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Pax3 and Zic1 trigger the early neural crest gene regulatory network by the direct activation of multiple key neural crest specifiers

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Abstract

Neural crest development is orchestrated by a complex and still poorly understood gene regulatory network. Premigratory neural crest is induced at the lateral border of the neural plate by the combined action of signaling molecules and transcription factors such as AP2, Gbx2, Pax3 and Zic1. Among them, Pax3 and Zic1 are both necessary and sufficient to trigger a complete neural crest developmental program. However, their gene targets in the neural crest regulatory network remain unknown. Here, through a transcriptome analysis of frog microdissected neural border, we identified an extended gene signature for the premigratory neural crest, and we defined novel potential members of the regulatory network. This signature includes 34 novel genes, as well as 44 known genes expressed at the neural border. Using another microarray analysis which combined Pax3 and Zic1 gain-of-function and protein translation blockade, we uncovered 25 Pax3 and Zic1 direct targets within this signature. We demonstrated that the neural border specifiers Pax3 and Zic1 are direct upstream regulators of neural crest specifiers Snail1/2, Foxd3, Twist1, and Tfap2b. In addition, they may modulate the transcriptional output of multiple signaling pathways involved in neural crest development (Wnt, Retinoic Acid) through the induction of key pathway regulators (Axin2 and Cyp26c1). We also found that Pax3 could maintain its own expression through a positive autoregulatory feedback loop. These hierarchical inductions, feedback loops, and pathway modulations provide novel tools to understand the neural crest induction network.

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Keywords

Neural crest; Pax3; Zic1; gene regulatory network; transcriptome; microarray; embryo; *Xenopus laevis*

INTRODUCTION

Patterning the embryo implies the precise orchestration of gene activities in time and space. This involves coordinated transcriptional and posttranscriptional regulations. Despite advances in the inference of complex transcriptional gene regulatory networks in invertebrate embryos (Busser et al.; Gohlke et al., 2008; Hertzano et al., 2011; Isern et al.; Lagha et al., 2010; Taher et al., 2011), this task remains challenging for early vertebrate embryogenesis. We focus on vertebrate neural crest induction, in which early transcriptional regulators activate a complex developmental network, and in which transcriptome analysis can be combined with *in vivo* experimental validation.

The neural crest arises between neural plate and epidermis at the “neural border”. Neural crest progenitors undergo an epithelial-to-mesenchymal transition (EMT) and generate migratory cells that populate many tissues and organs in the embryo. The neural crest cells form the peripheral nervous system, pigment cells, craniofacial cartilage and mesenchyme, endocrine cells and other derivatives (Le Douarin and Kalcheim, 1999). While neural crest migration and differentiation have been studied extensively, the molecular mechanisms that initiate neural crest development within the dorsal neural tube have remained elusive until recently. The neural border, which contains both neural crest and dorsal neural tube progenitors, is first patterned under the activity of secreted signals coming from the surrounding tissues: ectoderm, mesoderm, neural plate and notochord. FGF, Wnt and BMP signaling activate or enhance the expression of a first set of essential genes named the neural border specifiers (Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Monsoro-Burq et al., 2003; Saint-Jeannet et al., 1997; Villanueva et al., 2002, reviewed in Milet and Monsoro-Burq, 2012). These neural border specifiers include the transcription factors Pax3, Pax7, Gbx2, Msx1, Zic1, AP2, and Hairy2, which are essential for further neural crest development but not always maintained in the neural crest progenitors themselves (Basch et al., 2006; Li et al., 2009; Luo et al., 2003; Maczkowiak et al., 2010; Monsoro-Burq et al., 2005; Nichane et al., 2008; Sato et al., 2005). The combined activity of the neural border specifiers establishes a robust neural border territory during gastrulation (Basch et al., 2006; de Croze et al., 2011; Li et al., 2009). Some will then specifically induce the premigratory neural crest during neurulation (reviewed in Pegoraro and Monsoro-Burq, 2012). We have shown recently that Pax3 initiates neural crest development from pluripotent ectoderm, most efficiently when it is co-expressed with Zic1. Pax3 and Zic1 expressed together are sufficient to drive premigratory neural crest induction, EMT, migration and differentiation of multiple neural crest derivatives while Pax3 expression alone drives a modest induction, migration and differentiation (Milet et al., 2013).

To decipher the transcriptional responses activated by Pax3 and Zic1 during neural crest induction, we focused on genes activated as immediate early targets, i.e. in the absence of protein synthesis (Sive et al., 1984). Furthermore, since Pax3 and Zic1 also play roles in the development of other tissues such as muscles and cerebellum respectively (Nagai et al., 1997; Nakata et al., 2000; Nakata et al., 1997; Nakata et al., 1998; Relaix et al., 2004; Tremblay et al., 1998; Tremblay et al., 1996; Zhou et al., 2008), we also defined a large gene signature of the neural border and of the premigratory neural crest. This molecular signature provides the Pax3 and Zic1 targets likely to be relevant for neural crest

development. In addition, we assayed Pax3 either alone or together with Zic1, to determine whether they activate separate sets of target genes that would then cooperate, or if some novel targets are activated only when the two factors are combined. Finally, we asked whether Pax3 and Zic1 induced a subset of neural crest specifier genes, which would in turn switch on secondary targets, or if Pax3 and Zic1 simultaneously activate a large set of neural crest specifiers.

MATERIALS AND METHODS

Embryos, explants, in vivo injections and treatments

Xenopus laevis embryos were obtained using standard procedures (Sive et al., 2000). Neurula stage 12, 14 and 18 control embryos were used for neural plate, neural border and neural crest dissection (Figure 1A). For microinjections, two-cell stage embryos were injected into both blastomeres, aged until blastula stage 9 when the animal-most third of the animal hemisphere (the animal cap) was cut. Animal caps were aged in 3/4 Normal Amphibian Medium until the desired stage (Sive et al., 2000). Capped mRNAs for the previously described inducible pax3GR and zic1GR constructs (Hong and Saint-Jeannet, 2007) were synthesized *in vitro* using mMessage mMachine kits (Ambion). Pax3 and Zic1 antisense morpholino oligonucleotides were validated previously (de Croze et al., 2011; Monsoro-Burq et al., 2005). For neural crest induction in animal cap pluripotent ectoderm in the absence of protein synthesis, inducible Pax3 and Zic1 mRNAs were injected in whole embryos at the 2 cells stage as described previously (Hong and Saint-Jeannet, 2007; Milet et al., 2013). Animal caps were cut at stage 9. Cycloheximide (0,1mg/ml) was then applied to the healed animal caps, from stage 10 to 10.5 (i.e. for 30 min at 23°C), then dexamethasone (Kolm and Sive, 1995) was added at stage 10.5 to the cycloheximide-containing medium (Sive et al., 1984). Explants were rinsed and lysed after two additional hours at 23°C, i.e. when sibling embryos reached stage 11.5–12.

Microarray data analysis

For all microarray analysis, biological replicates were obtained from ~80 manually dissected explants from sibling embryos. Total RNA was extracted with Trizol (Invitrogen) and purified according to a standard protocol (Lin et al., 2004), modified to include a proteinase K digestion step to remove yolk phospholipoproteins. The detailed modified protocol is described in Supplemental Materials. Ten micrograms of total RNA were used to prepare cDNAs and labeled RNA probes that were hybridized to *Xenopus laevis* Affymetrix GeneChips using the standard Affymetrix protocol (UC Berkeley Affymetrix facility). *Xenopus laevis* 1.0 GeneChips were used to measure the neural border transcriptome and *Xenopus laevis* 2.0 GeneChips were used for the Pax3/Zic1 targets experiment. Raw expression level for each probeset was normalized with RMA (Robust Multi-array Average, Bioconductor Affy package) prior to analysis. The R programming environment with Bioconductor and LIMMA packages (Gentleman et al., 2004; Smyth, 2004), was used for the analysis. Probesets were selected according to the following criteria: expression threshold >7; significant differential expression between samples (logFold >1 and pvalue <2.5%). For hierarchical clustering, the data matrix was preprocessed by averaging gene expression over biological replicates of the same tissue, then centering and normalizing the expression vector. Hierarchical clustering was then computed using the centroid linkage algorithm and correlation distance as implemented in Gene Cluster 3.0 (de Hoon et al., 2004). Annotation of the Affymetrix Genechips was obtained from Xenbase (www.xenbase.org) and Affymetrix (www.affymetrix.com). Unannotated probesets were identified by similarity searches in the *X. laevis* genome (www.xenbase.org, version 6). Functional annotation with PIR keywords was conducted with DAVID (david.abcc.ncifcrf.gov) using the more thorough human orthologs annotations. The effect of Pax3 and Zic1 expression on the

blastocoel roof ectoderm transcriptome was modeled using the limma package taking into account biological replicate effect. Genes significantly overexpressed were identified using the limma package. Enrichment of neural crest signature genes in the identified targets was assessed using a chi-square test.

In situ hybridization

Embryos were stained by whole mount in situ hybridization using a procedure optimized for neural and neural crest tissues (Monsoro-Burq, 2007). They were then bleached, postfixed, and imaged using a MZFLIII stereoscope (Leica), Photoshop CS, and CombineZP software (hadleyweb.pwp.blueyonder.co.uk). cDNAs for novel genes were either purchased from Open Biosystems or cloned by nested PCR and inserted into the pGEM-T Easy vector (Promega). Primers and clone references are shown in Table S1.

RT-PCR, RT-qPCR, Electrophoresis Mobility Shift Assay (EMSA)

Reverse transcription was followed by semi-quantitative radioactive PCR or quantitative PCR. *Odc* was used as a reference to normalize measurements. Primers for *axin2*, *c3*, *cyp26c1*, *dusp5*, *ets1*, *pax3*, *pdgfra*, *snail1*, *snail2*, and *twist1* are described in Table S1.

Xenopus tropicalis genome browsers, Transfac, Jaspar, rVista and MacVector analyses (generegulation.com; jaspar.genereg.net; rvista.dcode.org; macvector.com) were used to identify putative Pax3 or Zic1-binding sites in *pax3*, *snail1* and *snail2* genome sequences upstream their transcription start site (TSS). 50-base length primers containing the putative binding site as well as their corresponding mutants (Table S1) were designed and used to perform EMSA according to standard procedures. Pax3 and Zic1 proteins were produced in HEK-293 cells, and lysates obtained by cryolysis. Controls were GFP-transfected cells. Pax3 monoclonal antibody (R&D Systems) and a nonspecific control antibody (Sigma P0498) were used for supershift assays.

RESULTS

Identification of a novel gene signature for premigratory cephalic NC

Five types of tissue samples were collected to identify neural crest signature transcripts, focusing on premigratory cephalic NC: the cephalic lateral neural border at stage 14 (3 biological replicates), the premigratory cranial neural crest at stage 18 with its overlying ectoderm (2 biological replicates), the anterior neural border at stage 18 (3 biological replicates), the neural plate including its lateral neural border at stage 12 (2 biological replicates), and animal cap explants cut at stage 9 and grown until stage 14 (1 sample) (Figure 1A). Transcripts were first selected using two threshold criteria (Figure 1B). (Criterion 1) Transcripts were required to exceed a defined expression threshold (7.0) in at least one neural border/NC sample. This threshold was selected because it is met by known neural crest regulators (*pax3*, *zic1*, *msx1*, *snail2*, *foxd3*), in order to select only robustly expressed genes. (Criterion 2) Transcripts needed to be significantly overexpressed (at least 2-fold with limma package) in neural border/NC samples (at stage 14 or 18) in comparison with anterior neural fold (stage 18) samples, which do not form neural crest. 206 genes met these criteria. Additionally, we grouped genes with similar expression in all tissues collected using hierarchical clustering. The cluster containing the known neural crest regulators *foxd3*, *pax3*, *snail1*, *snail2*, and *zic1* was considered as the putative neural crest signature cluster.

This cluster contains 83 genes including 44 (53%) genes known to be expressed at the neural border or in the neural crest (Table S2). In addition, the cluster contains 34 (41%) novel genes whose expression during neural crest induction has not been previously described (Table S2). Finally, five (6%) genes show no expression overlap with the neural border and

neural crest, three being expressed in the ectoderm overlying the neural border that was included in the dissections, and two being actin genes expressed in the underlying mesoderm. Functional annotation shows that DNA binding proteins, the Wnt signaling pathway, and proteins containing extracellular domains are significantly enriched in this cluster (Table 1 and 2).

We next validated a subset of the predicted early NC signature genes, by analyzing their expression pattern *in vivo*. Gastrula, neurula, and tailbud stage embryos were analyzed and compared to *pax3* and *snai2* expression (Figure 2 and data not shown). Transcripts for all the predicted genes were found robustly expressed at the lateral neural border or in the neural crest. Specifically, we found a first group of genes, including *greb1L*, *phnd*, and *tcf7*, which are enriched at the neural border in early neurulae. These transcripts are then either maintained in the dorsal neural tube and NC (*greb1L*, *tcf7*, Figure 2 E–F, I–J) or later excluded from the NC (*phnd*, Figure 2 G–H). This dynamic expression is similar to that of neural border specifiers such as *pax3* or *msx1* (Figure 2A–B, Monsoro-Burq et al., 2005). The second group of genes was expressed at later neurula stage: the stage 18 premigratory neural crest expressed *hapln3*, *kall1*, *mfap2*, and *odz4* (Figure 2 K–P and S–T). *Mmp28* transcripts were found at the edge of the neural crest domain (Figure 2 Q–R). At tailbud stage, *kall1* and *mfap2* were robustly expressed by the migrating cranial neural crest, while *odz4* and *mmp28* were found as discrete lines in the craniofacial structures (Data not shown). *Hapln3* remained expressed in the neural tube and around the eyes. Altogether, these observations validate the accuracy of the predicted gene signature for neural border and premigratory neural crest. This analysis thus provides 34 novel candidates for future analyses of NC development. Here, we have used this gene signature to identify gene targets of the two major neural crest inducers, Pax3 and Zic1.

Identification of putative Pax3 targets within the NC gene signature

To identify immediate-early targets of Pax3 and Zic1 within the neural crest signature using a transcriptomic approach, we analyzed 3 biological replicates for each of the following conditions: uninjected animal caps, animal caps injected with Pax3GR alone; or animal caps injected with Pax3GR and Zic1GR. We treated explants with cycloheximide, a translation inhibitor, to prevent secondary target induction by Pax3 and Zic1 targets, and with dexamethasone that induces nuclear translocation of GR fusion proteins (Figure 3A). We found 450 transcripts enriched more than 2-fold (but less than 4-folds) and 160 enriched more than 4-folds when Pax3GR was induced in the presence of cycloheximide, compared to the uninduced ectoderm (Figure 3B). Among these 610 transcripts, 21 belonged to the neural crest signature defined above. This represents a significant enrichment ($p < 0.001$) of neural crest signature genes among Pax3 targets. Among these, we found several known neural border specifiers (*tfap2b*, *pax3*, *zic1*) (reviewed in Pegoraro and Monsoro-Burq, 2012), known neural crest specifiers (*snail1*, *snail2*, *foxd3*, *twist1*, *ets1*) (reviewed in Rogers et al., 2012) and other neural border/crest regulators such as *irx1-3*, *cyp26c1*, *nrp1*, *pdgfra*, *olig4*, (Alarcon et al., 2008; Bellefroid et al., 1998; Hernandez-Lagunas et al.; Itoh et al., 2002; Liu et al., 2002; Martinez-Morales et al.; Reijntjes et al., 2004; Rodriguez-Seguel et al., 2009; Schwarz et al., 2009; Tallquist and Soriano, 2003). In addition, several transcripts such as *plekhn1*, *prtg*, *tfap2e*, *dact1*, *axin2*, and *ror2* remain to be analyzed in neural border/crest development. Interestingly, *dact1*, *axin2*, *ror2* participate in Wnt signaling which promotes neural crest development.

The neural crest is efficiently induced with the combination of Pax3 and Zic1 expression at appropriate relative levels (Hong and Saint-Jeannet, 2007; Milet et al., 2013). We tested the effect of Zic1 expression in Pax3GR-induced animal caps in the presence of cycloheximide. Among the 55 transcripts enriched more than 2-fold and less than 4-fold compared to Pax3

alone, two belonged to the NC gene signature (*dusp5*, *c3*) and among the 21 transcripts enriched more than 4-fold compared to Pax3 alone, three belonged to the NC signature (*glipr2*, *axin2*, *snail1*). This suggested that novel genes are activated when Zic1 is expressed (*glipr2*, *c3*, *dusp5*) and that Pax3 and Zic1 can synergize to drive *axin2* and *snail1* expression (Figure 3B).

We further validated these results by RT-qPCR, in order to confirm Pax3 targets, Zic1 targets and Pax3/Zic1 targets (Figure 3C and data not shown). These were done in several independent experiments in the same conditions as the ones analyzed on the microarray, plus the Zic1GR only condition. These analyses validated three main points (Figure 3C). Firstly, the main known neural crest specifiers, *snail1*, *snail2* and *twist1*, were highly and reproducibly activated either by Pax3 (*snail2*, *twist1*) or by Zic1 (*snail1*) in the presence of cycloheximide. Combining Pax3 and Zic1 seemed to have mild additive effects on *snail1* and *twist1*. Secondly, the novel putative targets *cyp26c1*, *dusp5*, *pdgfra*, and the known NC specifier *ets1* were activated by either Pax3 alone or Zic1 alone in the presence of cycloheximide, and their induction seemed potentiated when both factors are activated (*cyp26c1*, *dusp5*, *pdgfra*), although this effect was not obtained reproducibly for *dusp5* and *pdgfra*. Thirdly, *axin2* was a target of Zic1 alone and its expression was reduced when Pax3 and Zic1 were combined, yet remained significant (in agreement with its finding as a target of the Pax3/Zic1 combination compared to Pax3 alone). Together, these findings identify several novel immediate-early targets (thus potential direct targets) for Pax3 and Zic1 in neural crest induction.

NC signature genes are regulated by Pax3 and/or Zic1 *in vivo*

In order to validate the regulation of these novel direct targets by Pax3 and Zic1 further, we conducted knockdown experiments *in vivo*, using the previously validated Pax3 and Zic1 antisense oligonucleotide morpholinos (MO, Monsoro-Burq et al., 2005, De Crozé et al., 2011). Embryos were injected with the MOs into both blastomeres at 2-cell stage, grown until late neurula stage 17, lysed and analyzed by RT-qPCR (Figure 3D). We found that Pax3 depletion significantly reduced the expression of *snail2* as expected from previous studies (Monsoro-Burq et al., 2005), as well as the expression of *snail1*, *twist1*, *axin2*, *c3*, *cyp26c1*, *dusp5*, *ets1*, and *pdgfra*. This suggested that genes activated by Pax3 in the animal cap ectoderm, as immediate-early targets, such as *cyp26c1*, *dusp5*, *ets1*, *pdgfra*, *snail1*, *snail2*, and *twist1* (Figure 3C), were also regulated by Pax3 *in vivo* at this late neurula stage. In addition, genes which were not induced by Pax3 in the presence of cycloheximide in the blastocoele roof ectoderm, such as *axin2* and *c3*, were also regulated by Pax3, suggesting indirect regulations *in vivo*. Similarly, we found that Zic1 morphants displayed a reduced expression of *cyp26c1*, *dusp5*, *ets1*, *pdgfra*, *snail1*, and *snail2* (Figure 3D). All these genes were significantly activated by Zic1 alone in animal cap ectoderm treated with cycloheximide (Figure 3C). *Twist1* was neither activated by Zic1 alone, nor downregulated by Zic1 knock-down *in vivo*, suggesting that *twist* is regulated by Pax3 only. Finally, *axin2* was not decreased in Zic1 morphants, although it was robustly induced in animal cap ectoderm by Zic1 alone. Further studies will be needed to explore the details of this complex regulation.

Snail genes induction requires Pax3 and Zic1 *in vivo*, and their promoter contains putative Pax3 and Zic1 binding sites

Because *snail1* and *snail2* are two key neural crest specifiers, we have investigated in more details whether Pax3 and Zic1 were required *in vivo* for their early induction at the neural border and could also bind regulatory elements in their promoter. *Snail1* is expressed in the mesoderm during gastrulation and starts to be expressed at the neural border along with *snail2* at stage 11.5 (Essex et al. 1993). Accordingly, *snail1* and *snail2* transcripts increased

in whole wildtype embryos, from stage 11 to stage 14, when measured by RT-qPCR (Figure 4A, B). When knocking down Pax3, we observed that both *snail1* and *snail2* levels remained comparable to stage 11.5 levels, indicating that Pax3 is required for the induction of both genes at the neural border. In contrast, knocking down Zic1 prevented any significant increase in *snail1* expression, but only marginally affected *snail2* early activation (Figure 4A, B). Together with the potent induction of *snail1* by Zic1 and of *snail2* by Pax3 in cycloheximide-treated animal cap ectoderm (Figure 3C), these results indicate that Zic1 is essential for *snail1* induction at the neural border, while Pax3 is key to activate both genes *in vivo* during gastrulation and early neurulation, but activates efficiently only *snail2*, as an immediate-early target in ectoderm explants.

We next looked for putative binding sequences (BS) for these two transcription factors in the evolutionary conserved non coding regulatory elements (ECR, using rVista) located up to 3kb upstream of each coding sequence using the *X. tropicalis* genome. Transfac and Jaspar transcription factor binding motifs databases were used to predict potential binding sites. A putative Pax3 site (Pax3BS) was found in the *snail2* promoter, and a Zic1 putative site (Zic1BS) was found in a conserved element located 1,5kb upstream of *snail1* translation start site (TSS, Figure 4C). Double-stranded oligonucleotides were end-labelled for EMSA analysis. Optimal Zic1 and Pax3 binding sequences served as positive controls, while mutated Zic1 and Pax3 binding sequences served as negative controls (Vogan et al., 1996; table S1). *Snail1* and *snail2* oligonucleotides were designed according to the genomic sequences identified above, and *snail1* and *snail2* oligonucleotides were mutated on the putative binding sites served as negative controls. We showed that Pax3 binds efficiently to *snail2* oligonucleotide but much less to the oligonucleotide mutated in the putative binding site (Figure 4D). Moreover, an antibody against Pax3 induced a supershift of the *snail2* oligonucleotide, which did not occur in the presence of a non-specific antibody (Figure 4D). Similarly, we showed that Zic1 binds to *snail1* putative oligonucleotide but not to the *snail1* oligonucleotide mutated on the putative binding site (Figure 4E and not shown). Moreover, Zic1 binding on *snail1* oligonucleotide was efficiently competed by increasing amounts of cold *snail1* oligonucleotide but not by increasing amounts of the mutated *snail1* oligonucleotide, confirming the specificity of Zic1 binding (Figure 4E). Together, these data indicate that the *snail1* and *snail2* promoters contain putative binding sites for Zic1 and Pax3 respectively, which may mediate the direct activation of these immediate-early targets of Zic1 and Pax3.

Pax3 controls its own transcription

Finally, one intriguing result from the microarray analysis was the potential autoregulation of *pax3* gene transcription by Pax3 itself (Figure 3B). We designed *pax3* PCR primers located in the UTRs in order to detect endogenous *pax3* transcription but not the injected *pax3* mRNA (devoid of UTRs) (Table S1). Using these endogenous-*pax3*-specific primers, we showed that endogenous *pax3* transcription was activated in animal cap ectoderm injected with either Pax3 alone or Pax3+Zic1 combination and treated with cycloheximide (31 and 16-fold increase respectively compared to the cycloheximide-treated uninjected caps, Figure 5A), whereas Zic1 alone did not significantly induce *pax3*. Similar conclusions were also obtained using the other translation inhibitor emetine (not shown). While the addition of cycloheximide was needed to ensure that this induction did not require *de novo* protein translation, it also slightly modified endogenous *pax3* expression in the absence of mRNA injection. Moreover, it is well documented that cycloheximide produces a superinduction effect (Tadanoet et al., 1993). Thus, as a control, we also tested *pax3* induction in the absence of cycloheximide: either Pax3 alone or Pax3+Zic1 combination induced endogenous *pax3* expression (5 and 77-fold respectively compared to control

uninjected caps), whereas *Zic1* had no effect. These results indicate that *pax3* gene is an immediate-early target of its own protein product Pax3.

Using an *in silico* approach similar to the one described above, we looked for Pax3 binding sites in *pax3* gene regulatory sequences. We found several putative binding sites in ECRs, either 0.5 or 1.5kb upstream of *pax3* TSS (Figure 5B). Using EMSA, we show that Pax3 can bind to the conserved element located 1.5kb upstream of *pax3* TSS, but not to the equivalent oligonucleotide mutated on the putative Pax3 binding site (Figure 5C). In addition, the oligonucleotide was supershifted by the Pax3 antibody but not by a control antibody (Figure 5C). These data show that Pax3 exerts a positive autoregulation on its own transcription and suggest new putative regulatory elements mediating this regulation. This kind of regulation involving a positive feedback loop is novel in the neural crest gene regulatory network and may be used to stabilize the key neural border gene expression and lock cells in a neural border fate during early neurulation.

DISCUSSION

The initial regulatory steps of the neural crest gene network remain poorly understood. In particular, the links between neural border establishment and premigratory neural crest induction involve key transcription factors such as Pax3 and *Zic1*, but the genes directly targeted by those factors remained unknown. In this study, we have first defined a novel gene signature for the neural border and the premigratory cephalic neural crest in *X. laevis* embryos. Using a microarray-based approach, we then have devised methods to identify early regulators of neural crest induction. We have specifically focused on the establishment of the premigratory neural crest within the neural border and on the targets of Pax3 and *Zic1* since these two factors cooperate and are sufficient to trigger neural crest development (Milet et al., 2013). We have selected 81 genes robustly expressed at the neural border in mid-neurulae and in the premigratory neural crest, but not in the brain-forming anterior neural fold (Figure 1, Tables 1, 2, S2). Using this novel early NC signature, we have then looked for immediate-early targets of Pax3 and *Zic1* using three assays: neural crest induction in the pluripotent ectoderm of the animal cap (Figure 3A–C; Milet et al., 2013), *in vivo* validation using Pax3 and *Zic1* morpholinos (Figure 3D), and validation of putative binding sites *in vitro* (Figures 4, 5). These results allowed us to propose an extended gene regulatory network centered on Pax3, and comprising 21 novel Pax3 putative direct targets. In addition, we have validated three novel *Zic1* targets (Figure 3, 6). Finally, we have demonstrated Pax3 positive autoregulation (Figure 5), introducing a novel feedback loop in the neural border network, and we have evidenced a direct link between the neural border specifiers Pax3 and *Zic1* and the activation of several key actors of the epithelium to mesenchyme transition (EMT) in the neural crest, namely *snail1*, *snail2*, *foxd3*, and *twist1*.

Defining a premigratory neural crest gene signature

Two large scale transcriptomic approaches have previously been used to study neural crest development: both focused on the early migrating neural crest cells, either in chick or human embryos (Gammill and Bronner-Fraser, 2002; Thomas et al., 2008). While these studies highlighted the expression of many genes involved in cell shape, cell migration and adhesion, our study provides a novel group of known and putative regulators acting upstream of EMT, emphasizing that early induction involves a major proportion of transcription factors and cell signaling molecules. Indeed transcription factors and the Wnt signaling pathway were both significantly enriched ($p < 0.01$) in the neural crest signature. Using two complementary approaches, *i.e.* dissection of control embryo tissues and gain-of-function in animal cap pluripotent ectoderm, we have selectively identified genes relevant for neural crest development, although, despite well controlled dissections, two transcripts

out of 83 were encoding *cardiac actin* which is abundantly expressed in contaminating mesoderm (*actc1*, *acta1*; Hemmati-Brivanlou et al., 1990; Della Gaspera et al. 2012). Likewise, because superficial ectoderm was included with the neural crest explants, three superficial ectoderm markers were also found (*fkbp9*, *irg1*, *guk1*; Chalmers et al., 2006). However, the cluster that we chose as the “pre migratory neural crest signature” contains the main known neural crest specifiers (*ets1*, *foxd3*, *snail2*, *sox9/10*, *twist1*), the neural border specifiers (*msx1/2*, *pax3*, *tfap2a/b*, *zic1*) and genes involved in dorsal neural tube and neural crest patterning (*hoxa2*; *irx1/2/3*, *mafb*, *meis3*, *olig4*). This result validates our bioinformatics screening strategy and suggests similar roles for the novel transcription factors identified (*irf1*, *lmx1b.1*, *pou3f1*, *rara*, *tcf7*, *tfap2e*). Moreover, our pre migratory neural crest signature contains numerous factors with other functions than DNA binding, which also could display essential roles upstream of EMT. We have validated the expression of eight such novel genes at the neural border, some being expressed in the migrating neural crest as well (Figure 2).

By design, our study was limited to the 15000 probesets (about 10 000 genes) present on the Affymetrix arrays. This represents about one third of the frog genes. In order to define the full complement of genes activated at the neural border in the pre migratory neural crest, further analysis using next generation sequencing will be used. Moreover, we have focused on the genes expressed robustly by defining a minimal expression threshold prior to the unsupervised clustering analysis, and presenting a low p-value (< 2.5%) for the differential expression. Doing so, we may have overlooked important but weakly or broadly expressed regulators. Finally, our design has focused on genes enriched in the lateral neural border compared to the anterior neural fold, thus excluding potential regulators evenly expressed all around the neural border. Such genes, e.g. *zic2*, may nonetheless cooperate with neural crest-specific genes to activate EMT. In conclusion, we have limited the proposed signature to a group of 83 genes, which are likely to be most specific for neural border and pre migratory neural crest. Our 83-gene signature shares several neural crest specifiers with previous analyses in chick and human migrating neural crest (Gammill and Bronner-Fraser, 2002; Thomas et al., 2008). This signature will be useful to define the pre migratory neural crest step in differentiation protocols from stem cells (Bajpai et al., 2010; Mica et al.).

Defining an improved neural crest gene regulatory network centered on Pax3 targets

An initial gene regulatory network controlling neural crest development, composed of many epistatic relationships between neural border/crest regulators, was proposed based on expression patterns and functional studies *in vivo* (Meulemans and Bronner-Fraser, 2004). A few direct interactions have been documented (e.g. on *sox10*, *pax3*, *ecad* regulation, Betancur et al., 2010; de Croze et al., 2011; Cano et al., 2000). However, the direct targets of most transcription factors and signaling pathways remain to be identified (reviewed in Rogers et al., 2012). In our study, we focus on the putative direct targets of Pax3, because it is essential and, when combined to Zic1, sufficient to activate neural crest EMT, migration, and differentiation (Milet et al., 2013).

More specifically, we have focused on Pax3 immediate-early target genes belonging to the neural crest signature defined above (Figure 3). For example, muscle-specific Pax3 targets activated in the animal cap ectoderm were discarded (e.g. *myf5*). We found that several key neural crest specifiers were activated under these conditions: *foxd3*, *snail1*, *snail2*, and *twist1*. When Zic1 was added, *snail1* and *axin2* were better activated (Figure 3C). We confirmed that *snail1* is an immediate early target of Zic1 as shown in animal caps neuralized by Noggin (Cornish et al., 2009). In contrast, we found only two genes that belong to the neural crest signature which were downregulated when Pax3 was activated

(*glipr2*, *tub1a*; $p > 5\%$). This suggests that Pax3 plays mostly an activating role on neural crest signature genes.

In addition, we found binding sequences for Pax3 and Zic1 upstream of *snail1* and *snail2* coding sequences, which were validated for binding *in vitro*, suggesting that Pax3 and Zic1 may regulate *snail* genes directly *in vivo* (Figure 4). *Snail* genes cooperate with *foxd3* and *soxE* factors to activate EMT, survival and proliferation of neural crest cells (Cheung et al., 2005). One important question prior to this study was whether neural border specifiers Pax3 and Zic1 would directly activate several neural crest specifiers, or if they would trigger a cascade of gene activations, one being dependent upon the previous one. Our findings suggest the direct and parallel activation of several neural crest specifiers by the combined action of Pax3 and Zic1, thus explaining how these two factors are sufficient to trigger efficient neural crest development (Milet et al., 2013). In addition to the known neural crest specifiers, the role of the novel targets identified here in neural crest specification, EMT and migration awaits future studies. In particular, several of them may modulate Wnt (*axin2*, *dact1*) and other signaling pathways (*cyp26c1*, *pdgfra*, *dusp5*) in these processes.

Additional regulation must occur *in vivo* for the fine-tuning of the neural crest induction. Indeed, the timing of activation of these target genes is not simultaneous, neither *in vivo* nor in ectodermal explant assays. (Hong and Saint-Jeannet, 2007; Milet et al., 2013; Monsoro-Burq et al., 2005). The fine schedule of neural crest specifiers gene activation may be controlled by epigenetic regulations (Strobl-Mazzulla et al., 2010). Moreover these target genes are not activated in the entire neural border territory. Finally, some genes are found only weakly induced in our assays whereas they are robustly expressed *in vivo*, suggesting that additional regulators potentiate Pax3 and Zic1 action in the embryo.

Among Pax3 targets, *pax3* itself was found, as shown with specific assays for injected versus endogenous *pax3* transcripts, consistent with our findings of Pax3 binding sites in evolutionary conserved elements upstream of its own promoter (Figure 5). This finding indicates a novel positive feedback loop within the neural crest GRN, which suggests a mechanism for neural border maintenance as hypothesised in de Croze et al., 2011). Finally, we found that several novel targets, *i.e.* *c3*, *cyp26c1*, *dusp5*, *pdgfra*, and *twist1*, were better activated by the combined action of Pax3 and Zic1 rather than either one alone. This is the first indication of an additive effect of these two factors, on the expression of genes belonging to the premigratory neural crest signature.

Altogether, our results allow us to provide a much improved network described in Figure 6: at the neural border, Pax3 cooperates with Zic1, both factors being part of a “bottleneck” in the neural crest GRN since activating those two genes is both necessary and sufficient for neural crest development (Milet et al., 2013). Each factor activates several downstream neural crest specifiers or putative neural crest regulators, and both factors synergize to activate some common targets. These observations enrich our understanding of the larger neural crest gene regulatory network.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1. Identifying the premigratory neural crest transcriptome signature in frog.
2. Identifying immediate-early targets of the neural border specifiers Pax3 and Zic1
3. Defining novel hierarchical structures in the early neural crest GRN.

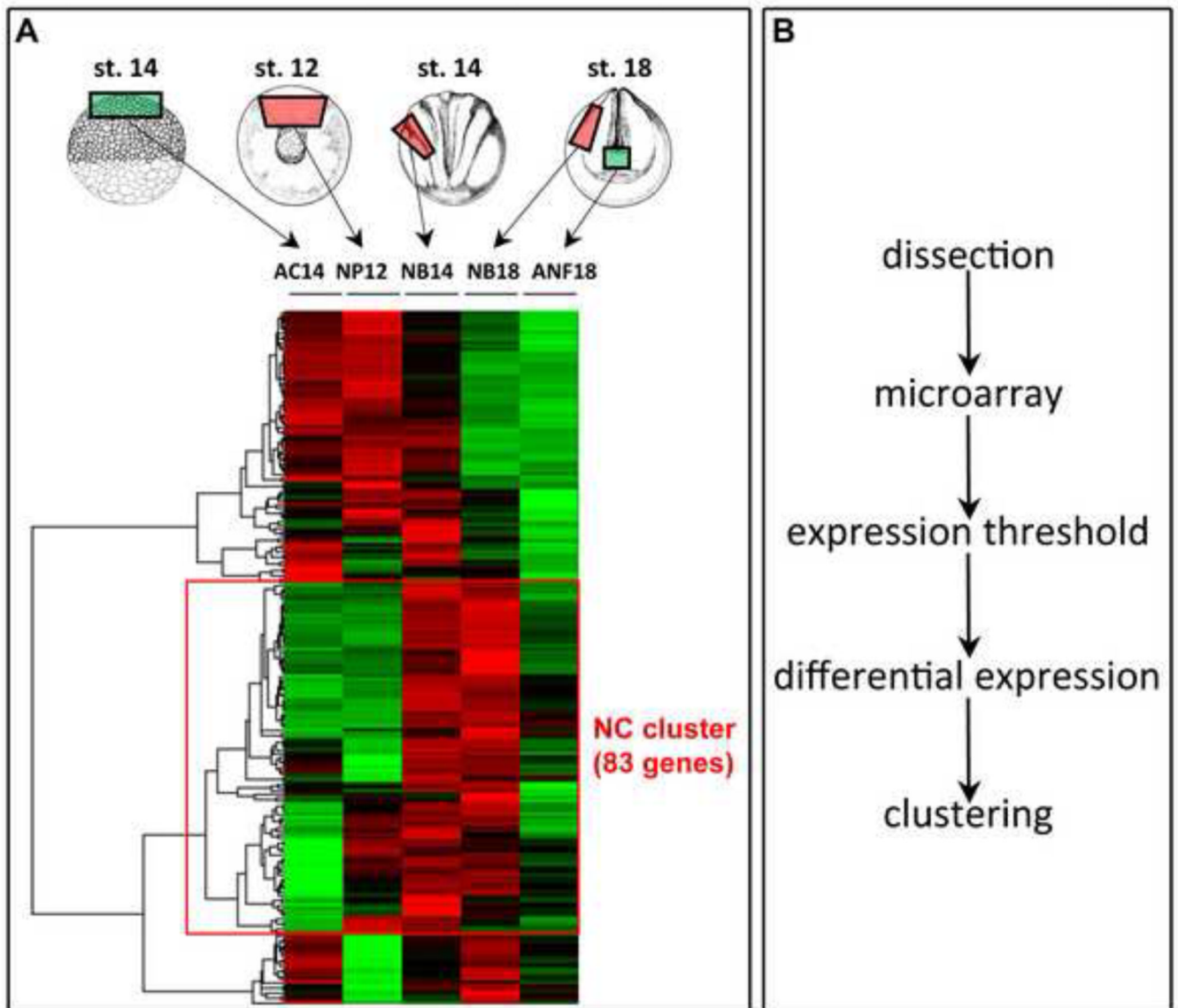


Figure 1. Identification of the premigratory neural crest transcriptome signature during neurulation

(A) Several types of early embryo explants were dissected for microarray analysis: the animal cap ectoderm, cut at blastula stage 9 and allowed to develop until stage 14 *in vitro* (AC14), the early neural plate at stage 12 (NP12), the lateral neural border at stage 14 (NB14), the premigratory cranial neural crest and its overlying ectoderm at stage 18 (NB18), and the anterior neural fold at stage 18 (ANF18). Expression level thresholding, differential analysis, and clustering defined a group of 83 genes enriched in neural border samples. (B) Outline of the experimental strategy used to identify the neural crest transcriptome signature.

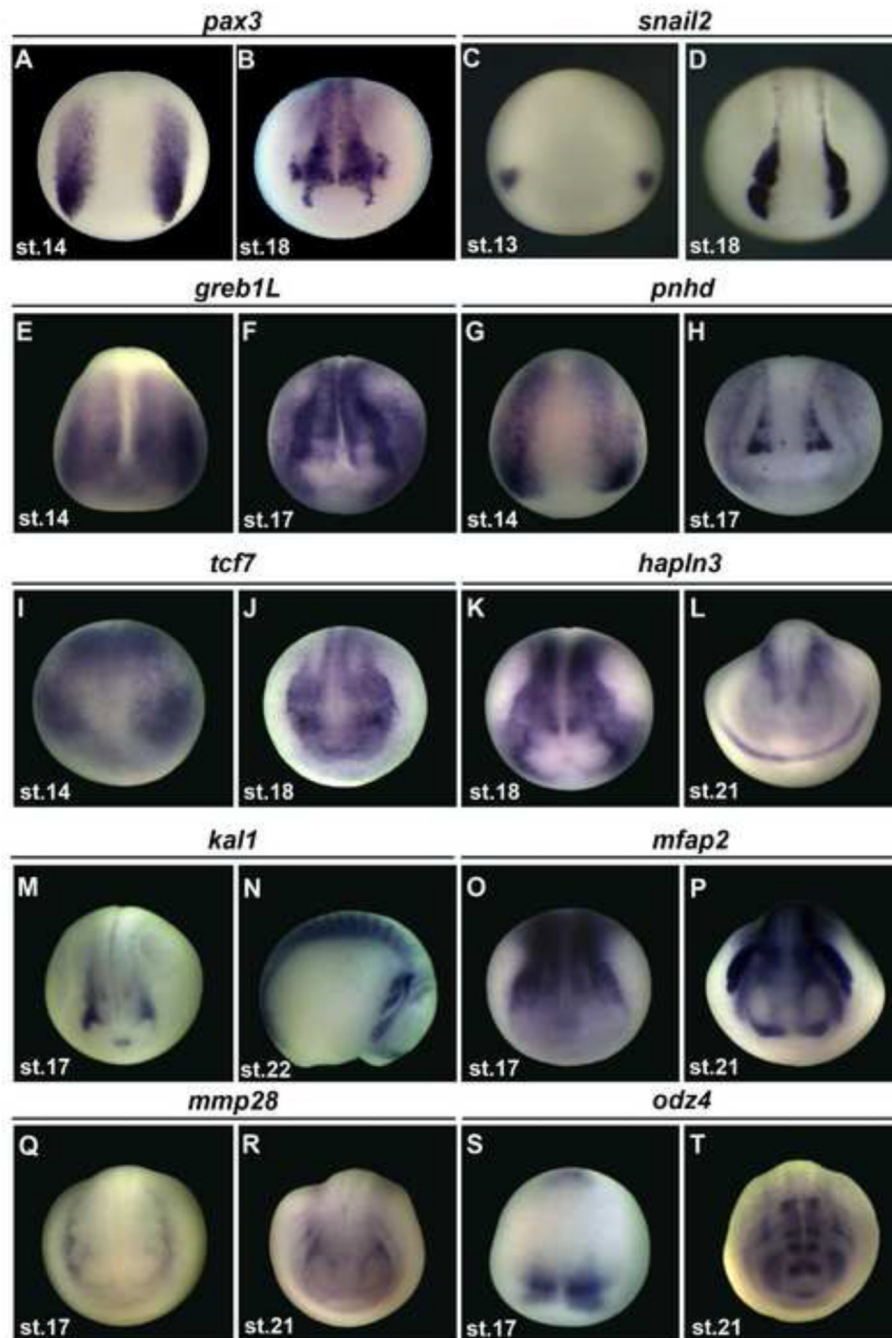


Figure 2. Developmental expression of a sample of novel genes belonging to the neural border/crest signature

Pax3 and *snail2* serve as markers for the lateral neural border and the premigratory neural crest respectively. Novel markers, identified in the neural border/neural crest signature, are shown at the most significant stages. (A–M, O–T) Dorsal views, with anterior to the bottom and posterior to the top. (N) Side view.

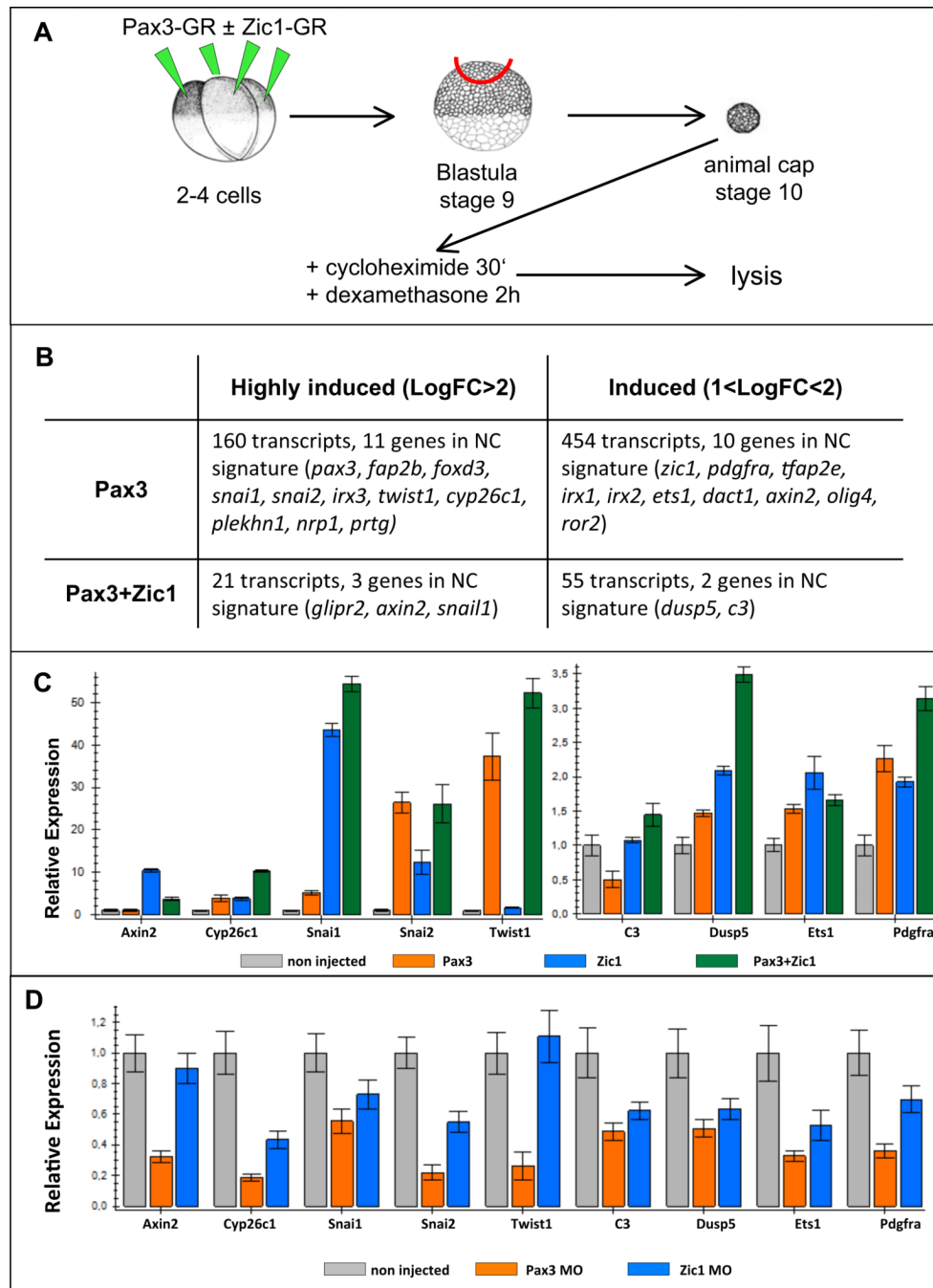


Figure 3. Pax3 and Pax3/Zic1 immediate early target genes in the neural crest signature
 (A) Experimental strategy used to identify Pax3 and Pax3/Zic1 immediate early target genes. (B) List of direct targets belonging to the neural crest signature. (C) Independent validation of a subset of targets by quantitative PCR. Embryos were injected as indicated, either with Pax3GR mRNA, or Zic1GR mRNA, or a combination of both. The animal cap ectoderm was treated with cycloheximide and dexamethasone as described in the text. (D) *In vivo* validation of the novel Pax3/Zic1 target genes using either Pax3 or Zic1 knockdown at stage 17. Abbreviations: LogFC, log₂ fold change.

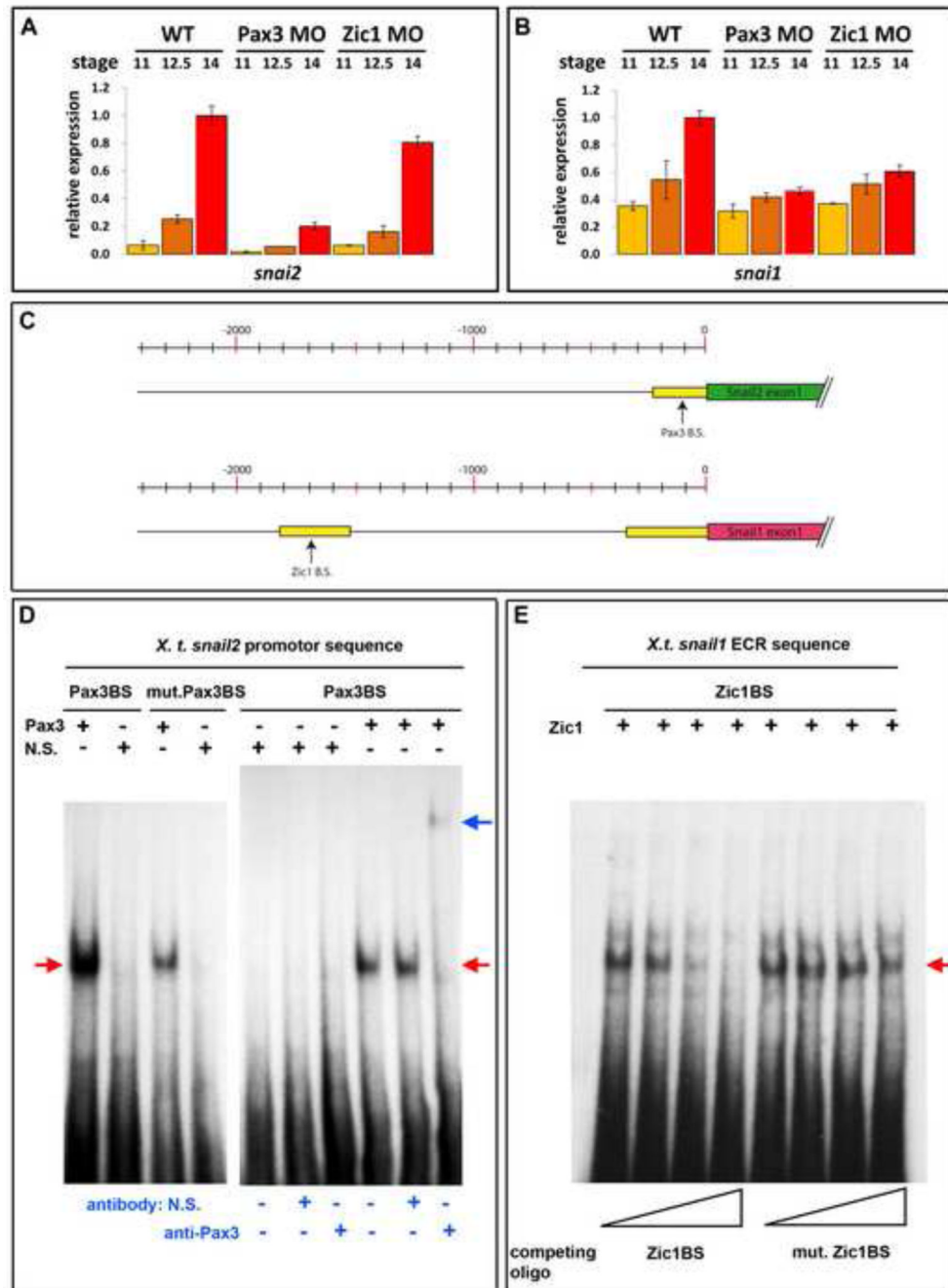


Figure 4. Identification of Pax3 and Zic1 binding sites on Snail1/2 promoters *in vitro*
 (A) Induction of *snail2* between stage 11.5 and 14 is strongly reduced by Pax3 knock-down, but only mildly affected by Zic1 knock-down. (B) The increase in *snail1* transcription between stage 11.5 and 14 is blocked by both Pax3 and Zic1 knock-down. (C) Location of the ECRs studied here (yellow boxes), containing Pax3 or Zic1 putative binding site (B.S.), in the genomic sequence upstream of *snail1* and *snail2* transcription start site (TSS). (D) Pax3 binds specifically to the motif identified in the *snail2* promoter ECR: the electrophoretic mobility of a radiolabeled oligonucleotide containing the putative Pax3 B.S. (Pax3BS) is shifted in the presence of Pax3-transfected cell extract, but not with the non-

specific (N.S) control (i.e. GFP-transfected cell extract). Intensity of the shift is reduced when the Pax3 binding site is mutated (mut. Pax3BS). In the presence of a specific anti-Pax3 antibody, we detect a mobility supershift, indicating that the Pax3 protein is indeed responsible for the observed shift. (E) Zic1 binds specifically to the motif identified in the *snail1* ECR: the electrophoretic mobility shift detected in the presence of Zic1-transfected cell extract can be competed by increasing doses of non-labeled oligonucleotide (Zic1BS), but only weakly by the mutated oligonucleotide (mut. Zic1BS).

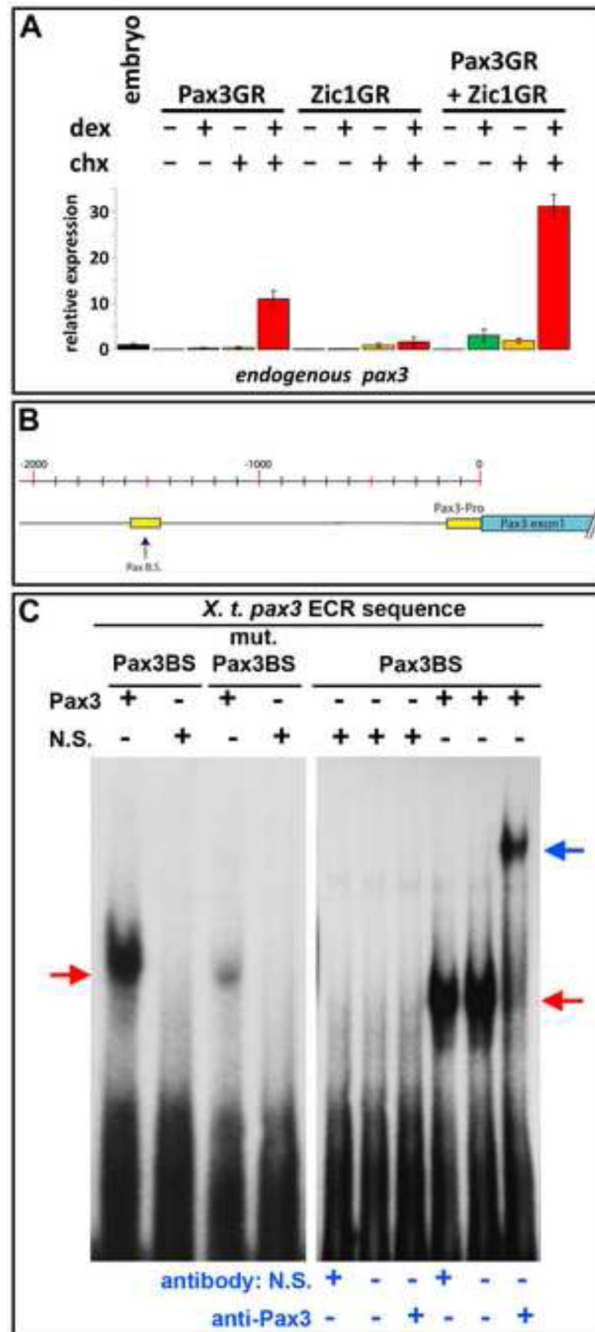


Figure 5. Identification of Pax3 binding sites on Pax3 promoters *in vitro*

(A) Pax3, but not Zic1 alone, can trigger synthesis of the endogenous Pax3 transcript (primers in 3'UTR, not amplifying the inducible form) either in absence of the translation inhibitor cycloheximide (induction is 5-fold compared to uninjected animal caps) or in the presence of cycloheximide (induction is 31-fold, compared to cycloheximide-treated uninjected animal caps). Endogenous *pax3* expression level in a stage 11 whole embryo is set at a relative value of 1 unit. After co-injection of Pax3 and Zic1, endogenous *pax3* was activated as an immediate-early target as well (77-fold compared to uninjected animal caps; and, in presence of cycloheximide, 16-fold when compared to cycloheximide-treated uninjected animal caps). (B) Location of the ECR containing Pax3 putative binding site in

the genomic sequence upstream of the Pax3 TSS. (C) Pax3 binds specifically to the motif identified in the *pax3* promoter ECR: an electrophoretic mobility shift of the radiolabeled Pax3 binding site (Pax3BS) oligonucleotide probe is detected in the presence of Pax3-transfected cell extract, but not with the GFP-transfected cell extract. Intensity of the shift is reduced when the Pax3 binding site is mutated (mut. Pax3BS). In the presence of a specific anti-Pax3 antibody, we detect a supershift, indicating that the Pax3 protein is indeed responsible for the observed shift.

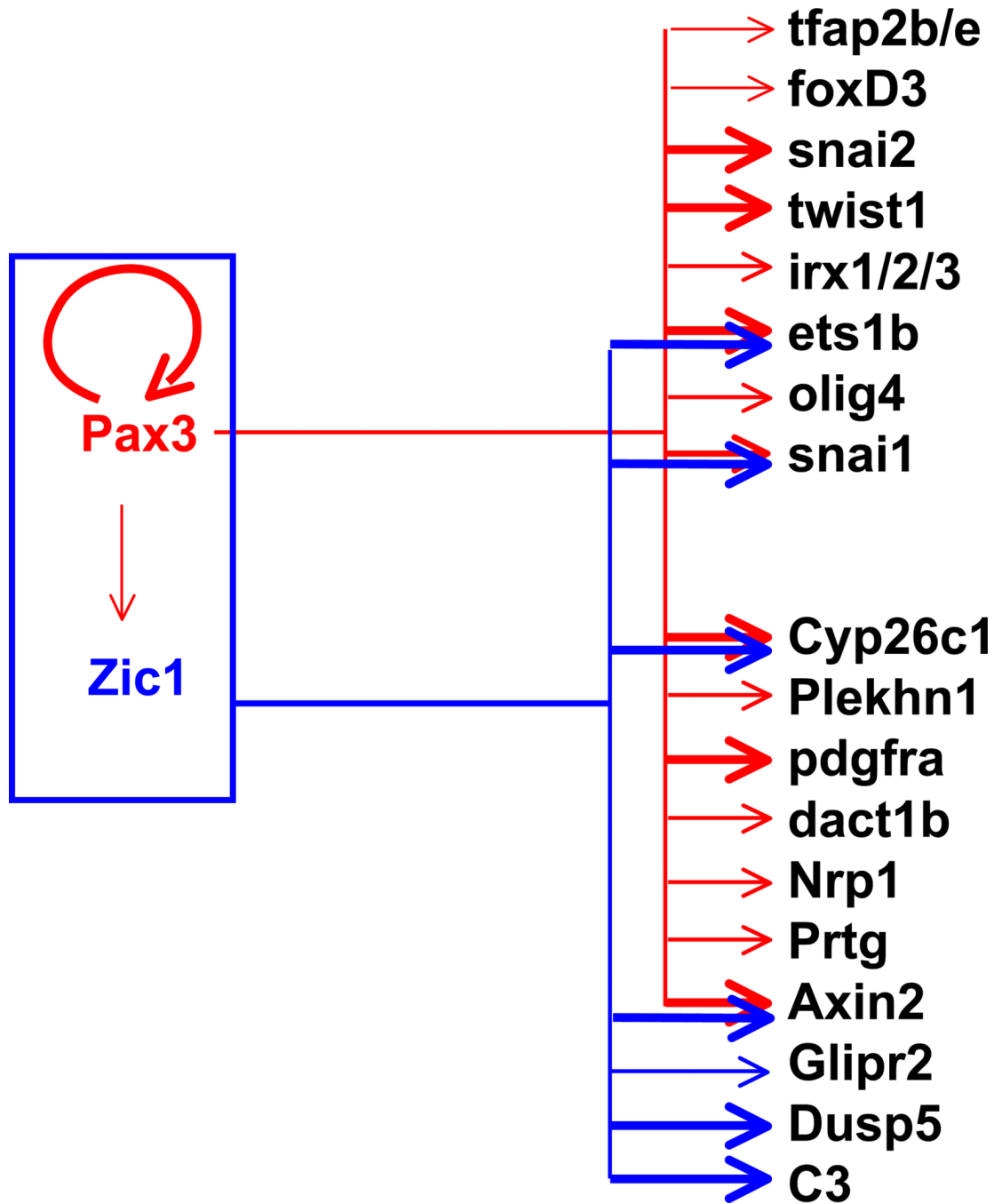


Figure 6. Model of the Pax3/Zic1-linked neural crest gene regulatory network

This model summarizes the targets of Pax3 and of Pax3 combined to Zic1, validated in this study. Red arrows indicate Pax3 targets, blue arrows indicate Zic1 targets. Bold arrows indicate that regulation by Pax3 or by Zic1 was confirmed by RTqPCR and/or *in vivo*.

Table 1

Functional terms enriched in the neural crest transcriptome signature

PIR keywords	Count	Adjusted p-value
Developmental protein	19 (27%)	2.7E-8
DNA binding	24 (34%)	5.1E-6
Homeobox	8 (11%)	1.3E-3
Disease mutation	18 (25%)	1.3E-3
Waardenburg syndrome	3 (4%)	5.9E-3
Wnt signaling pathway	6 (9%)	2.4E-3
Signal	23 (32%)	2.5E-2
Extracellular Matrix	6 (9%)	2.4E-2

Functional enrichment was computed using annotations of the frog genes human orthologs. Out of 83 genes, annotations were available for 71 of them, which represents the total count. The Signal category corresponds to proteins containing a signal peptide. Disease mutation corresponds to proteins found mutated in a genetic disease.

Table 2

List of genes in the neural crest transcriptome signature.

Molecular function	Genes
Transcription factor	<i>ets1, foxd3, hoxa2, irf1, irx1/2/3, lmx1b.1, mafb, meis3, msx1/2, olig4, pax3, pou3f1, rara, snail2, sox9/10, tcf7, tfap2a/b/e, twist1, zic1</i>
Wnt signaling	<i>axin2, dact1, fzd10, ror2, tpbp, wls, wnt1/8a/11b, xarp</i>
Other proteins with extracellular domain	<i>angpt4, btc, c3, capn8, col18a1, cypr1, fgfr4, fst, galnt1, hapln3, kall, kcne5.1, mfap2, mmp14/28, nipa2, nrp1, odz4, pcdh8l, pdgfra, pnhd, prtg</i>
Other intracellular proteins	<i>acta1/c1, bnip3, cyp26c1, dusp5, dynl1, elavl3, fkbp9, glipr2, greb1, guk1, hsp90aa1.1, irg1, loc100490918, MGC81667, myo10.2, myo1d, peli1, pfkfb4, plekhn1, pts, rab11fip4l, sdhb, tuba1a, zfp36l2</i>

Gene names follow the Xenbase nomenclature (<http://www.xenbase.org>).