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

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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 gene *Slug/Snail2* 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 hematopoietic 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 et al., 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 ectoderm. Sometime 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

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interesting to note that cMyb has been shown to be a direct input into the neural crest specifier gene *Sox10* (Betancur et al., 2010a), however its expression and function in the pre-migratory cranial neural crest have not been characterized in detail.

Over-expression of cMyb in the trunk neural tube upregulates *Msx1* and *Slug/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* and *Slug/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 HH4–6), we find that *cMyb* is expressed at the neural plate border and neural plate, as well as in presumptive blood islands (Fig. 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 (Fig. 1C and E). At HH10, transcripts are seen in neural crest cells delaminating and emigrating from the cranial neural tube (Fig. 1D and 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.

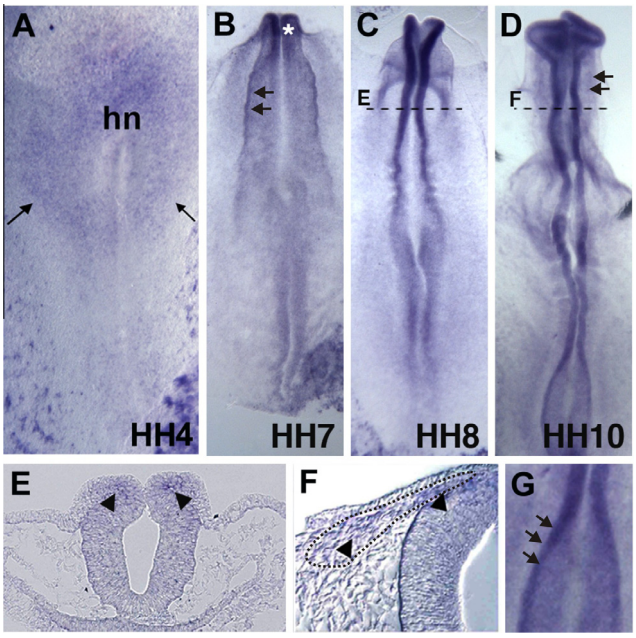

Fig. 1 – *cMyb* pattern of expression in the presumptive neural crest territory. (A) *cMyb* is detected in the neural plate and neural plate border (arrows) at HH4 as well as in blood islands. (B) At HH7, *cMyb* is expressed within the raising neural plate folds, which are beginning to close in the anterior end of the embryo (asterisk). (C) By HH8, *cMyb* is confined to dorsal neural folds containing precursors to cranial neural crest cells. (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.

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 *Slug/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 HH4 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