CHAPTER 3

Transcriptional Regulation at the Neural Plate Border

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Introduction

The neural crest (NC) is usually defined as a cell type arising at the border of the neural plate and the epidermis in vertebrate embryos. While accurate, this definition implies that the border exists as a distinct boundary, which is not really the case. Like other domains in the early embryo, the NC is not a sharply delimited territory, but rather is an overlapping zone of specification that has characteristics of both epidermis and neural plate, with additional characteristics of its own. This can be seen in the spatial pattern of regulatory factors that have been implicated in NC induction, many of which are shared.[1] For example Msx1 and AP2 are also expressed in the epidermis, at lower levels, and c-myc is likewise transcribed in neural plate in addition to NC. Nor is this limited to regulatory factors. The epidermal keratins, which constitute the intermediate filament cytoskeleton of epidermal cells are also expressed in NC, at a lower level than the epidermis. This can be seen by in situ hybridization (Fig. 1A) as a region of relatively weak but significant signal in the cranial neural crest region. Another indication of the fuzzy nature of NC comes from lineage mapping experiments in the chick embryo, which show that cells fated to differentiate as NC, neural plate, epidermis and placodal derivatives are all intermingled in the neural-epidermal boundary region.[2] Nor is there always a clear gap visible between neural plate and epidermal gene expression domains, for example in a double in situ with epidermal keratin and the pan-neural marker NCAM the two domains are contiguous (Fig. 1B). Expression of keratin genes is also evident in animal caps that have been dissected from embryos injected with BMP antagonists, such as chordin, along with a canonical Wnt signal molecule, such as Wnt3a. This strongly induces NC gene expression, but at the same time induces keratin gene expression, again to a level lower than that of epidermis, but much higher than the background seen in animal caps from embryos injected with BMP antagonist alone (Fig. 1C). The point of this discussion is that the premigratory NC is a somewhat ambiguously defined cell type, comprising overlapping and intermixed domains of gene expression, morphology and developmental fate. This should be kept in mind when thinking about the genes that control the NC program, i.e., regulatory mechanism giving rise to complex, overlapping and transient cellular identities will likely reflect this complexity. Indeed, many regulatory factors have been associated with NC,[1] a list that continues to grow. Even organizing the undoubtedly incomplete roster into a regulatory flow diagram results in a complex picture.[3] This review will focus primarily on two sets of transcription factors implicated in the earliest stages of NC induction, the Msx/Dlx and TFAP2 families.

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Neural Crest Induction and Differentiation, edited by Jean-Pierre Saint-Jeannet.

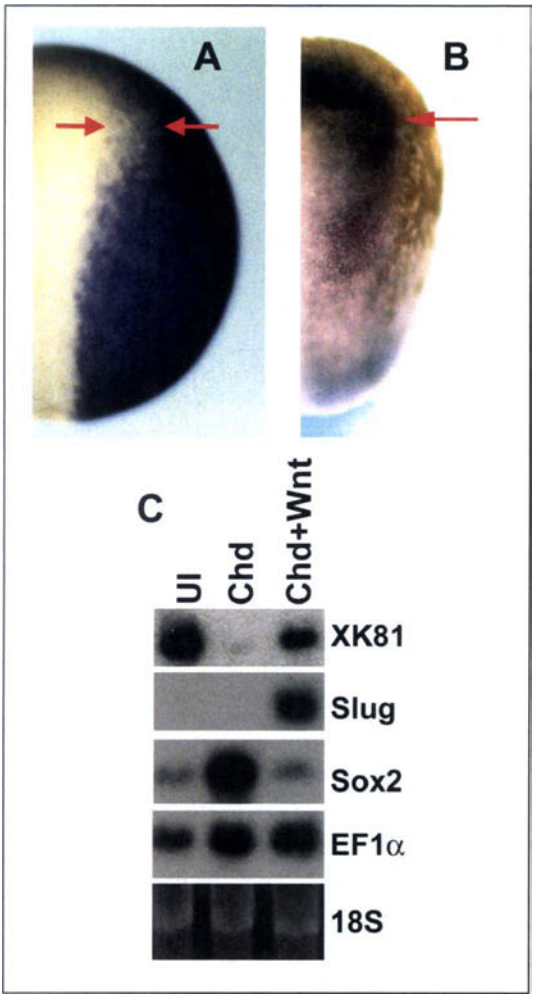

Figure 1. Overlap of epidermis and neural crest. A) Whole mount in situ hybridization to stage 14 (open neural plate) Xenopus embryo with an epidermal keratin probe (XK81). The arrows indicate the cranial NC region, where XK81 hybridization signal is clearly visible, although at a lower level than in the epidermis. B) Double in situ hybridization of similar staged embryo to neural plate (NCAM; purple color) and epidermal (XK81; brownish color) probes. The arrow indicates the cranial NC region, where no gap can be discerned between the epidermal and neural plate domains. Dorsal views, anterior towards top of figure. Panel B reproduced with permission from reference 61 and The Company of Biologists, Ltd. C) Northern blot analysis of an animal cap induction experiment. Embryos were injected with 250 pg of RNA encoding chordin (Chd) or a mixture of 250 pg each chordin and Wnt3a RNA (Chd+Wnt). Animal caps cultured to stage 14 show neuralization by Chd (Sox2 probe) and complete repression of epidermis (XK81 probe). NC is strongly activated by the chordin + Wnt3a treatment (Slug probe), which represses neural plate but also significantly reactivates the epidermal marker. EF1alpha and 18S RNA signals are shown as loading controls.

Dlx and Msx Genes

Dlx factors are homeodomain proteins, related to the Drosophila Distal-less (Dll) gene.[4] They behave as transcriptional activators, at least in coexpression assays.[5,6] In mouse and

human there are six Dlx genes, present in three pairs of convergently transcribed genes, each linked to a separate Hox cluster.[7] Only four Dlx genes have been described to date in Xenopus, of these three are known to be transcribed at the mid/late gastrula stage when NC is induced, these are Dlx3, Dlx5 and Dlx6.[8,9] Two other Dlx genes, Dlx1 and Dlx2, are also expressed in Xenopus NC, but later in development.[10] There are eight Dlx genes in zebrafish, with Dlx3b and Dlx4b representing the fish orthologs of the linked pair, Dlx3 and Dlx4, respectively.[11] There is no evidence for the existence of a Dlx4 ortholog in Xenopus either in EST databases or in the Xenopus tropicalis genome, which has been largely sequenced (http://genome.jgi-psf.org/Xentr3/Xentr3.home.html), so perhaps Dlx4 is absent in amphibian genomes. In mouse there are three Msx genes. These are also homeodomain factors, related to the Drosophila muscle segment homeodomain (msh) gene.[5] The Dlx and Msx homeodomains are quite similar, and bind to essentially identical DNA sequences in vitro.[6] Msx factors behave as transcriptional repressors. Msx1 can repress target gene transcription by binding to the TATA binding factor TBP,[12] and also by interacting with a linker histone H1b.[13] Importantly, Dlx and Msx factors can form heterodimers, and this interaction can antagonize the mutual effects on target gene activity.[14,15] In Xenopus, Msx1 and Msx2 are expressed beginning at or shortly following the midblastula transition, as are the Dlx genes. Both gene families are expressed in ventral and lateral tissues, Msx in mesoderm and ectoderm, and Dlx in ectoderm only.[5,9] This expression is regulated by BMP signaling. Msx1 is a direct target of such signaling, via a regulatory element in the 5' flanking DNA that associates with Smads factors.[16,17] Msx2 is likewise controlled by BMP signaling, and in the case of the mouse gene, the regulator element includes binding sites for both a Smads factor and an additional unknown protein that probably confers additional target specificity.[18] In contrast to Msx, the Dlx factors are not direct targets of the BMP pathway, requiring protein synthesis following BMP exposure.[6] In the ventral ectoderm, there is a graded dependence of Msx1, Dlx3 and Dlx5/6 on BMP signaling, which can be visualized by treating with increasing dosages of a BMP antagonist such as chordin. The lateral boundaries of Dlx3 and Dlx5/6 also differ, and this is consistent with the notion that a BMP signaling is locally weaker near the neural plate boundary compared to more laterally.[8]

There are several lines of evidence suggesting that Dlx and Msx play important roles in patterning the neural-epidermal border, including the NC. Before discussing these experiments, it is worth reviewing the tools that have been used in them. Gain of function has been accomplished by injecting synthetic mRNA into early cleaving embryos, which results in ectopic protein synthesis. For loss of function, two methods have been successfully employed; antisense and dominant negative overexpression. Most antisense work has been carried out with oligonucleotides based on morpholino backbone chemistry, which yields stable molecules that can base pair with target RNAs and either inhibit translation initiation or intron splicing. Dominant negative interference has been based on the concept that as transcriptional activators, Dlx factors can be antagonized by overexpressing derivatives in which the DNA binding domain has been fused to a transcriptional repressor, such as the repression domain from the Drosophila engrailed protein.[19] These experimental approaches can be quite informative, but they also have disadvantages and concomitant interpretational caveats. In the case of factor overexpression, the most obvious potential problem is that the level of the expressed protein is usually not known, and could be considerably higher than endogenous protein concentrations. This might lead to spurious interactions with genes or other factors, leading to regulatory artifacts. This is especially relevant to Dlx and Msx genes due to the similar DNA binding properties and opposite effects on target gene transcription. An illustrative example of this problem can be seen in the conclusion that Dlx5 functions in setting up the dorsoventral axis in Xenopus,[20] based on ectopic overexpression. Since Dlx5 is not expressed in mesoderm,[9] which is where the axial specification takes place, the ventralizing effect on this tissue is presumably an artifact.

Xenopus Dlx3 is excluded from the presumptive cranial neural crest domain, and when Dlx3 mRNA is delivered to this area by microinjection, repression of slug and other NC

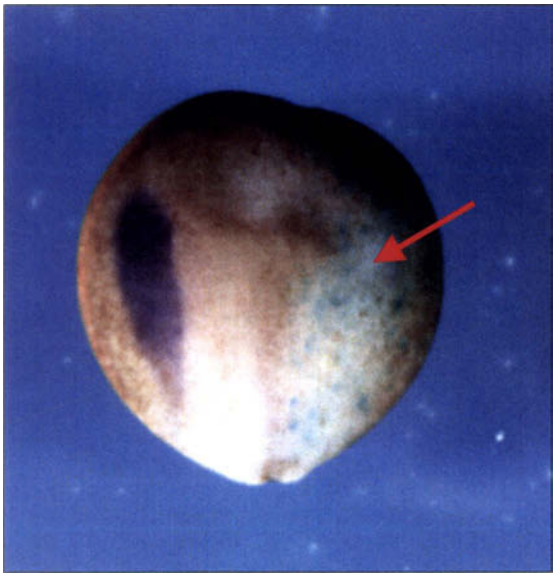

Figure 2. Repression of NC by truncated Dlx3. Stage 14 Xenopus embryo injected into one dorsal ectodemal cell at the eight-cell stage with 100 pg RNA encoding Xenopus Dlx3 that has been modified to delete amino acids 1-43 at the N-terminus and 200-277 at the C-terminus, leaving the homeodomain and about 60 residues N-terminal to it, and removing most of the transcriptional activation function of the Dlx3 protein.[6] LacZ RNA was also injected as a linear tracer (blue color). Hybridization with NC marker Slug shows complete inhibition on the injected side (arrow). Dorsal view, anterior towards top of figure. D. Khadka and T. Sargent, unpublished data.

markers is observed.[9] Dlx3 overexpression also inhibits expression of neural plate marker genes.[21] Dlx3 overexpression also antagonizes Wnt-beta catenin signaling, and this could be the basis for the negative effect on NC.[9,22] Similar results have been obtained using a zebrafish Dlx3 homeodomain fused to a VP16 activation domain, suggesting that this effect depends on target gene activation.[23] However, the inhibitory effect on NC is also observed with a truncated Dlx3 lacking the entire C-terminal and most of the N-terminal domains, a deletion construct that retains very little capacity to upregulate an artificial target gene[6] (Fig. 2). This makes it less likely that Dlx3 inhibits NC induction via a conventional transcriptional activation mechanism. Could Dlx3 be acting as an antagonist for Msx1 by competing for DNA binding, or interfering with Msx factors by protein-protein interactions? This model would predict a similar inhibitory effect for Dlx5, which is not what has been observed.[9] Furthermore, morpholino knockdown of Dlx3 alone does not seem to have much effect on the neural crest boundary, although there is a transient loss of some otic placode gene expression in Xenopus.[24] A similar effect on otic placode has also been observed in the zebrafish using morpholinos targeting Dlx3 and the linked Dlx7 genes,[25] with no obvious effect on neural crest boundaries. These results suggest that Dlx3 plays a role in ear development, but if this factor also functions in controlling the lateral limit of NC, there must be an alternate or alternates that are fully redundant. Dlx5 is not a good candidate in Xenopus, as its expression domain differs from that of Dlx3 in the NC. Another variation on the reduncancy theme is a model in which absence of Dlx3 from the NC domain is a permissive requirement for NC induction, while the limits of the induced NC domain could be determined by instructive signals.

Dlx5 is expressed more medially and overlaps with NC in the frog embryo, although this might be a bit misleading as the Dlx5-positive cells in the NC region are mostly limited to the

outer cell layer, whereas NC regulators such as Slug are mainly expressed in the inner, sensorial layer (Fig. 3). Overexpression of Dlx5 in Xenopus has not been tested extensively, although it has been shown to have little negative effect on NC induction compared to Dlx3 in animal cap assays.[9] In the chick embryo, ectopic Dlx5 expression, achieved by electroporation, alters the neural/nonneural boundary.[26] Artinger and colleagues used a dominant negative strategy to test Dlx function in frog embryos; the homeodomains from either zebrafish Dlx3 or Xenopus Dlx5 were fused to the engrailed repressor, intended to antagonize the normal activation function of Dlx factors. These constructs also included the ligand binding domain of glucocorticoid receptor to confer inducibility by dexamethasone.[27] This is important because mis-expression of Dlx factors in mesoderm results in severe axis disruption, as noted above. As already discussed, such a strategy also is essentially a combined loss of any and all Dlx function in the target embryos and tissues. In these experiments, both Engrailed fusions yielded loss of both neural plate and neural crest markers, or lateral shift of both.[23] This question has also been recently addressed in zebrafish by this group. In these experiments, loss of function was accomplished by the use of morpholinos designed to block translation or splicing of Dlx3a and Dlx4b, which are a linked pair in zebrafish and mammalian genomes. Other Dlx genes are not expressed at the relevant stages in the fish embryo.[28] Since these two genes have been shown to have largely redundant function in ear development,[25] they were treated in tandem in this study. Loss of Dlx3a and Dlx4b resulted in elimination of Rohon-Beard cells and trigeminal ganglia, and a rather slight effect on the neural crest, significant but considerably less extensive than what was observed using the dominant negative approach in Xenopus. Similar results were obtained with an Engrailed fusion in the zebrafish embryo, however, so this difference may reflect species individuality rather than pointing to a problem with the dominant negative approach per se. It would be interesting to see the effects of gene-specific knock-downs for Dlx genes in Xenopus, but this might require targeting three factors, Dlx3, Dlx5 and Dlx6, which might exceed the limits of morpholino oligonucleotides that the frog embryo can tolerate. However in the absence of such data, the interpretation of dominant negative overexpression in Xenopus should be considered somewhat tentative. In conclusion, Dlx genes are likely to be involved in the early specification of placodes, particularly the otic vesicle, but probably play a relatively minor role in NC induction, and are more important in fine-tuning the response to signals that determine the spatial limits of NC. Other regulators operate at a more upstream level in the initial formation of NC.

Two such factors may be the Msx1 and Msx2 homeoproteins. At the end of gastrulation, Msx1 and Msx2 RNAs, both of which are transcribed in epidermis, begin to accumulate at higher levels in the neural/epidermal border region. This pattern differs from that of most NC genes in that it is as strong or even stronger in the trunk region as in the cranial domain[29] (Fig. 4). This spatial difference suggests that the NC expression of Msx genes is controlled by somewhat different signaling compared to genes like Slug that are expressed more broadly in the anterior NC but in a narrow band in the trunk region. One possible difference is that Msx genes are more dependent upon canonical Wnt signaling, which would be expected to be locally stronger in the posterior, distant from Wnt antagonists elaborated by anterior tissues.[30] Using a microarray screening procedure, Willert et al[31] determined that in human embryonic carcinoma cells Msx1 and Msx2 were direct targets of this signaling pathway, and it is conceivable that this is also the case in the Xenopus embryonic NC. At any rate, the abundance (or conspicuous absence) of any regulatory factor in an embryonic domain is good preliminary evidence for a developmental role. This has been tested using the dominant negative approach by Mayor and colleagues.[29] These experiments were carried out in a similar manner to the Dlx experiments just described, except that to generate the negative interfering form of Msx1 the N-terminal third of the protein was deleted. Fusions to activation or repressor domains were not employed. As with the previous work, fusion to the glucocorticoid receptor ligand binding domain was used to enable dexamethasone induction, to avoid the axis disruption resulting from Msx1 overexpression during gastrulation. Overexpression of

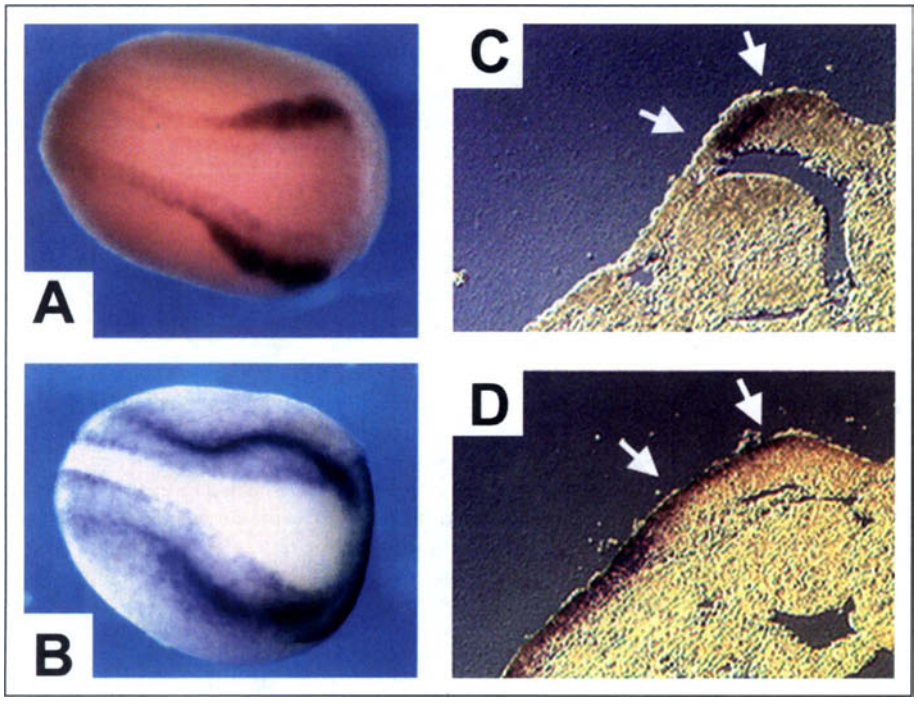

Figure 3. Deep versus superficial expression of Dlx5 and Slug genes. Panels A and B show whole mount in situ hybridizations of neurula stage Xenopus embryos to Slug and Dlx5 probes, respectively. Dorsal views, anterior to right. Transverse sections (dorsal side up) show that Slug is primarily transcribed in the deep ectodermal cells (C; arrows), whereas Dlx5 expression is restricted to the superficial cells in the NC domain (D; arrows). T. Luo and T. Sargent, unpublished data.

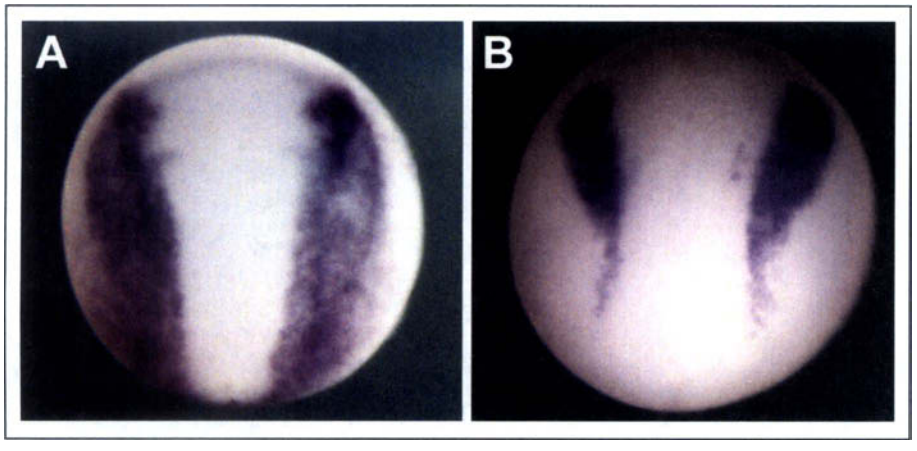

Figure 4. Comparison of Msx1 and Slug expression. Neurula stage Xenopus embryos hybridized in situ to Msx1 (A) and Slug (B) probes, showing that the Msx1 expression is much broader in the posterior region (bottom). D. Khadka and T. Sargent, unpublished data.

Msx1 in one side of Xenopus embryos after gastrulation resulted in an expansion of NC, visualized with FoxD3, slug and snail markers. This was accompanied by reduction in both epidermal (keratin XK81) and neural plate (Sox2) domains, although this effect is somewhat more difficult to appreciate due to the relatively large size of these domains compared to NC. The negative Msx1 had the opposite effect, reducing NC and expanding neural plate and epidermis. Interestingly, this loss of function phenotype could be significantly rescued by either slug or snail overexpression, while Msx1 was not able to rescue the effects of dominant negative forms of either factor. This suggests that Msx1 functions upstream from both slug and snail, possible acting as one of the first transcriptional effectors of NC induction. In subsequent work by Mayor and colleagues suggests that the transcriptional relationship between Slug and Msx ends by stage 15, after which time these two factors may act in an antagonistic manner to regulate apoptosis in NC cells.[32]

Thus we are left with the intriguing possibility that NC specification at the level of transcription is initiated by a repressor molecule. This of course raises the question of what is repressed, and how this in turn leads to the up-regulation of Snail and slug, which are also repressors. Another issue is that the use of dominant negative overexpression, as with the Dlx work, is open to the criticism regarding inappropriate targets and possible interference with other regulatory proteins. At this writing, we are completing a series of experiments on Msx1 and Msx2 loss of function using splice-inhibitory morpholino antisense oligonucleotides. These data confirm the importance of Msx genes in NC induction, although some aspects of NC seem to be independent of Msx function, as is epidermal development and dorsoventral axis specification (Khadka and Sargent, unpublished).

TFAP2

The transcription factor AP2 (TFAP2) is a family encoded by a total of five genes scattered at diverse sites in mouse and human genomes, designated alpha through epsilon, or TFAP2a, b, c, d and e. TFAP2 proteins have a basic helix-span-helix DNA binding and protein dimerization domain near the carboxy terminus. All five bind similar DNA elements with a consensus sequence of GCCNNNGGC[33] TFAP2 factors function as transcriptional activators via a proline/aromatic-rich activation domain located near the amino terminus. TFAP2 function also requires dimerization, and dominant interfering variants of TFAP2 have been generated by deletion of the activation domain, and also occur naturally, such as the variants associated with Char syndrome.[34] Members of the TFAP2 family have been implicated in many biological processes, including development and disease.[35] A number of directly regulated TFAP2 target genes have been identified, ranging in function from structural proteins such as keratin[36] to regulatory factors such as Hox genes.[37,38]

TFAP2a, the archetypical member of this family, has long been recognized as a characteristic factor expressed in neural crest.[39] Gene targeting in the mouse for TFAP2a was initially reported in 1996.[40,41] The null phenotype is a highly dysmorphic perinatal lethal. Among the most severely disrupted tissues were the facial bones, particularly the mandible, maxilla and frontonasal prominence. In addition, cranial ganglia and the heart outflow tract were abnormal. All of these derive largely from NC, supporting a role for TFAP2a in this lineage. TFAP2b and TFAP2c are also expressed in NC[35] but disruption of these genes does not have an apparent effect on NC induction or development in the mouse.[42,43]

An important aspect of the TFAP2a null phenotype is that the induction of NC and at least the early migratory processes do not seem to be affected much. Expression of Pax3 and Twist, two early NC markers, is essentially normal, and multiple aspects of NC migration can be observed.[41,44] Consequently, TFAP2a function in mouse appears to be more important in the terminal stages of NC cell differentiation.[40,41,44] Since TFAP2a is strongly expressed in NC, it might be expected that the loss of this factor would result in a largely cell-autonomous phenotype. However, this does not seem to be the entirely the case in the mouse, based on the results of a conditional TFAP2a knock out phenotype.[45] These embryos were generated by crossing a

line containing an TFAP2a allele including lox recognition sites with mice expressing Cre recombinase driven by a Wnt1 promoter,[46] resulting in ablation of TFAP2a expression in NC lineages (floxdel). Floxdel mice exhibited deficiencies in NC derivatives, reminiscent of the TFAP2a null phenotype, but with lower penetrance and with generally less severe craniofacial defects. For example, palatal shelves formed and elevated, but did not fuse, whereas in TFAP2a null pups these structures are absent.[40,41] Also, cranial ganglia were not affected by NC-specific TFAP2a loss, in contrast to severe reduction in the TFAP2a null phenotype. The less drastic phenotypic of floxdel TFAPa pups enabled further analysis of NC derivatives. For example, surviving floxdel pups exhibited defects in melanocytes that could not be evaluated in the original TFAP2a nulls due to the early lethality.[45] Similarly, the stapes bone, absent in the TFAP2a null, was particularly reduced compared to other middle ear ossicles. This is interesting in light of the TFAP2a null phenotype in zebrafish, discussed below. The most important lesson from these experiments is perhaps that TFAP2a functions in NC not only in a cell autonomous fashion, accounting for the similarities between the original null and the NC floxdel null phenotypes, but also in a noncell autonomous manner, accounting for the differences. Thus TFAP2a must regulate the expression of genes in NC-adjacent tissues encoding signaling factors that influence the terminal differentiation of NC cells. The identification of these TFAP2a-dependent genes and signals will be an exciting area of research in the coming years.

In zebrafish embryos, TFAP2a is first expressed in nonneural ectoderm, then is strongly upregulated in the NC. Interestingly the NC expression domain overlaps only partially with broadly expressed NC markers like Foxd3 and Sna2, which contrasts with the pattern in Xenopus (see below) and could account for some of the interspecies differences in loss of function phenotypes. TFAP2a is also expressed in intermediate and lateral plate mesoderm, which is not the case in Xenopus. Mutagenesis screens for zebrafish embryonic craniofacial anomalies led to the identification of two allelic recessive lethal mutations, montblanc (*mob*)[47] and lockjaw (*low*),[48] both of which are due to loss of function point mutations in the zebrafish TFAP2a gene.[49,50] The TFAP2a null phenotype in zebrafish is similar to the floxed TFAP2a mouse. The zebrafish TFAP2a null craniofacial skeleton is dysmorphic, but this is less devastating than in the TFAP2a null mouse and is mainly restricted to hypoplasia of derivatives of pharyngeal arches 2 (hyoid) through the branchial (gill) arches. Particularly affected is the hyosymplectic cartilage, which develops into a supporting bone in teleosts, but is the precursor to the stapes ossicle in mammals, and also greatly reduced in the floxed mouse. Also, like the floxed TFAP2 mouse, the zebrafish mutants exhibit reductions in pigments cells. This supports a phylogenetically conserved function for this factor in NC development, but implies some differences in the level of functional redundancy or the status of TFAP2a within the regulatory framework.

Both *low* and *mob* embryos have been analyzed using in situ hybridization with several molecular markers of NC, and the results are in basic agreement. Some early markers such as foxd3 and sna2 are not much affected, while others such as crestin and Sox9 show fairly conspicuous reductions in certain subdomains. At later stages migratory markers such as Dlx2 and EphA4 are reduced in the affected hyoid and more posterior arch NC.[38,49,50] This suggests that TFAP2 plays a less essential role during the early phases of NC development compared to later differentiation stages, and differs in function in different subsets of the NC. In the absence of TFAP2, affected NC cells undergo apoptosis and the target structures are absent or hypoplastic, similar to the mouse knockout data.

The question of cell autonomy for TFAP2 has been addressed in zebrafish by cell transplantation.[50] Wild type NC cells transplanted into the region of NC that gives rise to second pharangeal arch migrated and differentiated normally, and yielded some rescue of tissue missing in mutants. Mutant cells transplanted into wt recipients for the most part did not migrate normally into the branchial arches or contribute to cartilage. These results are consistent with the conclusion that TFAP2 functions in a subset of NC, but in a cell-autonomous manner. This is somewhat in contrast to the mouse, but an exact comparison is difficult due to the differences in experimental approaches.

In Xenopus the TFAP2 family is not as well characterized. All of the existing data have been obtained with the ortholog of TFAP2a. Other TFAP2 family members exist, however, represented as ESTs in Xenopus laevis and X. tropicalis databases. As of this writing our laboratory has characterized TFAP2b and TFAP2c, both of which are expressed at approximately the same time and place as TFAP2a (Y. Zhang, T. Sargent, unpublished). TFAP2a is first expressed throughout the ectoderm in Xenopus, then becomes cleared from the prospective neural plate during gastrulation. By the end of gastrulation TFAP2a is up-regulated in NC, but still remains expressed at a significant level in the epidermis, which continues throughout development.[36] Loss of function experiments have been done using both antisense and dominant negative approaches. Morpholino antisense oligonucleotides injected into one blastomere at the two-cell stage resulted in substantial, inhibition of Slug and Sox9 expression[51] but had little if any effect on Pax3, another early NC marker. Similar results have been obtained with a dominant negative TFAP2a lacking the activation domain. These results suggest that TFAP2a may be essential for early induction events in Xenopus, contrasting with zebrafish and mouse, where loss of TFAP2a leaves induction and migration largely unaffected. Another apparent difference between Xenopus and the other two species is the effect of global loss of TFAP2a, accomplished by radial injection of antisense olignonucleotide. These experiments were done using diethylethylene diamine (DEED) antisense oligonucleotides, which unlike the morpholino antisense strategy results in RNaseH-dependent cleavage of target RNAs.[52,53] Such embryos failed to gastrulate. The ectoderm was unable to engulf the mesoderm and collapsed into a convoluted mass on the animal hemisphere. This phenotype could be largely rescued by coinjection of a TFAP2a derivative with silent mutations rendering it resistant to the DEED oligonucleotide. A similar phenotype was also obtained using dominant negative TFAP2a. The gastrulation failure was accompanied by strong inhibition of epidermal marker genes (keratin). The dominant-negative TFAP2a, in which the activation domain had been replaced with a repressor domain from the Drosophila engrailed protein, also resulted in activation of neural genes concomitant with repression of epidermis. This was not observed with the DEED oligonucleotide, presumably indicating incomplete destruction of TFAP2a RNA. Thus in Xenopus it appears that TFAP2a is required for induction of NC, and also required earlier for epidermal function and gastrulation. One possibility is that the early loss of some NC markers in TFAP2a-inhibited embryos is a consequence of blocking the production of one or more inductive signals from epidermis that are needed for NC induction. This could be tested by targeting either a dominant negative TFAP2 or antisense specifically to the NC, avoiding expression in adjacent epidermis. This is impractical in Xenopus—even at the 32-cell stage, single blastomeres will give rise to both of these tissue types. In any case it is clear enough from the experiments outlined above that in the mouse there are TFAP2-dependent signals necessary for NC development that emanate from outside the NC, while this is apparently not the case in zebrafish based on the cell transplantation data. Thus it seems likely that TFAP2a, and perhaps other TFAP2's may function somewhat differently in various vertebrates.

In Xenopus it is relatively easy to carry out gain of function experiments by injecting synthetic mRNA into fertilized eggs, or into blastomeres fated to become particular tissues. TFAP2a RNA injected into one dorsal ectodermal blastomere at the 8 or 16 cell stage resulted in the expansion of Slug and Sox9 expression into the neural plate, accompanied by downregulation of neural markers indicated a neural to NC transfating had taken place as the result of ectopic TFAP2a activity.[51] This was also accomplished in animal cap explants. In these experiments, NC induction was achieved first by injecting synthetic mRNA for Wnt3a and for chordin.[54,55] When the chordin concentration was increased to very high levels, sufficient to extinguish BMP signaling entirely, as indicated by the loss of Dlx5 expression, the explanted ectoderm reverted to a posterior neural identity, as indicated by loss of all NC markers and activation of Sox2 expression. If the same treatment was performed with TFAP2a RNA added to the cocktail, the response was largely reversed. Most NC marker

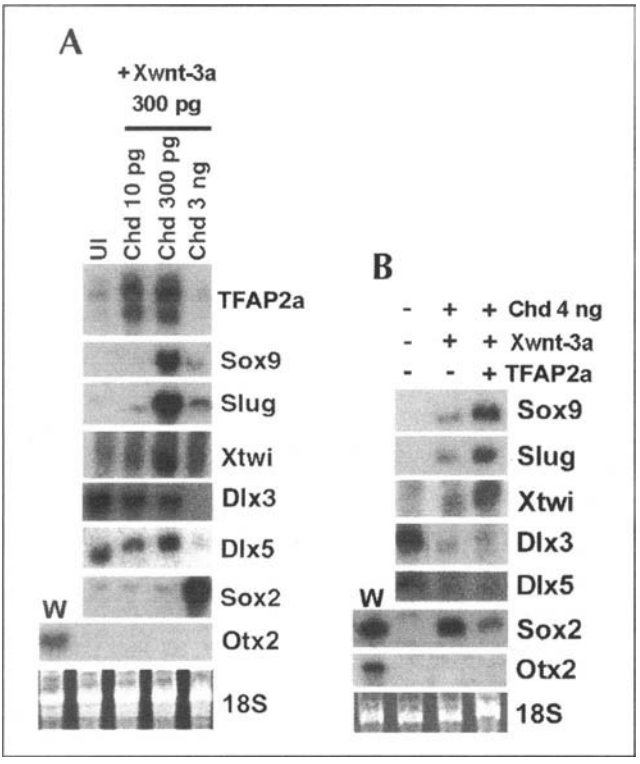

Figure 5. Dependence of NC induction on BMP signal strength and AP2 in Xenopus animal cap experiments. A) Increasing doses of RNA encoding chordin (Chd) from 10 pg to 3 ng, injected along with RNA encoding Wnt-3a induces NC gene expression (TFAP2a, Sox9, Slug, Xtwi) and represses Dlx genes and neural gene expression (Sox2, Otx2), except at the highest dose of Chd, which results in induction of the pan-neural Sox2, but not the anterior neural Otx2 genes, and silences the NC genes. B) Addition of TFAP2a reverses the NC to neural plate transition shown in (A). Modified from Luo et al, 2003.[51]

genes became active, neural genes were silenced (Fig. 5). This supports a model in which TFAP2a mediates the attenuated BMP signal which, in conjunction with a canonical Wnt signal (or FGF,[56] induces NC).

NC Regulatory Pathways Problems and Prospects

Does TFAP2 fit into a hierarchy of NC regulators? A good place to start is the epistatic pathway proposed for Xenopus by Mayor and colleagues in which Msx1 is upstream from Snail, followed by Slug.[29] In animal caps primed with Wnt/beta catenin signaling, and in embryonic neurectoderm, TFAP2a can induce Slug. There are no data regarding Msx1 or Snail, so for the sake of discussion we can place TFAP2a between Snail and Slug. TFAP2a is not a direct target of BMP signaling since it is not induced by BMP in the presence of cycloheximide,[36] and therefore there is presumably at least one activator factor upstream from TFAP2a that is synthesized in response to BMP. Both Msx1 and Snail are repressors, so this hypothetical TFAP2a activator (HTA) must be inserted in the pathway, i.e., downstream from Snail. Furthermore, as a repressor, the simplest way for Snail to activate HTA would be to repress a repressor of HTA (RHTA). Likewise, for Msx to positively regulate Snail, a repressor of Snail (RS) could be inhibited by Msx. This gives the following linear

pathway: Msx1 -| RS -|Snail -| RHTA -|HTA -> TFAP2 -> Slug. Additional steps downstream from Slug would presumably entail another repressed repressor event. Nor is it clear that another step is not required for TFAP2 to activate Slug. This pathway is complicated further by the apparent direct regulation of Slug by Wnt beta catenin signaling,[57] and the possibility that Msx is also a beta catenin target (see above). This is a complex event chain, all of which must take place within a fairly brief time interval, between late gastrula and early neurula. Indeed, it is difficult to discern any temporal differences in the expression of any of these known genes in the NC domain. Some steps, such as repression, could occur by protein interactions that might be essentially instantaneous, but it would seem that the linear model is probably too slow, cumbersome and too simple to account for all the available information.

Another argument against linear control of NC is the differences that exist between species. For example, Slug and Snail differ in expression pattern and presumably in function in various vertebrates.[58] Likewise, Msx1 and Msx2 null phenotypes in mouse are quite different,[59,60] and neither results in massive loss of NC. This could reflect redundancy, but could just as easily be due to differences in function in mouse compared to frog. There are other examples, and as data become available for orthologous genes in multiple species, this number will probably increase. A strict linear pathway would be inherently less tolerant of evolutionary change, compared to a network with plenty of redundancy and positive and negative feedback. Altering the status of one or a few regulatory factors in such a network could lead to alterations in the end product, as opposed to its elimination. This might help explain the tremendous variation in facial morphology among the vertebrates, a factor that has contributed very significantly to the adaptive success of this phylum.

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