Posteriorizing factor *Gbx2* is a direct target of Wnt signalling during neural crest induction

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I, Bo Li, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed…… Date……

Abstract

Wnt signalling is required for neural crest induction; however the direct targets of the Wnt pathway during neural crest induction remain unknown. I show here that the homeobox gene *Gbx2* is essential in this process and is directly activated by Wnt/β-catenin signalling. *Gbx2* has previously been implicated in posteriorization of the neural plate. Here I unveil a new role for this gene in neural fold patterning. Loss of function experiments using antisense morpholinos against *Gbx2* inhibit neural crest and expand the preplacodal domain, while *Gbx2* over expression leads to transformation of the preplacodal domain into neural crest cells. I show that the neural crest specifier activity of *Gbx2* is dependent on the interaction with *Zic1* and the inhibition of preplacodal genes such as *Six1*. In addition, I demonstrate that *Gbx2* is upstream of the neural fold specifiers *Pax3* and *Msx1*. My results place *Gbx2* upstream of the neural crest genetic cascade being directly regulated by the inductive molecules, and support the notion that posteriorization of the neural folds is an essential step in neural crest specification. I propose a new genetic cascade that operates in the distinction between preplacodal and neural crest territories.

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List of Abbreviation

BMP: Bone morphogenetic protein

DD1: Dsh dominant-negative

Dkk1: Dickkopf1

DMSO: Dimethyl sulfoxide

Dsh: Dishevelled

FDX: Fluorescein dextran

FGF: Fibroblast growth factor

Gbx2: Gastrulation brain homeobox 2

GFP: Green fluorescent protein

NC: Neural crest

MO: Morpholino

PFA: Paraformaldehyde

PPR: Preplacode

RA: Retinoic acid

RDX: Rhodamine dextran

Chapter One: Introduction

1.1 Neural crest

The neural crest is an embryonic cell population that arises at the neural plate border. As the neural plate folds over itself to form the neural tube, border regions of neural fold from opposite sides of the ectoderm come together and later fuse. Neural crest progenitors come to lie in or adjacent to the dorsal neural tube and later leave the neural tube after its closure to migrate through the body where they differentiate into a huge variety of cell types (Ledouarin and Kalcheim, 1999; Mancilla and Mayor, 1996; Marchant et al., 1998; Selleck and Bronner-Fraser, 1996; Theveneau and Mayor, 2012).

The neural crest was first described in the chick embryos by Wilhelm His (His, 1868) and it was referred to as the fourth germ layer because of its importance for vertebrate development and its ability to differentiate into many types of tissues.

Hörstadius was the first to recognize the neural crest as a remarkable embryonic structure. In his experiments, neural folds containing neural crest precursors were transplanted from one axial level of the head to

another resulting in the development of abnormal jaw and branchial cartilage. However, the skeletal structures produced by the progeny of the grafted neural crest cells closely resembled those that would have formed in their original location. This result suggested that the grafted neural fold were already specified as neural crest at the time of the transplant (Hörstadius, 1950).

In 1963, Weston demonstrated the ability of trunk neural crest cells to migrate and differentiate into melanoblasts and spinal ganglia by labelling the nucleus using radioisotope incorporation of nucleotides (Weston, 1963). However this was a transient method of cell labelling.

In 1970s, the limitation to label the neural crest cells was overcome upon introduction of the quail-chick chimaera, which was used to test the degree of determination of neural crest cells and their derivatives and to demonstrate the precise periodicity of the colonization of the primary lymphoid organ rudiments. It provided a stable tracing and allowed the study of the migration and fate of neural crest progenitors (Le Douarin, 1975).

Neural crest cells can be classified into two broad populations; the cephalic (or cranial) crest of the head and the trunk neural crest. The onset of neural crest migration proceeds in an anterior to posterior

fashion with the cephalic neural crest beginning their migration earliest. Cephalic neural crest cells migrate into the branchial arches in three distinct streams, termed the mandibular, hyoid and branchial streams, and ultimately differentiate to form the skeleton and cartilage of the head (Kontges and Lumsden, 1996). In addition, some cells from the mandibular stream also contribute to the cranial sensory ganglia and the cornea (Sadaghiani and Thiebaud, 1987). Trunk neural crest cells in most animals migrate along two distinct pathways. Some cells take a ventral pathway between the neural tube and the somites. These give rise to sensory and sympathetic ganglia, Schwann cells and Cromaffin cells (Le Douarin and Teillet, 1973). Others choose a lateral pathway between the somites and the ectoderm, eventually colonising the ectoderm to become the pigmented melanocytes (Ledouarin and Kalcheim, 1999). The majority of cells follow the ventral pathway, which can be recognised as streams of migrating cells in a segmented pattern through the somitic mesoderm (Bronner-Fraser, 1986; Rickmann et al., 1985; Teillet et al., 1987). One of the most notable features of neural crest cells is their ability to differentiate into such a wide range of tissues. Although derived from the ectodermal germ layer, neural crest cells are able to generate both typically ectodermal cells such as neurons as well as mesenchymal tissues like cartilage and muscle. This suggests a high level of pluripotency and it has been suggested that neural crest cells maintain a relatively undifferentiated stem cell-like

state for longer than cells in other tissues (Delfino-Machin et al., 2007; Knecht and Bronner-Fraser, 2002), see summary in Figure 1.1 (Knecht and Bronner-Fraser, 2002).

The neural crest has been considered an evolutionary innovation of vertebrates. Because of its contribution to the peripheral nervous system, the paired sensory organs and the craniofacial skeleton, the evolutionary introduction of neural crest at the dawn of vertebrates is thought to have been a driving force in the expansion of vertebrates, by facilitating a predatory lifestyle (Gans and Northcutt, 1983; Gostling and Shimeld, 2003; Northcutt and Gans, 1983). However, no migrating neural crest cells have been discovered in amphioxus (Holland and Holland, 2001; Meulemans and Bronner-Fraser, 2004), some cells with neural crest like properties such as the ability to migrate and form pigment cells, have been reported in the ascidians tunicate Ecteinascidia turbinate (Jeffery et al., 2004). Thus understanding the evolution of definitive neural crest cells is critical to understanding vertebrate origins; investigating the mechanisms of neural crest induction, migration, and differentiation is essential for understanding the normal development of the vertebrates.

Furthermore, defects in the processes of neural crest development result in a wide range of human diseases (Fuchs and Sommer, 2007).

For example, Waardenburg syndrome, which is a genetic disorder characterized by defects in structures arising from the neural crest (Bondurand et al., 2000) and Hirschsprung disease which is a failure of neural crest cell migration, results in the absence of peristaltic movement in the bowel (Iwashita et al., 2003). In addition, a number of identified molecular mediators of neural crest development appear to be misregulated in human cancers and a number of cancers are neural crest-derived, including neuroblastomas, melanomas and gliomas (Nakagawara and Ohira, 2004; Rosivatz et al., 2002; Rothhammer et al., 2004). Thus, by investigating the mechanisms of those factors that regulate normal development of neural crest cells, we will also gain important insight into their related roles in human diseases (Heeg-Truesdell and LaBonne, 2004).

1.2 Neural crest induction

As previously discussed, neural crest progenitors are induced at the border between the neural plate and the epidermis at the late gastrula stage. Later on, neural crest cells leave the neural tube to migrate and differentiate. This cell population is induced by signals produced by neural plate, epidermis and underlying mesoderm (Bonstein et al., 1998; Ledouarin and Kalcheim, 1999; Mancilla and Mayor, 1996; Marchant et al., 1998; Monsoro-Burq et al., 2003; Raven and Kloos, 1945; Selleck and Bronner-Fraser, 1996).

Development of the neural crest is a complex process, which requires the transformation from undifferentiated ectoderm into neural crest and occurs alongside neural induction (Ledouarin and Kalcheim, 1999; Mancilla and Mayor, 1996; Marchant et al., 1998; Monsoro-Burq et al., 2003). It has been suggested that the anterior-posterior axis of the neural plate is specified in a two step process (Nieuwkoop, 1952). First, neural tissue is induced as anterior neural plate, and second, this anterior neural plate is posteriorized by signals coming from the posterior end of the embryo generating the anterior-posterior differences of the neural plate (Nieuwkoop, 1952). It has also been proposed a three step signal model, in which an anterior source of “anteriorizing” signals is also involved (Stern, 2001). These models

have received support from molecular data in which the first anterior neural inducer signal has been proposed to be an inhibitor of BMP, while Wnt, FGF and retinoic acid (RA) have been proposed as the second posteriorizing factors (Gamse and Sive, 2000). It has been shown that for example specific midbrain and hindbrain markers can be induced by a combination of BMP inhibitors and Wnt signalling (Kiecker and Niehrs, 2001). The same combination of signals is involved in neural crest induction. Interestingly, only the medial-posterior, but not the anterior, neural folds give rise to neural crest cells, and the neural crest are induced by a combination of BMP inhibitors and posteriorizing factors, such as Wnt, FGF, RA (Bonstein et al., 1998; Ledouarin and Kalcheim, 1999; Mancilla and Mayor, 1996; Marchant et al., 1998; Monsoro-Burq et al., 2003; Raven and Kloos, 1945; Selleck and Bronner-Fraser, 1996). In fact, it has been proposed that the neural crest are induced by an initial induction of anterior neural fold, dependent on BMP signals, followed by a subsequent posteriorization of the anterior neural fold into neural crest, dependent on Wnt, FGF and RA signals (Papalopulu and Kintner, 1996; Villanueva et al., 2002). It has been reported that anterior neural fold that does not express neural crest markers can be transformed into neural crest by the activation of Wnt, FGF or RA (Carmona-Fontaine et al., 2007; Villanueva et al., 2002). The role of posteriorization in neural crest induction has been recently challenged. It has been shown that neural crest induction by

Wnt signalling is independent on the posteriorizing activity of Wnt as induction of neural crest by activation of Wnt does not affect the anterior-posterior axis of the neural plate (Wu et al., 2005).

1.3 BMP signalling

Prior to gastrulation, it has been shown in *Xenopus* and zebrafish embryos that high levels of BMP activity are observed throughout the entire ectoderm, and this activity is instructive of epidermal fates (Graham et al., 1994; Wilson and Hemmati-Brivanlou, 1995). At this time, the dorsal mesoderm releases anti-BMP molecules such as noggin, follistatin and chordin, that directly bind to BMPs, inhibiting their activity (Dale and Wardle, 1999; Glavic et al., 2004; Lemaire and Yasuo, 1998; Marchant et al., 1998; Neave et al., 1997; Wilson et al., 1997; Zimmerman et al., 1996). BMP signalling is inhibited by these antagonists in the region of the ectoderm that will form the neural plate (Wilson and Hemmati-Brivanlou, 1997; Wilson et al., 1997). In addition, the expression of BMPs is down regulated at the most dorsal ectoderm during gastrulation by a Wnt dependent mechanism (Baker and Bronner-Fraser, 1997). As a consequence of all these interactions a dorso-ventral gradient of BMP activity is generated. Formation of the neural plate would require lower BMP activity and non-neural ectoderm requires high level of BMP signalling (Dale and Wardle, 1999; Glavic et al., 2004; Lemaire and Yasuo, 1998; Marchant et al., 1998; Neave et al., 1997; Wilson et al., 1997; Zimmerman et al., 1996). The addition of BMPs to dissected neural crest induces the expression of neural crest

markers in chick, supporting a role for this molecule in this animal model as well (Liem et al., 1995).

Neural crest precursor cells arise at the border of neural plate and non-neural ectoderm, in regions of presumed intermediate BMP signalling levels (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Nguyen et al., 1998; Wilson et al., 1997). Evidence from both *Xenopus* and zebrafish suggests that intermediate level of BMP signalling at the neural plate border is necessary for neural crest precursor formation (LaBonne and Bronner-Fraser, 1998; Marchant et al., 1998; Neave et al., 1997; Nguyen et al., 1998; Schmid et al., 2000; Wilson and Hemmati-Brivanlou, 1997).

Thus, the elimination of BMP signalling in the neural plate seems to be a vital process of the early ectoderm patterning, however intermediate BMP signalling activity alone are not sufficient to induce neural crest fate, thus at least one additional signal appears to be required (LaBonne and Bronner-Fraser, 1998; LaBonne and Bronner-Fraser, 1999; Mancilla and Mayor, 1996; Wilson et al., 1997).

1.4 Posteriorizing factors

BMP signalling alone is not sufficient to induce neural crest fate. Posteriorizing signals including Wnts, FGFs and retinoic acid (RA) seem to be required for neural crest induction. These signals come from the posterior part of the embryo to regionalize the neural tube during AP patterning of the neuroectoderm (Darken and Wilson, 2001; Fredieu et al., 1997; Glinka et al., 1997; Kiecker and Niehrs, 2001; Kudoh et al., 2002). They are also required for neural crest induction of the posterior neural plate border (Chang and Hemmati-Brivanlou, 1998; Kengaku and Okamoto, 1993; LaBonne and Bronner-Fraser, 1998; Mayor et al., 1997; Villanueva et al., 2002).

Evidence demonstrated that Wnt signaling is one of the key elements in neural crest induction. Gain and loss of function experiments in chick, *Xenopus* and zebrafish show that Wnt signals are essential for neural crest induction (Chang and Hemmati-Brivanlou, 1998; Kengaku and Okamoto, 1993; LaBonne and Bronner-Fraser, 1998; Mayor et al., 1997; Villanueva et al., 2002). Neural crest makers can be induced by Wnt signalling molecules in neuralized animal caps (Deardorff et al., 2001; Sasai et al., 2001). Gain and loss-of-function experiments in whole embryos show that Wnt is both required for neural crest development and sufficient for an expansion of the neural crest domain

(Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997; Villanueva et al., 2002). However Wnt signals are not able to induce neural crest by itself in naive ectoderm, as they needed to work in combination with anti-BMP signals or in a previously induced neural plate (Bastidas et al., 2004; Deardorff et al., 2001; Garcia-Castro et al., 2002; LaBonne and Bronner-Fraser, 1998; Saint-Jeannet et al., 1997; Tribulo et al., 2003).

In *Xenopus* and zebrafish embryos Wnt8 is a good candidate to be a neural crest inducer. It is expressed in the mesoderm that is able to induce neural crest, and gain- and loss-of-function experiments show its role in neural crest induction (Bastidas et al., 2004; LaBonne and Bronner-Fraser, 1998; Steventon et al., 2009; Villanueva et al., 2002). A key downstream Wnt signalling component, β-catenin, plays an important role for mediating Wnt signalling in the induction of pre-migratory neural crest cells as well (Chapman et al., 2004; Deardorff et al., 2001; Dorsky et al., 1998).

FGF signals are also important in neural crest induction as shown by functional experiments (LaBonne and Bronner-Fraser, 1998; Mayor et al., 1997; Mayor et al., 1995; Monsoro-Burq et al., 2003). For example in two distinct experimental systems, dissociated cells (Kengaku and Okamoto, 1993) or intact ectoderm explants (Mayor et al., 1995), FGF

in combination with attenuation of BMP signalling was reported to induce pigment cells and *Snail2* expression, respectively. It has been recently shown that FGF works upstream of Wnt signaling during neural crest induction (Hong et al., 2008). Moreover, FGF signalling seems to be a direct regulator of Wnt8 expression in the mesoderm underlying the prospective neural crest cells, being Wnt8 the direct neural crest inducer (Hong et al., 2008).

In summary, two of the most well characterized signals involved in neural crest induction are attenuation of BMP and activation of posteriorizing signals. BMP attenuation is required to specify the neural plate border as anterior neural fold, while posteriorizing signals are needed for transformation of the anterior neural fold into neural crest (Chang and Hemmati-Brivanlou, 1998; Kengaku and Okamoto, 1993; LaBonne and Bronner-Fraser, 1998; Mayor et al., 1997; Villanueva et al., 2002).

1.5 Neural plate border specifier

Although many neural crest inducing factors have been recently characterized, the genetic cascade that specifies the neural crest at the neural plate border is still not clear. Neural crest formation genes have been categorized into two groups: neural plate border specifiers and neural crest specifiers, based on the spatial-temporal patterns of gene expression and their ability to induce ectodermal derivatives (Sauka-Spengler and Bronner-Fraser, 2008; Steventon et al., 2005).

In response to complex molecular signals from surrounding tissues, a set of transcription factors, called neural plate border specifiers, is up-regulated at the neural plate border and their combinatorial expression is thought to uniquely define this territory (Sauka-Spengler and Bronner-Fraser, 2008; Steventon et al., 2005). Neural plate border specifiers include *Zic1*, *Msx1*, *Pax3*, *Dlx* and *AP2* genes.

Recently, the role of border specifiers in the neural crest induction has been studied. Multiple *Zic* genes have been identified in mouse and *Xenopus* that are expressed in overlapping but distinct patterns (Mizuseki et al., 1998; Nakata et al., 1997; Sato et al., 2005). Disruption of mouse *Zic* genes leads to multiple defects in neural and neural crest derivatives (Nakata et al., 1998). In *Xenopus*, expression of

the *Zic* genes is an early response to neural inducing signals, and ectopic expression of these *Zic* genes leads to neural plate and neural crest formation (Mizuseki et al., 1998; Nakata et al., 1997; Sato et al., 2005). The direct regulation of *Zic1* by anti-BMP has been previously reported (Hong and Saint-Jeannet, 2007; Mizuseki et al., 1998; Rohr et al., 1999; Tropepe et al., 2006).

Msx1 is initially expressed in the non-neural ectoderm, and is later restricted to the neural fold region (Davidson, 1995; Suzuki et al., 1997). It was thought to be one of the earliest genes activated in the ectoderm fated to become neural crest (Tribulo et al., 2003). *Msx1* seems to play a key role in neural crest development, and epistatic experiments in *Xenopus* suggest that it works downstream of BMP and upstream of the Snail genes (Monsoro-Burq et al., 2005; Sato et al., 2005; Tribulo et al., 2003).

Another border specifier *Pax3* is expressed in the lateral neural folds during neural crest induction. Loss-of-function experiments show a key role in neural crest development (Monsoro-Burq et al., 2005; Sato et al., 2005). *Pax3* is initially induced by a posterior Wnt signal (Monsoro-Burq et al., 2005), although it also acts downstream of Msx1-mediated FGF8 signalling (Sato et al., 2005).

It has been proposed that Wnt controls neural crest induction through *Pax3* activity, while FGF8 requires *Msx1* and *Pax3* actions. Thus, Wnt and FGF8 signals act in parallel at the neural border and converge on *Pax3* activity during neural crest induction (Monsoro-Burq et al., 2005). It is also reported that induction of neural crest is mediated by the collective activity by *Pax3* and *Zic1*, and this co-activation of *Pax3* and *Zic1* is Wnt-dependent (Sato et al., 2005). In *Xenopus*, high levels of one of those transcription factors in the absence of the other tends to favour the induction of alternative neural plate border fates, such as the hatching gland which is promoted by *Pax3*, or the preplacodal fate which is promoted by *Zic1* (Hong and Saint-Jeannet, 2007). Thus, an appropriate balance of *Zic1* and *Pax3* gene products is required to induce the neural crest proper, see diagram Figure 1.2.

The neural plate border specifiers such as *Msx1*, *Pax3* and *Zic1* are independently induced by the secreted signals, but they interact with each other at the neural plate border to induce neural crest cells.

1.6 Neural crest specifiers

Following the initial specification of neural crest precursors at the neural plate border, a group of genes is set up that function to maintain these precursors. The genes transcribed at this step of neural crest induction are expressed only in the neural crest territory. Thus, these genes are often used as definitive markers of neural crest specification in many studies. They encode transcriptions factors of the *Snail* family and *Sox* family genes, and the genes *FoxD3, AP2, Twist, Id2*. Functional experiments are insufficient to predict a cascade of genetic interactions amongst these genes as the up or down regulation of one gene will affect the expression of others (Aybar et al., 2003; LaBonne and Bronner-Fraser, 2000; Pohl and Knochel, 2001; Sasai et al., 2001).

The *Snail* family of genes have two members *Snail* and *Snail2* (former *Slug*) and play key roles in neural crest development. Depending on the species, one or the other is highly specific to the neural crest, mice and fish express only *Snail* (Locascio et al., 2002), chickens express only *Snail2* (Del Barrio and Nieto, 2004) and *Xenopus* expresses both (Linker et al., 2000). In *Xenopus, Snail* is expressed in the prospective neural folds slightly earlier than *Snail2* (Linker et al., 2000). Based on animal caps and epistatic experiments, *Snail* seems to work upstream of *Snail2* in neural crest development. *Snail* and *Snail2* are functionally

equivalent (Aybar et al., 2003; del Barrio and Nieto, 2002). In addition, another important function of *Snail* genes is to control cell-cycle process in neural crest cells and to inhibit apoptosis in the crest cells (Tribulo et al., 2004; Vega et al., 2004).

The most studied *Sox* genes in the neural crest are *Sox9* and *Sox10*, which are specific to the premigratory neural crest and otic placode in frog and fish (Britsch et al., 2001; Cheng et al., 2000; Honore et al., 2003; Mori-Akiyama et al., 2003; Spokony et al., 2002). Murine *Sox9* is also expressed in premigratory and migratory neural crest (Mori-Akiyama et al., 2003), whereas *Sox10* is initially expressed just as neural crest migration initiates in chick and mouse (Britsch et al., 2001; Cheng et al., 2000). In *Xenopus*, *Sox9* or *Sox10* Mopholino knockdown inhibits neural crest specification (Honore et al., 2003; Spokony et al., 2002). However, mouse or fish *Sox9* mutants have no defects in neural crest formation or migration (Mori-Akiyama et al., 2003; Yan et al., 2002). Some results also showed another role of *Sox9* and *Sox10* as survival factor (Cheung et al., 2005; Kapur, 1999; Southard-Smith et al., 1998). Sox9-null mice mutants showed massive cell death in the trunk neural crest population prior to or shortly after delamination (Cheung et al., 2005) and in *Sox10* mutant embryos neural crest cells undergo apoptosis before they can differentiate (Kapur, 1999; Southard-Smith et al., 1998).

FoxD3 is another gene whose expression is specific to neural crest precursors in the ectoderm of all vertebrates (Dottori et al., 2001; Kos et al., 2001; Pohl and Knochel, 2001; Sasai et al., 2001). In *Xenopus* embryos, it has been shown that over-expression of *FoxD3* promotes neural crest development, whereas inhibition of *FoxD3* activity inhibits neural crest development (Dottori et al., 2001; Kos et al., 2001; Sasai et al., 2001).

1.7 Unsolved problems related to neural crest induction

Although considerable progress has been generated concerning neural crest induction, there are still many aspects of this process that remain unsolved. In this thesis I will focus on two of these problems:

1. Is posteriorization required for neural crest induction?
2. What are the direct targets of the neural crest inductive signals?

If posteriorization is involved in neural crest induction it is expected that this will be one of the earliest events in the process, and therefore it would be likely that neural crest inducing signals will control the expression of these posteriorizing factor directly. In consequence, the two questions mentioned above are related: analyzing the role of posteriorization in neural crest induction could lead us to identify the direct target of the inductive signals.

The two-step model of neural crest induction supposes that after the initial specification of the neural plate border, a second patterning event occurs that posteriorizes this border resulting in the expression of neural crest markers within a restricted anterior-posterior domain (Papalopulu and Kintner, 1996; Villanueva et al., 2002). However, the role of posteriorization as a general event that posteriorize the neural

plate and the neural crest has been recently challenged. It has been shown that neural crest induction by Wnt signalling is independent from the posteriorizing activity of Wnt as induction of neural crest by activation of Wnt does not affect the anterior-posterior axis of the neural plate (Wu et al., 2005). This issue needs to be investigated more deeply in order to understand neural crest induction.

Furthermore, several signals have been reported to be involved in neural crest induction including BMP, FGF and Wnt, see summary Figure 1.2. Neural crest induction by Wnt requires *Pax3* activity, while FGF8 requires *Msx1* and *Pax3* actions (Monsoro-Burq et al., 2005). However none of these transcription factors has been reported to be direct targets of Wnt or FGF.

In my thesis, I propose that *Gbx2* is a direct target of the neural crest inductive signals and it is involved in the posteriorization of the neural folds required for neural crest specification.

1.8 *Gbx2*

Gbx2 is gastrulation brain homeobox gene, (Hidalgo-Sanchez et al., 1999a; Joyner et al., 2000), involved in positioning of the mid-hindbrain boundary by interacting with *Otx2* (Hidalgo-Sanchez et al., 1999b; Irving and Mason, 1999; Katahira et al., 2000; Liu et al., 1999; Millet et al., 1999; Rhinn et al., 2003; Simeone, 2000). I propose *Gbx2* as a factor involved in neural crest induction, based in the following evidences:

First, in *Xenopus* embryos, the expression of *Gbx2* predominantly is in the dorsolateral ectoderm, with a gap in expression at the dorsal midline at early gastrulation stage. During neurulation, *Gbx2* is expressed dorsolaterally in the neural ectoderm and laterally-ventrally in the epidermis with sharp anterior expression borders in both tissues. The expression in the neural ectoderm persists throughout the early stages of development, in the region of the midbrain-hindbrain boundary (Byrd and Meyers, 2005; Su and Meng, 2002; Tour et al., 2002; von Bubnoff et al., 1996). Although not detailed analysis of *Gbx2* expression has been performed in the prospective neural crest, it is expected that its ectodermal expression includes the neural crest cells.

Second, *Gbx2* is controlled by posteriorization signals, such as FGFs, Wnt and retinoic acid in different species (Garda et al., 2001; Irving and

Mason, 1999; Liu et al., 1999; Rhinn et al., 2003). In mouse embryos, FGF8 can activate *Gbx2* and transform regions of the rostral mouse brain into a hindbrain fate (Liu et al., 1999). It has also been shown that cells near FGF8 soaked beads are induced to express *Gbx2* (Garda et al., 2001; Irving and Mason, 1999). Wnt1 misexpression affects *Gbx2* expression in chick embryos (Hidalgo-Sanchez et al., 1999a). *Gbx2* expression is induced by retinoic acid (RA) in animal caps, and RA treatment of whole embryos expands and enhances *Gbx2* expression in the ectoderm (von Bubnoff et al., 1996). Thus *Gbx2* might be required for neural crest induction as a posteriorizing factor in *Xenopus*.

Third, neural crest cell patterning and pharyngeal arch artery defects were found in *Gbx2* knock out mouse suggesting that *Gbx2* is required for neural crest pattering and differentiation (Byrd and Meyers, 2005). However no analysis of the role of *Gbx2* in early neural crest induction has been performed.

1.9 Hypothesis

Given that *Gbx2* is expressed within the neural plate and functions to posteriorize this tissue by working downstream of Wnt signals, I hypothesize that, see summary Figure 1.3:

First, *Gbx2* is required for neural crest induction as a posteriorizing factor in *Xenopus*.

Second, posteriorization is an early step of neural crest induction and therefore *Gbx2* is a direct target of the neural crest inductive signals.

Third, as neural crest is induced in posterior ectoderm by Wnt signals I propose that the posteriorizing activity of Wnt is mediated by *Gbx2*.

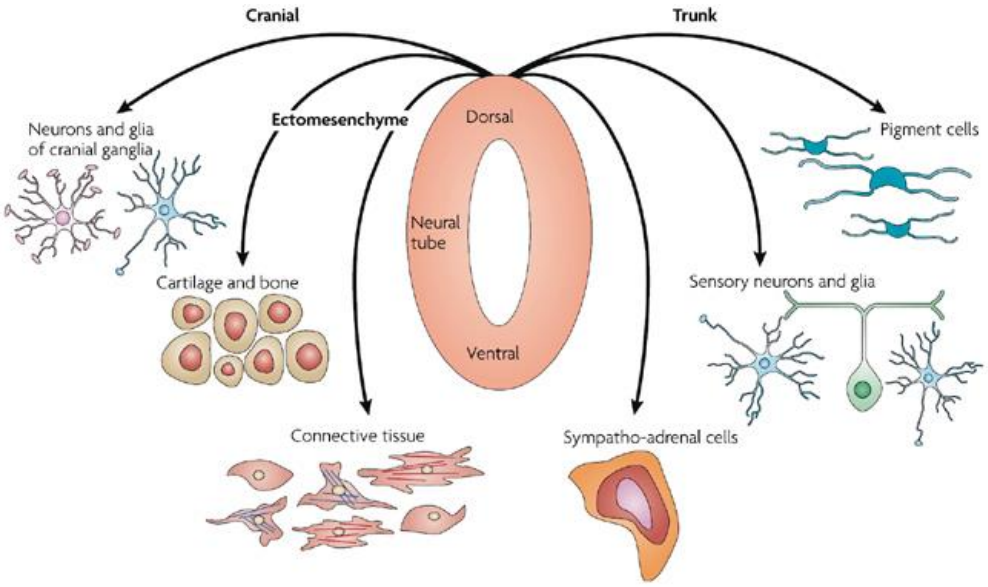

Figure 1.1 Summary of neural crest cells differentiating into different cell types Knecht and Bronner-Fraser, 2002

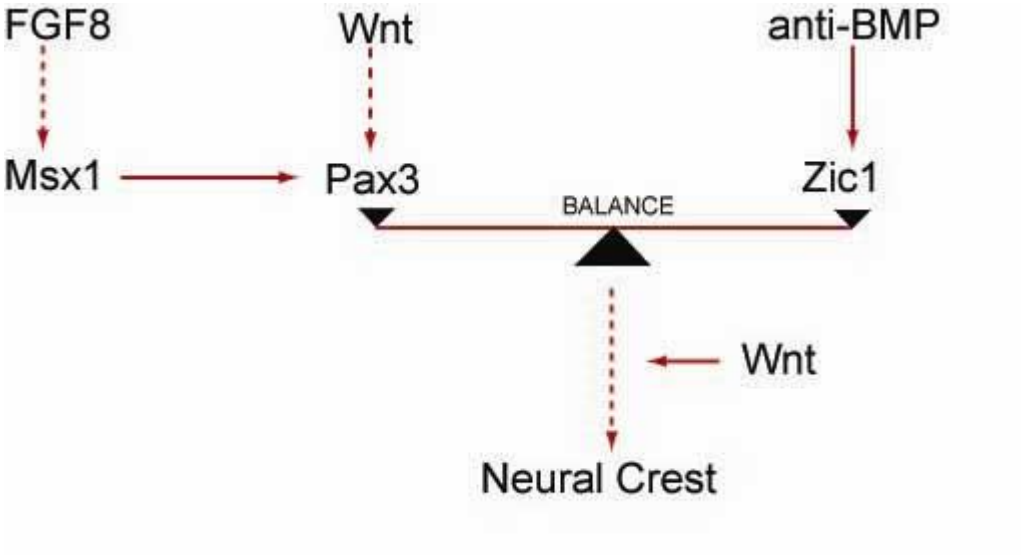

Figure 1.2 Summary diagram of neural crest induction

Msx1 is induced by FGF8; *Pax3* is indueced by Wnt; *Zic1* is induced by anti-BMP. *Msx1* is upstream of *Pax3*. Appropriate balance of *Pax3* and *Zic1* can induce neural crest together with Wnt.

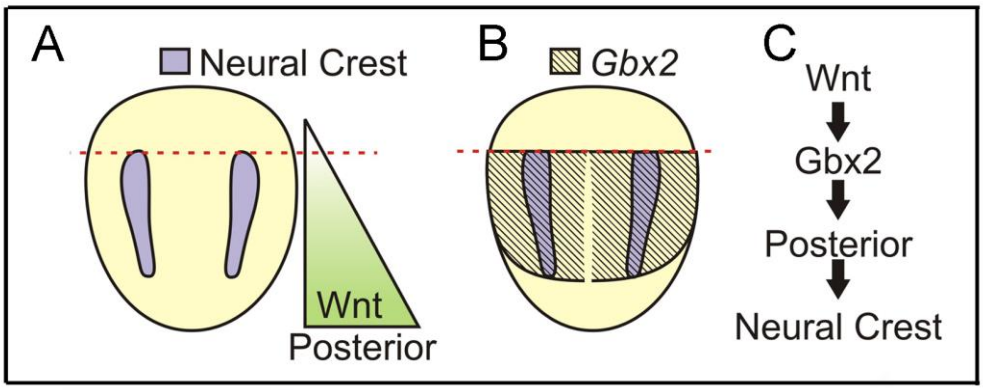

Figure 1.3 Hypothesis of neural crest induction by *Gbx2*

A: Posteriorizing factor Wnt signal is required in neural crest induction.

B: *Gbx2* is expressed the mostly posterior ectoderm including neural crest region.

C: *Gbx2* is required for neural crest induction as a posteriorizing factor in *Xenopus* and Gbx2 is activated by Wnt signal.

Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Hormones

Both lyophilized Folligon (Intervet) and Chorulon (Intervet) were stored at 4°C and reconstituted before use with the provided buffers. Aliquots of Folligon were prepared and stored at -20°C.

2.1.2 Materials and Solutions

All chemicals were obtained from Sigma, BDH or Fisher unless stated otherwise. Reagents for molecular biology including enzymes and buffers were acquired from Roche Biosystems or Promega.

Alkaline phospatase (AP) Buffer

100mM Tris-HCl pH9.5

100mM NaCl

50mM $MgCl_2$

0.1% Tween-20

Bleaching Solution

33% H_2O_2

5% Formamide

0.5 x SSC

Egg-laying medium (Ca2+ free)

110mM NaCl

2mM KCl

0.6mM Na_2HPO_4

15mM Tris base

2mM $NaHCO_3$

0.5mM $MgSO_4$

pH 7.6 (adjust with acetic acid)

Hybridization Buffer

50% Formamide

5 x SSC

1 x Denhardt's Solution

1mg/ml Ribonucleic acid

100µg/ml heparin

0.1% CHAPS

10mM EDTA

0.1% Tween-20

pH5.5

Maleic acid buffer (MAB)

100mM Maleic acid

150mM NaCl

0.1% Tween-20

MEMFA

4% Formaldehyde

0.1M MOPS

1mM $MgSO_4$

2mM EGTA

Normal amphibian medium (NAM) 1/10

11mM NaCl

0.2mM KCl

0.1mM $Ca(NO_3)_2$

0.1mM $MgSO_4$

0.01mM Disodium EDTA

0.2mM Na_2HPO_4 pH7.5

0.1mM $NaHCO_3$

50µg/ml Streptomycin

Normal amphibian medium (NAM) 3/8:

41mM NaCl

0.75mM KCl

0.36mM $Ca(NO_3)_2$

0.36mM $MgSO_4$

0.036mM Disodium EDTA

0.75mM Na_2HPO_4 pH7.5

0.1mM $NaHCO_3$

50µg/ml Streptomycin

Phosphate Buffered Saline (PBS)

137mM NaCl

2.7mM KCl

4.3mM Na_2HPO_4

1.4mM KH_2PO_4

pH 7.3

PBT

99.9% PBS

0.1% Tween-20

TE buffer

10mM Tris-HCl pH 7.5

1mM EDTA

SSC (20x)

3M NaCl

0.3M Tri-sodium citrate pH7.0

2.2 Methods

2.2.1 Obtaining *Xenopus* Embryos

Mature *Xenopus laevis* females were pre-primed by subcutaneous injection of 100 units of Serum Gonadotrophin (Intervet) 4 to 7 days before being used. 500 units of choronic gonadotrophin (Intervet) were injected 12-15 hours to stimulate ovulation. Frogs were then kept in Egg-laying solution (Ca2+ free), from which naturally laid, mature oocytes could be collected. Adult male frogs were terminally anaesthetized in 0.5% Tricaine (3-amino benzoic acid ethylester) and the testes were dissected and stored in Leibovitz L-15 medium (Invitrogen). For in vitro fertilization, a small piece of testis was macerated in dH_2O and the resulting sperm suspension was mixed with the oocytes in Petri dish. After cortical rotation, the dish was flooded with normal amphibian media NAM1/10. During 2-8 cell stage, a solution of 2% L-cysteine (pH8.2) was used to remove the jelly from embryos by vigorous agitation and embryos were rainsed by NAM1/10. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

2.2.2 *Xenopus* micromanipulation: animal cap

The vitelline membrane was physically peeled off using No. 5 Watchmakers forceps in NAM3/8. Animal caps were dissected from stage 9 embryos under a dissecting microscope (Leica) and were left to heal in NAM3/8. They were then cultured until the suitable stage before fixation.

2.2.3 *Gbx2* Morpholinos (MO)

Morpholino antisense oligonucleotides (MO) is uncharged polynucleotides that effectively bind to tmRNA and block its transcription without trapped in microsomal/lysosomal vesicles (Summerton, 1999; Summerton and Weller, 1997). In *Xenopus* embryo, treatment with a MO against β-catenin results in the repression of initial axis formation, which has been suggested by gain-of-function analysis (Heasman et al., 2000). Since then, MOs are largely accepted as the tools to block gene function in animal cells. Not only blocking of translation but also blocking of mRNA splicing has been reported (Morcos, 2007). MOs targeting the joint sequences lay in mRNA splicing site effectively block RNA splicing and largely result in a truncated protein.

A translation-blocking MO (ATG MO) and a splicing MO (Spl MO) against *Gbx2* were designed and both of them produced the same

neural crest phenotype. The sequence of the translation-blocking MO (ATG MO) is: 5'-GCTGAAAGGCTGCACTCATATAAGC-3'. A 7missmatch *Gbx2* mutant (7mut *Gbx2*) was generated to perform the rescue of the ATG MO. To amplify the 7mut *Gbx2* the following primers were used: forward primer: 5'-GAATTCACC *ATGtcgGCtGCtTTcCAa C*CCCCTCTCATGAT-3' and reverse primer: 5'-TTGACTCGAGTCAAGGTCTTGCTTGCTCCAGC-3'. The italic letters shows the morpholino target sequences. The lower case letters show the miss-matching nucleotides inserted by PCR without changing the amino acid sequences. The amplified *Gbx2* ORF cDNA was subcloned into the pCS2+ expression vector. To design the splicing MO we cloned the first intron of the *Xenopus laevis Gbx2* gene. We predicted the structure of *Xenopus laevis Gbx2* allele from that of *Xenopus tropicalis Gbx2*. The primers for exon1 (5 -GAGAAGAAGGAAACAAGACCTACAT-3') and exon2 (5'-AGTTTGGCAGGAGATATTGTCATCT-3') were designed to amplify 200 bp in ORF of cDNA, and 1.7kb in genomic DNA. The isolated genomic fragment was subcloned in pBluescript II vector, and the sequences of exon-intron boundary were read from several different clones. The sequence of the splicing MO is: 5'-GTGATGGTTGCTACACTTACCTAGA-3' (Gene Tools). To check the efficiency of splicing MO, we performed RT-PCR with exon1-exon2 primers as described above. The shifted RNA was not amplified by RT-

PCR using Go-Taq (Promega). A standard control morpholino was used: 5'CCTCTTACCTCAGTTACAATTTATA3'. Injection of this control MO in wild-type *Xenopus* embryos causes no defective phenotype.

2.2.4 Synthesis of mRNA for microinjection

Plasmid DNA was linearised with the restriction endonuclease (Promega), purified by chloroform extraction, ethanol precipitated and re-suspended in molecular biology DEPC H_2O with a final concentration of 1µg/µl. SP6 mMessage mMachine kit (Ambion) was used to synthesize capped mRNA by following instructions of manufacturer. mRNA was purified by the chroma-spin100 kit (Clontech) and re-suspended in DEPC H_2O. The following synthetic mRNAs were used during my study: dd1 (Sokol, 1996); Wnt8 (Sokol, 1996); β-catenin-GR (Domingos et al., 2001); *Gbx2* (Glavic et al., 2002); Gbx2EnR (Glavic et al., 2002); tBR (truncated BMP receptor); *Pax3* (Sato et al., 2005), HD-Msx1 (Tribulo et al., 2003); *Msx1* (Tribulo et al., 2003); *Zic1* (Nakata et al., 1998).

2.2.5 Microinjection

Microinjections were performed using a Narishige IM300 Microinjector under a Leica MZ6 or a Nikkon SMZ645 dissecting microscope.

Needles were pulled from AlSi thin-walled capillaries (AM systems). The needle was filled with mRNA and Fluoroscein dextran (FDX, as lineage label) and calibrated using an eyepiece graticule to inject 10nl into *Xenopus* embryos. Injections were carried out in a dish of 3% Ficoll. *Xenopus* embryos were injected at the 2, 8 or 32-cell stage and cultured in Ficoll until the onset of gastrulation, at which point they were transferred to NAM1/10. For injections specifically targeted to the neural crest, embryos were injected in the animal ventral blastomere at the 8-cell stage or into blastomeres A2 or B2 at the 32-cell stage.

2.2.6 Synthesis of antisense RNA probes for in situ hybridization

Plasmid DNA was cut by an appropriate restriction endonuclease (Promega) according to the manufacturer's instructions. The cut DNA was purified by chloroform extraction, ethanol precipitated and re-suspended in molecular biology H_2O (Ambion) to a concentration of 1µg/µl. In vitro transcription of anti-sense RNA was carried out using T3, T7 or SP6 RNA polymerase (Promega) using conditions suggested by the manufacturer. The NTP mix used for the reaction contained 0.35mM digoxigenin-labelled UTP, resulting in the production of a digoxigenin-labelled RNA. After transcription 1µl DNase (Promega RQ1 DNase) was added to degrade the template DNA and the quality of RNA was checked by gel electrophoresis. RNA was purified using the Chroma-

spin-100 kit (Clontech), resuspended in molecular biology H_2O and diluted in hybridization buffer to concentration of 1ng/µl for in situ hybridization. RNA probes for the following genes were used in this thesis: *Snail2* (formerly *Slug*; (Mayor et al., 1995); *FoxD3* (Sasai et al., 2001); *Gbx2* (von Bubnoff et al., 1996); *Otx2* (Blitz and Cho, 1995); *Sox2* (Kishi et al., 2000); *Six1* (Ghanbari et al., 2001); *Cpl1* (Richter et al., 1988); *En2* (Hemmati-Brivanlou et al., 1991); *Krox20* (Sasai et al., 1994); *Pax3* (Bang et al., 1997); *Msx1* (Suzuki et al., 1997) and cytokeratin *Xk81A* (Jonas et al., 1985).

2.2.7 Whole Mount in situ hybridization

In situ hybridization protocol was adapted from (Harland, 1991). For all solutions prior to washing off of the RNA probe, diethyl pyrocarbonate (DEPC) treated water was used to inhibit RNases that might otherwise degrade the probe.

A) Preparation of samples

Fixation of both whole embryos and explants was performed by one hour incubation at room temperature (RT) or overnight at 4°C in MEMFA and transferred to 100% methanol for indefinite storage at -20°C. Embryos were rehydrated with successive 5-minute washes of:

75% methanol, 50% methanol, 25% methanol and PBT. To remove pigment in embryos/explants they were placed into bleaching solution under bright light for 30 minutes or until pigment has disapperaed. Samples were then washed in PBT and re-fixed for 30 minutes in 4% paraformaldehyde (PFA) in PBS at room temperature, as bleaching can cause damage to whole embryos.

B) Probe hybridization:

After further washing in PBT, embryos were transferred to hybridization buffer and incubated for 2-5 hours at 62-65°C. The digoxigenin labelled probe was then added and incubated with embryos overnight at 62-65°C. To remove the probe, the following washes were performed for 10 minutes each at 62°C:

50% formamide / 2xSSC/ 0.1% Tween,

25% formamide / 2xSSC/ 0.1% Tween,

12.5% formamide / 2xSSC/ 0.1% Tween,

2xSSC / 0.1% Tween.

Followed by one final 30 minute wash with 0.2% SSC / 0.1% Tween.

C) Antibody staining

Samples were rinsed twice with PTW, then twice with maleic acid buffer (MAB) and then blocked in 2% BMBR (Boehringer Mannheim blocking reagent; Roche) in MAB for 1-2 hours at RT. Embryos/explants were incubated overnight at 4°C with an anti-digoxigenin-alkaline phosphatase (AP) conjugated antibody (Roche) diluted 1:5000 in 2% BMBR/MAB. Antibody was removed by six 30 minute washes with MAB at room temperature with gentle agitation. Samples were then transferred to AP (alkaline phosphatase) buffer where the AP reaction was developed using 75µg/ml BCIP (5-bromo- 4-chloro-3-indoyl-phosphate; Roche) and 150µg/ml NBT (4-nitro blue tetrazolium chloride; Roche) at 37°C. Embryos were then washed with 100% methanol to remove any background staining and transferred to 4% PFA for storage.

D) Antibody staining of fluorescein dextran

Staining was performed as for in situ hybridization above but with a 1:3000 dilution of anti-fluorescein-AP conjugated antibody (Roche) in 2% BMBR/MAB. To reveal a turquoise colour that is distinguishable from dark purple stain used for in situ, only 75µg/ml BCIP (Roche) was added to the AP buffer.

2.2.8 Double in situ hybridization

When it was necessary to compare the expression of two genes in one embryo, double in situ hybridization was carried out. Double in situ hybridization uses the same protocol described above, with a few minor alterations: a mixture of two RNA probes was added, one labelled with digoxigenin and another labelled with UTP-fluorescein. After blocking with 2% BMBR/MAB, embryos were incubated overnight at 4°C with an anti-fluorescein-AP antibody (Roche). For the first colouring reaction, only BCIP was used to develop a light green colour. To terminate the reaction, 100% methanol was added and embryos were incubated for 15 minutes at 62-65°C. Embryos were then transferred back to 2% BMBR/MAB and continue to develop the second colour. As simple in situ hybridization, the second colouring reaction, BCIP and NBT were added together to develop a colour of dark purple, which can be distinguished from the first one.

2.2.9 Cartilage staining

Embryos were fixed in MEMFA at stage 45 and washed with PBS. Cartilage was manually dissected to remove adherent tissues, dehydrated with ethanol and stained overnight in 0.2% Alcian Blue / 30% acetic acid in ethanol. It was then washed with ethanol several times, rehydrated through a series of ethanol/water and cleared in 2%

potassium hydroxide. The stained cartilage was further cleaned to remove residual tissues.

2.2.10 Photography of *Xenopus* embryos

Embryos were immobilized in an agarose coated dish and photographed with a Leica DFL420 camera attached to a Leica MZFLIII dissecting microscope using the IM50 software (Leica).

2.2.11 RT-PCR

Reverse transcriptase-polymerase chain reactions were used to analyze the expression of genes at various stages of embryogenesis.

A) RNA isolation (QIAGEN)

Whole embryos (2-3) or embryonic tissues (20-25) were collected into a 1.5 ml eppendorf tube without culture medium. They were then homogenized in 350 µl of lysis buffer (10µl β-mecaptoeth in 1ml buffer RTL from QIAGEN kit) by passing 10 or more times through needles (0.8, 0.55, 0.33 diameter) fitted to an RNase free syringe. The homogenate was centrifuged at 14000 rpm for 3 minutes; the supernatant was transferred into a fresh tube containing 350 µl of 70 %

ethanol and mixed by pipetting. The resulting solution was loaded onto a column and centrifuged at 10000 rpm for 15 seconds. The column was washed with 350 µl of buffer RW1 and centrifuged at 10000 rpm for 15 seconds. DNase digestion was performed on the column by pipetting 80 µl of DNase I solution (70 µl buffer RDD + 10 µl DNase I stock) onto it and incubating at 30°C for 30 minutes. The column was washed with 700 µl of buffer RW1 and centrifuged at 10000 rpm for 15 seconds, 700 µl of buffer RPE and centrifuged at 10000 rpm for 15 seconds. The last washing step was repeated, but centrifuged at 14000 rpm for 2 minutes. The RNA was eluted from the column in two steps, each with 20 µl of DEPC water and centrifuged at 10000 rpm for 1 minute.

B) Reverse transcription of cDNA

cDNA was synthesized from isolated RNA using Oligo dT (Promega) in the following total 20µl reaction

RNA	1µg
Oligo dT primers	0.5µl
dNTP mix(Promega)	1µl

RNase free H_2O to a final volume of 13.3µl

The reaction was placed at 65^0C for 3 minutes to denature any secondary structure of the RNA, and then cooled on the ice for 1 minute. To the reaction, added:

Reverse Transcriptase buffer (Promega)	4µl
Reverse Transcriptase (Promega)	0.5µl
$MgCl_2$	2µl
RNase inhibitor	0.2µl

The reaction was kept at room temperature for 2 minutes, before being placed at 42^0C for 1 hour. An aliquot of this reaction mix was used directly in the PCR reaction.

C) PCR reaction

The primers used in this study are summarized in Table 2.1.

PCR amplification using the following reaction, total volume of 20µl:

RT mix	2.1µl
RNase free H_2O	11µl
5X reaction buffer	4µl
$MgCl_2$	2µl
Primer Mix (Fwd.+Rev.)	0.5µl
dNTP mix	0.2µl
GoTag	0.2µl

The PCR reaction conditions were as follows:

denaturation 15 seconds at 96°C;

annealing 15 seconds at 52°C;

extension 60 seconds at 72°C.

Appropriate cycles (23-27 cycles) were performed for each primer.

As a control, PCR was performed with RNA that had not been reverse-transcribed to check for DNA contamination.

D) Analysis RT-PCR products

The PCR products were analyzed on 1.5% agarose gels at 150V for 30-45 minutes. 100-kilobase ladder was loaded along with the samples to confirm the molecular weight of each sample. The gel was photographed using the ChemiDoc XRS (BIO-RAD) system.

2.2.12 DNA sequencing

DNA sequencing was carried out using following protocol:

400 ng template DNA

1 µl 3.2 µM primer

RNase free H_2O to a final volume of 6 µl

Add 4 µl Big Dye Mix (ABI)

PCR reaction:

96 °C for 3min; 96 °C for 30sec; 50 °C for 15sec; 60 °C for 4min

Cycle 25 times in total

PCR reaction samples were precipitated by Sodium acetate (pH=5.2).

The pellet was washed and air dried.

Samples were loaded, and the sequences were obtained by Dr. Stuart Martin, Sequencing & Genotyping Facility, and Centre for Comparative Genomics, University College London,

Gbx2	U: 5'-AAACTGCCCACAAAGAGGAGGAC-3',	Cycles=27.
	D: 5'-TGGTGTTGGCTCCGTATGGCAAA-3';	
Six1 (Pandur and Moody, 2000);	U 5'-ATCCTTGTGGTATCTCC-3';	Cycles=25.
	D 5'-GGGCCCCTCACCTCCAGC-3';	
ODC (Heasman et al., 2000);	U: 5'-GCCATTGTGAAGACTCTCTCCATTC-3';	Cycles=23.
	D: 5'-TTCGGGTGATTCCTTGCCAC-3';	
Pax3 (Hong and Saint-Jeannet, 2007);	U 5'-GCTGTGTCTGATCCAAGCAA-3';	Cycles=27.
	D 5'-CAATAGGCCGAACTGCTCTC-3';	
Nrp1 (Richter et al., 1990);	U 5'-GGGTTTCTTGGAACAAGC-3';	Cycles =27.
	D 5'-ACTGTGCAGGAACACAAG -3';	
Snail2 (Aybar et al., 2003);	U 5'-GTTTACCAGGACTTATCACCTCC -3';	Cycles =23.
	D 5'-GCATTCCCTTAAACCCTTCTTGG-3';	
FoxD3 (Sasai et al., 2001);	U 5'-TCTCTGGGGCAATCACACTC-3';	Cycles=25.
	D 5'-GTACATTTGTTGATAAAGGG-3';	
Sox9 (Monsoro-Burq et al., 2003);	U 5'-AACAGGAGTTCCATCAATCCCC-3';	Cycles=25.
	D 5'-CTTTTGCTAAACCCCGTGTCAC-3';	
EpK (Jonas et al., 1989).	U 5'-CACCAGAACACAGAGTAC-3',	Cycles=27.
	D 5'-CAACCTTCCCATCAACCA-3';	

Table 2.1 Primer sequences

Chapter Three: Experiments and Results

3.1 *Gbx2* is expressed in the prospective neural crest

I am proposing that *Gbx2* is involved in neural crest induction and therefore I would expect that *Gbx2* is expressed in neural crest territory. In order to demonstrate that, *Gbx2* expression compared to the neural crest marker *Snail2* is shown in Figure 3.1.

At the late gastrula stage (Stage 12), when neural crest induction takes place (Mancilla and Mayor, 1996), *Gbx2* is expressed in the most posterior end of dorsolateral ectoderm at a distance from the blastopore lip and with a wide gap in the expression at the dorsal midline (Figure 3.1 B). *Gbx2* is clearly co-expressed with the neural crest marker *Snail2* at this stage (Figure 3.1 A, B). This result was further confirmed by double in situ hybridization for *Snail2* and *Gbx2* (Figure 3.1 C, D). Transverse histological sections show that *Gbx2* expression is restricted to the ectoderm and it is not expressed in the mesoderm (Figure 3.1 F). Lateral views show that, *Gbx2* is expressed in a wide domain of ectoderm, which includes the prospective neural crest domain (Figure 3.1 I-L) and interestingly it is absent only from the anterior-most region of the embryo (Figure 3.1 I-L).

At the neurula stage (Stage 16) *Gbx2* is strongly expressed in the prospective midbrain-hindbrain boundary in neural plate region and also in the epidermis region (Figure 3.1 N). There is a gap between the neural and epidermal expression of *Gbx2* (arrow in Figure 3.1 N). The most anterior cephalic neural crest has lost *Gbx2* at this stage. Double in situ hybridization with *Snail2* shows that this gap in *Gbx2* expression is adjacent to the neural crest (Figure 3.1 O-Q), corresponding presumably to the preplacodal region.

In summary, *Gbx2* is expressed in posterior ectoderm. At the time of neural crest induction *Gbx2* is expressed in the prospective neural crest territory.

3.2 *Gbx2* is essential for neural crest formation

It was confirmed that *Gbx2* is expressed in the prospective neural crest territory at the time of neural crest induction. Next I asked whether *Gbx2* is required for the neural crest induction.

In order to analyze *Gbx2* function in neural crest induction I first undertook a morpholino loss of function approach. MOs and fluorescein dextran were injected into one animal blastomere of an 8-cell stage embryo. In all injected embryos shown in this work, the injected side is shown on the right side. The small insert in each photo corresponds to the overlay of in situ hybridization and fluorescence to trace the lineage of injected site. While injection of 20ng control MO does not affect the expression of *Snail2* (Figure 3.2 A, I. n=38), injection of 20ng splicing blocking MO (Spl MO) or 16ng translation blocking MO (ATG MO) produced a strong inhibition of this neural crest marker (Figure 3.2 B, C, I. inhibition of *Snail2* expression by injection of Spl MO 68% n=38; ATG MO 77% n=35).

Follow these experiments, the gain of Gbx2 function experiments were performed. 1ng *Gbx2* mRNA was injected, which lead to a modest but consistent expansion of *Snail2* expression (Figure 3.2 D. expansion of *Snail2* 78% n= 39).

To confirm the *Gbx2* over-expression results, *Gbx2* homeobox domain was fused to Engrailed (EnR) which is transcriptional repressor domain of Drosophila. In addition to activated *Gbx2* construct constantly, to enable to control the timing of transcriptional regulation, I further fused the Gbx2EnR to the glucocorticoid receptor (GR). In the absence of any hormone, the glucocorticoid receptor resides in the cytosol complexed with Gbx2EnR, while in the presence of an agonist such as Dexamethasone (DEX), the agonist binds to the glucocorticoid receptor resulting in release and activation of Gbx2EnR. When 1ng of this Gbx2EnR-GR mRNA was injected and DEX was added to activate the construct at stage 10, a similar expansion of *Snail2* expression was observed as with *Gbx2* mRNA injection (Figure 3.2 F. expansion of *Snail2* by injection of Gbx2EnR-GR 68% n=37).

Also, to make sure the specificity of Gbx2 MO, a rescue experiment was done. To eliminate the possibility of *Gbx2* mRNA neutralizing the *Gbx2* MO, mRNA encoding 7 miss matches at ATG MO binding site of *Gbx2* mRNA (7mut *Gbx2*) was used in this work. The neural crest expansion was observed by injecting 1ng of 7mut *Gbx2* alone (Figure 3.2 H. expansion of *Snail2* by injection of 7mut *Gbx2* 75% n=39).

Then, the rescue experiment was performed to check the specificity of MO. Co-injection of 1ng Gbx2EnR-GR or 1ng of the mutated *Gbx2* mRNA was able to rescue the inhibition of *Snail2* expression produced by the translation blocking MO (Figure 3.2 E, G, I. expansion of *Snail2* expression by co-injection with Gbx2EnR-GR 13% n=39; with 7mut *Gbx2* 17% n=36), showing its specific effect on *Gbx2* RNA. Thus, this effect of *Gbx2* MO is specific for loss of endogenous *Gbx2* function.

To measure the efficiency of the MO treatment I analyzed the splicing of *Gbx2* mRNA when embryos were injected with 20ng of control MO or 20ng of *Gbx2* splicing MO. The primer for exon1 and exon2 was designed to demonstrate the effect of the splicing MO on RNA splicing. I expected that a 200 base pair band should be amplified from normally spliced RNA by RT-PCR, but that band would not be detected if the RNA was mis-spliced (Figure 3.2 H). Indeed, the 200 bp band was observed in presence of control MO, but this band disappeared when *Gbx2* splicing MO was injected (Figure 3.2 J). The results suggested that the injection of *Gbx2* splicing MO had a specific effect on the normal splicing of *Gbx2* RNA. As the splicing and translational MOs produced the same effect on neural crest induction I used the translational MO in most of the subsequent experiments (from now on referred to as *Gbx2* MO).

As *Gbx2* is expressed in the neural plate and epidermis in addition to the neural crest I performed targeted injections of *Gbx2* MO at the 32-cell stage into blastomeres fated to become neural plate (A1), neural crest (A2, A3) or epidermis (A4) (Figure 3.4 A-F). Only *Gbx2* depletion in the neural crest was able to inhibit *Snail2* (Figure 3.4 A, B, G. Snail2 75% n=40) and no effect was observed when injected in the neural plate (Figure 3.4 C, D, G. *Snail2* 11% n=38) or epidermis adjacent to the neural crest (Figure 3.4 E, F, G. *Snail2* 1% n=21). Thus, the effect on neural crest is due to cell autonomous function of *Gbx2* in neural crest, and not a secondary effect on neural plate or epidermis development.

To check whether inhibition of *Snail2* is transient or whether depletion of neural crest cells could also affect some neural crest derivatives, two of these derivatives were analyzed at later stages. The same 1ng injection of *Gbx2* MO produced a dramatic reduction of cartilage in stage 40 embryos (Figure 3.5 A, B, E. control MO n=30; reduction of cartilage by injection of *Gbx2* MO 87% n=39) and melanocyte in stage 35 embryos (picture not shown, data in Figure 3.5 E, control MO n=27; reduction of melanocyte by injection of *Gbx2* MO 80% n=32). Thus *Gbx2* MO affected the neural crest derivatives due to the depletion of neural crest cells. But interestingly, placode marker *Tbx2* shown optic placode was inhibited by over-expression of *Gbx2*. Embryos were injected with 1ng

of *Gbx2* mRNA, fixed at stage 26 (Figure 3.5 C, D, E. reduction of optic by injection of *Gbx2* mRNA 66% n=35, arrow in C, D: optic placode).

3.3 *Gbx2* works as a posteriorizing factor of the neural fold

Previous evidence demonstrates that *Gbx2* acts as posteriorizing factor in the neural plate (Wurst and Bally-Cuif, 2001). Therefore, I investigated whether *Gbx2* also interferes with anterior-posterior patterning of the neural folds when neural crest is induced by *Gbx2*.

Neural crest markers *Snail2* and *FoxD3* are inhibited by injection of 16ng *Gbx2* MO (Figure 3.6 A, C, *Snail2* 77%; n=35, *FoxD*3 68%; n=34), and slightly expanded by injection of 1ng *Gbx2* mRNA (Figure 3.6 B, D, R. *Snail2* 77% n=39, *FoxD3* 78% n=40). Interestingly, these effects are accompanied with changes in the expression of the preplacodal marker *Six1*and *Eya1*. The preplacodal domain forms in the outer border of the anterior neural fold and contributes to sense organs and cranial sensory ganglia (Schlosser, 2006; Streit, 2004). It has previously been reported that the preplacodal gene *Six1* and *Eya1* repressed neural crest fates and promoted preplacodal gene expression (Brugmann et al., 2004). Upon *Gbx2* MO injection, *Six1* and *Eya1* expression is expanded posteriorly (Figure 3.5 E, G. *Six1* 70% n=30, *Eya1* 66% n=32), and shifted anteriorly if *Gbx2* is over-expressed (Figure 3.5 F, H. *Six1* 63% n=32, *Eya1* 65% n=34). Also these results could explain the optic placode derivatives were inhibited by over-expression of *Gbx2* (Figure 3.5 C, D). All these data suggest that *Gbx2* plays an important role in

posteriorization of the neural fold in agreement with the transition of preplacode to neural crest cell.

These results indicate that *Gbx2* is a posteriorization factor for the neural fold. I then asked whether the anterior-posterior axis of the neural plate was also affected in these conditions. Surprisingly, I found that the anterior-posterior patterning of the neural plate was normal. Regionally restricted neural plate markers like *Cpl1, En2* and *Otx2* are expressed normally after similar *Gbx2* MO (Figure 3.6 I, K, M. no effect on *Cpl1* n=24; no effect on *En2* n=24 and no effect on *Otx2* n=22) or *Gbx2* mRNA injections (Figure 3.5 J, L, R no effect on *Cpl1* n=21; no effect on *En2* n=29 and no effect on *Otx2* n=20). In addition, *Gbx2* knock down does not change the expression of the pan neural plate marker *Sox2*, or the epidermal marker Keratin (Figure 3.5 O, P. *Sox2* 10% n=21; Keratin 15% n=20).

However, neural plate anterior-posterior patterning can eventually be affected by much higher levels of *Gbx2* MO (30ng) injection, compared to those that blocked neural crest markers (15ng). Neural plate marker *En2* was shifted and inhibited by this higher dose of *Gbx2* MO injection (Figure 3.7 A-C. *En2* shift 10%; inhibit 50% n=32). These results suggested that with high level of inhibition of *Gbx2*, neural plate anterior-posterior patterning was affected. Thus, taken together all

these results, Gbx2 is involved in posteriorization of neural fold and transform porsteriror of neural fold into neural crest. Porization of the neural fold can be dissociated from posteriorization of the neural plate, and that posteriorization of neural fold is required for neural crest induction.

Repression of the preplacodal marker *Six1* by *Gbx2* was further confirmed in explants experiments. Embryos were injected with different constructs into the four animal blastomeres at 8-cell stage; animal cap explants were dissected at stage 9 and cultured in NAM 3/8 until stage 17. Then total RNA was extracted and RT-PCR performed. Injection of a dominant negative form of the type II BMP receptor (tBR, 1ng) up-regulates the expression of preplacodal marker *Six1* in the animal cap (line 3 Figure 3.8 A; as shown in previous report (Brugmann et al., 2004). However, this effect is reverted when animal caps are co-injected with 1ng *Gbx2* mRNA and neural crest maker *FoxD3* is induced (line 4 Figure 3.8 A). These results suggest that *Gbx2* represses *Six1* and induces neural crest fate.

In conclusion, reduction of *Gbx2* produces an enlargement of the preplacodal domain at the expense of neural crest cells (Figure 3.8 B), while *Gbx2* over-expression has the opposite effect, transforming preplacodal domain into neural crest (Figure 3.8 C). Moreover, the

posteriorizing effect of *Gbx2* in the neural folds is independent of the anterior-posterior patterning of the neural plate.

3.4 *Gbx2* is a direct target of Wnt signaling in neural crest induction

It has been previously hypothesized (Figure 1.3) that one of the first steps in neural crest induction was posteriorization of anterior ectoderm by crest inducing signals (Aybar and Mayor, 2002). Since *Gbx2* specifies neural crest by posteriorizing the neural fold, I hypothesize that *Gbx2* could be one of the earliest factors activated in the neural crest induction cascade. Accordingly, I asked whether *Gbx2* was a direct target of crest inductive posteriorizing signals.

Activation of Wnt signalling by injection of Wnt8 into the prospective neural crest region leads to expansion of endogenous *Snail2* expression (Figure 3.9 A, 1ng Wnt8 injection, *Snail2* 69% n=35), Inhibition of Wnt signalling by expression of a dominant negative form of Dsh, DD1 (Tada and Smith, 2000) leads to neural crest inhibition (Figure 3.9 B, 1ng DD1, Snail 70% n=30). While a similar effect of *Gbx2* expression was observed after either activation or inhibition of the Wnt pathway by the same treatment (Figure 3.9 C, D. Wnt8 77% n=26, DD1 75% n=38). All these results suggest that *Gbx2* expression is controlled by Wnt signaling and neural crest induction by Wnt may be *Gbx2* dependent.

To test whether *Gbx2* is a direct target of Wnt signaling during neural crest induction, protein synthesis inhibitor cycloheximide (CHX) was used in animal cap explants experiment. Embryos were injected into animal four blastomeres at 8-cell stage with a combination of 1 ng tBR (a dominant negative form of the type II BMP receptor) and 1ng β-catenin-GR mRNAs to induce neural crest. The animal cap explants were dissected at stage 9 and cultured in NAM 3/8. Injected animal caps were treated with the protein synthesis inhibitor CHX at stage 11.5 and 30min later β-catenin activity was triggered by addition of dexamethasone (DEX). After 2 hrs culture, mRNA was extracted and RT-PCR was performed for *Gbx2* and other makers.

Neural crest induction by inhibition of BMP and activation of Wnt signalling was confirmed by *Sox9* and *Snail2* expression after DEX treatment (4th lane, Figure 3.9 E). In the same situation, *Gbx2* expression is induced (Figure 3.9 E). However, treatment with CHX completely blocks Sox9 expression (6th lane, Figure 3.9 E) and partly reduces *Snail2* expression, while no effect on *Gbx2* expression was observed in presence of CHX (6th lane, Figure 3.9 E). These results show that once Wnt signaling is activated, protein synthesis is not required for *Gbx2* up-regulation, indicating that *Gbx2* transcripts are directly regulated by β-catenin.

These results were further confirmed by the *Gbx2* promoter study. From the *Gbx2* promoter sequence, I distinguished three Lef binding site (Figure 3.10 A). *Gbx2* full length promoter construct including all three Lef binding site was fused to GFP coding region (Figure 3.10 B). 0.2ng of this Gbx2 full length GFP construct and 0.8ng of β-catenin-GR were injected into both sides of 2cell stage embryos. DEX was added after injection to activate Wnt/β-catenin pathway. GFP level were monitored throughout early development. At stage 17, a strong GFP expression was detected in neural crest cells with DEX group (Figure 3.10 C. DEX+ 81% n=26). While without DEX, endogenous Wnt signalling can drive the GFP expression but the percentage is very low (DEX- 20% n=34). This suggests that Gbx2 full length promoter contain the target site of Wnt signalling which can drive the constructs expression.

To confirm this more directly, an animal cap experiment was performed. 0.2ng of Gbx2 full length promoter GFP and 0.8 ng of β-catenin-GR were injected into all 4 animal blastmere of 8cell stage. The animal cap tissue was dissected at stage 9 and divided into two groups. One group of animal caps was treated with protein synthesis inhibitor CHX (1µg/ml) at stage 11.5; 30 minutes later DEX (1µg/ml) was added into both groups to activate Wnt/β-catenin pathway. At stage 20, both groups had the GFP fluorescence (Figure 3.10 D). Wnt signalling drove the promoter expression could not be inhibited by protein synthesis inhibitor

could not inhibit. It showed that Wnt signalling drove Gbx2 expression directly.

Next I tested whether neural crest induction by Wnt signalling is *Gbx2* dependent. Injection of 1ng β-catenin-GR (DEX stage 11) leads to an expansion of the neural crest markers *Snail2*, *Pax3* and *Msx1* (Figure 3.11 A-C, G, expansion of *Snail2* expression 73% n=41; *Pax3* 77% n=39; Msx1 84% n=32). This effect is reverted in the presence of *Gbx2* MO (Figure 3.11 D-F, G. inhibition of *Snail2* expression 50% n=34; *Pax3* 34% n=35; *Msx1* 50% n=30).

Similar experiments were performed by injecting of dominant negative Dsh (DD1, 1ng) to block the Wnt signalling pathway. Neural crest markers *Snail2, Pax3* and *Msx1* were inhibited (Figure 3.12 A-C. inhibition of *Snail2* expression 75% n=40; *Pax3* 73% n=37; *Msx1* 84% n=32). However, this inhibition of neural crest markers was rescued by co-injection of *Gbx2* mRNA (Figure 3.12 D-F, G. expansion of *Snail2* expression 38% n=39; *Pax3* 50% n=35; *Msx1* 34% n=31). Taken together these results demonstrate that neural crest induction by Wnt signalling is *Gbx2* dependent.

In summary, *Gbx2* is a direct target of Wnt activation and one of the earliest activated transcription factors of this pathway during neural crest induction.

3.5 *Gbx2* is upstream in the neural crest genetic cascade

As I discussed before, *Pax3, Msx1* and *Zic1* have been proposed as the first factors activated by secreted inducing signals in the genetic cascade controlling neural crest development (Mayor and Aybar, 2001; Meulemans and Bronner-Fraser, 2004; Steventon et al., 2005). Since *Gbx2* is a direct target of the neural crest inducer Wnt, I asked whether *Gbx2* was upstream of *Pax3, Msx1* and *Zic1*.

All the embryos in this part were injected in animal blastomeres at the 8-cell stage where indicated in the small inset, and fixed at stage 14-15. In situ hybridization was performed for different markers as indicated in each figure.

Loss of *Gbx2* (16ng *Gbx2* MO injection) leads to inhibition of early neural plate border specifiers *Msx1* and *Pax3* expression together with the inhibition of neural crest markers *Snail2* and *FoxD3* (Figure 3.13 A-D. *Snail2* 73% n=37; *FoxD3* 68% n=34; *Pax3* 74% n=35; *Msx1* 71% n=35), while over-expression of *Gbx2* mRNA (1ng *Gbx2* mRNA injection) produces an expansion of these markers (Figure 3.13 F-I. *Snail2* 74% n=39; *FoxD3* 78% n=40; *Pax3* 71% n=38; *Msx1* 65% n=40). However, there is no effect on *Zic1*expression after any of these loss and gain of *Gbx2* treatments (Figure 3.13 E, J. no effect on *Zic1* by

Gbx2 MO n=35; *Gbx2* n=40); which is consistent with the Wnt-independent regulation of *Zic1* (Hong and Saint-Jeannet, 2007; Sato et al., 2005).

Next, I performed a series of epistasis experiments aimed to test whether *Gbx2* was upstream of *Pax3*. Inhibition of *Pax3*, by injecting 20ng *Pax3* MO (Sato et al., 2005), generates loss of the neural crest marker *Snail2* but does not affect *Gbx2* expression (Figure 3.14 A, C, I. *Snail2* 77% n=35; *Gbx2* 0% n=35), while injection of *Pax3* mRNA produces an expansion of *Snail2* without changing *Gbx2* expression (Figure 3.14 B, D, I. *Snail2* 88% n= 33; *Gbx2* 0% n=30). These results suggest that *Pax3* might be downstream of *Gbx2* in neural crest induction. Furthermore, inhibition of neural crest markers *Snail2* and *FoxD3* by *Pax3* MO cannot be rescued by *Gbx2* co-injection (Figure 3.14 E, G, I. inhibition of *Snail2* 65% n=40; *FoxD3* 63% n=38), while *Pax3* mRNA injection rescues inhibition of neural crest markers by *Gbx2* MO (Figure 3.14 F, H, I. expansion of *Snail2* 74% n= 35; 67% n=39). All these experiments confirmed that *Pax3* is downstream of *Gbx2* in the neural crest genetic cascade (Figure 3.14 J).

Similar epistasis experiments were performed for *Msx1*. Inhibition of *Msx1* by expression of 1ng *Msx1* dominant negative, HD-Msx1 (Tribulo et al., 2003) represses neural crest induction without affecting *Gbx2*

expression (Figure 3.15 A, C, I. *Snail2* 68% n=37; *Gbx2* 0% n=33). In contrast, injection of 1ng *Msx1* mRNA expands neural crest markers expression without affecting *Gbx2* expression (Figure 3.15 B, D, I. *Snail2* 70% n=30; *Gbx2* 0% n=29). In addition, *Gbx2* is not able to rescue the effect of HD-Msx1 on neural crest markers *Snail2* and *FoxD3* (Figure 3.15 E, G, I. inhibition of *Snail2* 73% n=30; *FoxD3* 70% n=33), while *Msx1* rescues the expression of *Snail2* and *Foxd3* inhibited by *Gbx2* MO (Figure 3.15 F, H, I. expansion of *Snail2* 61% n= 31; 69% n=29). These results indicate that *Msx1* is downstream of *Gbx2* in the neural crest regulatory network (Figure 3.15 J).

3.6 *Gbx2* interacts with *Zic1* to induce neural crest

I have shown that *Gbx2* is essential for neural crest formation in the posterior neural folds. However, my data suggest that *Gbx2* is not sufficient for neural crest induction, as its over-expression can only expand the neural crest in the neural fold region. For this reason, I hypothesize that *Gbx2* may interact with another factor in the neural folds to induce neural crest. As there is evidence that attenuation of BMP signaling is necessary for neural fold specification (Marchant et al., 1998; Nguyen et al., 1998; Streit and Stern, 1999), I reasoned that a factor induced by BMP attenuation could be also required together with *Gbx2* for neural crest induction.

To test my hypothesis, the following experiments were undertaken. Embryos were injected into animal blastomeres at 8-cell stage with a series of combinations (1ng tBR, 1ng tBR + 1ng Wnt8, 1ng tBR + 1ng *Gbx2*, and 1ng tBR + 1ng Wnt8 + 10ng *Gbx2* MO). The animal cap explants were dissected at stage 9 and cultured in NAM 3/8 until stage 17. After mRNA was extracted, RT-PCR was performed for *Gbx2*, neural crest markers *Pax3* and *FoxD3*, the epidermal marker *EpK* and the neural marker *Nrp1*.

Only the neural marker *Nrp1* is induced in animal caps by BMP inhibition alone, without neural crest markers (lane 3, Figure 3.16 A). However, inhibition of BMP and Wnt activation leads to the induction of neural crest markers as well as *Gbx2* (lane 4, Figure 3.16 A). As I previously showed *Gbx2* is a direct target of Wnt, neural crest marker up-regulation is observed following BMP inhibition and in the presence of *Gbx2* (lane 5, Figure 3.16 A). The neural crest makers are completely abolished when *Gbx2* is inhibited by *Gbx2* MO (6th lane, Figure 3.16 A). Taken together these experiments indicate that attenuation of BMP induces a factor that in turn interacts with *Gbx2*, which is induced by Wnt signalling, to activate neural crest markers expression.

In order to identify the unknown factor I tested several candidate genes expressed in the early neural folds for their ability to induce neural crest markers when co-expressed with *Gbx2* in animal caps. From these, only *Zic1* was able to do so (Figure 3.16 B). Embryos were injected into animal four blastomeres at 8-cell stage with *Gbx2* and different amounts of *Zic1* mRNAs (amounts of *Gbx2* and *Zic1* mRNA is indicated in Figure 3.16 B). The animal cap explants were dissected at stage 9 and cultured in NAM 3/8 until stage 17. Then mRNA was extracted and RT-PCR was performed for preplacodal marker (*Six1*) and neural crest markers (*Pax3*, *Snail2* and *FoxD3*). Our results show that preplacodal marker *Six1*, but not neural crest markers, can be induced by *Zic1*

expression (lane 4, Figure 3.16 B), and this preplacodal specification can be transformed into neural crest specification by expression of *Gbx2* (lane 5-7, Figure 3.16 B). I tested different levels of *Zic1*; even the lowest level of *Zic1* in presence with *Gbx2* can induce neural crest markers. All these results suggest that *Zic1* is at least one of the factors that interact with *Gbx2* to induce neural crest in the posterior neural fold.

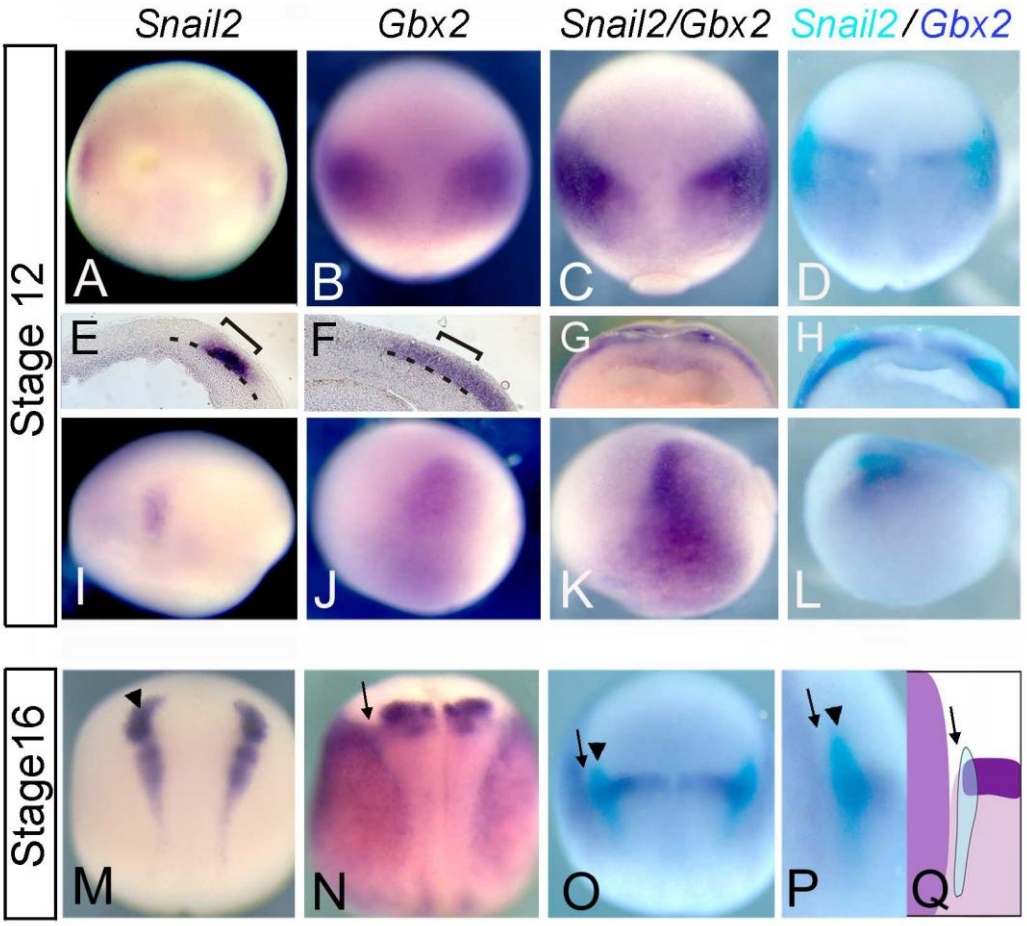

Figure 3.1 *Gbx2* is expressed in the prospective neural crest

Figure 3.1 *Gbx2* is expressed in the prospective neural crest.

(A-L) Stage 12 embryos; (M-P) Stage 16 embryos.

(A-D) Dorsal view, anterior to the top.

(E-H) Transverse sections. (E, F) Histological sections.

(I-L) Lateral view, anterior to the left.

(M-O) Dorsal view, anterior to the top. Arrowhead: neural crest; arrow: gap in *Gbx2* expression.

(A, E, I, M) In situ hybridization for Neural crest marker Snail2.

(B, F, J, N) In situ hybridization for Gbx2.

(C, G, K) In situ hybridization for Snail2 and Gbx2 together, Gbx2 domain are overlap with Snail2 domain.

(D, H, L, O, P) Double In situ hybridization for Snail2 and Gbx2; Snail2 light green and Gbx2 dark purple.

(P) Detail of the neural fold region in a lateral view, anterior to the top, midline to the right.

(Q) Summary of *Gbx2* and *Snail2* expression at stage 16. Anterior to the top, midline to the right. Different tones of purple denote different levels of *Gbx2* expression. Blue: neural crest. Note that there is a reduction in the level of *Gbx2* in the neural plate (pale purple), but this region still overlaps with the neural crest marker. A small gap (arrow) has been generated between the epidermal and the neural plate domains of *Gbx2* expression. The most anterior cephalic neural crest has lost *Gbx2* at this stage.

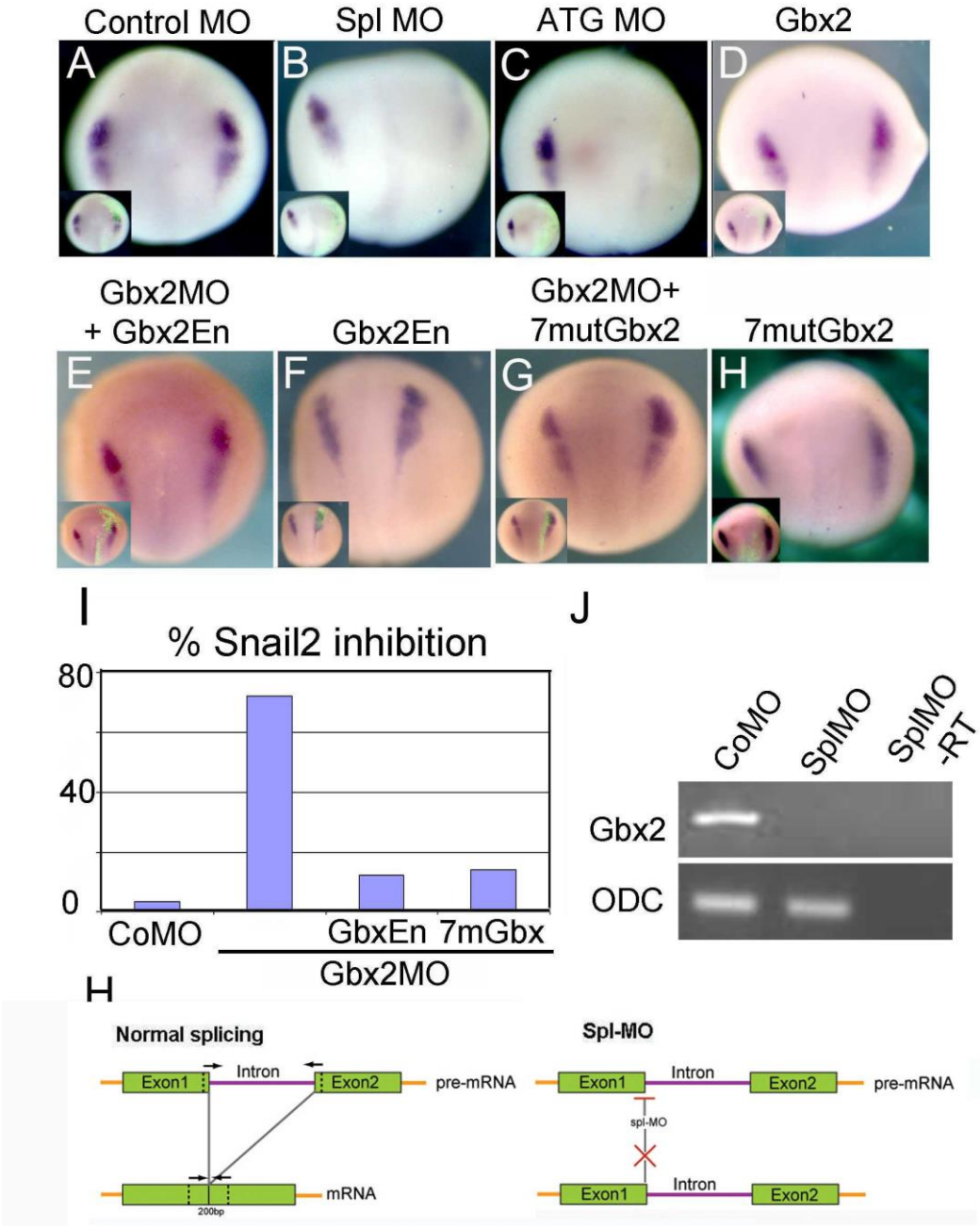

Figure 3.2 *Gbx2* is required for neural crest induction

Figure 3.2 *Gbx2* is required for neural crest induction.

Embryos were injected in animal blastomeres at the 8-cell stage with the indicated constructs. All *Snail2* in situ hybridizations are shown in dorsal view with anterior to the top. Inset: overlay of in situ hybridization and fluorescence to show the injected side to the right.

(A) 20ng of control MO.

(B) 20ng of Gbx2 Splicing MO.

(C) 16ng of Gbx2 translational MO.

(D) 1ng of Gbx2 mRNA.

(E) 16ng of Gbx2 translational MO and 1ng of Gbx2EnR-GR. Dex was added at stage 10.

(F) 1ng of Gbx2EnR-GR. Dex was added at stage 10.

(G) 16ng of Gbx2 translational MO and 1ng of 7 mismatches Gbx2 mRNA.

(H) 1ng of 7 mismatches Gbx2 mRNA.

(I) Summary of rescue experiment showing percentage of embryos with *Snail2* expression inhibition.

(J) Efficiency of splicing MO. RT-PCR of Embryos injected with 20ng of control MO or 20ng of Gbx2 Splicing MO. Gbx2 and ODC were analyzed. ODC: loading control.

(K) Mechanism of Splicing MO. 200bp band was produced by normal splicing. Exon1 was blocked by splicing MO.

Figure 3.3 Timing of *Gbx2* affect neural crest marker *Snail2*

Figure 3.3 Timing of *Gbx2* affect neural crest marker *Snail2*

1ng of Gbx2-EnR-GR was injected into half embryos at 2cell stage. Dex was added to activate this construct at 4cell stage; stage 10; stage 12; stage 14. Neural crest marker Snail2 was affected by early activation of Gbx2. But at stage 14 no affect.

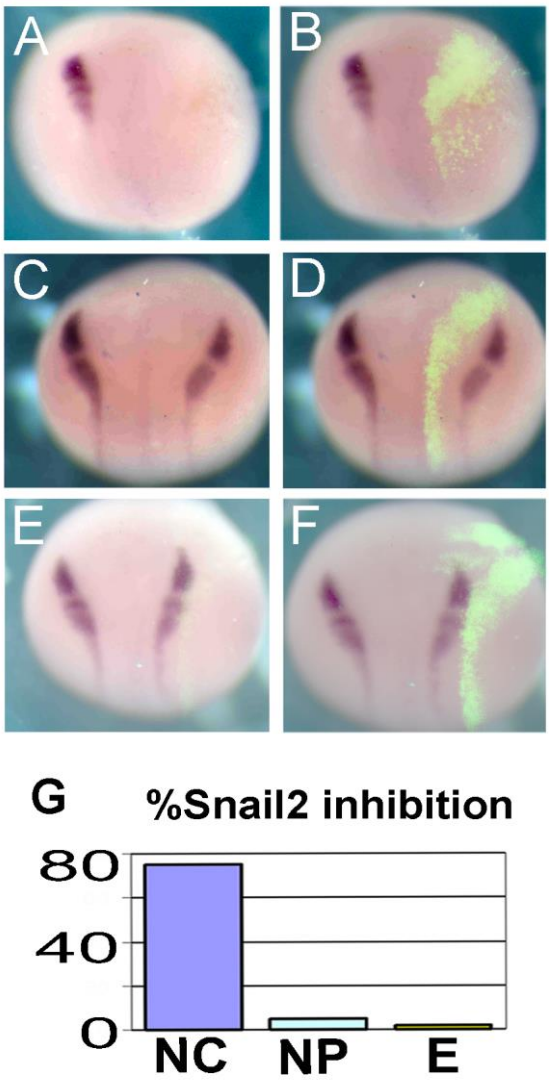

Figure 3.4 Targeted injection of Gbx2 translational MO

Figure 3.4 Targeted injection of Gbx2 translational MO

(A-C) Targeted injections of Gbx2 translational MO. A1, A3 or A4 blastomeres were injected with Gbx2 MO to target neural plate, neural crest or epidermis, respectively. All *Snail2* in situ hybridizations are shown in dorsal view with anterior to the top.

(A) Target neural crest injection. Snail2 was abolished by Gbx2; (B) Fluorescing showed the injected area of same embryos.

(C) Target neural plate injection. Snail2 has no effect. (D) Fluorescing showed the injected area of same embryos.

(E) Target epidermal injection. Snail2 has no effect. (F) Fluorescing showed the injected area of same embryos.

(G) Summary of targeted injection, showing % of Snail2 inhibition after injecting in prospective neural crest (NC), neural plate (NP) or epidermis (E) Only injected Gbx2 into neural crest region can affect Snail2 expression.

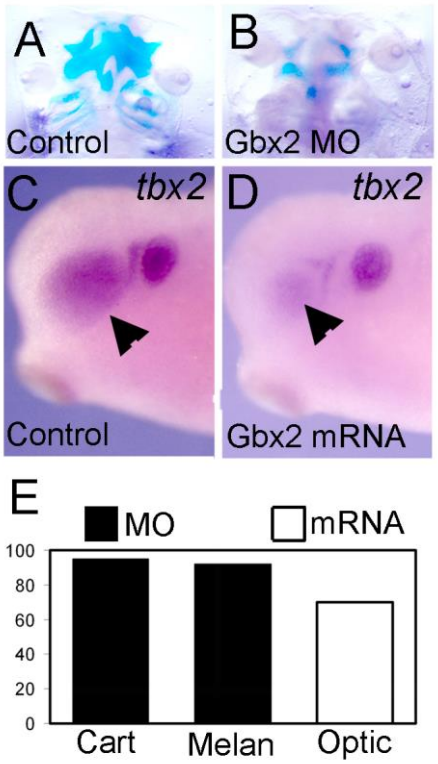

Figure 3.5 *Gbx2* is required for neural crest and posterior placode derivatives

Figure 3.5 *Gbx2* is required for neural crest and posterior placode derivatives

Embryos were injected at the 2-cell stage.

(A, B) Cartilage staining of stage 40 injected embryos.

(C, D) Placode marker Tbx2 of stage 28 injected embryos.

(A) Injected bilaterally with 20ng control MO, no effect on cartilage.

(B) Injected bilaterally with 16ng Gbx2 MO, reduction of cartilage.

(C, D) Injected into half of embryos with 16ng Gbx2 mRNA.

(C) un-injected side; (D) injected side, a reduction of optic placode. Arrow: optic placode.

(E) Summary of effect by Gbx2 mis-expression. Neural crest derivatives were reduced by loss of Gbx2. Optic placode was inhibited by Gbx2 gain of function.

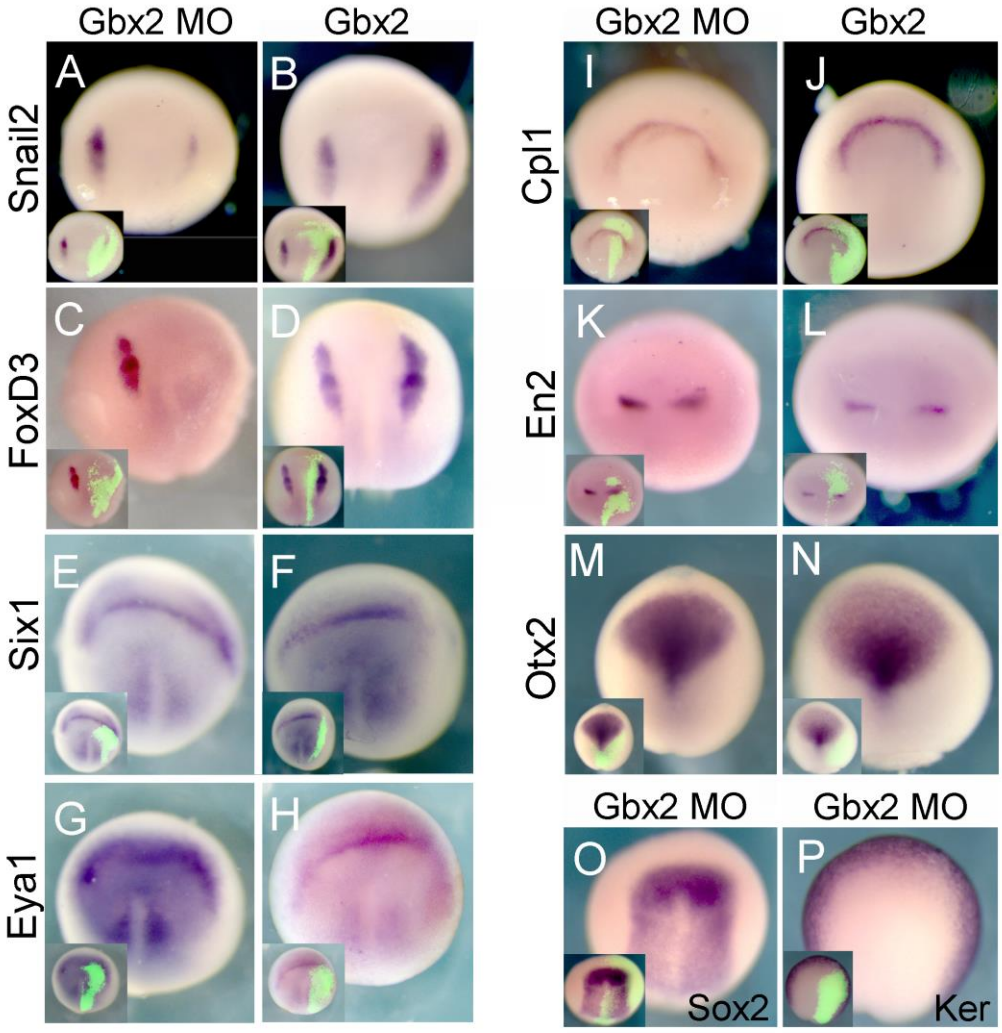

Figure 3.6 *Gbx2* is required for the posteriorization of neural folds

Figure 3.6 *Gbx2* is required for the posteriorization of neural folds

All embryos were injected in animal blastomeres at the 8-cell stage with 16ng Gbx2 MO or 1ng Gbx2 mRNA as indicated.

(A, B) Neural crest marker *Snail2* expression.

(C, D) Neural crest marker *FoxD3* expression.

(E, F) Preplacode marker *Six1* expression.

(G, H) Preplacode marker *Eya1* expression.

(I, J) *Cpl1* expression.

(K, L) *En2* expression.

(M, N) *Otx2* expression.

(O) *Sox2* expression.

(P) *Keratin* expression.

Note: Misexpression of Gbx2 have very strong effect on neural fold (neural crest and preplacode) markers, but no effect on neural plate markers and epidermal marker.

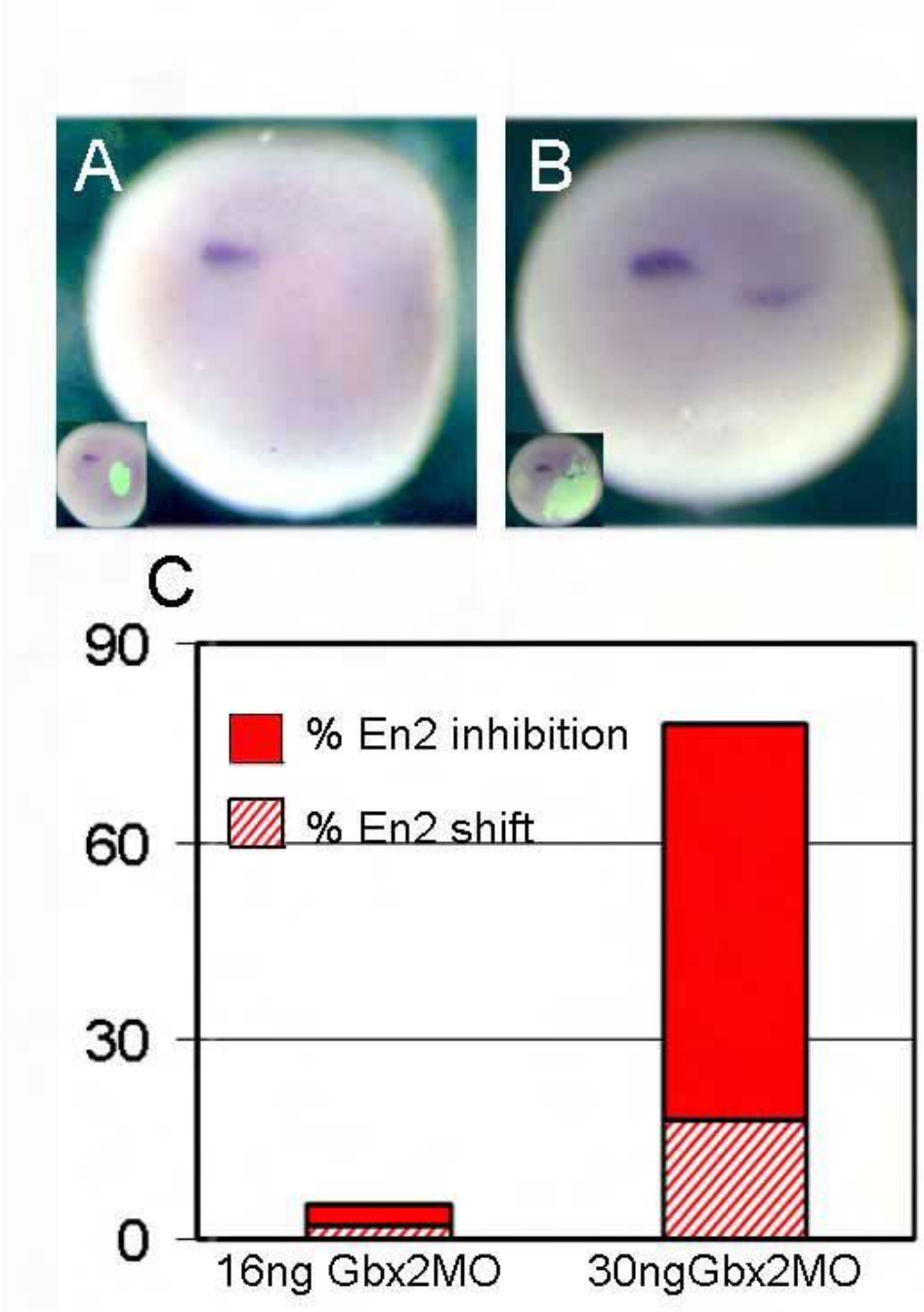

Figure 3.7 High levels of Gbx2 MO mis-lead the neural plate AP patterning

Figure 3.7 High levels of Gbx2 MO mis-lead the neural plate AP patterning

(A, B) 30ng of Gbx2 MO injection, *En2* expression

(C) Summary of Gbx2 MO affect on neural plate anterior-posterior pattering. 16ng Gbx2 MO affect was described in Figure 3.5. Note only the higher level of MO leads to effect on *En2* expression.

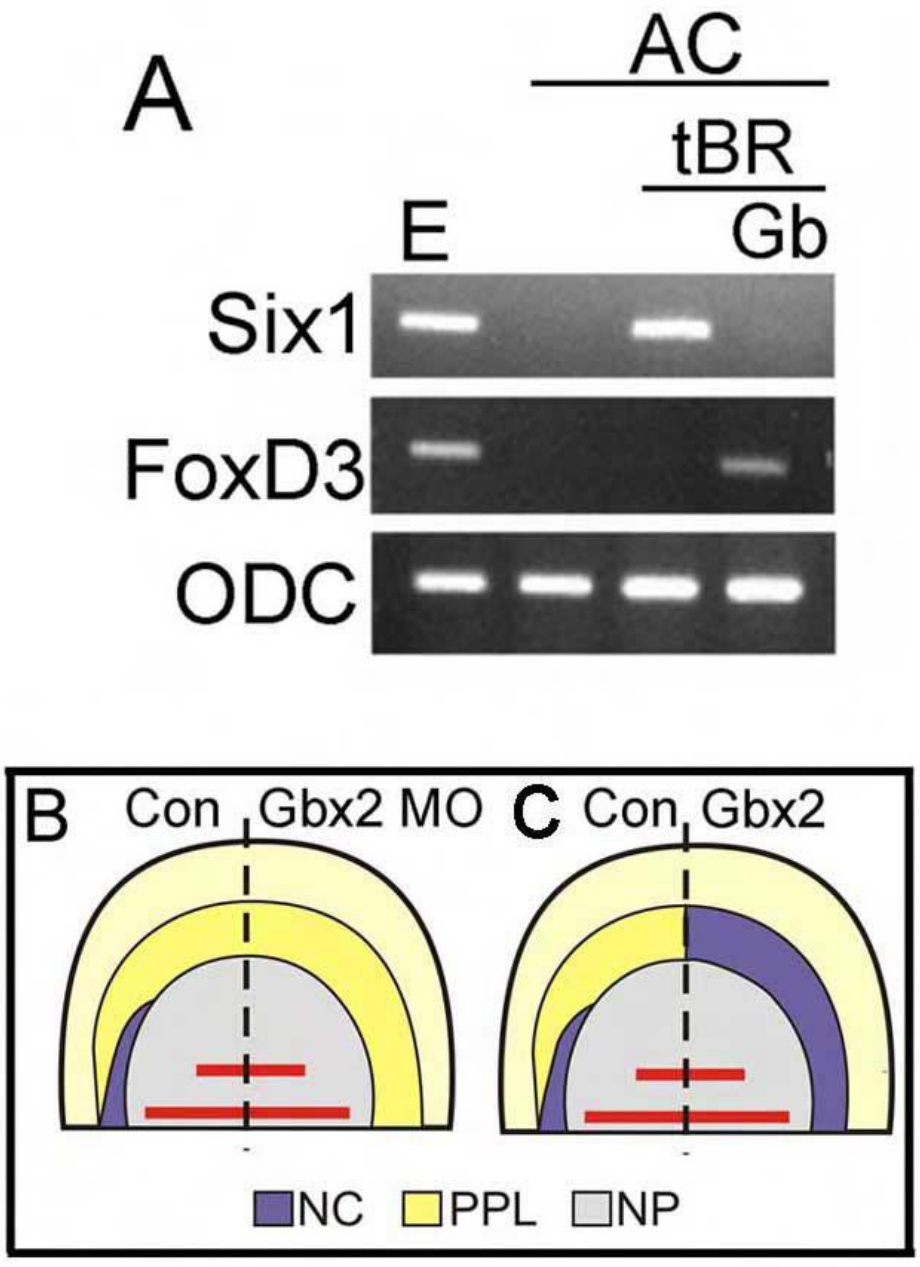

Figure 3.8 Gbx2 transform preplacodal into neural crest

Figure 3.8 Gbx2 transform preplacodal into neural crest

(A) RT-PCR of animal caps analyzing *Six1* and *FoxD3* expression. ODC: loading control. E: Whole embryo; AC: Animal cap; tBR: 2ng of dominant negative of BMP4 receptor; Gb: 1ng of Gbx2 mRNA.

(B, C) Summary of neural fold and neural plate phenotypes were produced by Gbx2 MO (B) and Gbx2 mRNA (C). Anterior part of the embryo is represented, with left side as control and right side as the injected one.

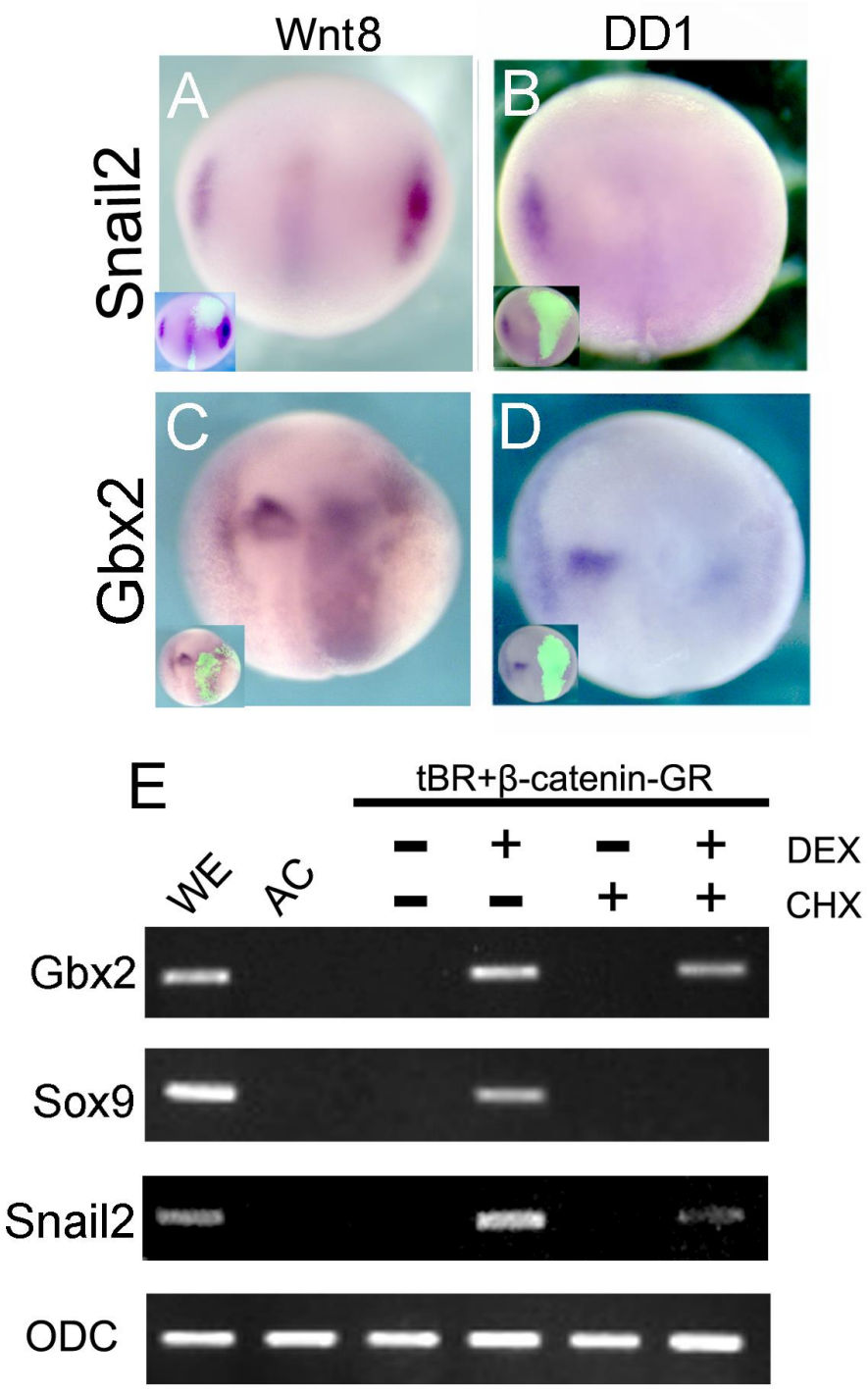

Figure 3.9 *Gbx2* is a direct target of Wnt signaling.

Figure 3.9 *Gbx2* is a direct target of Wnt signaling

(A, C) Embryo injected in animal blastomeres of an 8-cell stage embryo with 1ng of Wnt8 mRNA.

(B, D) Embryo injected with 1ng of DD1 mRNA into animal blastomere of an 8-cell embryo.

(A, B) *Snail2* expression.

(C, D) *Gbx2* expression.

(E) RT-PCR of animal caps analyzing *Gbx2, Snail2* and *Sox9* expression. ODC: loading control. WE: Whole embryo; AC: Animal cap; tBR: 1ng of dominant negative of BMP4 receptor; β-catenin-GR: 1ng of inducible β-catenin. DEX added at stage 11.5. CHX added 0.5hr before DEX.

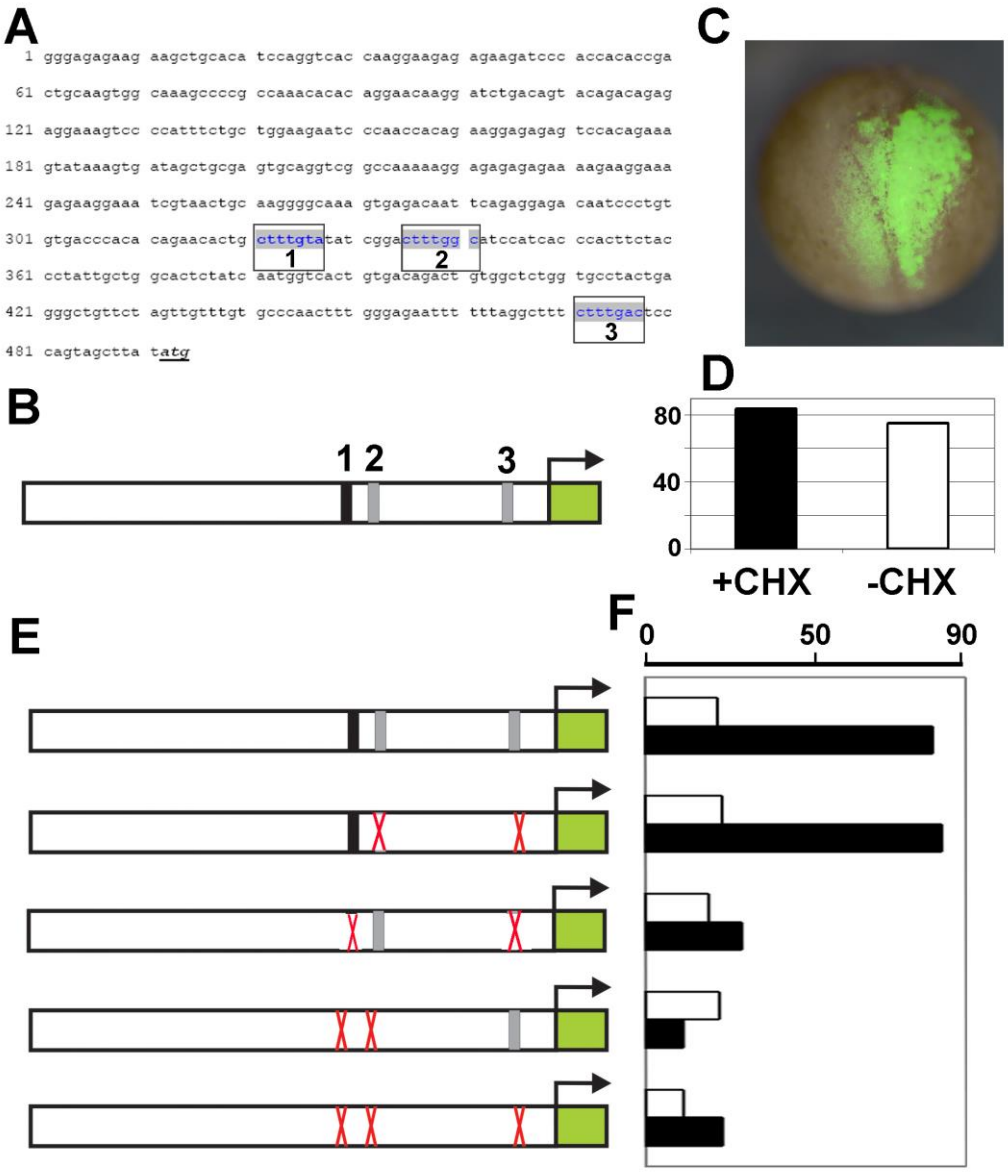

Figure 3.10 Neural crest induction by Wnt is Gbx2 dependent

Figure 3.10 Neural crest induction by Wnt is Gbx2 dependent

Embryos were injected in all 4 animal blastomeres at the 8-cell stage.

(A) Sequence of Gbx2; 1, 2, 3: Tcf binding side.

(B) Full length of Gbx2, contain all three Tcf binding side, fused with GFP domain.

(C) Embryos were injected with 1ng construct showed in B. Florescence could be seen at stage 15.

(D) Embryos were injected with 1ng construct showed in B and 0.2 ß-cartnin-GR. Animal cap was dissected at stage 9, CHX was added at stage 11.5 and 30min later DEX was add, check the florescence of animal cap at stage 20. CHX: protein synthesis inhibitor.

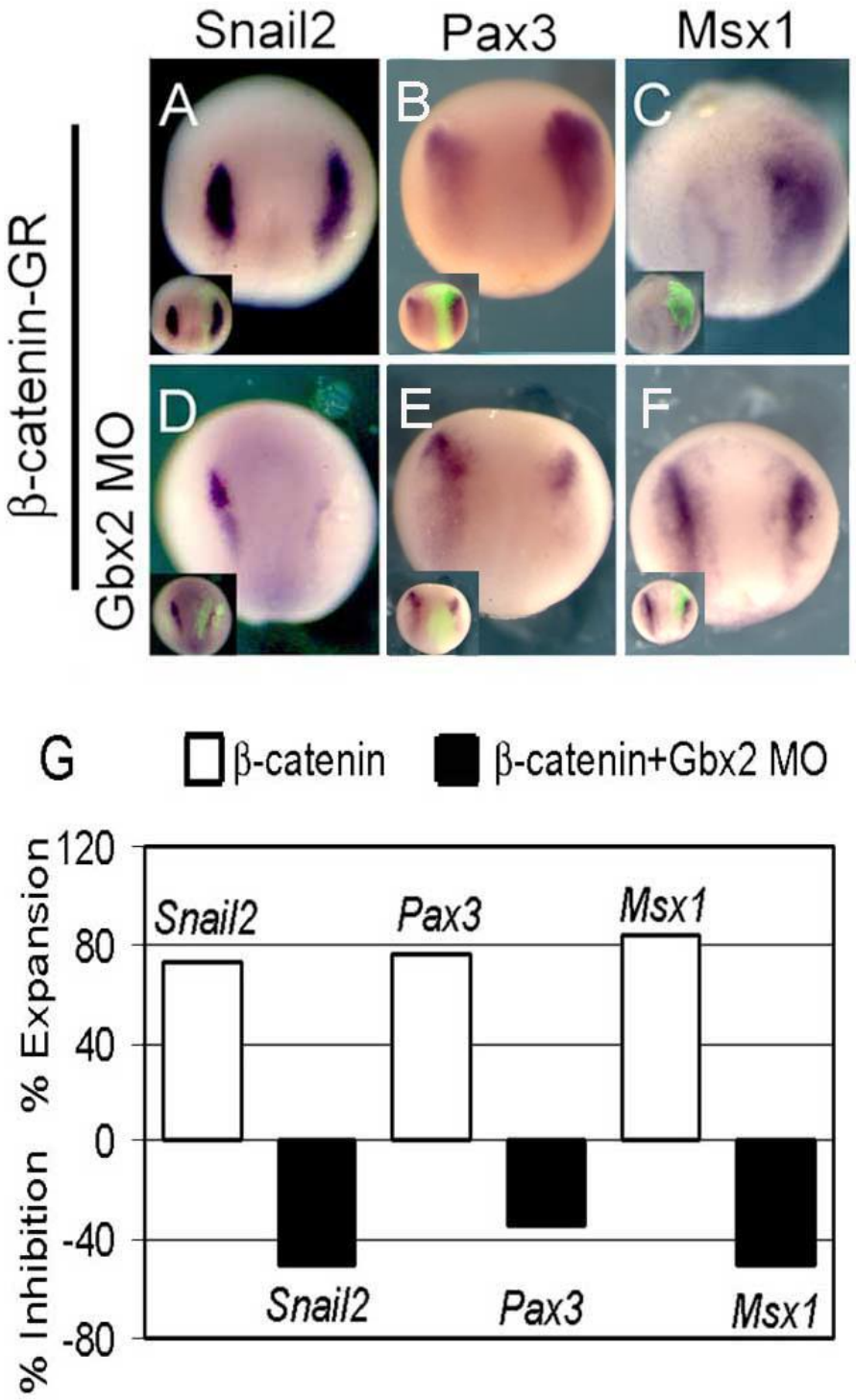

Figure 3.11 Gbx2 is downstream of Wnt in neural crest induction

(A)

Figure 3.11 Gbx2 is downstream of Wnt in neural crest induction (A)

Embryos were injected in animal blastomeres at the 8-cell stage as indicated.

(A-C) 1ng of β-catenin-GR injection and induced at stage 10 with DEX.

(D-F) 1ng of β-catenin-GR injection, induced at stage 10 with DEX and 16ng of Gbx2 MO.

(A, D)The expression of *Snail2*

(B, E) The expression of *Pax3*

(C, F) The expression of *Msx1*

(G) Summary the percentage for expansion or inhibition of different neural crest markers. Note that the expansion of neural crest markers induced by β-catenin is reversed by co-injection with Gbx2 MO.

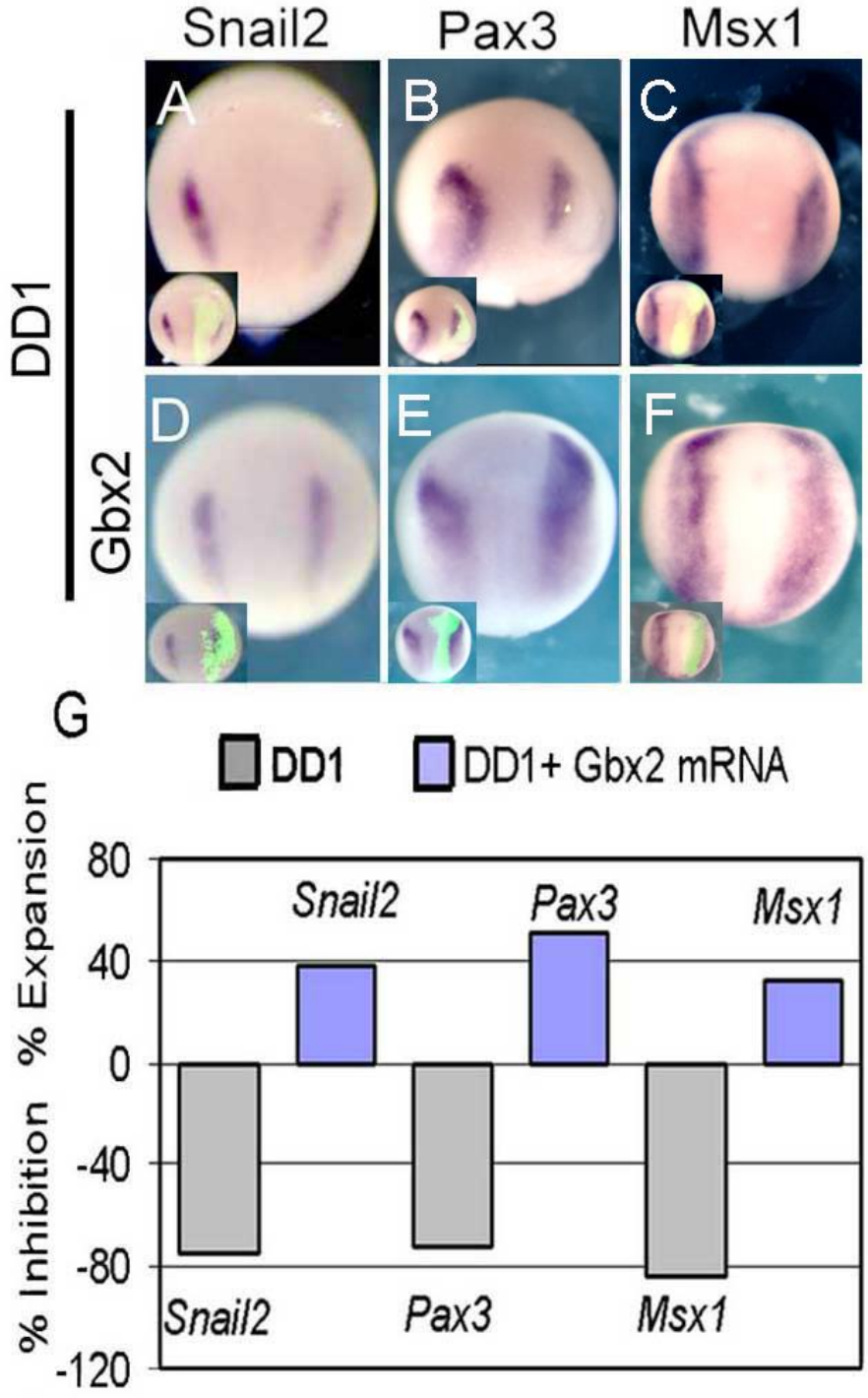

Figure 3.12 Gbx2 is downstream of Wnt in neural crest induction

(B)

Figure 3.12 Gbx2 is downstream of Wnt in neural crest induction (B)

Embryos were injected in animal blastomeres at the 8-cell stage as indicated.

(A-C) 1ng of Dsh dominant negative DD1 injection

(D-F) 1ng of Dsh dominant negative DD1 and 1ng of Gbx2 mRNA injection

(A, D) The expression of *Snail2*

(B, E) The expression of *Pax3*

(C, F) The expression of *Msx1*

(G) Summary the percentage for expansion or inhibition of different neural crest markers. Note that the inhibition of neural crest markers produced by DD1 is reversed by co-injection with Gbx2 mRNA.

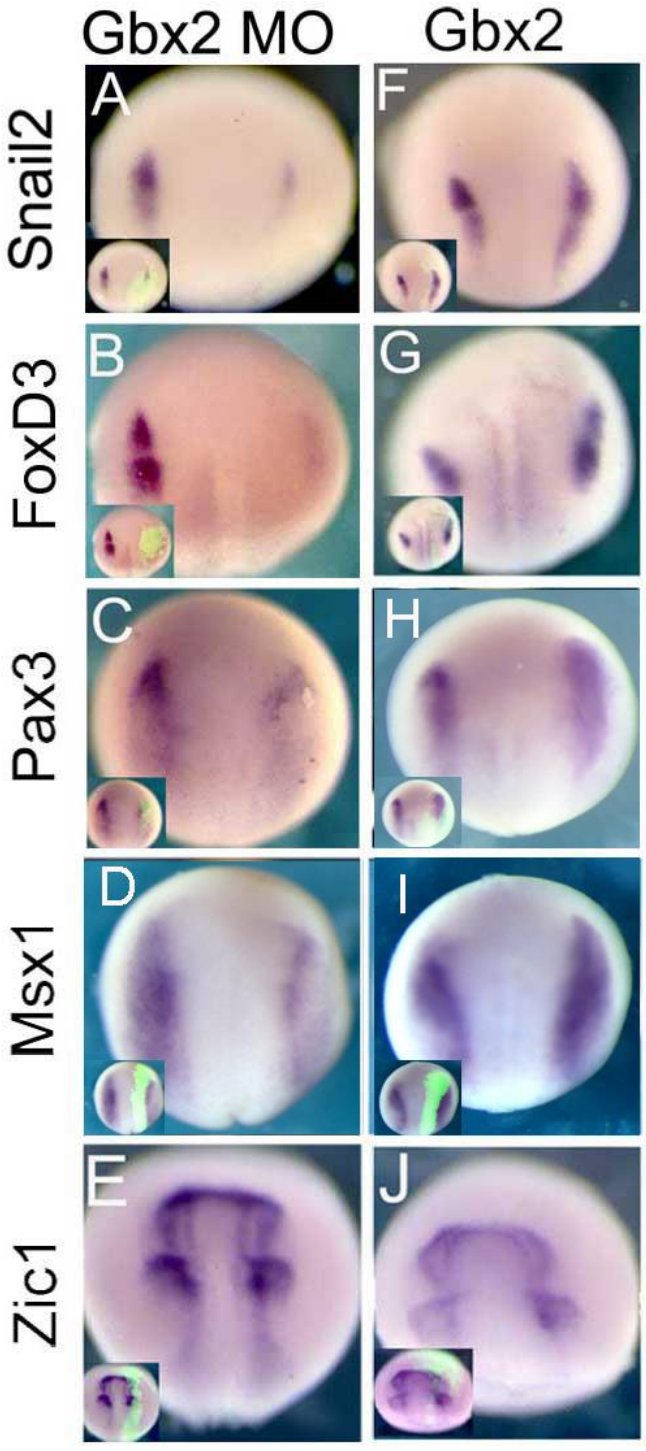

Figure 3.13 *Gbx2* is required in neural crest induction genetic cascade

Figure 3.13 *Gbx2* is required in neural crest induction genetic cascade

Embryos were injected in animal blastomeres at the 8-cell stage as indicated.

(A-E) 16ng of Gbx2 MO injection.

(F-J) 1ng of Gbx2 mRNA injection.

(A, F) The expression of *Snail2.*

(B, G) The expression of *FoxD3.*

(C, H) The expression of *Pax3.*

(D, I) The expression of *Msx1.*

(E, J) The expression of *Zic1.*

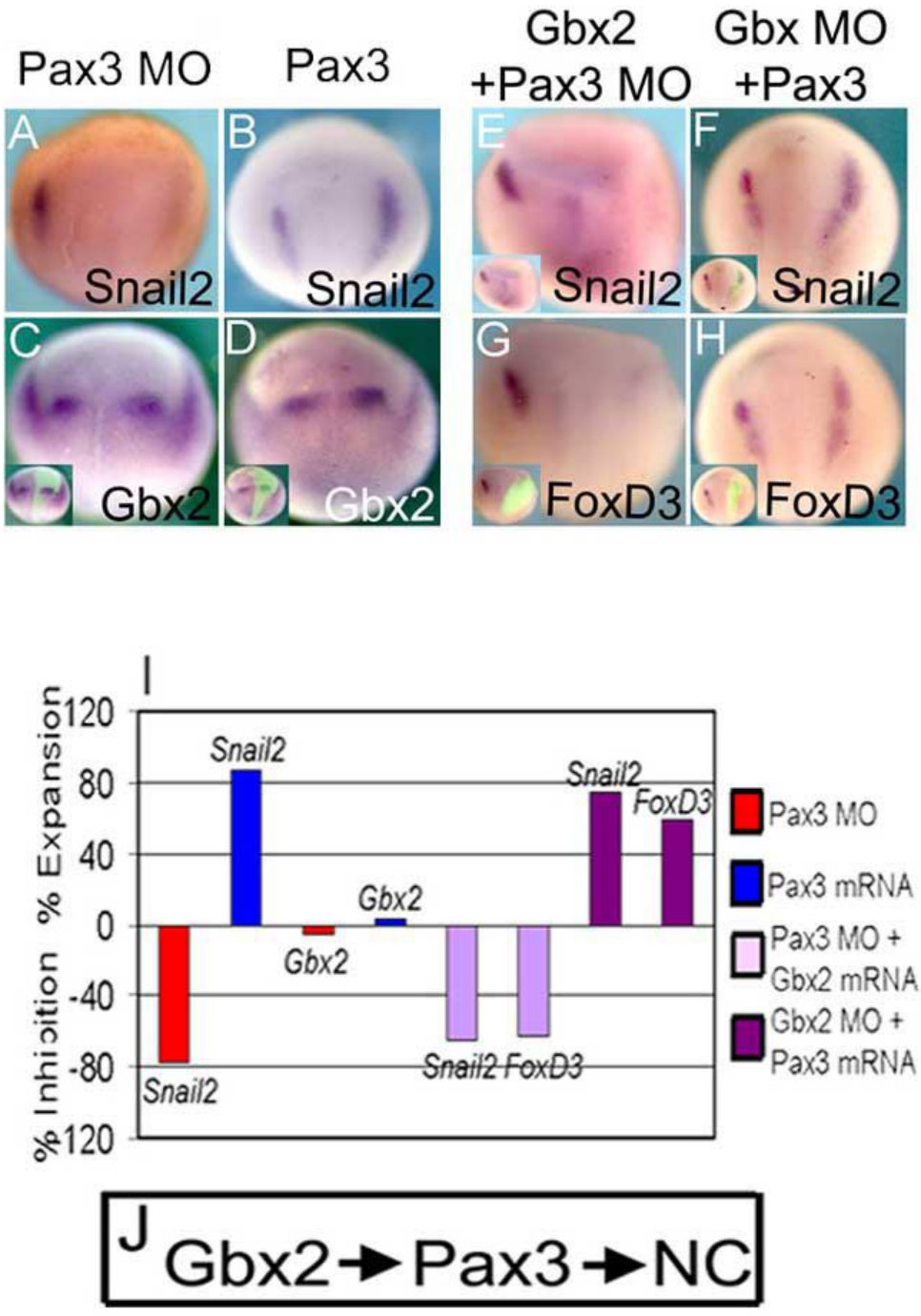

Figure 3.14 *Gbx2* is upstream of *Pax3* in neural crest induction genetic cascade

Figure 3.14 *Gbx2* is upstream of *Pax3* in neural crest induction genetic cascade

Embryos were injected in animal blastomeres at the 8-cell stage as indicated.

(A, C) 20ng of Pax3 MO injection.

(B, D) 1ng of Pax3 mRNA injection.

(E, G) 20ng of Pax3 MO and 1ng of Gbx2 mRNA injection.

(F, H) 16ng of Gb2 MO and 1ng of Pax3 mRNA injection.

(A, B) The expression of *Snail2.*

(C, D) The expression of *Gbx2.*

(E, F) The expression of *Snail2.*

(G, H) The expression of *FoxD3.*

(I) Summary percentage of phenotypes in epistasis experiments.

(J) Summary neural crest induction genetic cascade: *Gbx2* is upstream of *Pax3*.

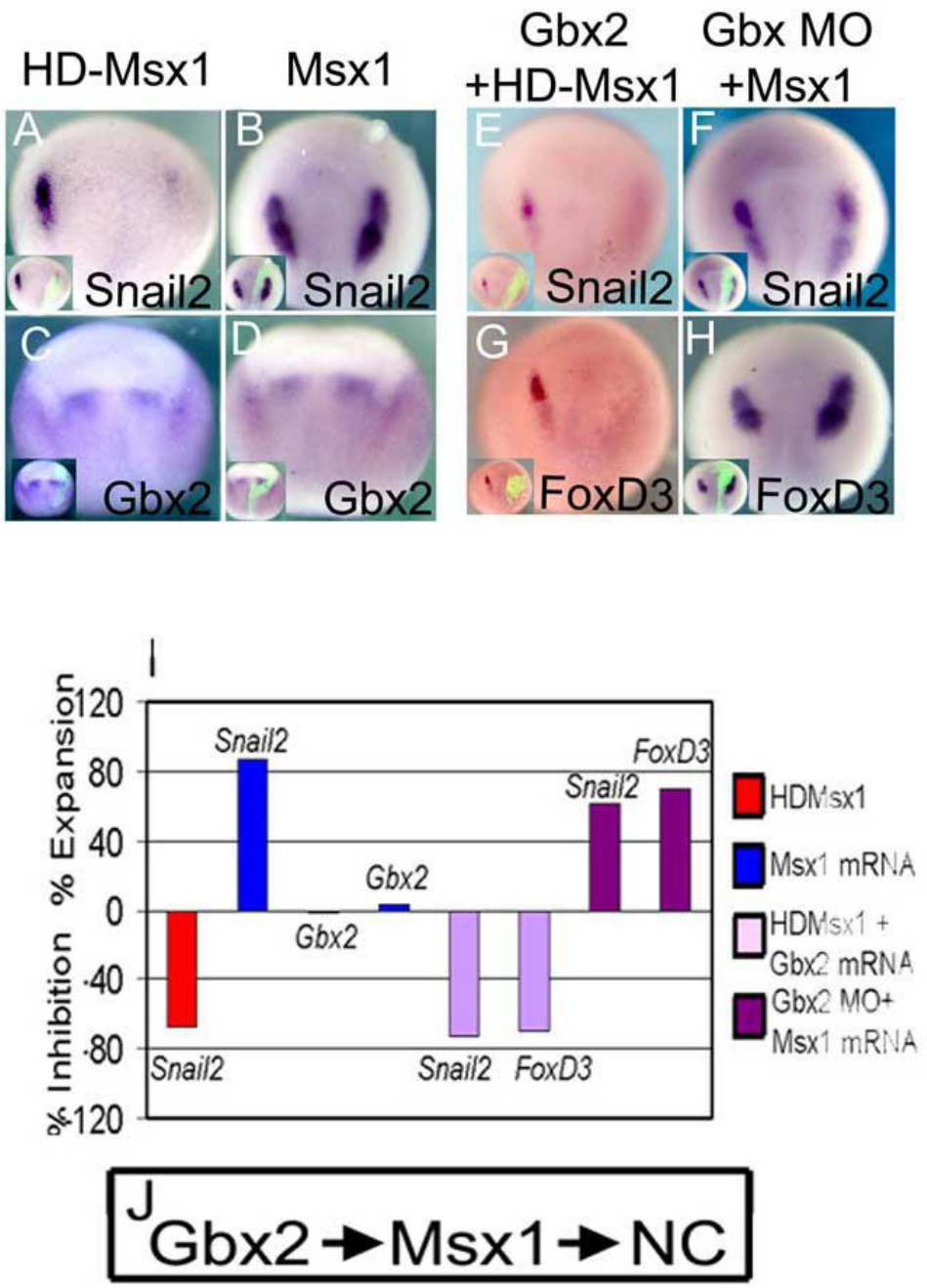

Figure 3.15 *Gbx2* is upstream of *Msx1* in neural crest induction genetic cascade

Figure 3.15 *Gbx2* is upstream of *Msx1* in neural crest induction genetic cascade

Embryos were injected in animal blastomeres at the 8-cell stage as indicated.

(A, C) 1ng of HD-Msx1 mRNA injection.

(B, D) 1ng of Msx1 mRNA injection.

(E, G) 1ng of HD-Msx1 mRNA and 1ng of Gbx2 mRNA injection.

(F, H) 16ng of Gbx2 MO and 1ng of Msx1 mRNA injection.

(A, B) The expression of *Snail2.*

(C, D) The expression of *Gbx2.*

(E, F) The expression of *Snail2.*

(G, H) The expression of *FoxD3.*

(I) Summary percentage of phenotypes in epistasis experiments.

(J) Summary neural crest induction genetic cascade by *Gbx2*: *Gbx2* is upstream of *Msx1*.

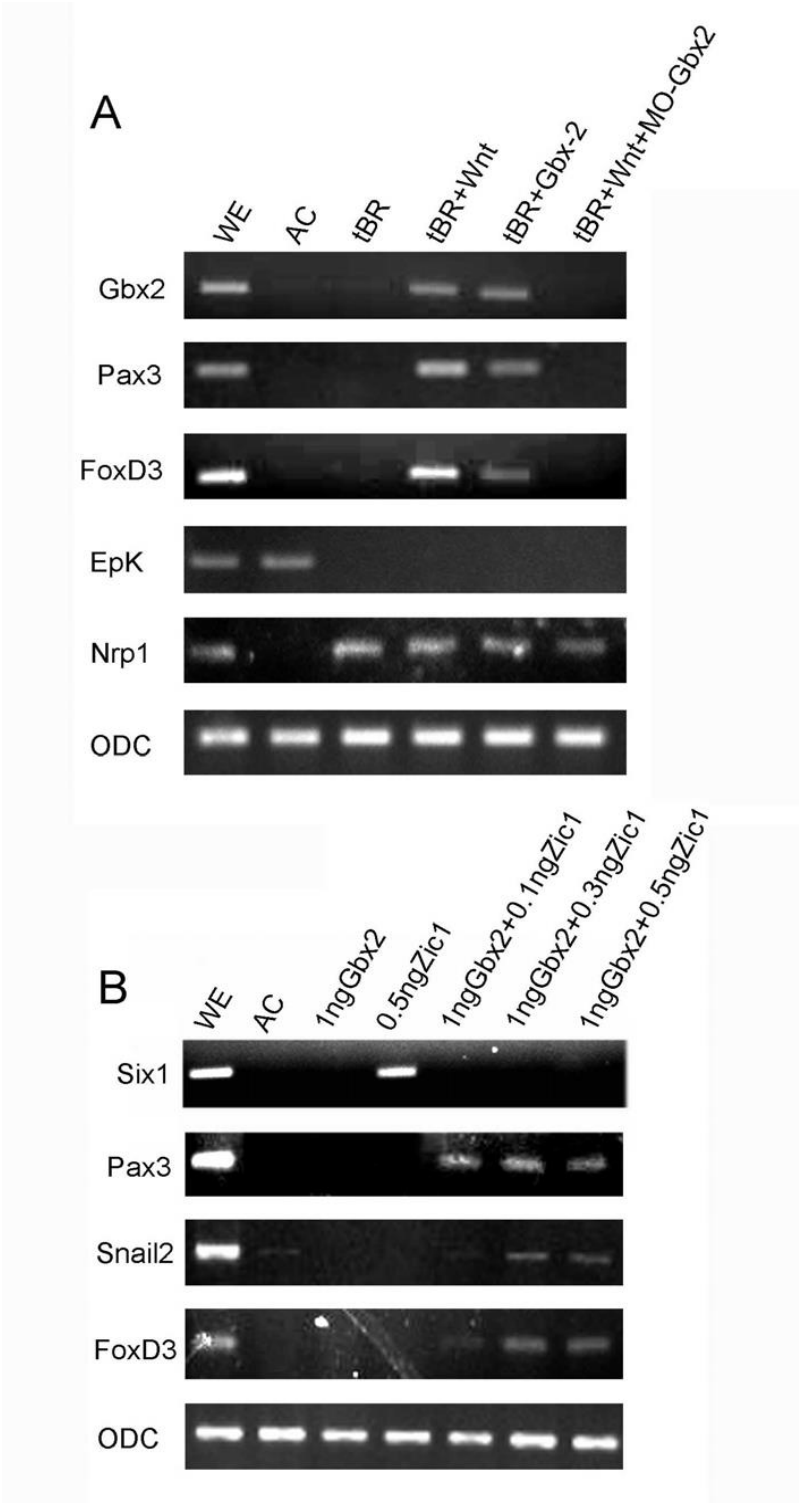

Figure 3.16 Interaction between *Gbx2* and *Zic1* induces neural crest

Figure 3.16 Interaction between *Gbx2* and *Zic1* induces neural crest

(A, B) RT-PCR of animal caps analyzing the expression of the indicated genes. ODC: loading control. WE: Whole embryo; AC: Animal cap

(A) *Gbx2* interacts with a factor induced by attenuation of BMP activity. tBR: 1ng of dominant negative of BMP4 receptor; Wnt: 1ng of Wnt8 mRNA; *Gbx2*: 1ng of *Gbx2* mRNA; Gbx2 MO: 8ng of Gbx2 MO.

(B) Interaction of *Gbx2* and *Zic1* induces neural crest. Animal caps expressing the indicated amounts of Gbx2 and Zic1 mRNA.

Chapter Four: Discussion

4.1 Neural crest induction model

I propose the following model of neural crest induction (Figure 4.1 A-D). Initially, a specific level of BMP activity induces *Zic1* along the entire neural plate border (Figure 4.1 A, D). The direct regulation of *Zic1* by BMP has been previously reported (Hong and Saint-Jeannet, 2007; Mizuseki et al., 1998; Rohr et al., 1999; Tropepe et al., 2006). Then, Wnt signalling directly induces the expression of *Gbx2* in the posterior region of the embryo (Figure 4.1 B, D). *Zic1* by itself induces *Six1* and specifies the preplacodal domain next to the anterior neural folds (Figure 4.1 C, D); while *Zic1* in combination with *Gbx2* induces neural crest in the posterior neural folds, where both genes are co-expressed (Figure 4.1 C, D). In addition, *Gbx2* inhibits *Six1* expression and the PPR domain (Figure 4.1 D). In summary the presence of *Gbx2* at the neural plate border defines the region that becomes neural crest and its absence the region develops into placode territory.

4.2 *Gbx2* is upstream of the neural crest genetic cascade

Since the discovery of the transcription factor *Slug* (now named as *Snail2*) more than 10 years ago (Mayor et al., 1995; Nieto et al., 1994), more than a dozen transcription factors required for neural crest development have been identified. Several attempts to organize these factors in a genetic cascade have been performed (Mayor and Aybar, 2001; Mayor et al., 1999; Meulemans and Bronner-Fraser, 2004; Sauka-Spengler and Bronner-Fraser, 2008; Steventon et al., 2005). From all these proposals the idea emerges that secreted molecules (BMPs, Wnts and FGF) activate the expression of a first set of transcription factors, among these *Pax3* and *Msx1*. Evidence that any of these factors, or any other known transcription factors, are directly regulated by the inducing signals has so far been lacking. Here I show for the first time that *Gbx2* is the earliest element of the cascade, which is directly regulated by Wnt signals and participates in neural crest induction.

Several lines of evidence indicate that *Gbx2* is one of the most upstream factors in the neural crest genetic cascade. First, activation of *Gbx2* by Wnt signalling does not require protein synthesis. Second, *Pax3* and *Msx1*, usually described as the most upstream factors in the genetic cascade (Meulemans and Bronner-Fraser, 2004; Sauka-

Spengler and Bronner-Fraser, 2008; Steventon et al., 2005), are downstream of *Gbx2*. Third, β-catenin/TCF/LEF factors seem to bind directly to the *Gbx2* enhancer. It is interesting to note that in our CHIP analysis we found that β-catenin/TCF/LEF does not bind to the *Snail2* promoter during early neural crest induction, but it binds at later stages. This observation is consistent with the *Snail2* promoter study that shows activity only after stage 14 (Vallin et al., 1998), and with a large number of publications that clearly show *Snail2* as a factor downstream of *Pax3*, *Msx1* and *Zic1* during early neural crest induction (Hong and Saint-Jeannet, 2007; Monsoro-Burq et al., 2005; Sato et al., 2005; Tribulo et al., 2003; Zhao et al., 2008). These data suggest that inductive signals such as Wnt are likely to work at different steps during neural crest development, such as in the early neural crest induction by controlling genes such as *Gbx2* and later during neural crest maintenance by controlling genes such as *Snail2*. This idea is consistent with the recent identification of an early induction and a later maintenance step for neural crest specification and with the demonstration that both steps are Wnt dependent (Steventon et al., 2009).

Gbx2 has been implicated in the formation of the midbrain-hindbrain boundary (MHB) and in the posteriorization of the neural plate (Hidalgo-Sanchez et al., 1999b). In this work I show that during gastrulation,

when neural crest induction starts (Mancilla and Mayor, 1996), *Gbx2* is expressed in a broad domain of the ectoderm including the prospective neural crest. Later, at neurula stages, when some additional interactions are required to refine the position of the induced neural crest, *Gbx2* is absent from the most anterior neural crest domain. This loss of *Gbx2* is likely to be due to the repressor activity of *Otx2* in the MHB (Glavic et al., 2002). It is known that at gastrula stages *Gbx2* and *Otx2* expression overlaps in a domain wider than the MHB (Garda et al., 2001; Glavic et al., 2002; Steventon et al., 2012). At this stage, *Gbx2* is expressed in the entire neural crest population and I show here that it plays an essential role in early neural crest induction. *Gbx2* knockout mice exhibit defects in neural crest derivatives, such as heart and head (Byrd and Meyers, 2005). However, this phenotype has been explained as a defect in neural crest patterning or migration, instead of neural crest induction. My results support a very early role for *Gbx2* in the specification of neural crest cells that explain the reported deficiencies in neural crest derivatives in these mutants.

4.3 *Gbx2* works as a neural fold posteriorizing factor

Gbx2 has also been implicated in posteriorization of the neural plate and its role in MHB specification has been widely studied (Hidalgo-Sanchez et al., 1999b; Millet et al., 1999). My results support a similar role for *Gbx2* as a neural fold posteriorizing factor. I show here that anterior neural fold, defined by the expression of the preplacodal marker *Six1*, is transformed into neural crest by the action of *Gbx2* corresponding to the posteriorization process (Cox and Hemmati-Brivanlou, 1995; Nieuwkoop, 1952; Villanueva et al., 2002). Furthermore, I show that *Gbx2* is sufficient to repress the expression of *Six1* and promotes the expression of the neural crest markers in vitro. My results provide molecular support to the hypothesis that neural crest induction requires posteriorization of the neural fold (Aybar et al., 2002; Villanueva et al., 2002). Surprisingly I found that neural fold and neural plate posteriorization can be dissociated: anterior-posterior patterning of the neural fold is possible without affecting this process in the neural plate. Moreover, the neural fold seems to be more sensitive to the posteriorizing agents *Gbx2* and Wnt than neural plate (this work; (Carmona-Fontaine et al., 2007). The different sensitivity offers an explanation to apparently contradictory results. It has previously shown that posteriorizing factors, like Wnt, FGF and RA, also can transform the anterior neural fold into neural crest, supporting a role for

posteriorization in neural crest induction (Villanueva et al., 2002). This has been challenged by the observation that activation of Wnt signalling induced neural crest markers without any effect in the anterior-posterior axis of the neural plate, concluding that neural crest induction was independent of posteriorization (Wu et al., 2005). The results presented here show that neural crest induction is independent from neural plate posteriorization, and that posteriorization of the neural fold can be dissociated from posteriorization of the neural plate. I propose that neural crest induction at the anterior neural fold described by Wu (Wu et al., 2005) is the result of neural fold posteriorization.

4.4 *Gbx2* makes the distinction between neural crest and PPR

The preplacodal domain forms in the outer border of the anterior neural fold and contributes to sense organs and cranial sensory ganglia (Schlosser, 2006; Streit, 2004). A recent model for its induction has been proposed in which inhibition of Wnt signaling is an essential component (Brugmann et al., 2004; Litsiou et al., 2005). This model is consistent with my results showing that *Gbx2* is an inhibitor of PPR and is a Wnt target. Inhibition of *Zic1* leads to a depletion of the neural crest and PPR population (Hong and Saint-Jeannet, 2007). However, activation of *Zic1* inhibits neural crest induction and promotes PPR development (Hong and Saint-Jeannet, 2007). The observation that activation and inhibition of *Zic1* leads to neural crest inhibition can be explained with my finding that *Zic1* plays a dual role that is context dependent. In the posterior region of the embryo, *Zic1* interacts with *Gbx2* to promote neural crest specification, while anteriorly *Zic1* induces *Six1* required for specification of the placode territory (Brugmann et al., 2004). In addition, different levels of *Zic1* may be also important to specify different territories, as it has been shown for the distinction between hatching gland, neural crest and placodal (Hong and Saint-Jeannet, 2007) and by the direct up-regulation of neural crest markers (Sato et al., 2005).

It has previously shown that *Dkk1* is required to inhibit neural crest specification at the anterior neural fold by inhibiting cell proliferation (Carmona-Fontaine et al., 2007). The data shown here suggest that in addition to cell proliferation Wnt signaling are controlling the specification of neural crest versus anterior placode. Interestingly, we have previously shown that placode markers are the only genes affected by Wnt/*Dkk1* (Carmona-Fontaine et al., 2007). Taken together, these observations suggest that *Dkk1* could work as an inhibitor of *Gbx2* at the anterior neural fold region.

I show that injection of *Gbx2* leads to a modest lateral expansion of neural crest. I propose that this expansion could be due to the overlap of *Gbx2* and *Zic1*. It is known that *Zic1* is expressed in a wider domain than neural plate border which includes the neural crest and the PPR region (Hong and Saint-Jeannet, 2007). PPR marker *Six1* is expressed in anterior of the neural plate border, however at posterior end of *Six1* domain, which is expressed outside of the neural crest (Glavic et al., 2004). I show before *Gbx2* makes the distinction between neural crest and PPR, *Gbx2* is expressed in neural crest but lacking in PPR domain (Figure 3.1). Thus, injection of *Gbx2* represses PPR and generates a new region of *Gbx2/Zic1* co-expression. This new interaction expands the neural crest expression not only anteriorly along the neural plate border, but also laterally where *Six1* is expressed outside of neural

crest (Figure 3.1, 3.5 and 3.7). It is interesting to notice that the same genetic network specifies both the anterior-posterior and medio-lateral border between neural crest and PPR.

The model presented here supports the idea that initial patterning of the ectoderm is determined by positional information dependent on two orthogonal gradients. A medio-lateral BMP gradient specifies neural plate, neural plate border and epidermis, while an antero-posterior Wnt gradient divides the neural plate border into PPR and neural crest. Interestingly, a similar orthogonal double gradient of decapentaplegic (the invertebrate homologous to BMP) and Wingless (the invertebrate homologous to Wnt) specify the antero-posterior and medio-lateral axis of the Drosophila imaginal disc (Strigini and Cohen, 1999), suggesting that a BMP/Wnt orthogonal gradient is an ancient mechanism to generate positional information (see Figure 4.2).

4.5 Summary

I found that *Gbx2* plays an essential role in neural crest induction and propose a new genetic cascade that operates to distinguish between preplacodal and neural crest territories. *Gbx2* resides at the top of the neural crest induction genetic cascade, being directly activated by the neural crest inducer Wnt. Several lines of evidence support this idea:

1) *Gbx2* is expressed in the prospective neural crest at the time of its induction.

2) Inhibition of *Gbx2* expression leads to a complete loss of the neural crest region, concomitant with an expansion of the preplacodal region (PPR).

3) *Gbx2* is a direct downstream target of Wnt/β-catenin signalling during neural crest induction.

4) *Gbx2* is upstream of the earlier transcription factors, *Pax3* and *Msx1*, in the neural crest genetic cascade.

5) Interaction between *Gbx2* and *Zic1* is sufficient to induce neural crest and inhibit PPR in animal caps.

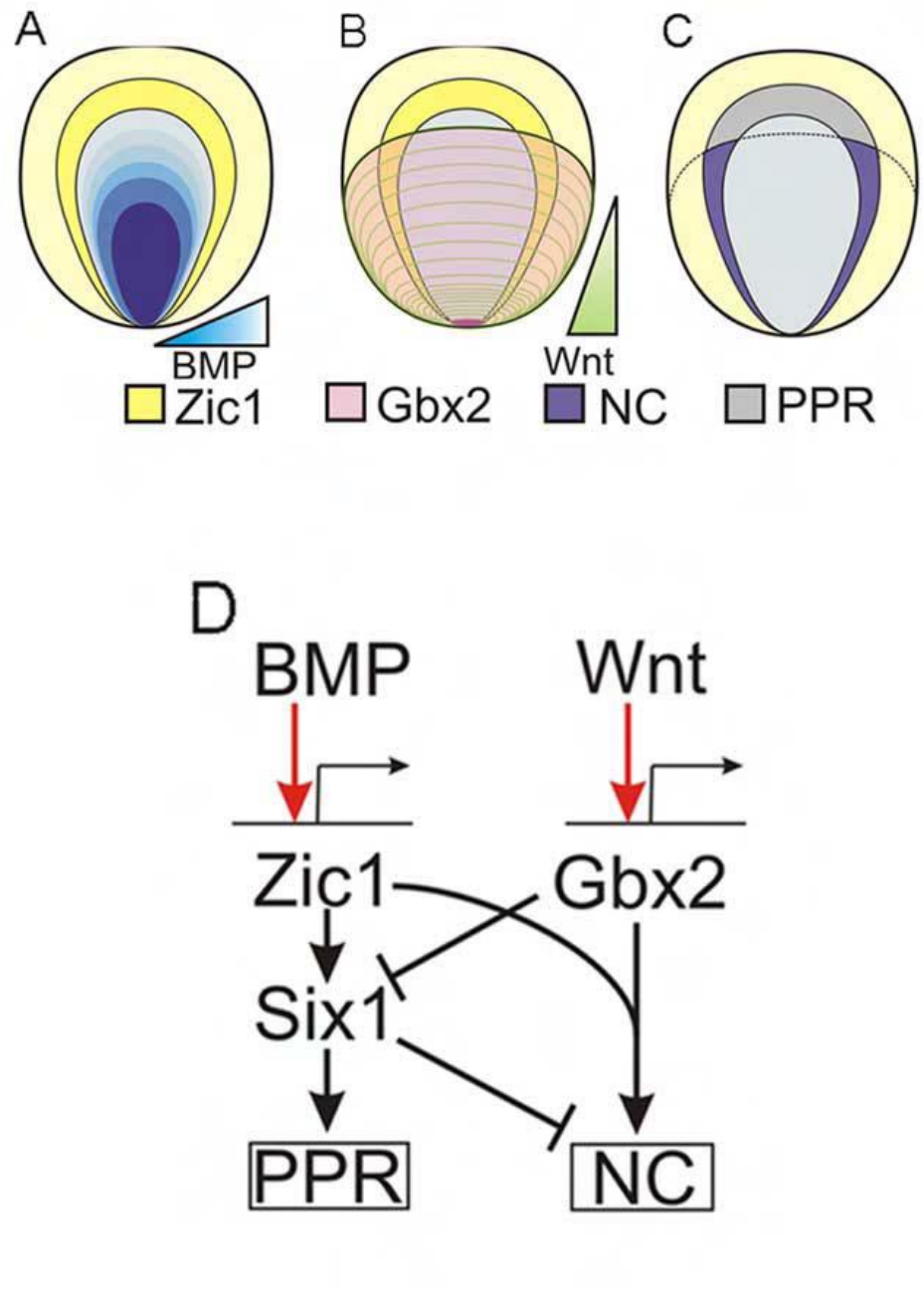

Figure 4.1 Model of neural crest induction by Gbx2

Figure 4.1 Model of neural crest induction by *Gbx2*.

(A) A precise level of BMP activity (blue) induces *Zic1* (yellow) in the entire neural plate border.

(B) Posteriorizing signal Wnt (green) induces *Gbx2* (pink) in the posterior embryo, including the neural plate border. Thus the posterior neural fold is the only region where *Gbx2* and *Zic1* are co-expressed.

(C) *Zic1* controls the expression of *Six1* and specifies preplacodal (PPR, grey) territory, while that *Zic1* plus *Gbx2* activates the neural crest genetic cascade in the posterior neural fold, specifying the neural crest (NC, purple) territory.

(D) Network of genetic interactions that specify PPR and NC. Red arrows: direct regulation of *Zic1* by BMP (Tropepe et al., 2006) and of *Gbx2* by Wnt (this work).

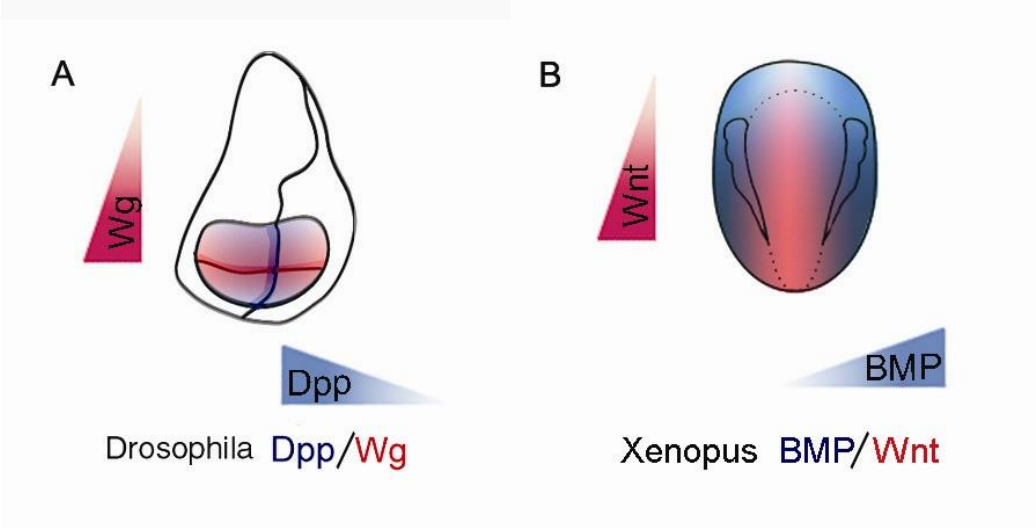

Figure 4.2 Double gradient model of the Drosophila imaginal disc and Xenopus neural crest

Chapter Five: References

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