

Chapter 2:

**Comprehensive Spatiotemporal Analysis of
Early Chick Neural Crest Network Genes**

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ABSTRACT

Specification of neural crest progenitors begins during gastrulation at the neural plate border, long before migration or differentiation. Neural crest cell fate is acquired by progressive activation of discrete groups of transcription factors that appear to be highly conserved in vertebrates; however, comprehensive analysis of their expression has been lacking in chick, an important model system for neural crest development. To address this, we analyzed expression of ten transcription factors that are known specifiers of neural plate border and neural crest fate and compared them across developmental stages from gastrulation to neural crest migration. Surprisingly, we find that most neural crest specifiers are expressed during gastrulation, concomitant with and in similar domains as neural plate border specifiers. This suggests that interactions between these molecules may occur much earlier than previously thought in the chick, an important consideration for interpretation of functional studies.

INTRODUCTION

Neural crest cells are a transient, multipotent population of migratory cells that arise during development in dorsal neural tissue. After emigrating from the neural tube, they travel extensively throughout the body to form diverse derivatives in the periphery (Sauka-Spengler and Bronner-Fraser, 2008). Many of the processes and molecules that govern neural crest induction, commitment, migration, and differentiation have been uncovered over the past several decades through experimentation on a number of vertebrate models. In particular, the *Xenopus* embryo has been invaluable to our understanding of neural crest induction and underlying gene cascades. In addition, genetic studies using mouse and zebrafish have resolved many of the molecular interactions operating during neural crest development. Due to its easy accessibility to manipulation as well as optical clarity, the chick embryo has added much to our knowledge of neural crest formation and migration. In addition, its relatively slow development compared to other vertebrates and recently available genome have been advantageous for resolving early genetic events in neural crest development (Le Douarin and Kalcheim, 1999; Le Douarin, 2004).

Neural crest cells acquire their identity early in development, at gastrula stages, and often retain stem cell-like properties during migration (Le Douarin, 2004; Basch et al., 2006; Crane and Trainor, 2006). Fate map studies in the chick show that cells originating from the junction of neural and non-neural ectoderm, known as the presumptive “neural plate border” region, are progenitors of dorsal neural folds, dorsal neural tube, and migrating neural crest cells (Ezin et al., 2009). Furthermore, when explanted from the embryo, tissue from this region

executes a neural crest cell program in the absence of exogenous factors (Basch et al., 2006). Therefore, progenitor cells in the prospective neural plate border have already received instructive signals that specify them as neural crest in the gastrula. However, the nature of these early signals remains largely unknown.

Comparison of molecular data from several model organisms has led to the formulation of a putative neural crest gene regulatory network (NC-GRN) to help explain the signaling and transcriptional events underlying neural crest development (Meulemans and Bronner-Fraser, 2004; Sauka-Spengler and Bronner-Fraser, 2008). The NC-GRN suggests that hierarchical relationships between distinct groups of genes contribute to progressive acquisition of neural crest cell fate. As such, the first signals are received during gastrulation, when diffusible “induction factors” (BMP, FGF, Wnt) subdivide the ectoderm into neural plate and non-neural ectoderm. A specific, finely tuned combination of such signals at the junction between neural and non-neural tissues induces a cadre of transcription factors that specify this region as the neural plate border (Bang et al., 1997; Suzuki et al., 1997; Pera et al., 1999; Streit and Stern, 1999; Luo et al., 2001a; Tribulo et al., 2003; Monsoro-Burq et al., 2005). These “neural plate border specifiers” (*Msx1*, *Zic1*, *Pax7*, *Dlx5*, *Dlx3*) cooperate to delineate the neural plate border, which contains a heterogeneous population of progenitor cells including those fated to become neural crest, placodes, and dorsal neural tube (McLarren et al., 2003; Tribulo et al., 2003; Woda et al., 2003; Sato et al., 2005; Hong and Saint-Jeannet, 2007; Merzdorf, 2007). Acquisition of these distinct cell fates depends on particular combinations of downstream molecules. Specifically in neural crest progenitors, the aforementioned “neural plate border specifiers” induce a group of “neural crest specifiers” (*FoxD3*, *Snail2*, *c-myc*, *N-myc*, *AP-2 α* ,

Sox9, Sox10, among others) that function to impart bona fide neural crest characteristics, such as migratory ability on these progenitors. In addition, these genes play a crucial role in cell survival and differentiation, and their expression in pre-migratory and migrating neural crest is maintained by extensive cross- and auto-regulation (Wakamatsu et al., 1997; LaBonne and Bronner-Fraser, 2000; Dottori et al., 2001; Kos et al., 2001; Sasai et al., 2001; Bellmeyer et al., 2003; Cheung and Briscoe, 2003; Luo et al., 2003; McKeown et al., 2005; Sakai et al., 2006; Stewart et al., 2006; Taneyhill et al., 2007; Teng et al., 2008). Finally, the neural crest specifiers activate effector genes that regulate differentiation of distinct neural crest derivative lineages. These include *Col2a* (chondrocyte), *Trp* (melanocyte), *c-Ret* (enteric neuron), and many others (Sauka-Spengler and Bronner-Fraser, 2008).

Comparative studies of NC-GRN in modern vertebrates (zebrafish, frog, chick and mouse), basal vertebrate (lamprey), and non-vertebrate chordates (amphioxus and ascidian) suggest that neural plate border genes are remarkably conserved throughout chordate evolution but that expression of neural crest specifier genes at the neural plate border is unique to vertebrates (Meulemans and Bronner-Fraser, 2004; Sauka-Spengler et al., 2007; Yu et al., 2008). Recent studies in the lamprey suggest that some neural crest specifier genes, such as *AP-2* and *c-myc*, may be activated much earlier than previously thought, concomitant with neural plate border specifiers during gastrulation (Sauka-Spengler et al., 2007). Likewise in *Xenopus*, neural crest specifiers *Snail2* and *FoxD3* are co-expressed in the neural plate border with *Msx1* and *Pax3* during gastrulation (Huang and Saint-Jeannet, 2004). Orthologs of these genes have been identified and studied individually, but there is little information regarding their onset,

duration, or overlap of expression in the chick. Although expression patterns of chick NC-GRN members have been examined in detail at neurula stages, much less is known about their early deployment. Such information represents a critical backdrop for interpretation of functional perturbation studies.

Here, we characterize and carefully compare the expression patterns of transcription factors of the neural plate border and neural crest specifier category in chick embryos from stages of gastrulation to neural crest migration. Surprisingly, we find that a number of neural crest specifier genes are co-expressed with neural plate border specifiers during gastrulation in remarkably similar expression domains, implying possible early regulatory relationships. This suggests that interactions between chick specifier genes in the NC-GRN may be more complex and occur earlier than previously thought.

MATERIALS AND METHODS

Chick embryo incubation

Fertilized chicken eggs were obtained from AA Enterprises (Ramona, CA) and incubated at 38°C in a humidified incubator (Lyon Electric, Chula Vista, CA). Embryos were staged according to the Hamburger and Hamilton chick staging system (Hamburger and Hamilton, 1992).

Whole-mount *in situ* hybridization

Chick embryos were dissected in Ringer's solution and fixed in 4% paraformaldehyde at 4°C overnight. Whole-mount *in situ* hybridization was performed as described previously (Nieto et al., 1996; Xu and Wilkinson, 1998), with some modifications involving more extensive washing adapted from a lamprey *in situ* protocol (Sauka-Spengler et al., 2007). Stained embryos were photographed in 50% glycerol on a Zeiss Stemi SV11 microscope using AxioVision software (Release 4.6) and processed using Photoshop 7.0 (Adobe Systems).

Cryosectioning

To obtain transverse sections for histological analysis, embryos were equilibrated in 15% sucrose (in phosphate-buffered saline (PBS)) for 2 hours at room temperature, then transferred to 30% sucrose and incubated overnight at 4°C. Embryos were embedded in O.C.T. Compound (Tissue-Tek, catalog #4583) and frozen at -80°C. Sections 20 or 25 µm thick were obtained by cryosectioning at a

temperature of -23°C on a Microm HM550 cryostat. For imaging, slides were washed twice for 10 minutes in PBS with 0.1% Tween, rinsed in double-distilled water, and mounted with PermaFluor Mountant Medium (Thermo Electron Corporation, catalog #434990). Sections were imaged on a Zeiss Axioskop 2 Plus and processed as described for whole-mount images.

***In situ* mRNA probes**

Many of the templates for mRNA probe synthesis were obtained from the BBSRC ChickEST Database (<http://www.chick.umist.ac.uk>). The following clones were used: Msx1 (ChEST900p21), Zic1 (ChEST459n6), c-myc (ChEST191o11), N-myc (ChEST895e1), AP-2 α (ChEST765g1), and Irx1 (ChEST523e4). The other template plasmids used were: Pax7 (Basch et al., 2006), Dlx5 (Bhattacharyya et al., 2004), Dlx3 (Brown et al., 2005), Snail2 (Nieto et al., 1994), and FoxD3 (Kos et al., 2001). Linearized DNA was used to synthesize digoxigenin- and fluorescein-labeled antisense probes with Promega buffers and RNA polymerases (Promega Corp). RNA probes were purified with illustra ProbeQuant™ G-50 Micro Columns (GE Healthcare, product code 28-9034-08).

RESULTS

Here, we present a detailed analysis of the expression of ten transcription factors that are part of the putative neural crest gene regulatory network in chick embryos using whole-mount mRNA *in situ* hybridization. We compare their expression patterns across each stage of development from gastrulation (Hamburger and Hamilton (HH) stage 4) to cranial neural crest migration (HH stage 10; Hamburger and Hamilton, 1992).

HH4

The expression patterns of known neural plate border specifiers were compared with early expression domains of neural crest specifiers during chick gastrulation. Surprisingly, the results show that many neural crest specifiers are already present in the gastrula that and their distribution patterns are strikingly similar to those of canonical neural plate border specifiers. When compared at stage 4, these markers loosely group into three expression categories: 1, neural plate border and posterior epiblast; 2, neural plate, neural plate border, and anterior epiblast; and 3, non-neural ectoderm adjacent to and including part of the neural plate border.

Msx1 and *c-myc* fall into the first category. Similar to observations in *Xenopus* and zebrafish and previous work in the chick, we find that *Msx1* is expressed in posterior epiblast (ventrolateral ectoderm and mesodermal progenitors) and in the posterior and lateral edges of the neural plate (Fig. 2.1B,B'; Streit and Stern, 1999; Tribulo et al., 2003; Phillips et al., 2006). *c-myc* is also expressed at high levels in prospective mesoderm progenitors in the

posterior epiblast. Its anterior boundary of expression encompasses the neural plate border, similar to *Msx1* (Fig. 2.1C,C').

Zic1, *FoxD3*, *N-myc*, and *Dlx3* fall in the second category. *Zic1* is a known neural specifier that is also expressed in the neural plate border and in the anterior epiblast region which contains placodal progenitors (Fig. 2.1D,D'; Merzdorf, 2007). Interestingly, neural crest specifiers *FoxD3* and *N-myc* are also expressed during gastrulation in a domain remarkably similar to that of *Zic1*. Namely, *FoxD3* transcripts are present in anterior neural plate, where their rostral-most boundary of expression is adjacent to and slightly overlapping with that of *Dlx5*, the neural plate border and placodal specifier (Fig. 2.1F,F'; Fig. 2.3B). However, *FoxD3* does not overlap with *Msx1* in the posterior neural plate border (Fig. 2.3A). Some of the *FoxD3*-positive cells anterior to the node are likely notochord progenitors, since *FoxD3* functions in development of this structure at later stages (Odenthal and Nusslein-Volhard, 1998). However, *FoxD3* appears to be mainly restricted to the neural plate, as it does not overlap with ectodermal specifier *AP-2 α* (data not shown). The oncogene *N-myc* is expressed at very high levels in the neural plate border, and to a lesser extent, in anterior epiblast surrounding the presumptive neural plate (Fig. 2.1E,E'). It overlaps in the neural plate border with *Msx1* (Fig. 2.3C). *Dlx3* is a known specifier of ectoderm and placode fates and also functions indirectly to position the neural plate border by repressing adjacent neural fates (Woda et al., 2003). Therefore, we were surprised to find low levels of chick *Dlx3* transcripts in a large swath of epiblast tissue encompassing the presumptive neural plate border and lateral portions of the prospective neural plate, suggesting an additional novel role for *Dlx3* in the chick. *Dlx3* transcripts are concentrated at higher levels in the anterior epiblast,

which contains the pre-placodal region (Fig. 2.1G,G'). In contrast, *Dlx3* in the frog is never expressed in the neural plate border (Luo et al., 2001b).

The third category of neural crest gene expression at stage 4 includes *Dlx5* and *AP-2 α* (henceforth referred to as *AP-2*). We find that *Dlx5* is expressed during gastrulation in anterior epiblast adjacent to the neural plate border that contains the pre-placodal region, consistent with previously published frog and chick studies (Fig. 2.1H,H'; Yang et al., 1998; Luo et al., 2001b; McLaren et al., 2003; Woda et al., 2003; Bhattacharyya et al., 2004). Chick *AP-2* is expressed broadly throughout non-neural ectoderm at all axial levels (Fig. 2.1I,I'). It is co-expressed anteriorly with *Dlx5* in the pre-placodal region that is defined by *Irx1* expression (Fig. 2.3E,F). Surprisingly, *AP-2* transcripts also overlap in the posterior neural plate border with *Msx1* (Fig. 2.3D).

HH5

At stage 5, when the primitive streak begins to regress and leave notochord tissue in its wake, network genes become more clearly resolved at the neural plate border. *Msx1* is maintained in posterior epiblast but its expression at the neural plate border becomes more refined, and also begins to extend anteriorly (Fig. 2.1K). In the lateral and posterior portions of the neural plate border, *Msx1* expression domain is identical to that of the border specifier *Pax7*, which becomes upregulated at this stage (Fig. 2.1J,J'). *c-myc* is maintained in posterior epiblast and posterior neural plate border, but unlike *Msx1*, does not extend anteriorly (Fig. 2.1L). *Zic1* persists in anterior neural tissue and begins to accumulate more strongly at the edges of the neural plate (Fig. 2.1M). Likewise, *N-myc* expression is maintained in the neural plate and its border, where it

extends posteriorly as the embryo elongates (Fig. 2.1N). The expression domain of *Dlx3* is almost identical to that of *N-myc* (Fig. 2.1P). Conversely, *FoxD3* is no longer expressed in the neural plate or its border and is almost exclusively localized to the notochord (Fig. 2.1O). *Dlx5* and *AP-2* remain in the non-neural ectoderm, with *AP-2* marking ectoderm at all axial levels and *Dlx5* being concentrated anteriorly (Fig. 2.1Q,R).

HH6 and HH7

At stages 6 and 7, neural folds begin to thicken and elevate, allowing for better resolution of gene expression at their edges. During this time, *Pax7* is expressed exclusively in cells at the neural plate border caudal to the anterior prominence of the neural folds (Fig. 2.1S,S',BB). Likewise, *Msx1* becomes refined in the neural plate border both anteriorly (similarly to the *Dlx* genes) and posteriorly, and it is also maintained in open neural plate in the tail (Fig. 2.1T,T',CC). With progressive development, the neural plate closes anteriorly, while staying open at the caudal end of the embryo where the streak has not yet fully regressed. Gene expression at the open neural plate level recapitulates events that occur earlier at rostral levels. Expression of *c-myc* in the caudal open neural plate is markedly similar to *Pax7* and *Msx1*, but it is not expressed more anteriorly prior to the definitive appearance of the neural folds (Fig. 2.1U,U'). However at stage 7, very low levels of *c-myc* transcripts begin to accumulate in the anterior-most neural folds, becoming more distinct at later stages (Fig. 2.1DD). *Zic1* and *N-myc* are both maintained in the neural plate and upregulated at its edges, although *Zic1* becomes primarily restricted to the anterior neural folds (Fig. 2.1V,V',W,W',EE,FF). A stripe of enhanced *N-myc* expression is visible

in the region of the first forming somite at stage 7 (Fig. 2.1FF). *FoxD3* transcripts continue to mark the notochord, but also begin to accumulate at low levels in the anterior neural folds beginning at stage 6 (Fig. 2.1X,X',GG). *Dlx3* is expressed most prominently in the anterior neural ridge and placode region where it shares its domain with *Dlx5* (Fig. 2.1Y,Y',HH). Low levels of *Dlx3* remain in neural tissue at all axial levels. *Dlx5* and *AP-2* are expressed in ectoderm directly adjacent to the neural folds (Fig. 2.1Z,Z',AA,AA',II, JJ).

HH8

At the 3 to 6 somite stage (HH stage 8), neural folds are markedly elevated and begin to fuse anteriorly. At this time, most of the genes examined are co-expressed in the dorsal neural folds. *Msx1* and *Pax7* show almost identical expression in dorsal neural folds and border of the open neural plate (Fig. 2.2A,A',B,B'). Strong *Snail2* expression is present in the dorsal neural folds at the mid- and hindbrain level (Fig. 2.2C,C'). Likewise, *FoxD3* is recruited to the dorsal neural folds, where it is co-expressed in the mid- and hindbrain with *Snail2* and in the forebrain with *Zic1*, *N-myc*, and *c-myc* (Fig. 2.2D,D'). *Zic1* and *N-myc* have almost identical expression patterns in the anterior neural folds, though *N-myc* is also found in heart mesoderm and in posterior lateral plate mesoderm (Fig. 2.2E,E',F,F'). At this stage, we first see clear expression of *c-myc* in the anterior-most neural folds (Fig. 2.2G,G'). Intriguingly, its expression seems to be divided into two completely separate domains: anterior neural folds and posterior lateral plate mesoderm. The expression domain of *Dlx3* also resolves cleanly at this stage. It has disappeared from the neural folds and is expressed exclusively in the anterior neural ridge and surrounding ectoderm, where it is co-expressed

with *Dlx5* (Fig. 2.2H,H'). However, the *Dlx5* expression domain extends more caudally to the level of the hindbrain and medially into the lateral-most edge of the dorsal neural folds (Fig. 2.2I,I'). At this stage, *AP-2* transcripts are also recruited to the lateral edge of the neural folds (Fig. 2.2J,J').

HH9

At the 7 to 9 somite stage (HH stage 9) the neural folds have completely fused in the head, and neural crest precursors that arose from the neural plate border come to lie in the dorsal neural tube and begin to emigrate. Many of the neural plate border and neural crest specifiers now mark pre-migratory neural crest in the dorsal neural tube and emigrating cranial neural crest cells. The expression domains of *Msx1* and *Pax7* are identical (Fig. 2.2K,L), marking neural crest progenitors in the head, trunk and tail. *Snail2* and *FoxD3* also exhibit similar expression patterns in emigrating cranial neural crest and trunk dorsal neural tube (Fig. 2.2M,N). However, they are not expressed in the open neural plate like *Msx1* and *Pax7*. At this time, *Zic1*, *Dlx3*, and *Dlx5* expression patterns are similar; their transcripts are almost exclusively restricted to olfactory progenitors in the anterior forebrain (Fig. 2.2O,R,S). However, *Zic1* is additionally expressed in a specific stripe in the hindbrain, and *Dlx3* is present in cranial placogenic ectoderm and prospective otic placode. *N-myc* is maintained in anterior neural tissue at high levels, and is also expressed in lateral plate mesoderm at the level of the heart and in the tail (Fig. 2.2P). *c-myc* is expressed in the forebrain and in emigrating neural crest cells at the level of the midbrain, where its expression is highly similar to that of *Snail2* and *FoxD3*. It is additionally expressed in the blood islands (Fig. 2.2Q). Finally, at this stage *AP-2* is recruited to the dorsal

neural tube at the cranial and vagal trunk levels. Its transcripts also persist in non-neural ectoderm adjacent to the open neural plate (Fig. 2.2T).

HH10

At the 9 to 11 somite stage (HH stage 10), migrating neural crest cells can be identified by their characteristic “cobra hood” pattern in the head. Many neural crest specifiers are expressed in these cells, including *Snail2*, *FoxD3*, *c-myc*, and *AP-2* (Fig. 2.2W,W',X,X',AA,AA',DD,DD'). They are also maintained in pre-migratory trunk neural crest. Likewise, the neural plate border specifiers *Pax7* and *Msx1* persist in migrating cranial and pre-migratory trunk neural crest cells, suggesting a role in maintenance of neural crest traits (Fig. 2.2U,U',V,V'). However, the other specifier genes are excluded from neural crest at this stage and are instead expressed in other cell types such as placodes (*Zic1*, *Dlx3*, *Dlx5* (Fig. 2.2Y,BB,BB',CC,CC')) and neural tissue and somites (*Zic1* (Fig. 2.2Y,Y')). *N-myc* is also not expressed by migrating neural crest cells despite transcripts being concentrated in the dorsal neural tube (Fig. 2.2Z,Z'). It has been shown that *N-myc* plays a role in neural crest migration at much later stages (Wakamatsu et al., 1997).

DISCUSSION

Expression patterns of ten transcription factors that are members of the chick NC-GRN were compared between HH stages 4 to 10. We show that transcripts of most neural crest specifiers are already expressed in or around the presumptive neural plate border at gastrulation stages. Thus, this work challenges our current formulation of gene interactions within the chick NC-GRN. The remarkable similarities in expression patterns of neural plate border and neural crest specifiers at gastrulation suggests that regulatory events are occurring much earlier than previously thought. For instance, the shared expression domain of *Zic1* and *FoxD3* hints at a direct interaction between these two molecules at HH4. In addition, the overall similarity in expression of most specifiers at the neural plate border and in the dorsal neural tube support data from ongoing studies that demonstrate extensive cross- and auto-regulation between these genes. Importantly, comparison of NC-GRN specifier expression patterns as they are resolved throughout development lays crucial groundwork for functional studies aimed at understanding the interactions between these transcription factors. Namely, such comprehensive and comparative expression data provide key information on when and where genes should be inactivated or activated during functional studies. Finally, resolving the overlap between all of these molecules brings us closer to defining the “neural plate border” and the location of neural crest progenitors within it, which is summarized in Fig. 2.5A. Interestingly, there is strong conservation of early expression patterns of neural crest network genes between chick and *Xenopus* (Fig. 2.5B), suggesting that early

specification of neural crest progenitors is likely to be conserved across vertebrates.

While comparing the expression patterns of neural plate border and neural crest specifiers at each stage of early neural crest development, we found some interesting trends in terms of which genes are maintained and which are differentially expressed from stage to stage. For instance, the canonical neural plate border specifiers *Msx1* and *Pax7* are continuously expressed in neural crest progenitors from gastrulation to migration, suggesting a role in both induction and maintenance of other neural crest genes. In contrast, *Zic1*, *Dlx5*, and *Dlx3* are expressed in or near the neural plate border region at early stages but are later recruited to other regions of the embryo, such as neural tissue, placodes, and somites. This suggests that they play several spatiotemporally separable roles during development, only one of which is specific to neural crest.

Changes in expression patterns of neural crest specifiers are even more interesting. *N-myc* is maintained in neural crest progenitors throughout development like *Msx1* and *Pax7*, but it is also expressed in other areas of the embryo, such as neural, ectodermal, and mesodermal tissues. Being a proto-oncogene, *N-myc* is likely playing a role in proliferation and maintenance of all of these progenitor cell types throughout development. It is surprising, however, that *N-myc* is not expressed in migrating neural crest cells at HH10 since those cells proliferate extensively. In contrast, although *AP-2* is continuously expressed in the gastrula and neurula, it does not definitively appear in neural crest progenitors until HH9, when it is recruited to the dorsal neural tube. Its early role as an ectodermal specifier is highly conserved among chordates, but its recruitment to the dorsal neural tube is a vertebrate-specific event. It is likely that

this event in vertebrates has occurred as a result of the evolution of a novel crest-specific *AP-2* regulatory element that is distinct from the element(s) driving its expression in the ectoderm. We hypothesize that it may also have an earlier function in neural crest development by maintaining ectodermal fate and repressing neuronal markers, therefore contributing to the placement of the prospective neural plate border. It has been shown that the *Dlx3/5* genes function to position the neural plate border in this manner (McLarren et al., 2003; Woda et al., 2003). In addition, we have also found that *AP-2* transcripts overlap in a small portion of the posterior neural plate border, suggesting a more direct role in the formation of this region. Likewise, *Snail2* is not expressed in the neural plate border at HH4 but instead plays a role in epithelial-to-mesenchymal transition of ingressing mesoderm cells during gastrulation (Fig. 2.4A; Nieto et al., 1994). *Snail2* transcripts begin to accumulate in dorsal neural folds only around early HH8 (Fig. 2.4E,E'). Intriguingly, we find two very specific stripes of *Snail2*-positive mesodermal cells directly under the forming neural plate border at HH stages 5-8 (Fig. 2.4B,B',C,C',D, E, E''). We conjecture that that *Snail2* may also play an early role in neural crest specification by participating in a feedback loop to maintain neural plate border specifier expression via signals from underlying mesoderm. The expression pattern of *c-myc* is intriguing in that it is present in the neural plate border and mesoderm progenitors at gastrulation, but is then exclusively expressed in mesoderm until HH8, when it strikingly appears in the anterior neural folds. Likewise, *FoxD3* is expressed in the neural plate at HH4, after which it becomes restricted to the notochord and does not definitively reappear in neural crest progenitors until HH8. This suggests that *c-myc* and *FoxD3* may play temporally distinct roles in specification and maintenance of cell fates.

These data are highly suggestive of the presence of distinct enhancer elements that drive expression of neural plate border and neural crest specifiers in temporally and spatially separable domains. Identification of such elements is key to elucidating the regulatory interactions between these genes as well as to providing insights into regulatory events that have facilitated neural crest evolution. In conclusion, these results represent an important first step in examining regulatory interactions between transcription factors in the NC-GRN and for comparative analysis with other species.

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Figure 2.1: Early expression of neural plate border and neural crest specifiers

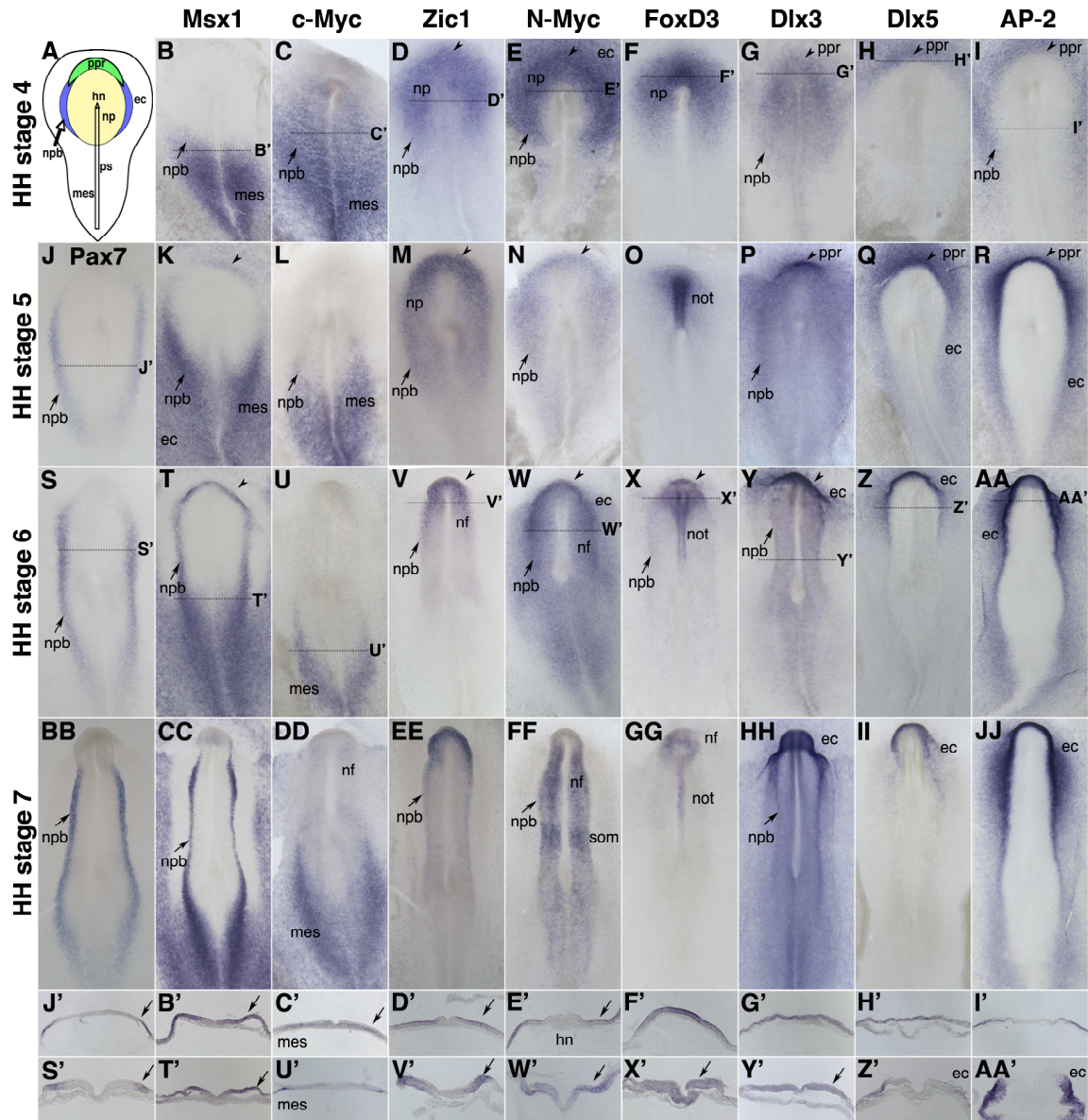


Figure 2.1. A-JJ: Neural plate border and neural crest specifier gene expression during early development. Hamburger and Hamilton (HH) stage 4 (B-I), HH5 (J-R), HH6 (S-AA) and HH7 (BB-JJ) embryos were analyzed by whole-mount *in situ* hybridization using digoxigenin (DIG)-labeled RNA probes for chick *Pax7* (J, S, BB), *Msx1* (B, K, T, CC), *c-myc* (C, L, U, DD), *Zic1* (D, M, V, EE), *N-myc* (E, N,

W, FF), *FoxD3* (**F, O, X, GG**), *Dlx3* (**G, P, Y, HH**), *Dlx5* (**H, Q, Z, II**), and *AP-2 α* (**I, R, AA, JJ**). Transverse sections were performed on HH4 (**B'-I'**) and HH6 (**S'-AA'**) embryos as indicated. A rough schematic of a HH4 embryo showing the respective positions of the primitive streak, Hensen's node, neural plate, neural plate border, pre-placodal region, non-neural ectoderm, and mesodermal progenitors is shown in **A**. Gene expression at the neural plate border and pre-placodal region is indicated by arrows and arrowheads, respectively. Ec, non-neural ectoderm; hn, Hensen's node; mes, mesoderm; nf, neural fold; not, notochord; np, neural plate; npb, neural plate border; ppr, pre-placodal region; ps, primitive streak; som, somite.

Figure 2.2: Neural crest network gene expression during late neurulation

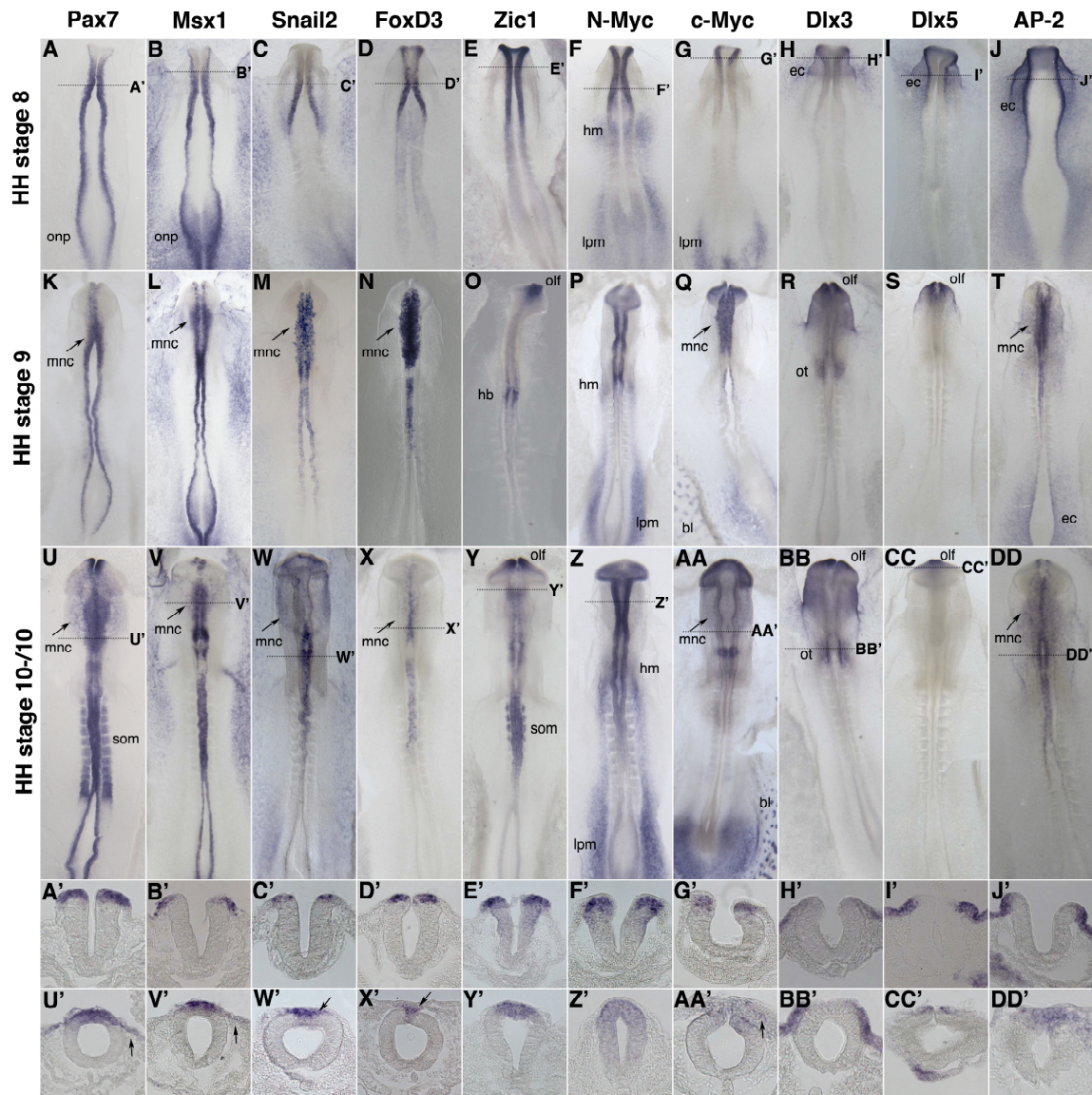


Figure 2.2. Neural plate border and neural crest specifier gene expression during neurulation and neural crest migration. HH8 (A-J), HH9 (K-T) and HH10-/10 (U-DD) embryos were analyzed by whole-mount *in situ* hybridization using digoxigenin (DIG)-labeled RNA probes for chick *Pax7* (A, K, U), *Msx1* (B, L, V), *Snail2* (C, M, W), *FoxD3* (D, N, X), *Zic1* (E, O, Y), *N-myc* (F, P, Z), *c-myc* (G, Q,

AA), *Dlx3* (**H, R, BB**), *Dlx5* (**I, S, CC**), and *AP-2a* (**J, T, DD**). Transverse sections were performed on HH stage 8 (**A'-J'**) and HH stage 10 (**U'-DD'**) embryos as indicated. Gene expression in migrating neural crest cells is indicated by arrows. Bl, blood islands; ec, non-neural ectoderm; hb, hindbrain; hm, heart mesoderm; lpm, lateral plate mesoderm; mnc, migrating neural crest; olf, olfactory placode/pit; onp, open neural plate; ot, otic placode; som, somites.

Figure 2.3: Overlap of gene expression at the neural plate border

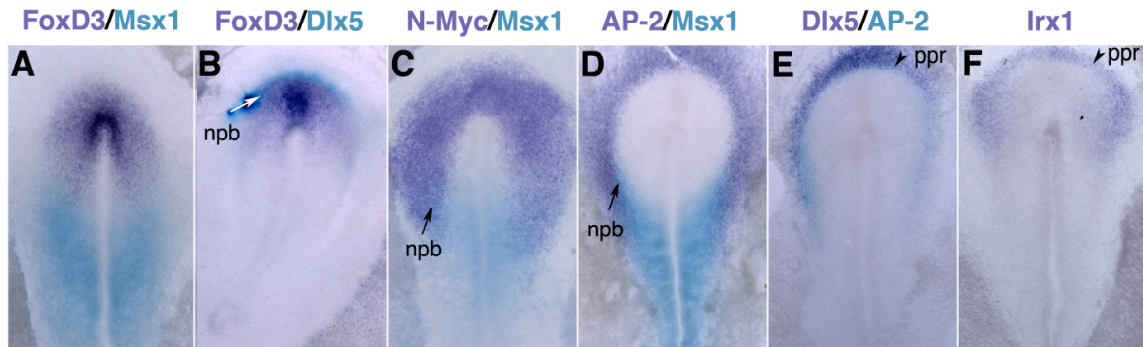


Figure 2.3. Co-expression of neural crest network genes in the chick gastrula. Stage HH4 or HH4+ embryos were analyzed by whole-mount double *in situ* hybridization using digoxigenin (DIG)- and fluorescein (FITC)-labeled RNA probes for chick *Msx1*, *AP-2 α* , *FoxD3*, *N-myc*, *Dlx5*, and *Irx1*. **A.** Expression of *FoxD3* and *Msx1* is complementary. *FoxD3* (purple) is expressed in anterior neural plate while *Msx1* (blue) is expressed in posterior epiblast and neural plate border. **B.** *FoxD3* expression domain (purple) in the anterior neural plate border (arrow) lies adjacent to and slightly overlaps with pre-placodal marker *Dlx5* (blue). **C.** *N-myc* (purple) is co-expressed in the posterior neural plate border (arrow) with *Msx1* (blue). **D.** *AP-2* (purple) is co-expressed in posterior neural plate border (arrow) with *Msx1* (blue). **E.** *Dlx5* (purple) and *AP-2* (blue) are co-expressed in the pre-placodal region (arrowhead). **F.** Expression of the placodal marker *Irx1* at stage 4+ is shown for comparison with E. Npb, neural plate border; ppr, pre-placodal region.

Figure 2.4: *Snail2* expression during early development

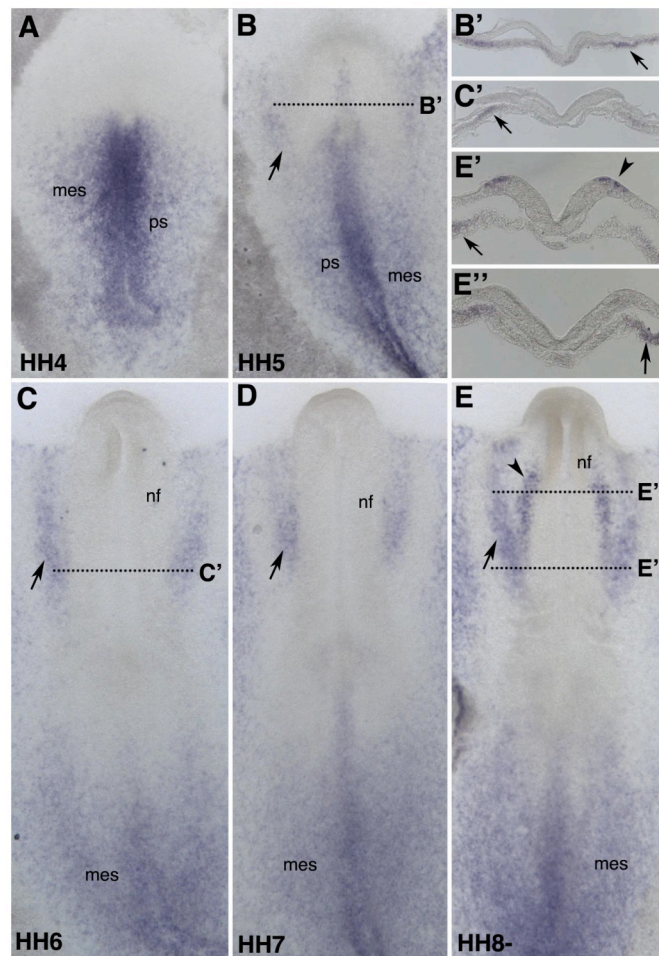


Figure 2.4. *Snail2* expression was examined in HH4-8- chick embryos by whole-mount *in situ* hybridization. **A.** At HH4 *Snail2* is expressed by mesodermal progenitors ingressing through the primitive streak. **B.** By HH5, two *Snail2*-positive stripes are observed in mesoderm beneath the neural plate border (**B'**). **C** and **D.** During HH6 and HH7, *Snail2* expression in mesoderm underlying the neural plate border becomes more obvious (**C'**). **E.** By early HH8, *Snail2* is upregulated strongly in dorsal midbrain neural folds (**E'**) and persists in the mesoderm(**E''**).

Figure 2.5: Conservation of early neural crest gene expression in vertebrates

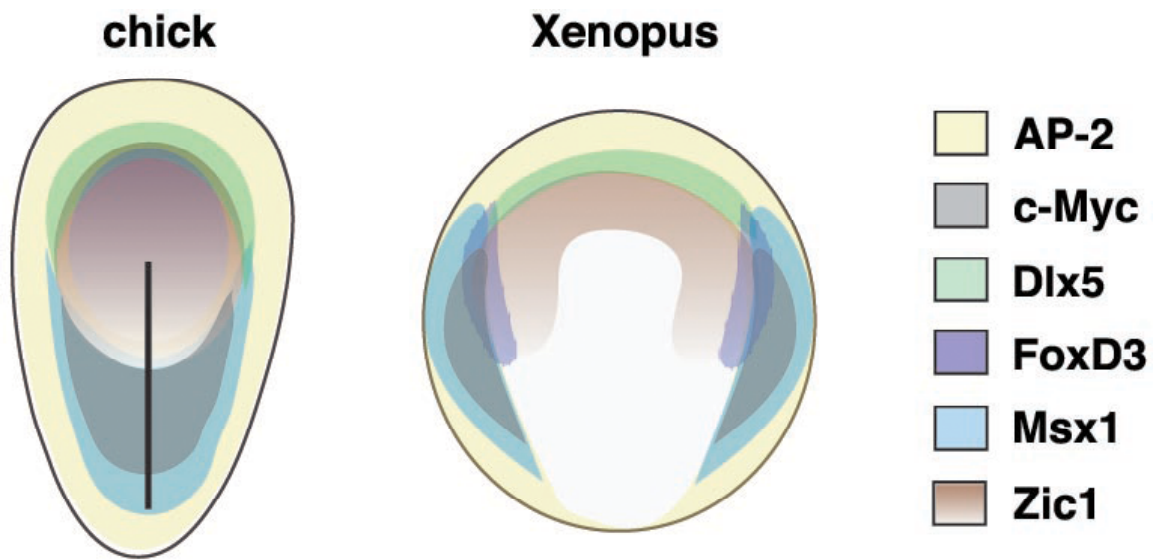


Figure 2.5. Schematic comparison of overlap in neural plate border and neural crest specifier expression domains during mid-gastrulation in chick and *Xenopus*. Expression of *AP-2* (yellow), *c-myc* (gray), *Dlx5* (green), *FoxD3* (purple), *Msx1* (blue) and *Zic1* (brown) are illustrated. During neurulation, expression of these genes overlaps in the neural plate border and dorsal neural tube in both organisms.