NORTHWESTERN UNIVERSITY

Molecular Underpinnings of Pluripotency Suggest a Novel Origin for Neural Crest Stem Cells and a New Theory for Evolution of Vertebrates

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Elsy Cristina Buitrago Delgado

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ABSTRACT

Neural crest cells are a population of multipotent stem cells that are unique to vertebrates and give rise to a wide range of derivatives in the developing embryo, including elements of the craniofacial skeleton, pigmentation of the skin and peripheral nervous system. Although these cells reside in the ectoderm, they generate cell types typically categorized as mesodermal, and their broad developmental potential persists past the time when most ectoderm-derived cells have become lineage restricted. We recently proposed a new model for the developmental and evolutionary origins of neural crest cells based on the strikingly conserved molecular underpinnings of potency observed in these cells and pluripotent blastula stem cells. We suggest that neural crest cells may have evolved as a consequence of a subset of pluripotent blastula cells retaining the activity of much of the regulatory network underlying pluripotency through gastrula stages.

A striking difference in the regulatory factors expressed in pluripotent blastula cells and neural crest cells is the deployment of different sub-families of Sox transcription factors. Whereas SoxB1 factors are known to play central roles in pluripotent blastula and ES cells, neural crest cells express high levels of SoxE family transcription factors. I explored the role that this molecular "hand-off" of Sox factor activity, from SoxB1 to SoxE, play in the retention of pluripotency and the subsequent biasing of cells to contribute to specific neural crest-derived lineages by probing the shared and distinct activities of these factors in early *Xenopus* embryos.

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Dedication

This thesis is dedicated to my family

In memory of Edward Buitrago-Delgado's life

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Chapter 1

General Introduction

All embryos, from fruit flies to humans, start life as a single cell, the fertilized egg that divides repeatedly to form a hollow ball of cells termed the blastula embryo. At this time in development only a few hours after fertilization have passed and although all the cells in the embryo look very similar, they all, in fact, have received signals designating which tissues each cell will become. At the end of the blastula stage the embryo is comprised of three basic cell types called "germ layers", the ectoderm, the mesoderm, and the endoderm, which give rise to all the tissues in the body. The ectoderm will ultimately form the skin and the nervous system of the embryo, but initially, (at blastula stages) it exists as a stem cell population called embryonic stem cells. After blastula stages are complete, dramatic cell movements occur during a process that we call gastrulation and neurulation. During this time most of the body plan is established including the new cell type unique to vertebrates called the neural crest (Figure 1.1).

Despite that only 5% of species of animals in the world are vertebrates, they possess the most complex body plans found in the animal kingdom. Vertebrates are distinguished from the nearest relatives, the non-vertebrate chordates, by the appearance of a novel basic cell type in many ways equivalent to a fourth germ layer, the neural crest (Hall, 2009a; Le Douarin and Dupin, 2014). Although they form in the ectoderm, these cells exhibit broad multi-germ layer developmental potential, and they have fascinated scientists since their discovery by Wilhelm His 150 years ago.

In animal evolution, neural crest cells allow for increasing levels of organization and cell specialization. Vertebrate chordates evolved locomotion, support, specialized feeding, and reproduction. The emergence of these properties is the result of multiple processes that occur

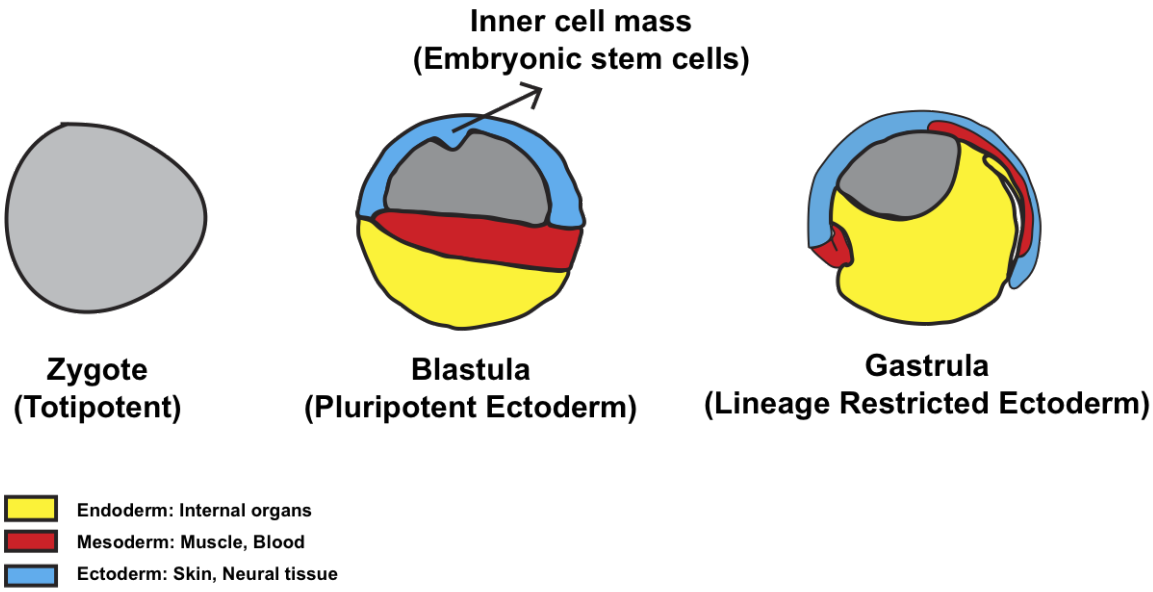


Figure 1.1 The formation the three germ layers
Schematic representation of embryonic development and the three germ layer specification; embryos depicted from a zygote to a gastrula stage showing the formation and specification of the endoderm (yellow) mesoderm (red) ectoderm (blue).

at a cellular and molecular level. The emergence of neural crest cells and its regulatory network allow the new tissues to form and new functions that are associated to cellular and molecular processes that include proliferation (organismal growth), migration (cellular movements), adhesion (cellular matrix), and differentiation (emergence of specialized cell types from stem cells and progenitors).

My thesis work mainly focuses on understanding the origin of evolutionary patterns and complex systems by investigating how neural crest cells formed and maintained multipotency through development. Elucidating neural crest origins, will allow us understand how neural crest contributes to the evolution of vertebrate chordates. Neural crest are an excellent system for studying how stem cells are formed and maintained, and later how their differentiation and migration is regulated. Here I present evidence that supports a new model for neural crest formation in which instead of being a cell population that gain potential at neurula stages, these cells are derived from pluripotent blastula cells and maintain developmental potential due to a shared regulatory program between embryonic stem cells and neural crest cells. I further explored a molecular mechanism that neural crest cells might use to maintain pluripotency, I proposed a model in which SoxB1 and SoxE transcription factors, may play important roles in the retention of pluripotency and the subsequent biasing of cells to contribute to specific neural crest-derived lineages by probing the shared and distinct activities of these factors in *Xenopus* embryos.

Neural crest cells

Neural crest cells arise from the developing ectoderm, during the end of gastrulation and beginning of neurulation, migrate through the embryo to differentiate into a diverse set of derivatives including cells that are important for the pigmentation of the skin, craniofacial skeleton, portions of the cranial ganglia, other elements of the peripheral nervous system such as the enteric ganglia the sensory nervous system and cardiac smooth muscle, among other derivatives (Figure 1.2) (Barembaum and Bronner-Fraser, 2005; Bronner and LeDouarin, 2012; Mayor and Aybar, 2001)

Traditional models of neural crest formation suggest that neural crest arise from the ectoderm as a subpopulation of cells located between the neural (neural plate) and non-neural ectoderm (epidermis). It has been established that during early neurulation, neural plate border cells are induced to gain potential, and give rise to two sister populations of cells, the cranial placodes and the neural crest (Figure 1.3) (LaBonne and Bronner-Fraser, 1998a; Barrallo and Nieto, 2006; Milet and Monsoro-Burq, 2012; Stuhlmiller and Garcia-Castro, 2012). After induction of neural crest cells during late gastrula stages, the neural tube closes and cells start to delaminate and migrate extensively through the embryo due to the activation of a molecular program termed the epithelial-to-mesenchymal transition (EMT), during this process cells change characteristics and migrate through the body to distant places where they start to differentiate into different cell types.

Multiple studies have been focused in understanding neural crest formation, delamination and differentiation from a gene regulatory network perspective (Martik and Bronner, 2017; Prasad et al., 2012; Sauka-Spengler and Bronner-Fraser, 2008a; Simoes-Costa and Bronner, 2015). Many

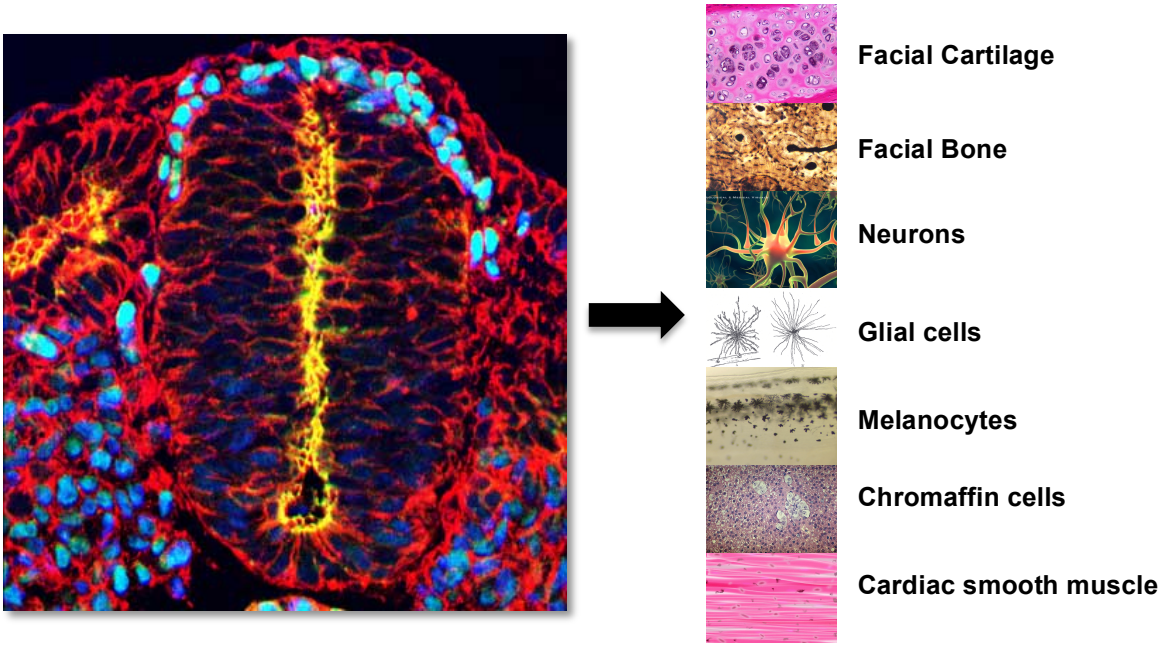


Figure 1.2 Neural crest derivatives
Neural crest cells are present in the ectodermal germ layer, on the left, a cross-section of a chicken embryo, neural crest cells are depicted in blue, once neural tube closes, neural migrate and differentiate into multiple cell types typical of two different germ layers. In the right, different cell types that neural crest cells can form for example muscle and cartilage are mesodermal cell types while neurons and glia are ectodermal cell types.

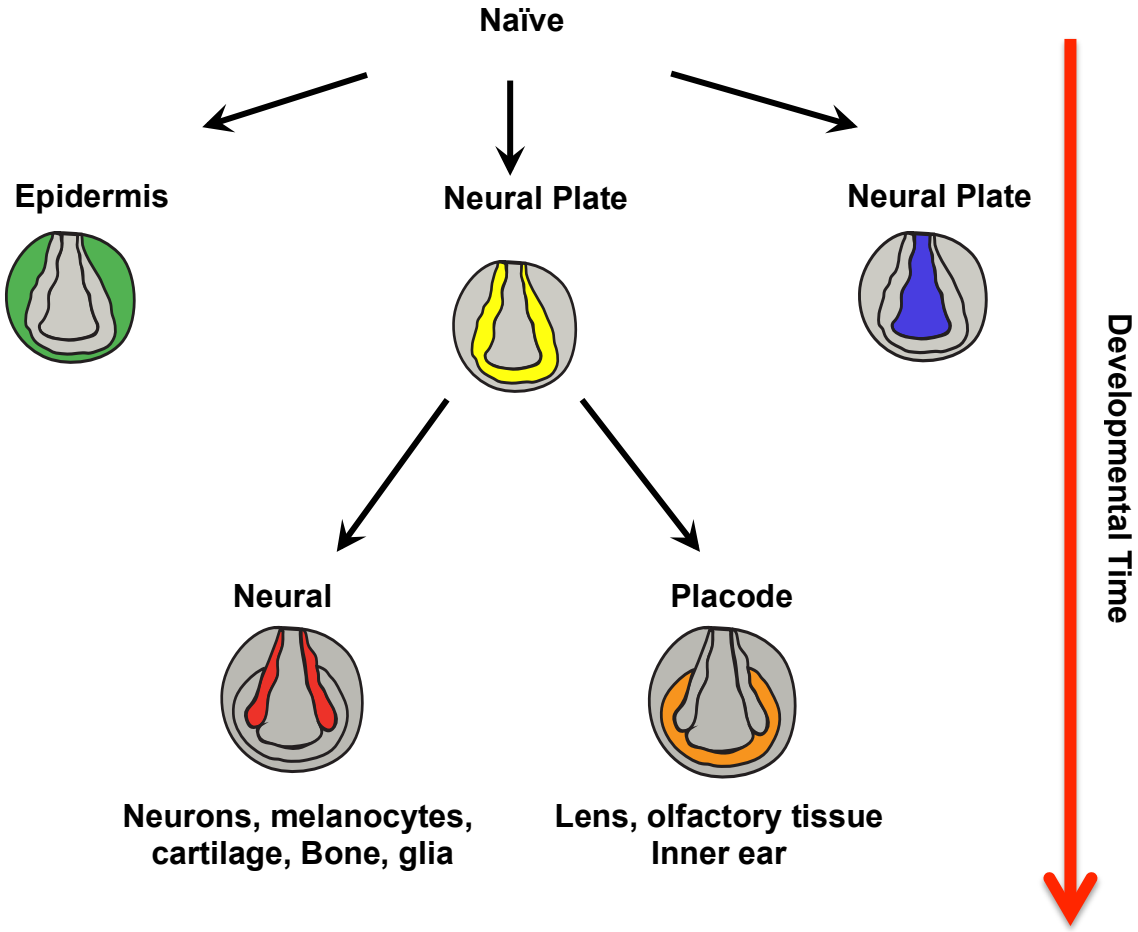


Figure 1.3 Neural crest development
Once the ectoderm becomes lineage restricted during gastrulation, it will be divided into three main domains. The neural plate will form the early central nervous system, the epidermis will form the skin, and only in vertebrates the border region between these cell types give rise to two population of cells that are very important – the cranial placodes and a stem cell population called the neural crest.

proteins that comprise a gene regulatory network have been organize in complete maps that described how cascade of genes controls neural crest development and described in detail the logic control of the induction, migration and differentiation of neural crest cells have been identified (Akiyama et al., 2002; Haldin and LaBonne, 2010; Kim et al., 2003; LaBonne and Bronner-Fraser, 1998a; LaBonne and Bronner-Fraser, 1998b; Light et al., 2005; Nordin and LaBonne, 2014; Ochoa et al., 2012; Simoes-Costa et al., 2015; Theveneau and Mayor, 2012)

Importantly, crest cell migration is mimicked during early stages of metastasis, as cells start to change its properties and migrate adopting invasive behaviors. It is possible that at the molecular level, the parallels between neural crest development and tumor progression are extensive (Vernon and LaBonne, 2004). The relationship between neural crest cells and tumor cell behavior provides a platform to compare how normal developmental programs can be reactivated to contribute tumor invasion and progression. It has been reported that many of the tissues derived from neural crest cells utilize these same mechanisms to contribute to malignancy in different types of cancer such as neuroblastomas, glioblastomas, and melanomas (Figure 1. 4).

The neural crest: brief historical perspective

The neural crest was first described as a unique population of cells in 1868 by Swiss anatomist Wilhelm His who called them the zwischenstrang “the intermediate cord” as the junction between the fold of the neural ectoderm and the non-neural ectoderm at the neurula stage of chicken embryo (His, 1868). He observed a belt of cells that were morphologically different to those in the neural plate or in the presumptive epidermis regions. It wasn’t until 1878

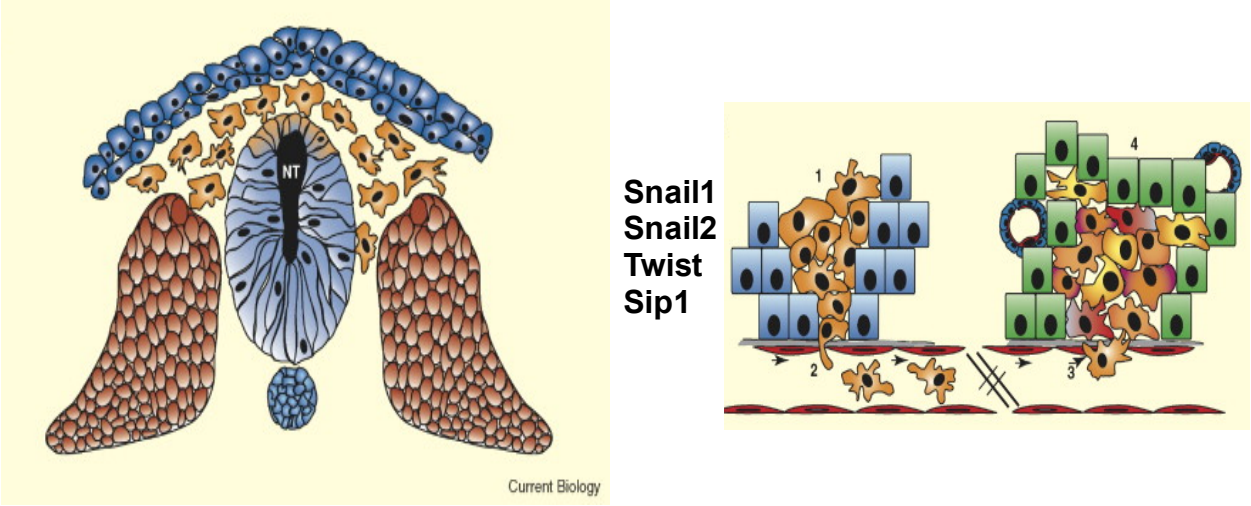


Figure 1.4 Regulatory programs important for cell migration contribute to neural crest formation and tumor metastasis
During embryo development, EMT is involved in the delamination and migration of neural crest cells (left). The same EMT process is also present during tumor progression and metastasis (right) adapted from (Vernon and LaBonne, 2004).

that cells were officially named *the neural crest* by Arthur Milnes Marshall (Marshall, 1879). The name was given due to the anatomical morphology and position in the neural tube of the developing embryo.

During the first 10 years of neural crest research, the origin of cranial and spinal ganglia and neurons from neural crest cells was identified, but, it was not until 1891 that Julia Platt proposed that cells from the craniofacial cartilage and the odontoblasts have neural crest origins. Conferring to the neural crest a multilayer potential as they could give rise to derivatives typically associated to the ectoderm such as neurons and melanocytes, but also from the mesoderm such as cartilage (Platt, 1891). Platt's contributions were controversial because her findings were a clear contradiction of the three germ layer specification theory proposed by Von Baer in 1828. It wasn't until 1946 that detailed descriptions of neural crest cells differentiating into cartilage of the visceral arches and odontoblasts were presented using photomicrographs of preparations made from wilt type embryos of axolotl. The cells derived from neural crest were followed by morphology and the presence of pigmented granules that were observed using picro-indigo carmine or iron-alum cochineal. This was the first evidence demonstrating that neural crest cells have a multilayer potential in vivo.

The demonstration of the multilayer potential of neural crest cells lead researchers to study neural crest cells and its derivatives and found a vast contribution from the neural crest cells to different tissues in the body. It was around the same time that it was found that most of the tissues in the head of vertebrates have a neural crest origin and soon, neural crest cells were considered as an important innovation and central component in vertebrate evolution. Several

publications highlighted its important contributions specifically related with the formation of the new head in vertebrates. (De Beer, 1947; Glenn Northcutt, 2005; Hall, 2009a; Hall, 2013).

Beginning in 1960, the focus on neural crest cell research shifted to understand migration and differentiation, the discoveries of labeling techniques and grafting experiments, made possible the visualization of migrating neural crest cells and fate maps were developed at a single cell level. (Johnston, 1966; Weston, 1963). The first chimeric quail- chicken embryo, made possible the tracking of the behavior of neural crest cells in a host environment and lead to the study of neural crest cell migration, differentiation and morphogenesis in vivo. (Bronner and Cohen, 1979; Bronner-Fraser and Cohen, 1980; Hall, 2009a; Le Douarin, 1984; Le Douarin and Kalcheim, 1999; Le Douarin, 1974; Le Douarin and Teillet, 1974). These discoveries enabled defects in neural crest to be identified as the likely source of multiple different developmental abnormalities and, soon parallels between migration of neural crest cells and the migration of cancer cells were made.

It wasn't until 1980s, with the advance of new imaging techniques that neural crest cell researchers started to investigate the dynamics of these cells in different model organisms (Bronner-Fraser and Fraser, 1988; Krotoski et al., 1988; Serbedzija et al., 1990; Teillet et al., 1987). Soon after the integration of molecular biology, classic developmental biology, and evolution; mechanisms of induction, migration and differentiation of neural crest cells were starting to be elucidated and maps of the molecules that are involve in neural crest formation were made (Bronner and LeDouarin, 2012; Bronner-Fraser and Fraser, 1989; LaBonne and Bronner-Fraser, 1998a) More recently, researchers have devoted efforts toward understanding

the neural crest development from a gene regulatory network and systems biology perspective. These new approaches led scientist to build refined maps of signaling molecules and genes grouped in modules that explain neural crest induction, delamination, migration and differentiation (Green et al., 2015; Prasad et al., 2012; Sauka-Spengler and Bronner-Fraser, 2008b; Simoes-Costa and Bronner, 2015,). Lately, neural crest research is focused on understanding the molecular signatures that confer multilayer potential to neural crest cells, and the regulatory programs and modules that are implicated in each step of development. Now, studies involve high throughput analysis and single-cell resolution using quantitative approaches to understand the dynamic of multiple regulatory programs that are essential to understand the biology of neural crest cells (Lignell et al., 2017; Roellig et al., 2017)

Induction of neural crest cells

Traditional models of neural crest formation propose that neural crest "induction" first involves generating a broad zone of competence/potency in a region of the early ectoderm known as the neural plate border. Neural crest formation traditionally represented an increase in developmental potential or stemness and, therefore, these cells are an excellent system to understand the molecular basis of multipotent stem cell potential (Figure 1.5). At the end of gastrulation, the combined action of several signaling pathways leads to the formation of the three major domains of the ectoderm in *Xenopus* embryo: the epidermis, the neural plate, and the neural plate border. (Hong and Saint-Jeannet, 2007).

The midline (the neural plate) of the embryo at this stage is a flat tissue. During neurulation, the neural plate folds and give rise to the presumptive nervous system, and transform into the

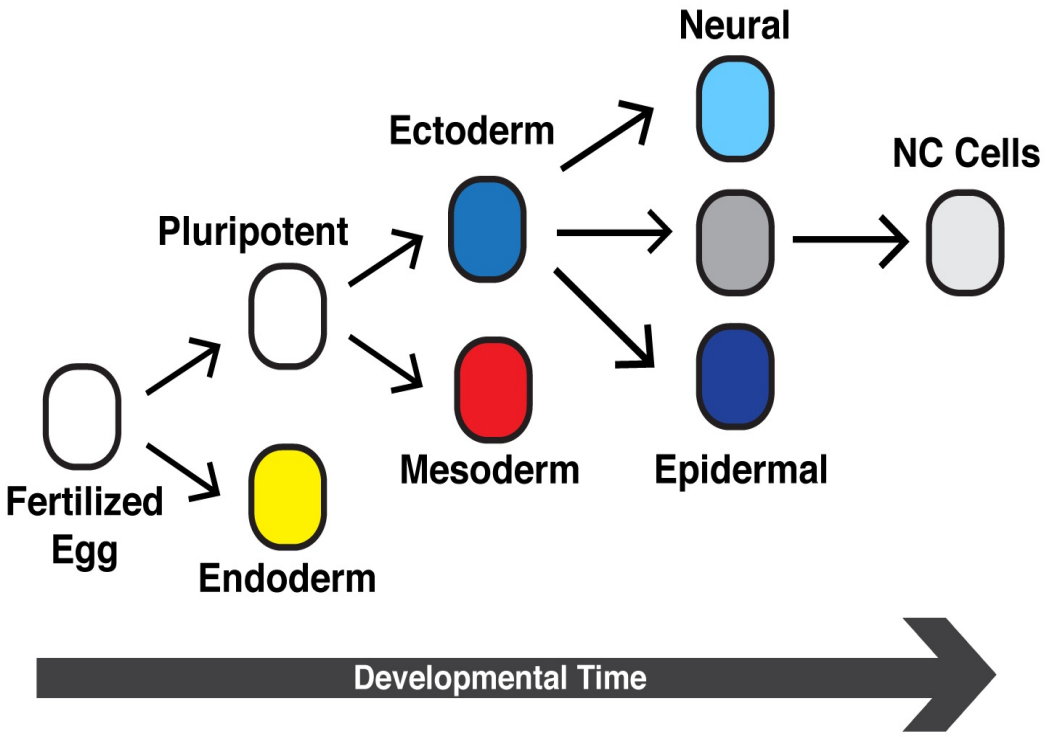


Figure 1.5 Traditional model of neural crest formation
Neural crest cells arise from the ectodermal germ layer as a population of cells between the presumptive epidermis and presumptive neural tissue. Neural crest cells gain potential to give rise to mesodermal and ectodermal derivatives

cylindrical neural tube that will give rise to the brain. Lateral to the neural plate is the non-neural ectoderm that will form the epidermis. Between the presumptive epidermis and the presumptive neural tissue, there is stem cell population called the neural plate border that will give rise to the presumptive neural crest cells and the cranial placodes and is localized at the most dorsal aspect of the neural tube (Figure 1.6). Each of these cell populations contributes a number of derivatives with important specialized functions in the developing embryo. (Gammill and Bronner-Fraser, 2003; Le Douarin, 2004). During the induction of the potential, the position of the cells within the embryo is a crucial determinant of which cells adopt a neural plate border state. Is only a specific group of cells responsive to the highly dynamic interaction of different signaling pathways that promote the activation of a cascade of transcription factors that specify the neural plate border and further neural crest cells.

Signaling involved in neural crest induction

Multiple signaling pathways and transcription factors are involved in neural crest induction; signals from the adjacent ectoderm and the paraxial mesoderm are integrated at the neural plate border to induce neural crest cells. Studies using multiple model organisms determined that regulatory molecules in neural crest development appear to participate in the fate determination of this lineage. Low levels of Bone morphogenetic protein (BMP), along with fibroblast growth factors (FGF), Wnt, and Notch ligands, turn on the cascade of genes that will specify the neural plate border and the neural crest (Cornell and Eisen, 2005; Delaune et al., 2005; Hemmati Brivanlou and Thomsen, 1995; LaBonne and Bronner-Fraser, 1998a; LaBonne and Bronner-Fraser, 1999; Monsoro-Burq et al., 2003), (Figure1.7).

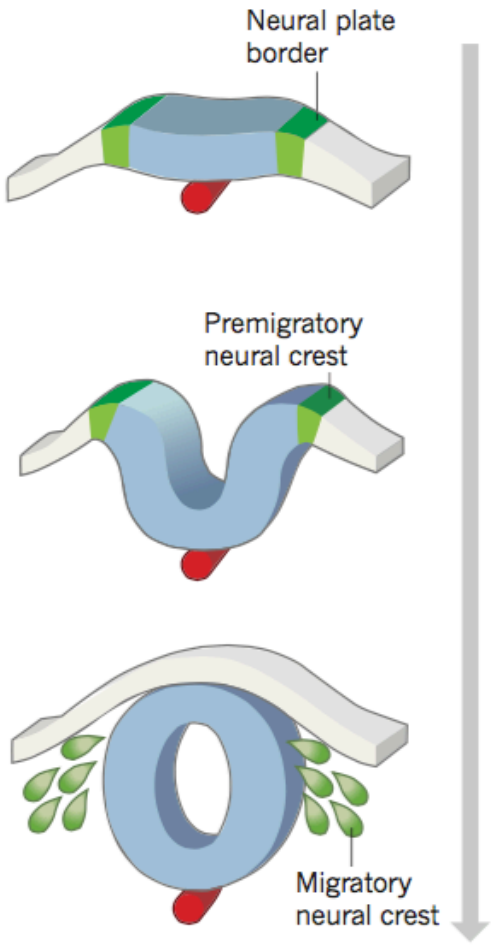


Figure 1.6 Cartoon of the neural crest development
Schematic representation of neural crest development, during vertebrate development, the neural plate is subdivided into neural ectoderm (blue), non-neural ectoderm (white) and neural crest cells (green). At the end of neurulation, after the neural plate closure, neural crest cells undergo EMT to delaminate and migrate throughout the embryo. Adapted from (Green et al., 2015)

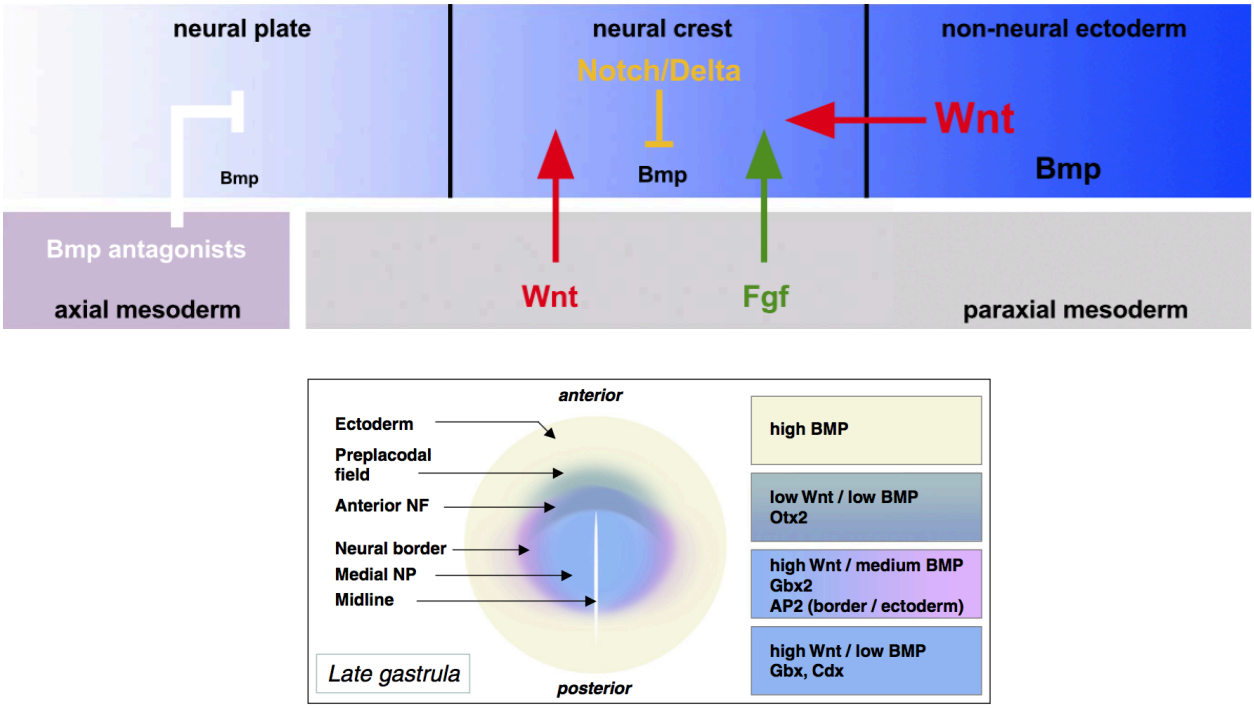


Figure 1.7 Schematic representation of signaling involve in neural crest induction at the neural plate border

BMP, Wnt, FGF, and Notch are the major signals in neural crest induction. A BMP gradient is established to pattern the ectoderm, with intermediate levels of BMP required for neural crest formation. In *Xenopus*, both Wnt and FGF signals originate from the underlying paraxial mesoderm to induce the neural crest. Notch signaling acts in the ectoderm. Adapted from (Huang and Saint-Jeannet, 2004), (Bae and Saint-Jeannet, 2014; Huang and Saint-Jeannet, 2004).

Evidence from *Xenopus* suggests that neural crest is induced during gastrulation, and its early development can be explained in a two-step process that starts at mid gastrula stages and persists until neurulation, around the time when the neural tube closes (LaBonne and Bronner-Fraser, 1998a). An initial phase of FGF and Wnt signaling during gastrulation induces the neural crest in the prospective neural plate border, and the second phase of Wnt and BMP signaling during neurulation maintains the stemness of the neural crest cell population (Ben Steventon et al., 2009; García-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998a).

The establishment of a BMP gradient, in *Xenopus*, is modulated by BMP inhibitors (i.e. Chordin, Noggin, follistatin), and a separate signal mediated either by canonical Wnt or FGF (García-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998a; Stuhlmiller and García-Castro, 2012; Wu et al., 2005; Yanfeng et al., 2003). Interestingly these signaling pathways integrate early in development to induce the expression of a set of transcription factors, which specify the neural plate border, the precursors of the neural crest cells. High levels of BMP induce epidermal cell fate, whereas low levels are necessary for induction of neural fate. Only the cells with intermediate levels of BMP will be competent to induce neural plate border (Ben Steventon et al., 2009). FGF signals are also implicated in the induction of neural crest cells mediating neural crest induction by indirectly up-regulating Wnt ligand in the mesoderm of *Xenopus* (LaBonne and Bronner-Fraser, 1998a; Monsoro-Burq et al., 2003). The regulation of the induction of neural crest cells mediated by FGF differs among diverse model systems. It seems that FGF is important only during gastrulation for induction of neural plate border in chicken (Stuhlmiller and García-Castro, 2012), but in mouse embryos lacking FGF receptors, neural crest development appears to be normal (Prasad et al., 2012).

Activation of the canonical Wnt pathway is critical for neural crest cell specification. A number of Wnt ligands are proposed to be involved with induction, and development of neural crest; knockdown of Wnt1 is sufficient to block the formation of neural crest cells in chicken embryos (Stuhlmiller and García-Castro, 2012), and *Xnkd* an antagonist of canonical Wnt signaling , blocks neural crest formation in *Xenopus* embryos (Wu et al., 2005). Active canonical Wnt signaling is necessary for neural crest induction but is not sufficient to induce neural crest state in the naïve ectoderm; it also requires the correct levels of BMP signaling (Heeg-Truesdell and LaBonne, 2006). In addition to Wnts, FGFs, and BMPs, Notch signaling has been proposed to act upstream of BMP. Notch signaling directly activates *Hairy2*, attenuating the expression of *BMP4* in the ectoderm. In *Xenopus,* constitutively active Notch prevents the formation of neural crest and epidermis; in contrast, it enhances the expression of neural fates (Coffman et al., 1993).

In response to these signaling events mediated mainly by BMP, FGF, Wnt, and Notch, a sequence of transcription factors are activated. The first set of genes, known as neural plate border specifiers, and their expression domain is located in the presumptive neural crest cell population. The molecules and interactions important for induction of neural crest cells are known and have been integrated into a gene regulatory network. (Sauka-Spengler and Bronner-Fraser, 2008a, Betancur et al., 2010, Nikitina:2009eg, Prasad et al., 2012, Simoes-Costa and Bronner, 2015, Martik and Bronner, 2017) (Figure 1.8). It seems that timing and expression levels of signaling molecules and transcription factors are, in fact, critical for conferring to the neural plate border cells the ability to "induce" stemness of the precursors of neural crest cells. Future work understanding dynamics, levels, timing and sequence that these signaling events occur to induce the precursor population of neural crest cells is needed.

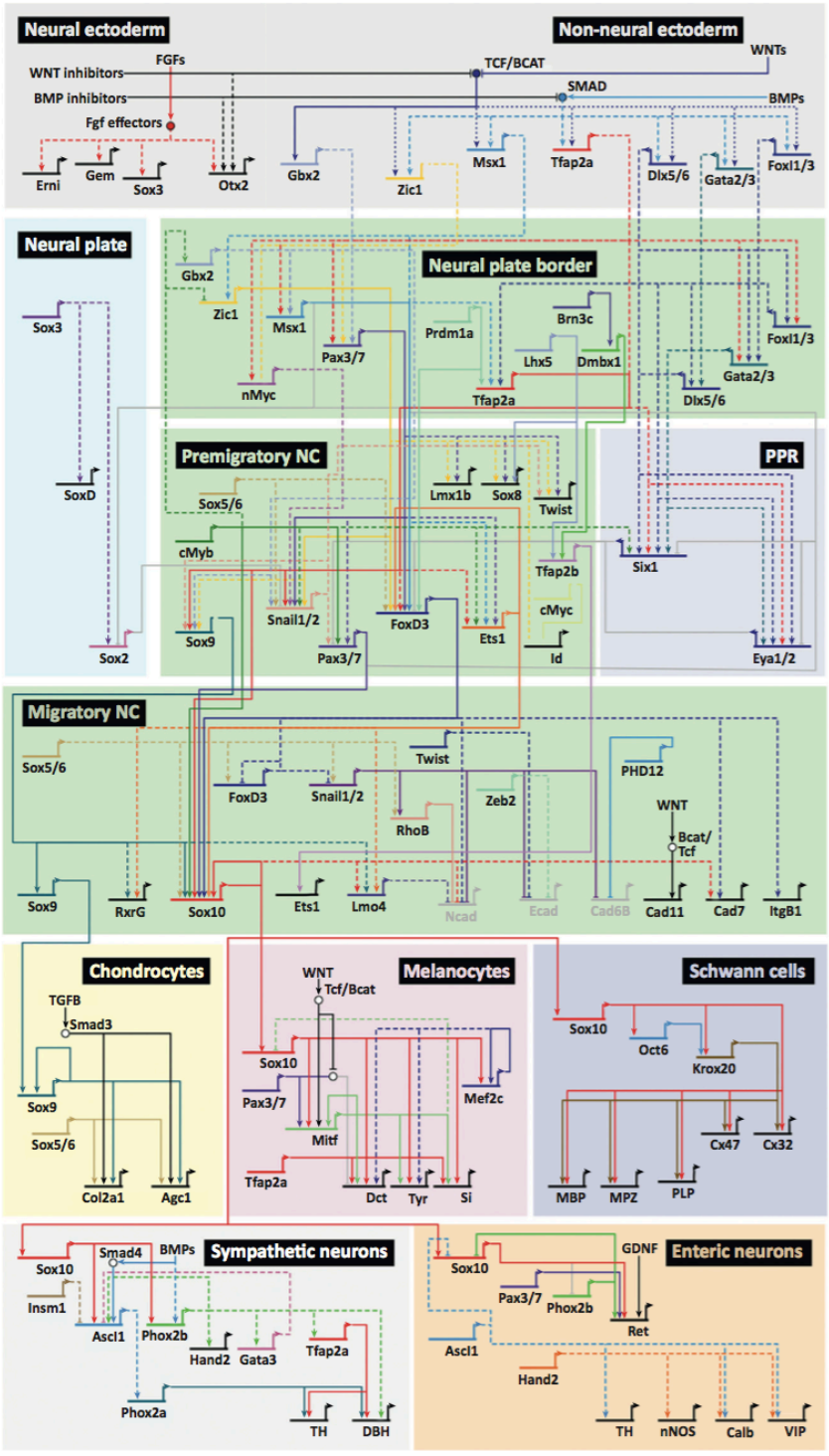


Figure 1.8 Current neural crest gene regulatory network
Current view of the neural crest gene regulatory network subdivided into modules important for "induction", migration and differentiation of neural crest cells in vertebrates (Martik and Bronner, 2017)

Specification of the neural plate border

To understand the origin of neural plate border cells, researchers have focused on investigating the response of the cells to the signals previously described. The combinatorial signaling events function together to induce and position the presumptive neural crest cells in the developing embryo. As result, a cascade of genes that are part of a gene regulatory network important for the specification of the neural plate border is up-regulated. Among the earliest genes involved in the induction of the neural plate border cells include *Msx1*/2, *Pax3*/7, *Zic1*, *Gbx2*, *Hairy2*, *Id3,* and *TF-Ap2*. Loss-of-function experiments of neural plate border genes have demonstrated that neural plate border factors are required for neural crest formation (Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2005; Sato et al., 2005; Li et al., 2009)

Several studies have demonstrated that the dynamic action of different signaling pathways is necessary to induce the neural plate border genes. For instance, *Gbx2* is one of the first genes activated in the neural plate border region and is an immediate direct target of canonical Wnt signaling (Li et al., 2009). FGF signaling has been shown to regulate the induction of *Msx1/2* early neural plate border markers (Monsoro-Burq et al., 2003, Monsoro-Burq et al., 2005). The most well known signaling molecule implicated in the induction of neural crest cells is BMP. Researchers have demonstrated that BMP is the signal responsible for the induction of many neural plate border genes such as *Zic1*, *Dlx5*, *Msx1*/3 and *Id3* (Bae and Saint-Jeannet, 2014; Ben Steventon et al., 2009; Prasad et al., 2012; Stuhlmiller and García-Castro, 2012).

Interestingly the regulatory sequences of some of these genes contain response elements for the signaling pathways involved in the process. For example *Pax3* and *Zic1* have enhancers that are

regulated by BMP, Wnt, and FGF (Garnett et al., 2012). The expression pattern of neural plate border genes broadly marks the cells that will be competent to give rise to the neural crest, placodes and rohon-beard primary neurons. While neural plate border factors are thought to be essential for the formation of neural crest cells, much still remains to be learned about the direct or indirect regulation of the transition from neural plate border to neural crest cells. Neural crest inductive genes in general display broad overlapping expression at the neural plate border, but little is known about the individual and combined contributions to neural crest formation. One hypothesis is that these factors cooperate to delineate the precise region of the ectoderm where the neural crest will form. Another hypothesis is that the inductive factors set up the neural plate border regions earlier in development and with a combination of signaling pathways and epigenetic modifications, neural crest specification will occur.

Neural crest specification

Traditional studies propose that the induction of neural crest cells occurs at mid to late gastrula stage, indicating that important patterning events occur prior to these stages. Genes such *Msx1/2, Pax3/7* and *Zic1, Id3, Ap2,* and *Ets1*, along with combinations of BMP, Wnt, and FGF have been implicated in promoting the initial expression of the neural crest specifier genes *Foxd3* and *Snail1*. These and many other factors are expressed as the ectoderm is patterned, and they are the first markers that distinguish the neural crest cells from other cell types in the ectoderm. (LaBonne and Bronner-Fraser, 2000, Lander et al., 2011).

Foxd3 is a winged helix transcription factor that is expressed in the pre-migratory neural crest cells and is an important regulator of neural crest development. Early studies demonstrated that

Foxd3 is essential for the maintenance of pluripotency of embryonic stem cells, and later it was found to be important for the regulation of the induction, maintenance and differentiation of neural crest cells (Mundell and Labosky, 2011; Sasai et al., 2001; Stewart et al., 2006; Teng et al., 2008). Loss of function of *Foxd3* results in a loss of the expression of multiple neural crest factors, and reduction of the ability to differentiate into different cell types (Stewart et al., 2006; Teng et al., 2008). On the other hand, gain of function experiments resulted in the expansion of the neural crest domain causing ectopic expression of *Snail2*, *Foxd3*, *Ets1* and *TF-AP2* (Sasai et al., 2001).

The members of the Zinc finger transcription factors *Snail1*/2 play critical roles in the specification of the neural crest. Functional studies in *Xenopus* demonstrate that gain-of-function resulted in an expansion of neural crest territory, while loss-of-function prevent neural crest formation (Aybar et al., 2003; LaBonne and Bronner-Fraser, 1998b). *Snail1* is expressed in the prospective neural crest cells earlier than *Snail2* suggesting that *Snail1* is the upstream regulator of *Snail2* (Aybar et al., 2003). Importantly, *Snail2* in combination with WNT is sufficient to induce neural crest formation in ectodermal explants (Heeg-Truesdell and LaBonne, 2006; LaBonne and Bronner-Fraser, 1998b)

Following Snail factors, SoxE factors *Sox8, Sox9, Sox10,* and, *Twist,* among others start to be expressed. The onset of expression of these factors varies among species, but their expression pattern is conserved (LaBonne and Bronner-Fraser, 1998a, Hong and Saint-Jeannet, 2005; Kelsh, 2006).

The dynamic expression of these specifier genes is important to confer to the neural crest cells its identity. Specifier genes appear to both auto-regulate and cross-regulate each other to maintain their collective expression. Neural crest specifier genes regulate a diverse set of effector genes and are reiteratively used in the process of development and are critical for different aspects of neural crest formation such us specification, maintenance, subsequent migration, and differentiation.

Neural crest migration and differentiation

Neural crest cells are a very dynamic and heterogeneous cell population. After specification, cell migration and differentiation occur. There are interesting differences along the body axes in terms of the derivatives that the neural crest cells can form (Lignell et al., 2017; Theveneau and Mayor, 2012). Cranial neural crest give rise to craniofacial bone and cartilage whereas trunk neural crest cells give rise to glia, neurons and melanocytes. After neural crest specification, precursor genes maintain the multipotency of neural crest cells for some time whereas combination of neural crest specifier markers along with signaling molecules triggers a massive expansion and differentiation of neural crest cells (Nieto, 2011; Lignell et al., 2017; Newgreen and Gibbins, 1982).

After neural tube closure, neural crest cells delaminate and undergo an epithelial to mesenchymal transition (EMT). By losing their epithelial connections, they gain the motility and invasive properties seen in mesenchymal cells (Lim and Thiery, 2012; Prasad et al., 2012; Sauka-Spengler and Bronner-Fraser, 2008a) (Figure 1.9 top). EMT allows the neural crest cells to migrate from the neural tube throughout the embryo following distinct pathways and signals, to get to

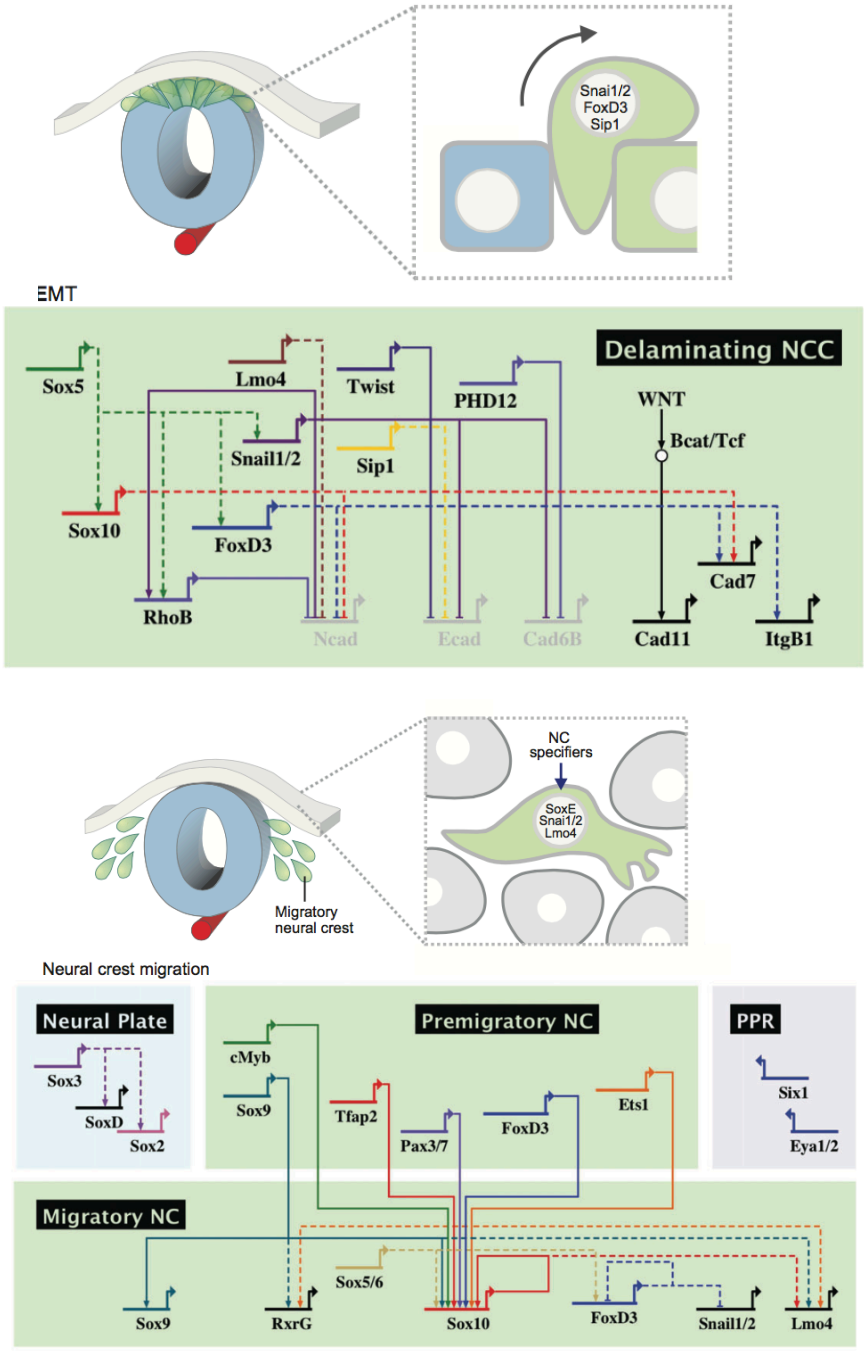


Figure 1.9 Pre-migratory and migratory neural crest cell gene regulatory networks
Neural crest cells after "induction" undergo dramatic cell behaviors that include delamination (Top) and migration (Bottom). For both both processes the gene regulatory network has been widely studied. Adapted from (Simoes-Costa and Bronner, 2015)

multiple places in the periphery to form different derivatives along the body axes (Theveneau and Mayor, 2012, Lim and Thiery, 2012, Martik and Bronner, 2017).

Pre-migratory neural crest cells undergo dramatic changes to allow the cells to migrate from the neural tube. Some of the changes include loss of epithelial polarity, alterations in cell adhesion, changes in cytoskeletal arrangements, dissociation of tight junctions, and remodeling extracellular matrix (Figure 1.9 top) (Lim and Thiery, 2012, Ridley et al., 2003). These changes confer to neural crest cells the ability to delaminate and migrate extensively and ultimately differentiate into a diverse set of cell types, In fact, after neural tube closure, neural crest cells disperse away from the neuroepithelium, and organize into discrete streams in response to guidance cues from the surrounding environment (Figure 1.9 Bottom) (Lim and Thiery, 2012; Theveneau and Mayor, 2012). A combination of environmental signals, such as chemotactic signals, cooperative behaviors due to cell-cell interactions, and distribution of extracellular matrix will allow to promote the formation of subpopulations of neural crest cells along distinct paths. Often the path of migration and the signals the neural crest cells encounter along the way influence differentiation and fate determination (Burns, 2003; Vermeren, et al, 2003; Kirby and Hutson, 2010).

During migratory stages, an EMT gene regulatory network is activated. Neural crest cells then have to respond to environmental cues that will trigger migration but simultaneously maintain their stemness. This gene regulatory network is comprised of several neural crest specifier genes such *Foxd3*, *Snail1/2, Sip1,* SoxE, and *Twist* (LaBonne and Bronner-Fraser, 2000; Rogers et al., 2013; Cheung et al., 2005; Lander et al., 2011; Newgreen and Gibbins, 1982; Sauka-Spengler

and Bronner-Fraser, 2008a) (Figure 1.9). Interestingly, these same factors that are important for conferring the cells a stem cell-like state at pre-migratory stages, will later be reiteratively used for triggering NCC migration and subsequent differentiation. Much work is needed to understand how this switch in utilization of these factors occurs.

Snail family transcription factors that are crucial for the induction of neural crest cells, they are also important partners of cell adhesion molecules such E Cadherin that will ultimately trigger changes in cell polarity and promote mesenchymal characteristics (Vernon and LaBonne, 2004; Lander et al., 2011). Although research has advanced in this field, much work remains to be done to fully understand how cell polarity and directionality are regulated during neural crest migration. One potential explanation is that the dynamics of those Snail factors are very important for regulating cell state. The LaBonne lab that demonstrated that levels of *Snail1*/2 and relative concentrations of those factors are important for carrying on different functions during neural crest development (Lander et al., 2011; Vernon and LaBonne, 2006).

The separation of the population into cranial, vagal, trunk and sacral neural crest cells is a process that requires a complex array of signaling molecules and the dynamic expression and function of SoxE factors. Depending on the environmental cues, individual subpopulations will respond to a number of instructions at a particular time and place in the embryo to differentiate. It has been demonstrated that the final differentiation of these cells will depend on signals, history of trajectories and final position into the embryo (Cheung et al., 2005; Le Douarin and Teillet, 1974), (Kuo and Erickson, 2011; Martik and Bronner, 2017).

The Sox family of transcription factors

The Sox transcription factors are evolutionary conserved and are found in all the species of the animal kingdom. They carry out multiple diverse functions during development, including roles in: pluripotency, germ layer formation, gastrulation, induction, specification and determination of many cell types including the neural crest. (Bowles et al., 2000; Guth and Wegner, 2008; Heenan et al.,2016). Given these different roles, the expression patterns of Sox factors are dynamic and diverse among tissues. Many of the members of the sox transcription factors are essential for the normal development. Functional assays of Sox proteins often result in developmental defects and congenital diseases

Sox transcription factors are known as transcriptional regulators that have a HMG (high mobility group) domain that mediates DNA-binding. The HMG-box domains in Sox family proteins are subdivided into two subfamilies based upon differences in protein structure. The first subfamily has multiple HMG domains that bind DNA in a sequence nonspecific manner, whereas the second subfamily contains only a single HMG domain that binds in a sequence specific manner. These two subfamilies are grouped in eight different classes (A-H) according to the similarities in their encoded amino acid sequences and the homology of the HMG domain (Figure 1.10) (Guth and Wegner, 2008; Bowles et al., 2000).

The HMG domain has about 79 amino acids and is highly conserved among all the members of the family. Outside the HMG domain, the sequences of Sox genes are very variable (Figure 1.11) (Guth and Wegner, 2008). Sox factors from different groups have acquired distinct biological

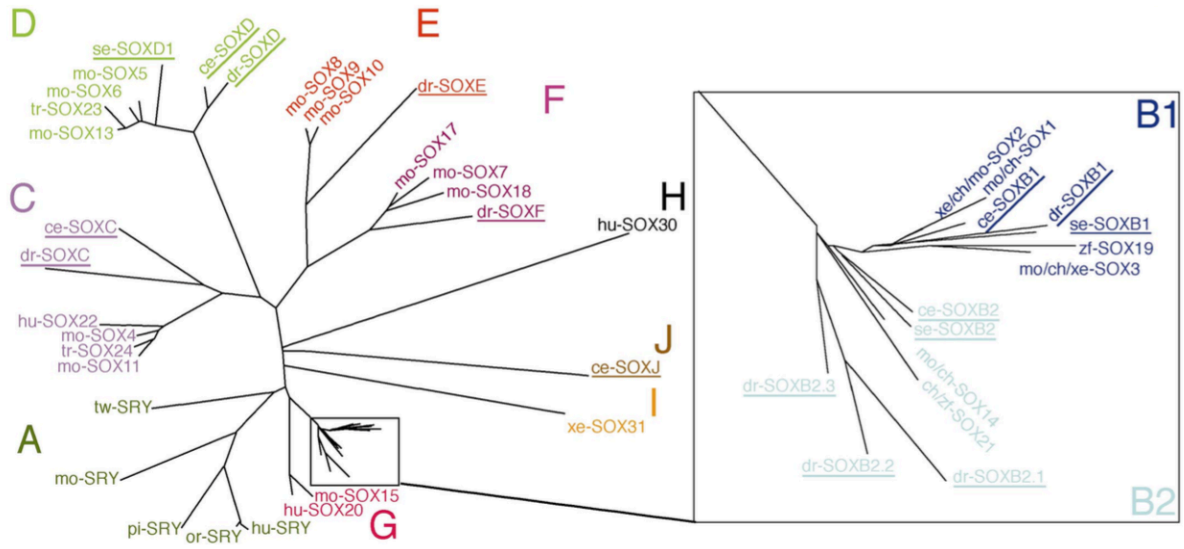


Figure 1.10 Un-rooted phylogenetic tree for the SOX HMG domain
Branch lengths are representative of the extent of divergence. The various groups are highlighted by use of color. The inset shows group B in enlarged format. B1 and B2 clades are colored differently. Invertebrate sequences are underlined. From (Bowles et al., 2000)

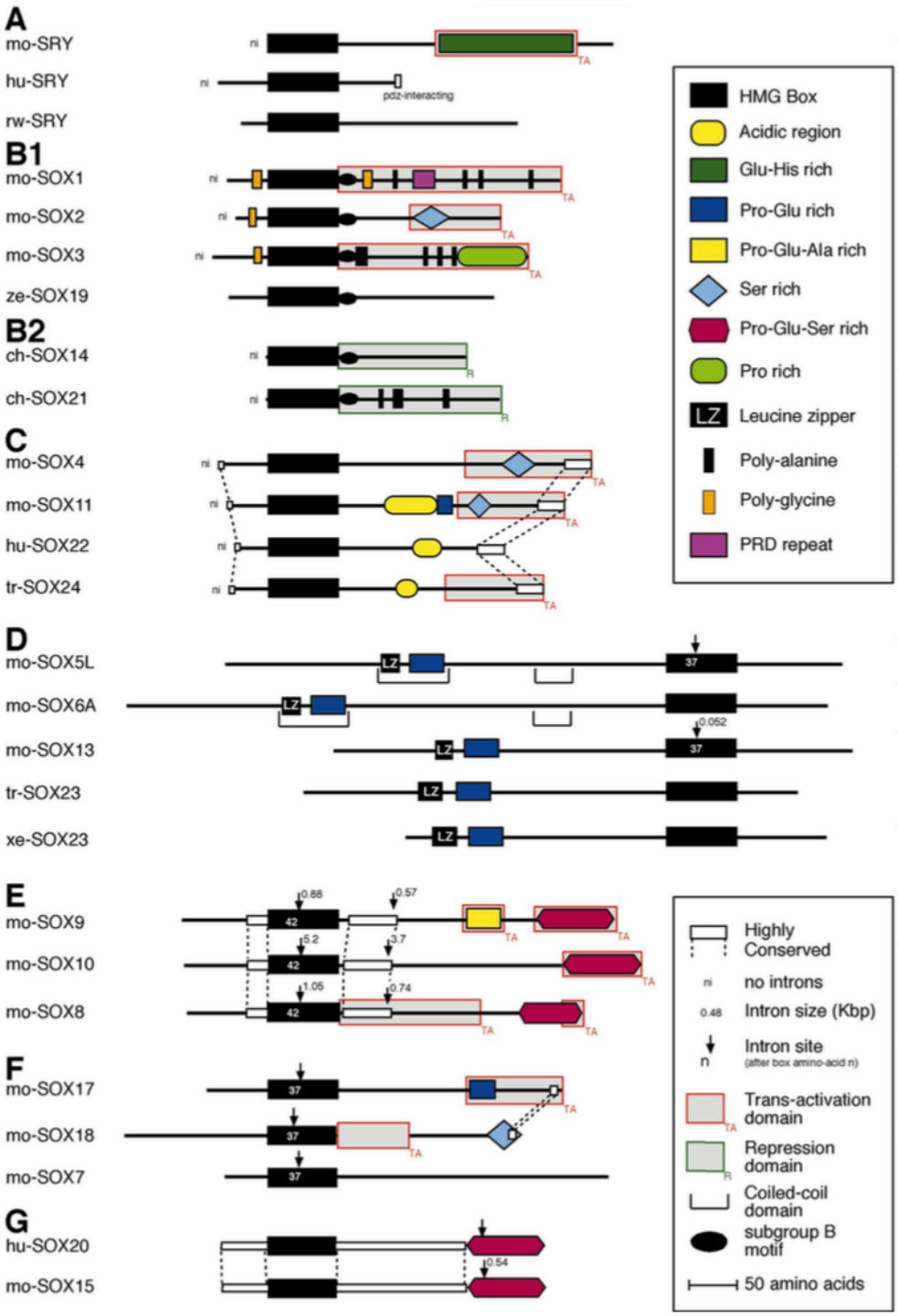


Figure 1.11 Schematic representation of SOX protein

Sox family of proteins (groups A-G) highlighting protein conservation within SOX family groups, proteins are arranged in groups as defined by HMG domain sequence. Various structural features, motifs, and functional regions are shown. From (Bowles et al., 2000)

functions despite recognizing the same DNA consensus motif. For example, HMG domains of groups B, C and E are highly conserved but, there are differences in the flanking regions, the conserved region in group B is located in the C-terminal whereas in groups C and E is located in the N-terminal region (Sarkar and Hochedlinger, 2013; Wegner, 1999). Nevertheless, differential affinity for particular sequences next to consensus Sox sites, homo or hetero-dimerization among Sox proteins, posttranslational modifications, or interaction with other cofactors as well spatial-temporal expression can select similar or different target genes by different groups.

SoxB1 factors and its importance in development

SoxB1 transcription factors include *Sox1, Sox2,* and *Sox3* genes, play diverse roles many developmental processes (Figure 1.12). They are best known for their role in pluripotency and self-renewal, but have also important contributions in neural specification and terminal differentiation of the central nervous system (Kamachi and Kondoh, 2013). In *Xenopus,* SoxB1 *Sox2,* and *Sox3* factors have strong maternal expression, indicating an essential role in the very early stages of development. Later these factors are restricted to the neural plate and placodal domains where they play important roles maintaining progenitor populations. (Collignon et al., 1996; Sarkar and Hochedlinger, 2013; Guth and Wegner, 2008). It might be possible that a precise developmental clock regulates the dynamics of decisions used to generate different fates in the embryo, and the concentrations in which SoxB1 factors are present in the cells could be essential for the maintenance of pluripotency and for of the differentiation of neural fates (Mandalos and Remboutsika, 2017).

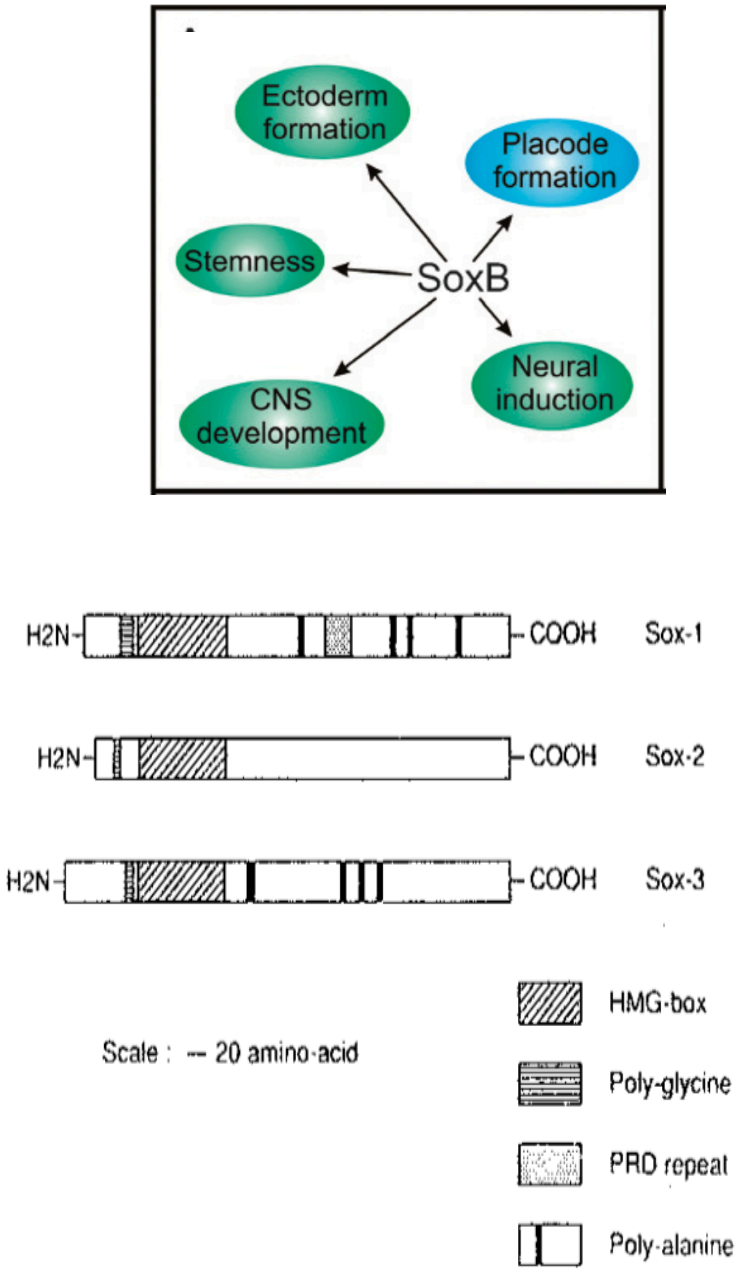


Figure 1.12 SoxB1 proteins functions and schematics (Top), ancient (green) and newly acquired, vertebrate-specific (blue) SoxB1 gene functions. (Bottom), schematic representation of the predicted functional domains of SoxB1 proteins. Adapted from (Collignon et al., 1996; Guth and Wegner, 2008)

Expression of *Sox2* and *Sox3* in *Xenopus* can be visualized at the two-cell stage of development and it persists throughout blastula stages where they are strongly expressed in the inner cell mass cells or embryonic stem cells. Later in development *Sox2* and *Sox3* will restrict their expression to the neural plate where they play important functions in the specification and formation of the early central nervous system from invertebrates to vertebrates.

In *Xenopus,* SoxB1 transcription factors are expressed in a wide, overlapping manner in the neural tube. This expression persists until later in development suggesting a functional significance in the maintenance of the progenitor program during the development of the central nervous system to prevent differentiation. Later SoxB1 transcription factors activate pro-neuronal genes such as *Nestin* that later will interact with *Pou* proteins (*Brn1*, *Brn2,* and *Brn3*) to promote pro-neuronal differentiation (Avilion et al., 2003; Bergsland et al., 2011; Keramari et al., 2010; Niwa, 2007).

SoxE proteins and the importance in development

SoxE proteins Sox8, Sox9, and Sox10 are central components of several processes in vertebrate embryogenesis including neural crest development, inner ear development, skeletal development and sex determination (Figure 1.13). SoxE transcription factors are involved in maintaining pluripotency of neural crest cells at pre-migratory stages, and later, in promoting the differentiation of glial cells, melanocytes and cartilage (Haldin and LaBonne, 2009; Haldin and LaBonne, 2010; Hong and Saint-Jeannet, 2005).

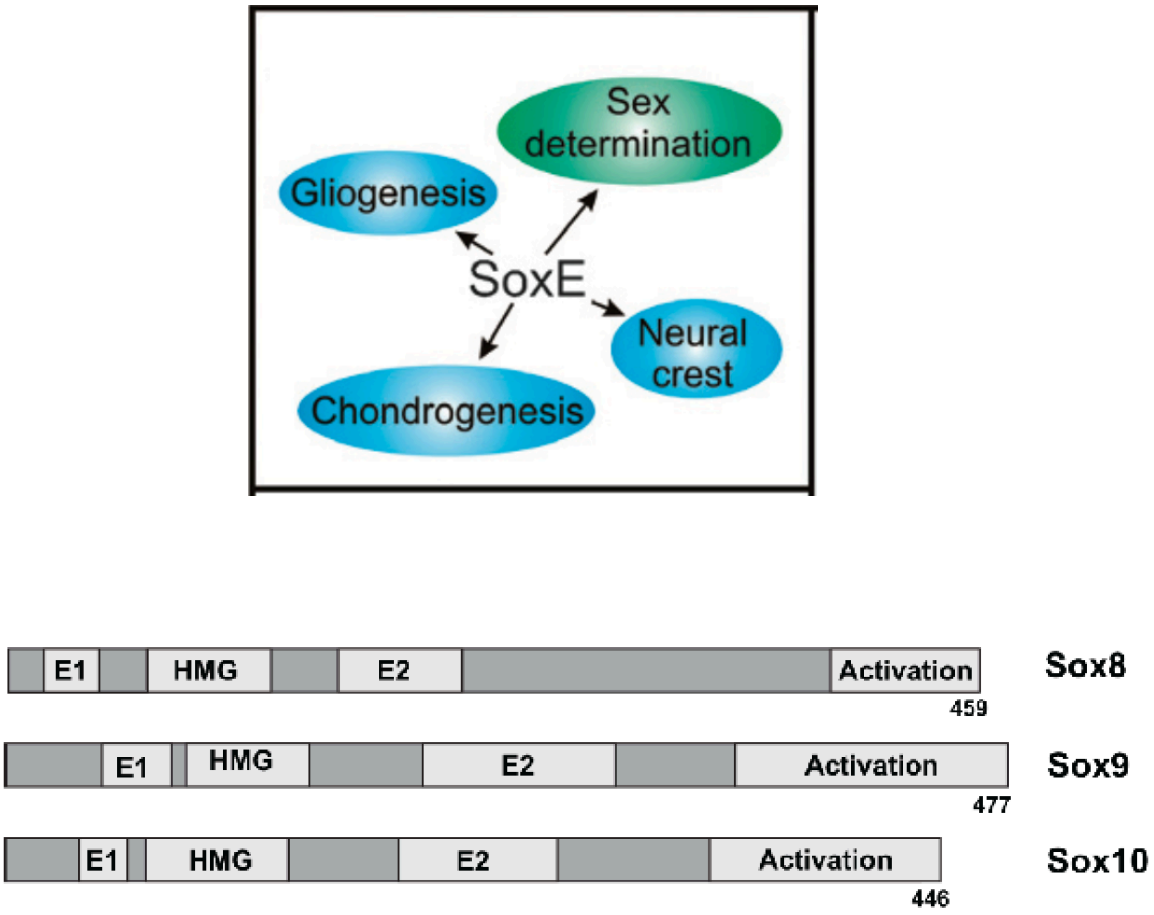


Figure 1.13 SoxE proteins functions and schematics (Top), ancient (green) and newly acquired, vertebrate-specific (blue) SoxE gene functions. (Bottom) schematic representations of the predicted domains for SoxE proteins. Adapted from (Guth and Wegner, 2008; McDowall et al., 1999; Pusch et al., 1998; Schepers et al., 2000)

SoxE factors function at pre-migratory stages is analogous to those of SoxB1 factors in the very early stages development. Their expression *Sox8* is first detected in *Xenopus* in the prospective neural crest cells at mid gastrula stages, followed by the expression of *Sox9* at the end of gastrulation, while *Sox10* is activated at the beginning of neurulation in the neural crest cells. Morpholino of SoxE factors in *Xenopus* resulted in the loss of expression of several neural crest specifier genes such as *Snail2*, *Foxd3,* and *Twist.* Consequentially, neural crest cells failed to develop (Cheung et al., 2005; Lee et al., 2011; Spokony et al., 2002; Taylor and LaBonne, 2005). Gain of function for SoxE factors leads to the expansion of neural crest domain inhibiting or delaying the differentiation of neuronal, glial and melanocyte lineages (Bondurand et al., 2006; Kim et al., 2003; McKeown et al., 2005, Aoki et al., 2003; Taylor and LaBonne, 2005). Suggesting that SoxE factors are required for the formation and survival of neural crest cells.

Importantly, SoxE factors regulate the transition from pre-migratory to migratory neural crest cells. During migratory stages, *Sox9* is an essential regulator of the initiation of EMT (Cheung and Briscoe, 2003), and, at these stages, *Sox10* keeps the migratory neural crest cells in a proliferative state, and helps them to survive and maintain their potency (Kim et al., 2003). This suggests that SoxE factors might sequentially regulate different processes in order to maintain the heterogeneity of neural crest cells during development. This reiterative use can be seen as a switch in functions that could drive neural crest cells towards differentiation and at the same time to maintain in a pluripotent state part of the population. The LaBonne lab has explored extensively the differences in the activity of all three SoxE genes in *Xenopus*.It has been proposed that posttranslational modifications by SUMOylated forms of Sox9 and Sox10 play

context dependent roles in neural crest inducing activity and promoting ear formation by recruitment of transcriptional co-regulator factors (Lee et al., 2012; Taylor and LaBonne, 2005).

SoxE factors are not only required for formation, maintenance of pluripotency and migration of neural crest cells, but are also essential for the differentiation of a variety of neural crest cell derivatives. *Sox9* plays an essential role in chondrocyte formation and cartilage development. During this process *Sox9* turns on the cascade of genes that are required chondrocyte specification. (Akiyama et al., 2002; Bi et al., 1999; Lefebvre and Dvir-Ginzberg, 2017, Akiyama et al., 2002; Bi et al., 1999; Spokony et al., 2002). It has been proposed that *Sox9* plays dual role during chondrogenesis: promoting chondrogenic cell fate while preventing ossification (Lefebvre and Dvir-Ginzberg, 2017; Lefebvre et al., 1997; Suzuki et al., 2006). Loss of function of Sox9 leads to failed formation of neural crest showing a down regulation of neural crest specifier markers such *Snail1/2, Pax3* and *FoxD3*, later in defects in cartilage formation are observed due to the lack of expression of chondrocyte marker genes such as *Col2a1*, *Col9a2* and *Agc1* (Bi et al., 1999; Akiyama et al., 2002; Mori-Akiyama et al., 2003; Spokony et al., 2002). Interestingly, lack of cartilage formation do not affect the formation of other derivatives such melanocytes and trunk neural crest cells.

The best example studied of cell lineage specification in neural crest cells, is melanocyte differentiation. *Sox10* is required for the induction of the regulatory program that induces melanoblasts (Bondurand et al., 2000; Elworthy et al., 2003; Ludwig et al., 2004), and also cooperates with terminal differentiation of pigment cells (Potterf et al., 2000). In *Xenopus*, overexpression of Sox10 leads to an increased number of melanocytes, suggesting a positive role

of SoxE in the process of regulating enhancer regions of genes such as *Dct* and *Trp1* (Aoki et al., 2003; Cheung and Briscoe, 2003; Taylor and LaBonne, 2005). *Sox10* has been shown to cooperate with *Mitf* to activate downstream target genes essential for melanocyte development (Aoki et al., 2003; Jiao et al., 2004; Murisier et al., 2006). Loss of function experiments shows defects in the development of melanocytes. Suggesting that *Sox10* is required for the induction and regulation of the molecular program involve in melanocyte formation (Taylor and LaBonne, 2005)

Neural crest cells undergo dynamic changes in morphology cell behavior, expression and regulation of transcription factors in the course of development. Despite several groups have work to understand neural crest cells from different perspectives, much is still to explore and discover about the molecular mechanisms underlying the origin of the potency of these cells to understand the evolution and development of the complex structures and cell types that emerge during the evolutionary history of vertebrate chordates.

Xenopus laevis as a model system

Xenopus laevis commonly known as the African clawed frog is a vertebrate that lives in freshwater. *Xenopus laevis* is one of the traditional model systems used in biology research. This model system has lead to important discoveries in cell biology, molecular biology, biochemistry and developmental biology including work on the cell cycle, cell reprogramming, and DNA damage response.

The reasons for its worldwide usage in research lie in the high conservation of most essential cellular and molecular mechanisms. Furthermore, it is inexpensive to maintain, embryos are easily manipulated and large amounts of material can be readily obtained and fertilized for a variety of experimental procedures.

One of the most powerful advantages of *Xenopus* embryos is its size; the eggs measure 1mm in diameter approximately, that offer plenty of material (Protein, DNA, RNA) for biochemical experiments. Because the embryos are big, it is easy to dissect animal caps for competence experiments.

What is unique about *Xenopus* is that after the first cell division the Left / Right are separated allowing for manipulation of one half of the embryo without disturbing the other side – leaving it as our perfect internal control. These manipulations are very useful for several functional analyses of proteins of interest. Another advantage of Xenopus embryos is that they grow outside the mother and they develop fast; so we can study development from two cells to a tadpole in a short period of time (3 days) (Figure 1.16).

In this chapter I presented a compilation of the history of neural crest research from its discovery until recent years, I explained in detail molecular processes that are involved in the induction, migration, and differentiation of neural crest cells making special emphasis in the participation of Sox proteins. At the end I presented the evolutionary implications of the appearance of neural crest in evolution. In the next chapters I will describe the molecular underpinnings of pluripotency and how we suggest a novel origin for neural crest stem cells.

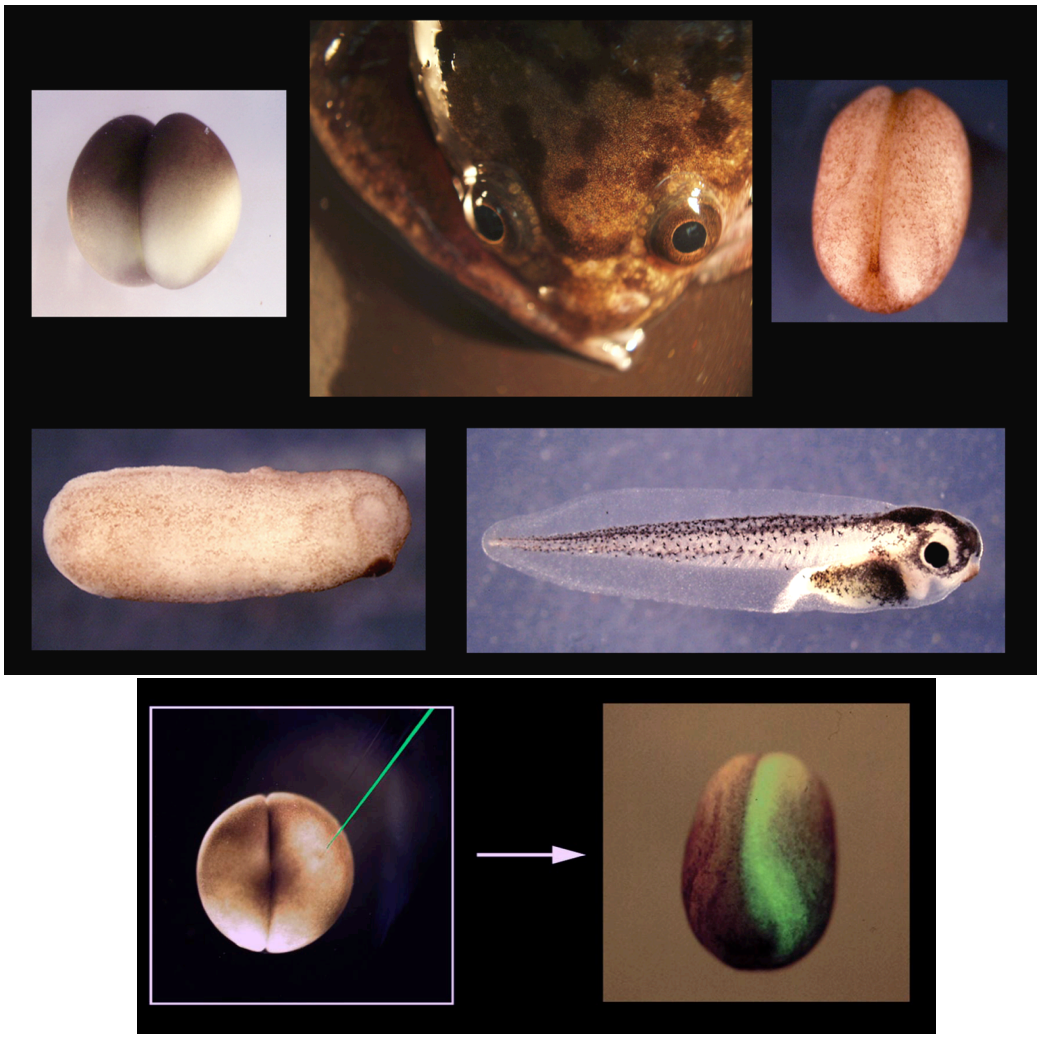


Figure 1.14 *Xenopus laevis* embryos a powerful system to study developmental biology
Top and intermediate panels: Xenopus in different stages of development from a two-cell embryo to an adult. Bottom panel manipulation of the right cell of the two-cell embryo with GRF mRNA, the effect is restricted to one half of the embryo and leave the other half as an internal control.

Chapter 2

Shared regulatory programs suggest a retention of blastula-stage potential in neural crest cells

Summary

Neural crest cells are a unique cell type to vertebrates, they arise in the ectoderm but can generate cell types that are typically categorized as mesodermal. This broad developmental potential persists past the time when most ectoderm-derived cells become lineage restricted. The ability of neural crest to contribute to mesodermal derivatives and to the complexity of the body plan in vertebrates has raised the question about how this apparent gain of potential is achieved. Here, I describe shared molecular underpinnings of potency in neural crest and blastula cells. I show that in *Xenopus*, key neural crest regulatory factors are also expressed in blastula animal pole cells and promote pluripotency in both cell types. I suggest that neural crest cells may have evolved as a consequence of a subset of blastula cells retaining activity of the regulatory network underlying pluripotency.

Introduction

Embryogenesis initiates with a fertilized egg whose cells are totipotent. As development proceeds, progressive restrictions in cellular potential take place. After fertilization, several cell divisions occur and only few hours after fertilization the embryo is a ball of cells called blastula. At blastula stages, chordate embryos possess populations of cells capable of differentiating into all somatic cell types. In mammals these inner cells are part of the inner cell mass, whereas in *Xenopus* they are the deep/inner cells of the blastula roof, also termed animal pole cells (Furue and Asashima, 2004). The pluripotency of blastula cells is transient; as embryogenesis proceeds into gastrulation, their potential becomes rapidly restricted into one of three cell types: ectoderm, mesoderm, and endoderm. In all vertebrate species, a population of stem cell–like progenitors,

called neural crest cells, represents an exception to this loss of potential. These cells arise from ectoderm positioned at the neural plate border. However, in addition to ectodermal cell types, neural crest cells can also differentiate into cartilage, bone, connective tissue, smooth muscle, pericytes, and adipocytes, all of which are also formed by the mesoderm. Neural crest cells represent a major vertebrate innovation, collectively contributing to many of the features that distinguish vertebrates from nonvertebrate chordates, including much of the craniofacial skeleton, the chromaffin cells of the adrenal medulla, and spinal nerve (dorsal root) ganglia. Despite its ectodermal origin, neural crest forms numerous cell types considered mesodermal. As result, the neural crest has been described as a fourth germ layer that renders vertebrates quadroblastic, and endows them with the potential to form a diversity of new cell types (Hall, 2013; Le Douarin and Dupin, 2014).

Much effort has been directed toward determining the mechanisms via which neural crest induction leads to the formation of cells with greater potential than those they were derived from. In recent decades several research groups have focus on understanding the gene regulatory network that allow the neural crest cells to have this grater developmental potential. Their work is based on the classic model of neural crest formation, which suggests that neural crest cells regain developmental potential before they give rise to different germ layer derivatives (Martik and Bronner, 2017; Prasad et al., 2012; Sauka-Spengler and Bronner-Fraser, 2008; Simoes-Costa and Bronner, 2015). An alternative, more parsimonious, model for the origins of these cells might be that they retain activity of the regulatory circuitry underlying the broad developmental potential of their blastula precursors.

Previous research from the LaBonne lab, suggests that certain factors known to be required for pluripotency of blastula cells, such as *Myc* and its target *Id3*, are also required for neural crest development (Bellmeyer et al., 2003; Cartwright et al., 2005; Light et al., 2005; Ying et al., 2003). Also, the LaBonne lab recently found that the transcription factor *Sox5,* is first expressed in blastula cells where it functions as a BMP R-Smad co-factor (Nordin and LaBonne, 2014). This evidence and results from a preliminary screen of pluripotency factor expression led me to question whether neural crest cells and pluripotent stem cells share regulatory programs to maintain a greater developmental potential. In this chapter I will show evidence demonstrating in *Xenopus* that both cell states, namely neural crest and pluripotent blastula cells, share indeed a common pluripotency regulatory programs. Based on the observations made here, an alternative and more parsimonious model for neural crest development in *Xenopus* is proposed. Finally I discuss possible implications of these findings in the context of stem cell biology.

Results

Neural crest shares regulatory circuitry with pluripotent blastula cells.

In mammals, *Pou5F1* (*Oct4*), *Sox2* and *Nanog*, constitute a core pluripotency network essential for maintaining the uncommitted state of blastula cells (Avilion et al., 2003; Chambers et al., 2003; Mitsui et al., 2003; Nichols et al., 1998; Niwa et al., 2000; Young, 2011). In *Xenopus*, the *Pou5F1* factors expressed in ectoderm are *Pou5F3*.1 (*Oct91*), *Pou5F3*.2 (*Oct25*) and *Pou5F3*.3 (*Oct60*) (Frankenberg et al., 2014; Morrison and Brickman, 2006). The functional role of *Nanog* in *Xenopus* is assumed by the Ventx factors (*Vent1*/2) (Scerbo et al., 2012). These factors, along with *Sox2*, and the closely related *Sox3*, are expressed in blastula cells (Rogers et al., 2013) (Figure 2.1). I first asked if factors essential for the genesis/potency of neural crest

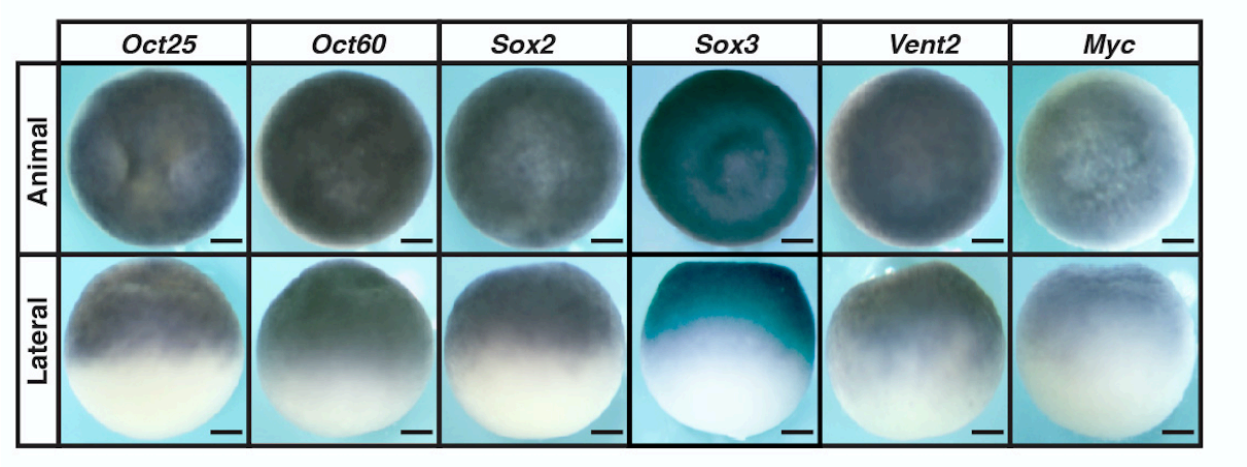


Figure 2.1 Wild type expression of pluripotency factors at blastula stage
In situ hybridization of wildtype blastula (stage 9) *Xenopus* embryos examining the expression of genes associated with pluripotency. Scale bars 250 μm.

cells are also present in blastula cells, and found that *Id3*, *TF-AP2, Ets1, FoxD3* and, *Snail1* were co-expressed with the core pluripotency factors (Figure 2.2). *FoxD3* and *Snail1* are also expressed in murine embryonic stem cells (Lin et al., 2014; Liu and Labosky, 2008), providing further molecular links between neural crest factors and pluripotency. While both neural crest and pluripotency factors exhibit broad expression during blastula stages, their expression becomes progressively restricted during gastrulation as lineage determination progresses. Several factors including *Oct60, Sox3, Vent2, Ets1, Zic1*, *Pax3*, and *Snail1*, show enhanced mRNA expression at the neural plate border by late gastrula stages (Figure 2.3 and 2.4). I found *Vent2* expression co-localized with *Snail2* at late gastrula/neurula stages when neural crest cells retain their full developmental potential. However, *Vent2* is down-regulated at the time when these cells begin to migrate and lose multipotency (Figure 2.5).

Explanted blastula animal pole cells retain full developmental potential until the onset of gastrulation when they lose competence to form mesoderm and endoderm (Jones and Woodland, 1987; Grainger and Gurdon, 1989). I thus examined whether mRNA expression of regulatory factors present in pluripotent blastula cells is lost as these cells age and their developmental potential becomes restricted. *Oct60, Sox3*, *FoxD3*, and *Myc* expression is high in blastula cells but reduced by late gastrula stages, correlating with loss of developmental potential (Figure 2.6). Not all potency factors were down-regulated as these cells lost plasticity; expression of *Vent2* and *Id3* was unchanged as explants aged from blastula to gastrula stages (Figure 2.6). This suggests that a concentration-dependent signature of regulatory factors may be essential to retaining broad developmental potential and preventing lineage restriction, This notion is consistent with findings in mouse showing that specific threshold concentrations of Oct4 (50-

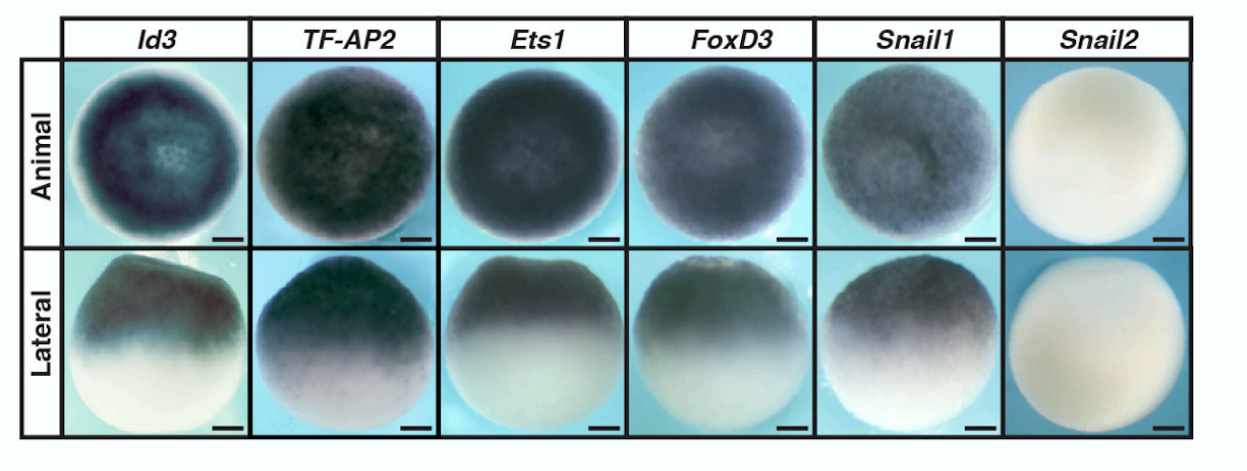


Figure 2.2 Wild type expression of pluripotency factors at blastula stage
In situ hybridization of wildtype blastula (stage 9) *Xenopus* embryos examining the expression of genes associated with neural crest development. Scale bars 250 μm.

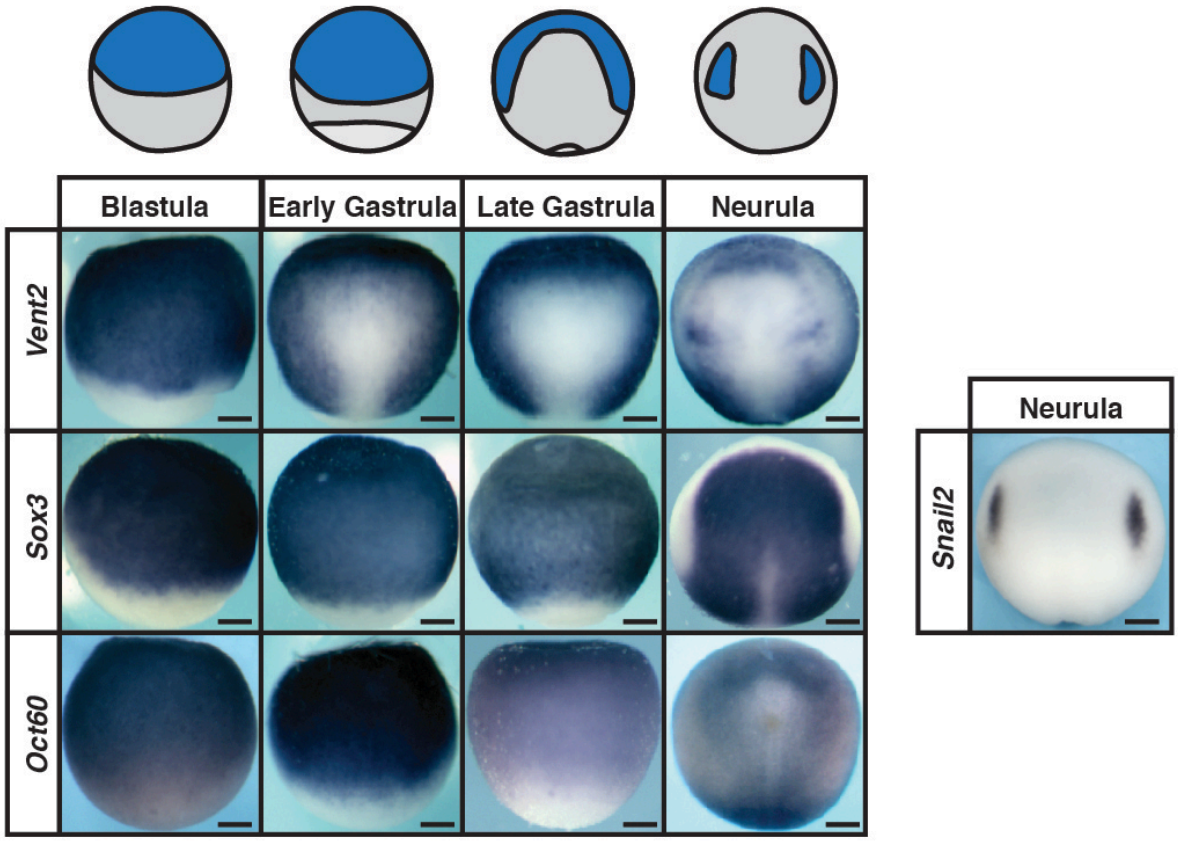


Figure 2.3 Expression of core pluripotency factors becomes progressively restricted to the neural plate border

In situ hybridization of a time series wild type *Xenopus* embryos from blastula (stage 9) to neurula (Stage 13) examining the expression of genes associated with pluripotency. Scale bars 250 μm.

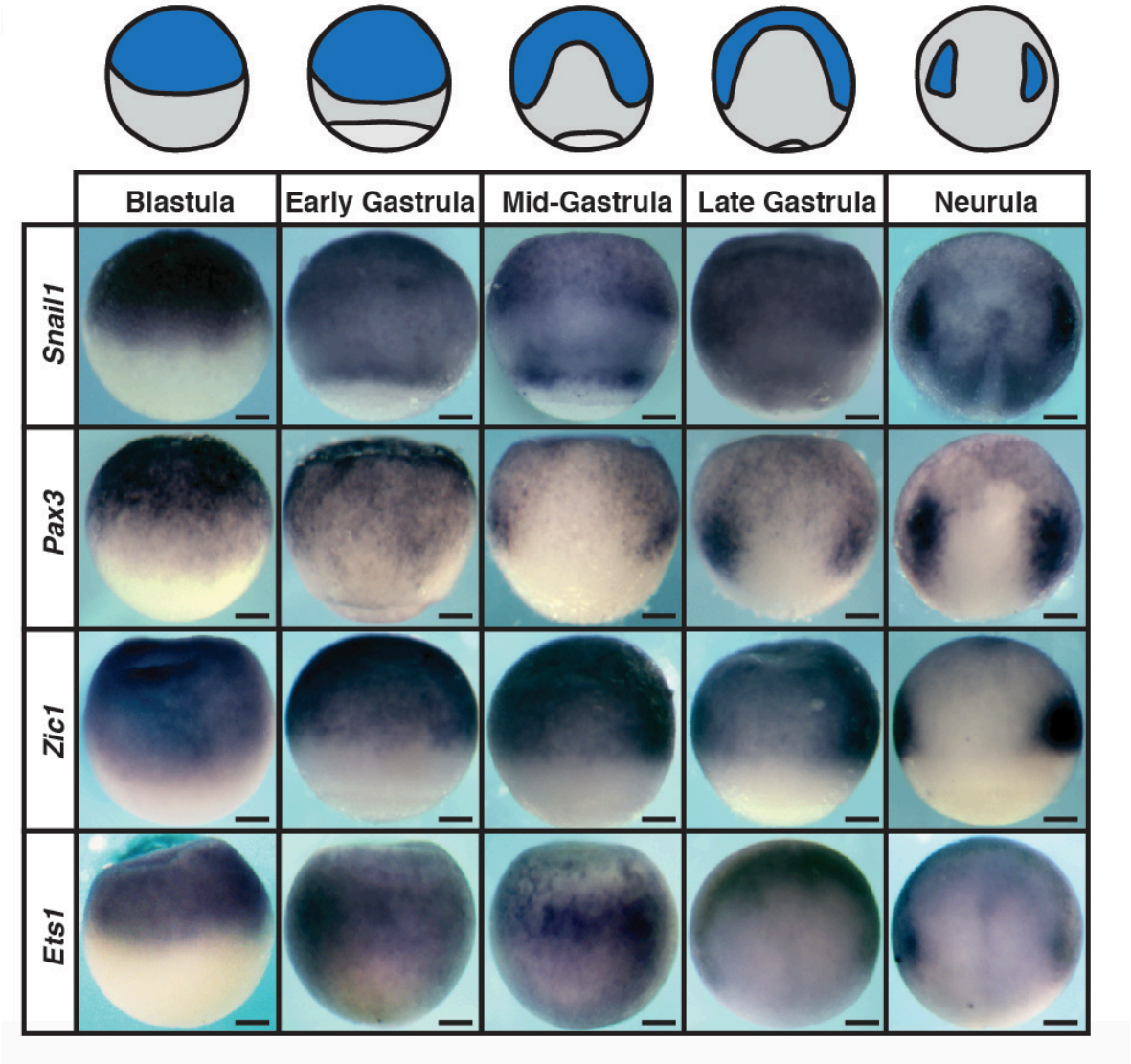


Figure 2.4 Expression of neural crest factors becomes progressively restricted to the neural plate border

In situ hybridization of a time series wild type *Xenopus* embryos from blastula (stage 9) to neurula (Stage 13) examining the expression of genes associated neural crest development. Scale bars 250 µm.

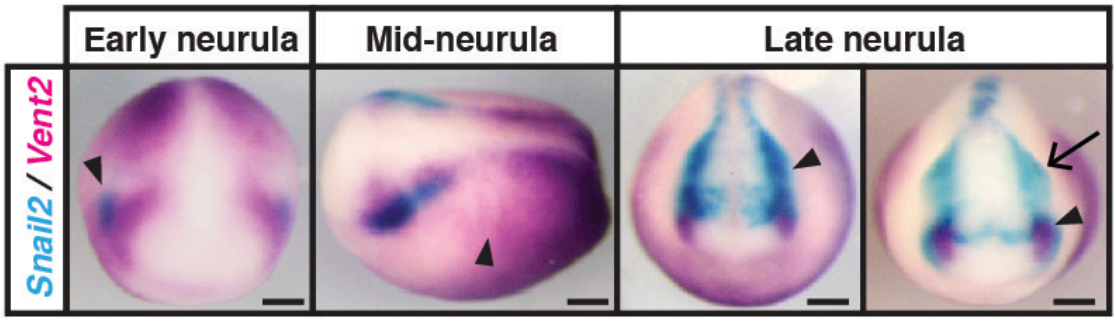


Figure 2.5 The pluripotency factor *Vent2* expression co-localized with the neural crest factor *Snail2.* Double *in situ* hybridization, demonstrating *Vent2* and *Snail2* expression overlap in at early/mid-neurula stages. At late neurula stages expression becomes non-overlapping. Arrowhead denotes overlap in expression. Arrow denotes expression only of *Snail2*. Scale bars, 250 μm. (Double in situ hybridizations were performed by Ann Vernon)

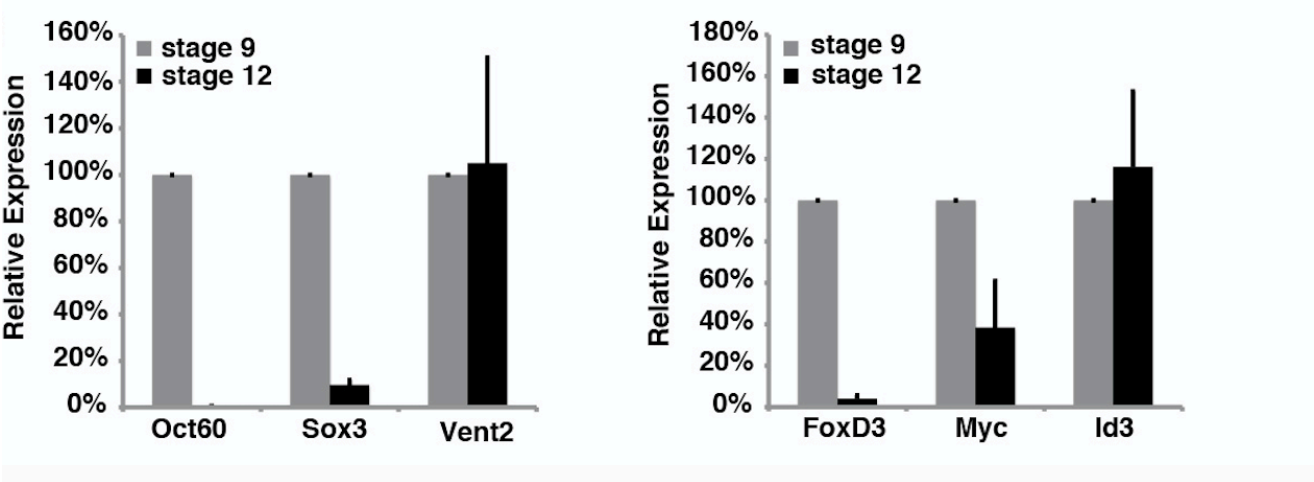


Figure 2.6 Quantification of pluripotency factors and neural crest factors at different stages of embryonic development

qRT-PCR of wildtype ectodermal explants examining relative expression of pluripotency genes (*Oct60*, *Sox3,* and *Vent2*) and neural crest genes (*FoxD3*, *Myc,* and *Id3*) over developmental time. These experiments were performed by Kara Nordin.

150% of endogenous levels) support pluripotency, while levels outside this range result in differentiation (Chambers and Tomlinson, 2009; Niwa et al., 2000).

Neural crest factors are required for pluripotency of blastula cells

Given that neural crest potency factors are co-expressed with core pluripotency promoting factors in blastula cells, I asked if neural crest factors were required to maintain expression of the core pluripotency factors at blastula stages of development. Experiments to answer this question were done in collaboration with Kara Nordin and Anjali Rao from the LaBonne Lab. Blocking *Snail* function using dominant negative forms of Snail proteins in blastula cells led to a loss of factors linked to the neural crest state, such as *TF-AP2* and *Id3* (Figure 2.7 and 2.8). Interestingly, expression of *Oct/Sox/Vent* network components was also lost (Figure 2.7 and 2.8). Most importantly, we performed the same experiments using translation blocking morpholinos for Sox5 and, and similar results were obtained (Figure. 2.9 and 2.10). Thus, neural crest regulatory factors are not only expressed in pluripotent blastula cells but are necessary and function there to maintain expression of core pluripotency factors.

Peter Nieuwkoop first demonstrated the developmental plasticity of amphibian animal pole cells. His famous recombinant assay drove current understanding of mesendoderm formation (Figure 2.11) (Nieuwkoop, 1969). As neural crest factors such as Snail1 are required for maintaining expression of factors linked to pluripotency, I hypothesized that cells depleted of Snail1 would lack competence to respond to endogenous inducing signals.

To test this hypothesis, animal pole explants from control blastulae, or blastulae in which Snail1

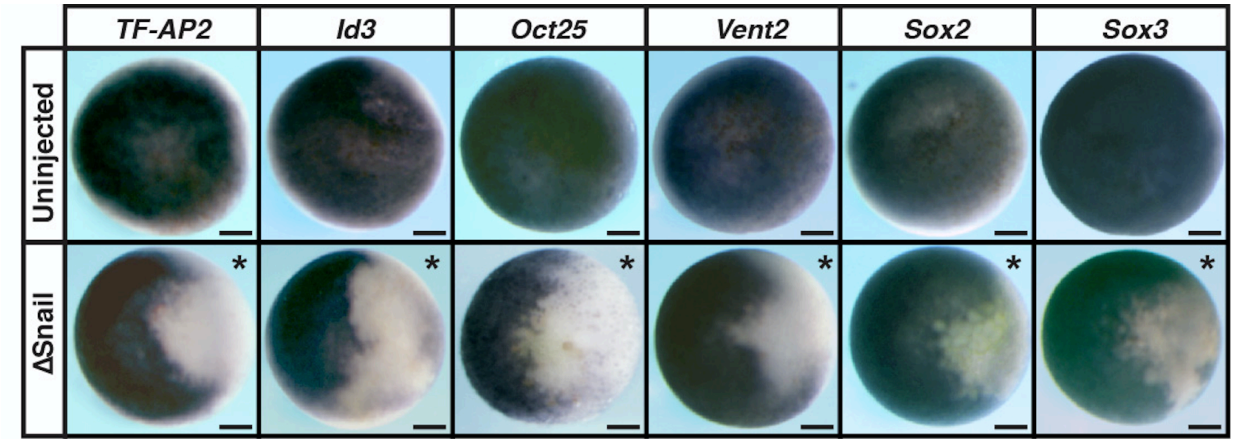


Figure 2.7 Neural crest factors Snail1/2 are required for the expression of blastula pluripotency factors

In situ hybridization of embryos injected with ΔSnail Embryos were collected at blastula stages (stage 9) and examined for expression of genes associated with pluripotency/neural crest formation. Asterisk denotes injected side with β-gal staining (red) serving as a lineage tracer. Scale bars, 250 μM, (experiments from the figure were performed by Anjali Rao)

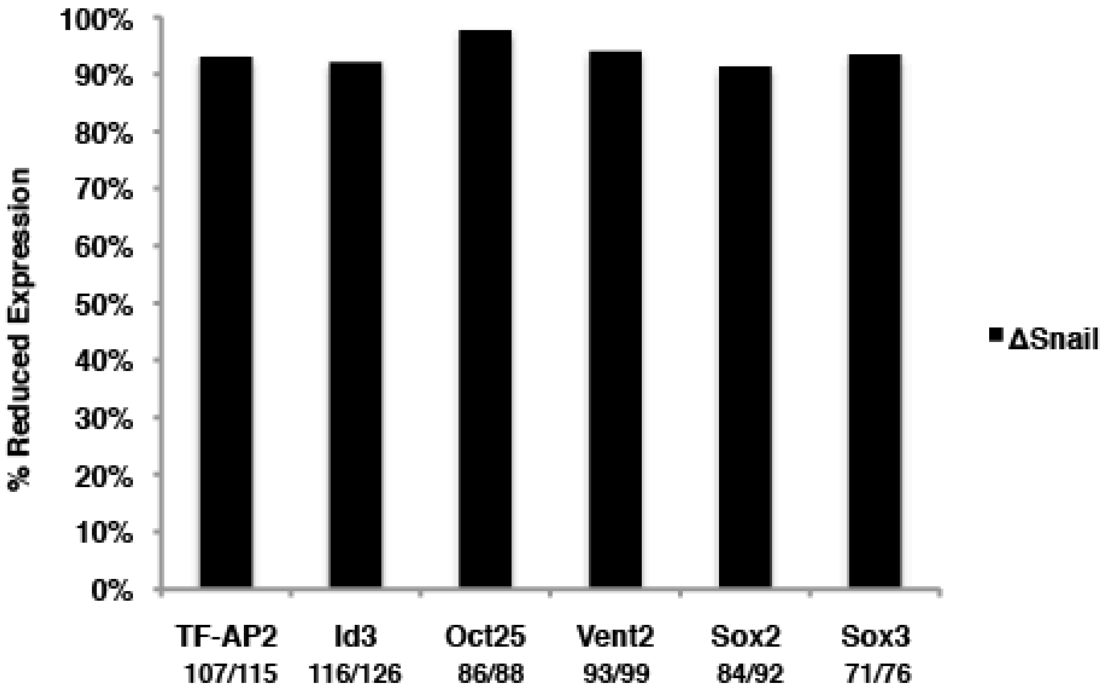


Figure 2.8 Quantification of Snail1/2 loss of function experiments
Quantification of the % reduced expression of pluripotency and neural crest genes in whole embryos that were injected with ΔSnail mRNA, (from figure 2.7).

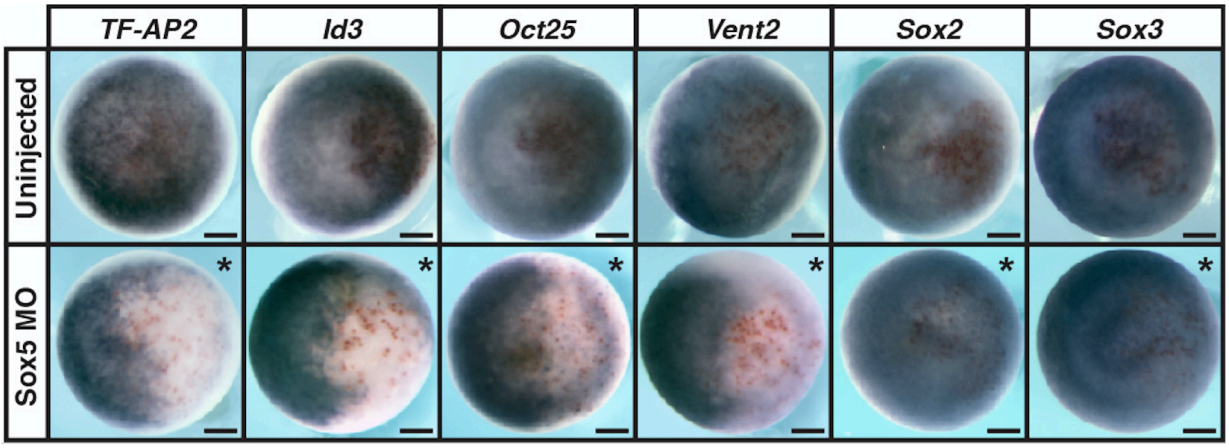


Figure 2.9 Neural crest factor *Sox5* is required for the expression of blastula pluripotency factors.
In situ hybridization of embryos injected with ΔSnail Embryos were collected at blastula stages (stage 9) and examined for expression of genes associated with pluripotency/neural crest formation. Asterisk denotes injected side with β-gal staining (red) serving as a lineage tracer. Scale bars, 250 μM, (experiments from the figure were performed by Kara Nordin).

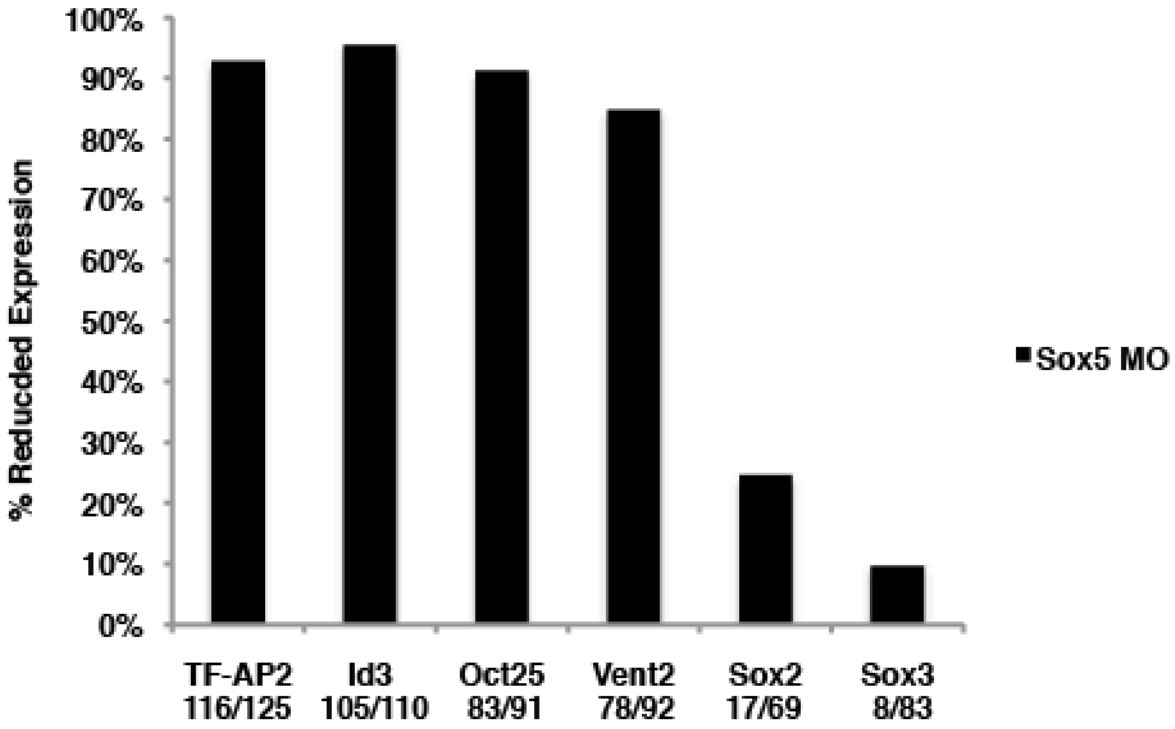


Figure 2.10 Quantification of *Sox5* loss of function experiments in whole embryos
Quantification of the % reduced expression of pluripotency and neural crest genes in whole embryos that were injected with Sox5 MO (from figure 2.9).

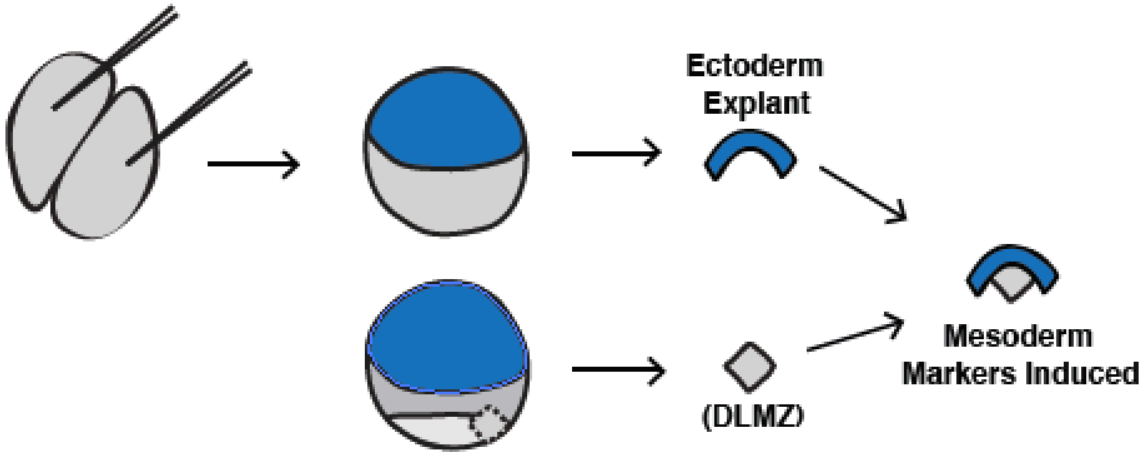


Figure 2.11 Schematic representation of the Nieuwkoop recombinant assay.

Animal pole ectoderm of injected or control embryos is dissected at blastula stages and conjugated to a dorsal lateral vegetal pole explant from a stage 10 embryo. Vegetal cells send, inducing signals (Nodal) to responding cells in the ectoderm.

function had been blocked, were conjugated to vegetal tissue from sibling controls. Recombinants robustly expressed mesodermal markers *Brachyury* and *MyoD*, whereas cells

blocked for *Snail* function showed dramatically diminished responsiveness (Fig. 2.12 and 2.13). Similar results were observed with cells depleted of *Sox5* (experiments on this section were performed by Kara Nordin)(Figures 2.12 and 2.13).

As with conjugation to vegetal tissue, treatment of pluripotent blastula cells with low/moderate doses of activin instructs them to form mesoderm (Figure 2.14), and this responsiveness is also lost in cells depleted of *Snail* or *Sox5* function (Figures 2.15 and 2.16). Since Snail factors have roles in mesoderm formation endogenously, a more demanding test of their contributions to pluripotency in blastula cells is to ask if cells lacking Snail retain the capacity to form endoderm. Blastula explants adopt endodermal fates in response to high activin, expressing endoderm markers *Endodermin* and *Sox17,* but blastula explants depleted of Snail function can no longer form endoderm (Figures 2.17 and 2.18). Snail proteins are neither expressed in, nor function in endoderm endogenously, thus loss of activin-mediated endoderm induction likely reflects a general lack of competence of Snail depleted animal pole cells to respond to lineage restricting signals. Similar results were found when Sox5 was depleted from blastula cells (Experimets were performed by Anjali Rao and Kara Nordin)(Figures 2.17 and 2.18).

Reprogrammed neural crest cells can form endoderm

Given that neural crest potency factors are expressed in pluripotent blastula cells and required for expression of core pluripotency factors, I further explored the link between the neural crest state and the pluripotent blastula state. Specifically, I asked if establishing a neural

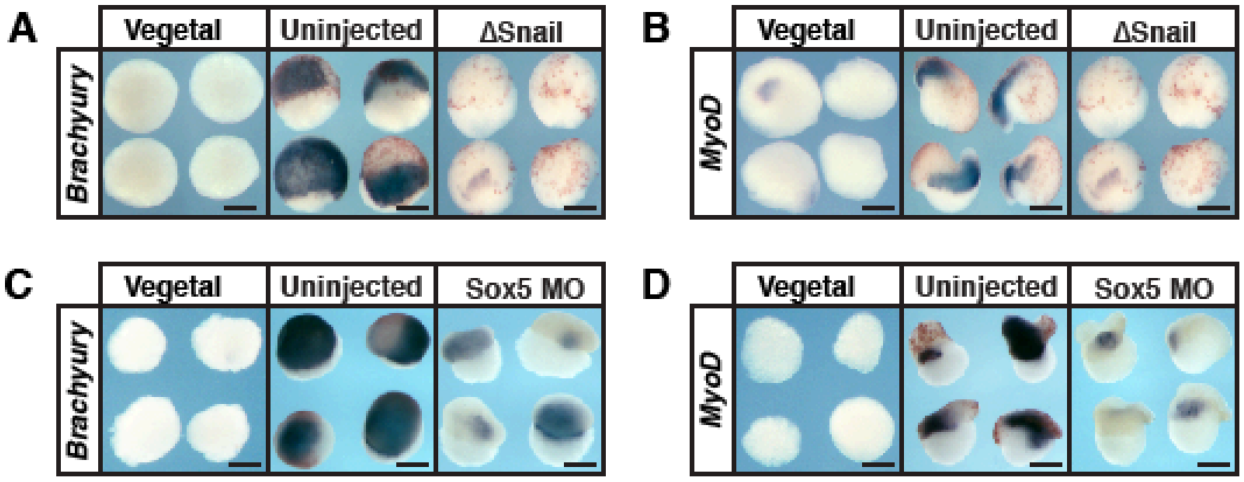


Figure 2.12 Neural crest factors Snail1 and Sox5 are required for the competence of animal pole cells to form mesoderm

Nieuwkoop recombinant assay, examining the expression of *Brachyury* (A and C) and *MyoD* (B and D) after depleting Snail1 (A and B) and Sox5 function (C and D). Recombinants were harvested at gastrulation stages for *Brachyury* expression (stage 11.5) and at early neurula stages (stage 13/14) for *MyoD* expression. Scale bars, 250 μM, (experiments in this figure were performed by Kara Nordin).

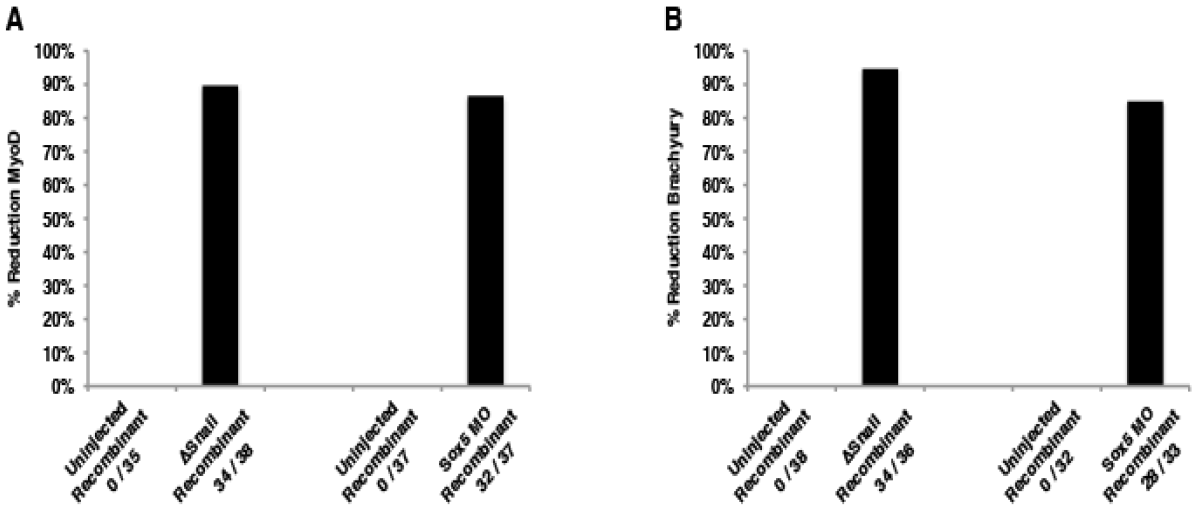


Figure 2.13 Quantification of *Sox5* loss of function experiments in Nieuwkoop experiments. Quantification of the % reduced expression of *MyoD* (A) and *Brachyury* (B) in Nieuwkoop recombinant assays (Figure 2.12) analyzing the effect of depleting Snail1 or Sox5.

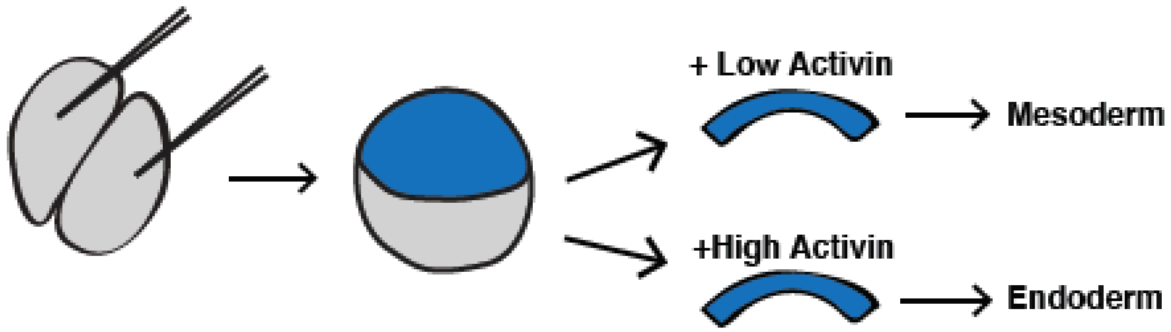


Figure 2.14 Schematic representation of activin treatment of ectodermal explants Ectoderm from the animal pole of donor/ injected embryos is explanted at blastula stages and treated with low or high amounts of activin to induce either mesoderm or endoderm formation, explants were cultured until gastrulation stages for *Endodermin, Sox17* and *Brachyury* and collected at early neurula stages for *MyoD*.

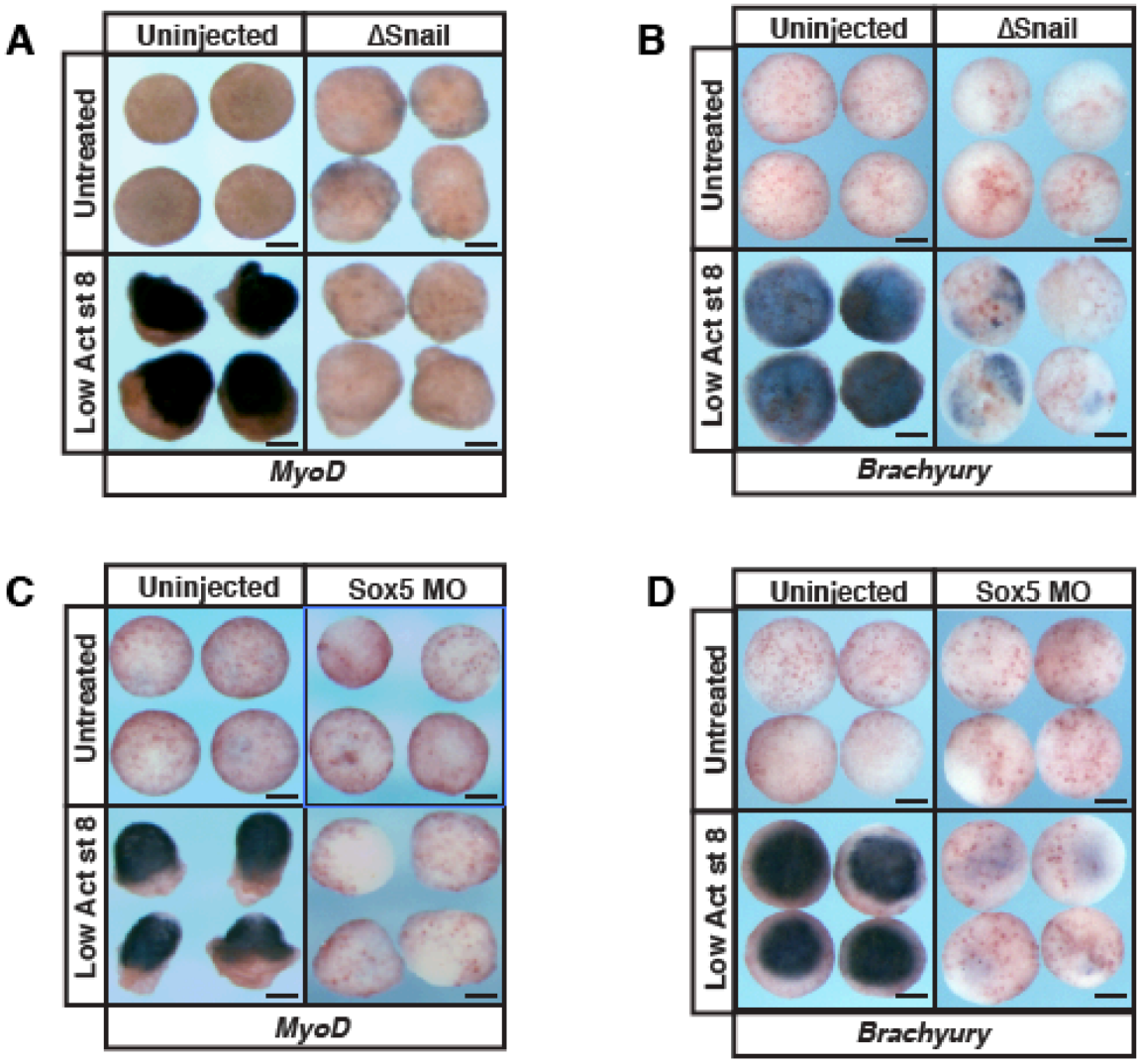


Figure 2.15 Snail1 and Sox5 are required for pluripotency of blastula ES cells to form mesoderm

Ectodermal explant assay examining the expression of *MyoD* (A, C) and *Brachyury* (B, D). Explants were injected with ΔSnail (A, B) or Sox5 morpholino (C, D) and cultured with or without activin until early neurula stages for *MyoD* expression (stage 13/14) and early gastrula stages (stage 11.5) for *Brachyury* expression. Scale bars, 250 μM, (experiments in this figure were performed by Kara Nordin and Anjali Rao).

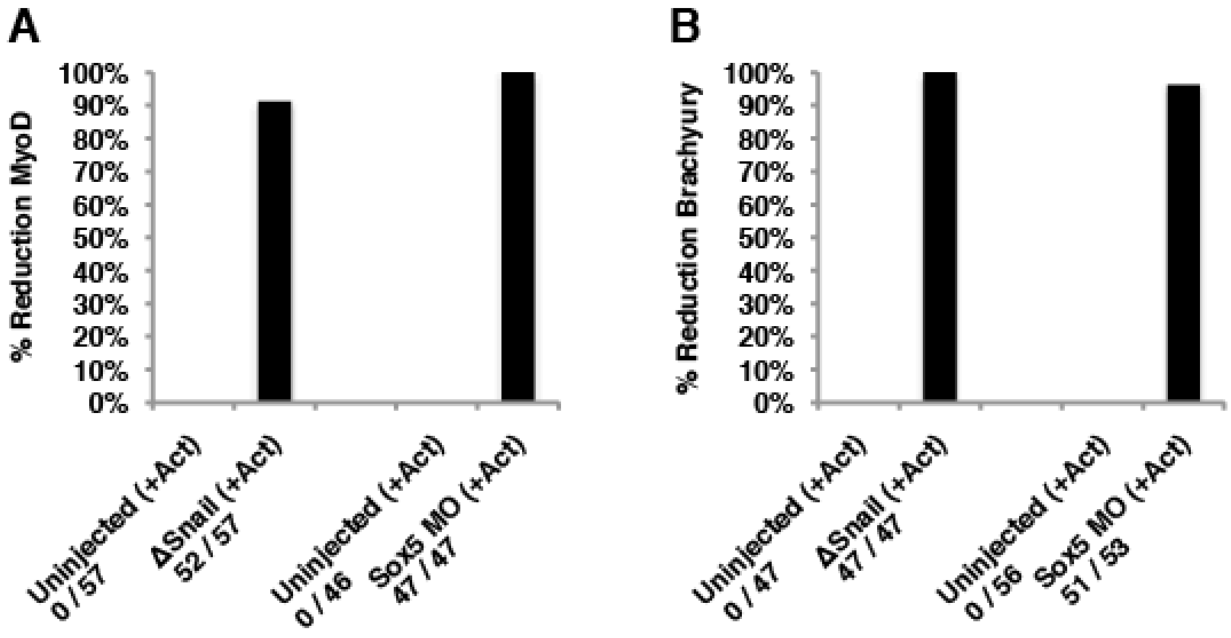


Figure 2.16 Quantification of *Snail1* and *Sox5* loss of function experiments in explants
Quantification of the % reduced expression of *MyoD* (A) and *Brachyury* (B), in ectoderm explant assays examining the effect of depleting Snail1 or Sox5 (Figure 2.15). N values listed beneath each gene/condition.

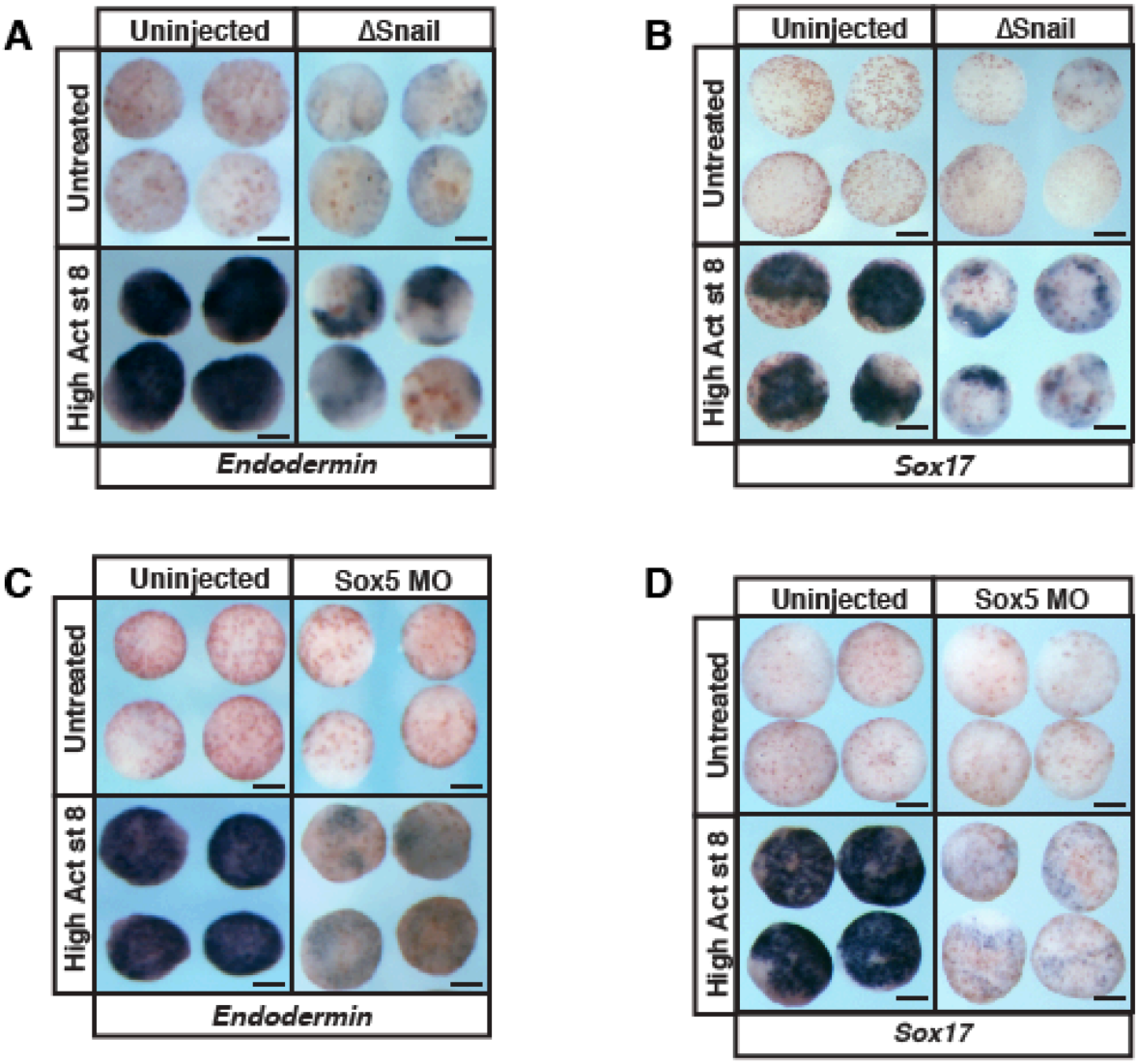


Figure 2.17 Snail1 and Sox5 are required for pluripotency of blastula ES cells to form endoderm

Ectodermal explant assay examining the expression of *Endodermin* (A, C) and *Sox17* (B ,D). Explants were injected with ΔSnail (A, B) or Sox5 morpholino (C, D) and cultured with or without activin until early gastrula stages (stage 11.5) for *Endodnermin and Sox17* for 250 μM, (experiments in this figure were performed by Kara Nordin and Anjali Rao).

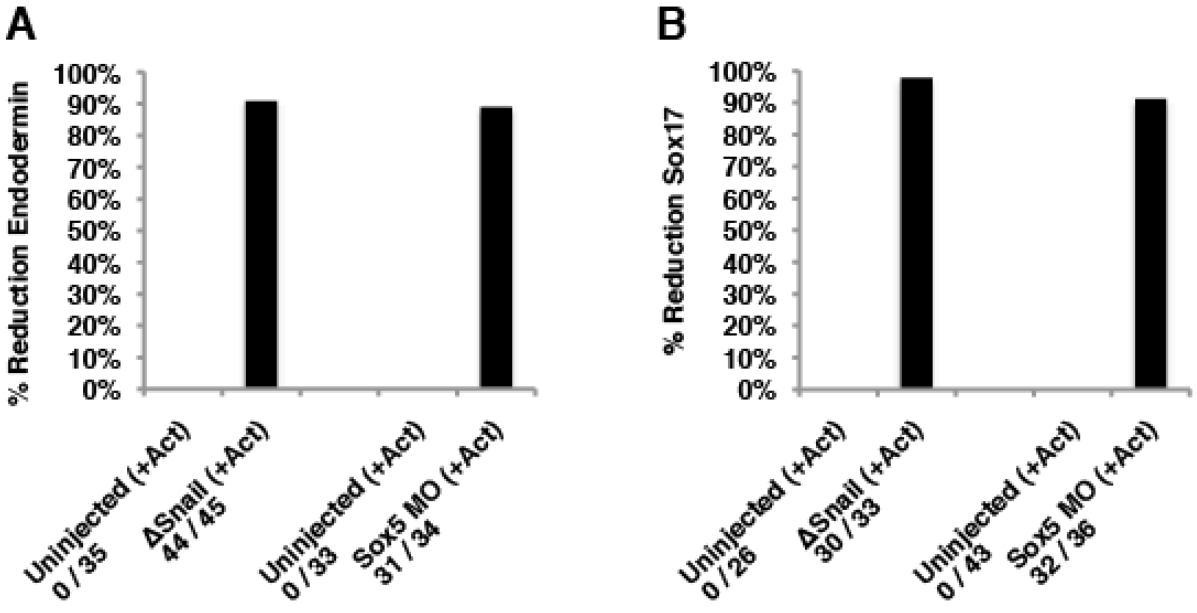


Figure 2.18 Quantification of *Snail1* and *Sox5* loss of function experiments in explants. Quantification of the % reduced expression of *Endodermin* (A) and *Sox17* (B), in ectoderm explant assays examining the effect of depleting Snail1 or Sox(Figure 2.15). N values listed beneath each gene/condition.

crest state is sufficient to confer pluripotency on or prevent loss of pluripotency in, descendants of blastula animal pole cells. Established protocols exist for converting blastula animal pole explants to a neural plate border or neural crest state. Combined expression of Pax3 and Zic1 efficiently converts explants to neural plate border (Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2005; Sato et al., 2005) whereas Snail2 together with Wnt signaling is sufficient to establish a neural crest state *in vitro* (LaBonne and Bronner-Fraser, 1998; Taylor and LaBonne, 2007). Animal pole cells explanted at blastula stages are competent to give rise to all somatic cell types but lose pluripotency by gastrula stages. I, therefore, asked if converting these explants to a neural plate border or neural crest state would be sufficient to prevent loss of competence and extend the developmental plasticity of these cells (Figure 2.19).

Explants treated at blastula stages with mesoderm-inducing concentrations of activin robustly express *MyoD*, but if aged to gastrula stages before treatment no longer form mesoderm (Figure 2.20). By contrast, cells converted to a neural plate border state retain potency and form mesoderm at both stages (Figure 2.20 and 2.21). When blastula-derived cells were treated with endoderm-inducing doses of activin, identical results were achieved (Figure 2.22). Explants treated with high activin at blastula states expressed the endodermal markers *Endodermin* and *Sox17* but were unable to do so when treated at gastrula stages (figure 2.22 and 2.23). By contrast, Pax3/Zic1 programmed explants retain the ability to form endoderm even when treated at gastrula stages (Figure 2.22 and 2. 23). Similarly, blastula-derived cells programmed to a neural crest state with Snail2/Wnt8 retain competence to form mesoderm (Figure 2.24 and 2.25) and endoderm (Figure 2.26 and 2.27) through gastrula stages. The ability of neural plate border/neural crest factors to prevent loss of pluripotency in animal pole derived cells, combined

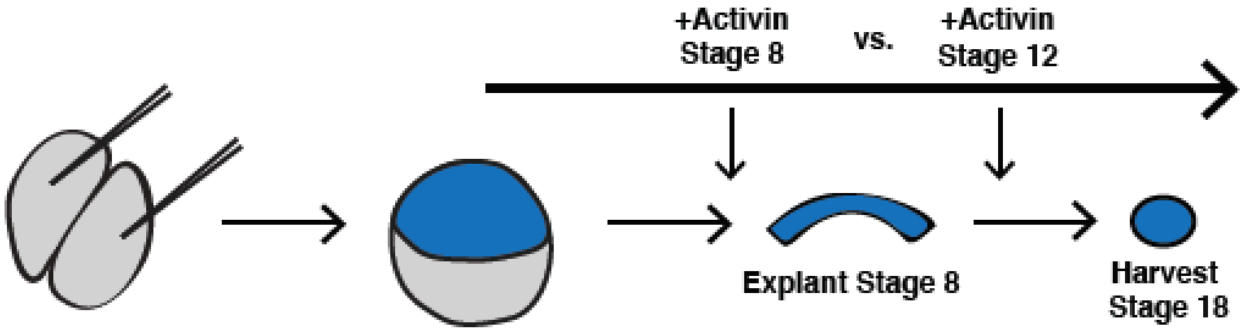


Figure 2.19 Schematic representation of activin treatment of ectodermal explants conferring neural crest state

Ectoderm from the animal pole of donor/ injected embryos is explanted at blastula stages and treated with low or high amounts of activin to induce either mesoderm or endoderm formation. Treatments were added at different stages of development, either at stage 8 or after explants had been cultured until stage 12. Explants were cultured until late neurula stages (stage 18)

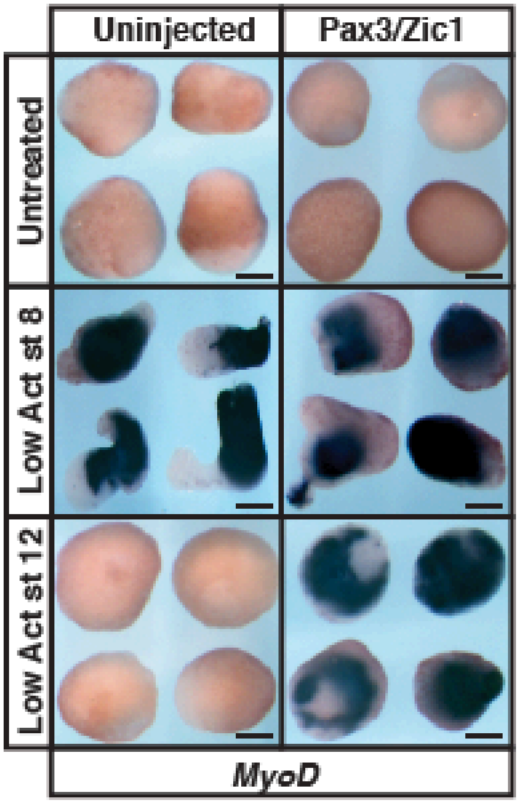


Figure 2.20 Establishing neural crest state to the animal pole cells maintains the competence of the cells to form mesoderm
Ectodermal explant assay examining the expression of *MyoD*. Embryos were injected with Pax3-GR/Zic1-GR mRNA. Explants were treated with activin at either stage 8 or 12 and cultured until late neurula stages (stage 18). Scale bars, 250 μM.

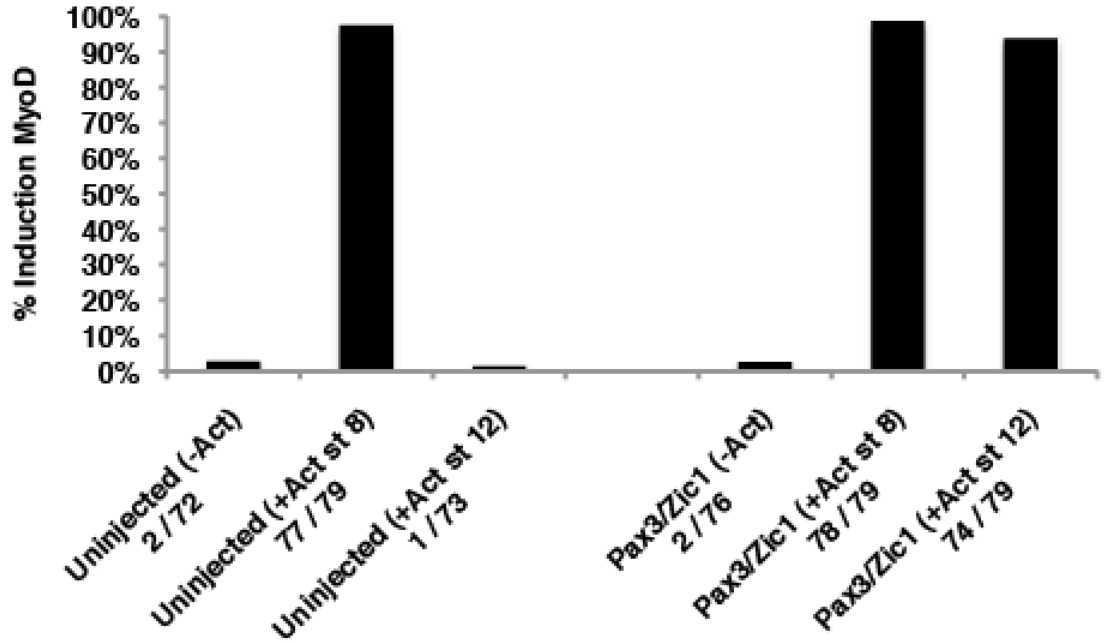


Figure 2.21 Quantitative analysis of the capacity of reprogrammed and endogenous neural crest for mesoderm formation
Quantification of the % induction in expression of *MyoD* in ectodermal explant assays that were injected with Pax3-GR/Zic1-GR mRNA and were treated with activin at either stage 8 or 12 (Figure 2.20), n values listed beneath each gene/condition.

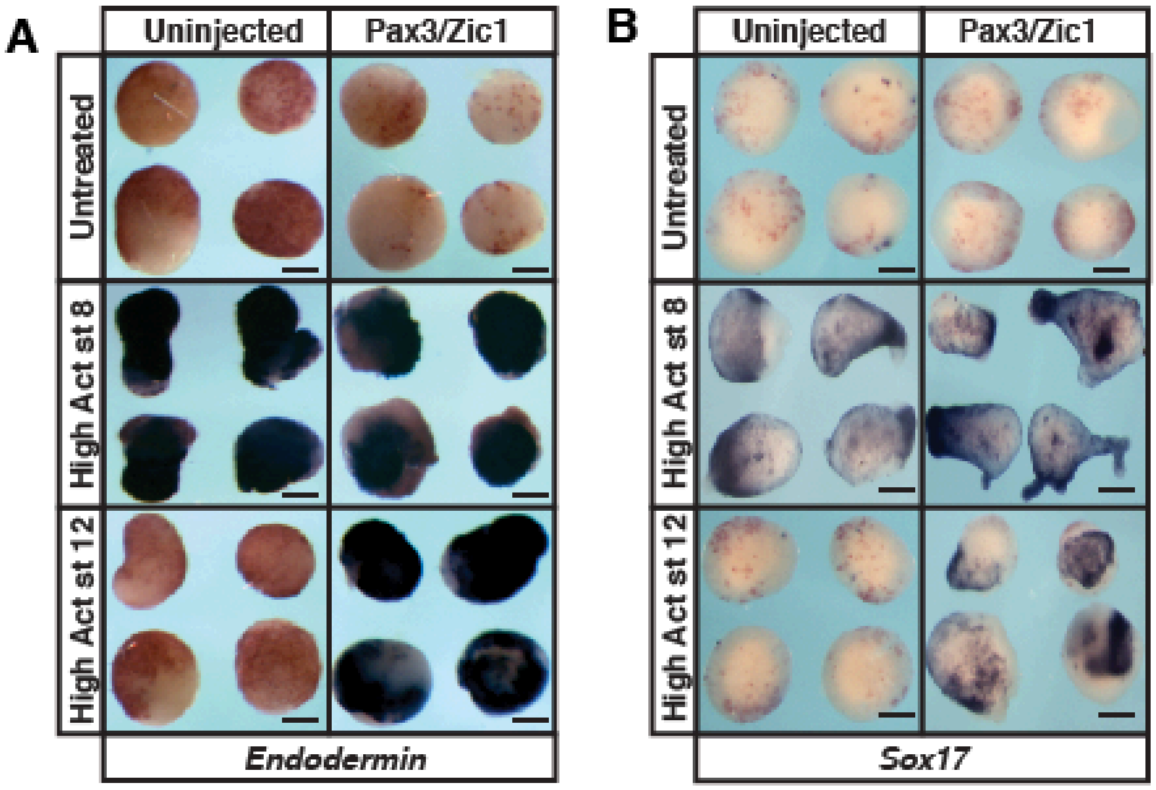


Figure 2.22 Establishing neural crest state to the animal pole cells maintains the competence of the cells to form endoderm

Ectodermal explant assay examining the expression of *Endodermin* (A) and *Sox17* (B). Embryos were injected with Pax3-GR/Zic1-GR mRNA. Explants were treated with activin at either stage 8 or 12 and cultured until late neurula stages (stage 18). Scale bars, 250 μM.

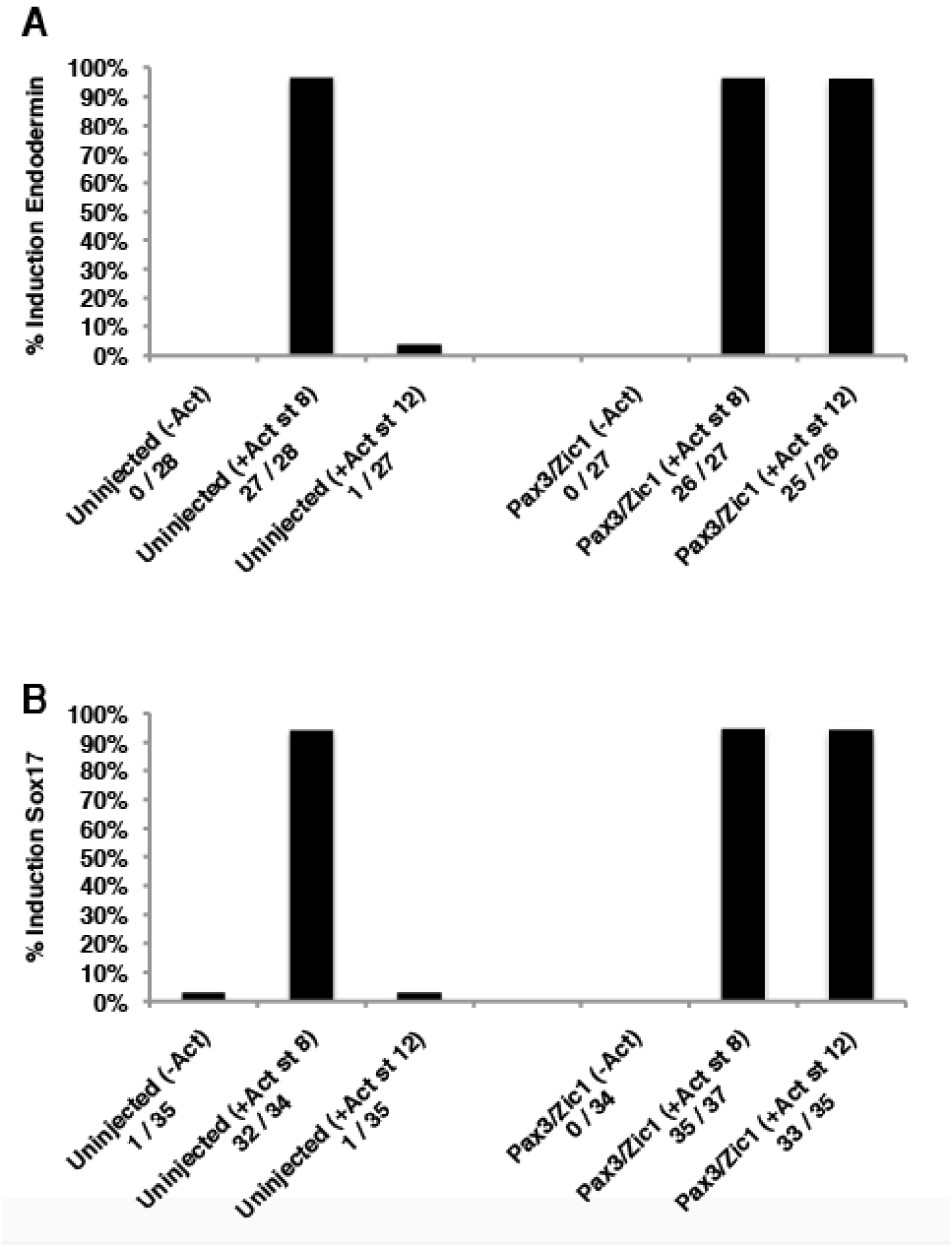


Figure 2.23 Quantitative analysis of the capacity of reprogrammed and endogenous neural crest for endoderm formation.
Quantification of the % induction in expression of *Endodermin* (A) and *Sox17* (B) in ectodermal explant assays that were injected with Pax3-GR/Zic1-GR mRNA and were treated with activin at either stage 8 or 12 (Figure 2.22), n values listed beneath each gene/condition.

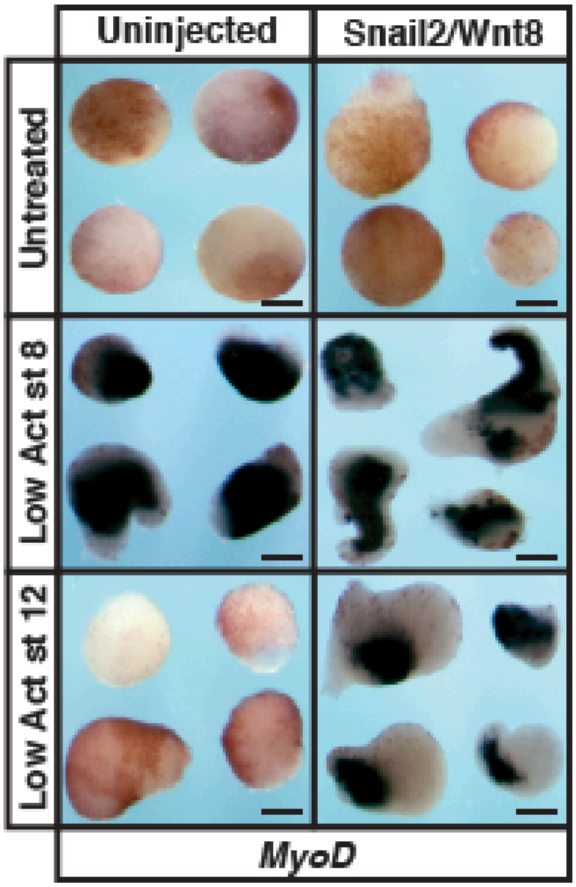


Figure 2.24 Establishing neural crest state to the animal pole cells maintains the competence of the cells to form mesoderm
Ectodermal explant assay examining the expression of *MyoD*. Embryos were injected with Snail2/Wnt8 mRNA. Explants were treated with activin at either stage 8 or 12 and cultured until late neurula stages (stage 18). Scale bars, 250 μM.

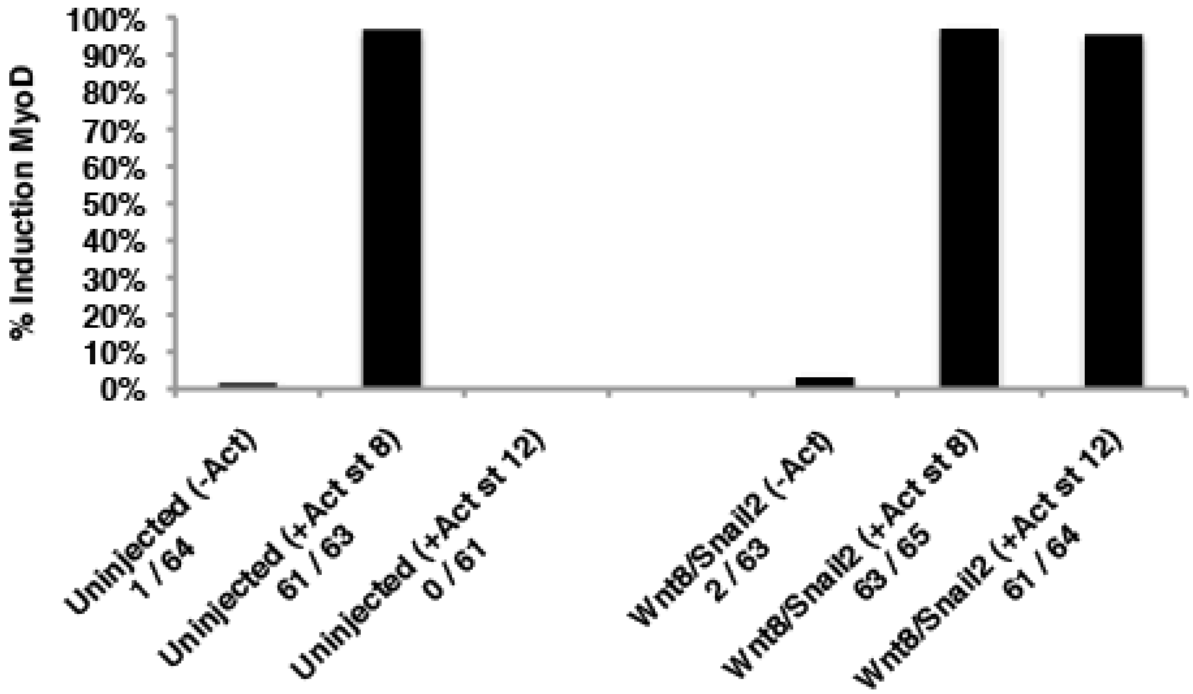


Figure 2.25 Quantitative analysis of the capacity of reprogrammed and endogenous neural crest for mesoderm formation.
Quantification of the % induction in expression of *MyoD* in ectodermal explant assays that were injected with Snail2/Wnt8 mRNA and were treated with activin at either stage 8 or 12 (Figure 2.24), n values listed beneath each gene/condition.

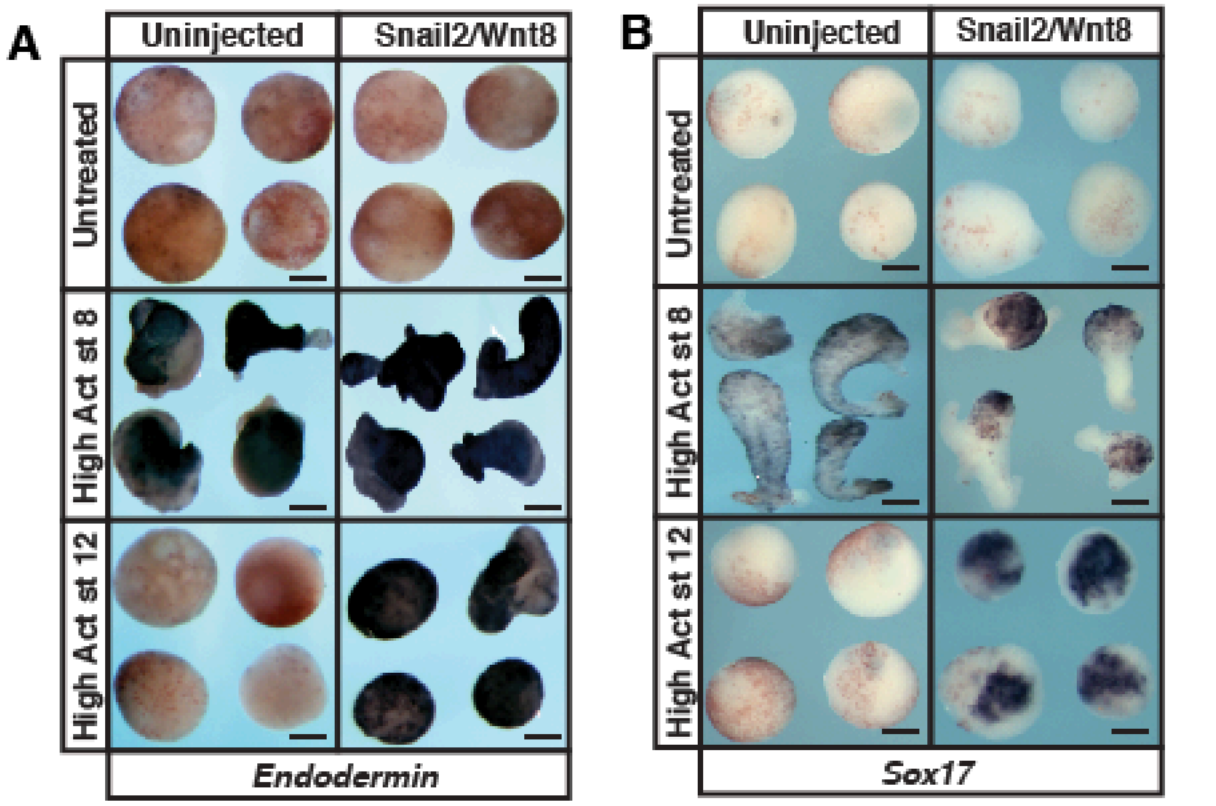


Figure 2.26 Establishing neural crest state to the animal pole cells maintains the competence of the cells to form endoderm

Ectodermal explant assay examining the expression of *Endodermin* (A) and *Sox17* (B). Embryos were injected with Snail2/Wnt8 mRNA. Explants were treated with activin at either stage 8 or 12 and cultured until late neurula stages (stage 18). Scale bars, 250 µM.

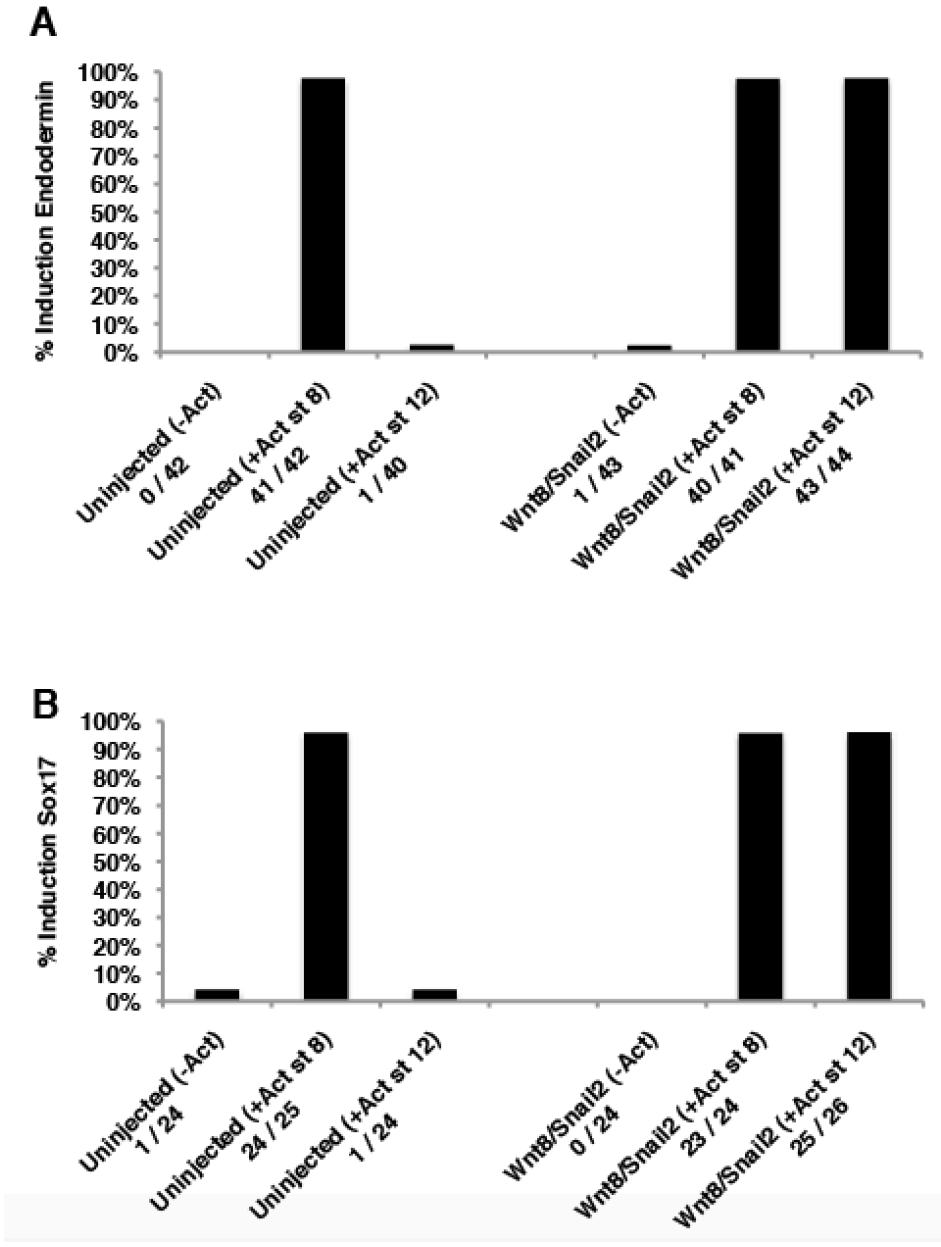


Figure 2.27 Quantitative analysis of the capacity of the reprogrammed neural crest for endoderm formation.
Quantification of the % induction in expression of *Endodermin* (A) *and Sox17* (B) in ectodermal explant assays that were injected with Snail2/Wnt8 mRNA and were treated with activin at either stage 8 or 12 (Figure 2.26), n values listed beneath each gene/condition.

with the requirement of these factors for the normal plasticity of these cells at blastula stages, suggests a close link between the molecular networks controlling the potency of neural crest and blastula cells.

Endogenous neural crest can form endoderm

The capacity of explants reprogrammed to a neural plate border/neural crest state to form endoderm, despite lack of evidence that these cells ever do so during normal development, led me to further probe the plasticity of the endogenous neural crest. I dissected neural plate border cells from a developing embryo (Figure 2.28) and cultured neural plate border cells isolated from neurula stage embryos that do not express the mesodermal markers *MyoD* or *Brachyury,* or the endodermal markers *Endodermin* or *Sox17* (Figure 2.29). However, treatment of neural plate border explants with concentrations of activin that induce mesoderm or endoderm in pluripotent blastula cells elicited strong expression of mRNA of all these genes (Figure 2.29 and 2.30). These findings demonstrate that endogenous neural crest cells possess a much greater degree of display during normal development, even including an unexpected capacity for endoderm formation. Overall, neural crest cells are competent to form derivatives from all three germ layers in vitro.

Discussion

Long-standing models for neural crest formation posit that inductive interactions endow these cells with greater developmental potential than that of the cells they were derived from, developmentally or evolutionarily (Figure 2.31) (Hong and Saint-Jeannet, 2007; LaBonne and Bronner-Fraser, 1998; Monsoro-Burq et al., 2005; Taylor and LaBonne, 2007).

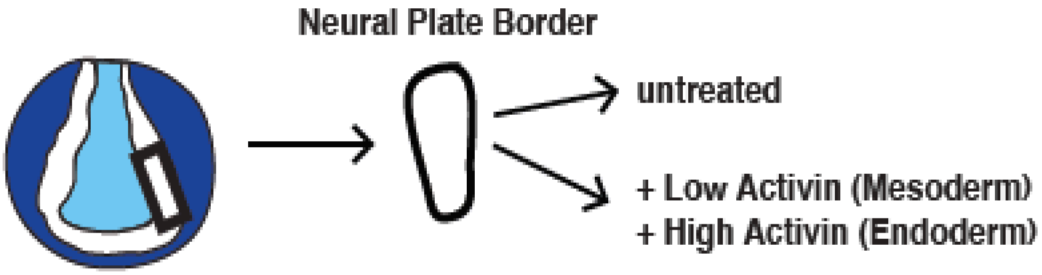


Figure 2.28 Schematic representation of neural plate border/neural crest isolation.
Neural folds are dissected at early neurula stages (stage 14/15) and cultured with or without activin until late neurula stages (stage 18).

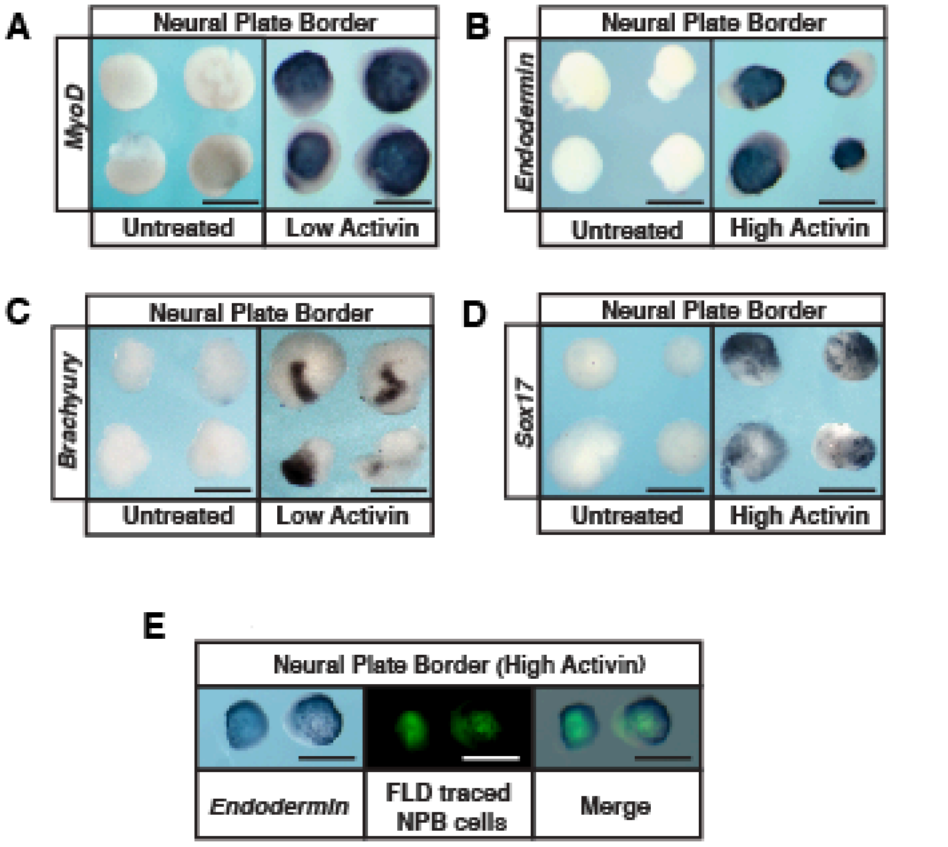


Figure 2.29 Endogenous neural crest cells have the full capacity to form all three germ layers

In situ hybridization examining the expression of mesodermal markers *MyoD* (A), *Brachyury* (C) and endodermal markers *Endodermin* (B), *Sox17* (D) in NPB tissue treated with or without activin and cultured until late neurula stages (stage 18). In situ hybridization examining the expression of *Endodermin* on neural plate border tissue that was lineage traced with fluorescein-labeled dextran (FLD) and cultured with or without activin until late neurula stages (stage 18). Scale bars, 250 μM.

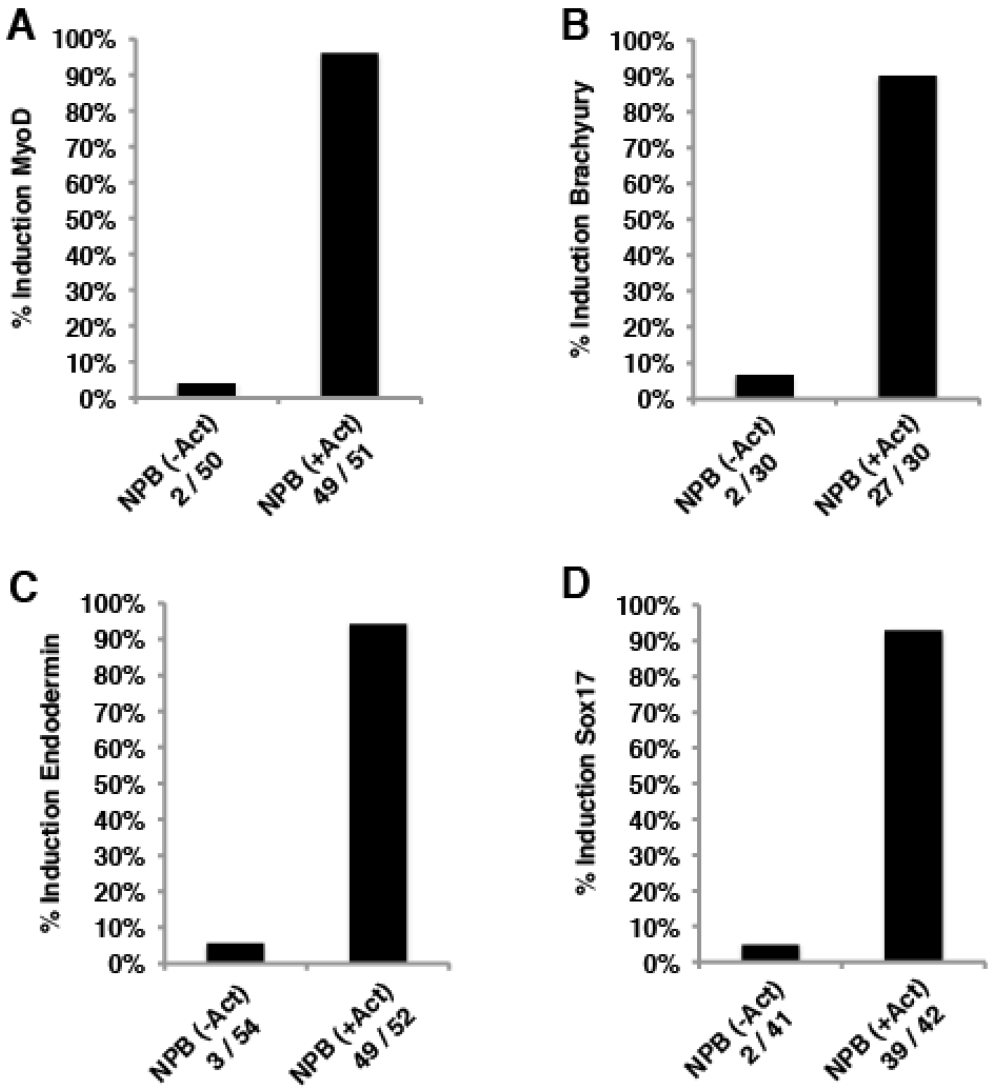


Figure 2.30 Quantitative analysis of the capacity of the endogenous neural crest for mesoderm and endoderm formation

Quantification of the % induction in expression of *MyoD* (A), *Brachyury* (B), *Endodermin* (C), and *Sox17* (D) in NPB tissue treated with or without activin (Figure2.29), n values listed beneath each gene/condition.

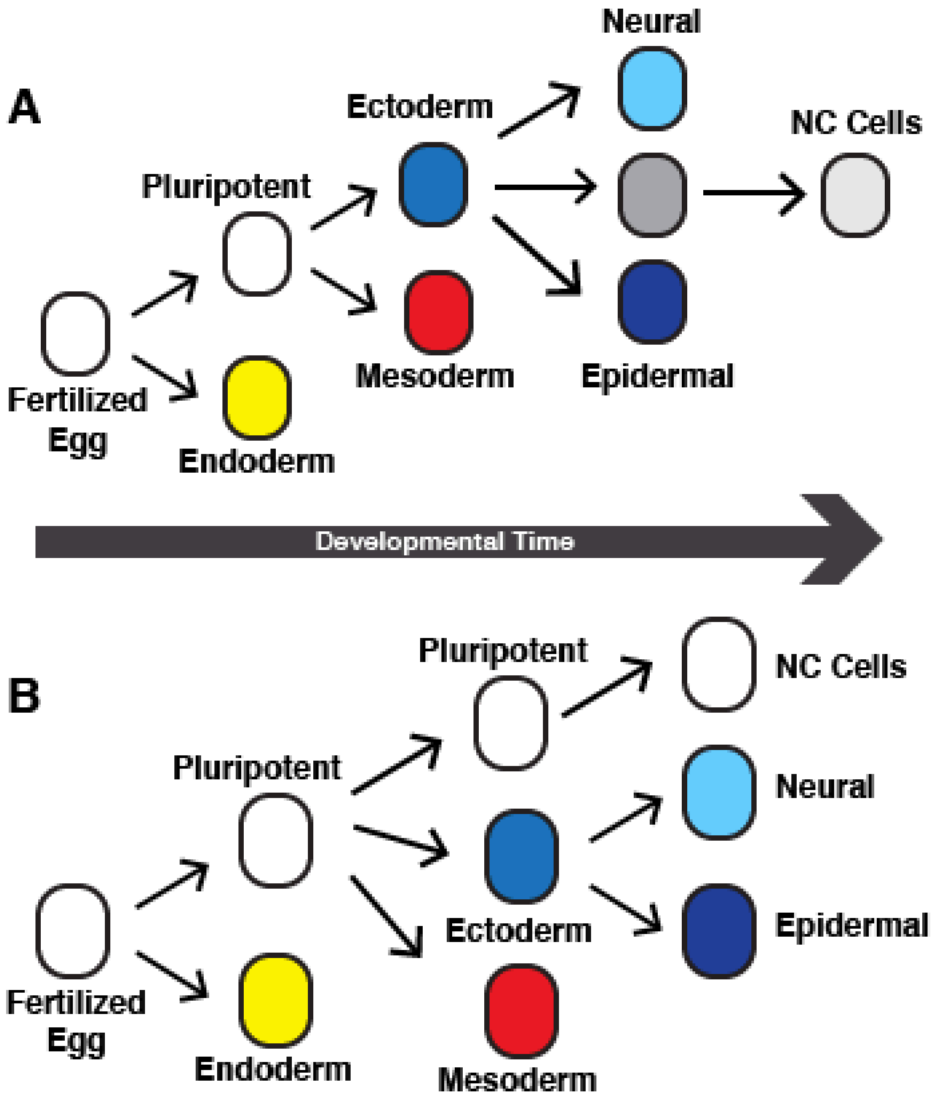


Figure 2.31 Traditional Vs new models that explain neural crest formation

Historical model for NC 'induction', the NC was thought to 'gain' potential from predecessor cells (A). Proposed new model for the generation of NC cells via retention of the pluripotency transcriptional regulatory network (B).

Waddington proposed a metaphor in which development is seen as a process where cells, represented by spheres, roll downhill in a landscape of developmental potential. According to such metaphor, as cells roll down they lose developmental potential and become lineage restricted (Waddington, 1942). The classic view of neural crest development implies that, as the neural crest cells develop, they undergo a distinct reversal (an up-hill ontogenetic shift) of the cell trajectory in Waddington's landscape of progressive restriction of developmental potential.

Based on our findings reported here, we suggest instead a revised model in which neural crest cells are an example of cellular neoteny (Anderson, 2016). Neoteny occurs when maturing organisms retain during development features of earlier developmental stages. In the case of neural crest development, such neoteny occur since cells with the pluripotent potential characteristic of the blastula state persist to neurula stages, where they can be induced to form the highly diverse lineages that derive from the neural crest (Figure 2.31). This retention of pluripotency long after other cells have become fate-restricted has endowed the neural crest with the capacity to contribute the novel attributes characteristic of vertebrates to the simple chordate body plan. Mechanistically, we propose that neural crest cells arose as *a consequence* of their retention of all or part of a regulatory network that controls pluripotency in the blastula cells from which they were derived.

Our model is consistent with, and helps explain an earlier study of avian embryos that detected expression of genes associated with the neural crest state, such as *Pax7*, in the medial epiblast at early gastrula stages (Basch et al., 2006). Those findings were interpreted at the time as evidence that neural crest induction occurs earlier than previously believed. On the contrary, we suggest that expression of factors such as *Pax7* reflects the retention of pluripotency in a subset of avian

epiblast cells. Our work further suggests that transcription factors such as Pax7 or Snail1, which were previously considered and defined as neural plate border or neural crest factors, should instead be viewed as pluripotency maintenance factors. The discovery that loss of Snail1 and Sox5 results in the absence of expression of factors that have been traditionally considered "core" pluripotency conferring proteins (such as POU/Oct25,60,91, Sox2/3, Nanog/Vent2, and Myc) implies that Snail1 and Sox5 must also be considered *bona fide* core pluripotency promoting factors. It remains to be determined if *Klf4* expression also requires Snail1 and Sox5 for its endogenous expression at the blastula stage. In any case, these findings imply that the architecture of the pluripotency network requires inputs from Snail1 and Sox5 for its normal function, and suggests that the pluripotency network is indeed larger and more complex than was initially thought. To this end, it will be interesting to test if factors that were thought to be specific to neural crest, such as Snail1 or Sox5, could also be used to or as part of reprograming protein sets that convert adult differentiated cells into induced pluripotent stem cells (iPSCs). Future studies should address how factors such as neural crest pluripotency factors function to retain potential to contribute to all three germ layers in cells that will become neural crest.

The previously unrecognized capacity of neural crest cells to express endodermal markers in culture raises the question of whether they also contribute endodermal cell types endogenously. Perhaps neural crest contributions to otherwise endodermal organs, such as the parafollicular cells of the thyroid (Le Douarin and Kalcheim, 1999), should be considered a contribution of endoderm.

In this chapter I have shown that neural crest cells express pluripotency promoting factors and that pluripotent blastula cells express proteins thought to be neural crest-specific. Although is

expected that neural crest cells do have molecular differences with blastula pluripotent stem cells, these findings imply that neural crest might be a new *bona fide* pluripotent cell population that could be used to study how pluripotency is maintained and lost during cell differentiation. Accordingly, it would be interesting to further determine additional similarities and differences between the pluripotent stem cells and neural crest cells. For example, it will be important to find if neural crest cells isolated from a developing embryo can be reprogramed to achieve multiple fates, and more importantly, to evaluate if neural crest cells can self renew in vitro while they maintain their prime pluripotent state.

The model for the formation of neural crest cells proposed here provides a framework for future studies in basal chordates to probe the earliest evolutionary origins of these cells. Ascidians, for example, possess a cell lineage that arises from the neural plate border and expresses genes such as *Snail*, *Id*, *FoxD*, and *Ap2*, all of which we find shared between pluripotent blastula cells and neural crest. This a9.49 lineage in ascidians may be homologous to the neural crest lineage in vertebrates (Abitua et al., 2012). Investigating shared and divergent aspects of pluripotency network components in these and other protochordate and basal vertebrate models should, therefore, shed light on when and how pluripotency was retained in cells that become neural crest and thus provide insight into the evolutionary origins of the vertebrates.

Materials and methods

Embryological methods

Collection, injection and *in situ* hybridization of *Xenopus* embryos were performed as previously described (Bellmeyer et al., 2003) using digoxigenin-labeled RNA probes detected with BM Purple AP Substrate (Roche). For double *in situ* hybridizations, probes were labeled

either with digoxigenin or fluorescein and were detected either with BCIP (Roche) or Magenta Phosphate (Biosynth). mRNA for microinjection was produced *in vitro* from linearized plasmid templates using the SP6 Message Machine kit (Ambion). β-galactosidase mRNA was co-injected as a lineage tracer and detected with Red-Gal substrate (Research Organics). All results shown are representative of at least three independent experiments. Ectodermal explants were manually dissected from the animal pole of blastula (stage 8) embryos previously injected at the two-cell stage with the indicated mRNA or morpholino. Explants were cultured at room temperature in 1X MMR in agar-coated dishes until the stage indicated, and fixed in formaldehyde for 30 minutes before being processed for *in situ* hybridization. For Nieuwkoop recombinants, dorsal lateral vegetal explants were dissected at stage 10 and conjugated to an ectoderm explant (stage 8) previously injected with ΔSnail mRNA or Sox5 MO. Recombinants were cultured until stage 16 and fixed in 4% formaldehyde for 30 minutes before *in situ* hybridization.

Activin and Dexamethasone treatment on animal caps and neural folds

Ectodermal explants were manually dissected from the animal pole of blastula (stage 8) embryos previously injected at the two-cell stage with the indicated mRNA or morpholino. Zic1-GR and Pax3-GR injected explants were cultured in 0.1X MMR and 10 μM Dexamethasone (Sigma) at stage 8. Explants were treated with Activin at the indicated stage. To induce mesoderm and endoderm fates, precise amounts of activin were added to the 1X MMR media in conjunction with 1X BSA (Sigma) to aid in delivery (0.5 μl of activin for mesoderm induction and 5 μl of activin for endoderm induction). Explants were cultured at room temperature in 1X MMR in agar coated dishes until the indicated stage, and fixed in formaldehyde for 30 minutes before being processed for *in situ* hybridization. Neural folds were dissected from *Xenopus*

embryos at stage 15 and treated with activin immediately after dissection (0.5 µl of activin for mesoderm induction and 5 µl of activin for endoderm induction). Neural folds were cultured until stage 18 and fixed in formaldehyde for 30 minutes before being processed for *in situ* hybridization.

RNA Extraction, cDNA synthesis, and qRT-PCR

Animal caps were lysed in 300 µl of Buffer A (50mM NaCl, 50mM Tris-Cl pH 7.5, 5mM EDTA pH 8.0, 0.5% SDS and 200ug/mL proteinase K) and incubated at 37°C for 1 hour. Samples were phenol/chloroform extracted twice and ethanol precipitated. mRNA was then incubated with RQ1 DNAse (Promega) at 37°C for 1 hour. Samples were subsequently phenol/chloroform extracted and ethanol precipitated. 1ug of mRNA was then used as a template for synthesizing cDNA using the Superscript VILO MasterMix Kit (Life Technologies). qPCR was performed using SYBR Premix (Clontech #RR820W). Primer sequences are described below. Fold expression was normalized to ornithine decarboxylase (ODC) and relative to stage 9. The ΔΔCT method was used to calculate fold expression and represented as a mean from three separate biological replicates with error bars representing S.E.M.

DNA constructs and primers

The ΔSnail construct acts as a dominant negative to deplete Snail family protein function. It only includes the zinc finger region spanning amino acids D117-H266 as previously described (LaBonne and Bronner-Fraser 2000). Morpholino oligonucleotides were designed to target the translation-initiation site of Sox5 (Gene Tools). Two Sox5 morpholinos were designed with the following sequences: 5'CTGGGGCTCAGTGAGCATTTCTGGG-3' (initiation codon

underlined) and 5'CTGCCTCCTCCTCCGCTTCCCGACT-3' (upstream of initiation codon). Both morpholinos were used at a concentration of 5ng unless otherwise noted as described in Nordin and LaBonne 2014). Primers used in this study are listed below

Gene	Forward Sequence	Reverse Sequence
Oct60	ACT ACA ATG CCC CGT CCT ACC C	ACT CCC CGG CGT TTC TTC CTC
Sox3	CAC AAC TCG GAG ATC AGC AA	TCG TCG ATG AAG GGT CTT TT
Vent2	GCT ACA CAG GGA CAC AAC CTC	GCC TGA GTC AGT GCT AGT GC
FoxD3	AAG GAG ATC CCC AGG AGT CC	AGG CTG TTC TTG GGC TTG TT
Myc	GAG AGG CAG CCT GTG GAT TT	CTT TCC TGC CAT GCG ATT GG
Id3	GTT ATT TGC CAC CCC ATC TG	TTA CTA GCC AAG CCC CAC AC
ODC	TGA AAA CAT GGG TGC CTA CA	TGC CAG TGT GGT CTT GAC AT

Chapter 3

A transition from SoxB1 to SoxE transcription factors is essential for progression from pluripotent blastula cells to neural crest cells

Summary

The neural crest is a stem cell population unique to vertebrate embryos that give rise to derivatives from multiple embryonic germ layers. The molecular underpinnings of potency that govern neural crest potential are highly conserved with that of pluripotent blastula stem cells, suggesting that neural crest cells may have evolved through retention of aspects of the pluripotency gene regulatory network (GRN). A striking difference in the regulatory factors utilized in pluripotent blastula cells and neural crest cells is the deployment of different sub-families of Sox transcription factors; SoxB1 factors play central roles in the pluripotency of naïve blastula and ES cells, whereas neural crest cells require SoxE function. Here I explore the shared and distinct activities of these factors to shed light on the role that this molecular hand-off of Sox factor activity plays in the genesis of neural crest and the lineages derived from it. Our findings provide evidence that SoxB1 and SoxE factors have both overlapping and distinct activities in regulating pluripotency and lineage restriction in the embryo. I hypothesize that SoxE factors may transiently replace SoxB1 factors to control pluripotency in neural crest cells, and then poise these cells to contribute to glial, chondrogenic and melanocyte lineages at stages when SoxB1 factors promote neuronal progenitor formation.

Introduction

The neural crest is a major evolutionary innovation of vertebrates, allowing for the generation of many vertebrate-specific features (Green et al., 2015; Hall, 2008; Le Douarin et al., 2008). First described by Wilhelm His 150 years ago, neural crest cells are distinguished by their ability to contribute a diverse array of cell types associated with multiple germ layers to the vertebrate body plan (Le Douarin et al., 2008). Referred to as the "Zwischenstrang" by His (His,

1868), acquisition of these cells allowed a myriad of novel structures, including a "new head", to be layered onto the simple chordate body plan (Hall, 2008). Within developing embryos, neural crest cells retain broad, multi-germ layer potential even as neighboring cells become lineage restricted (Prasad et al 2012). Ultimately, neural crest cells give rise to a diverse array of derivatives that includes chondrocytes, melanocytes, and neurons and glia of the peripheral nervous system (Bronner and LeDouarin, 2012; Bronner-Fraser and Fraser, 1989; Taylor and LaBonne, 2007). While much has been learned about the signaling pathways and transcriptional responses required for formation of the neural crest, and the subsequent lineage diversification of these cells, how these factors contribute to the broad developmental potential of neural crest cells remains unclear.

A core network of transcriptions factors controls pluripotency in the blastula, and among these are Sox (SRY-related high mobility group (HMG)-box) family transcription factors (Takahashi and Yamanaka, 2006). Sox proteins are highly conserved and contain a DNA-binding domain known as the HMG-box. Based upon homology within the HMG domain and other structural regions of the protein, Sox factors are divided into nine subfamilies (Bowles et al., 2000; Julian et al., 2017; Schepers et al., 2002). Two subfamilies of particular importance for pluripotency in blastula and neural crest cells are SoxB1 and SoxE factors, respectively. SoxB1 factors (Sox1/2/3), are essential regulators of the stem cell state in both blastula cells and ES cells (Guth and Wegner, 2008; Sarkar and Hochedlinger, 2013, Avilion et al., 2003, Masiu et al., 2007, Abdelailm et al., 2014). SoxB1 proteins are maternally expressed and highly enriched in the pluripotent cells (inner cell mass) of the blastula, consistent with a role during the early stages of development (Avilion et al., 2003; Buitrago et al., 2015). SoxB1 proteins can act as

transcriptional activators of genes essential for pluripotency. For example, SOX2 functions with OCT4, another core pluripotency factor, to initiate a gene regulatory network involved in maintaining a stem cell state (Takahashi and Yamanaka, 2006). Subsequent to their functions in pluripotent blastula cells, expression of SoxB1 factors becomes restricted to the prospective neuroectoderm, where they are essential to establishing a neural progenitor state (Guth and Wegner, 2008; Rex et al., 1997; Streit et al., 1997).

Interestingly, while neural crest cells and pluripotent blastula cells display remarkable similarity in their gene expression profiles (Buitrago-Delgado et al., 2015), in neural crest cells the Sox factors involved in stem cell maintenance are SoxE factors (Sox8/9/10) rather than SoxB1. SoxE proteins first play important roles in establishing the neural crest stem cell population, and subsequently direct the formation of a subset of neural crest cell lineages (Cheung and Briscoe, 2003; Haldin and LaBonne, 2010; Hong and Saint-Jeannet, 2005; Kim et al., 2003). For example, Sox9 is required for formation of chondrocytes, while Sox10 is essential for melanocyte and glial cell formation (Akiyama et al., 2002; Aoki et al., 2003; Haldin and LaBonne, 2010; Mori-Akiyama et al., 2003). While distinct roles for SoxE factors in directing these neural crest cell lineage decisions have been defined, little is understood about their contributions to maintaining the neural crest stem cell state. One hypothesis is that SoxE factors play an analogous role in the neural crest to that of SoxB1 factors in naïve blastula cells with respect to maintaining a pluripotent state. However, this raises the question of why a hand-off from SoxB1 to SoxE factors is necessary and/or advantageous.

Here, I examine the activities of SoxB1 and SoxE factors in the neural crest and in the pluripotent blastula cells of early Xenopus embryos. I demonstrate that SoxB1 and SoxE factors exhibit both unique and redundant activities in these cell types. Our findings suggest a model in which the essential role of SoxB1 in regulating the blastula stem cell state is handed off to SoxE factors which function in part to retain the developmental potential of the neural crest.

Results

SoxB1 and SoxE factors are expressed sequentially during embryo development

I recently demonstrated that much of the regulatory network that controls the pluripotency of blastula stem cells/animal pole cells is shared with neural crest cells, shedding new light on the origins of the neural crest cells and the evolution of vertebrates (Buitrago-Delgado et al., 2015). I found that factors that have long been considered neural crest potency factors are first expressed in blastula animal pole cells, and are required for the pluripotency of these cells. Importantly, however, a subset of neural crest factors does not show prior expression in pluripotent blastula cells. These factors represent true evolutionary novelties whose cooption into the neural crest GRN may have played a key role in endowing neural crest cells with the ability to transform the vertebrate body plan. Prominent among these factors are the SoxE family transcription factors, Sox9 and Sox10.

I compared the expression of SoxB1 and SoxE transcription factors from blastula to late neurula stages using *in situ* hybridization to better understand the transition in their expression. The SoxB1 factors *Sox2* and *Sox3* are robustly expressed in the animal pole region of blastula embryos, where pluripotent cells reside (Figure 3.1. top). By contrast, expression of the SoxE

factors *Sox9* and *Sox10* cannot be detected until late gastrula or early neurula stages respectively, when they mark the neural crest populations at the neural plate border (Figure 3.1 bottom). A third SoxE factor, *Sox8*, is expressed at low levels in blastula stage embryos, but is not detectible by the onset of gastrulation. By mid-gastrula stages (stage 11) *Sox8* expression can be detected in neural crest regions as previously reported (O'Donell, 2006) as well as in anterior prospective ectoderm regions where *Sox2* expression has been diminished (Figure 3.1 A). By late gastrula/early neurula stages, expression of *Sox2* and *Sox3* has been restricted to the prospective neural plate, marking the transition of their role in pluripotency to their subsequent roles in maintaining neuronal progenitor cells. The expression of SoxB1 and SoxE factors overlap at late gastrula stages, when both are expressed in neural crest regions of the neural plate border, but by early neurula stages their expression is distinct, with *SoxE* factors marking the neural crest (and in the case of *Sox9* and *Sox10* also the otic placode), and *SoxB1* factors marking the neural plate and preplacodal region. (Figure 3.1)

Premature SoxE activity interferes with proper pluripotency gene expression

The distinct deployment of SoxB1 and SoxE factors in pluripotent blastula cells and neural crest respectively suggests that these proteins may have distinct activities that favor their function in these different contexts. In particular I was interested in whether exclusion of SoxE factors from pluripotent blastula cells was essential to maintain a stem cell state. To determine this, mRNA encoding Sox9 or Sox10 was injected in once cell of two cell embryos. Injected embryos were cultured to blastula stages when expression of four key genes expressed in pluripotent cells,

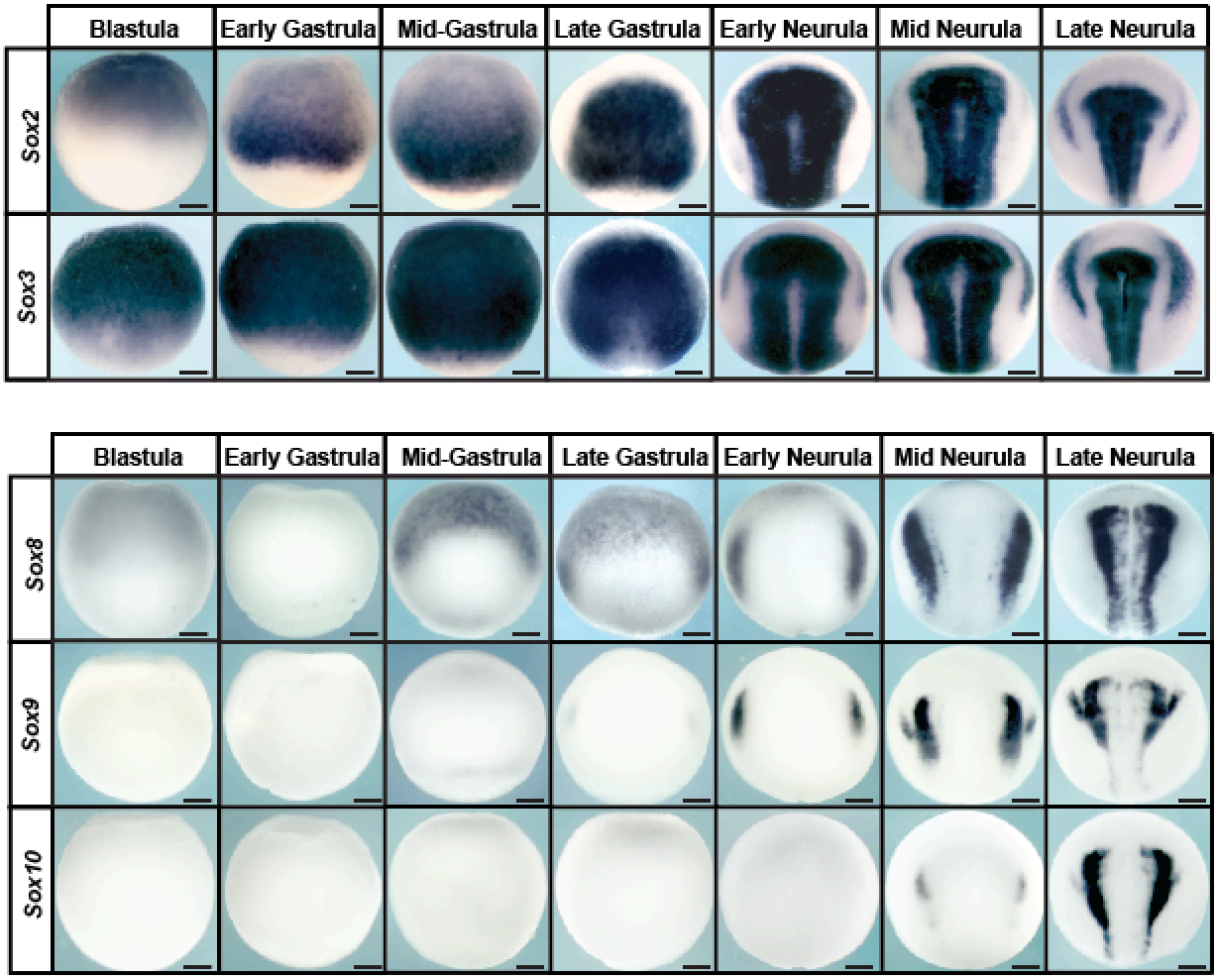


Figure 3.1 Sequential expressions of SoxB1 and SoxE factors during embryo development *In situ* hybridization of wild type *Xenopus* embryos of SoxB1 factors *Sox2* and *Sox3* and SoxE factors *Sox8*, *Sox9,* and *Sox10*. Sox2/3 and Sox8/9 expression appear to overlap only at late gastrula stages. The domains are differentially expressed from early to mid neurula stages. Scale bars, 250 μm.

Oct25, Vent2, Id3, and *TF-AP2,* was examined by *in situ* hybridization. A premature activity of either Sox9 or Sox10 led to strong down-regulation of these genes on the injected side of the embryo (Figure 3.2 A,B, 3.3 A-D). This results suggested that SoxE function at these stages may be incompatible with normal development of pluripotent blastula cells. In parallel experiments, Sox2 or Sox3 were expressed in blastula cells at protein levels matched to Sox9 and Sox10. While this too led to disruptions in gene expression, these changes were less pronounced than those observed in response to equivalent levels of Sox9 or Sox10 (Figure 3.2 A,B, 3.3 A-D). The changes in blastula gene expression in response to up-regulation of SoxB1 factors is consistent with findings in mouse and human ES cells, where both increases and decreases in Sox2 activity can lead to loss of pluripotency (Kopp et al., 2008; Boer et al., 2007; Yamaguchi et al., 2011; Takahashi &Yamanaka, 2006; Thomson et al, 2011). These findings suggest that pluripotent blastula cells, while sensitive to changes in the levels of Sox protein activity, are more sensitive to increases in the activity of SoxE family transcription factors.

I next asked if a specific domain of these SoxE proteins mediated the down-regulation of blastula pluripotency genes in response to Sox9 or Sox10. I use mutant forms of these proteins in which either the activation or the HMG DNA binding domain had been deleted (Figure 3.4 A, 3.5A). These mutants were expressed at equivalent levels to the wild type protein as determined by western blot (Figures 3.4 C, 3.5 C), and their ability to down-regulate *Id3* or *Vent2* expression at blastula stages was examined with in situ hybridization. I found that mutants lacking the

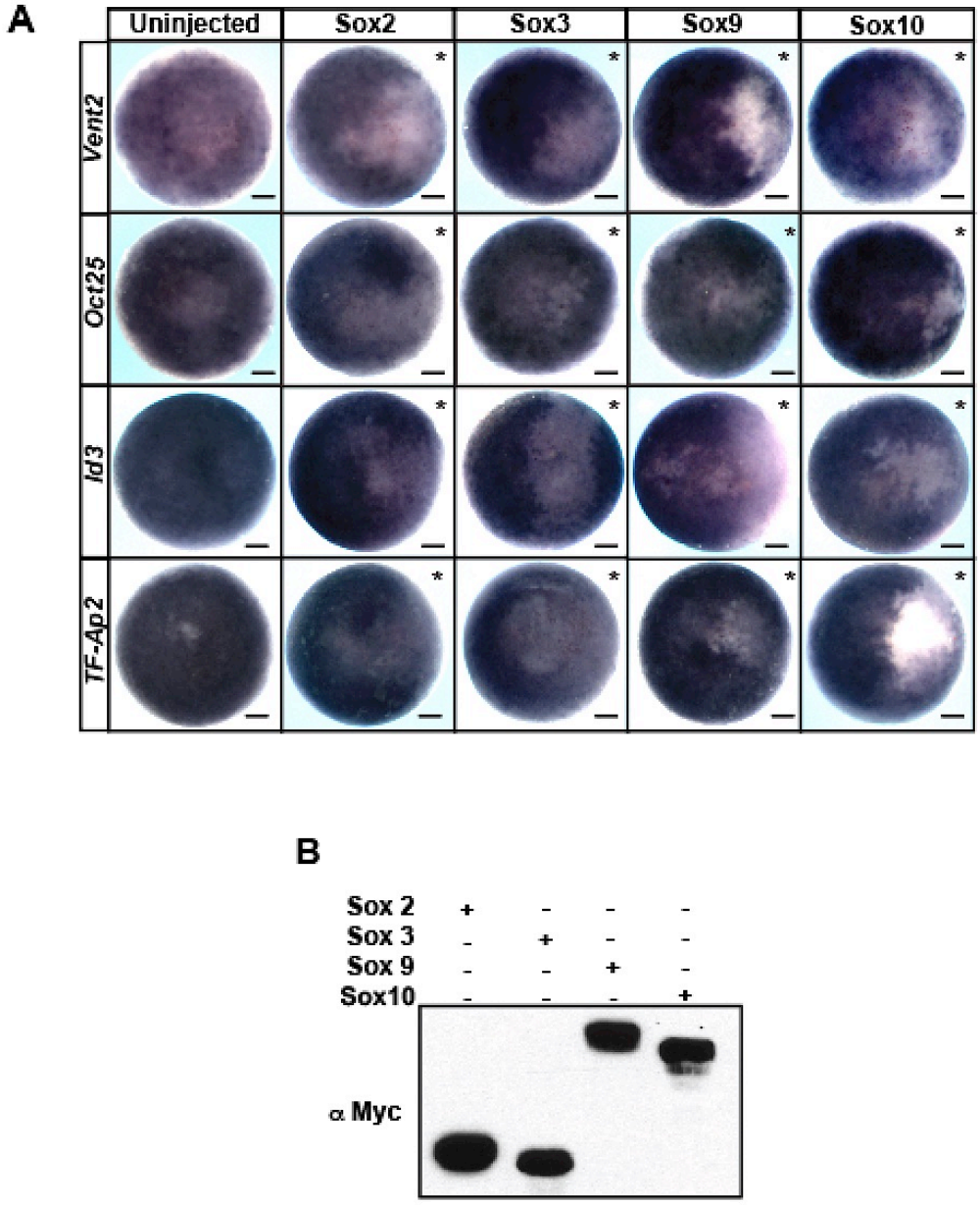


Figure 3.2 SoxB1 and SoxE factors overexpression results in the loss of expression of pluripotency factors in blastula animal pole cells

(A) *In situ* hybridization of *Xenopus* embryos injected with either SoxB1 factors (Sox2, Sox3) or SoxE factors (Sox9 and Sox10). Embryos were collected at blastula stages (atage9) and examined the expression of genes associated with pluripotency and neural crest formation. (*Oct 25, Vent2, Id3, Tf-Ap2*) Scale bars, 250 μm. (B) Western blot using lysates from embryos injected either with Sox2, Sox3, Sox9 or Sox10 to examine levels of mRNA injected.

A **B**

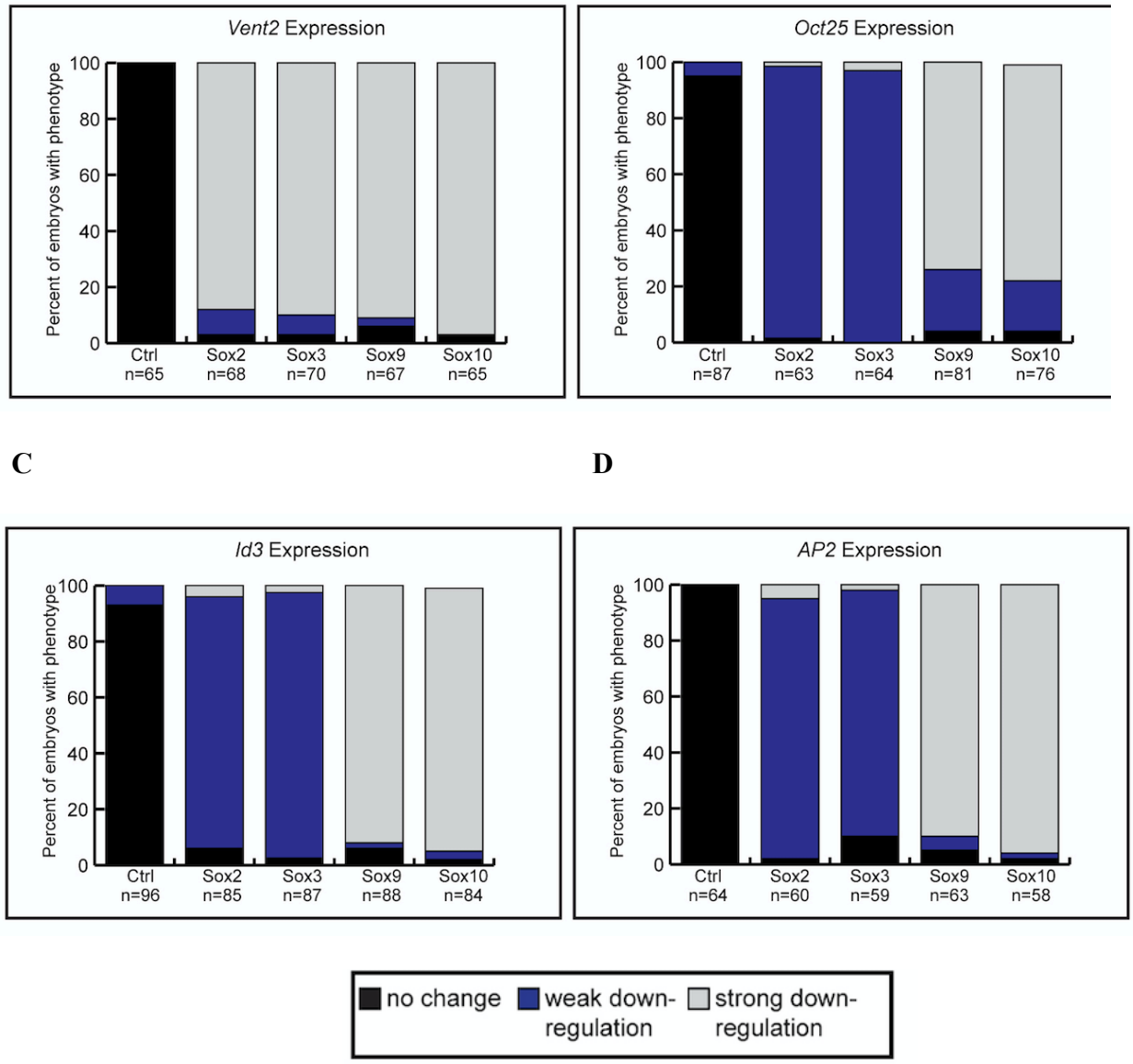


Figure 3.3 Quantification of SoxB1 and SoxE overexpression blastula embryos
Quantification of the % reduced expression of pluripotency genes in whole embryos that were injected with SoxB1 (Sox3 and Sox3) and SoxE (Sox9 and Sox10) mRNA.(A) *Vent2*, (B) *Oct25*, (C) *Id3*, (D) *TF-AP2* (from figure 3.2).

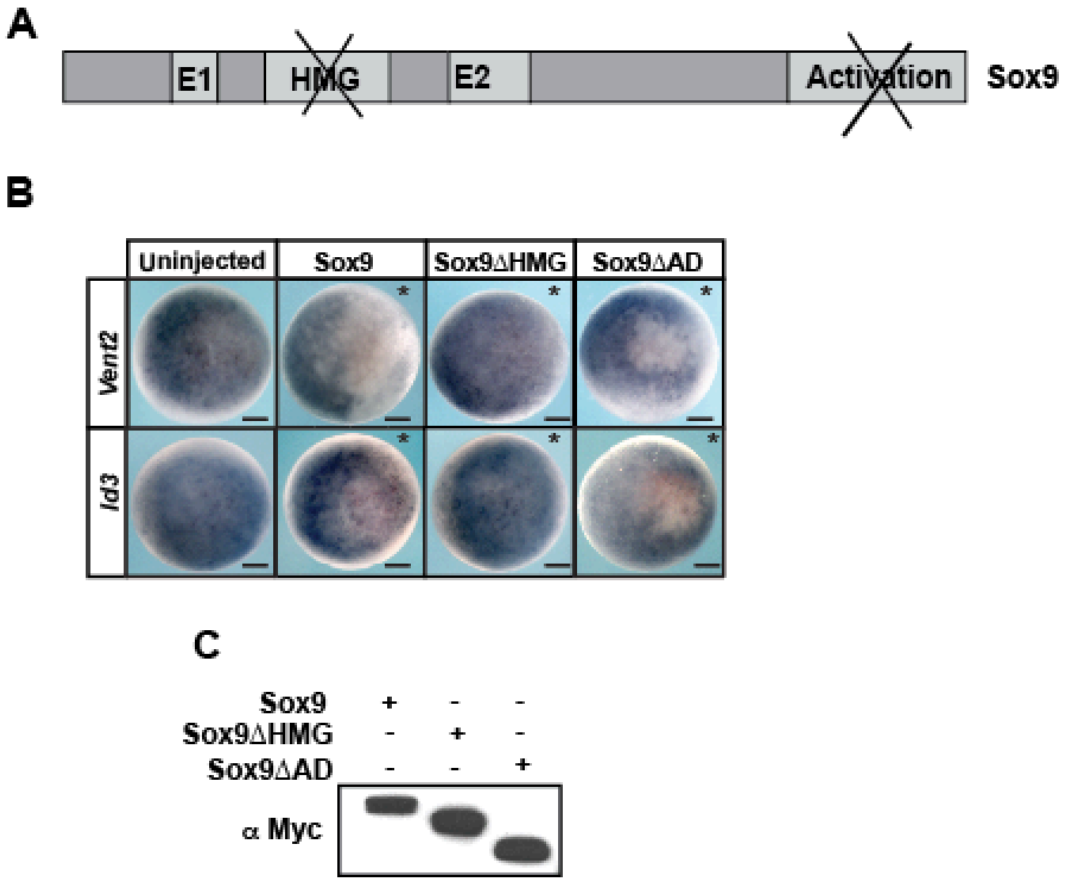


Figure 3.4 HMG domain is necessary for the function of the Sox9 protein in blastula animal pole cells

(A) Schematic diagram showing Sox9 deletion constructs. (B) In situ hybridization of blastula stage *Xenopus* embryos examining the expression of *Vent2* and *Id3*. Embryos were injected with Sox9, Sox9 ΔHMG domain or Sox9Δ Activation domain mRNAs. (C) Western blot using lysates from embryos injected with same mRNAs confirming relatively equivalent levels of proteins expressed. Scale bars, 250 μm.

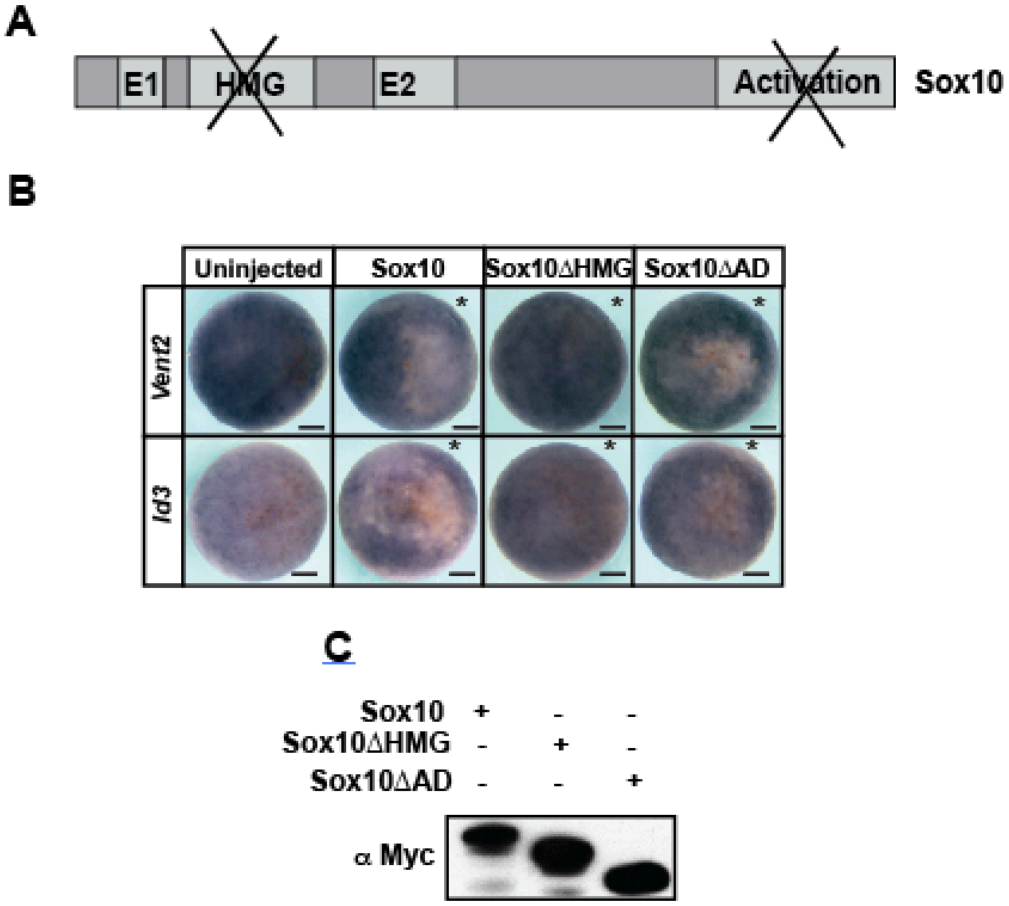


Figure 3.5 HMG domain is necessary for the function of the Sox10 protein in blastula animal pole cells

(A) Schematic diagram showing Sox10 deletion constructs. (B) In situ hybridization of blastula stage *Xenopus* embryos examining the expression of *Vent2* and *Id3*. Embryos were injected with either Sox10, Sox10 ΔHMG domain or Sox10Δ Activation domain mRNAs. (C) Western blot using lysates from embryos injected with same mRNAs confirming relatively equivalent levels of proteins expressed, Scale bars, 250 μm.

activation domain could still down-regulate *Vent2* and *Id3* (Figure 3.4 B, 3.5 B, and figure 3.6). By contrast, Sox9 and Sox10 mutants lacking the HMG domain had no effect on blastula gene expression *Vent2*, and *Id3*. (Figure 3.4B, 3.5 B, and, figure 3.6), suggestinßg that the observed down-regulation of pluripotency genes is dependent on DNA binding.

SoxB1 and SoxE factors have distinct effects on neural crest and epidermis at neurula stages.

Given that premature expression of SoxE factors in pluripotent blastula cells disrupts normal gene expression, we wished to determine if a prolonged expression of SoxB1 factors in the cells that will become neural crest has effects distinct from SoxE activity. Importantly, however, comparing the effects of SoxB1 and SoxE activity in neural crest cells necessitates bypassing their effects on blastula cells. To accomplish this we expressed glucocorticoid receptor fusion proteins of both SoxB1 (*Sox2* and *Sox3*) and SoxE factors (*Sox9* and *Sox10*). These fusion proteins remain functionally inactive until embryos are treated with dexamethasone, allowing temporal control of their function in the developing embryo. Embryos were injected with mRNA encoding these factors in one cell at the two-cell stage. Injected embryos were treated with dexamethasone at stage 10 and cultured to neurula stages when they were harvested for analysis by *in situ* hybridization (Figure 3.7) Interestingly, we found that inducing SoxB1 activity at the neural plate border at these stages led to down-regulation of neural crest factors *Foxd3* and *Snail2* (Figure 3.8 A-C and 3.9). By contrast, when SoxE proteins were similarly activated they enhance the expression of these neural crest markers (Figure 3.8 A-C, and 3.9)

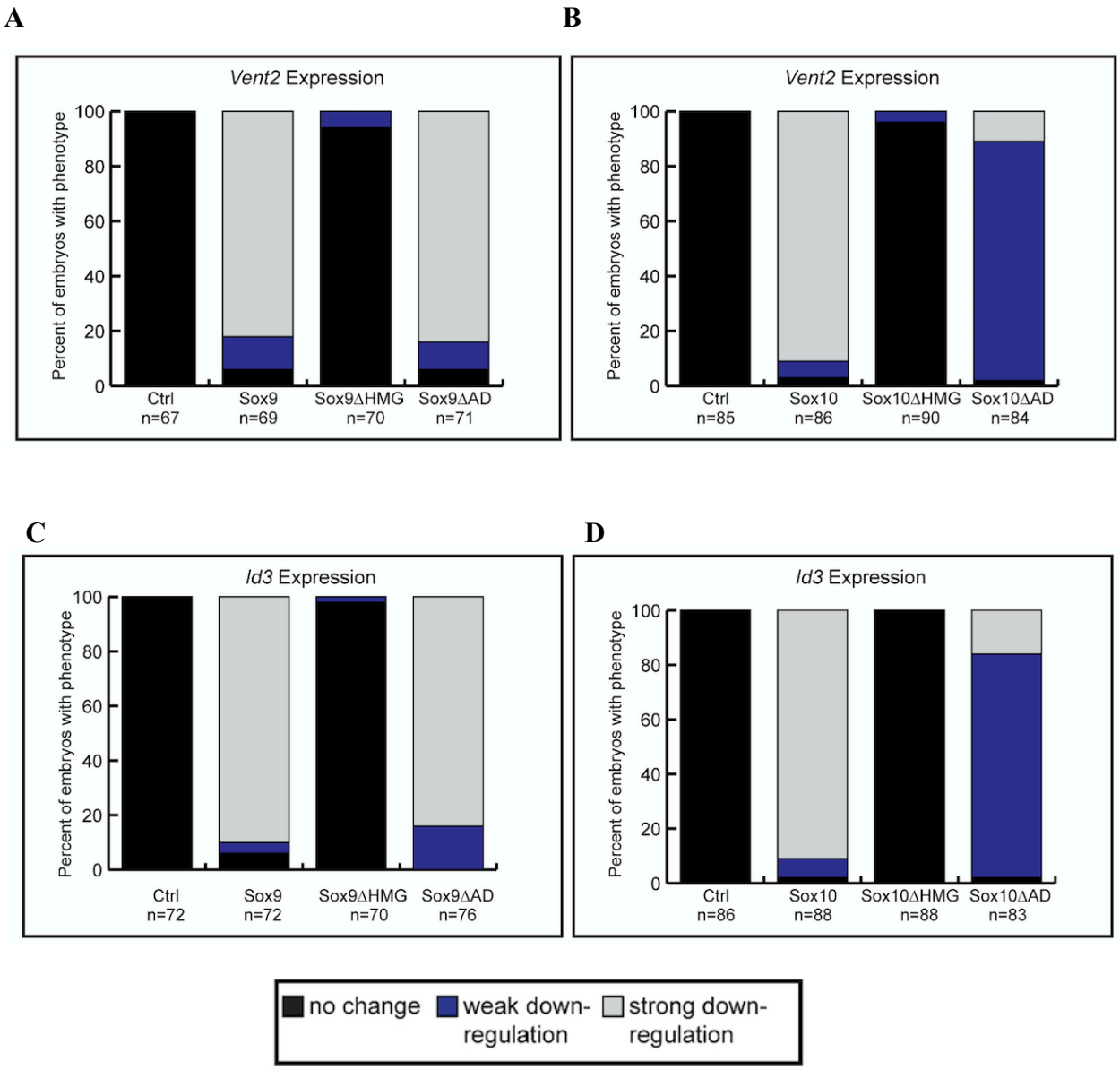


Figure 3.6 Quantification of Sox10, Sox10 ΔHMG domain or Sox10Δ Activation domain embryos

Quantification % reduced expression of pluripotency factors (A) *Vent2* expression in whole embryos injected with Sox9, Sox9 ΔHMG domain or Sox9 Δ Activation domain mRNAs (B) *Vent2* expression in whole embryos injected with Sox10, Sox10 ΔHMG domain or Sox10 Δ Activation domain mRNAs (C) *Id3* expression in whole embryos injected with Sox9, Sox9 ΔHMG domain or Sox9 Δ Activation domain mRNAs (D) *Id3* expression in whole embryos

injected with Sox10, Sox10 ΔHMG domain or Sox10 Δ Activation domain mRNAs (Figure 3.4 and 3.5) n values listed beneath each gene/condition

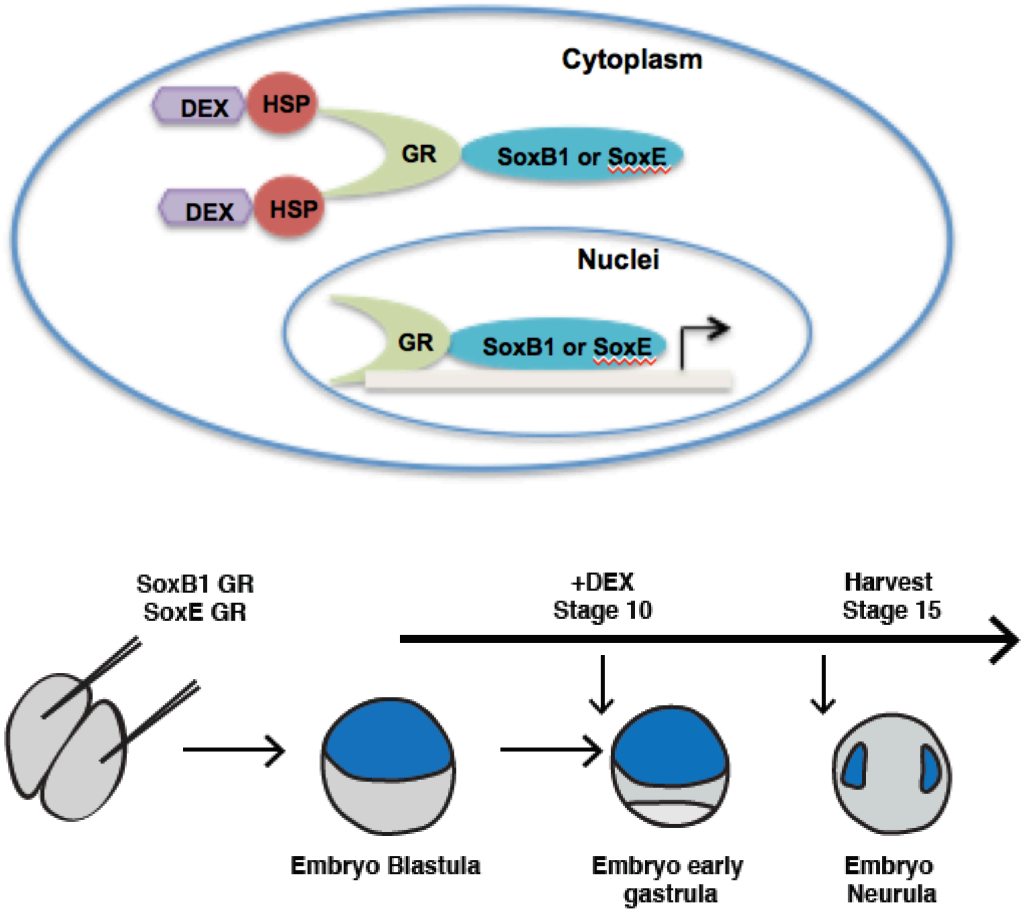


Figure 3.7 Schematic representation of glucocorticoid receptor experiments (GR)
Proteins that are fused to a glucocorticoid receptor (GR) will be occluded from the nucleus due to binding of heat shock proteins (HSP90). When dexamethasone (DEX) is added, the heat shock proteins will be released from the GR protein and then the fusion protein will be able to translocate into the nucleus. This method is a great way to temporally control protein function (Top). We used this system, to bay pass effects in blastula stages *Xenopus* embryos. Embryos were injected with either SoxB1 or SoxE GR fusion mRNAs and collected at mid-neurula stages (stage 17). Embryos were treated with dexamethasone at early gastrula stages (stage 10). Control embryos were treated with the vehicle control (ethanol) at the same stages.

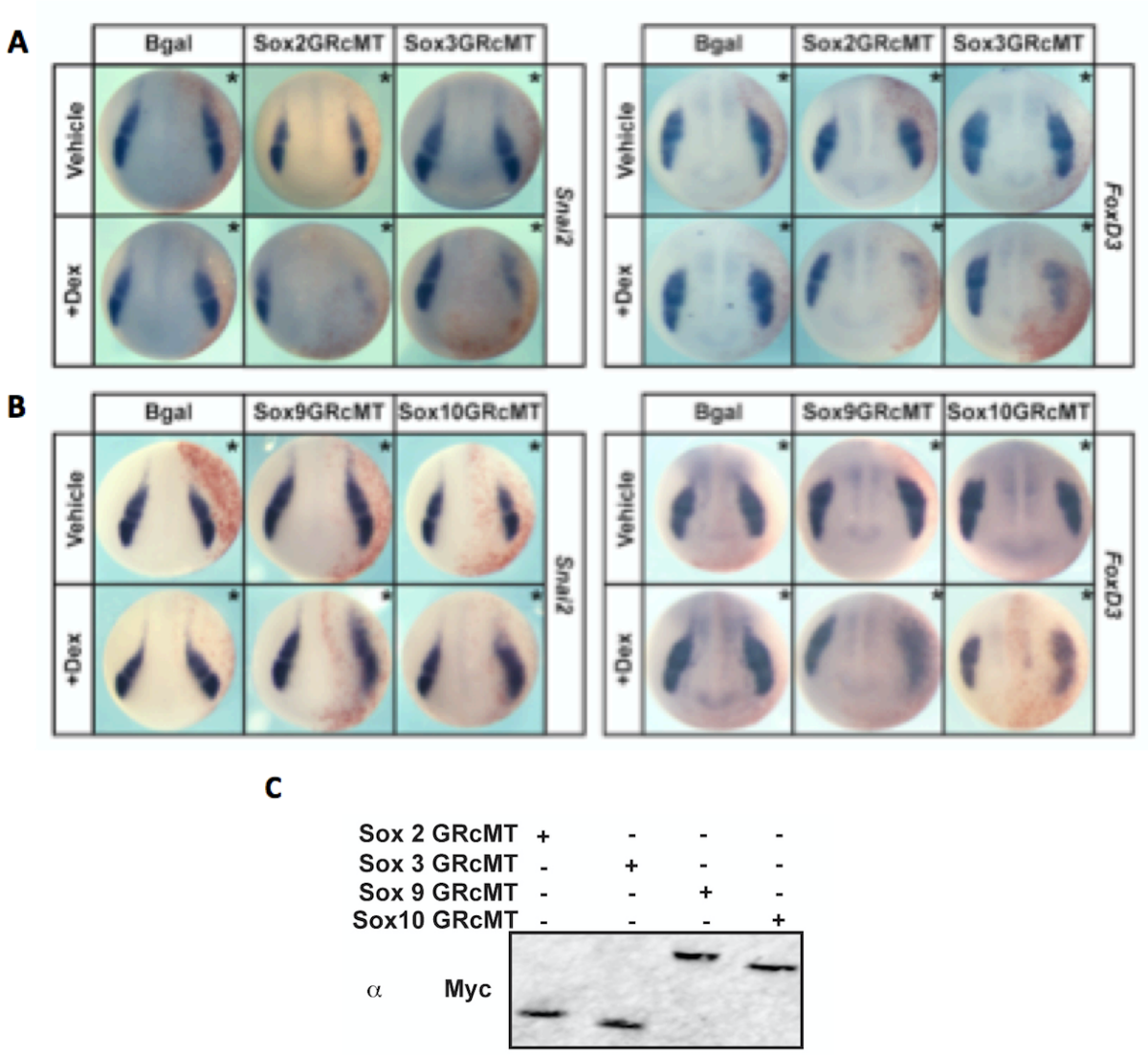


Figure 3.8 SoxB1 and SoxE proteins differentially regulate neural crest lineages
(A) Overexpressing SoxB1 proteins shows inhibition of the expression of neural crest markers *FoxD3* and *Snail2,* in contrast*,* (B) Overexpressing SoxE protein shows ectopic expression of neural crest markers *FoxD3* and *Snail2*. Embryos were induced with dexamethasone treatment at early gastrula stages and co-injected with β-gal that serves as a lineage tracer. Scale bars, 250 μm. Control treated embryos at early gastrula stages do not affect the expression of neural crest markers *FoxD3* and *Snail2.* (C) Western blot using lysates from embryos injected with same mRNAs confirming relatively equivalent levels of proteins expressed, (experiments for the figure were performed by Elizabeth Schock)

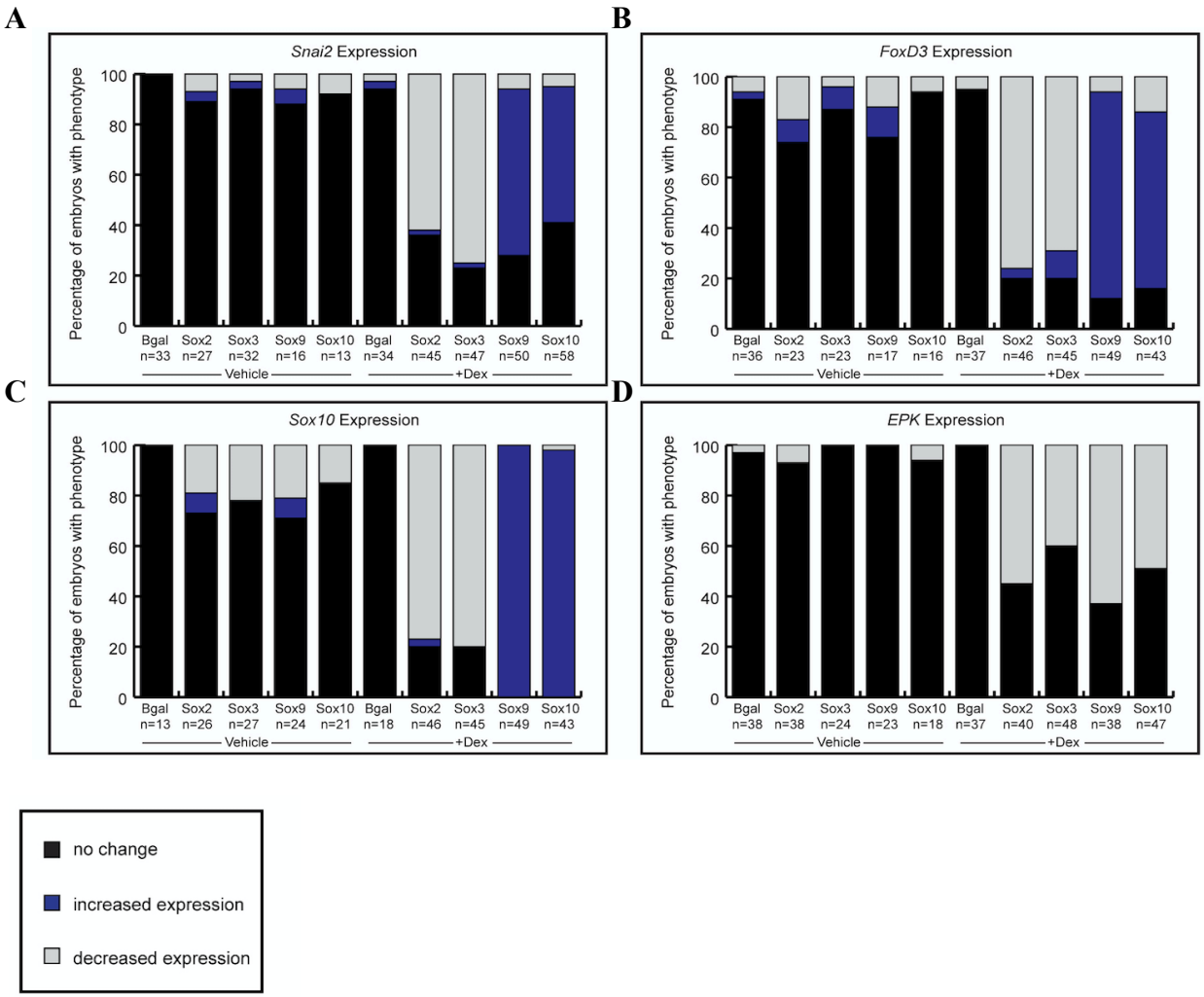


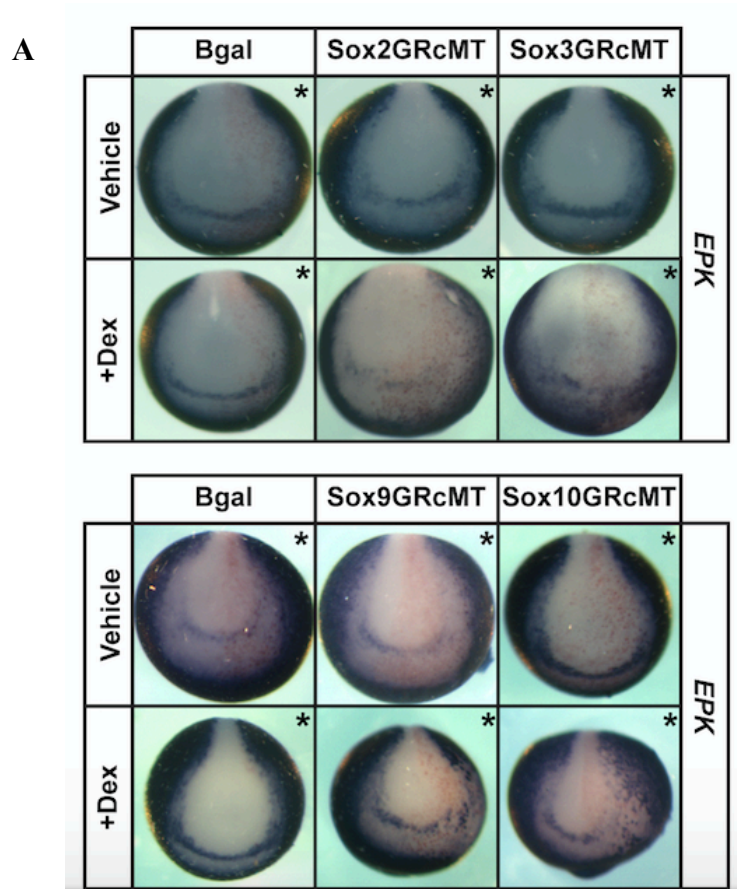
Figure 3.9 Quantification of the effects of SoxB1 and SoxE on the regulation of ectodermal cell fates

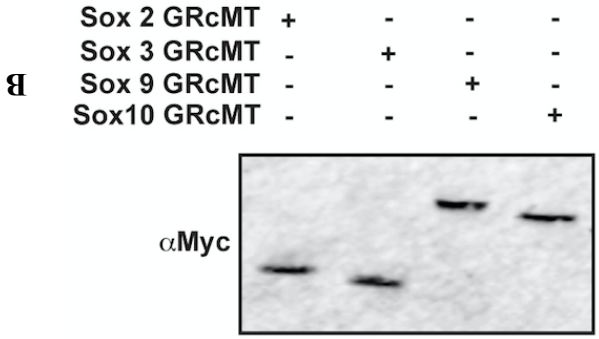
Quantification of the phenotypes of changes in GR experiments the expression of neural crest genes (A) *Snail2* (B) *FoxD3,* and the epidermal marker (D) *EPK* in whole embryos that were injected with Sox2GR, Sox3GR, Sox9GR or Sox10GR, treated with dexamethasone and collected at neurula stages (stage 15) (from figures 3.9 and 3.11). N values are listed beneath each gene/condition (quantifications of experiments for this figure were performed by Elizabeth Schock)

consistent with previous reports (Aoki et al.,2003; Saint-Germain et al., 2004; Taylor and LaBonne, 2005). These findings demonstrate that SoxE and SoxB1 activity have very different effects on the expression of neural crest markers at the neural plate border that are consistent with the spatiotemporal expression of these factors. To determine if these functional differences correlate with developmental timing (gastrula/neurula stage ectoderm) or cell type (neural crest) we also compared the effects of SoxB1 and SoxE activation on expression of the epidermal marker *Epidermal keratin*. Activation of either SoxB1 or SoxE factors downregulated the expression of this epidermal marker (Figure 3.10 A-B and 3.9) suggesting that in this context, SoxB1 and SoxE factors have similar activities.

Increased SoxB1 or SoxE activity interferes with pluripotency

Given the observed down-regulation of key potency genes in pluripotent blastula cells, I next determine the consequences of SoxE and SoxB1 activation for pluripotency. Animal pole cells of blastula stage (stage 8-9) embryos are pluripotent, and explants of these cells can give rise to cell types of all three germ layers given proper instruction (Ariizumi & Asashima, 2001). For example, treatment of animal pole cells with activin instructs them to adopt mesoderm or endoderm fates in a dose dependent manner (Asashima et al., 1990a; Asashima et al., 1990b; Lamb et al., 1993; Sasai et al., 1995). Using this system I asked whether SoxE and SoxB1 activity interfered with the ability of animal pole explants to adopt these fates in response to activin. Embryos were injected with mRNA encoding Sox2, Sox3 Sox9, or Sox10 in both cells at the 2-cell stage. Explants were manually dissected at blastula stage 8 and cultured with or without activin until early gastrula stages (stage 11.5) (Figure 3.11).





3.10 SoxB1 and SoxE proteins similarly regulate epidermal lineages

(A) Overexpressing either SoxB1 or SoxE proteins at early gastrula stages shows inhibition of the expression of the epidermal markers *Epk* . Embryos were co-injected with β-gal that serves as a lineage tracer, Control treated embryos at early gastrula stages do not affect the expression of the epidermal markers *Epk* (B) Western blot using lysates from embryos injected with same mRNAs confirming relatively equivalent levels of proteins expressed, (experiments for the figure were performed by Elizabeth Schock)

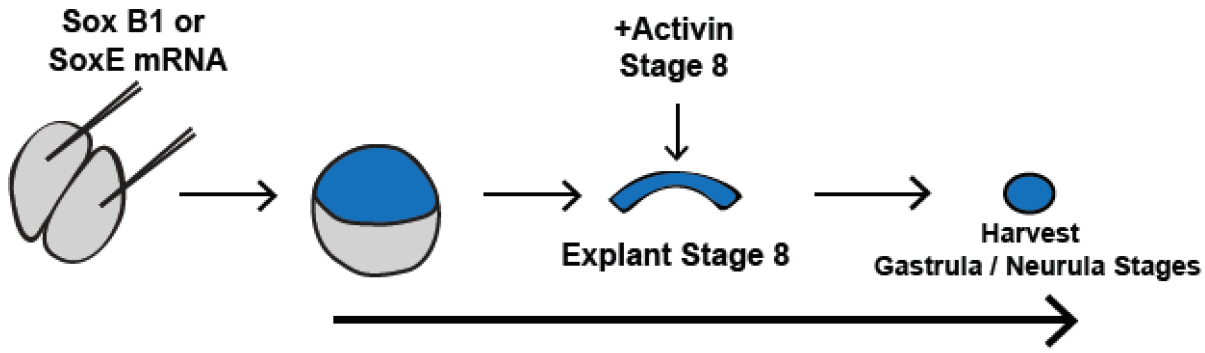


Figure 3.11 Schematic representation of activin treatment of ectodermal explants Ectoderm from the animal pole of donor/ injected embryos is explanted at blastula stages and treated with low or high amounts of activin to induce either mesoderm or endoderm formation. Explants were cultured and collected until gastrula stages for *Brachyury* and *Sox17* expression.

While control explants showed robust expression of the mesodermal marker *Brachyury* in response to low doses of activin, overexpression of SoxB1 or SoxE factors interfered with this response (Figure 3.12 A-B, and 1.13) Similarly, SoxB1 or SoxE overexpression interfered with induction of endoderm in response to high doses of activin, as evidenced by loss of *Sox17* expression (Figure 3.14 A-B, and 3.15). Thus, consistent with effects on pluripotency gene expression, up-regulation of Sox activity interfered with the functional pluripotency in these cells, and therefore in this context SoxB1 and SoxE factors exhibit similar activities.

Both SoxB1 and SoxE1 proteins can maintain pluripotency.

The ability of the core pluripotency factors, including Sox2, to maintain developmental potential is concentration dependent (Kopp et al., 2008; Boer et al., 2007; Yamaguchi et al., 2011), and this may explain the diminished ability of animal pole explants to form mesoderm or endoderm when SoxB1 or SoxE factors are overexpressed. To more rigorously compare the ability of SoxB1 and SoxE factors to mediate pluripotency I carried out a molecular replacement experiments (Figure 3.16). I used previously characterized translation blocking morpholinos targeting Sox2 and Sox3 to deplete the function of SoxB1 in animal pole cells (Schlosser et al., 2008). Cells depleted of SoxB1 factors are no longer competent to form mesoderm or endoderm in response to activin treatment, as assayed by expression of *Brachyury* and *Endodermin* (Figure 3.17 A-B, and 3.19A-B), confirming that SoxB1 function is essential for pluripotency in blastula stem cells. Interestingly, the ability the ability of these cells to adopt mesodermal or endodermal states could be rescued not only by expressing Sox2 or Sox3, but also expressing Sox9 or Sox10 (Figure3.17- 3.20). Thus, although they are not normally expressed in pluripotent cells of the

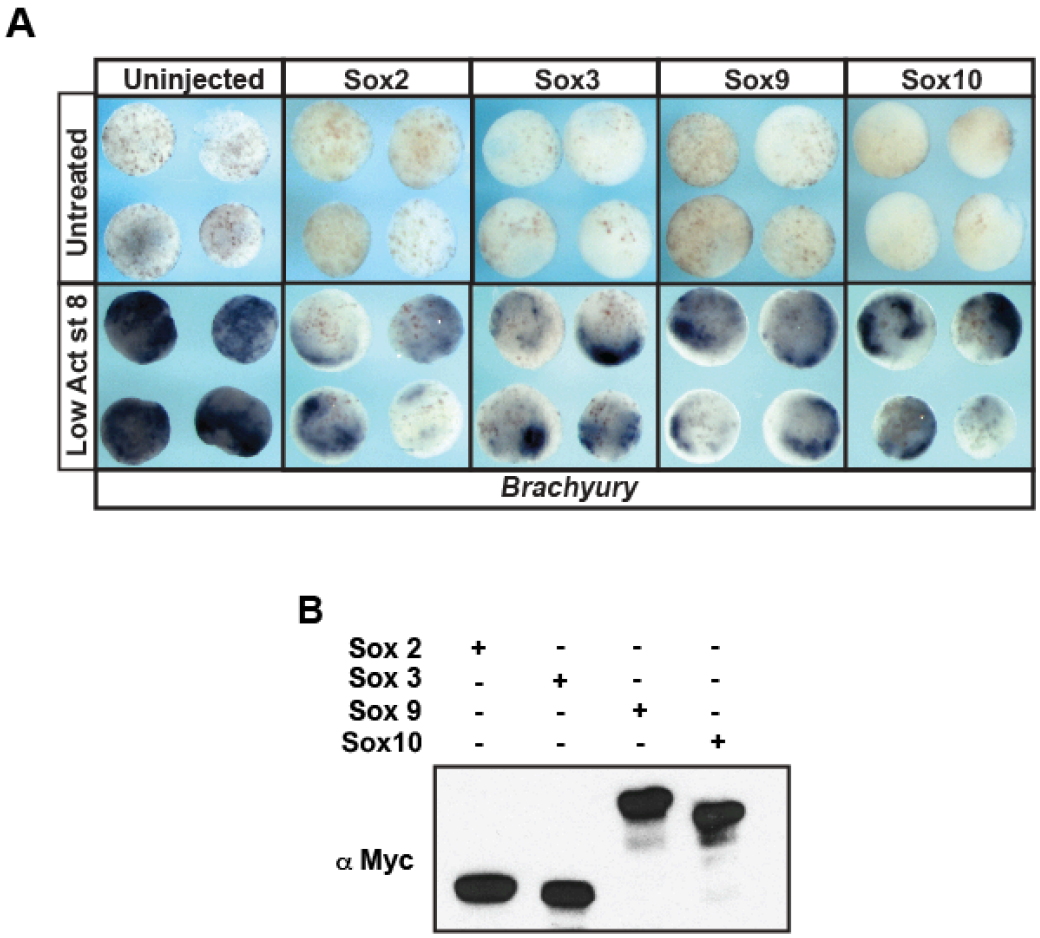


Figure 3.12 SoxB1 and SoxE factors inhibit activin mediated mesoderm formation
(A) *In situ* hybridization of an ectodermal explant assay examining the expression of *Brachyury*. Explants were injected with Sox2, Sox3, Sox9, or Sox10 mRNA and cultured with or without activin until early gastrula stages (stage 11.5) (B) Western blot analysis using lysates from animal caps injected with Sox2, Sox9, Sox3 or Sox10 mRNAs confirming relatively equivalent levels of proteins expressed.

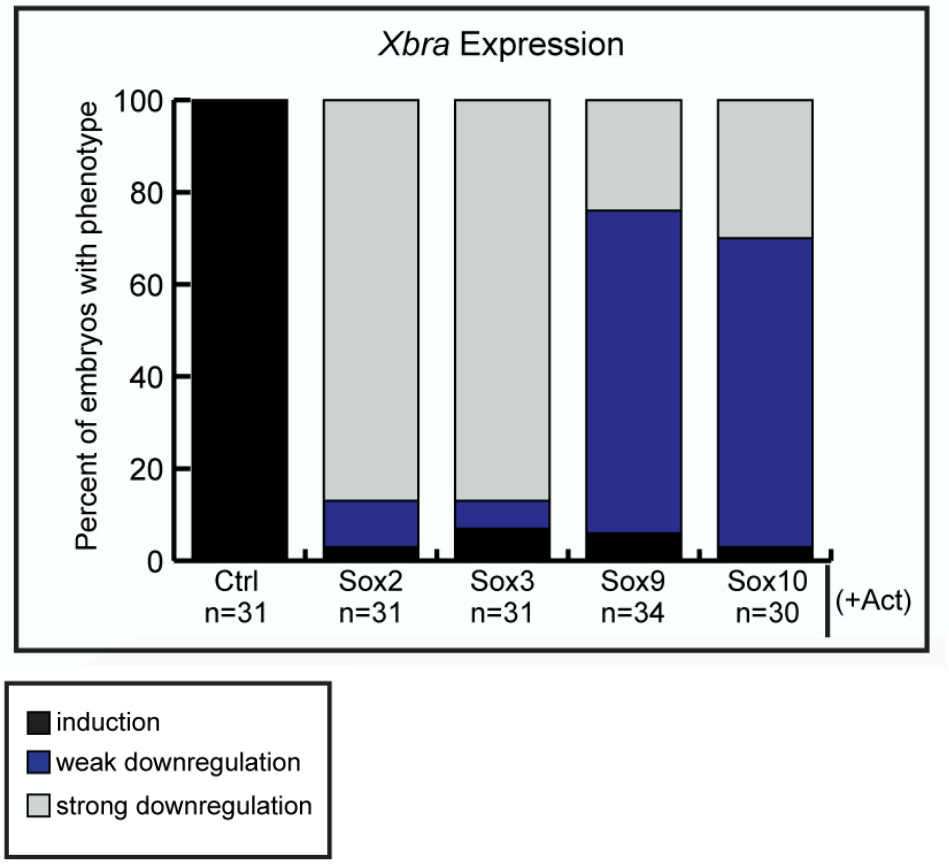


Figure 3.13 Quantification of gain of function experiments in ectodermal explants evaluating the competence to form mesoderm

Quantification of the % induction in expression of *Brachyury* in ectodermal explant assays that were injected with Sox2, Sox3, Sox9 or Sox10 mRNA and treated with activin at stage 8-9 for gain of function experiments (Figure 3.15).

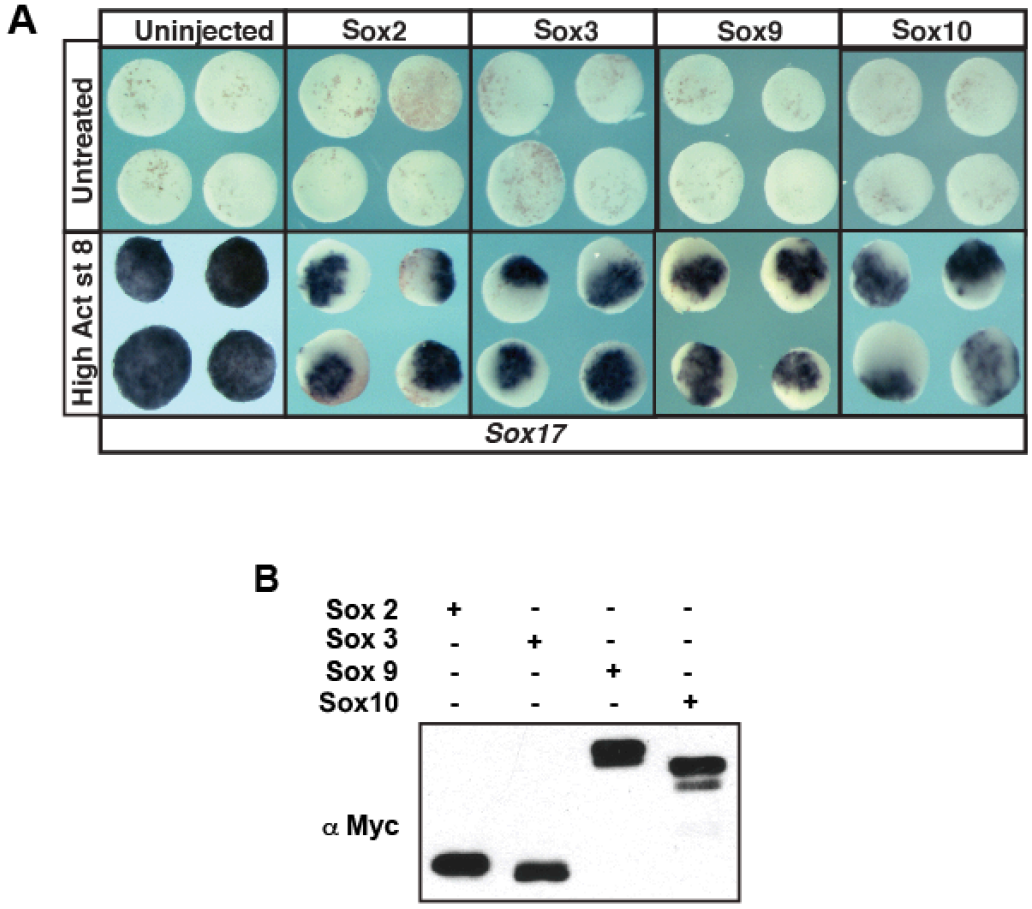


Figure 3.14 SoxB1 and SoxE factors inhibit activin mediated endoderm formation (A) *In situ* hybridization of an ectodermal explant assay examining the expression of *Sox17.* Explants were injected with Sox2, Sox3, Sox9, or Sox10 mRNA and cultured with or without activin until early gastrula stages (stage 11.5) (B) Western blot analysis using lysates from animal caps injected with Sox2, Sox9, Sox3 or Sox10 mRNAs confirming relatively equivalent levels of proteins expressed.

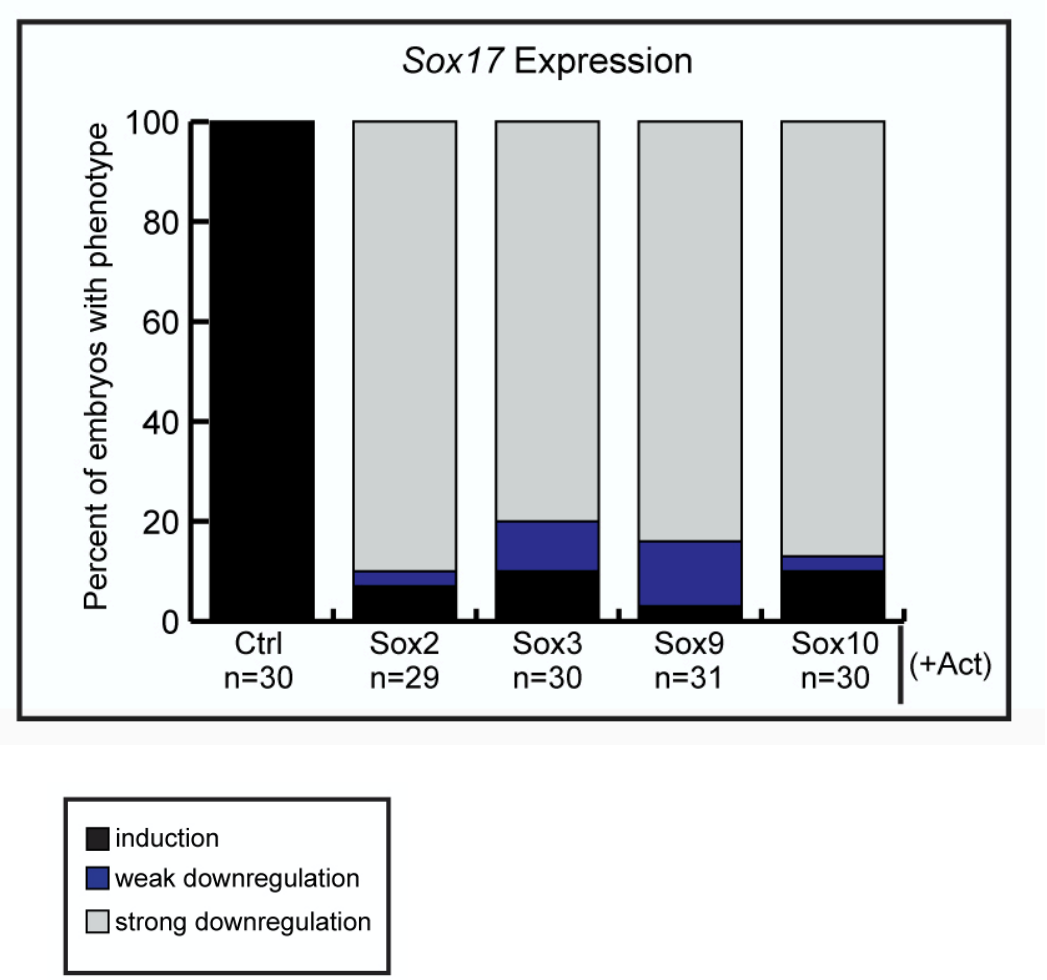


Figure 3.15 Quantification of gain of function experiments in ectodermal explants evaluating the competence to form endoderm

Quantification of the % induction in expression of *Sox17* in ectodermal explant assays that were injected with Sox2, Sox3, Sox9 or Sox10 mRNA and treated with activin at stage 8-9 for gain of function experiments (Figure 3.16).

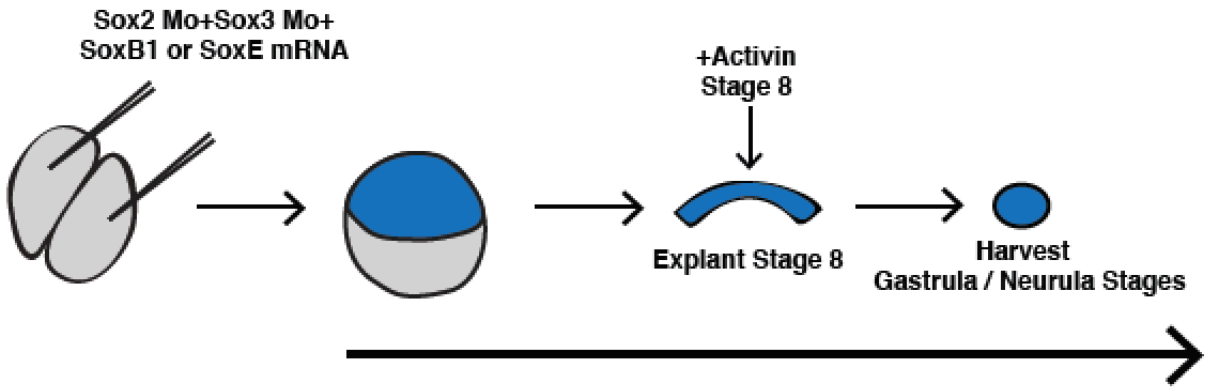


Figure 3.16 Schematic representation of the experimental approach, rescue experiments
Embryos were injected at two cell stage with either Sox2 and Sox3 translation blocking Morpholinos or Morpholinos along with c terminal tag versions of Sox2, Sox3, Sox9 or Sox10 mRNAs to perform rescue experiments. Explants were dissected at blastula stages and cultured with or without activin to induce either mesodermal or endodermal formation until early gastrula stages (stage 11.5).

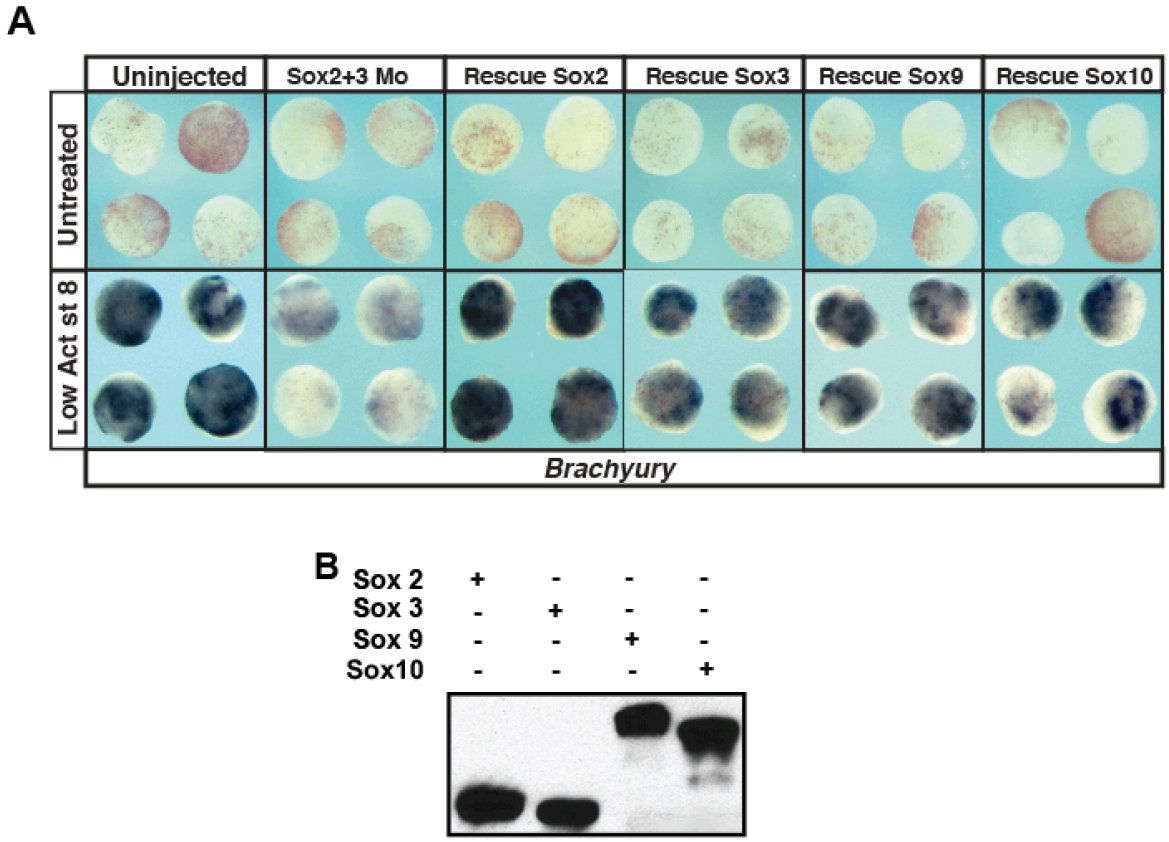


Figure 3.17 SoxB and SoxE factors rescue competence of animal pole cells to form mesoderm

(A) *In situ* hybridization of an ectodermal explant assay examining the expression of *Brachyury*. Explants were injected with SoxB1 morpholino along with Sox2, Sox3, Sox9, or Sox10 mRNA and cultured with or without activin until early gastrula stages (stage 11.5) (B) Western blot analysis using lysates from animal caps injected with Sox2, Sox9, Sox3 or Sox10 mRNAs confirming relatively equivalent levels of proteins expressed.

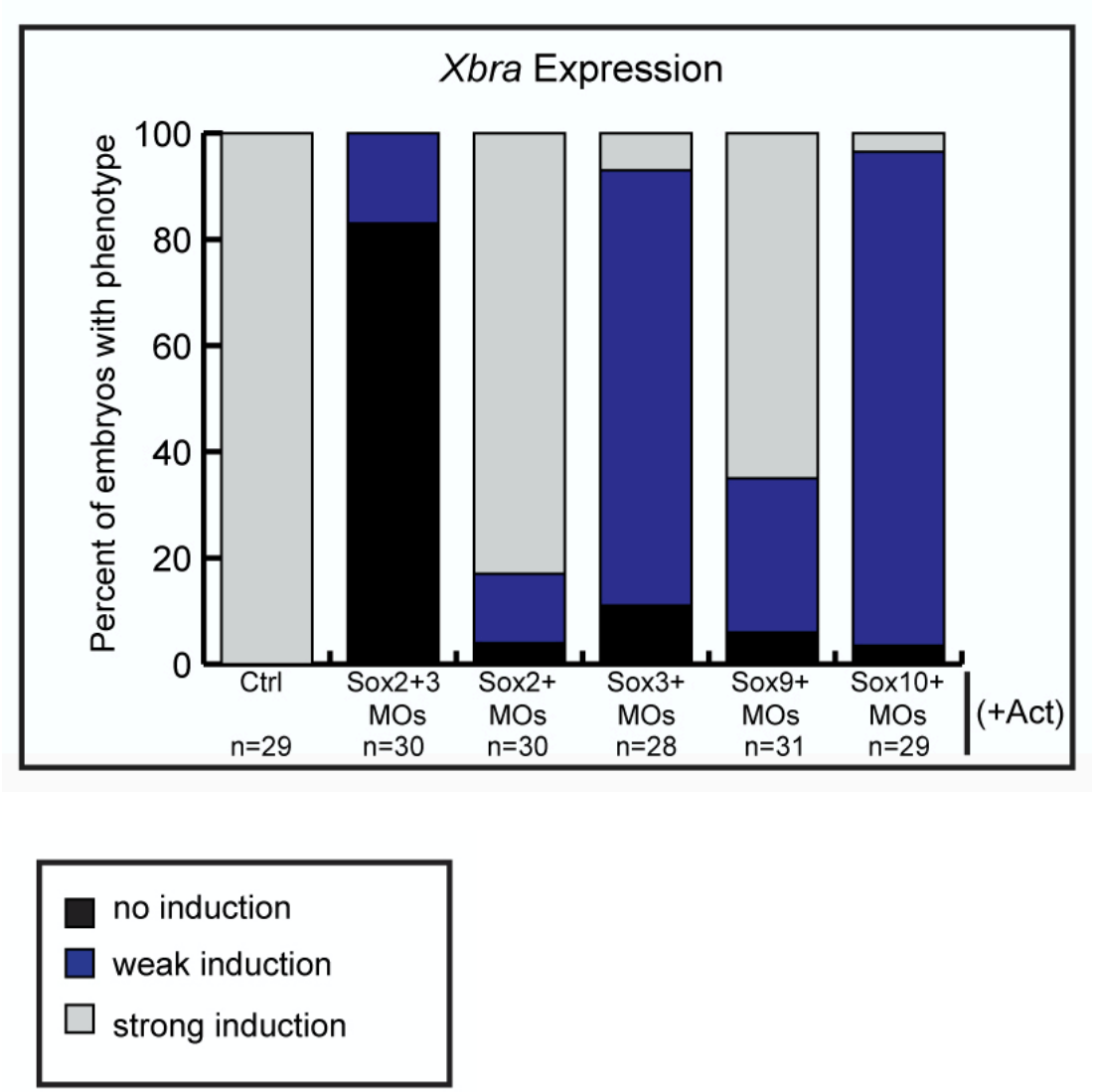


Figure 3.18 Quantification of gain of function experiments in ectodermal explants evaluating the competence to form mesoderm

Quantification of the % induction in expression of *Brachyury* in ectodermal explant assays that were injected with Sox2, Sox3, Sox9 or Sox10 mRNA and treated with activin at stage 8-9 for gain of function experiments (Figure 3.15).

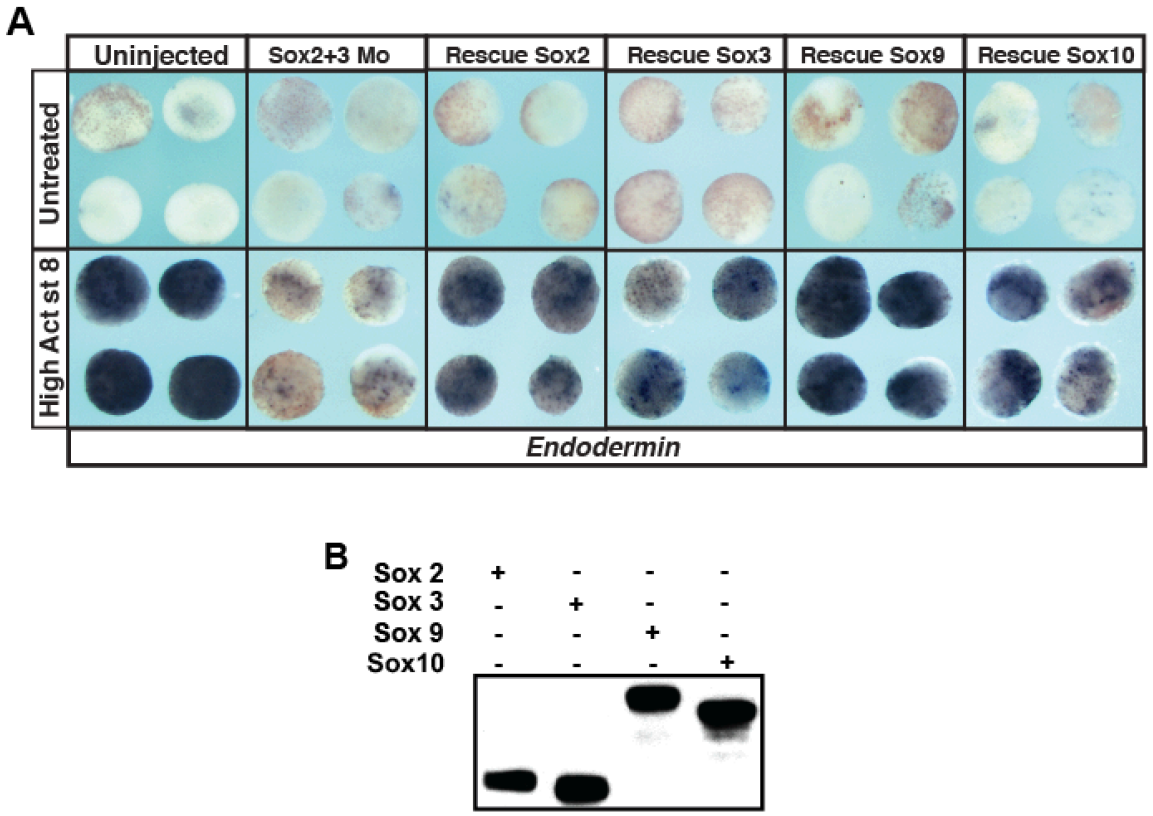


Figure 3.19 SoxB and SoxE factors rescue competence of animal pole cells to form endoderm

(A) *In situ* hybridization of an ectodermal explant assay examining the expression of *Endodermin*. Explants were injected with SoxB1 morpholino along with Sox2, Sox3, Sox9, or Sox10 mRNA and cultured with or without activin until early gastrula stages (stage 11.5) (B) Western blot analysis using lysates from animal caps injected with Sox2, Sox9, Sox3 or Sox10 mRNAs confirming relatively equivalent levels of proteins expressed.

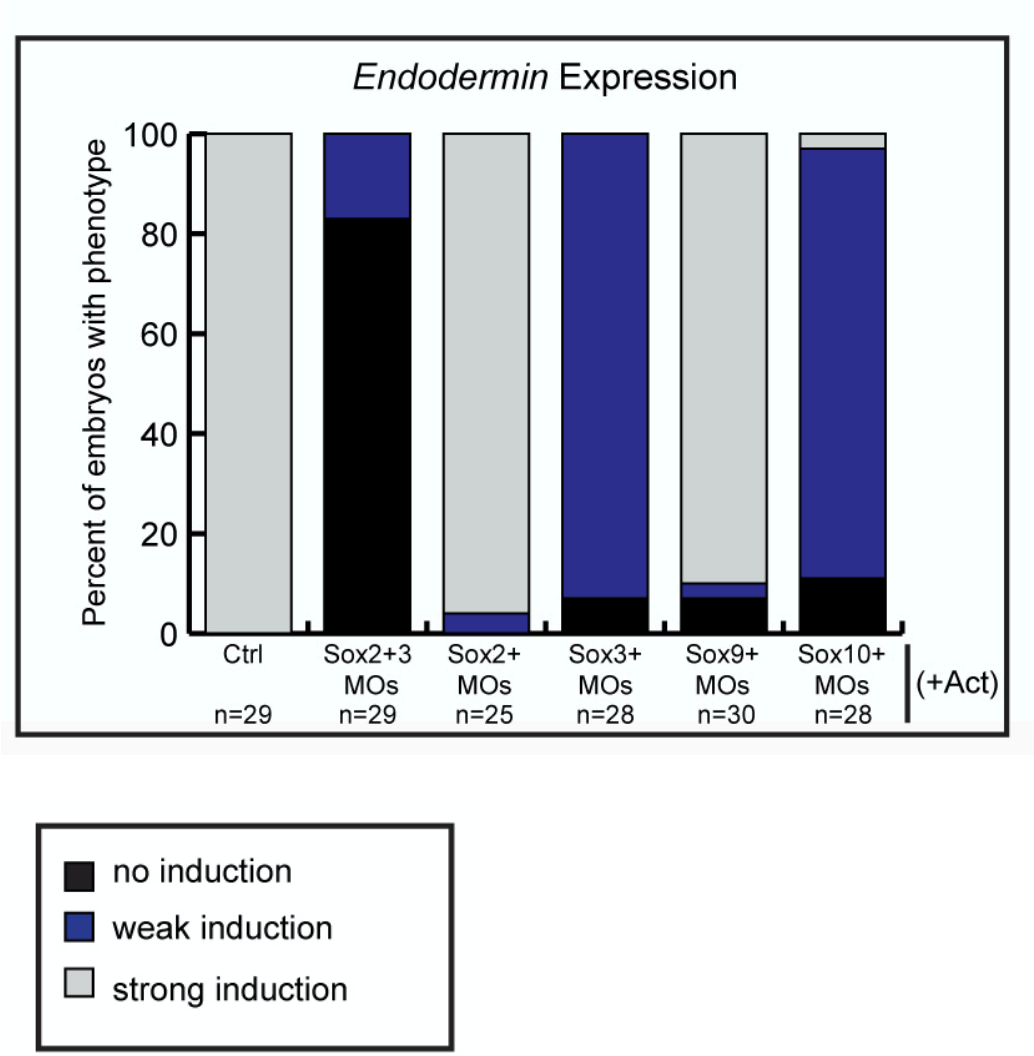


Figure 3.20 Quantification of gain of function experiments in ectodermal explants evaluating the competence to form endoderm

Quantification of the % induction in expression of *Sox17* in ectodermal explant assays that were injected with Sox2, Sox3, Sox9 or Sox10 mRNA and treated with activin at stage 8-9 for gain of function experiments (Figure 3.15).

blastula, Sox9 and Sox10 have the ability to promote pluripotency, but may do so less robustly than the SoxB1 factors do.

SoxB1 proteins, but not SoxE proteins, can maintain a neuronal progenitor state.

The ability of SoxE factors to replace SoxB1 factors in maintaining pluripotency raises the question of why a hand-off from SoxB1 to SoxE factors is necessary or advantageous. Given their later expression patterns, I hypothesized that these two families of Sox transcription factors might differentially poise cells for distinct lineage decisions. For example, subsequent to their roles in maintaining pluripotency in blastula stem cells, SoxB1 factors become restricted to the neural plate and are essential for the formation of neuronal progenitor cells. Thus I wondered if SoxE factors could replace the function of SoxB1 factors in promoting a neural progenitor state. Animal pole explants can be induced to adopt a neural state in response to the BMP antagonist chordin (Figure 3.21). I first showed that morpholino-mediated depletion of Sox2 and Sox3 prevented chordin-mediated neural induction (Figure 3.22 A-B). I then compared the ability of SoxB1 and SoxE factors to rescue the adoption of a neural state. Explants from morphant embryos co-injected mRNA encoding Sox2, or Sox3 expressed the neural marker *Sox11* in response to Chordin (Figure 3.23, 3.24 A-D and, 3.26). By contrast, neither Sox9 nor Sox10 equivalently rescued neural induction (Figure 3.24 A-B and, 3.25) Indicating that SoxB1 factors have a great ability to promote neural progenitor formation than do SoxE factors, consistent with their deployment in these cells at neurula stages.

SoxE proteins, but not SoxB1 proteins, can promote the neural crest state.

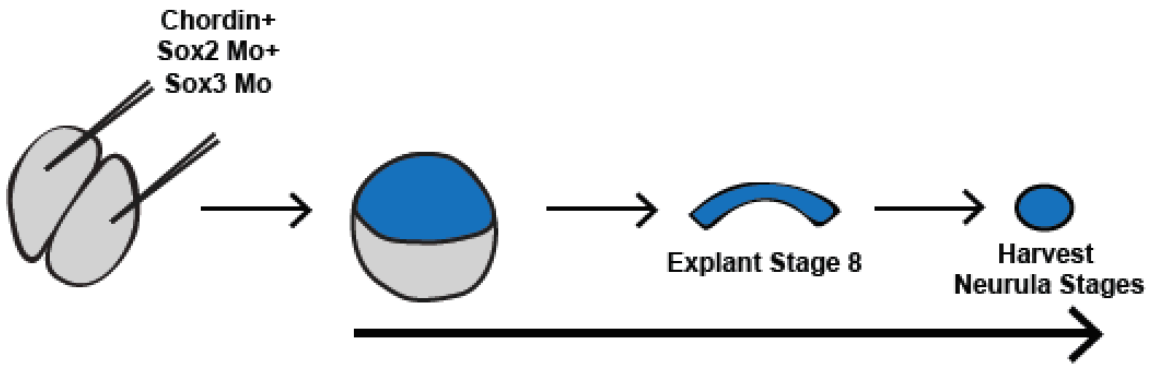


Figure 3.21 Schematic representation of the experimental approach, loss of SoxB1 factors function in explants

Embryos were injected at two-cell stage with either Sox2 and Sox3 translation blocking Morpholinos or Morpholinos, to perform competence experiments. Explants were dissected at blastula stages and cultured until neurula stages (stage 17).

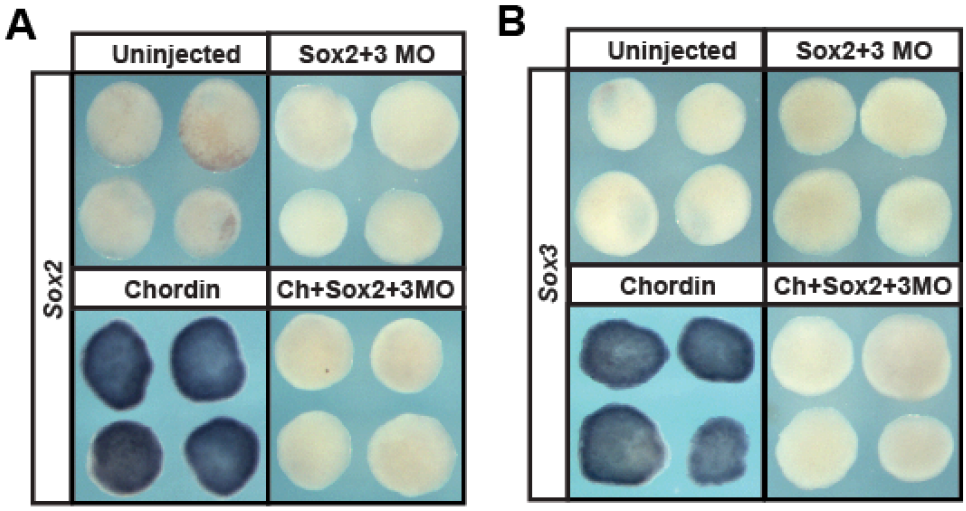


Figure 3.22 *Sox2* and *Sox3* are necessary for the commitment of neural fates in explants

(A-B) Explant assay examining *Sox2* and *Sox3* expression in Chordin induced explants that were injected with Sox3 and Sox3 morpholino. Explants were collected alongside sibling embryos, and cultured until neurula stages (stage 14). In situ hybridization was performed examining the expression of (A) *Sox2* and (B) *Sox3*.

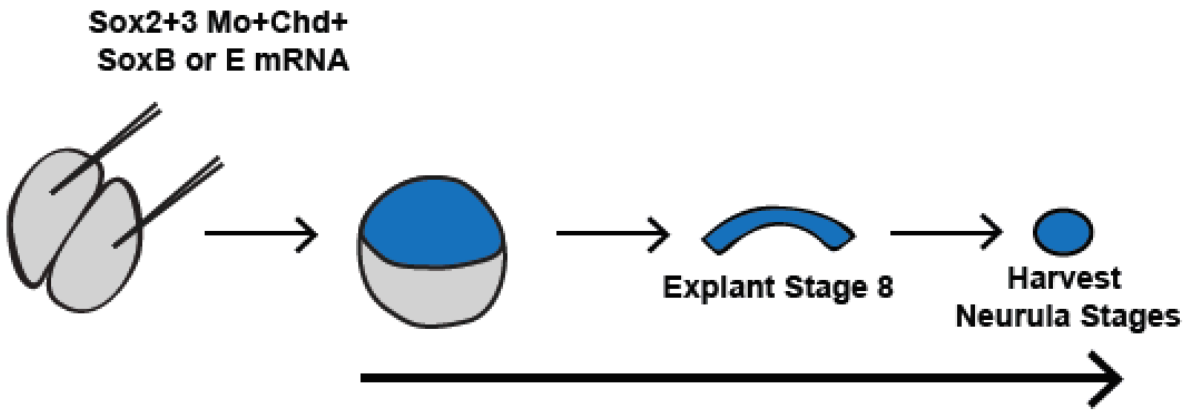


Figure 3.23. Schematic representation of the experimental approach, functional replacement experiments

Embryos were injected at two cell stage with either Sox2 and Sox3 translation blocking morpholinos or morpholinos along with c terminal tag versions of Sox2, Sox3, Sox9 or Sox10 mRNAs to perform functional replacement experiments. Explants were dissected at blastula stages and cultured until neurula stages (stage 14).

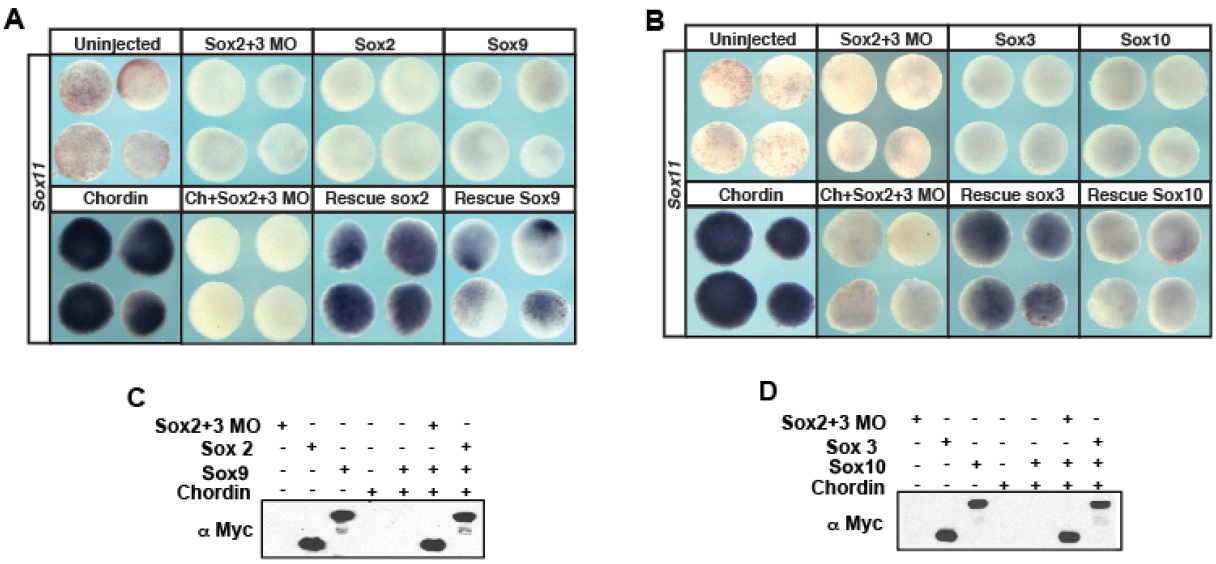


Figure 3.24 SoxE factors can partially replace the functions of SoxB1 factors by rescuing the induction of neural fates in explants

(A-B) *In situ* hybridization of explant assays examining *Sox11* expression in *Chordin* induced explants that were injected with Sox2 and Sox3 morpholino and rescuing functions with both SoxB1 and SoxE factors**.** Explants were collected alongside sibling embryos, cultured until neurula stages (stage 14). (C-D) Western blot analysis using lysates from animal caps injected with Sox2, Sox9, Sox3 or Sox10 mRNAs confirming relatively equivalent levels of proteins expressed.

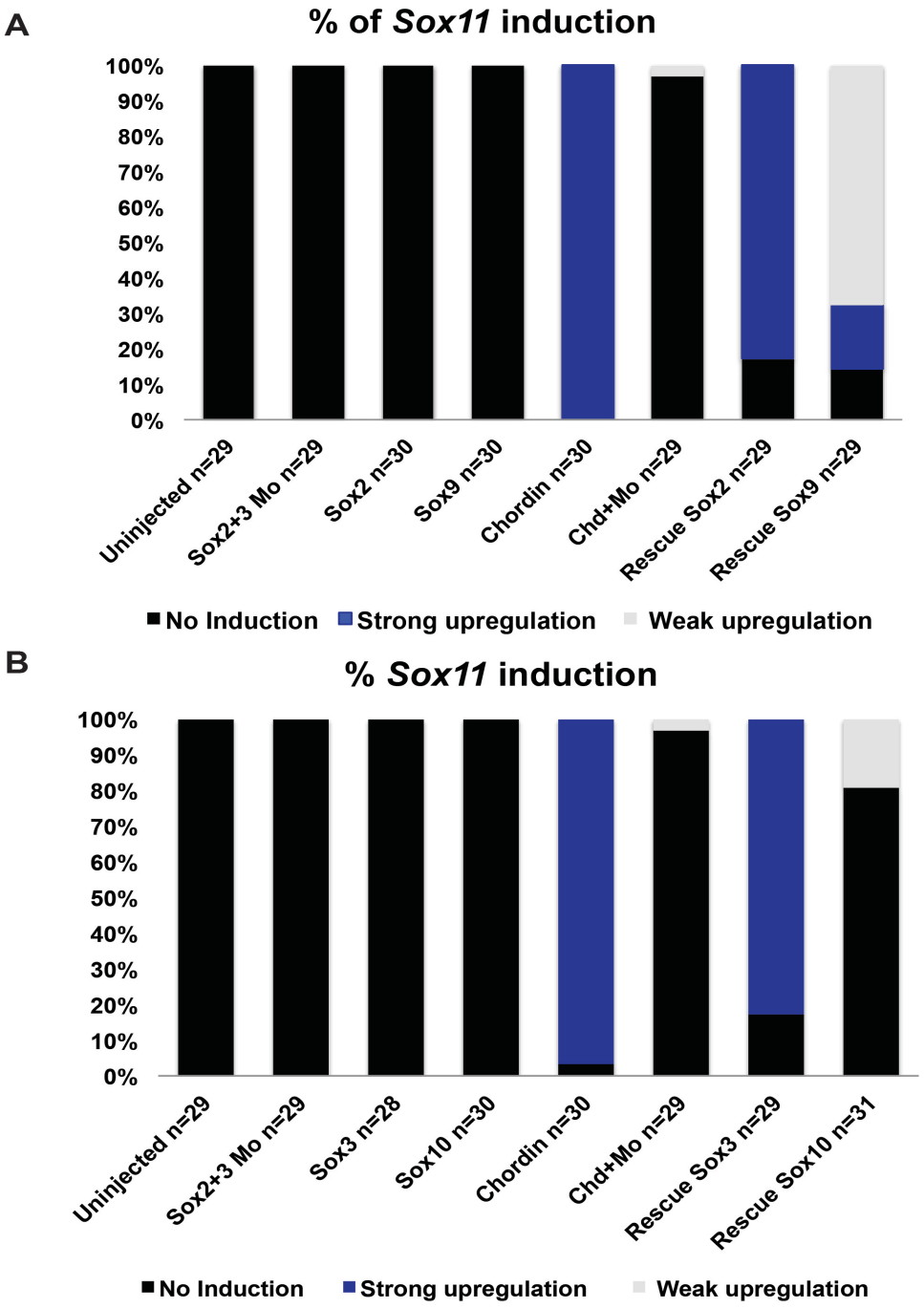


Figure 3.25 Quantification of functional replacement inducing neural fate experiments
Quantification of the % induction in expression of *Sox11* in ectodermal explants that were injected with either Chordin, Sox2 and Sox3 morpholinos and rescue with (A) Sox2, Sox9 or (B) Sox3 or Sox10 mRNA.

Given these findings, I next tested whether SoxB1 factors could replace SoxE functions in establishing a neural crest state. Animal pole cells can be reprogrammed to a neural crest state by expression of Wnt8 and Chordin (LaBonne & Bronner-Fraser, 1998, Figure 3.26 and, 3.27).

Sox10 is required for establishing the neural crest state (Aoki et al., 2003; Honoré et al., 2003; Taylor & LaBonne, 2005) and morpholino-mediated depletion of Sox10 prevents expression of the neural crest markers *Sox9*, *Sox10* and *Foxd3* in these explants (Figure 3.27 A-D) Co-expression of either Sox9 or Sox10 could rescue the induction of the neural crest marker *FoxD3* (Figure 3.29 A-D and, 3.30) in these explants. By contrast, Sox2 or Sox3 showed little or no ability to rescue *Foxd3* expression. These findings suggest that SoxB1 factors have only a limited ability to replace SoxE function in promoting formation and maintenance of neural crest cells.

Discussion

One hundred and fifty years after the discovery of the neural crest, the primary synapomorphy of vertebrates, by Wilhelm His in 1868, much remains to be learned about these cells. The neural crest is distinguished by its retention of stem cell attributes long past the time when neighboring cells in the early embryo have undergone lineage restriction, as well as by the broad and diverse set of derivatives to which these ultimately contribute. While there is considerable overlap in the GRNs controlling pluripotency in blastula stem cells and neural crest cells (Buitrago et al., 2015), a major difference between these cell types is the sub-type of Sox family transcription factors deployed (Figure 3.1).

A dramatic change in the expression of SoxB1 and SoxE factors is observed as embryos

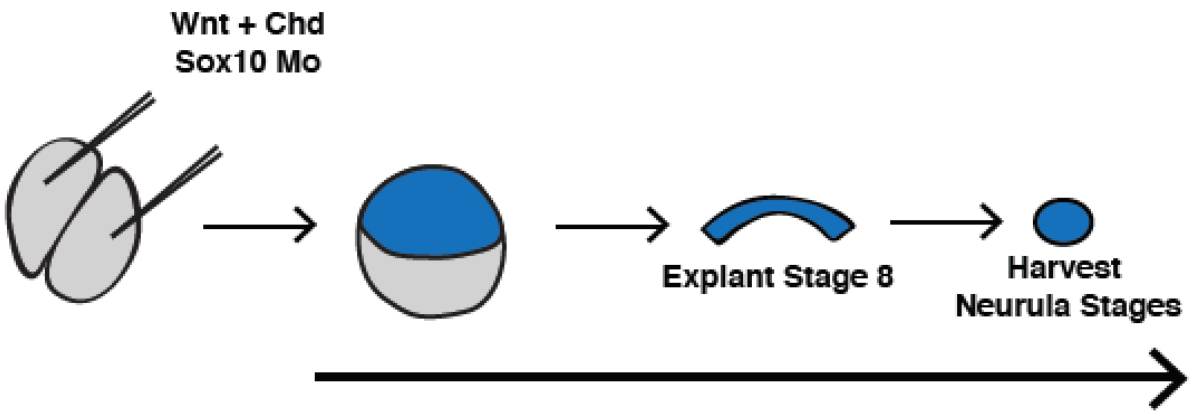


Figure 3.26 Schematic representation of the experimental approach, loss of SoxE factors function in explants
Embryos were injected at two-cell stage with Sox10 translation blocking Morpholino to perform competence experiments. Explants were dissected at blastula stages and cultured until neurula stages (stage 17).

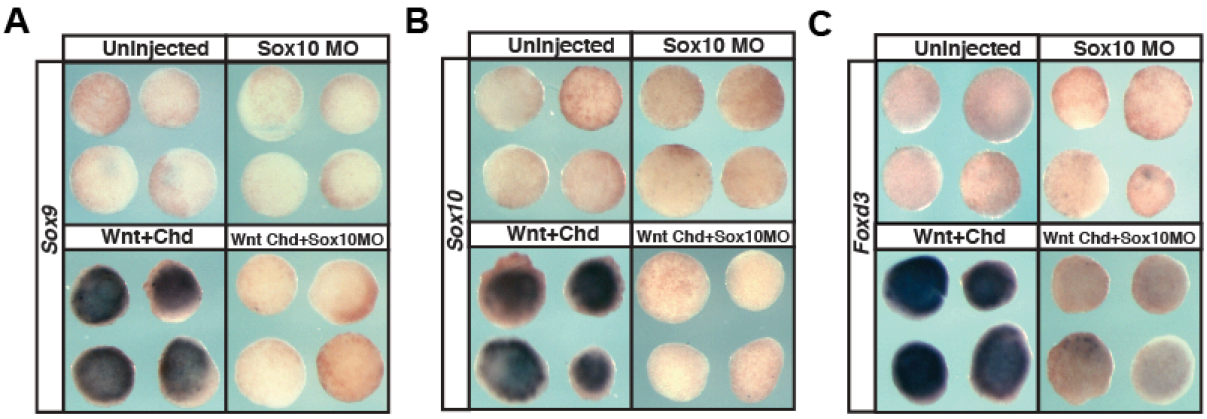


Figure 3.27 *Sox10* is necessary for the commitment of neural crest fates in explants
(A-B) Explant assay examining *Sox9, Sox10* and *FoxD3* expression in Wnt8 + Chordin induced explants that were injected with Sox10 morpholino. Explants were collected alongside sibling embryos, cultured until neurula stages (stage 17). In situ hybridization was performed examining the expression of (A) *Sox9* (B) *Sox10* and (C) *FoxD3*

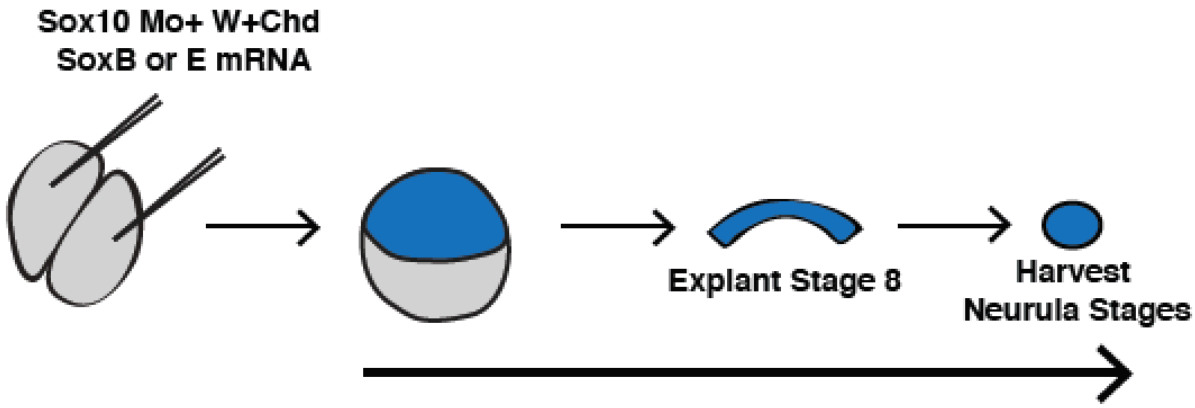


Figure 3.28 Schematic representation of the experimental approach of functional replacement experiments
Embryos were injected at two-cell stage with Sox10 translation blocking Morpholinos or Morpholinos along with c terminal tag versions of Sox2, Sox3, Sox9 or Sox10 mRNAs to perform functional replacement experiments. Explants were dissected at blastula stages and cultured until neurula stages (stage 17).

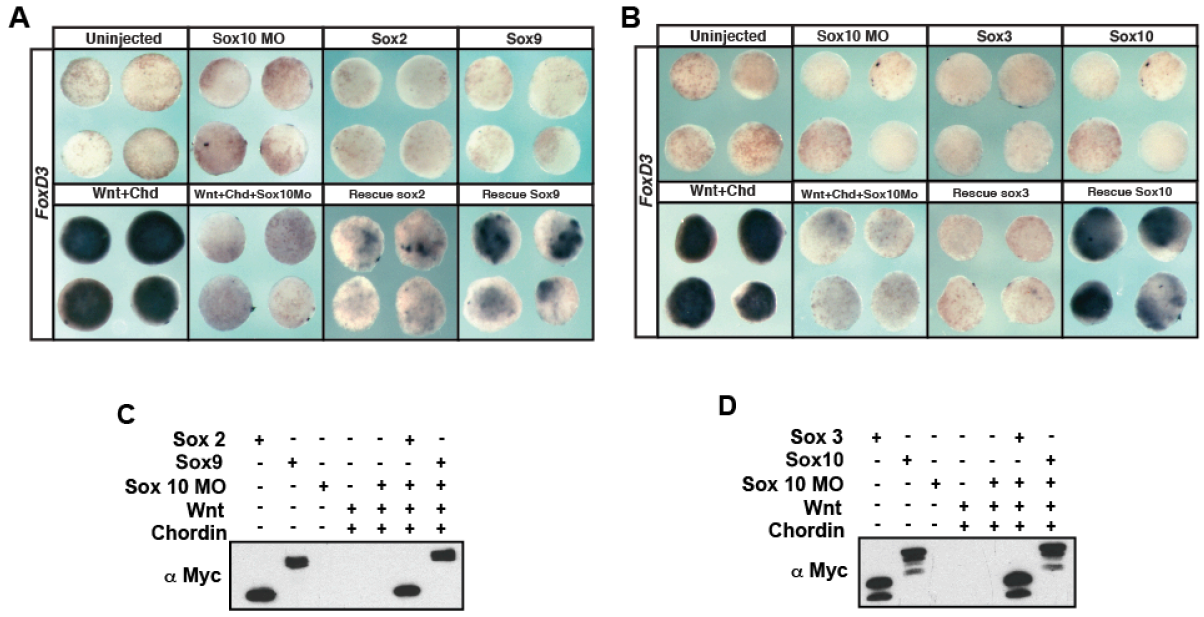


Figure 3.29 SoxE factors can partially replace the functions of SoxB1 factors rescuing the induction of neural fates in explants

(A-B) *In situ* hybridization of explant assays examining *FoxD3* expression in Wnt8 + *Chordin* induced explants that were injected with Sox10 morpholino and rescuing functions with both SoxB1 and SoxE factors**.** Explants were collected alongside sibling embryos, cultured until neurula stages (stage 17). (C-D) Western blot analysis using lysates from animal caps injected with Sox2, Sox9, Sox3, or Sox10 mRNAs confirming relatively equivalent levels of proteins expressed

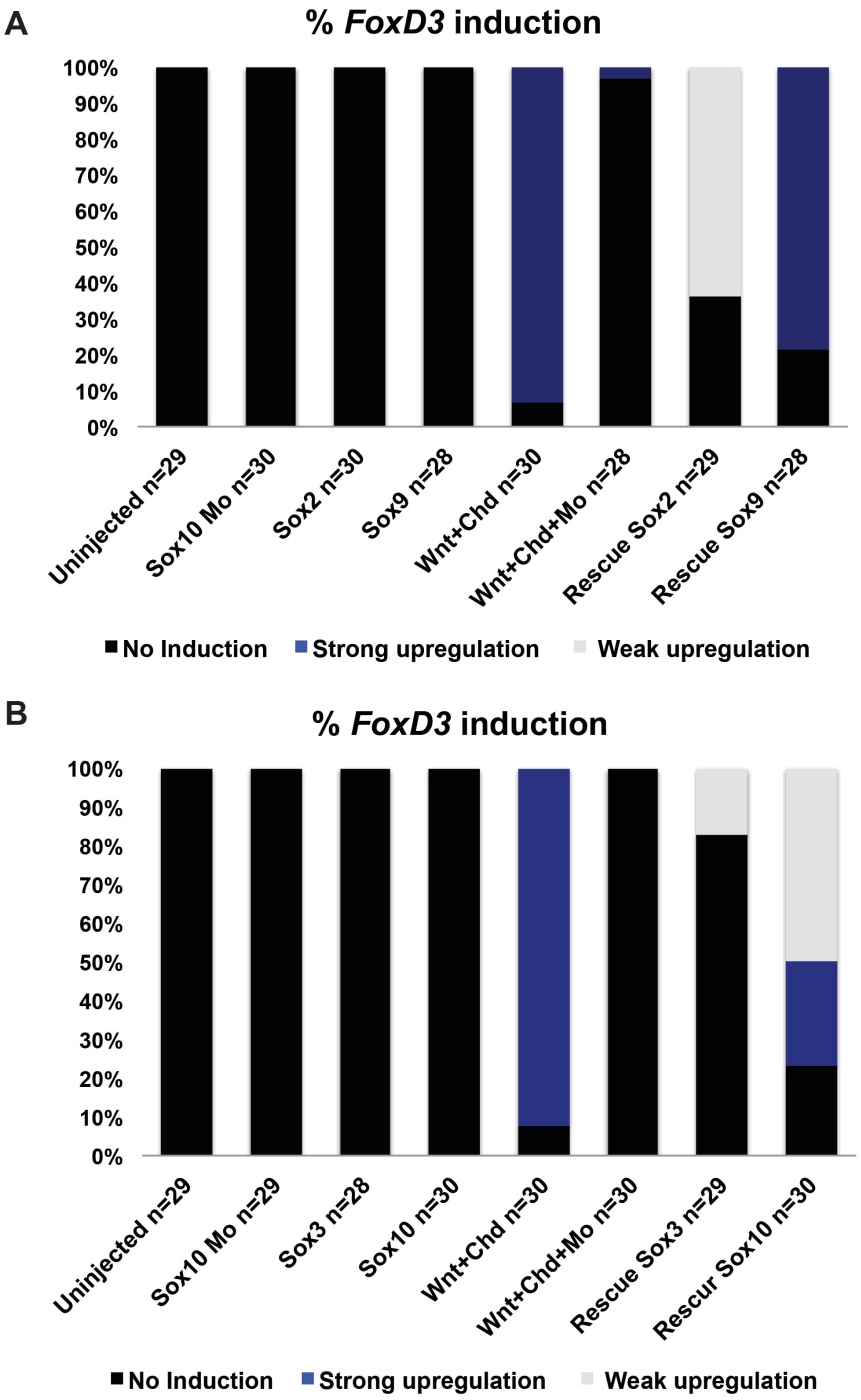


Figure 3.30 Quantification of functional replacement inducing neural fate experiments
Quantification of the % induction in expression of *FoxD3* in ectodermal explants that were injected with Wnt8/Chordin, Sox2 and Sox3 morpholinos and rescue with (A) Sox2, Sox9 or (B) Sox3, Sox10 mRNA

progress from early cleavage and blastula stages, a time in development when populations of pluripotent cells are present, to neurula stages when significant lineage restriction has occurred, and definitive neural crest cells are present. The SoxB1 factors, *Sox2* and *Sox3,* are highly expressed in early pluripotent cells, but become restricted to the medial neural plate following gastrulation as animal pole cells become progressively lineage restricted (Figure 3.1 top panel). By contrast, expression of the SoxE factors *Sox9* and *Sox10* is absent from naïve blastula cells but becomes up-regulated at the neural plate border (NPB) by late gastrula stages, making the definitive neural crest stem cell population (Figure 3.1 bottom panel). By early neurula stages, the expression domains of SoxB1 factors and SoxE factors have become mutually exclusive.

This is likely due, at least in part, to the repressive activity of *Snail2* on *Sox2* expression (Acloque et al., 2011). The sequential deployment of different sub-families of Sox factors is reminiscent of what has been observed for Fox family transcription factors (Charney et al., 2017; Xu et al., 2009), suggesting that the SoxB1 to SoxE transition could serve as a paradigm for understanding the temporal, and sequential utilization of related transcription factors during development. Furthermore, these findings suggest that with respect to the evolution of neural crest, the co-option of SoxE factors into the GRN represents one of the true novelties that correlated with or drove the acquisition of this cell type at the base of the vertebrates.

Why might it be important to deploy distinct sub-families of Sox transcription factors to maintain pluripotency in neural crest cells versus naïve blastula cells? One clue may be derived from the cells types that deploy SoxB1 and SoxE factors during later events in

embryogenesis. By neural plate stages, the expression of SoxB1 factors becomes restricted to neural progenitor cells where they play an essential role in maintaining the neural progenitor state (Bergsland et al., 2011). Neural crest cells retain their broad developmental potential through neurulation, and the onset of migration (Light et al., 2011), before ultimately differentiating and contributing to a broad set of derivative cell types (Taylor and LaBonne, 2007; Prasad et al., 2012). At these later stages, SoxE factors function to promote the formation of a subset of neural crest derivatives. For example, Sox9 plays a key role in the formation of cartilage, whereas Sox10 is essential for the formation of melanocytes and glia (Akiyama et al., 2002; Aoki et al., 2003; Britsch et al., 2001, Lee et al., 2011; Taylor and LaBonne, 2005). The restricted expression of SoxB1 versus SoxE factors suggests that as cells exit from pluripotency, SoxB1 function might be better suited to promote the establishment of a neural progenitor state, whereas SoxE function better poises cells to adopt non-neuronal neural crest states such as cartilage, glia, and melanocytes. Consistent with important sub-functionalization, I find that SoxE factors promote the neural crest cell state whereas SoxB1 factors inhibit the formation of neural crest cells (Figure 3.8 A-B, and 3.27).

Interestingly, during neural differentiation of embryonic stem cell cultures, SoxB1 factors take part in an additional relay event (Wegner, 2011). Following SoxB1-mediated maintenance of the neural progenitor state, SoxC family transcription factors (Sox4/11/12) function to promote neural differentiation (Bergsland et al., 2006; Bergsland et al., 2011; Hoser et al., 2008). It has been proposed that in this context Sox2 functions as a pioneer factor to both activate pluripotency genes and poises neural precursor genes. As cells

assume a neuronal progenitor state, Sox3 replaces Sox2 occupancy of neural progenitor genes and poises neuronal differentiation genes for later expression. Finally, during neuronal differentiation, the SoxC protein, Sox11, replaces Sox2/3 and promotes expression of neural differentiation targets, including *Lhx2*, *Pax2*, and *Tubb3* (Bergsland et al., 2011). Our results show that the SoxB1 factors, Sox2 and Sox3, can rescue chordin-mediated neural induction to a much greater degree than can Sox9 and Sox10 (Fig.ure 3.24, and 3.25). By contrast, SoxE factors can rescue Wnt/chordin-mediated neural crest induction more potently than can SoxB1 factors (Figure 3.29, and 3.30). I hypothesize that SoxB1 and SoxE factors can occupy an overlapping set of regulatory elements on target genes, and may assemble distinct regulatory complexes in some contexts. Precedence for this is found in the regulation of neural crest derived oligodendrocytes, where both Sox10 and Sox2 can bind the regulatory elements for myelin binding protein, but with different transcriptional outputs (Hoffmann et al., 2014). Going forward it will be important to examine the dynamics of SoxB1 and SoxE protein occupancy across the genome as cells progress from a naïve blastula to a neuronal progenitor or neural crest state.

Sox factors are a highly versatile family of transcription factors that play multiple reiterative roles at different stages during development (Akiyama et al., 2002; Kim et al., 2003; Sarkar and Hochedlinger, 2013). Post-translational modifications can further contribute to the functional versatility of these factors, for example, SoxE proteins can function as activators or repressors depending upon whether they have been modified by Sumoylation (Lee et al., 2012; Taylor and LaBonne 2005). SUMOylated SoxE factors recruit transcriptional repressors to inhibit genes important for melanogenesis, including *Dct* (Lee

et al., 2012). SoxE function can also be tuned by context dependent interactions with the SoxD factor, Sox5, which enhances SoxE-mediated activation of cartilage genes such as *Col2a1*, but inhibits SoxE mediated activation of melanocyte and glial specific genes (Lefebvre et al., 1998; Nordin & LaBonne, 2014; Stolt et al., 2006; Stolt et al., 2008). Thus, multiple distinct mechanisms could contribute to tuning SoxB1 function for pluripotency and neuronal progenitor functions, and SoxE factors for neural crest progenitors and glial, melanocyte and cartilage fates.

Consistent with other studies, I found that levels of Sox proteins expressed were an important determinant of their function. In mouse and human ES cells, either increased or decreased Sox2 expression leads to loss of pluripotency (Kopp et al., 2008; Boer et al., 2007; Yamaguchi et al., 2011; Takahashi &Yamanaka, 2006; Thomson et al, 2011). Similarly, I found that increased levels of Sox2 or Sox3 could inhibit gene expression in pluripotent blastula cells, although these cells appeared more sensitive to increases in SoxE expression (Figure 3.2 A-B). Interestingly, whereas SoxB1 factors inhibited the expression of neural crest markers and SoxE factors promoted this expression, both inhibited expression of *epidermal keratin* (Figure 3.10). There remains much to be learned about the distinct and overlapping regulatory activities of these two classes of Sox Factors, and how their differential deployment contributes to pluripotency and lineage restriction decisions in the early embryo.

Understanding the mechanisms underlying the evolution of gene regulatory networks is a subject of great interest and importance. Evolution of these networks has been facilitated by gene and genome duplications that increased the number and type of network

components that could be deployed. The Sox superfamily has proven particularly 'evolutionarily pliable,' having undergone multiple rounds of duplication, divergence, sub-functionalization and neo-functionalization (Guth and Wegner, 2008; Heenan et al., 2016; Tai et al., 2016). In contrast to most neural crest potency factors (including *Snail1*, *Myc*, *Foxd3*, *Ets1*, *Ap2*, and *Vent2*), *Sox9* and *Sox10* are not first expressed in pluripotent naïve blastula cells. SoxE expression commences in definitive neural crest cells at the neural plate border only once SoxB1 factors have become restricted to the neural progenitor pool. Our findings suggest a model in which the essential role of SoxB1 in regulating the blastula stem cell state is handed off to SoxE factors which function in part to retain the developmental potential of the neural crest. I hypothesize that this switch in Sox factor deployment facilitates the subsequent lineage restriction of neural crest cells to non-neural cell types including cartilage, melanocytes, and glia (Figure 3.31).

Material and Methods

Embryological methods.

Collection, manipulation and *in situ* hybridization of *Xenopus* embryos were performed as previously described (Bellmeyer et al. 2003) using digoxigenin- labeled RNA probes detected with BM Purple AP Substrate (Roche). mRNA for microinjection was synthesized in vitro from can occupy the same target genes that *Sox2* occupied along with core pluripotency factors when they are regulating stem cell maintenance. linearized plasmid templates using the SP6 Message Machine kit (Ambion). β-galactosidase mRNA was co-injected as lineage tracer and was detected with Red-Gal substrate (Research Organics). All results shown are representative of at least three biological replicates of independent

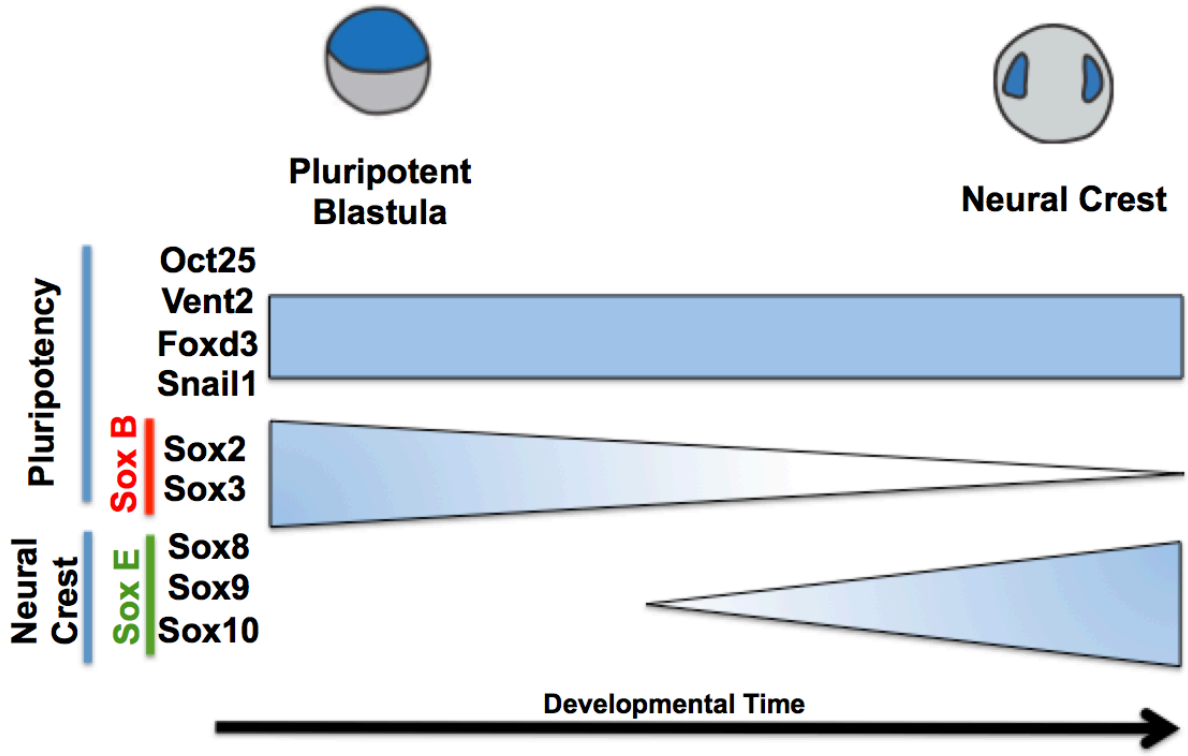


Figure 3.31 Model of transcription factor functional replacement to maintain pluripotency in neural crest cells

Pluripotency factors and neural crest factors (*Oct25*, *Vent2, FoxD3,* and *Snail*1) are present in blastula animal pole cells and resolve into the neural crest progenitor cell population. In contrast, Sox2 and Sox3 transcription factors are present at early stages of development and their expression is restricted to the progenitor neural progenitor population from mid-gastrula to neurula stages. In contrast, Sox8 and Sox9 start to be expressed at late gastrula stages and later during early neurula stage SoxE factors are enriched in the neural crest cell population where they play important roles as regulators of cell potency.

experiments. Explants of naïve ectoderm were manually dissected from the animal pole of blastula (stage 8-9) embryos previously injected at two-cell stage with the indicated mRNA or morpholino. Explants were cultured at room temperature in 1X MMR on agar coated dishes until indicated stage, and fixed in formaldehyde for 40 minutes before being processed for *in situ* hybridization.

Activin treatment on animal caps

For Activin treatment of animal pole explants explants of naïve ectoderm were manually dissected from animal pole of blastula (stage 8-9) embryos previously injected with mRNA or morpholino. Injected explants were culture in 1X MMR. Activin prepared from R&D Systems Human/Mouse/Rat Activin A Recombinant Protein concentrated to an effective stock concentration of 20 µg/ml. Explants were treated with Activin at the stage indicated in 1X MMR supplemented with 0.1% BSA as carrier. Concentrations of 20ng/µl or 200 ng/µl were used to induce mesoderm or endoderm respectively. Explants were cultured at room temperature in 1X MMR on agar coateddishes until the indicated stage, samples were fixed in formaldehyde for 40 minutes before being processed of *in situ* hybridization.

Dexamethasone treatment of whole embryos.

Embryos were injected with Sox2-GR, Sox3-GR, Sox9-GR, Sox10-GR at two cell stage and cultured in 0.1X MMR. A solution of 10ml of 0.1X MMR with 10µl Dexamethasone (Sigma) was applied at stage 10 to induce and bypass blastula stages. Embryos were cultured until stage 15 (neurula) and, fixed in formaldehyde 1 hour before being processed

for *in situ* hybridization.

Western blot analysis.

For western blot tag versions of Sox2, Sox3, Sox9 and Sox10 and deletion mutants of Sox9 and Sox10 were injected, 5 embryos were collected at stage 10 and lyse in lysis buffer (PBS +1% NP-40) supplemented with a protease inhibitor cocktail (Roche), phenylmethylsulfonyl fluoride, aprotinin, leupeptin, N-ethylmaleimide and, idoacetamide. Proteins were detected using a primary antibody against epitope tag: Myc 1:3000 (9E10, Santa Cruz Biotechnology), and a secondary antibody conjugated to Horseradish peroxidase (HRP) and detected by chemiluminescence (GE Healthcare).

DNA constructs

Morpholino antisense oligonucleotides against the 5'UTR and coding region of *Xenopus* Sox2 (5'-GCGGAGCTACATGTCGTACTACCTC-3'), Sox3 ACTTCGAGGTTTACATATCGTACAA-3') were previously described (Schlosser, G., et al., 2008). Embryos were injected with 5ng of each morpholino to inhibit the Sox2 and Sox3 expression. Morpholino targeting Sox10 (AGCTTTGGTCATCACTCATGGTGCC- 3') was described (Taylor, K and LaBonne, C. 2005) 10ng of Sox10 Morpholino was injected. For rescue experiments, mRNA epitope tagged forms of Sox2, Sox3, Sox9, and Sox10 was co-injected with mRNA encoding lineage tracer β-gal. Animal caps were dissected and cultured to the indicated stage and fixed for 40 minutes in formaldehyde for *in situ* hybridization

Chapter 4

General discussion

Neural crest cells are a multipotent stem cell-like population unique to vertebrates that gives rise a wide array of cell types in the organism. These cells are distinguished from others by their broad potential and stem cell-like attributes past the time when other cells in the embryo become lineage restricted. During early vertebrate embryogenesis, cells maintain a pluripotent state due to a balance in the expression of a set of pluripotency factors. It is believed that initial differences in the spatial distribution of these factors are fate determinants and ultimately underlie key developmental decisions such as the first steps of the transit from an undifferentiated to a differentiated state. During the process of differentiation, numerous cell types with distinct functions and morphologies arise due to the increased spatial and cell heterogeneity over time. Vertebrate embryos are highly complex, many distinct cell types appear due to molecular and evolutionary changes. Neural crest cells are one of the major innovations of vertebrate chordates, because they display multi germ layer developmental potential, they have been referred to as the fourth germ layer (Hall, 2009).

For 150 years, neural crest research has been focused on understanding the unique multilayer properties of neural crest cells. Maps of gene regulatory networks that control different aspects of neural crest cell biology have been built based on a wide variety of experimental systems and strategies performed in diverse vertebrate taxa and (Ben Steventon et al., 2009; LaBonne and Bronner-Fraser, 1998a; Martik and Bronner, 2017; Prasad et al., 2012; Sauka-Spengler and Bronner-Fraser, 2008). While these maps have been important to understand neural crest development, the developmental and evolutionary origins of neural crest cells remained poorly understood. This thesis proposes a novel hypothesis for the origin of neural crest cells, and advances our understanding of the mechanism underlying the potency of these cells.

Traditional models of neural crest formation attribute the multi germ layer potential of neural crest cells to an "induction" event that occurs at mid-gastrula stages (Bae and Saint-Jeannet, 2014; LaBonne and Bronner-Fraser, 1998a; LaBonne and Bronner-Fraser, 1998b; Prasad et al., 2012; Sauka-Spengler and Bronner-Fraser, 2008). In this framework, neural crest cells multipotency is gained after an initial restriction of potential. The new neural crest cells then can originate multiple cell fates across germ layers past the time when neighboring cells in the early embryo are restricted. The "induction" first involves generating a broad zone of competence in a region of the early ectoderm that is called the "neural plate border." This zone represents an increase in developmental potential or "stemness." At late gastrula stages, signaling events involving BMP, Wnt, FGF and, Notch are orchestrated to induce the GRN that describes neural crest cells (Martik and Bronner, 2017; Prasad et al., 2012; Sauka-Spengler and Bronner-Fraser, 2008; Simoes-Costa and Bronner, 2015). The response to these signaling events is the induction of a cohort of genes that confers identity of neural crest cells.

A new theory for the origin of neural crest cells, and the diversity of the vertebrates

Evidence suggesting that similar transcriptional networks regulate neural crest cells and embryonic stem cells potency are presented in this thesis. Recent research has found that neural crest induction might occur earlier than what was documented; instead of being a process that takes place at neurula stages, it might occur during gastrulation (Basch et al., 2006). Specifically it has been proposed that a region of the chicken epiblast is specified to form neural crest cells, as evidenced by the early expression of the neural crest transcription factor *Pax7* that is known to contribute during neural crest formation and migration. Further, *Pax7* is required for the

formation of the neural crest. Loss of *Pax7* function inhibits the expression of inducing factors *Snail2*, *Sox9*, *Sox10* and, *HNK*-1, suggesting that neural crest induction happens earlier than previously thought (Basch et al., 2006).

I found that as the neural crest forms, expression *Foxd3, Myc, Id3, TF-Ap2, Vent2, Ets1, Snail1,* and *Oct25* is shared at the neural plate border regions (Buitrago-Delgado et al., 2015). Surprisingly, the same core neural crest and pluripotency genes are expressed at blastula stages. These findings led me to hypothesize that these "pluripotency factors" work in synergy to maintain the cells in a naïve state by retaining the molecular underpinnings that control pluripotency, in contrast with the classical view in which ectodermal tissue acquire multipotency during early neurulation. This new model is based on the co-expression of some of the pluripotency factors (*Oct60*, *Vent2* and, *Sox2*) and "neural crest factors" (such as *Snail1*, *Sox5*, *Id3*, *Ap2*, *FoxD3*, *Myc* and, *Ets1*). In addition, I demonstrated that pluripotent blastula cells require the function of neural crest regulatory factors indicating that these factors are required for the pluripotency of blastula and neural crest cells.

Seminal work demonstrates that neural crest cells could share multiple characteristics with embryonic stem cells, including its capacity to self-renew (Anderson, 1993; LaBonne and Bronner-Fraser, 1998b; Mundell and Labosky, 2011; Stemple and Anderson, 1992; Trentin et al., 2004). Interestingly, neural crest cells precursors seem to have multiple stem cell phenotypes along the derived lineages in which a class of pluripotent neural crest stem cells controls the ability to derive a diversity of cell types from neural crest cells (Trentin et al., 2004). Supporting this idea, recent findings using multiplex single-cell resolution in situ hybridization describe

neural crest cells as an heterogeneous population with multiple developmental stages, specifically demonstrating expression of core pluripotency factors (*Oct4*, *Sox3/2* and, *Nanog*) in pre-migratory neural crest cells further confirming their role in regulating pluripotency at neural crest stages (Lignell et al., 2017).

A set of neural crest factors, important for the induction of multipotency of neural crest cells were previously reported. Some of these factors include *FoxD3*, *Ap2*, *Id3* and *Myc* (Mundell and Labosky, 2011; Sasai et al., 2001; Teng et al., 2008),(Light et al., 2005; Ying et al., 2003). The new wider shared regulatory program that was found to be essential for the pluripotency of neural crest and blastula stem cells, led me to hypothesize that it was retention of potential from the earlier blastula cells that conferred on neural crest cells their special attributes. To test this hypothesis, I used an elegant gain of function experiment. I utilized explants from pluripotent blastula stem cells of an embryo. These explants are pluripotent at early stages of development and can be induce to form any embryonic germ layer. The pluripotency of these explants is transient, as time pass, explants lose pluripotency and become lineage restricted; by default they will adopt epidermal fates in a time window that can be compared to neurula stages in a developing embryo. It has been described that these explant can also be induce to form neural crest state (LaBonne and Bronner-Fraser, 1998a, Sato et al., 2005). I therefore induce neural crest state in explants by injecting a concentration of *Pax3* and *Zic1*. Blastula explants were cultured in isolation until neurula stages (stage 13) when fate restrictions are made, at this time different concentrations of activin were added to induce either mesodermal or endodermal fates. Using this gain of function experiment I observed that neural crest induced explants remained competent to form derivatives from the three germ layers. These experiments let me demonstrate

that neural crest cells, in fact retain potential and that they are competent to form not only mesoderm and ectoderm, but also endoderm, retaining a wider potential that could be compared with the potential seen in embryonic stem cells.

Results presented and discussed here let me propose that the network that controls pluripotency in the neural crest cells and in the pluripotent stem cells, has many more elements than we previously realized and that this network can be reused in different developmental processes to control pluripotency. Additional studies are necessary to compare the ability of neural crest cells to form different derivatives. The findings in this thesis represent a paradigm shift to explain the origin of the pluripotency of neural crest cells. Instead a model that involves a reversal trajectory of the Waddington's landscape, I propose that retention of developmental potential is the mechanism that neural crest cells used to maintain pluripotency. Based on the evidence presented here, a new and more parsimonious model is presented: neural crest cells retain potential that persists from their blastula ancestors as an example of a cellular neoteny, in which a select group of cells with pluripotent potential characteristic of the blastula state persist to neurula stages where they can be induced to form the highly diverse lineages that derive from the neural crest.

These discoveries open new questions about how this retention of potential is achieved. One of the major differences that we found in the regulatory factors expressed in pluripotent blastula cells and neural crest cells is the deployment of different subfamilies of Sox transcription factors. While SoxB1 factors are known to play central roles in regulating pluripotency of blastula and embryonic stem cells. SoxE transcription factors are unique to neural crest cells and regulate multiple aspects pre-migratory, migratory and differentiation stages of neural crest development.

Given these differences, I explored the role of SoxB1 and SoxE factors in a molecular hands-off activity that could explain how the pluripotency of neural crest cells is maintained. I hypothesize that Sox factors might transiently replace SoxB1 factors in the control of pluripotency in neural crest cells, and the poise these cells to contribute to glial, chondrogenic and melanocyte linages at stages when SoxB1 factors promote neuronal progenitor formation.

The Sox transcription factors and the progression from pluripotent blastula cells to neural crest

SoxB1 and SoxE transcription factors are differentially expressed during development of *Xenopus* embryos. At blastula stages of development SoxB1 factors (*Sox2* and *Sox3*) are strongly expressed in the presumptive ectoderm in which they play functions to keep poised genes that are fundamental for differentiation and the maintenance of pluripotency (Niwa, 2007), (Avilion et al., 2003). Later in development, at neurula stages, SoxB1 gene expression is restricted to the neural-ectoderm, SoxB1 factors then become essential for the induction of regulatory programs that control the development of the early central nervous system and placodal development (Sarkar and Hochedlinger, 2013), (Guth and Wegner, 2008).

On the other hand, SoxE transcription factors (*Sox8*, *Sox9* and, *Sox10*) expression is restricted to neural crest cells and starts to be noticeable at mid-gastrula stages persisting until adulthood. SoxE factors are transcriptional regulators essential for both maintenances of pluripotency, and differentiation of neural crest cells into a subset of derivatives (Cheung and Briscoe, 2003; Haldin and LaBonne, 2009), (Kim et al., 2003), (Hong and Saint-Jeannet, 2005). After migration

of neural crest cells, SoxE proteins are essential to drive cell fate decisions. It has been shown that *Sox9* is essential the epithelial to mesenchymal transition (EMT) and subsequent chondrocyte and cartilage formation (Akiyama et al., 2002), (Haldin and LaBonne, 2009). Interestingly, *Sox10* is relevant in the development of cranial neural crest cells and is mainly important to maintain a proliferative and multipotent state of neural crest derived cells during pre-migration steps (Kim et al., 2003). After migration, *Sox10* will become essential for melanocyte formation (Aoki et al., 2003) and later will be important for the differentiation of glial cells (Britsch et al., 2001).

Testing for functional similarities and differences between SoxB1 and SoxE transcription factors, I observed that these two groups of proteins could both regulate pluripotency at blastula stages of development. Intriguingly, I observed differences in the down-regulation of pluripotency factors such as *Vent2*, *Oct25*, *Ap2,* and *Id3*. Suggesting that although SoxE factors are not endogenously expressed at blastula stages, they have the potential to regulate expression of pluripotency factors upon overexpression.

Using functional experiments I compared the abilities of SoxB1 and SoxE factors to regulate ectodermal fates at neurula stages. I found that SoxB1 and SoxE factors differentially regulate neural crest fates. Overexpressing SoxB1 factors inhibited the expression of *Snail2* or *FoxD3*, by contrast, SoxE factors enhance the expression of neural crest markers. These results demonstrate distinct functions at neurula stages where SoxE factors play roles in promoting neural crest cells while SoxB1 repress it. Interestingly, evaluating epidermal derivatives by looking at the expression of the epidermal marker *Epidermal keratin* I found that both SoxB1 and SoxE factors

down-regulate its expression, suggesting that in this context SoxB1 and SoxE factors have similar activities regulating epidermal fates. There is much to be learned about the differential and similar activities of these two classes of Sox transcription factors and how their functions in different contexts lead perhaps to the regulation of similar enhancers at different times during development, that allows them to play similar functions at different stages of development. It will be interesting to follow these phenotypes over time, and evaluate the differential ability of embryos to develop cartilage/bone, glial cells, and melanocytes in organogenesis stages.

Since overexpressing either SoxB1 or SoxE factors inhibited the expression of *Oct25*, *Vent2*, *Id3*, and *TF-Ap2* in pluripotent blastula cells, I hypothesized that SoxE factors might regulate pluripotency. In order to determine whether SoxB1 and SoxE factors play a role in regulating pluripotency, I use gain and loss of function experiments in explants. Using Animal pole explants, I demonstrated that SoxE and SoxB1 factors levels are critical for blastula animal pole cells to remain competent to form endoderm or mesoderm upon activin signaling. Explants overexpressed with either SoxB1 or SoxE factors failed to possess the full competency to induce mesoderm or endoderm markers upon induction of activin.

I can speculate that the levels in which these factors are present either in blastula stages (SoxB1) or neural crest stages (SoxE) are critical to play functions as regulators of pluripotency and that SoxB1 and SoxE factors role is to maintain cells in a precursor state. To test whether both SoxB1 and SoxE factors can rescue pluripotency, I used translation-blocking morpholino for both Sox2 and Sox3. Cells depleted for these B1 factors are no longer competent to form mesoderm or endoderm. Surprisingly, this loss of competency can be rescued by expression of SoxB1 factors

but only partially rescued by SoxE factors. Suggesting that these two families of Sox transcription factors could play similar roles regulating pluripotency but that they do so at different times and contexts during development. These findings support the hypothesis of a relay model in which SoxB1 factors regulate pluripotency at early stages of development pass the functions to SoxE factors regulate pluripotency at neurula stages specifically in neural crest cells.

I hypothesized that there might be a hand off of Sox activity from SoxB1 to SoxE factors; To test this hypothesis I performed functional replacement experiments in explants. I examined the ability of pluripotent cells to form neural tissue in response to *chordin*, using translation-blocking morpholinos against Sox2 and Sox3, inhibits induction of neural markers. Adding back to the system mRNA of *Sox2* and *Sox3* can recue the loss of neural induction in explants, however *Sox9* or *Sox10* only partially do demonstrating that in this context there are differences in the activities of soxB1 and SoxE factors. In a similar way I induced neural crest tissue in response to *Snail2* and Wnt using translation-blocking morpholinos against Sox10, inhibits the induction of neural crest markers. Adding back to the system mRNA of *Sox9* and *Sox10* I fully rescued the loss of neural crest induction in explants but Sox2 and Sox2 are able to only partially rescue neural crest markers.

Results from these experiments support the hypothesis that SoxE factors replace the function of SoxB1 factors to maintain pluripotency in neural crest cells, and that this event happens during the transition from late blastula to early gastrula stage. This differential activity demonstrates that pluripotency is maintained over time due to a relay of functions from SoxB1 to SoxE factors,

demonstrating a molecular mechanism that helps to explain the remarkable ability that neural crest cells have to remain pluripotent.

Similar mechanisms have been reported for SoxB1 and SoxC factors; for example Sox11 replaces the functions of Sox2/3 during neuronal development, and promotes expression of neural differentiation targets (Bergsland et al, 2011). It will be interesting to characterize in detail how this functional replacement occurs by looking at the potential targets that both of the groups of proteins can share. A ChiP-Seq could be used to determine if SoxB1 and SoxE factors bind to similar promoters at different times in development to determine whether or not they can regulate the similar target genes, and will clarify whether differential binding activity during development is the mechanisms that these proteins are using to switch their functions.

Together these findings demonstrate that while SoxB1 and SoxE factors have some shared activities, there are also functional differences between these factors that may begin to explain why utilization of these factors changes as cells progress from a pluripotent blastula state to a neural crest state. The most striking examples of these differences are that SoxB1 factors, which are essential in neuronal progenitor cells, have more potent neural inducing activity, whereas SoxE factor activity is more compatible with establishing a neural crest state. Important next steps will be to examine the ability to form neural crest derived fates such as cartilage or melanocytes when Sox E factors are replaced by SoxB1 factors.

Final considerations

In this thesis, I propose a new model of neural crest formation in which a small population of cells that retain pluripotency from early stages of development and become the neural crest. I hypothesize that the neotenic retention of the pluripotency program in neural crest cells is linked to a dramatic expansion of the number of adult cell fates and to a greater tissue complexity present in chordate vertebrates.

Why did the evolution of the vertebrate body plan involve the creation of a complex mechanism in which cells first retain their pluripotency for a prolonged amount of time and then migrate to a multitude of locations in the body? It could be possible that combination of signals and transcription factors needed for the formation of different cell types that arise from neural crest cells are not present in the timing when pluripotent stem cells start to differentiate into the three germ layers. It is possible neural crest cells retain its potential until the unique combinatorial signaling input needed to originate the diverse neural crest derivatives is activated neurula stages. To this end, coupling a delay in the relative timing of differentiation with a subsequent cell migration of the population that differentiated last, which now migrates in a more complex signaling environment, could be a general mechanism that has expanded phenotypic complexity during animal evolution.

In this framework, major remaining questions for future research involves identifying in a much larger scale where, when and how variation in the molecular and cellular programs that are directed by neural crest cells leads to differentiation. Addressing this question has important implications for understanding both evolution and the molecular mechanisms that drive neural crest development. Further investigation is needed to test the hypothesis of retention of potential

rigorously. Single-cell RNA-seq and RNA velocity at different stages of development will be necessary not only to know which other transcripts are present from blastula to neural crest stages but also to reconstruct developmental trajectories that define neural crest cells from pluripotent stem cells. Likewise, a more multi-dimensional strategy that accounts effects of changes in levels and spatial distribution of gene expression over developmental time are needed, additional approaches that incorporate genome-wide differences in the regulation of pluripotency of embryonic stem cells and neural crest cells could have a high potential to elucidate this issues. Lastly, detailed studies to understand the role of changes in chromatin state or post-translational modifications will provide valuable information to understand the mechanism underlying the retention of pluripotency.

The retention of the potential of neural crest cells provides a novel conceptual framework in the field to explain the remarkable differentiation potential that will have a high impact of how we interpret vertebrate evolution and ultimately will have an impact in regenerative medicine, and raises the possibility that much more remains to be discovered in embryonic development.

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