurs indirectly, such that each factor can induce a neural crest like transcriptional program including the induction of a complete suite of factors necessary for Sox10E2 activity. Alternatively, repressors that suppress module activity may be present in other locations. As case in point, the element controlling down regulation of endogenous Sox10 expression within the branchial arches appears to be missing in the 3.5 kb Sox10E construct such that Sox10E2 and 10E1 enhancers activate reporter expression the first two, and last three arches, respectively.

Ets1, Sox9 and cMyb directly bind to Sox10E2 enhancer in vivo and are necessary for regulation of Sox10 expression in neural crest cells

Endogenous cMyb, Sox9 or Ets1 factors are necessary for Sox10E2 enhancing activity in the cranial neural crest. Inactivation of these upstream regulators, together with mutation of their binding motifs and chromatin immunoprecipitation, clearly demonstrate that Ets1, Sox9 and cMyb converge onto the Sox10E2 *cis*-regulatory element and directly regulate onset of *Sox10* expression in delaminating neural crest cells. Morpholino-mediated inactivation of individual upstream regulators completely abolishes Sox10E2 reporter activity, while greatly reducing endogenous Sox10 expression. Importantly, simultaneous inactivation of all three factors leads to a complete loss of initial *Sox10* expression (at HH8+) indicating that Sox9, cMyb and Ets1 transcription factors are responsible for initial activation of *Sox10* expression in cranial neural crest. Later, activation of different regulatory elements likely activates and maintains Sox10 expression in migrating neural crest. The direct binding of cMyb, Sox9 and Ets1 to the Sox10E2 element, shown by chromatin immunoprecipitation, gel shift assays, combined

with mutational analysis of their binding sites within not only the enhancer but also a much larger genomic region, indicate that these factors directly control the onset of *Sox10* expression in the cranial neural crest via the core regulatory element Sox10E2.

cMyb regulates Ets1 expression during NCC specification

Knock-down of cMyb not only affects *Sox10* expression, but also causes down-regulation of other neural crest specifier genes. Most notably, there was complete elimination of *Ets1* expression within the neural crest territory. An intriguing possibility is that cMyb first induces *Ets1* and together they act to directly activate *Sox10*. A regulatory battery of similar dynamics is used during melanocyte differentiation, where Sox10, in addition to directly regulating the expression of microphthalmia-associated transcription factor (*Mitf*), acts synergistically with Mitf to directly control the expression of dopachrome tautomerase (*Dct/Trp2*) (Sauka-Spengler and Bronner-Fraser 2008a).

cMyb in combination with Sox8 and Pea3 activate Sox10 gene expression in otic placode

In the otic placode, however, the onset of *Sox10* expression through Sox10E2 is mediated by cMyb in combination with Sox8 and Pea3. Whereas regulation of *Sox10* in neural crest cell individually requires each of the Sox10 activators, cMyb, Sox9 and Ets1, elimination of individual factors in the otic placode reduces but fails to eliminate Sox10E2 activity or *Sox10* endogenous expression. However, simultaneous depletion of the three factors completely abolishes *sox10* endogenous expression during otic placode

development; and later *Sox10* expression appears to recover, when the otic vesicle begins to shape, probably mediated by other *Sox10 cis*-regulatory regions with different inputs. It is not yet know if the onset of *Sox10* expression is controlled directly by the binding of Sox8 and Pea3 to the corresponding SoxE and Ets binding motifs within Sox10E2 enhancer. Future experiments will utilize chromatin immunoprecipitations and gel shifts to test for direct binding similar to what has been done for cMyb, Sox9 and Ets1 with Sox10E2 in neural crest cells. Ets1 and Pea3 are both known to recognize the same Ets binding motifs (Fisher et al. 1991). Moreoever, both Ets1 or Pea3 can regulate expression of the TGF-beta receptor (*TbetaR-II*) gene through the same enhancer, as demonstrated in embryonal carcinoma cells (Kopp et al. 2004). This suggests that Ets1 and Pea3 may activate *Sox10* expression, through the shared Ets functional binding motif within the enhancer Sox10E2. Similarly, there is evidence that Sox9 and Sox10 can share the same binding motifs within other Sox10 regulatory regions in mouse (Werner et al. 2007). Thus, it seems likely that Sox8 has the ability to bind to SoxE binding sites within the Sox10E2 enhancer.

In summary, in-depth interrogation of the newly identified Sox10E2 regulatory module has allowed identification of two new neural crest specifier, cMyb and Ets1 that are involved in regulating early gene expression in the neural crest cells. Together with Sox9, Ets1 and cMyb converge onto and directly bind to the Sox10E2 enhancer to regulate reporter and endogenous *Sox10* expression in the cranial neural crest. Interestingly, cMyb, Sox8 and Pea3 activate this same enhancer in the otic placode. Such identification and perturbation of regulatory sequences and the candidate upstream regulators controlling them provides a powerful tool for demonstrating direct binding interactions and testing

regulatory outcomes *in vivo*. Our analysis of novel regulatory regions provides new information that has expanded our knowledge of the gene regulatory interactions that occur during formation of both the vertebrate neural crest and developing ear.

Possible conserved role for the SoxE group of transcription factors in neural crest cells and otic placode during evolution

Previous studies have shown that *Sox8, Sox9* and *Sox10* genes, belonging to the SoxE family of transcription factors are expressed by both neural crest and otic placode cells across different vertebrate taxa. However, the timing of the onset of expression for different SoxE paralogs in the two populations varies across species. For instance while in *Xenopus* neural crest progenitors *Sox8* expression is followed by *Sox9* and *Sox10*, in chicken neural crest, *Sox9* is the first SoxE factor to appear closely followed by Sox8 and *Sox10* (Hong and Saint-Jeannet 2005). Conversely, in the otic region of the chicken embryo, *Sox9* is expressed only later, in the formed vesicle, whereas the early forming territory is marked first by *Sox8* and then by *Sox10* expression. Despite different paralogue usage across species, the shared expression of SoxE genes in both neural crest and otic placodes is a common feature of vertebrates. It is even conserved in the basal vertebrates, such as lamprey, where *SoxE3* expression resembles that of *Sox10* and *SoxE2* is similar to that of *Sox9* (Fig. 1). Intriguingly, my thesis work shows that the onset of *Sox10* expression is regulated by one common neural crest and otic placode enhancer. Similarly, other *Sox10* enhancers characterized in mouse and zebrafish, display activity in both population of cells as well (Tables 1 and 2 detail the chicken Sox10 enhancers activity compared to *Sox10* enhancers identified in other species). Such enhancers with a dual function in neural crest

and otic placode have also been previously shown to control Sox9 expression in mouse. Bagheri-Fam and colleagues showed that enhancer E3 regulates weak initial expression of *Sox9* in cranial neural crest cells and otic placode as early as 8.5 dpc, when initial endogenous *Sox9* expression is first observed. Altogether, this information, may suggest a close evolutionary connection between neural crest cells and otic placode cells, marked firstly, by the required expression of the SoxE family of genes in both population of cells and secondly by the utilization of the same enhancers to activate and maintain SoxE expression uniquely in both populations of cells. While the requirement for SoxE function in both neural crest and placodal populations is indicative of a common cellular function, the reiterative use of a single *cis*-regulatory element to regulate gene expression in both cell populations could reflect a common evolutionary origin. In the case of chick neural crest and otic development, it appears that the *Sox10* and *Sox9* enhancers have retained their ability to respond in two different cellular environments with little or no rearrangement at the *cis*-regulatory level.

A common neural crest and placode evolutionary origin has been proposed before based on fate maps drawn from zebrafish, *Xenopus*, mouse and chicken that show that during gastrulation the ectoderm is subdivided into neural and non-neural ectoderm and, within the intermediate region where both overlap (Fernandez-Garre et al. 2002, Garcia-Martinez et al. 1993, Keller 1975, 1976, Kimmel et al. 1990, Tam 1989), precursors for different placodes including otic are still widely dispersed and intermingled with future neural crest, neural and epidermal cells (Garcia-Martinez et al. 1993, Hatada and Stern 1994). At early neurula stage a contiguous stripe of ectoderm (termed the neural plate border) co-expresses pre-neural and non-neural ectoderm markers. Within the border

region, precursors for neural, neural crest, epidermis and placodes remain interspersed (Streit 2002). Thus in this region, similar sets of inductive signals affect the cell fate specification of neural crest vs placode cells, and both populations are exposed to FGF8, BMPs and Wnts, which establish the neural plate border. However, in the chicken embryo, the underlying future heart mesoderm secretes BMP and Wnt antagonizers. As this signaling source is in proximity to placodal precursors within the border, it has been proposed that these antagonizers protect future placode cells from Wnt and BMP influences, thought to induce neural crest or epidermis progenitors. It is plausible that in a hypothetical vertebrate ancestor both cell populations could have been exposed to the same inductive signals. Subsequent evolutionary changes to the neighboring environment may have then rendered one cell subpopulation, in this case placodal cells, responsive to new signals secreted by newly adjacent regions. The signaling switch would have resulted in upregulation of different, possibly novel combinations of regulatory factors, creating a different transcriptional output and ultimately changing the fate of the cell. Such changes likely intervene at the *cis*-regulatory level by turning on new modules and turning off or changing the response of existing active elements. This could explain why the Sox10E2 regulatory region, responsible for the onset of *Sox10* in neural crest cells, responds to cMyb, Sox9 and Ets1 inputs, while in otic placode cells it is activated by a combination of cMyb with different SoxE and Ets paralogs, Sox8 and pea3. The new combination of regulatory factors upregulates *Sox10* in the otic placode cells, which otherwise present many characteristics distinguishing them from neural crest.

A hypothesis proposing a common neural crest and placode origin would agree with Gans and Northcutt's idea that vertebrate-specific characters arose mainly from neural

crest and cranial placode cells, providing respective derivatives with adaptations for a mobile predatory life style (Baker 2008, Northcutt and Gans 1983). They also argued that the chordate common ancestor probably had a neural crest/placode precursor involved in sensory tissue development. This precursor would have been part of a diffuse ectodermal nerve plexus, such as that seen in some modern deuterostomes, including echinoderms and hemichordates and did not form condensations characteristic of vertebrate placodes. Evidence for this has come from analysis of ascidians and cephalochordate molecular embryology (Baker and Bronner-Fraser 1997). The ascidian neurohypophyseal duct that connects the cerebral ganglion to the oral cavity is neurogenic and produces migratory cells. Thus, it has been proposed as a good candidate homolog for both neural crest and for the combined olfactory and adenohypophyseal placodes (Burighel et al. 2003, Manni et al. 2004).

Alternatively, it is possible that the neuroepithelial layer, containing neural crest and placode progeny, coopted similar gene batteries and then each population evolved independently influenced by their environments, which in turn created differential expression of paralog genes. For instance, in the most basal chordate, Amphioxus (a cephalochordate), most neural plate border specifiers are found in the neural plate border while most neural crest specifiers, with the exception of *SoxE* found in the neural tube, are expressed in the underlying mesoderm (Baker 2008, Meulemans and Bronner-Fraser 2005, 2007). It is possible that "the neural crest specifier genes" were recruited by some cells in the neuroepithelial domain (future neural crest), while *SoxE* was coopted simultaneously by presumptive neural crest and otic placode cells found in the same domain.

The possible common origin of the neural crest and placode cells is a subject of great controversy. First, there is no fossil record of an ancestral organism that suggests such a common origin. Also it should be considered that while the Sox10E2 neural crest and otic placode conserved enhancer is highly conserved across amniotes, it has not been found to be conserved to anamniotes, perhaps because the location in the genome has been changed and yet, genomic comparisons have not been done to more basal vertebrates, such as lamprey. Identifying a conserved region that is equivalent to Sox10E2 in these species will give more insight regarding the conserved function of this enhancer in neural crest and placode cells. In addition, it is important to bear in mind that such regions may never be found if the same comparative protocols used to search for conserved regions are applied. Due to evolutionary distance, many non-essential regions may have been added or lost. Moreover, while the essential functional motifs have been conserved, changing distances between and/or order motifs, may make it difficult to recognize conserved regions.

Further data arguing against the common-origin hypothesis comes from single cell labeling experiments which strongly indicate that neural crest and placode cells at the neural plate territory are not a mixed population but are already preprogrammed to adopt one of the fates (Selleck and Bronner-Fraser 1995). Finally, to date there is no evidence of molecular factors that are exclusively expressed by neural crest and placode cells at the border territory. Markers that are expressed by both cell populations are expressed broadly and are also found in the neural plate or non-neural ectoderm future epidermis. Interestingly, the one exemptions is members of the SoxE family, although not expressed

when both populations of cells are intermingling at the neural plate border, but later when neural crest cells are localized in the dorsal folds of the neural tube and the otic placode cells are located outside the neural domain.

Rather than supporting the hypothesis of a common evolutionary origin of neural crest and placodes, it has been strongly suggested that all placodal populations come from a single preplacodal cell state. This implies that in an ancestral organism, primitive neural crest cells were a separate entity from primitive placode cells (Schlosser 2008). This is supported by the expression pattern of certain genes like *Eya* and *Six* family members, which are expressed uniquely by all placodal progenitors (Streit 2007). A further argument supporting the common preplacode origin are based on otic induction experiments in chicken, which demonstrate that when explanted and exposed to an environment that can induce a preplacodal state (FGF signal), cells with epidermal fate can express otic placode markers. If the presumptive epidermal cells do not acquire a preplacodal state first, they fail to express otic markers (Martin and Groves 2006). These experiments suggest a common specification mechanism for all placodal populations, but, it does not address their possible common evolutionary origin. Thus, the possibility of a common evolutionary origin of placodes and neural crest remains an open argument.

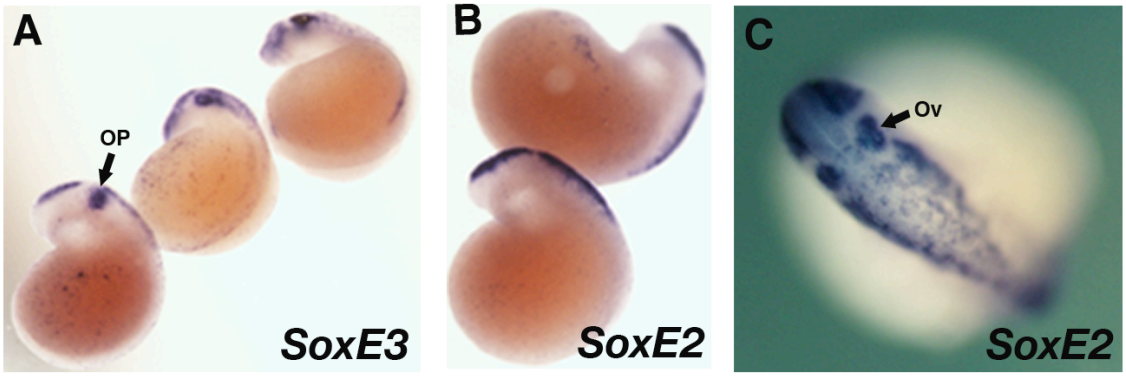

Figure 1. *SoxE3* and *E2* in lamprey are concomitantly expressed by neural crest and otic placode cells. (A) The Pattern of expression of lamprey *SoxE3* is similar to that of *Sox10* in other vertebrate embryos. Expression is observed in migrating cranial neural crest and in the otic placode. Expression of *SoxE2* resembles early expression of *Sox9* in other vertebrates. When neural crest cells have begun migration, no SoxE2 expression is observed in the otic placode (B). Later on, when neural crest cells are extensively migrating, *SoxE2* expression appears in the otic as it shapes into a vesicle (C).

	CRE	Size bp	Delaminating CNCC	OP	Migrating CNCC	Cranial ganglia	OV	Ectopic
Chick	**E1**	**600**	-	-	-	+	+	-
Mouse	**D6**	**229**				++	++	-
Chick	**E2**	**264**	++	++	++	++	++	++ (BA)
Mouse	**D7**	**235**				-	-	-
Chick	**L8**	**3000**	-	-	+		+	
Mouse	**U3**	**396**				++	-	+ (H & DSC)
Mouse	**U1**	**190**				++	++	-
	U2	**190**				++	-	-
	U4	**149**				-	-	+ (BV)
	U5	**170**				++	-	-
	MCS3	**759**				-	-	
	MCS8	**667**				++	+	+ (CS)
	MCS9	**665**				++	-	
Zebra-fish	**pSox10 1252D(+159 +1862)**	**> 1000**	-	++	+	+	++	+ (NT)
Xeno-pus								

Table 1. Sumary of Sox10 enhancers activity at the cranial level and ectopic areas

++ Strong activity
+ weak activity
- No activity
CRE *Cis*-regulatory element
OP Otic placode
OV Otic vesicle
CNCC Cranial neural crest cells
BA Branchial arches
BV Blood vessels
H Heart
DSC Dorsal spinal cord
NT Neural tube
CS Craniofacial skeleton

For E1 and D6 (in blue), E2 and D7 (in green), L8 and U3 (in purple) the enhancers have equivalent sequences.

	CRE	Size bp	Delaminating V&T NCC	Migrating V&T NCC	DRG	ENS	AG	Melanocytes
Chick	**E1**	**600**	-	+	+			
Mouse	**D6**	**229**			+	-	-	-
Chick	**E2**	**264**	-	-	-			
Mouse	**D7**	**235**			-	-	-	-
Chick	**L8**	**3000**						
Mouse	**U3**	**396**			-	+	-	+
Mouse	**U1**	**190**			+	+	+	-
	U2	**190**			+	-	-	-
	U4	**149**			-	-	-	-
	U5	**170**			+	-	-	-
	MCS3	**759**			-	-		-
	MCS8	**667**			-	+		-
	MCS9	**665**			-	-		+
Zebra-fish	**pSox10 1252D(+159 +1862)**	**> 1000**	+	+	+			-
Xenopus								

Table 2. Summary of Sox10 enhancers activity at vagal and trunk level

++ Strong activity
+ weak activity
- No activity
CRE *Cis*-regulatory element
V&T NCC Vagal and trunk neural crest cells
DRG Dorsal root ganglia
ENS Enteric nervous system
AG Adrenal gland

For E1 and D6 (in blue), E2 and D7 (in green), L8 and U3 (in purple) the enhancers have equivalent sequences.

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