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Expression and function of transcription factor *cMyb* during cranial neural crest development

Paola Betancur[1], **Marcos Simões-Costa**, **Tatjana Sauka-Spengler**[2], and **Marianne E. Bronner**[*]
Division of Biology 139-74, California Institute of Technology, Pasadena, CA 91125

Abstract

The transcription factor *cMyb* has well known functions in vertebrate hematopoiesis, but little was known about its distribution or function at early developmental stages. Here, we show that *cMyb* transcripts are present at the neural plate during gastrulation in chick embryos. *cMyb* expression then resolves to the cranial neural folds and is maintained in early migrating cranial neural crest cells during and after neurulation. Morpholino-mediated knock-down of cMyb reduces expression of *Pax7* and *Twist* at the neural plate border, as well as reducing expression of neural crest specifier genes *Snail2* and *Sox10* and completely eliminating expression of *Ets1*. On the other hand, its loss results in abnormal maintenance of *Zic1*, but little or no effect on other neural crest specifier genes like *FoxD3* or *Sox9*. These results place *cMyb* in a critical hierarchical position within the cranial neural crest cell gene regulatory network, likely directly inhibiting *Zic1* and upstream of *Ets1* and some, but not all, neural crest specifier genes.

1. Introduction

cMyb is an important transcriptional regulator with diverse functions in the hematopoeitic system. It maintains T cells and other progenitors in a proliferating and immature state (Allen et al. 1999). Interestingly, neural crest cells in the developing embryo share many transcription factors with other stem cell populations, particularly those used in the hematopoietic and immune systems.

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 (Betancur, 2010b; Milet and Monsoro-Burq, 2012). In the cranial region, these events are initiated by inductive signals like BMPs and Wnts that set up a domain at the neural plate border that expresses marker genes including *Msx*, *Pax3/7*, *Twist* and *Zic genes*. These cumulatively render the border region distinct from the neural plate and non-neural

[*]Author for editorial correspondence: mbronner@caltech.edu.
[1]Present address: Institute for Stem Cell Biology & Regenerative Medicine, 265 Campus Dr., Stanford, California 94305
[2]Present address: Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DS

ectoderm. Some time later, cranial neural crest cells first become identifiable as a discrete population of premigratory cells within the dorsal neural tube, by their expression of a combination of neural crest specifier genes including *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. Direct connections between neural plate border genes like *Pax7* and neural crest specifier genes like *FoxD3* are only now becoming elucidated (Simoes-Costa et al., 2012). In this context, it is interesting to note that cMyb has been shown to be a direct input into the neural crest specifier gene *Sox10* (Betancur et al., 2010a), though it had not been previously known to be present or functional in the premigratory cranial neural crest.

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 to 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).

To better define cMyb's role in the context of neural crest gene regulation, focusing on the cranial neural crest, we have performed a detailed analysis of its expression and function from gastrulation throughout neurulation. The results show that loss of cMyb reduces expression of neural plate border genes *Pax7* and *Twist*, increases *Zic1*, and reduces or eliminates neural crest specifier genes, *Ets1*, *Sox10* and *Snail2*, but not *FoxD3* or *Sox9*, expression levels. These findings help identify the position of cMyb within the hierarchy of the cranial neural crest gene regulatory network.

2 Results

2.1 Isolation of full-length chick cMyb

As a first step in understanding the possible early functions of this gene in embryonic patterning, we isolated the chick homologue of *cMyb* and examined its expression from gastrulation through early stages of nervous system development in the chick. The full-length chick homologue of *cMyb* was obtained by a degenerate RT-PCR approach. Comparative analysis of the aligned sequences reveals that chick *cMyb* shares 80% identity with the mouse protein, 82% with human, and 75% identity with the *Xenopus* homolog at the amino acid level.

2.2 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 beginning at gastrulation to early organogenesis stages. From gastrulation through early neurulation (stages 4–6), we find that *cMyb* is expressed at the neural plate border and neural plate, as well as in presumptive blood islands (Figure 1A). As neurulation begins, it is expressed in the elevating neural folds (arrows on Fig. 1B), as well as more faintly in the neuroepithelium. Expression is maintained in the presumptive neural crest forming region, accumulating in the neural folds by HH7-8, with strongest expression at the dorsal margins containing neural crest precursors (Figs.1C, E). At HH10, transcripts are seen in neural crest cells delaminating and emigrating from the cranial neural tube (Fig. 1D,

F). In the trunk, *cMyb* expression is detected in the elevating neural folds (arrows in Fig. 1G) as well as the neural plate. Thus, *cMyb* is expressed early at the neural plate border and in presumptive neural crest cells.

2.3 Effects of cMyb knock-down on neural plate border and neural crest specifier expression

cMyb protein production was perturbed by unilaterally electroporating FITC-tagged antisense morpholino oligonucleotide into one side of HH4 embryo and assaying the subsequent effects on expression of neural crest markers. In particular, we focused on those whose expression precedes that of *Sox10*. While there was no strong effect on expression of *Sox9* (Fig. 2A; n=10/12) or *FoxD3* (Fig. 2B; n=8/11), the results show that knock-down of cMyb decreases *Snail2* expression (Fig. 2C n=9/13). Even more striking, cMyb loss completely abolishes *Ets1* expression (Fig. 2D; n=11/12). To demonstrate specificity of the effect, we co-electroporated cMyb-morpholino with a *cMyb* mRNA expression construct; the results show that this largely rescues the loss of *Ets1* expression caused by cMyb morpholino alone (100% of embryos rescued, n=3/3) (Fig. 2E).

Since *cMyb* is expressed very early at the neural plate border, we investigated whether perturbing cMyb at stage 4 using antisense morpholino alters expression of other genes expressed at the neural plate border. To test this at a multiplex level, we employed NanoString technology which allows us to quantify the effects of cMyb knock-down of numerous genes simultaneously, using a probe set comprised of 45 neural plate, neural plate border, non-neural ectoderm, and ectodermal placode genes. We directly compared the levels of gene expression on the morpholino injected to the contralateral side of the same embryo. The results show that cMyb knock-down increases the expression of *Zic1* while decreasing the expression of *Gbx2, Six1, Twist* and *Pax7* neural plate and neural plate border specifier genes (Fig. 3A). No changes were observed on the expression of *GapDH* and *SDHA* among other housekeeping genes and negative control genes. Furthermore, to verify the results and obtain spatial information regarding neural plate border genes, *Twist, Pax7* and *Zic1*, we performed *in situ* hybridization. The results supported the data obtained with the NanoString analysis and show that both *Pax7* and *Twist* expression are reduced on the morpholino-injected side of the embryo (Fig. 3B, B′, C and C). Interestingly, cMyb knockdown resulted in an increase of *Zic1* expression in the dorsal neural folds, which is more prominent in the stage HH9 (Fig. D and D′).

3 Discussion

By examining the expression pattern of *cMyb* in the early avian embryo, we find that it is expressed at the neural plate border region. Previously, we identified *cMyb* as a direct input into the initiation of *Sox10* expression at stage 8+ (Betancur 2010a). The present results show that its expression commences much earlier than *Sox10. cMyb* transcripts are observed during gastrulation, concomitant with early neural crest specifiers such as *Pax7*, *AP-2* or *c-Myc*. It remains expressed in premigratory neural crest cells within the dorsal head neural folds, as well as in migrating cranial neural crest cells. This raised the intriguing possibility that cMyb may regulate additional genes in the neural crest gene regulatory network. Here, we demonstrate that cMyb differentially regulates expression of several genes, whose onset

of expression precedes that of *Sox10*. cMyb knock-down reduces the expression of *Snail* and eliminates expression of *Ets1*, but does not effect other neural crest specifier genes, like *Sox9* and *FoxD3*. The complete loss of *Ets1* expression indicates that cMyb is necessary for the expression of *Ets1*. However, it does not appear to be a primary input into a recently reported cranial crest enhancer for *Ets1* (Barembaum and Bronner, 2013), raising the possibility that its regulation of *Ets1* is indirect or functions through a different enhancer. Our previous data show that cMyb and Ets1 cooperate to activate *Sox10* expression in cranial neural crest cells. The present data suggest that cMyb helps activate *Ets1* and they then both help to initiate and maintain expression of *Sox10*. Later in development, *cMyb* functions to specify melanocyte fate by regulating *c-kit* (Karafiat et al., 2007), suggesting that cMyb plays multiple spatially and temporally distinct roles in the neural crest.

Our NanoString data suggest that cMyb is involved in the regulation of neural and neural plate border genes *Gbx2*, *Six1*, *Twist* and *Pax7* in the late gastrula embryo. Interestingly, we find that loss of cMyb results in abnormal maintenance of *Zic1* in the cranial neural folds, which is normally only expressed at low levels in the head compared with the trunk neural crest. *Zic1* is a member of the Zinc-finger containing family of proteins and a well known neural plate border specifier, also expressed in the neural plate border region and in the anterior ectoderm region containing placodal progenitors (Khudyakov et al., 2009). Much of the information about the role of *Zic1* in the neural crest gene regulatory network comes from experiments performed in frog and lamprey (Sato et al., 2012; Nikitina et al). The finding that loss of Myb results in upregulation of *Zic1* in the cranial neural folds raises the intriguing possibility that cMyb may normally function to repress *Zic1* expression in the head. Recently, it has been shown that *Zic1* plays an important role in regulating trunk neural crest expression of *FoxD3* (Simoes-Costa et al., 2012). Thus the differential effects of cMyb on *Ets1* and *Zic1* may partially explain the reciprocal expression pattern of *Ets1* in the cranial neural folds and *Zic1* in the trunk neural folds (Simoes-Costa et al., 2012). We speculate that different levels of *Zic1* and *Ets1* expression may contribute to differences in developmental potential between cranial and trunk neural crest cells.

In contrast to *Zic1*, loss of cMyb causes a decrease in expression of *Gbx2*, and *Six1*. *Gbx2*, is a homeobox gene, important for the anterior-posterior patterning of the neural axis, previously reported to be expressed in the ectodermal regions that includes the future neural plate border in *Xenopus* embryos (Li et al., 2009). *Six1*, on the other hand, is expressed in the anterior neural plate, marking a region containing mostly placodal precursors (Streit, 2004). More recently, Sato and colleagues (2012) detected seven functional *Six1* enhancers and through mutational analysis, identified inputs from Sox, Pax, Fox, Wnt/Lef1 and an autoregulatory input from Six1 that potentially integrate through these enhancers to regulate *Six1* expression at different times of placode development (Sato et al., 2012). Nevertheless, their study did not identify a functional binding motif for cMyb, suggesting that perhaps the reduction on *Six1* expression observed in our NanoString data might be indirect and possibility mediated by other transcription factors, such Pax7.

In summary, our data help to elaborate the gene regulatory underlying neural crest formation (Figure 4). Most notably, we show that cMyb functions early in neural crest development by partially regulating *Pax7* and *Twist* at the neural plate border. These factors are part of a

subgroup of transcription factors that include *AP-2a, Snail1/2, Id, c-Myc*, among others, and that are expressed well before neural crest progenitors become apparent. Most notably, our data suggest that cMyb plays a critical role in regulating cranial neural crest expression of the neural crest specifier gene, *Ets1*, which together with cMyb itself, is a direct activator of initial *Sox10* expression in the premigratory and emigrating cranial neural crest cells.

This new information allows us to place cMyb within the gene regulatory network hierarchy underlying neural crest cell formation. Furthermore, combining morpholino knockdown and multiplex NanoString plus in situ hybridization assays guides in the establishment of linkages between the genes within the neural crest cell gene regulatory network (Fig. 4), thus helping to unravel the complex interacting events for testing by in depth *cis*-regulatory analyses.

4 Experimental Procedures

4.1 *In situ* hybridization

Sox9 and *Sox10* 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). *Sox10* template was digested with HindIII, while all EST clones were linearized using NotI restriction enzyme. All antisense RNA probes were synthesized using T3 RNA polymerase, according to standard protocols. Whole-mount *in situ* hybridizations were performed as previously described (Wilkinson, 1992). Fluorescent *in situ* procedure using GFP probe was adapted from Acloque et al. (2008).

4.2 Embryos culture and electroporation

Fertilized chicken eggs were obtained from AA Laboratories, Westminster, CA and Chino Valley Ranchers, Arcadia, CA and incubated at 37–38°C for approximately 24–40hrs, depending on the desired stage. The embryos were staged according to the criteria of Hamburger and Hamilton (1992).

Chicken embryos were electroporated at stages HH4-8 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 ranged from 1–2 mM.

4.3 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 as follows:

cMyb_5′-ATGGCCGCGAGCTCCGCGTGCAGAT-3′;

Control_5′-ATGGCCTCGGAGCTGGAGAGCCTCA-3′.

4.4 NanoString nCounter

Embryos at primitive streak stage were unilaterally electroporated with antisense morpholino against cMyb on the right side of the embryo and control morpholino on the left side. Embryos were cultured ex vivo and allowed to grow for 24 hours. Next, each individual half of the embryo was processed and the total RNA obtained from each side was prepared as previously described (Strobl-Mazzulla et al., 2010).

4.5 Microscopy and inmunohistochemistry

The electroporated embryos were collected at stages 8–10, 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 2x 10 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.

Acknowledgments

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Highlights

cMyb is expressed at the neural plate border and presumptive neural crest region

cMyb knock-down results in decrease of neural crest specifier genes *Ets1*, *Sox10* and *Snail2*

Repression of *Zic1* in the cranial neural folds is mediated by cMyb

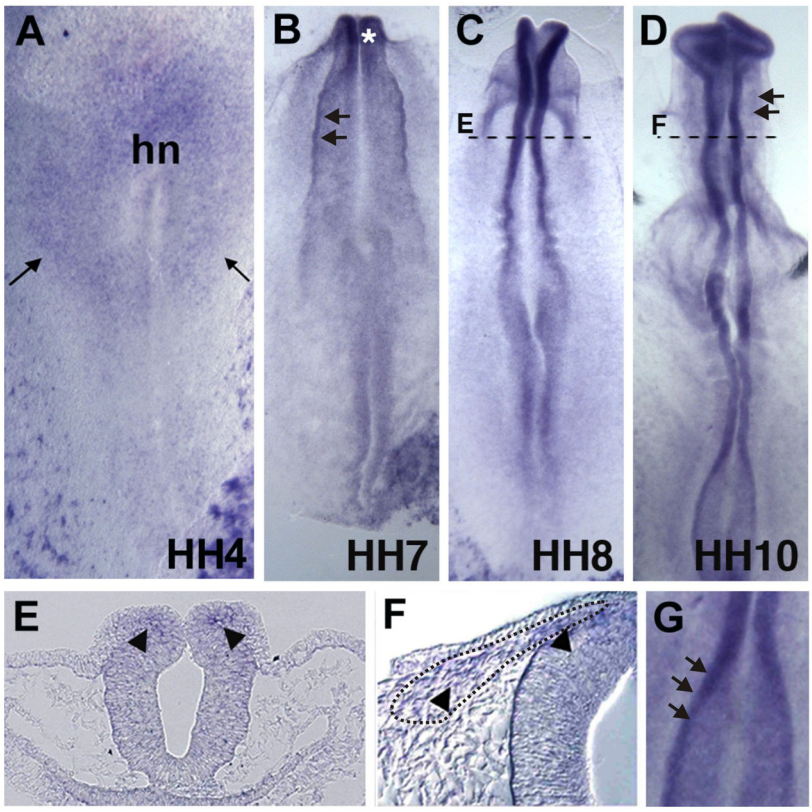

Figure 1. *cMyb* pattern of expression in the presumptive neural crest territory

A) cMyb is detected in the neural plate and neural plate border (arrows) at HH4 as well as in blood islands. B) At HH7, *cMyb* is expressed within the raising neural plate folds, which are beginning to close in the anterior end of the embryo (asterisk). C) By HH8, *cMyb* is confined to dorsal neural folds containing precursors to cranial neural crest cells by HH8. D) At HH10, *cMyb* is observed in migrating neural crest. E) Section at dotted line of C showing expression of *cMyb* in the neural folds (arrowheads). F) Section through dotted line of D showing *cMyb* expression in migrating neural crest cells (arrowheads). G) Expression of cMyb in the trunk neural folds of a HH10 chicken embryo. Hn = Hensen's node.

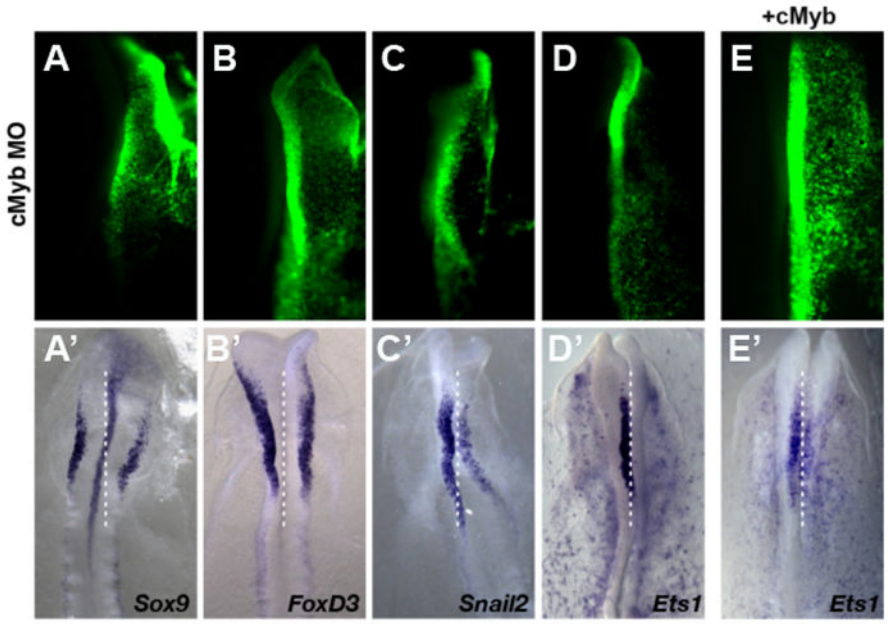

Figure 2. Effects of cMyb mediated morpholino knock-down on neural crest specifier gene expression analyzed at embryonic stage HH8 to HH8+

FITC-labeled morpholino to cMyb (A–E) was introduced onto the right side of each embryo, with the contralateral side serving as an internal control. Loss of cMyb does not affect the expression of *Sox9* (A′) or *FoxD3* (B′) but causes reduction of *Snail2/Slug* (C′) and completely abolishes *Ets1* (D′) expression. Co-electroporation of cMyb morpholino plus mRNA encoding cMyb partially rescued to loss of *Ets1* expression (E′) on the right injected side.

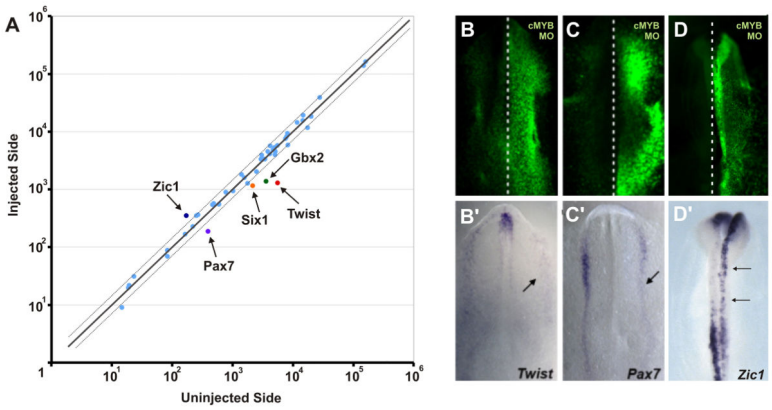

Figure 3. cMyb knock-down decreases expression of neural plate border genes

A) Nanostring multiplex analysis shows that loss of cMyb causes downregulation of neural plate border genes *Pax7* and *Twist1*, as well as placode markers. In contrast, *Zic1* is upregulated. In situ hybridization confirms that the right morpholino-treated side (B, C) has less *Pax7* (B′) and *Twist1* (C′) expression (arrow) than the control side of the same embryo. Expression of *Zic1* is increased in the cranial neural folds of embryos electroporated with *cMyb* morpholino (D′).

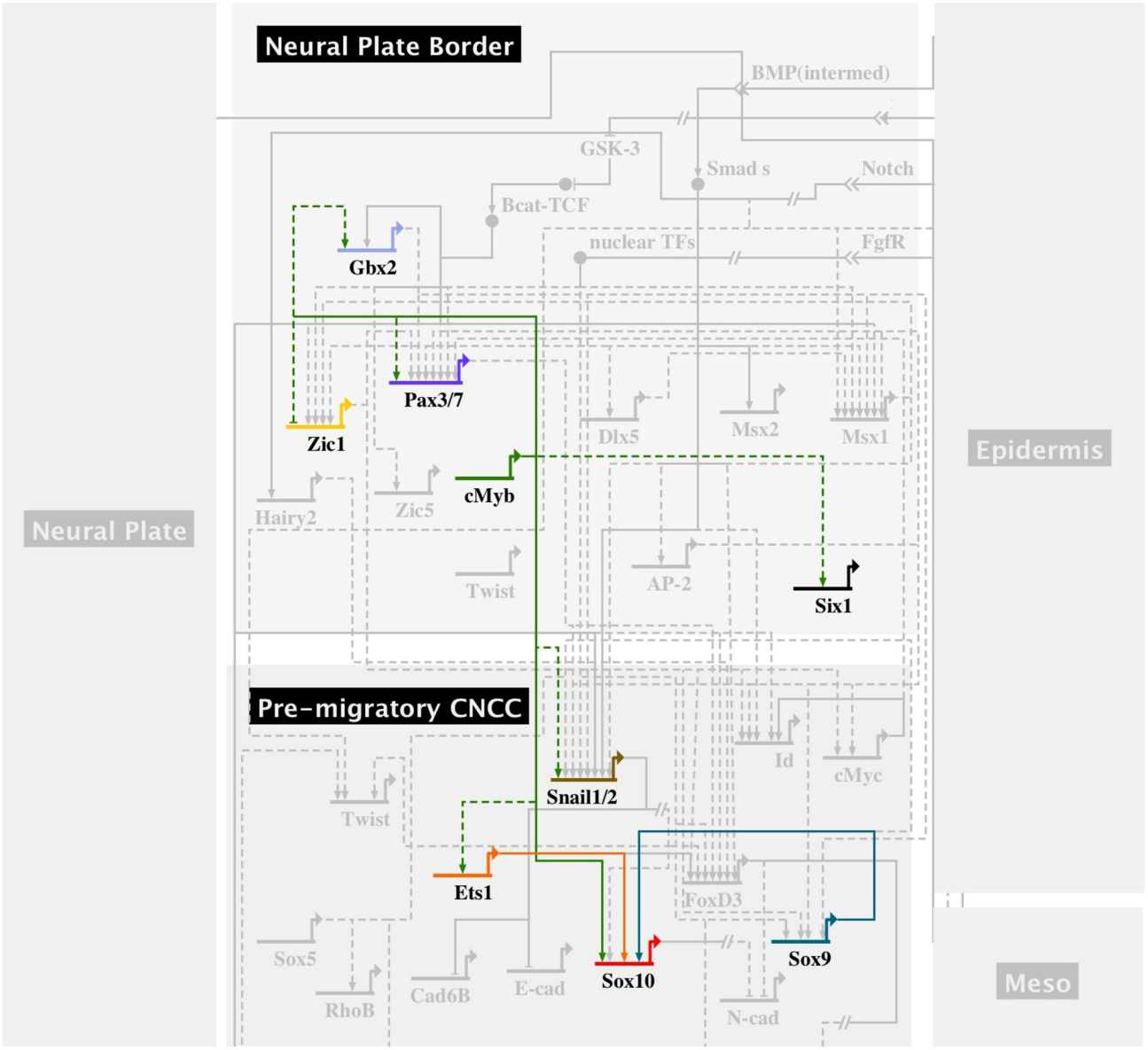

Figure 4.

Schematic representation of the neural crest gene regulatory network illustrating the position of cMyb therein.