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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

Jean-Louis Plouhinec [a,b], Daniel D. Roche [a,b], Caterina Pegoraro [a,b], Ana Leonor Figueiredo [a,b], Frédérique Maczkowiak [a,b], Lisa J. Brunet [c], Cécile Milet [a,b], Jean-Philippe Vert [d,e,f], Nicolas Pollet [g,h], Richard M. Harland [c], Anne H. Monsoro-Burq [a,b,*]

[a] Université Paris Sud, Centre Universitaire, F-91405 Orsay, France
[b] Institut Curie, CNRS UMR3347, INSERM U1021, Centre Universitaire, F-91405 Orsay, France
[c] University of California at Berkeley, Department of Molecular and Cell Biology and Center for Integrative Genomics, USA
[d] Centre for Computational Biology, Mines ParisTech, 77300 Fontainebleau, France
[e] Institut Curie, F-75248 Paris, France
[f] INSERM U900, F-75248 Paris, France
[g] CNRS, iSSB, F-91000 Évry, France
[h] Univ. Evry, iSSB, F-91000 Évry, France

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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.

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., 2012; Gohlke et al., 2008; Hertzano et al., 2011; Isern et al., 2011; 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

* Corresponding author at: Institut Curie, CNRS UMR3347, INSERM U1021, Centre Universitaire, F-91405 Orsay, France.
E-mail address: anne-helene.monsoro-burq@curie.fr (A.H. Monsoro-Burq).

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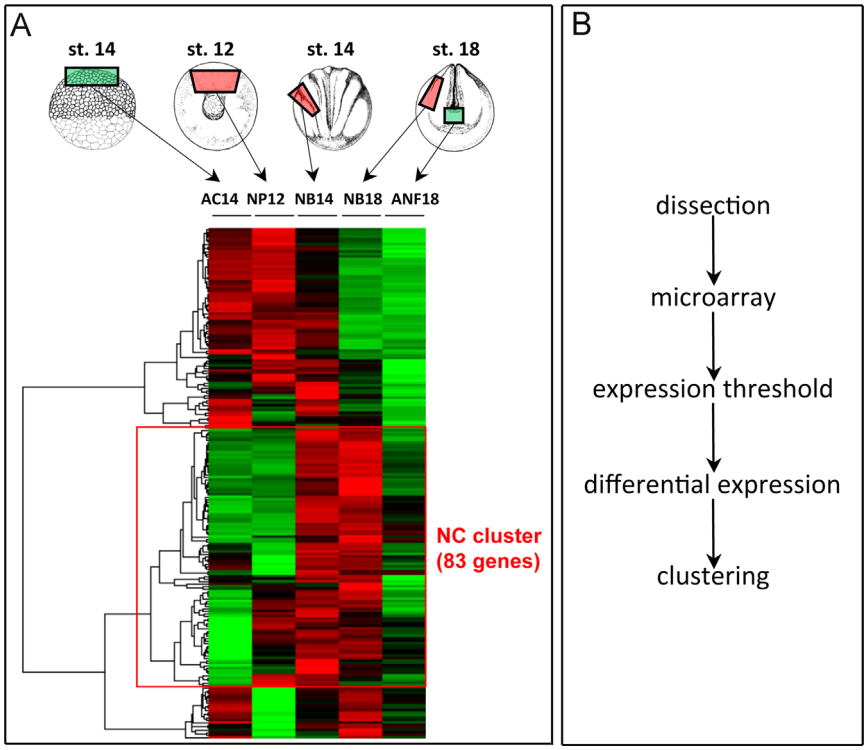

Fig. 1. Identification of the premigratory neural crest transcriptome signature during neurulation (A) Several types of early embryo explants were dissected for microarray analysis: the animal cap ectoderm, cut at blastula stage 9 and allowed to develop until stage 14 *in vitro* (AC14), the early neural 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.

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, 2013). 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., 1997, 1998, 2000; Relaix et al., 2004; Tremblay et al., 1996, 1998; 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 (Fig. 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 cultured 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 (0,1 mg/ml) was