AN EXTRA-EMBRYONIC Wnt SIGNALING EVENT CONTROLS GASTRULATION IN MICE

A Dissertation Presented

By

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For my grandmother
(*in memoriam*) whose life was dedicated to helping people in need.
This work is also dedicated to my wife and daughter for sharing their lives with me.

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Abstract

The formation of the anterior-posterior axis requires a symmetry-breaking event that starts gastrulation. Ultimately, the morphogenetic movements of gastrulation reshape the embryo to its final tri-dimensional form. In mouse embryos, the identity of the molecule that breaks the bilateral symmetry and sets in motion gastrulation remains elusive. The Wnt signaling pathway plays a pivotal role during axial specification and gastrulation in metazoans. Loss-of-function experiments have demonstrated a requirement of *Wnt3* for gastrulation in mice. But because *Wnt3* is expressed sequentially in two tissues, the visceral endoderm and the epiblast, its tissue specific requirements remain uncertain. Here, we report that embryos lacking *Wnt3* specifically in the visceral endoderm do not form a primitive streak, mesoderm, endoderm or any derivatives. Visceral endoderm-specific *Wnt3* mutants also lack primordial germ cells. Moreover, we provide data demonstrating that *Wnt3* carries out its actions in the epiblast via the canonical Wnt pathway. Together, these data suggest that the posterior visceral endoderm via *Wnt3,* regulates the development of mouse embryos in a similar fashion to the amphibian Nieuwkoop center. Next, we conditionally ablated *Wnt3* locus in the epiblast to investigate whether *Wnt3* expression is also required in that tissue. Embryos lacking *Wnt3* expression in the epiblast, but retaining its expression in the visceral endoderm, show delayed but not absent gastrulation. We conclude that the expression of *Wnt3* in the epiblast is required for maintenance but not initiation of gastrulation in mouse embryos. Furthermore,

we used *in vitro* and *in vivo* approaches to demonstrate that the *Wnt3*-mediated activation of the canonical Wnt pathway leads to β-catenin occupancy followed by transcription of key loci, including the *Wnt3* locus itself, during gastrulation in mice. Our data indicate the presence of an autoregulatory loop in which *Wnt3* controls its own expression and orchestrates the process of gastrulation in the mouse embryo.

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CHAPTER I:

General Introduction.

One of the most interesting questions in biology is how a single cell gives rise to an entire organism. In humans for instance, a single fertilized cell gives rise to about 400 different cell types present in an adult individual (Vickaryous and Hall 2006). Each cell type is different from the others, yet they all share a common ancestor, the zygote. More importantly, these different cell types have to be arranged in a proper way to give rise to a functional individual. The understanding of how a single progenitor cell gives rise to many different cell types is rather difficult and laborious, yet solving this problem will not only further increase our understanding of our own development but also help to develop new medical therapies that could be used to help people in need.

To a large extent, development relies on differentiation of naïve precursor cells towards more specified cell types. Differentiation implies formation of at least two distinct cell types from a single progenitor. In mammals, this process is morphologically evident at the blastocyst stage when two distinct populations of cells emerge from a compacted morula.

In mice, the model of study used for this work, the blastocyst is formed at embryonic day 3.5 (E3.5). At some point between E3.5 and E4.5, the ICM differentiates itself into two distinct tissues: the primitive endoderm, and the epiblast. At this point the embryo has moved down the oviduct and is now in the uterus. Here, the embryo implants itself in the uterine wall and the trophectoderm cells that are in direct contact with the ICM (polar trophectoderm) give rise to a new tissue, the extraembryonic ectoderm (Arnold and Robertson 2009;

Beddington and Robertson 1999; Tam, Loebel, and Tanaka 2006). The growth of the newly formed extraembryonic ectoderm, together with the growth of the epiblast, pushes the embryo even more into the blastocoel cavity. At around E5.0 the mouse embryo looks like a cylinder, and sometimes this stage of the development is referred as egg cylinder stage (Skreb, Solter, and Damjanov 1991). At day E5.0, the cells of the epiblast undergo very rapid cell cycles and increase their number from about 120 cells to 660 at E6.5, in roughly 24 hours (Snow and Bennett 1978; Snow 1977). This accelerated increase in cell numbers in the epiblast primes the embryos for gastrulation, the next big step of development. The next day, at E6.5, the epiblast of the mouse embryo undergoes gastrulation (Arnold and Robertson 2009; Beddington and Robertson 1999). Gastrulation is a period of development that is crucial for the formation of the definitive metazoan body plan. It is defined as a period of embryonic development in which the single-layered blastula is re-organized into a tri-laminar structure known as gastrula (Skreb, Solter, and Damjanov 1991; Snow and Bennett 1978; Tam, Loebel, and Tanaka 2006). Gastrulation is widely studied in a variety of animal models, and provides an opportunity for researchers to understand morphogenetic phenomena such as cell differentiation, cell migration, epithelial to mesenchymal transition (EMT) and morphogenesis. Also, in the regenerative medicine field, the study of gastrulation gives to researches a unique opportunity to understand mechanisms of development needed for

generation and regeneration of different tissues that could be used later to develop new medical treatments for people in need.

In this dissertation I investigate the mechanism that control the initiation of gastrulation in mice. In the following section some of the research that have been applied to understand gastrulation in different model systems will be discussed.

Introduction to Gastrulation.

Gastrulation is the period of development in which the single-layered blastula is re-organized into a tri-laminar structure known as the gastrula. The germ layers newly formed during gastrulation serve as building blocks for all the tissues in the embryo (Stern 2004; Wolpert 2002). Because of that, Lewis Wolpert once said “it is not birth, marriage or death, but gastrulation which is the truly most important time in your life” (Wolpert 1991).

The German embryologist Ernst Haeckel coined the term gastrulation in the 1800’s when he was mapping the development of calcareous sponges. In these organisms the term gastrulation refers to the formation of the archenteron (i.e., a sort of primitive digestive tube present in lower metazoans) (reviewed by Leys and Eerkes-Medrano, 2005). The word gastrulation is derived from the Greek word *Gaster* meaning belly or stomach. Thus, the term gastrulation actually implies the formation of a primitive digestive system, a tube running through the interior of the animal (Leys and Eerkes-Medrano 2005; Leptin 2005).

The morphogenetic events of gastrulation are not conserved across different species. One might therefore speculate that this happened due to environmental changes or due to specific adaptations among distinct species (Leptin 2005). It has been suggested that development of a multilayered gastrula allows for the existence of semiautonomous developmental units, with each layer favoring the differentiation in many different tissues in an organized and modular way (Nielsen 2008; Degnan and Degnan 2010).

Interestingly, however the basic molecular footprint of gastrulation appears to be conserved across different species. A very similar network of genes and proteins is conserved from lower organisms to higher vertebrates suggesting the presence of a high degree of conservation of a primitive, yet efficient apparatus active during this process (Degnan and Degnan 2010). The cnidarian fresh water hydra, with a very simple body organization (Martin et al. 1997; Nakamura et al. 2011), appears to use some of the same basic signaling pathways involved in axial specification and gastrulation present in higher metazoans (Nakamura et al. 2011; Petersen and Reddien 2009).

These days, the definition of gastrulation often includes the expression of a group of genes involved with the acquisition of posterior/dorsal identity (molecular identity) Due to its importance for the development of the entire organism it has been extensively studied (Ang and Constam 2004; Arnold and Robertson 2009; Beddington and Robertson 1999; Kemp et al. 2005; Kimelman 2006). A common theme during gastrulation of different species is the presence

of the Wnt signaling pathway (Cadigan and Nusse 1997; Klaus and Birchmeier 2008; Logan and Nusse 2004). In the next section we will describe the mechanism of gastrulation and axial specification in some of the most common research model organisms and how the Wnt signaling pathway helps to shape the embryos during this processes.

Mechanism for Initiation of Gastrulation in vertebrates.

Amphibians.

Amphibians were some of the first animals to be subject to in-depth studies in developmental biology (Wolpert 2002). There are several reasons for this, such as: large eggs (~1mm, whereas a mouse oocyte is ~80µm), easy to obtain and handle in high quantities; rapid embryonic development, do not require sophisticated facilities to be maintained, embryos can be cultured in reasonably simple medium and they can be visualized with relatively simple methods. These characteristics played a key role for studies on pattern formation and axial specification in the 1900's (Beetschen 2001; Wolpert 2002). The bias towards the use of the amphibian embryo in the developmental biology field, can be tracked back to the seminal research conducted by Hans Spemann who, in the beginning of the 20th century, made significant contributions to the understanding of inductive processes that shape the vertebrate embryo during

development (Beetschen 2001; Harland and Gerhart 1997; De Robertis and Kuroda 2004).

A theme that is re-visited often in amphibian developmental research is the study of axial specification and gastrulation (De Robertis and Kuroda 2004; Gerhart 1997; Vonica and Gumbiner 2007). Axial specification is the determination of the definitive body plan of the embryo, when cell populations are allocated in order to form a ventral or dorsal, anterior or posterior and proximal or distal structures in the embryo (Zernicka-Goetz 2002). It occurs early in development and is a pivotal event for the proper development of all living things. In amphibians, when the egg is laid, even before fertilization, it already has polarity. It has two easily distinguishable poles, a pigmented surface named the animal pole that can be easily distinguished from the unpigmented, yolk-rich surface, the vegetal pole (Wolpert 2002; Moon and Kimelman 1998). At fertilization, the sperm may enter the egg and fertilize it anywhere at the animal pole. Fertilization starts a series of molecular events that break the radial symmetry of the egg and define the dorsal-vegetal pole of the embryo, with the dorsal side forming opposite 180˚ from the sperm entry site (Heasman 2006; Meinhardt 2001).

Later studies found that the sperm entry causes a re-arrangement of components of the cytoskeleton of the embryo, a process known as cortical rotation, which in turns drives the formation of a signaling center known as the

Nieuwkoop center (Weaver and Kimelman 2004; Gerhart 1999; Vonica and Gumbiner 2007).

Essentially, the phenomenon of cortical rotation leads to accumulation of maternally inherited RNAs on the dorsal-most, vegetal-most side of the embryo. This leads to compartmentalized enrichment of signaling molecules, forming a signaling center able to change the fate of target cells at the animal pole in the embryo (Guger and Gumbiner 1995; Harland and Gerhart 1997; Meinhardt 2001). This signaling center was named in honor of Pieter Nieuwkoop, who in the 60's performed a series of transplantation experiments in order to demonstrate that the formation of mesoderm and the Spemann organizer are the results of inductive signals (Gerhart 1999). The Nieuwkoop center comprises a small population of cells located at the dorsal-most, vegetal-most part of the embryos that can induce the formation mesendoderm and an organizer (Gerhart 1999; Guger and Gumbiner 1995). The results acquired by Nieuwkoop and others were later used to build a three-signal model for mesodermal induction and patterning in amphibians (De Robertis and Kuroda 2004; Vonica and Gumbiner 2007). This model predicts that all vegetal cells are capable to produce a general mesoderm-inducing signal responsible for mesoderm induction in the boundary region between animal and vegetal poles (first signal). But cells located at dorsal-vegetal region in addition to the general mesoderm signal also produce a dorsalizing signal required for the formation of the organizer (the second signal sometimes referred as the Nieuwkoop signal). Ultimately the organizer itself

secretes the third signal that is responsible for patterning of the dorsal-ventral axis (De Robertis and Kuroda 2004; Vonica and Gumbiner 2007; Niehrs 2004).

So, what is an organizer? In beginning of the 20th century, Hans Spemann and Hilde Mangold performed pivotal experiments that defined the concept and origin of the amphibian organizer (Moon and Kimelman 1998; Niehrs 2004). The experiments consisted of transplantation of a piece of the gastrula stage of the salamander embryo (the dorsal lip of the blastopore) into the ventral side of another embryo. This newly transplanted piece was able to release inductive signals capable of changing the fate of the neighboring tissue, re-specifying it to different cellular lineages (dorsal lineages). Interestingly, the cells of the organizer itself did not participate in the newly formed tissue, indicating induction and not merely differentiation of the organizer tissue (Gerhart 1999; Moon and Kimelman 1998; Niehrs 2004).

Despite being sought by many different labs, the signal responsible for induction of the Spemann organizer remained elusive for many years, and it was simply referred as “Nieuwkoop signal” (De Robertis and Kuroda 2004; Gerhart 1999). Several lines of evidence have suggested that Wnt signaling played a role in axial specification and gastrulation in amphibians (De Robertis and Kuroda 2004; Weaver and Kimelman 2004). Only recently, maternally inherited *Wnt11* mRNA was shown to be the main factor derived from the Nieuwkoop center able to induce formation of an organizer (Tao et al. 2005). *Wnt11* RNA becomes more abundant at the dorsal side than at the ventral side after cortical rotation.

Maternally derived *Wnt11* mRNA is necessary and sufficient to activate the canonical Wnt pathway and guide axis formation in *Xenopus laevis* embryos. Furthermore, loss of *Wnt11* results in ventralized embryos, reinforcing the argument that *Wnt11* is indeed the dorsalizing factor that emanates from the Nieuwkoop center (Tao et al. 2005). Nowadays, there is a consensus among researches that activation of the canonical Wnt pathway is a key event for the formation of the organizer and axial specification in amphibians, and it is fairly well accepted that *Wnt11* is the Wnt ligand that initiate formation of the organizer in amphibians.

Fish.

Most of the research carried out on fish development is performed on zebrafish. The zebrafish (*Danio rerio*) is a tropical freshwater species originally found in southeastern Asia. It began to be used as a model system in the late 1960's. The idea to applying genetic analysis to study zebrafish embryonic development originated with George Streisinger in the late 60's (Grunwald and Eisen 2002). The ability to conduct classical forward genetics analysis, in addition to its small size, year-round breeding capability and large yield of gametes and embryos made the zebrafish a robust scientific model used by many scientists worldwide (Grunwald and Eisen 2002; Wolpert 2002).

The embryonic development of a zebrafish has been divided into seven distinct stages: the zygote, cleavage, blastula, gastrula, segmentation,

pharyngula and hatching periods. These stages comprise the major developmental process accomplished by the zebrafish embryo over a period of three days (Kimmel et al. 1995). We will focus our discussions on the blastula to gastrula transition and gastrula stage. In these stages, the zebrafish organizer is formed and gastrulation takes place (Rohde and Heisenberg 2007; Schier and Talbot 1998).

The zebrafish embryo at the end of the blastula stage contains four tissues: an outer epithelial monolayer named enveloping layer; an underlying group of cells named the deep cells of the blastoderm; a large group of membrane-enclosed nuclei that lies on top of the yolk mass named yolk syncytial layer, and the yolk mass itself (Kimmel et al. 1995). The enveloping layer, the deep cells of the blastoderm and the yolk syncytial layer undergo epiboly (thinning and spreading of a cell layer) at the transition between the blastula and gastrula stages. When this epiboly movement reaches the equator of the embryos (i.e.: 50% epiboly), a portion of the deep cells of the blastoderm moves inward through the margin of the blastoderm and forms the hypoblast. This movement forms a bi-laminar ring and a structure named “the shield” at the dorsal side (D'Amico and Cooper 1997; Kimmel et al. 1995).

The equivalent of the Spemann organizer in zebrafish is believed to be the shield (Kanki and Ho 1997; Kodjabachian, Dawid, and Toyama 1999; Langdon and Mullins 2011). The shield is a structure formed at the boundary of the animal-vegetal pole on the dorsal side of the zebrafish embryo. It shares the

homologous location of the Xenopus organizer (Kanki and Ho 1997; Langdon and Mullins 2011; Montero et al. 2005). Morphologically it looks like an asymmetrically positioned thickening of the marginal zone or germ ring that is formed approximately 6 h after fertilization in zebrafish embryos (Kimmel et al. 1995; Montero et al. 2005).

Transplantation experiments have indicated that the zebrafish shield is sufficient to re-specify cell fate and drive ectopic formation of an axis in zebrafish embryos (Driever et al. 1997; Shih and Fraser 1996). Conversely, ablation of the shield yields embryos with loss of dorsal structures (Shih and Fraser 1996).

In zebrafish, as in *Xenopus laevis,* the formation of the organizer depends on an ordered system for transportation and deposition of maternally inherited RNAs on the prospective dorsal side of the embryo (Kanki and Ho 1997; Kodjabachian, Dawid, and Toyama 1999). It has been postulated that the activation of the canonical Wnt signaling marks the formation of the shield region and the initialization of gastrulation in these embryos (Langdon and Mullins 2011; Montero et al. 2005; Schier and Talbot 1998). Interestingly, *Wnt8a* appears to be the vegetal maternally inherited dorsal determinant in zebrafish embryos (Lu, Thisse, and Thisse 2011). Whereas, *Wnt11* has been shown to be the maternally inherited dorsal determinant in *Xenopus laevis* (Tao et al. 2005). This difference in the selection of distinct Wnt ligands might reflect an evolutionary character intrinsic to speciation. It is worth noting that although different Wnt ligands have

been co-opted during evolution of these two species, the activation of the canonical Wnt pathway is still the main goal to be achieved in both.

Birds.

Unlike amphibians, the sperm entry site does not determine polarity in chicken embryos. Chicken embryos and perhaps all avian embryos in general are polyspermatic. It has been suggested that anywhere between five and twenty-six sperm may enter a single chicken egg at the time of fertilization (Stern 2004).

Chicken eggs have a very large quantity of yolk, and cleavage in these embryos is restricted to the animal pole. Thus, cleavage in avian embryos is meroblastic (mero = partial) (Stern 2004). As in fish, which also have meroblastic cleavage, it is likely that maternally inherited dorsal determinants may exist in chicken embryos. However, the most accepted hypothesis is that gravity plays a role in axial specification in chicken (Stern 2004; Wolpert 2002). In chicken, it takes twenty hours from fertilization to laying and when the egg is laid it already contains about 20,000 cells (Stern 2004; Eyal-Giladi 1997). During this time, the egg slowly rotates around its long axis and this rotation is thought to promote separation of components inside the embryo, driving the establishment of the anterior-posterior axis (Stern 2004; Eyal-Giladi 1997).

The chick embryo resembles an almost flat disc, the blastoderm that rests on top of the yolk. An inner, lighter colored *area pellucida* is easily distinguishable

from a more peripheral ring, named *area opaca* (Zahavi, Reich, and Khaner 1998; Wolpert 2002). The epiblast, the embryonic part of the conceptus that will give rise to the embryo proper, is a simple epithelial layer that extends over the two areas. The basic difference is that the area opaca refers to the blastoderm cells that have not shed their deep cells (Zahavi, Reich, and Khaner 1998). Another tissue present in the chick embryos is the hypoblast. The hypoblast is a tissue located beneath the epiblast formed from the fusion of a population of small islands of yolk cells that resides under the *area pellucida*. Finally, the epiblast cells that reside in the narrow region between the *area opaca* and the *area pellucida* form the structure known as the marginal zone (Stern 2004).

The posterior side marginal zone (posterior marginal zone) of chicken embryos is thought to contain organizer activity. This is due to its ability, in transplantation experiments, to form an ectopic axis (Mikawa et al. 2004; Bachvarova 1999; Bachvarova, Skromne, and Stern 1998). Here, once again, the Wnt signaling pathway seems to play a pivotal role for organizer formation and establishment of the anterior-posterior axis and gastrulation. Interestingly, a canonical Wnt, *Wnt8C,* seems to form a gradient from the posterior/concentrated to the anterior/less-concentrated side of the embryo (Skromne and Stern 2001).

Genes downstream of the canonical Wnt pathway such as, *β-catenin, left1, Brachyury*, are more highly expressed in the posterior marginal zone and primitive streak region of the embryo (Skromne and Stern 2001; Chuai and Weijer 2008). Furthermore, ectopic expression of chicken *Wnt8C* in mouse

embryos leads to ectopic axis formation (Popperl et al. 1997), reinforcing the argument that *Wnt8C* expression is sufficient to activate the canonical Wnt pathway and drive formation of embryonic axes.

Mammals.

In mammals, most of the molecular studies on axial specification and gastrulation have been conducted in mice (*Mus musculus*), therefore, I will limit my analysis to data obtained from the murine model system.

In mice, axial specification and gastrulation take place after implantation (Arnold and Robertson 2009; Beddington and Robertson 1999; Camus and Tam 1999). The murine blastocyst implants itself into the uterine wall at around E4.5, (Beddington and Robertson 1999; Stern 2004). At E5.0 the mouse embryo looks like a cylinder comprised of four tissues: the ectoplacental cone, the interface that connects the embryo to the uterus blood supply; the extraembryonic ectoderm, a tissue that emerged from the trophectoderm and will contribute to extra-embryonic lineages; the epiblast, a pseudostratified, single layered epithelium that will give rise to the embryo proper; and the visceral endoderm, a tissue descendent from the primitive endoderm, involved with nutrition and patterning of the embryo (Figure 1.1) (Beddington and Robertson 1999; Snow 1977; Skreb, Solter, and Damjanov 1991). Immediately, after implantation the epiblast of the mouse embryo undergoes very rapid cell cycles in order to increase cell numbers before gastrulation starts (Snow 1977). At E.5.0 the

epiblast contains about 120 cells, however a day later its cellular content increases to 660 (Snow and Bennett 1978; Snow 1977).

At the gastrula stage the mouse embryo is bilaterally symmetric (embryo E5.5, Figure 1.1). The two major events that mark axial specification in mouse embryos are: the positioning of the anterior visceral endoderm on the prospective anterior side of the embryo (embryo E5.7.5, Figure 1.1) (Arnold and Robertson 2009; Rivera-Perez, Mager, and Magnuson 2003; Thomas and Beddington 1996); and second, the formation of the primitive streak on the posterior side of the embryo (embryo E6.5, Figure 1.1) (Beddington and Robertson 1999; Tam, Loebel, and Tanaka 2006). The anterior visceral endoderm comprises a small population of cells within the visceral endoderm located on the anterior side of the embryos at E6.0 and it has been suggested to play a role in axial specification in mouse (Srinivas 2006; Perea-Gomez, Rhinn, and Ang 2001). The primitive streak is the hallmark of gastrulation. It forms on the prospective posterior side of the embryo, and it is defined as a region where massive epithelial-to-mesenchymal transition (EMT) occurs. EMT is crucial for the formation of mesoderm and endoderm cells that migrate out of the streak in order to be allocated somewhere else along the anterior-posterior axis of the embryo (Arnold and Robertson 2009; Beddington and Robertson 1999; Kimelman 2006). Together with the ectoderm, the mesoderm and the endoderm will serve as building blocks for all tissues in the developing embryo (Arnold and Robertson 2009).

Figure 1.1

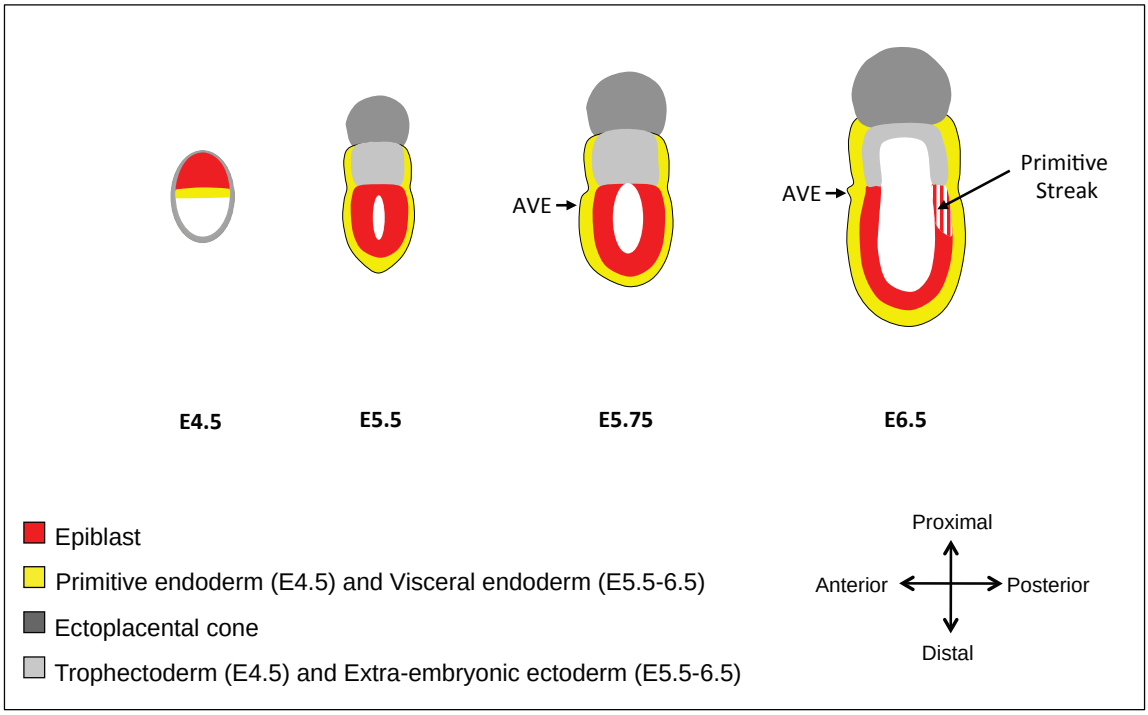
AVE
AVE
Primitive Streak
E4.5
E5.5
E5.75
E6.5
Epiblast
Primitive endoderm (E4.5) and Visceral endoderm (E5.5-6.5)
Ectoplacental cone
Trophectoderm (E4.5) and Extra-embryonic ectoderm (E5.5-6.5)
Proximal
Anterior
Posterior
Distal

Figure 1.1: Anterior-posterior axis establishment in mice. Cartoon depicts two days of early post-implantation development of mouse embryos. The different colors (red, yellow and grey) mark the fate commitment of specific cell lineages represented from E4.5 to E6.5. The anterior side of the mouse embryo becomes evident with the shift of the anterior visceral endoderm (AVE) cells from the distal tip of the embryo (E5.5) to the prospective anterior side of the embryo (5.75). The formation of the primitive streak diametrically opposite to the AVE is the first morphological hallmark of gastrulation and it is noticeable as early as E6.5. Modified after Rivera-Perez, 2007.

In mice the location of the equivalent to the Spemann organizer has been suggested to be the node (Beddington 1994) or the primitive streak (whole streak or at least part of it) (Robb and Tam 2004; Tam et al. 1997).

The node is a bilaminar and circular structure that forms at the anterior part of the primitive streak in the midline of the E7.5 mouse embryo (Beddington and Robertson 1999; Joubin and Stern 2001). The hypothesis that the node is the murine equivalent of the organizer was largely based on transplantation experiments in which the node could induce partial axial duplication in another embryo that had received the transplanted tissue. The newly induced axis lacked anterior structures however (Beddington 1994). Thus, although exciting, these experiments only indicated that the node was not sufficient to induce a complete new axis, unlike the dorsal lip of the blastopore (Harland and Gerhart 1997).

An alternative hypothesis proposes that a small population of cells residing in the posterior epiblast, anterior to the newly formed primitive streak comprises the mouse organizer at around E6.5 (Kinder et al. 2001; Robb and Tam 2004). This small cell population (about 40 cells) is termed the early gastrula organizer (EGO), and when the EGO is transplanted into another embryo it induced an ectopic axis (Tam and Steiner 1999). However, the induced ectopic axis once again lacks anterior structures, indicating that a complete axial induction requires also head organizer activity that most likely resides elsewhere outside the EGO (Tam and Steiner 1999; Robb and Tam 2004).

Besides the EGO, the mouse gastrula also contains another region with organizing activity named the mid-gastrula organizer (MGO). The MGO is formed by a population of cells residing in the anterior end of the primitive streak (Robb and Tam 2004), and unlike the EGO, the MGO can induce a complete axis, with posterior and anterior structures (Robb and Tam 2004; Tam et al. 1997). Also, the MGO expresses more organizer related genes than those of the EGO, suggesting the MGO as a stronger candidate to be the mouse homologous structure of the amphibian Spemann's organizer (Camus and Tam 1999).

If the primitive streak or at least part of it is the murine equivalent of the amphibian organizer, then what is the murine equivalent of the Nieuwkoop center that is responsible for the induction of the organizer?

Two tissues in the mouse embryo have been suggested to be the equivalent to the amphibian Nieuwkoop center: the extra-embryonic ectoderm and the posterior visceral endoderm. The extra-embryonic signaling hypothesis states that Bmp4 released from the extraembryonic ectoderm induces the expression of *Wnt3* in the adjacent epiblast, and *Wnt3* in turn controls the formation of the primitive streak (Figure 1.2) (Ben-Haim et al. 2006). This hypothesis largely relies on the ability of Bmp4 derived from the extraembryonic ectoderm to activate *Wnt3* in the epiblast. These studies, however, lack direct evidence for such a regulatory loop involving Bmp4 and Wnt3. The model proposes that *Bmp4* expression in the extraembryonic ectoderm is induced by Nodal originating from the epiblast at early gastrula stage (Ben-Haim et al. 2006;

Whitman 2001). Nodal belongs to the TGF-β superfamily of secreted molecules and carries out its effects in a Smad-dependent manner (Shen 2007). Nodal expression is regulated by dual mechanism. In pre-streak mouse embryos, Nodal is expressed in the epiblast and visceral endoderm, this expression is governed by an asymmetric intronic enhancer that seems to be independent of the canonical Wnt pathway (Norris and Robertson 1999). At later time points, during gastrulation, Nodal is expressed in the posterior epiblast. This expression pattern is controlled by a proximal promoter element, which contains specific *cis*-regulatory TCF-binding sites that are downstream targets of the activation of the canonical Wnt pathway (Norris and Robertson 1999; Shen 2007).

Although the literature lacks direct evidence that the canonical Wnt pathway directly modulates Nodal expression, it is well accepted that Nodal expression in the posterior epiblast of gastrulating mouse embryos is epistatically downstream *Wnt3* expression, since *Wnt3* null embryos do not have Nodal expression in the posterior epiblast (Liu et al. 1999; Shen 2007). Thus, the idea that Nodal emanates from the epiblast and controls *Bmp4* expression in the extraembryonic ectoderm, and that Bmp4 emanates from the extraembryonic ectoderm to activate the expression of *Wnt3* in the posterior epiblast lacks support. Moreover, in accordance with this hypothesis one should expect *Bmp4* null embryos to halt their development at pre-streak stages. However some *Bmp4* knockout embryos gastrulate without major problems and some embryos

can advance all the way to head folds and even early somite stages (Lawson et al. 1999; Winnier et al. 1995). Therefore, *Bmp4* cannot be the Nieuwkoop signal.

An alternative hypothesis states that the posterior visceral endoderm contains the Nieuwkoop signal needed to induce organizer and mesoderm formation (Rivera-Perez and Magnuson 2005). The posterior visceral endoderm can re-specify anterior ectoderm to a posterior mesendodermal fate, and this reprograming mechanism does not depend on cell-cell interaction. It seems to rely on a short-range diffusible molecule (Belaoussoff, Farrington, and Baron 1998), in a similar fashion to what occurs in the amphibian transplantation experiments when the Nieuwkoop center is transplanted to a region of neural ectoderm (Gerhart 1999).

In *Xenopus*, zebrafish and perhaps birds the Nieuwkoop signal appears to be a Wnt molecule (Tao et al. 2005; Langdon and Mullins 2011; Boettger et al. 2001; Lu, Thisse, and Thisse 2011). Thus, from an evolutionary point of view it makes sense to propose that Wnt signaling would be conserved as the murine Nieuwkoop signal.

Wnt3 is a canonical Wnt expressed sequentially in the posterior visceral endoderm at E5.5, and later in the epiblast at E5.75 (Rivera-Perez and Magnuson 2005). Because of its pattern of expression and genetic requirement *Wnt3* is an appropriate candidate to be the Nieuwkoop signal in mouse embryos. One of the goals of this work is to test whether the expression of *Wnt3* in the posterior visceral endoderm governs the formation of the primitive streak.

Figure 1.2

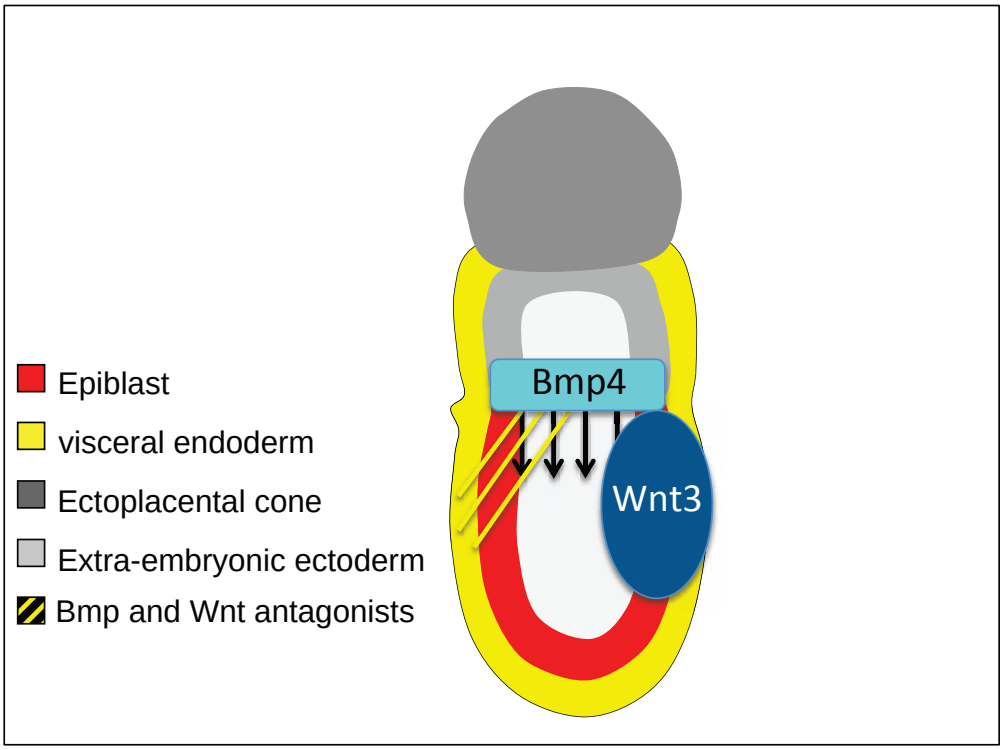
Epiblast
visceral endoderm
Ectoplacental cone
Extra-embryonic ectoderm
Bmp and Wnt antagonists
Bmp4
Wnt3

Figure 1.2: Current model for initiation of gastrulation in mice. Cartoon represents an E6.5 mouse embryo in which Bmp4 emanating from the extra-embryonic ectoderm signals to activate *Wnt3* and initiates primitive streak formation in the posterior epiblast. The expression of *Wnt3* is restricted to the posterior side because the AVE cells secrete Bmp and Wnt antagonists preventing posteriorization of cells committed to anterior lineage. Based on Ben-Haim et al., 2006.

The Wnt signaling pathway is conserved across metazoans and this pathway has been co-opted for axial specification in multiple animal species from Hydra to vertebrates (Tao et al. 2005; Langdon and Mullins 2011; Boettger et al. 2001; Lu, Thisse, and Thisse 2011; Bode 2011; Nakamura et al. 2011). In the following section an extensive review of the literature will be presented aimed at detailing the complex mechanism and current models of activation of Wnt signaling pathway.

The Wnt Signaling Pathway.

Wnts are secreted glycoproteins broadly studied due to their importance in processes such as embryonic development, regeneration, cancer and cellular differentiation. (Cadigan and Nusse 1997; Fearon and Cadigan 2005; Kimelman 2006; Klaus and Birchmeier 2008; Lickert et al. 2005; Petersen and Reddien 2009; Tanaka et al. 2011; van Amerongen and Berns 2006). The first Wnt gene to be described was found after analysis of a *Drosophila melanogaster* recessive mutation characterized by segmentation defects leading to absence of wings, named *wingless* accordingly (Sharma 1973). Later, mapping of the integration site of the mouse mammary tumor virus (MMTV) led to the realization that the site of integration (*int1*) was the locus of the mouse orthologue of the *wingless* gene (Rijsewijk et al., 1987). Thus, the term Wnt was coined as a composite of the *Int1* for integration 1 and *wingless* (Nusse et al. 1991; Nusse and Varmus 1992; Rijsewijk et al. 1987).

Wnts seem to occur across different phyla of the animal kingdom (Holstein 2012; Ryan and Baxevanis 2007; Sidow 1992). They are present in animals as simple as sponges (Phylum porifera) (Nichols et al. 2006) and the fresh water polyp, *Hydra* (Pylum Cnidaria) (Nakamura et al. 2011), all the way to planarias (Pylum Platyhelminthes) (De Robertis 2010) and flies (Phylum Arthropoda) (Nusse and Varmus 1992; Sharma 1973) and vertebrates (Cadigan and Nusse 1997).

Vertebrates have an intricate set of Wnt genes as well as genes associated with Wnt signaling (Cadigan and Nusse 1997; Nusse and Varmus 1992; Peter and Davidson 2011). In vertebrates, Wnts are known to play pivotal roles in development as well as regeneration (Nakamura et al. 2011; Logan and Nusse 2004; Gurley et al. 2010; Guger and Gumbiner 1995; De Robertis 2010; Cadigan and Nusse 1997; Nichols et al. 2006). In mice, 19 Wnt genes have been identified (Cadigan and Nusse 1997; Klaus and Birchmeier 2008). They have been separated into two groups: the canonical Wnts (*Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt8* to name some) (Cadigan and Nusse 1997; Klaus and Birchmeier 2008) and the non-canonical Wnt (*Wnt5a, Wnt11, Wnt7a*) (Barrow 2006; Kuhl 2004). However, some Wnts, such as *Wnt11* seem to defy this classification since it has been shown to activate both canonical and non-canonical pathways (Kofron et al. 2007; Tao et al. 2005). Independent of the branch of Wnt signaling activated, a general mechanism of signal transduction is observed, that is, a given Wnt ligand binds to receptors located on the plasma membrane and it

leads to activation of intracellular machinery which in turn regulates gene expression. In the following section the main players of the Wnt signaling pathway will be described.

Wnt Ligands.

Wnt ligands range in length from 350-400 amino acid with a predicted molecular weight around 38 kDa, but that varies accordingly to amino acid sequence length and post-translation modifications present in the Wnt protein such as N-termini-located glycosylation and lipid modifications (Cadigan and Liu 2006; Chien, Conrad, and Moon 2009; Komekado et al. 2007). Wnt proteins contain 23-24 conserved cysteine residues that are thought to mediate protein-protein interactions either with receptors or secreted proteins (Sidow 1992; Willert et al. 2003). Lipid modifications of Wnt proteins occur in conserved residues (C77 and S209 in Wnt3a) and are thought to play a role in protein activity essential for binding to receptors, in the case of C77, and secretion, in the case of S209 (Cadigan and Liu 2006; Komekado et al. 2007; Lorenowicz and Korswagen 2009; Willert et al. 2003). The lipid modification step is thought to be carried out by the membrane-bound O-acyltransferase (MBOAT) family member named porcupine (Biechele, Cox, and Rossant 2011; Port and Basler 2010; Willert et al. 2003).

After the lipid modification are done, in the endoplasmic reticulum, the Wnt ligand is transported to the Golgi apparatus where it is glycosylated and sorted to

the secretory pathway (Port et al. 2008). *Wntless*/Gpr177 is yet another molecule that acts in the secretion route of Wnt ligands. It is an integral membrane protein that cycles between the plasma membrane and the Golgi and its activity is necessary for Wnt ligand secretion (Port et al. 2008; Yang et al. 2008). Interestingly, the activity of *Wntless*/Gpr177 appears to be conserved across vertebrates, being also required for Wnt secretion (Fu et al. 2009).

In addition to Wnt ligands other Non-Wnt molecules have been shown to be capable to activate the Wnt signaling (Binnerts et al. 2007; Hendrickx and Leyns 2008). R-Spondins for instance are a family of secreted proteins that can bind to LRP5/6 Wnt co-receptors preventing their internalization and activating the canonical Wnt pathway (Binnerts et al., 2007). Norrin, also called Norrie disease protein (NDP) is another protein that has being found to bind and activate Wnt receptors at the plasma membrane and mutations in it are known to affect the formation of the vasculature of the retina and cochlea, (Hendrickx and Leyns 2008).

Wnt Receptors.

Two major classes of receptors play substantial roles in activation of Wnt signaling. The primary Wnt receptors are named Frizzled receptors, and belong to a very specific G-protein coupled receptor (GPCR) sub-family (Schulte and Bryja 2007). Wnts also bind to members of the low-density lipoprotein receptor-related protein (LRP) family, such as LRP5/6 (Angers and Moon 2009).

The Frizzled receptors are a distinct family within the GPCR super family. They have 7 transmembrane-spanning domains, a large N-terminal domain that contains both a signal sequence directing the protein to the plasma membrane and a cysteine-rich domain (CRD), which mediates extracellular ligand binding. A large C-terminal that mediates interaction with intracellular proteins such as Disheveled (Dsh), G-proteins, Arrestins and a number of intracellular loops that have been found to contain phosphorylation sites for intracellular kinases (Cadigan and Liu 2006; Foord et al. 2005; Schulte 2010; Schulte and Bryja 2007).

Mammals have ten different genes encoding Frizzled receptors, *D. melanogaster* and *X. laevis* have four and eleven isoforms respectively (Schulte 2010). The name Frizzled was derived from a recessive mutation in *D. melanogaster*, which gives rise to a very specific phenotype. It yields flies with curly and disorganized bristles and cuticular hair. This is most likely due to the role of Frizzled receptors in the planar cell polarity pathway (Klaus and Birchmeier 2008; Vinson and Adler 1987; Vinson, Conover, and Adler 1989). The Frizzled receptors range in length from 500 to 700 amino acids with a molecular weight of about 71kDa, again due to differences in sequence and post-translational modifications (Dann et al. 2001; Vinson, Conover, and Adler 1989; Huang and Klein 2004).

The Low-density lipoprotein receptor-related proteins (LRP) comprise another receptor family involved in Wnt signaling. There are 10 genes in the

mammalian genome encoding Low-density lipoprotein receptor-related proteins (LRP), however, LRP5 and LRP6 are the most studied members due their role in Wnt signaling (Cadigan and Nusse 1997; He et al. 2004). LRP5/6 are co-receptors for Wnts and they are absolutely necessary for the activation of the canonical Wnt pathway (Hsieh et al. 2003; Kelly, Pinson, and Skarnes 2004), as is their orthologue *arrow* in *D. melanogaster* (Cadigan and Nusse 1997; He et al. 2004). A role in Wnt signaling for LRP/arrow came from genetic studies that demonstrated that mutations in arrow phenocopied the *wingless* phenotype in flies (Wehrli et al. 2000). It was also this study that classified *arrow* as an orthologue of LRP5/6 and its epistatic position in the Wnt pathway (Wehrli et al. 2000). Later, a series of studies involving knockout of either or both LRPs in mice also confirmed the LRP requirement for activation of the Wnt pathway (Kelly, Pinson, and Skarnes 2004). Additionally, a number of studies comprising overexpression, deletion and domain-specific deletion of LRPs have helped to understand their function in Wnt signaling (He et al. 2004).

LRPs are type I single-pass transmembrane proteins with a long extracellular N-terminus that mediates interaction with extracellular ligands and/or antagonists of Wnt signaling and a short C-terminus that mediates interaction with intracellular protein and also contains target motifs for kinase phosphorylation that are thought to be important for regulation of Wnt signaling (Hsieh et al. 2003; Kelly, Pinson, and Skarnes 2004; He et al. 2004). Arrow/LRP5/LRP6 have a small variation in length containing 1678, 1615 and

1613 amino acid residues, respectively, with calculated molecular weights of about 200 kDa. Their extracellular region contains four EGF-like domains and three LDLR repeats needed to mediate ligand binding (Angers and Moon 2009; Wehrli et al. 2000). The intracellular portion contains the proline-rich (PPPSP) motif, which is the sequence target of GSK3β phosphorylation required for activation of the canonical Wnt pathway (Angers and Moon 2009; He et al. 2004).

Wnt Antagonists.

Wnts have still another level of regulation that ensures confinement of the signal to specific locations. To accomplish this, cells secrete molecules that either bind to the Wnt ligand themselves, preventing binding to their receptors, such as Wnt inhibitory factor (WIF1), secreted Frizzled related protein (sFRP) and Cerberus or molecules that bind to the Wnt receptors preventing the receptors from responding to the Wnt ligands, such as Sclerostin, Dickkopf (DKK) and Wise (Angers and Moon 2009; Chien, Conrad, and Moon 2009; Malinauskas et al. 2011; Schulte 2010). The Dkk family contains four members in vertebrates (Dkk1-4) (Niehrs 2006). Dkks are secreted glycoproteins ranging in length from 255-350 amino acids with molecular weights ranging from 24-29 kDa for Dkk1,2,4 and a predicted molecular weight of 38 kDa for Dkk3 (Niehrs 2006). Dkks are thought to play a role in Wnt inhibition because of their ability to bind to LRP5/6 Wnt co-receptors and to recruit yet another single-pass transmembrane protein, Kremen (Mao et al. 2001; Bafico et al. 2001). Dkk brings together

LRP5/6 and Kremen, which in turn, leads to internalization of the LRP5/6 receptors via the endocytic pathway, therefore shutting down the canonical Wnt pathway (Mao et al. 2002).

Sclerostin and Wise are two other molecules involved with inhibition of the canonical Wnt pathway. Wise was identified in a screen to find factors that can change anteroposterior identity of neural tissues (Itasaki et al. 2003). Although, Wise is considered to be an antagonist of Wnt due to its ability to bind to LRP5/6 receptors and prevent activation of the canonical Wnt pathway, this view has been proven to be context-dependent (Angers and Moon 2009; Chien, Conrad, and Moon 2009; Itasaki et al. 2003). For instance, Wise can counteract the posteriorizing effects of Wnt8 when *Wise* RNA is co-injected with *Wnt8* RNA in *X. laevis* (Angers and Moon 2009; Chien, Conrad, and Moon 2009; Itasaki et al. 2003). However, this antagonism has been shown to be context-dependent, since injection of Wise RNA alone in animal caps of *X. laevies* leads to activation of the canonical Wnt pathway (Itasaki et al. 2003). Perhaps, these apparently contradictory effects of Wise reflect how the environment can shape the activation response of specific signaling pathways.

Sclerostin is product of the *SOST* locus. This locus encodes a secreted glycoprotein, which has an inhibitory effect on Wnt signaling (Angers and Moon 2009; Chien, Conrad, and Moon 2009; Semenov, Tamai, and He 2005). Mutation in the *SOST* locus causes abnormal bone formation, similar to gain-of-function mutations in LRP5 (Semenov, Tamai, and He 2005; Weidauer et al. 2009).

SOST knockout mice have a high bone mass phenotype confirming the role of *SOST* in bone formation and Wnt signaling (Li et al. 2008). Structural analysis of Sclerostin revealed that the mechanism of Wnt signaling inhibition is based on its ability to complex with LRP5/6, thereby inhibiting activation of the canonical Wnt pathway (Weidauer et al. 2009).

The other group of Wnt antagonists comprises WIF1, sFRP and Cerberus. The mechanism of action of this group is based on their ability to bind to Wnt ligands, thereby sequestering the Wnt ligand away from the Wnt receptors (Angers and Moon 2009; Chien, Conrad, and Moon 2009). The mechanism of inhibition of players in this group, however, is slightly different. In the case of the Wnt inhibitory factor (WIF1), it binds to the lipid-derived appendages of the Wnt molecule, thus preventing it to bind to its cognate receptors (Chien, Conrad, and Moon 2009; Malinauskas et al. 2011; Hsieh et al. 1999). WIF1 is highly conserved across vertebrate families and it encodes for a protein of 379 amino acids that contains a WIF domain (Hsieh et al. 1999). The WIF domain presents itself in the mature protein as a hydrophobic pocket that binds to the acyl chains of secreted Wnt proteins (Malinauskas et al. 2011). Mice with targeted deletion of WIF1 locus are viable and fertile but show increased likelihood of developing osteosarcoma (Kansara et al. 2009).

The soluble/secreted Frizzled-related protein (sFRP) family comprises 5 known members (sFRP1-5) (Chien, Conrad, and Moon 2009). Unlike WIF1, sFRPs contain a cysteine-rich domain that is thought to mediate binding not only

to Wnts but also to Frizzled receptors (Bafico et al. 1999; Cadigan and Liu 2006; Chien, Conrad, and Moon 2009). When the sFRP molecule binds to Wnt ligands, it prevents the Wnt ligand from interacting with the Frizzled receptor and activating Wnt signaling. But, when the sFRP molecule bind to the Frizzled receptor it most likely forms a non-functional complex with the Frizzled receptor which cannot activate Wnt signaling. This interaction is mediated by the cysteine-rich domain present on both molecules (Bafico et al. 1999; Chien, Conrad, and Moon 2009; Hoang et al. 1998).

Cerberus is another Wnt antagonist that also targets the Wnt ligands themselves. It was first isolated in *X. laevis* and it is expressed in the anterior endoderm as well as the organizer (Bouwmeester et al. 1996). It received its name based upon its ability to induce formation of ectopic heads in *X. laevis* (Kawano and Kypta 2003; Bouwmeester et al. 1996). Cerberus protein contains a cysteine-knot domain that is thought to be important for its function (Kawano and Kypta 2003). The mouse orthologue of Cerberus (Cerl-1) does not seem to have a high degree of conservation and it is debatable whether it indeed plays a role for Wnt inhibition (Belo et al. 2000; Bouwmeester et al. 1996).

β-Catenin and the β-Catenin Destruction Complex.

β-catenin plays a major role in Wnt signaling. It is a key factor in Wnt signaling that transduces proximal events from the plasma membrane to the nuclear compartment (Cadigan and Liu 2006; Chien, Conrad, and Moon 2009).

Mouse β-catenin contains 781 amino acids and its molecular weight is 88 kDa. Its structure consists of an N-terminal region of 150 amino acids which contains phosphorylation sites for CK1α and GSK3-β, a central part of ~520 amino acids which contains 12 armadillo (arm) repeats known to mediate protein-protein interactions and a 100 residue C-terminus that contains predicted phosphorylation sites thought to regulate protein-protein interaction as well (Shapiro and Weis 2009; Staal et al. 2002).

In the absence of Wnt ligands β-catenin mainly exists as part of the adherents junction complex, and its cytosolic concentrations are kept low by the β-catenin destruction complex (Archbold et al. 2012; Cadigan 2012). The β-catenin destruction complex readily phosphorylates β-catenin molecules that detach from the adherents junction, and this phosphorylation event tags β-catenin to the proteasome mediated degradation pathway (Arce, Yokoyama, and Waterman 2006; Archbold et al. 2012; MacDonald, Tamai, and He 2009). The basic scaffold of the β-catenin destruction complex comprises four proteins: Axin, a scaffolding protein that serves as an anchor site for β-catenin and the other proteins of the complex, Adenomatous polyposis coli (APC), a binding protein that brings together β-catenin and Axin and casein kinase 1 alpha (CK1α) and glycogen synthase kinase 3 beta (GSK3-β), two kinases that sequentially phosphorylate β-catenin tagging it for ubiquitination and subsequent degradation (Archbold et al. 2012; Cadigan and Nusse 1997; MacDonald, Tamai, and He

2009; Kimelman and Xu 2006). Although the exact order of β-catenin phosphorylation remains unclear, it seems that the phosphorylation of S45 by CK1α is permissive to subsequent phosphorylation on S33, S37 and T41 by GSK3-β (Archbold et al. 2012; Liu et al. 2002). This sequential phosphorylation event on β-catenin creates recognition sites for the β-transducin repeat-containing protein (β-TrCP), which is a member of the E3 ubiquitin ligase, which in turn carries out polyubiquitination of β-catenin driving it to the proteasome-mediated degradation pathway (Zeng et al. 2005; Spiegelman et al. 2000).

The Canonical Wnt Pathway.

The canonical or β-catenin-dependent Wnt pathway is the most extensively studied branch of the Wnt signaling pathway (Cadigan and Nusse 1997; Klaus and Birchmeier 2008). Its is defined by the following events: 1) binding of a Wnt ligand to frizzled and LRP receptors; 2) activation of intracellular machinery that prevents β-catenin degradation leading to cytosolic accumulation and nuclear translocation of β-catenin; and 3) the nuclear accumulation of β-catenin, which leads to a change in the transcription rate of genes in a TCF/LEF-dependent manner (Figure 1.3) (Cadigan and Liu 2006; Kimelman and Xu 2006; Taelman et al. 2010; Archbold et al. 2012).

How is the β-catenin destruction complex inhibited by the Wnt ligands?

Here, we need to introduce another member of the Wnt signaling pathway, the Disheveled (Dsh) protein. Dsh is thought to play a key role in inhibition of the β-catenin destruction complex and activation of the canonical Wnt signaling pathway (Archbold et al. 2012; Angers and Moon 2009; Cadigan and Liu 2006; Chien, Conrad, and Moon 2009). *Disheveled* was first identified as a recessive mutation in *D. melanogaster* that could phenocopy the Frizzled phenotype, therefore indicating some sort of epistatic relationship between the two genes (Wallingford and Habas 2005; Chien, Conrad, and Moon 2009). There are three homologues for the *Disheveled* gene in vertebrates (Dsh1-3) (Wallingford and Habas 2005). Disheveleds are modular proteins raging in length from 500 to 600 amino acids. Near its N-terminus, Disheveled contains a domain that mediates interaction with Axin (DIX, Disheveled Interaction with Axin) (Schwarz-Romond, Metcalfe, and Bienz 2007). At the central part of the protein lies a PDZ domain (, there is required for interaction with Frizzled receptors and other PDZ-containing proteins (Schulte and Bryja 2007). The proximal part of the C-terminal domain contains a DEP domain, which also mediates protein-protein interactions including disheveled itself and pleckstrin (Wong et al. 2000). *Disheveled* has been shown to be required for *wingless* function in flies (Cadigan and Nusse 1997; Chien, Conrad, and Moon 2009; Wallingford and Habas 2005). However, in mice, knockout experiments have questioned its primary requirement for activation of the canonical Wnt signaling, since activation of the canonical Wnt pathway seems to occur even in the absence of two of the three Dsh isoforms

(van Amerongen and Berns 2006). A plausible explanation for this could be due to some degree of redundancy among the different isoforms of Dsh present in mice. A *Disheveled* triple knockout animal is yet to be generated.

A classical model for the activation of the canonical Wnt pathway dictates that the binding of a Wnt ligand to its cognate receptors (Frizzled and LRP5/6) would lead to a phosphorylation-mediated activation of cytosolic pools of Disheveled protein. Phosphorylated Disheveled binds to Axin and recruits it to the plasma membrane, resulting in dissolution of the β-catenin destruction complex (Figure 1.3) (Cadigan and Liu, 2006; Angers and Moon, 2009). Also, at the plasma membrane level, GSK3-β-mediated phosphorylation of LRP5/6 in PPPSP motifs creates docking sites for physical interactions between GSK3-β and LRP5/6 (Zeng et al. 2005; Angers and Moon 2009). This interaction partially inhibits GSK3-β, which in turn leads to cytosolic accumulation and nuclear translocation of β-catenin. In the nuclear compartment β-catenin promotes transcriptional activation of genes containing TCF-binding sites (Angers and Moon 2009; Cadigan and Liu 2006; Cadigan and Nusse 1997).

This model although widely accepted often fails to explain results obtained from different groups (Blitzer and Nusse 2006; Taelman et al. 2010; Wu and Pan 2010). For instance, the model predicts that the β-catenin destruction complex would either stay at the plasma membrane in an inactivated mode, or fall apart and no longer being able to phosphorylate β-catenin. However, previous studies have demonstrated that the complex does not fall apart, and endocytosis of the

complex is absolutely required for activation of the Wnt signaling pathway (Blitzer and Nusse 2006). Furthermore, it has been shown that the proposed destabilization mechanism for inhibition of GSK3-β is rather inefficient and does not seem to be a reliable mechanism for GSK3-β inhibition (Cselenyi et al. 2008). Finally, the activity of GSK3-β is not affected in detergent permeabilized cells, even though this model would predict the inhibition to occur (Taelman et al. 2010).

A recent publication has addressed these disagreements by proposing a new view of activation of canonical Wnt signaling (Taelman et al. 2010). In this new model, the first steps remain the same (i.e. in the presence of Wnt ligand, the β-catenin destruction complex is recruited to the plasma membrane in a disheveled-dependent manner). Once at the plasma membrane, the β-catenin destruction complex and the Wnt-Frizzled-LRP complex undergoes endocytosis, forming an early endosome vesicle that eventually fuses with a multivesicular body. This step results in separation of the β-catenin destruction complex from the cytosolic environment by a double membrane layer, which prevents GSK3-β-mediated β-catenin phosphorylation and degradation from occurring, leading to β-catenin accumulation and translocation to the nuclear environment, where it performs its functions as a transcription activator (Taelman et al. 2010).

Figure 1.3

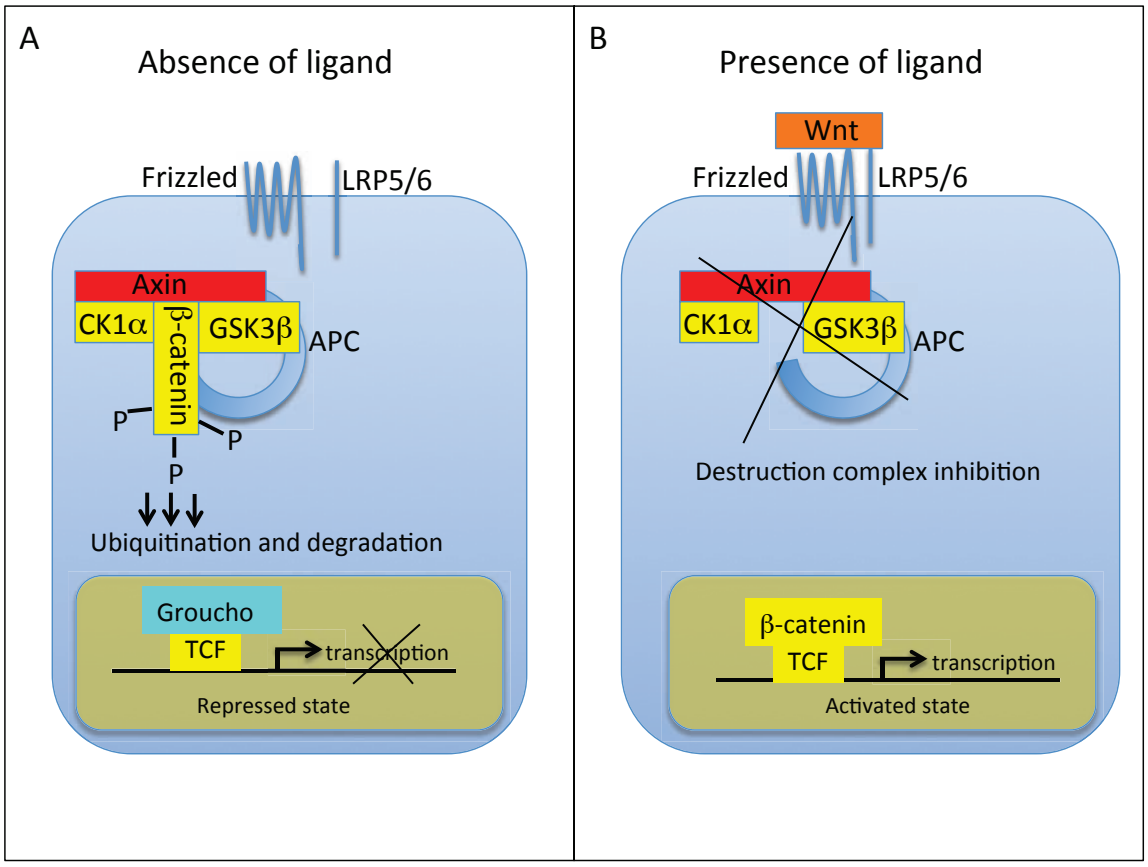
A
Absence of ligand
Frizzled
LRP5/6
Axin
CK1α
β-catenin
GSK3β
APC
P
P
P
Ubiquitination and degradation
Groucho
TCF
transcription
Repressed state
B
Presence of ligand
Wnt
Frizzled
LRP5/6
Axin
CK1α
GSK3β
APC
Destruction complex inhibition
β-catenin
TCF
transcription
Activated state

Figure1.3: The canonical Wnt pathway. A. In the absence of a Wnt ligand the levels of cytosolic β-catenin are kept low by the action of the β-catenin destruction complex (Axin, Ck1α, GSK3-β and APC) that phosphorylates and targets β-catenin to the proteasome-mediated degradation pathway. B. In the presence of a Wnt ligand, the β-catenin destruction complex is inhibited, and β-catenin gets accumulated in the cytosol and it is readily translocated into the nucleus of the cell where it acts as a transcriptional co-activator, increasing the transcription of target genes. Adapted from Eisenmann, 2005.

β-Catenin and Activation of the Transcriptional Machinery.

In the nuclear compartment β-catenin plays a pivotal role in assembling the nuclear protein complex that is required for transcriptional activation of Wnt target genes (MacDonald, Tamai, and He 2009). The primary DNA-binding proteins in the complex are T-cell factor/lymphoid enhancer factor (TCF/LEF) family members (Arce, Yokoyama, and Waterman 2006; MacDonald, Tamai, and He 2009). In vertebrates the TCF/LEF family comprises four genes, however due to the existence of several splice variants there are numerous slightly different protein isoforms (Arce, Yokoyama, and Waterman 2006; Okamura et al. 1998; Hoppler and Kavanagh 2007). TCFs are members of the high mobility group (HMG) box proteins which upon binding cause DNA bending that is thought to facilitate recruitment of other factors by making DNA more accessible to the transcriptional machinery (Arce, Yokoyama, and Waterman 2006; Atcha et al. 2007).

All TCF/LEF family members bind to a conserved DNA sequence CCTTTGWW (“W” means weak “A” or “T” base at any given position) at the core of a 16 base-pair long motif named TCF-binding site (Arce, Yokoyama, and Waterman 2006; Atcha et al. 2007). In mice deletion of TCF family members show different phenotypes, probably due to different expression patterns (Arce, Yokoyama, and Waterman 2006; van Amerongen and Berns 2006; Hoppler and Kavanagh 2007). For instance, double knockout of TCF1 and LEF1 yields a phenotype similar to deletion of Wnt3a, that is, defects in paraxial mesoderm,

limb bud development and several problems with neural tube development (Galceran et al. 1999). TCF3 knockout embryos have defects in A-P axis formation such as axial duplication and also duplication of structures such as node and notochord (Merrill et al. 2004).

In the absence of β-catenin, TCF proteins form a complex with the transducing-like enhancer of split (TLE/groucho) repressor (Logan and Nusse 2004; Chien, Conrad, and Moon 2009). The TLE/TCF complex recruits agents such as histone deacetylases (HDAC), causing chromatin condensation and gene silencing (Courey and Jia 2001). Conversely, when the canonical Wnt pathway is activated, β-catenin is translocated into the nuclear environment where it causes the displacement of TLE and forms a complex with TCF proteins bound to DNA, this complex recruits transcription activators to that particular DNA site (Archbold et al. 2012; Atcha et al. 2007; Graham et al. 2000).

Binding of β-catenin to TCFs is not sufficient to start transcription. It is rather a first step that leads to the recruitment of factors that also bind to β-catenin in order to create a transcription “hot-spot” (Courey 2001; Courey and Jia 2001; Graham et al. 2000). B-cell lymphoma 9 (*BCL-9/B9L/legless*) is yet another protein recruited to the transcription machinery. It was identified in B-cell cancers as a gene commonly upregulated due to chromosome translocations (Itoyama et al. 2002; Willis et al. 1998). Around that time *legless,* the *D. melanogaster* homologue of BCL-9, was identified and suggested to be a binding partner of *pygopus* (Kramps et al. 2002; Thompson 2004). *Pygopus* is another

binding partner of β-catenin and although it has been shown to be required for wingless signaling in flies, its requirement for Wnt signaling in vertebrates remains uncertain (Hoffmans, Stadeli, and Basler 2005; Brembeck et al. 2004; Chien, Conrad, and Moon 2009; Thompson 2004). Additionally, β-catenin through its C-terminal binds to the histone acyltransferases CBP and p300, which promote chromatin unpacking and recruit RNA pol II to the site of transcription, in order to start the transcription of genes downstream the canonical Wnt pathway (Parker et al. 2008; Hecht et al. 2000).

The Non-Canonical Wnt Pathway.

The so-called non-canonical Wnt pathway refers to the Wnt-activated cell signaling in which LRP5/6 and β-catenin are not involved (Archbold et al. 2012; Cadigan 2012; Cadigan and Nusse 1997). The non-canonical Wnt pathway is divided into three branches. The first is the planar cell polarity (PCP) pathway, which has been characterized in *D. melanogaster* where it controls gastrulation via convergent extension movements in developing embryos. Additionally, it controls hair polarity, spindle orientation and orientation of inner ear hair cell development (Barrow 2006).

The second branch is called Wnt/Ca^{2+} pathway. In this pathway, Wnt signaling controls the activation of two kinases, Ca^{2+}-dependent protein kinase (PKC) and Ca^{2+}/calmodulin-dependent protein kinase (CaMKll), by modulating intracellular concentrations of calcium ions via Frizzled receptors (Cha et al.

2008; Klaus and Birchmeier 2008; Kuhl 2004). It has been debated whether the Wnt/ Ca^{2+} pathway is indeed a distinct pathway or whether it is an extension of the PCP pathway in vertebrates, mainly because some molecules such as disheveled are indistinguishably activated in both (Veeman, Axelrod, and Moon 2003).

The third branch of the non-canonical Wnt pathway is mediated by Wnt ligands binding to atypical transmembrane tyrosine kinases receptors Ryk and Ror2 (Hendrickx and Leyns 2008; Veeman, Axelrod, and Moon 2003). In fact, it was the discovery of *Derailed*, a Ryk homologue in flies that opened this new venue within Wnt signaling (Yoshikawa et al. 2003). Monomeric G-proteins such as Rho and Rac, c-jun N-terminal kinase, PKC, CaMK, nuclear factor of activated T-cell (NFAT) are some of the intracellular effectors that also have been implicated with activation of the non-canonical Wnt pathway (Chien, Conrad, and Moon 2009).

The activation of one intracellular pathway over another is highly dependent on cellular context such as, the presence of receptors, different Wnt ligands, and intracellular effectors that may or may not be present in a particular cell type.

Rationale and Aims of the Dissertation.

Multiple lines of evidence have suggested that the Wnt signaling pathway has been evolutionarily selected to orchestrate axial specification and gastrulation across different phyla in metazoans.

In mammals knockout studies have indicated that the Wnt signaling pathway is at the core of the process of axial specification and gastrulation. However, the mechanism by which the organizer is formed and whether it is governed by Wnt signals remains elusive.

In mice, it has been shown that the posterior visceral endoderm can re-specify cell fate in a similar fashion to the amphibian organizer and Wnt signals are detected in the posterior visceral endoderm immediately before they appear in the epiblast.

The main goal of this dissertation is to investigate whether a posterior visceral endoderm-derived Wnt-mediated signal controls axial specification in mice. The specific aims of this Dissertation are:

- To determine whether Wnt signals emanating from the posterior visceral endoderm are required for the formation of the primitive streak *in vivo*.
- To investigate whether Wnt3 carries out its functions via activation of the canonical Wnt pathway in the epiblast of early post implantation mouse embryos.

- To investigate the mechanism by which posterior visceral endoderm controls the activation of the *Wnt3* locus in the epiblast and therefore gastrulation.
- To investigate the importance of epiblast-derived Wnt3 for gastrulation in mice.

During animal development the formation of the three germ layers is a spatially and temporally organized process. Understanding how Wnt signals help to shape the embryos and generate new tissues will improve our knowledge on how our own body is shaped and help us to gather new cues on how inductive signals can help to shape complex structures in the embryo.

CHAPTER II:

Wnt3 Originating in the Posterior Visceral Endoderm is Required for the Formation of the Primitive Streak in Mice.

Preface.

Wnt3 derived from the Posterior Visceral Endoderm is Required for the Formation of the Primitive Streak in Mice.

This chapter comprises the analysis of the results of the specific deletion of *Wnt3* in the visceral endoderm of early post-implantation mouse embryos. All results described in this chapter are currently part of a manuscript in preparation. In order to delete *Wnt3* in the visceral endoderm we took advantage of distinct *Wnt3* alleles. The $Wnt3^{lacZ}$ allele used in this work was generated by Dr. Maki Wakamya and Dr. Richard Behringer (Baylor College of Medicine, TX) and gifted to us as part of collaborative efforts between the two groups. Due to this collaboration MW and RB will co-author this manuscript. The $Wnt3^{c}$ (conditional) allele was generated by Dr. Jeff Barrow and Dr. Andy McMahon (Harvard University, MA) and gifted to us by that same group. Dr. A.K Hadjantonakis and Gloria Kwon (Memmorial Sloan-Kettering Cancer Center, NY) generated the Ttr-Cre transgenic line used in this work, and gifted it to us as part of on-going collaboration between the two groups. AKH and GK will co-author this manuscript.

Abstract.

Wnt3, a secreted Wnt ligand, is required for gastrulation in mouse embryos. *Wnt3* null mouse embryos fail to gastrulate, form mesoderm, endoderm or any derivative. *Wnt3* is expressed in the Posterior Visceral Endoderm (PVE) at E5.5 and subsequently in the adjacent epiblast (E5.75). Due to its pattern of expression and genetic requirement we hypothesized that *Wnt3* signals derived from the PVE induce the formation of a primitive streak in mouse embryos. To test this hypothesis, we first determined whether Wnt3 was required in the VE for formation of the primitive streak. We generated mouse embryos lacking *Wnt3* specifically in the visceral endoderm. Embryos with a visceral endoderm-specific knockout of *Wnt3* phenocopy the *Wnt3* null mouse phenotype, that is, they fail to form a primitive streak and gastrulate. Interestingly, we also observed lack of formation of primordial germ cells in embryos with a visceral endoderm-specific knockout of *Wnt3* at E7.5. Furthermore, we show that Wnt3 signaling propagates in the epiblast via activation of the canonical Wnt pathway. These experiments indicate that *Wnt3* utilizes the canonical Wnt pathway during gastrulation and that *Wnt3* function in the PVE is essential for gastrulation in mice.

Background.

Axial specification is a process by which the main body plan is laid down. It is intrinsically linked to a symmetry-breaking event that sets in motion the formation of an organizer. An organizer is a structure that is capable of influencing and changing the fate of target cells, therefore orchestrating/organizing the development of an entire organism (Harland and Gerhart 1997; Wolpert 2011).

At the beginning of the twentieth century Hans Spemann and Hilde Mangold conducted transplantation experiments in amphibian embryos and discovered that the dorsal lip of the blastopore when transplanted to the ventral side of another embryo was able to organize embryonic development by instructing neighboring cells not only to differentiate into specific cell types but also by guiding their spatial organization and orientation (Harland and Gerhart 1997; Heasman 2006). In the 1960's Pieter Nieuwkoop discovered that the dorsovegetal region of the amphibian blastula was responsible for the induction of the organizer described by Spemann (Gerhart 1997, 1999). Today, this region is referred as the "Nieuwkoop center" in his honor (Gerhart 1999).

In *Xenopus*, the Nieuwkoop center activates the canonical Wnt signaling pathway in the dorsal/vegetal most area of the embryos (Fagotto, Guger, and Gumbiner 1997; Guger and Gumbiner 1995). Wnt11 has been suggested to be the main Wnt ligand utilized in this process (Heasman 2006; Tao et al. 2005).

In mammals, the existence of a Nieuwkoop center equivalent remains elusive. The posterior visceral endoderm (PVE) is a tissue that covers the epiblast at its junction with the extraembryonic ectoderm. The posterior visceral endoderm is capable of re-specifying neural ectoderm to a posterior/mesodermal fate (Belaoussoff, Farrington, and Baron 1998). *Wnt3*, a canonical Wnt, is first expressed in the PVE and subsequently in the epiblast (Rivera-Perez and Magnuson 2005). *Wnt3* null embryos fail to form a primitive streak, mesoderm, endoderm and all derivatives (Liu et al. 1999). Because of its pattern of expression and genetic requirements, we hypothesized that Wnt3 emanating from the posterior visceral endoderm is the inducer of the primitive streak. A logical extension of this hypothesis is that the posterior visceral endoderm would be the murine equivalent of the amphibian Nieuwkoop center.

Here we show that *Wnt3* function in the PVE is essential for the formation of the primitive streak. The canonical Wnt signaling pathway mediates Wnt3 effects in the epiblast. These results suggest the presence of a Nieuwkoop center equivalent in mammals. Comparison with *X. laevis* and Zebrafish reveals that signaling through the canonical Wnt signaling pathway has been conserved in vertebrate gastrulation, however, because different Wnt ligands control gastrulation in *X. laevis,* zebrafish and mice, it appears that different Wnt ligands have been co-opted during the evolution of axial development in vertebrates.

Results.

Wnt3 Expression in the Visceral Endoderm is Necessary for Primitive Streak Formation.

Wnt3 expression is first observed in the PVE of embryos dissected at E5.5 and spreads to the underlying epiblast by E5.75, approximately eighteen hours before the appearance of the primitive streak (Rivera-Perez and Magnuson 2005). These observations led to the hypothesis that *Wnt3* activation in the PVE is the first event that leads to the induction of the primitive streak. If *Wnt3* function in the PVE is essential for the establishment of the primitive streak, embryos whose *Wnt3* has been conditionally inactivated in the visceral endoderm should phenocopy the *Wnt3*-null phenotype and fail to form the primitive streak. To test this hypothesis, we took advantage of two *Wnt3* alleles, a conditional allele that has *loxP* sites flanking exons 3 and 4 (Barrow et al. 2003) and a newly generated null allele that carries a *lacZ* gene inserted into unique *ClaI* site within exon 4 (Figure 2.1A), provided by Dr. Richard Behringer. After Cre-mediated recombination exons 3 and 4 exons of the conditional $Wnt3^{c}$ allele are excised creating a null allele of *Wnt3*, named $Wnt3^{\Delta 3,4}$ (Barrow et al. 2003). A schematic representation of these different alleles of *Wnt3* is provided in figure 2.1A.

To inactivate *Wnt3* in the visceral endoderm, we used Ttr^{Cre} transgenic mice (Kwon, Viotti, and Hadjantonakis 2008). In this strain, Cre recombinase is expressed under the control of the *Transthyretin* (*Ttr*) promoter.

Figure 2.1

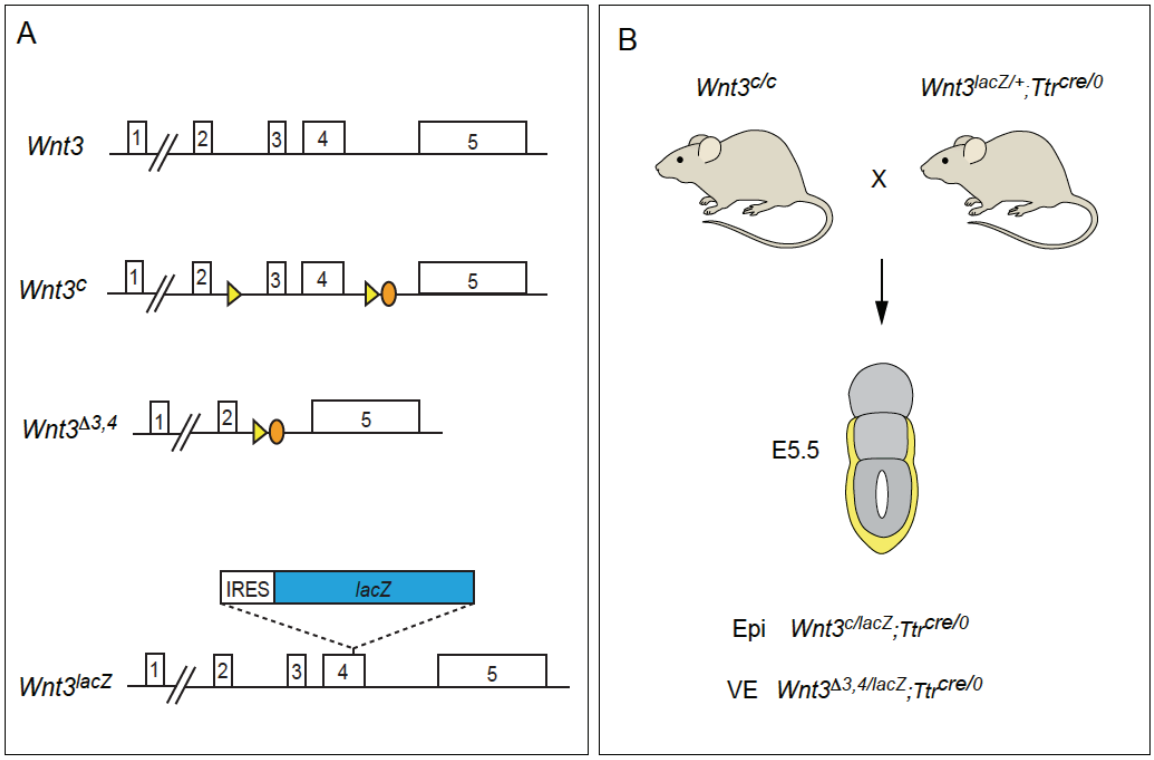
A
Wnt3
Wnt3c
Wnt3Δ3,4
IRES
lacZ
Wnt3lacZ
1
2
3
4
5
B
Wnt3c/c
Wnt3lacZ/+;Ttrcre/0
X
E5.5
Epi Wnt3c/lacZ;Ttrcre/0
VE Wnt3Δ3,4/lacZ;Ttrcre/0

Figure 2.1: Schematic representation of *Wnt3* alleles and breeding strategy utilized to ablate *Wnt3* in the visceral endoderm. A. Schematic representation of *Wnt3* alleles. *Wnt3*, wild-type; $Wnt3^{c}$, floxed allele. The *loxP* sites are marked as yellow triangles and a residual *FRT* site is marked as an orange oval. $Wnt3^{\Delta 3,4}$, recombined null allele; $Wnt3^{lacZ}$, null allele. An *IRES-lacZ* cassette was inserted in the unique ClaI site present in the exon 4 of the *Wnt3* locus creating a null allele. B. Breeding strategy to generate embryos lacking Wnt3 specifically in the visceral endoderm.

This promoter is exclusively expressed in the visceral endoderm of early post-implantation embryos (Kwon, Viotti, and Hadjantonakis 2008; Kwon and Hadjantonakis 2009). We crossed male mice heterozygous for *Wnt3*^{lacZ} and hemizygous for the *Ttr*^{Cre} transgene with females homozygous for the *Wnt3*^{c} floxed allele (Figure 2.1B). This cross generates embryos with a conditional ablation of the *Wnt3* locus in the visceral endoderm, however, because *Wnt3* is expressed only in the posterior visceral endoderm, it is basically a knockout of *Wnt3* in the posterior visceral endoderm.

Morphological analysis of embryos carrying a visceral endoderm-specific knockout of *Wnt3* at E6.5 and E7.5 revealed an absence of a primitive streak (Figure 2.2A,B), as is the case with *Wnt3* null embryos (Liu et al. 1999). At E6.5 the visceral endoderm mutant embryos fail to undergo anterior to posterior elongation and the primitive streak is not evident (Figure 2.2A). Analysis of the primitive streak was facilitated by the use of *Brachyury* (*T*) probe (black arrow Figure 2.2A). *T* is an early marker of the primitive streak and its precursors in mouse embryos (Herrmann 1991; Rivera-Perez and Magnuson 2005). Visceral-endoderm specific *Wnt3* knockout embryos fail to show *T* expression in the epiblast at E6.5 (Figure 2.2A). At E7.5, visceral endoderm-specific *Wnt3* knockout embryos lacked a primitive streak (black arrow Figure 2.2B), amnion (red arrow Figure 2.2B) and allantois (yellow arrow Figure 2.2B).

Figure 2.2

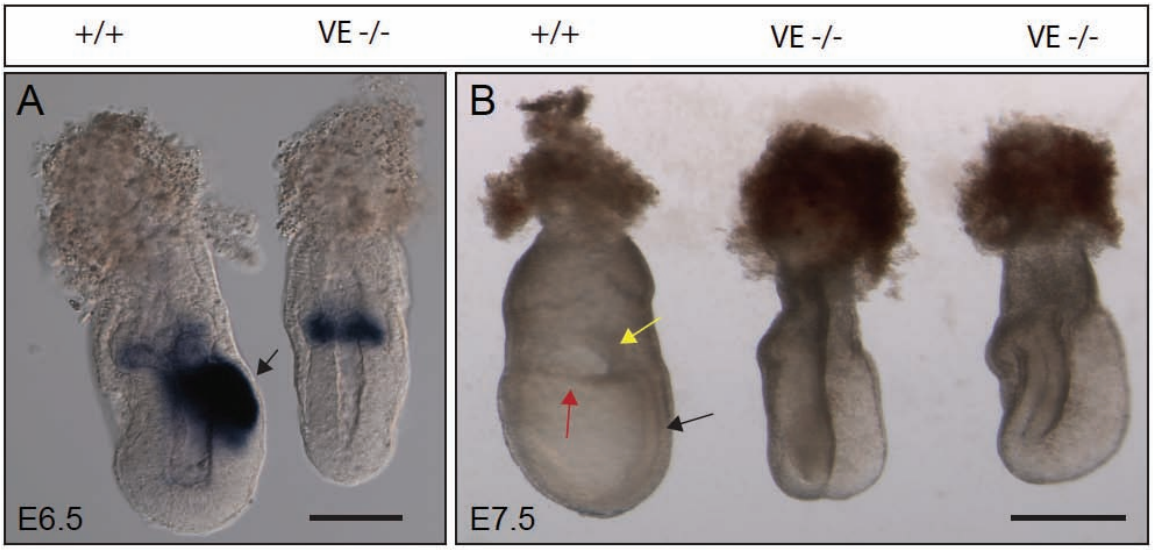

Figure 2.2: Visceral endoderm-specific *Wnt3* null embryos fail to gastrulate. A. The absence of a primitive streak was detected using riboprobes for *Brachyury* (Black arrow). B. Visceral endoderm-specific Wnt3 null embryo dissected at E7.5. Mutant embryos evolve into a “spoon shape” structure that lacks gastrulation hallmarks observed in the control embryo, such as primitive streak (black arrow), amnion (red arrow) and allantois (yellow arrow). Scale bar in A 100 µm and in B 250 µm

These mutant embryos also failed to undergo an anterior to posterior elongation, evolved into “spoon-shaped” embryos (Figure 2.2A,B), and were mostly resorbed by E9.5.

In order to confirm the observed results were due *Ttr*^{Cre} activity solely confined to the visceral endoderm and not due to an ectopic activation of this transgene in the epiblast, we crossed *Ttr*^{Cre} males to *ROSA26-lacZ* reporter (*R26*^{lacZR}) females. *R26*^{lacZR} harbor the bacterial *lacZ* gene downstream a neo cassette and stop codon flanked by *LoxP* sites, inserted in the *ROSA26* locus (Soriano 1999). Cre-mediated recombination puts the *lacZ* gene under control of the *ROSA26* promoter. The *ROSA26* locus is ubiquitously expressed in mouse embryos (Soriano 1999), therefore the tissue specificity of *cre* recombinase expression dictates where the *lacZ* will be expressed. In E6.5 double transgenic embryos (n=10), we detected recombination of *R26*^{lacZR} exclusively in the visceral endoderm layer of the conceptus (Figure 2.3A), confirming that the *Ttr*^{Cre} line indeed drives Cre recombinase expression exclusively in the visceral endoderm.

To determine whether a primitive streak had been specified in the visceral endoderm-specific *Wnt3* knockout embryos we analyzed the expression of well characterized primitive streak markers such as *T (Brachyury)*, *Axin2*, *Fgf8*, *Mixl1*, *Sp5*, and *Cripto* (Ding et al. 1998; Hart et al. 2002; Herrmann 1991; Jho et al. 2002; Sun et al. 1999; Treichel, Becker, and Gruss 2001).

Figure 2.3

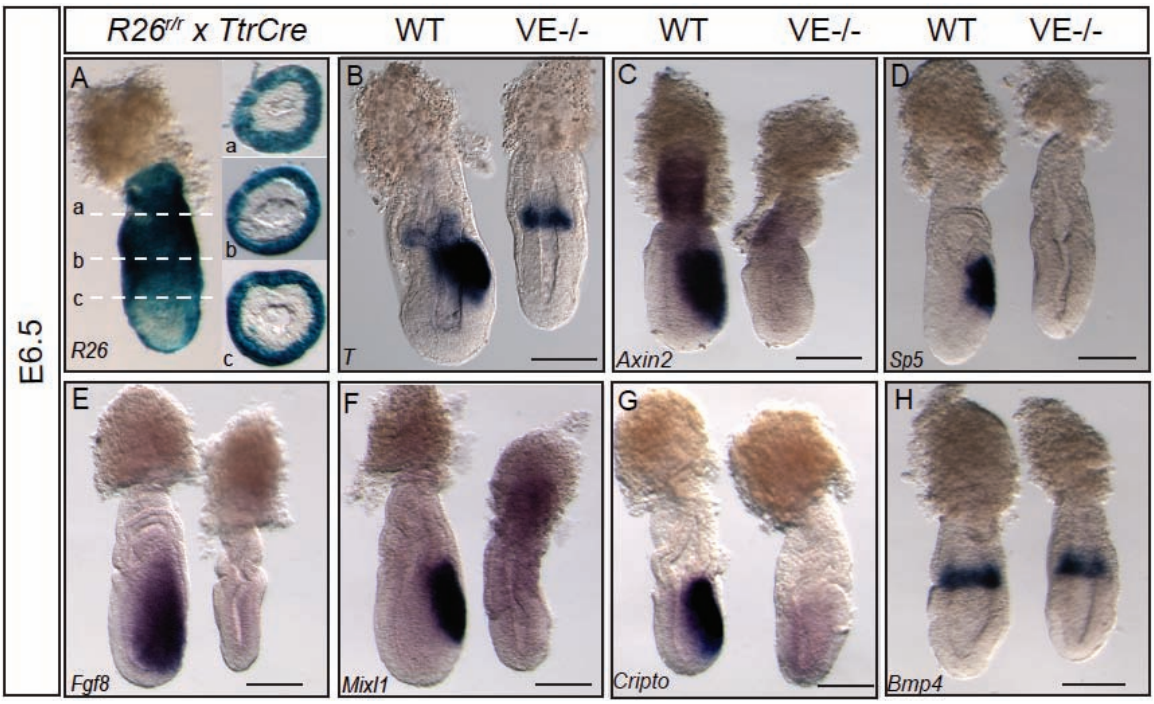
R26r/r x TtrCre
WT
VE-/-
WT
VE-/-
WT
VE-/-
E6.5
A
a
b
c
R26
B
T
C
Axin2
D
Sp5
E
Fgf8
F
Mixl1
G
Cripto
H
Bmp4

Figure 2.3: Molecular analysis of visceral endoderm-specific *Wnt3* null embryos at E6.5. A. Embryo dissected at E6.5 showing Ttr^{Cre} recombination of the R26 reporter exclusively in the visceral endoderm layer. B-G. *Wnt3*-VE mutant (VE -/-) and control (WT) embryos dissected at E6.5 and hybridized with markers of the primitive streak. H. Embryo dissected at E6.5 showing normal expression of *Bmp4* in the extra-embryonic ectoderm. Scale bar 100µm.

Figure 2.4

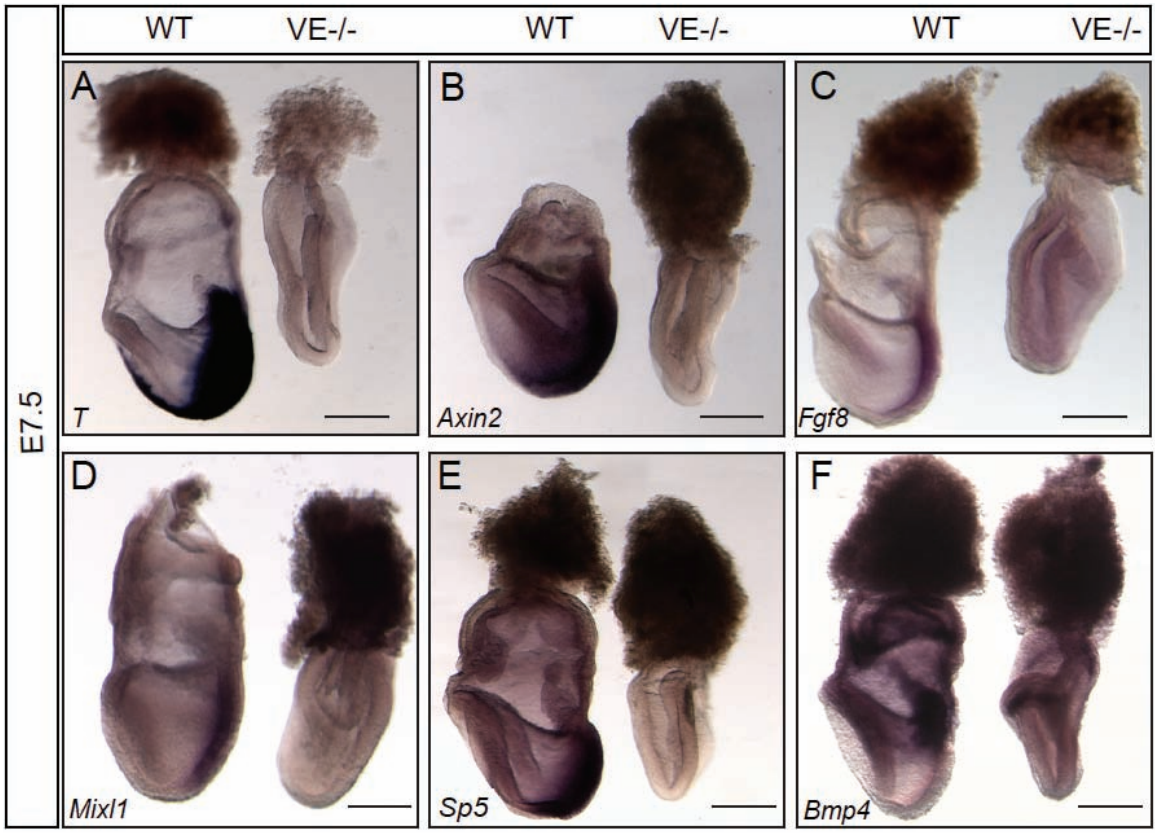

Figure 2.4: Molecular analysis of visceral endoderm-specific *Wnt3* null embryos at E7.5. A-F. *Wnt3*-VE mutant (VE -/-) and control (WT) embryos dissected at E7.5 and hybridized with markers of the primitive streak. *Wnt3*-VE mutant embryos lack expression of *Brachyury, Axin2, Fgf8, Mixl1, Sp5* and *Bmp4*. Scale bar 200µm.

These genes mark the primitive streak and its precursors in the epiblast. *T* also marks the distal part of the extra-embryonic ectoderm adjacent to the proximal epiblast (Perea-Gomez et al. 2004; Rivera-Perez and Magnuson 2005).

VE-specific Wnt3 knockout embryos analyzed at E6.5 and at E7.5 did not show expression of *T* (n=13), *Axin2* (n=12), *Fgf8* (n=17), *Mixl1* (n=10), *Sp5* (n=12), and *Cripto* (n=9) (Figure 2.3B-G and Figure 2.4A-F) respectively. Expression of *T* in the extraembryonic ectoderm was present but reduced showing that although its initial expression in this tissue is independent of *Wnt3* function in the visceral endoderm, *Wnt3* appears to play a role in the maintenance of *T* expression in the extraembryonic ectoderm (Figure 2.3A).

From this set of experiments we conclude that *Wnt3* function in the posterior visceral endoderm is necessary for the specification of the primitive streak in the mouse embryo.

Bmp4 is not Sufficient to Induce a Primitive Streak.

It has been suggested that Bmp4 emanating from the extraembryonic ectoderm induces the formation of the primitive streak on the posterior epiblast of E6.5 mouse embryos (Ben-Haim et al. 2006). Therefore, we wondered whether *Bmp4* expression was affected in VE-specific *Wnt3* knockout embryos. To address this question, we carried out wholemount *in situ* hybridization studies to analyze the expression of *Bmp4* in VE-specific *Wnt3* knockout mouse embryos

dissected at E6.5 and E7.5. All the mutant embryos tested (E6.5 n= 5, and E7.5 n= 4), had expression levels of *Bmp4* in the distal part of the extraembryonic ectoderm comparable to control embryos (Figure 2.3H and Figure 2.4F). Thus, despite having a functional copy of *Wnt3* in the epiblast, no primitive streak is observed in VE-specific *Wnt3* knockout embryos indicating that Bmp4 is not sufficient for the formation of the primitive streak.

Visceral Endoderm-Derived *Wnt3* Regulates its Own Expression in the Epiblast.

The sequential expression of *Wnt3,* first in the posterior visceral endoderm, and then in the adjacent epiblast (Rivera-Perez and Magnuson 2005) suggested that *Wnt3* activity in the posterior visceral endoderm was required for its own expression in the epiblast. To test this hypothesis, we analyzed the expression of *Wnt3* in embryos with conditional ablation of *Wnt3* specifically in the visceral endoderm, dissected at E6.5. To this end we used a mixture of two riboprobes probes for detection of *Wnt3* RNA described as following: a cDNA probe spanning exons 2 through 5 of the coding sequence (Liu et al. 1999) and a second probe that maps to the 3'UTR region of the *Wnt3* locus. We did not observe *Wnt3* expression in the posterior visceral endoderm or epiblast of mutant embryos with these probes (n=6) (Figure 2.5A).

Figure 2.5

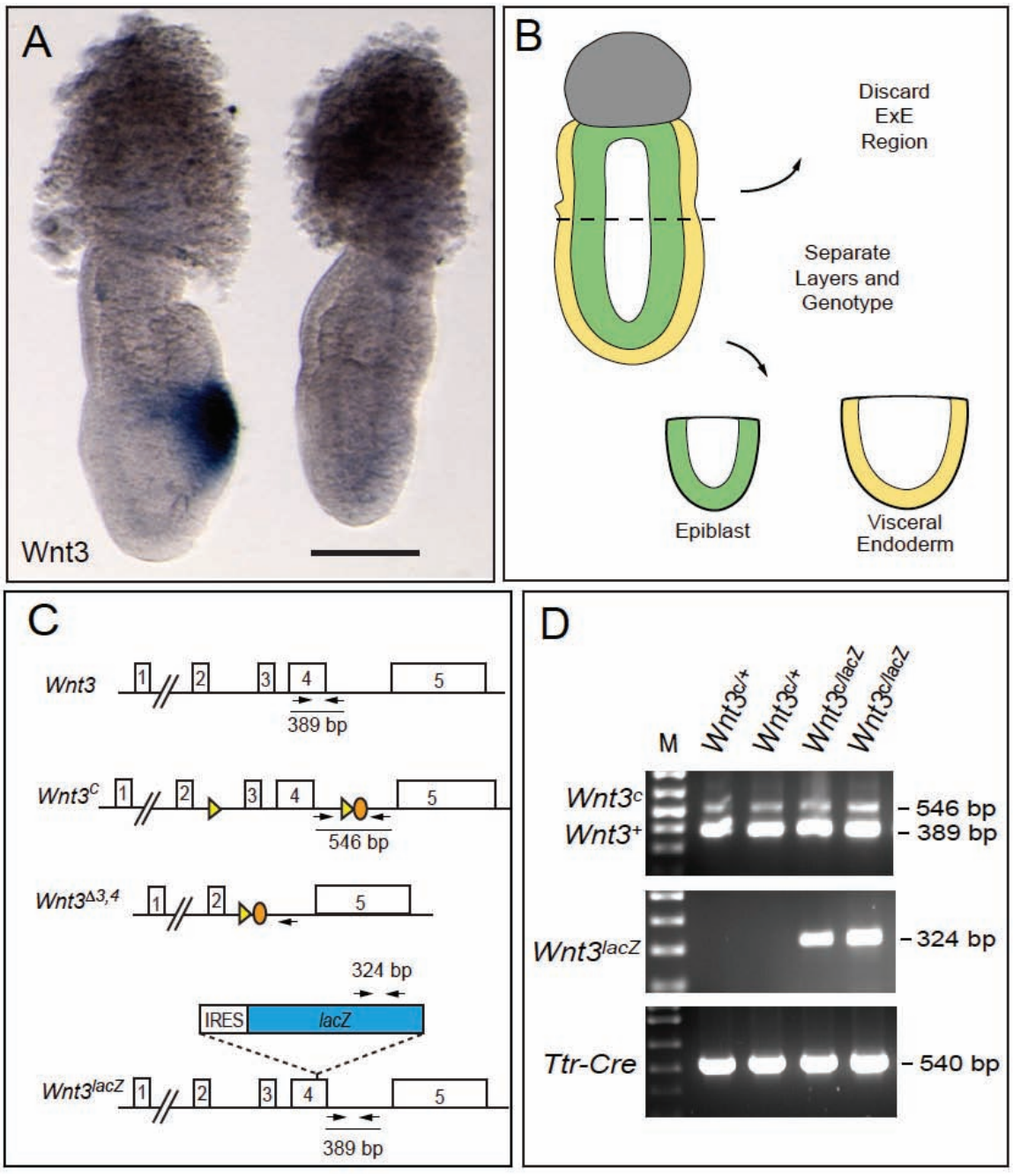
A
Wnt3
B
Discard
ExE
Region
Separate
Layers and
Genotype
Epiblast
Visceral
Endoderm
C
Wnt3
1
2
3
4
5
389 bp
Wnt3C
546 bp
Wnt3Δ3,4
324 bp
IRES
lacZ
Wnt3lacZ
389 bp
D
M
Wnt3c/+
Wnt3c/+
Wnt3c/lacZ
Wnt3c/lacZ
Wnt3c
Wnt3+
546 bp
389 bp
Wnt3lacZ
324 bp
Ttr-Cre
540 bp

Figure 2.5: Germ layer genotyping of visceral endoderm *Wnt3* null embryos. A. *Wnt3* expression in control and *Wnt3*-VE mutant embryos dissected at E6.5. B. Cartoon depicting the strategy utilized to isolate the epiblast from the *Wnt3*-VE mutant embryos. C. Alignment of primers to specific Wnt3 alleles and their respective amplicoms. D. PCR-Genotyping of the epiblast from two *Wnt3*-VE mutant and two heterozygous embryos. The *Wnt3*-VE mutant epiblast retains the non-recombined $Wnt3^{c}$ allele (546 bp) along side the $Wnt3^{lacZ}$ (324 bp) and Ttr^{Cre} transgene (540 bp). Scale bar 100 µm.

To exclude the possibility that the *Wnt3*^{c} allele was recombined in the epiblast, we genotyped the epiblast region of mutant *Wnt3*-VE embryos and control littermates. Pre-primitive streak embryos dissected at E6.5 were severed transversely at a plane distal to the epiblast-extraembryonic ectoderm boundary to avoid contamination with extraembryonic ectoderm (Figure 2.5B). The distal piece was treated enzymatically to separate the epiblast from the visceral endoderm layer as described previously (Nagy et al. 2003). The epiblast layer was then subjected to PCR genotyping using primers designed to amplify the floxed (*Wnt3*^{c}) allele, and *Ttr*^{Cre} transgene and the *Wnt3*^{lacZ} allele (Figure 2.5C). Using this strategy we were able to show that the visceral endoderm-specific *Wnt3* null embryos retain a functional allele of *Wnt3* in the epiblast (Figure 2.5D). These results support the idea that *Wnt3* is required in the visceral endoderm for its own expression in the epiblast.

To confirm that *Wnt3* expression in the epiblast requires *Wnt3* activity in the visceral endoderm, we conducted β-galactosidase assays in visceral endoderm-specific *Wnt3* null embryos. Since these VE-mutant embryos carry a copy of the *Wnt3*^{lacZ} allele we reasoned that if *Wnt3* was required in the visceral endoderm for its own expression in the epiblast, we should not detect β-galactosidase activity in the epiblast in VE-specific *Wnt3* null embryos. At the same time, we should observe β-galactosidase activity in the posterior visceral endoderm were *Wnt3* is first activated during development.

Embryos obtained from crosses between *Wnt3*^{lacZ};*Ttr*$^{Cre/0}$ males and *Wnt3*$^{c/c}$ females were assayed for β-galactosidase activity. Wild type embryos show expression of β-galactosidase in the primitive streak and derivatives as expected (Figure 2.6A). Conversely, VE-specific *Wnt3* mutant embryos (n=9) lacked β-galactosidase activity in the epiblast. They showed however β-galactosidase activity in the posterior visceral endoderm (Figure 2.6B). When VE-specific *Wnt3* mutant embryos were severed across the region where the stain was detected we could verify that β-galactosidase staining was restricted to the posterior visceral endoderm adjacent to the posterior epiblast (Figure 2.6C). These results indicate that *Wnt3* function is required in the posterior visceral endoderm for its own activation in the epiblast.

The Heterozygosity of the *Wnt3* Locus in the Epiblast does not Affect the Phenotype of VE-Specific *Wnt3* Nulls Embryos.

In the crosses above described (i.e.: *Wnt3*$^{lacZ/+}$;*Ttr*$^{Cre/0}$ males to *Wnt3*$^{c/c}$ females) (Figure 2.1B), the VE-specific *Wnt3* null embryos harbored a heterozygous epiblast for the *Wnt3* locus (*Wnt3*$^{lacZ+c}$), that contains a copy of the floxed *Wnt3* allele (*Wnt3*^{c}) and a copy of a null allele of *Wnt3* (*Wnt3*^{lacZ}), alongside with a visceral endoderm that contains two distinct null alleles of *Wnt3* (*Wnt3*$^{lacZ/\Delta 3,4}$).

Figure 2.6

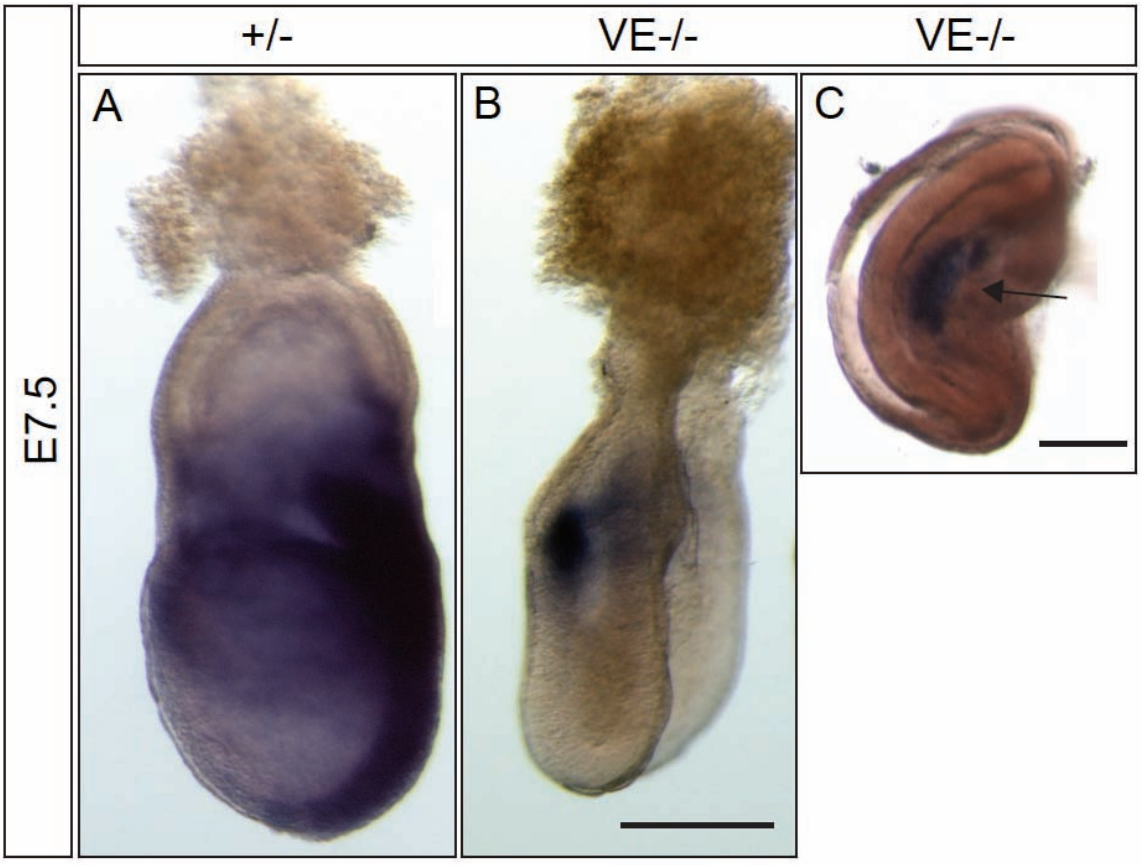

Figure 2.6: Activation of *Wnt3* locus in visceral endoderm-specific *Wnt3* null embryos. Analysis of β-galactosidase activity in *Wnt3*$^{lacZ/+}$ heterozygous and visceral endoderm-specific *Wnt3* mutant embryos dissected at E7.5. A. β-galactosidase activity marks the primitive streak and its derivatives in embryonic and extra-embryonic tissues in the heterozygous embryos. B. In visceral endoderm *Wnt3*$^{lacZ/D3,4}$ mutants, β-galactosidase activity is restricted to a small area of visceral endoderm. C. *Wnt3*$^{lacZ/D3,4}$ mutant embryo severed at junction between embryonic and extraembryonic region shows β-galactosidase activity in the visceral endoderm only (arrow). Scale bar in A and B 200 µm, scale bar in C 100 µm.

Thus, there is the possibility that the effect of the ablation of the *Wnt3* locus in the visceral endoderm is potentiated by the heterozygosity of the *Wnt3* locus in the epiblast. To address this possibility we crossed $Wnt3^{c/+}$;$Ttr^{Cre/0}$ males to $Wnt3^{c/c}$ females. In this scenario the epiblast of the VE-specific *Wnt3* null embryos harbored two functional *Wnt3* alleles ($Wnt3^{c/c}$). Generation of VE-specific *Wnt3* null embryos this way yielded embryos that were morphologically identical to the VE-specific Wnt3 null embryos analyzed before (compare Figs 2.2 and 2.4 with Figure 2.7). They failed to form a primitive streak and any derivative such as amnion and allantois (Figure 2.7A,B). Moreover, judging by the lack of expression of primitive streak markers such as *T* (n= 5) and *Sp5* (n=6) these mutants also fail to establish the molecular identity of the primitive streak (Figure 2.7A,B). These VE-specific Wnt3 null embryos gave rise to an amorphic structure in the epiblast that folded inwards on the posterior side, being eventually resorbed at around E9.5, like the VE-specific *Wnt3* knockout embryos analyzed previously. These set of data led us to conclude that the heterozygosity of the epiblast does not have any effect on the phenotype of the ablation of *Wnt3* exclusively in the visceral endoderm and that the phenotype of the visceral endoderm-specific Wnt3 mutant embryos is due solely to its absence in the visceral endoderm.

Figure 2.7

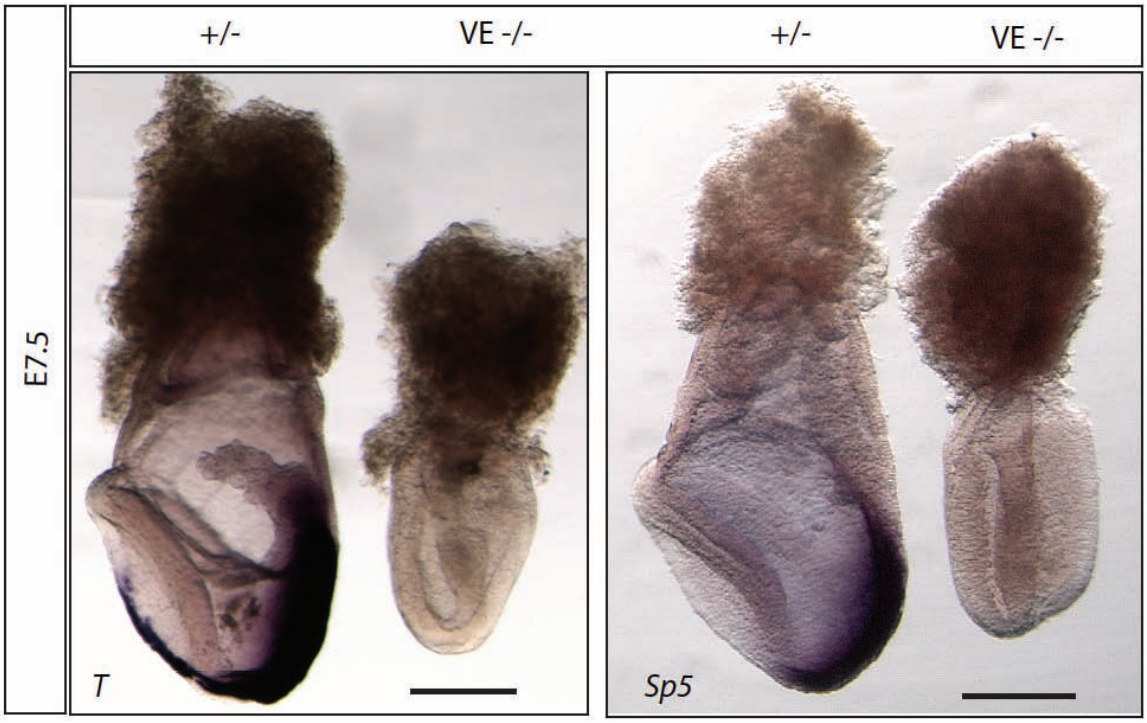

Figure 2.7: Number of functional *Wnt3* alleles in the epiblast does not affect penetrance of phenotype of visceral endoderm *Wnt3* null embryos. A-B mutant embryos generated from *Wnt3*$^{c/+}$*; Ttr*^{cre} male x *Wnt3*$^{c/c}$ female crossings have two functional copies of the Wnt3^{c} allele in the epiblast yet fail to gastrulate. *Wnt3*-VE mutant, on the right, fails to gastrulate and express primitive streak makers. Scale bar 200 µm.

Wnt3 Activates the Canonical Wnt Pathway in the Epiblast of Developing Mouse Embryos.

Our results showed that visceral endoderm-specific *Wnt3* null embryos lacked expression of *T, Axin2* and *Sp5,* three genes known to be under the control of the canonical Wnt signaling pathway (Jho et al. 2002; Yamaguchi et al. 1999; Weidinger et al. 2005). These results suggested that activation of the canonical Wnt pathway in the epiblast depends on Wnt3 signaling emanating from the posterior visceral endoderm.

To test this possibility, we analyzed the activity of the *BAT-Gal* transgene in *Wnt3* mutant embryos. *BAT-Gal* is a reporter for the activation of the canonical Wnt signaling pathway. It contains seven TCF-binding sites upstream a *lacZ* sequence that leads to expression of β-galactosidase upon activation of the canonical Wnt pathway. This reporter is active in the posterior epiblast and primitive streak of early post-implantation embryos (Maretto et al. 2003). To obtain *Wnt3* mutant embryos hemizygous for *BAT-Gal*, we crossed $Wnt3^{\Delta 3,4}$ heterozygous mice *inter se* with one of the parents also hemizygous for the *BAT-Gal* transgene. A total of 48 embryos obtained from these crosses were dissected at E7.5 and assayed for β-galactosidase activity (Figure 2.8A,B). After PCR genotyping, we identified five *Wnt3* null embryos that were also hemizygous for *BAT-Gal*. None of these embryos expressed the *BAT-Gal* transgene (Figure 2.8A). These results show that *Wnt3* is required for activation of the *BAT-Gal* transgene, and thus activation of the canonical Wnt pathway *in vivo*.

Figure 2.8

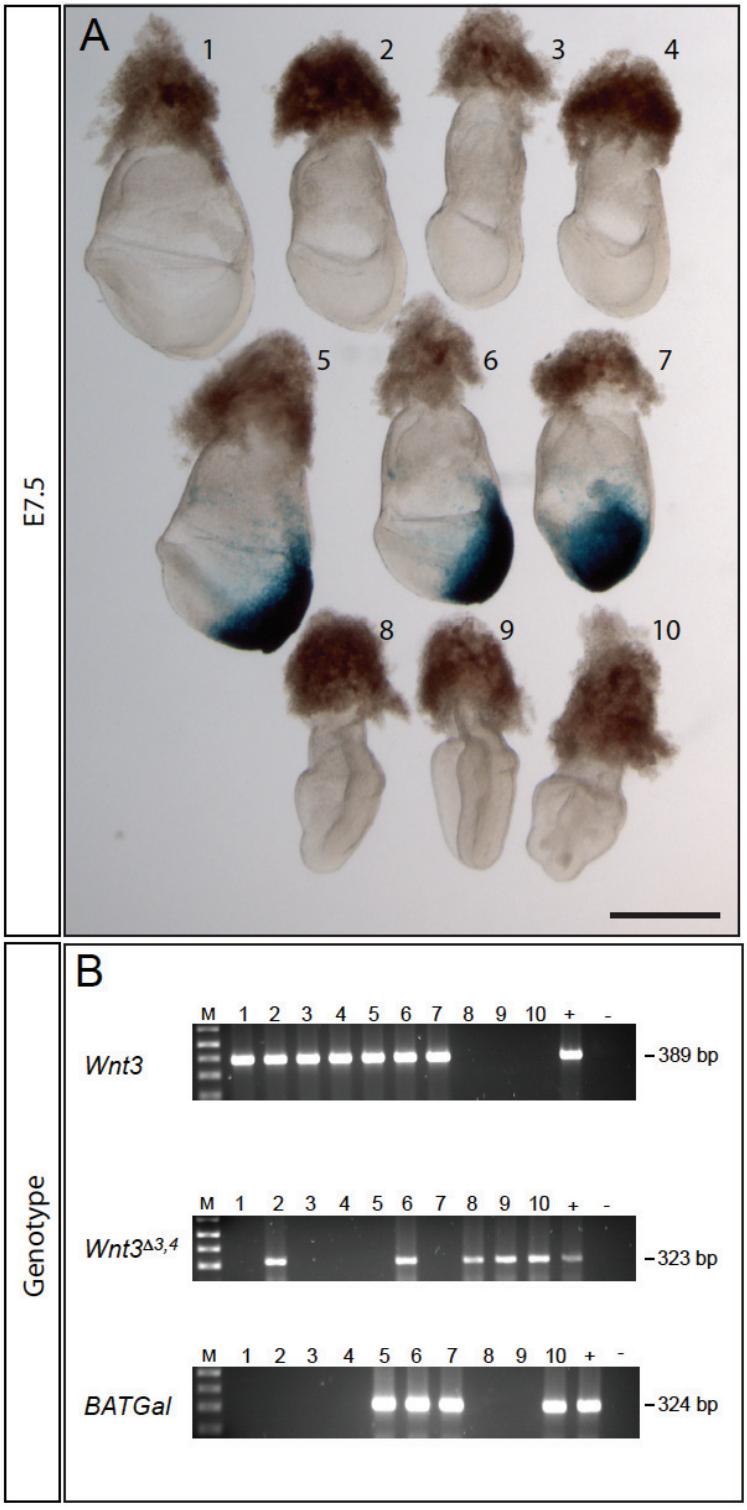
A
1
2
3
4
5
6
7
8
9
10
E7.5
B
M 1 2 3 4 5 6 7 8 9 10 + -
Wnt3
389 bp
M 1 2 3 4 5 6 7 8 9 10 + -
Wnt3Δ3,4
323 bp
M 1 2 3 4 5 6 7 8 9 10 + -
BATGal
324 bp
Genotype

Figure 2.8: Visceral endoderm *Wnt3* null embryos fail to activate the canonical Wnt pathway. A. Litter obtained from *Wnt3*$^{\Delta 3,4/+}$ x *Wnt3* $^{\Delta\ 3,4/+}$; BAT-Gal hemizygous crosses dissected at E7.5 and assayed for β-galactosidase activity. Three *Wnt3*$^{D3,4/D3,4}$ mutant embryos are shown at the bottom of the figure. B. PCR genotyping of embryos shown in A. Embryos are numbered from left to right and from top to bottom. The embryo at bottom right (number 10) is a *Wnt3*$^{D3,4/D3,4}$ mutant hemizygous for the BAT-Gal transgene, yet it lacks β-galactosidase activity showing a *Wnt3* requirement to activate the BAT-gal transgene *in vivo*. Scale bar, 500 µm.

Visceral Endoderm-Specific Wnt3 Null Embryos Fail to Form Primordial Germ Cells.

Primordial germ cells (PGCs) are the cells that give rise to the germ line cells in males and females. Previous studies have established a dependence on Bmp/Smad signaling for PGC formation (Saitou and Yamaji 2010). Interestingly, Wnt signaling also seems to be required for PGC formation, since *Wnt3* null embryos fail to specify PGCs (Ohinata et al. 2009).

Our data suggest that the lack of *Wnt3* specifically in the visceral endoderm recapitulates the phenotype of *Wnt3* null embryos. Thus, we decided to investigate whether the formation of PGCs was another process affected in visceral endoderm-specific *Wnt3* null embryos. To do so, we crossed $Wnt3^{lacZ/+};Ttr^{Cre/0}$ males to $Wnt3^{c/c}$ females in order to generate visceral endoderm-specific *Wnt3* null embryos. We then performed wholemount in situ hybridization using RNA probes for two PGC markers, *Fragilis* and *Stella.* These genes mark a population of PGC cells residing in the extraembryonic mesodermal niche committed with PGC lineage at around E7.5 (Saitou and Yamaji 2010).

Control embryos show clear expression of *Fragilis* in cells located at the base of the allantois at the boundary of the epiblast with the extraembryonic ectoderm at E7.5 (Figure 2.9A), whereas visceral endoderm-specific *Wnt3* mutant embryos do not show any detectable level of Fragilis staining (n=5) (VE-/- embryos Figure 2.9A), suggesting that the initial signaling for the specification of

PGCs was compromised. To confirm these initial observations we probed the visceral endoderm-specific *Wnt3* null embryos for the presence of *Stella*. *Stella* is a definitive marker of PGC specification that marks a population of approximately forty lineage restricted PGCs cells in the base of the allantois of E7.5 embryos (Ohinata et al. 2009; Saitou 2009). Visceral endoderm-specific *Wnt3* null embryos do not show expression of *Stella* (n=6) (Figure 2.9B), indicating a lack of PGCs in these mutant embryos. These observations once again support the hypothesis that visceral endoderm-derived Wnt3 is essential for the induction of the primitive streak and the initial signal that drives gastrulation.

Discussion.

In mice, genetic studies have indicated that Wnt signaling is at the core of gastrulation. Embryos with loss of function mutations of *Wnt3* (Liu et al. 1999), *Mesd* (Hsieh et al. 2003; Lighthouse et al. 2011), and a double mutation of *Lrp5* and *Lrp6* (Kelly, Pinson, and Skarnes 2004) fail to gastrulate. In addition, mutations in *Axin*, a negative regulator of the canonical Wnt signaling pathway, leads to duplications of the primitive streak (Zeng et al. 1997). Moreover, ectopic expression of chicken *Wnt8C* can induce an ectopic primitive streak in mouse embryos (Popperl et al. 1997). Our demonstration that PVE-derived *Wnt3* is necessary to induce a primitive streak and that Wnt3 activates the canonical Wnt signaling pathway provides a framework that links these discoveries into the chain of events that leads to gastrulation in mammals.

Figure 2.9

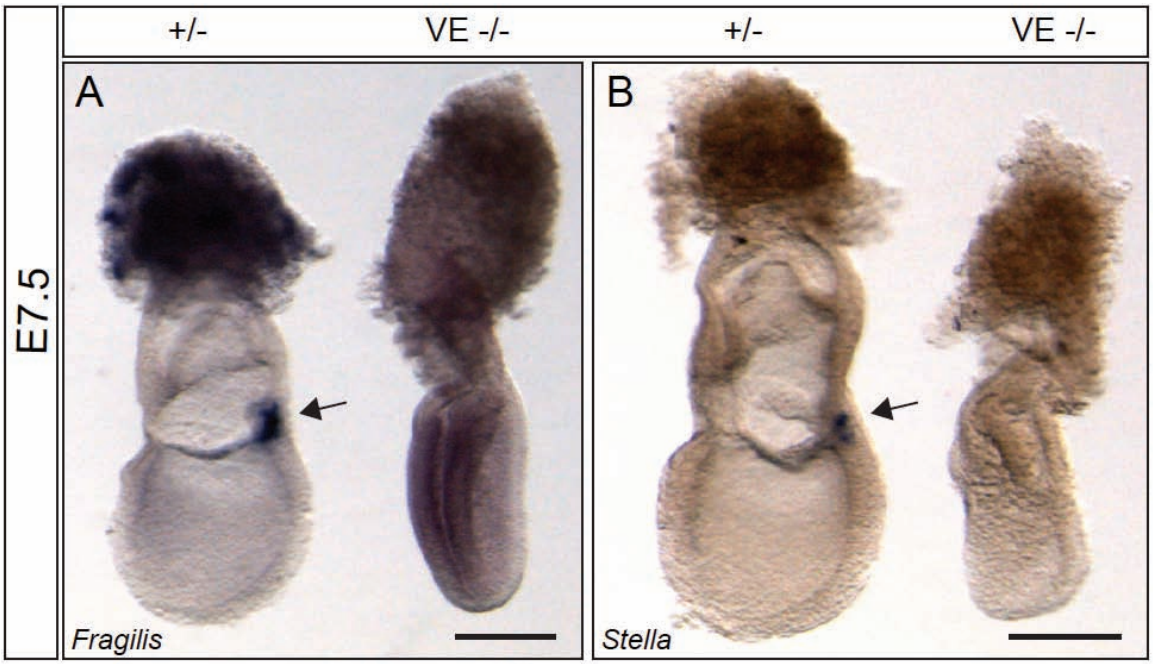

Figure 2.9: Visceral endoderm *Wnt3* null embryos lack primordial germ cells at E7.5. A-B. Visceral-endoderm *Wnt3* mutant embryo dissected at E7.5 fail to show expression of the primordial germ cell makers *Fragilis* and *Stella* (black arrow). Scale bar 200 µm.

Based on *Wnt3* expression (Rivera-Perez and Magnuson 2005) and the data presented here we propose that *Wnt3* emanating from the posterior visceral endoderm induces its own expression and that of other markers of the primitive streak in the adjacent epiblast tissue in embryos at ~E5.75-E6.0.

Based on comparative embryology and molecular evidence, the extra-embryonic ectoderm has been proposed to be the equivalent of the Nieuwkoop center in mice (Beddington and Robertson 1999; Ben-Haim et al. 2006). In this model, Bmp4 emanating from the extra-embryonic ectoderm signals to the adjacent epiblast, to activate primitive streak markers (Ben-Haim et al. 2006). Our results do not support this view. Our data shows that embryos with a *Wnt3* mutation in the visceral endoderm do not form a primitive streak despite having a functional allele of *Wnt3* in the epiblast and normal expression of *Bmp4* in the extraembryonic ectoderm. Previous data also showed that *Bmp4* mutants can form a primitive streak albeit with reduced penetrance (Winnier et al. 1995).

Using mouse chimeras, a previous study suggested that *Wnt3* function in the visceral endoderm is dispensable during gastrulation (Barrow et al. 2007). Our data does not support this conclusion. This may be the due to differences in the experimental strategies used. Previous experiments relied on E9.5 chimeras generated by aggregation of morulae derived from *Wnt3* heterozygous parents and wild-type ES cells, and retrospective genotyping using the visceral endoderm layer of the visceral yolk sac (Barrow et al. 2007). Our results are based on the generation of a tissue specific knock out of *Wnt3* in the visceral endoderm. In

these experiments we have demonstrated a requirement of *Wnt3* in the visceral endoderm while retaining a functional copy in the epiblast. Evidence for a requirement of *Wnt3* in the visceral endoderm is also supported by the inability to activate the *Wnt3*^{lacZ} allele in the epiblast of *Wnt3*-VE mutant embryos.

Another interesting observation obtained from the studies of *Wnt3* null embryos is their inability to specify primordial germ cells (Ohinata et al. 2009). Interestingly, embryos lacking *Wnt3* specifically in the visceral endoderm fail to specify PGC at E7.5 again recapitulating the phenotype of *Wnt3* null embryos.

Overall our data indicated that the expression of *Wnt3* in the visceral endoderm breaks the bilateral symmetry of the embryo at E5.5 and it is an essential event for the formation of the primitive streak and initiation of gastrulation in the epiblast. Furthermore, we provided data *in vivo* evidence that Wnt3 carries out its functions in the epiblast via activation of the canonical Wnt pathway.

Materials and Methods.

Embryo staging: Embryos were staged based on morphological landmarks as previously described (Downs and Davies 1993; Rivera-Perez, Jones, and Tam 2010) or in terms of dissection time. Noon of the day that a mating plug was observed was considered embryonic day 0.5 (E0.5) of gestation. All embryos were derived from mice maintained as mixed stock.

Generation of *Wnt3*^{lacZ} knock-in mice: Maki Wakamiya and Richard Behringer carried out the generation of this mouse strain in Behringer's lab. These animals were gifted to us as part of an ongoing collaboration. Briefly, the targeting vector carried a 6 kb NotI-BamHI genomic DNA fragment (129/SvEv) containing exons 3-4 of the *Wnt3* locus. A *lacZ* cassette and a floxed PGK*neo*bpA cassette were inserted into the ClaI site in exon 4. The *IacZ* cassette contained an internal ribosomal entry site (IRES) and an SV40 polyA signal sequence. An HSV-*tk*pA cassette was added to the 5'-homologous arm. Gene targeting in ES cells was performed as previously described (Mishina et al. 1995). A total of 192 G418; FIAU double-resistant ES cell colonies were screened for homologous recombination by Southern blotting using a *Wnt3* 3' UTR probe (Liu et al. 1999). Twenty-seven lines were positive in the initial screening, and two lines gave rise to germ-line chimeras. Heterozygous mutants were normal and fertile. The phenotype of the *Wnt3*^{lacZ} homozygous mutants was indistinguishable from that of *Wnt3* null mutants previously reported (Liu et al. 1999; Barrow et al. 2007), indicating that the *Wnt3*^{lacZ} allele is a null allele.

Germ layer isolation: To separate the epiblast from the visceral endoderm we followed the germ layer separation protocol previously described (Nagy 2003). Briefly, E6.5 mouse embryos were dissected in dissection medium (DMEM; 10% FBS; 1X penicillin/streptomycin). Next, we use a 22-gauge needle to separate

the extraembryonic ectoderm from the epiblast covered by visceral endoderm. The epiblast piece was washed in serum free medium and subsequently treated with a cocktail of proteolytic enzymes (pancreatin/trypsin) for about 8 minutes or until the visceral endoderm started to loosen from the adjacent epiblast. These embryo pieces were passed through a fine glass Pasteur pipet a few times (average 5 times) until the visceral endoderm layer had completely fallen off of the epiblast. These two separated tissues were washed in dissection medium and kept in separate tubes for genotyping.

Whole-mount *in situ* hybridization: Dissection and fixation: Embryos were dissected with forceps and fixed in 4% paraformaldehyde pH 7.4 at 4^{o}C overnight. Dehydration: Embryos were dehydrated through 25%, 50% and 75% methanol/PBT (PBS, 0.1% Tween 20) series, twice in 100% methanol and stored in methanol at -20^{o}C. Rehydration, proteinase K treatment and refixation: Embryos were rehydrated in methanol/PBT series in the reverse order, and washed twice for 10 min. in PBT. They were then treated with 10 mg/ml proteinase K in PBT, rinsed once and washed twice in PBT for 10 min. E6.5 and E7.5 embryos were treated for 5 or 10 min. in proteinase K, respectively. The embryos were then refixed in 4% paraformaldehyde/0.2% glutaraldehyde in PBT, rinsed once for 20 minutes and washed twice in PBT. Prehybridization and Hybridization: Embryos were incubated in a 1:1 mixture of PBT/hybridization solution at room temperature until settled in the bottom of the plate. They were

rinsed twice and prehybridized in hybridization solution for a minimum of 2 h at 70^{o}C and hybridized overnight at 70^{o}C in hybridization solution containing 0.1 – 1 mg/ml digoxigenin labeled probe. The hybridization solution contained: 50% formamide (Sigma, F7503), 5 mM EDTA, 0.195M NaCl, 0.0195 M sodium citrate, 0.2% Tween 20, 50 mg/ml yeast tRNA (Sigma, R5636), 0.5% CHAPS (Fisher BP5715) and 100 mg/ml heparin (Sigma, H9399). After hybridization, the embryos were rinsed twice in hybridization solution and washed twice in hybridization solution for one hour each at 70^{o}C. The embryos were then rinsed twice and then washed twice for 30 min. in MABT (0.1M maleic acid, 0.15 M NaCl, 0.1% Tween 20). Blocking and antibody incubation: Embryos were blocked for 1h at room temperature in MABT containing 2% Roche blocking reagent, (Roche, 1096176) and for an additional hour in the same solution containing 10% normal goat serum. The embryos were then incubated at 4^{o}C overnight with anti-digoxigenin antibody (Roche, 11093274910) diluted 1:2000 in blocking solution containing normal goat serum. After the antibody incubation, the embryos were rinsed twice and washed four times for 30 min. in MABT at room temperature. Color reaction: Embryos were stained in NTMT (0.1 M NaCl, 0.1 M Tris-HCl pH9.5, 0.05 M $MgCl_2$, 1% Tween 20) containing 20 ml/ml NBT/BCIP (Roche,1681451) for up to three days at room temperature. After the color reaction was completed embryos were rinsed in PBT, refixed in 4% paraformaldehyde and cleared in glycerol. Probes: *Wnt3* (904 bp cDNA fragment containing a piece of exon 2, exons 3 and 4 and a portion of exon 5. *Wnt3*-3’

(1,050 bp 3’ UTR fragment). *Brachyury* (full length cDNA, 1,784 bp) (Herrmann 1991). *Cripto* (full length cDNA, 983 bp), *Mixl1* (1,515 bp fragment containing a piece of exon 2 and most of 3’ UTR) (Robb et al. 2000), *Bmp4* (1,063 bp cDNA piece containing a portion of exon 1, exons 2 and 3 and a portion of exon 4)(Jones, Lyons, and Hogan 1991), *Fgf8* (full length cDNA, 1,100 bp), *Axin2* (2,420 bp cDNA piece containing part of exon 2, exons 3 – 9 and a portion of exon 10), *Sp5* (full length cDNA, 1,540 bp). All riboprobes were prepared using a digoxigenin RNA labeling kit (Roche Cat. No. 1175025).

β-Galactosidase staining protocol: Embryos were dissected using forceps and fixed for 5 minutes in freshly prepared fixation solution (0.2% glutaraldehyde, 2% formalin, 5 mM EGTA, 2 mM $MgCl_2$ and 0.1 M phosphate buffer pH 7.3). After fixation, embryos were rinsed three times for 20 minutes each in wash solution (0.1% deoxycholate, 0.2% NP40, 2 mM $MgCl_2$ and 0.1 M phosphate buffer pH 7.3) and stained overnight at 37°C in staining solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide The next day, embryos were rinsed in wash solution and refixed in 4% paraformaldehyde at room temperature for 20 min. Stained embryos were cleared in glycerol or mounted in OCT (Fisher, 1437365) for cryosectioning.

Genotyping: The genotype of the embryos was determined retrospectively after wholemount *in situ* hybridization or after the β-galactosidase assay. Each litter

was pictured before genotyping to allow unique identification of each embryo. Embryos were placed in 15-20 ml of PCR lysis buffer (50 mM KCl, 10 mM Tris-HCl pH8.3, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% IGEPAL and 0.45% Tween 20) containing 100 mg/ml Proteinase K and incubated overnight at 56^{o}C. After lysis, the proteinase K was inactivated at 95^{o}C for 5 min and 1 ml of the sample was use for the PCR reaction. Mice were genotyped at postnatal day 10 using a 2 mm tail tip piece.

PCR for *Cre*, *LacZ* and *Wnt3*^{c} allele was carried out using GoTaq Flexi DNA polymerase (Promega cat no. M8295) using the following PCR cycle conditions: 30 sec. at 95^{o}C, 40 sec. at 60^{o}C and 40 sec. at 72^{o}C repeated 31 times with an initial denaturation cycle of 5 min. at 95^{o}C and a final elongation step of 7 min. at 72^{o}C. The *Wnt3*$^{\Delta 3\text{-}4}$ allele was amplified using the following cycle: 1 min at 95^{o}C, 1 min at 58^{o}C and 1 min at 72^{o}C were repeated 35 times.

PCR reaction were conducted using the following oligonucleotides: *Wnt3* wild-type and *Wnt3*^{c} alleles, Wnt3F3 5’ TGG CTT CAG CAT CTG TTA CCT TC 3’ and Wnt3R6 5’ AAG ATC CCC ATA CTG CCA TCA C 3’; *Wnt3*^{lacZ} and BAT-Gal alleles, LacZF 5’TGG CGT TAC CCA ACT TAA TCG 3’ and LacZR 5’ATG TGA GCG AGT AAC AAC CCG 3’; *Wnt3*$^{\Delta 3\text{-}4}$ allele, LoxPF 5’ GTA TAA TGT ATG CTA TAC GAA G 3’ and Wnt3R6 5’ AAG ATC CCC ATA CTG CCA TCA C 3’; *Ttr*^{Cre} allele, CreF 5’ TCC AAT TTA CTG ACC GTA CAC CAA 3’ and CreR 5’ CCT GAT CCT GGC AAT TTC GGC TA 3’.

CHAPTER III:

Wnt3 Function in the Epiblast is Required for the Maintenance but not the Initiation of Gastrulation in Mice.

Preface.

Wnt3 Function in the Epiblast is Required for the Maintenance but not the Initiation of Gastrulation in Mice.

The results presented in this chapter are part of a manuscript recently accepted for publication in *Developmental biology* (DOI 10.1016/j.ydbio.2012.10.013). This chapter describes the outcome of the ablation of *Wnt3* in the epiblast and also describes the existence of a Wnt3-dependent autoregulatory loop at work during gastrulation. In order to delete the *Wnt3* locus in the epiblast we used an epiblast-specific cre driver (Sox2cre) obtained from the Jackson Laboratories. This line was originally generated in Dr. McMahon's Lab (Harvard University, MA). Additionally, we used 2 different *Wnt3* alleles for this work. The $Wnt3^{lacZ}$ allele used in this work was generated by Dr. Maki Wakamya and Dr. Richard Behringer (MD Anderson Cancer Center, TX) and provided to us as part of collaborative efforts between the two groups. The $Wnt3^{c}$ (conditional) allele was generated by Drs. Jeff Barrow and Andy McMahon (Harvard University, MA) and gifted to us by that same group. Dr. Alexandre JC Quaresma and Dr. Jeff Nickerson assisted with the confocal microscopy experiments for the analysis and interpretation of nuclear accumulation of β-Catenin in MEF cells. Both of them are co-authors of this paper. Dr. J.M. Hernandez-Hernandez and Dr. Tony Imbalzano assisted with the gene expression (pPCR) and ChIP experiments. Dr. J.M. Hernandez-Hernandez and Dr. Tony Imbalzano are coauthors of this paper.

Abstract.

Wnt3 is expressed sequentially in two distinct areas of pre-streak mouse embryos: first in the posterior visceral endoderm and soon after in the adjacent posterior epiblast. Hence, although the requirement of *Wnt3* for gastrulation is well established, its temporal and tissue specific requirements remain unclear. Here, we conditionally inactivate *Wnt3* in the epiblast in order to determine its requirement for gastrulation. Our data shows that embryos lacking *Wnt3* specifically in the epiblast are able to initiate gastrulation and advance to late primitive streak stages but fail to thrive and are rebsorbed by E9.5. Furthermore, we demonstrate that Wnt3 directly activates the canonical Wnt pathway to drive the expression of primitive streak makers. Moreover, using chromatin immunoprecipitation we show that β-catenin, the effector of *Wnt3* signaling, occupies the promoter region of primitive streak markers and the promoter region of Wnt3 during gastrulation. This occupancy is followed by activation of specific loci. Hence, our data suggest that *Wnt3* function in the epiblast is not essential for the initiation of gastrulation but it is required for its maintenance. Also our results suggest the presence of an autoregulatory loop that is at work during gastrulation involving expression of Wnt3 in different tissues of the developing embryo.

Background.

The establishment of the main body axes, in particular the anteroposterior axis, is a fundamental aspect of embryonic development of vertebrates. In mice, the formation of the primitive streak on the posterior side of the embryo, together with the positioning of the anterior visceral endoderm cells at a diametrically opposite site, are the most robust morphological markers of anteroposterior specification (Arnold and Robertson 2009).

Several studies have implicated the Wnt signaling pathway in axial specification and primitive streak formation in mice (reviewed by van Amerongen and Berns, 2006). Ablation of *Wnt3*, leads to complete absence of the primitive streak (Liu et al. 1999) as well as simultaneous inactivation of the Wnt co-receptors *Lrp5* and *Lrp6* (Kelly, Pinson, and Skarnes 2004) or absence of their chaperone *Msd* (Hsieh et al. 2003; Kelly, Pinson, and Skarnes 2004). Conversely, ablation of *Axin*, a negative regulator of the canonical Wnt signaling leads to duplication of the primitive streak (Zeng et al. 1997). This is also the case in transgenic mice expressing the chick *Wnt8C* gene, a canonical Wnt (Popperl et al., 1997).

Wnt3 is expressed in the early post-implantation mouse embryo in a sequential manner. First, it is observed in the posterior visceral endoderm at around embryonic day 5.5 (E5.5), and six hours later, at ~E5.75, *Wnt3* expression is evident in the epiblast (Rivera-Perez and Magnuson, 2005). The

expression in the epiblast is restricted to a region directly abutting the posterior visceral endoderm (Rivera-Perez and Magnuson 2005).

Because of its dual pattern of expression, in the posterior visceral endoderm and in the epiblast, the role of *Wnt3* in anteroposterior axis formation cannot be assigned to either tissue in standard knockout experiments. Moreover, the molecular events downstream of *Wnt3* signaling that control gastrulation in the developing embryo are not completely understood. In order to determine the function of *Wnt3* in the epiblast we conducted a tissue specific knockout using $Sox2^{Cre}$, an epiblast specific *cre* driver (Hayashi et al. 2002), and a conditional allele of *Wnt3* (Barrow et al. 2003). We also investigated the ability of *Wnt3* to activate the canonical Wnt pathway and control the transcription rate of its own locus and other loci involved in gastrulation.

Results.

Initiation of Gastrulation Occurs Independently of *Wnt3* Expression in the Epiblast.

Because of the dual expression pattern of *Wnt3* in the posterior visceral endoderm at E5.5 and subsequently in the epiblast at E5.75 (Rivera-Perez and Magnuson 2005) one cannot attribute the requirement of Wnt3 to either tissue. In this chapter we describe experiments designed to examine the requirement of *Wnt3* expression in the epiblast focusing on its actions for primitive streak specification and the process of gastrulation.

Figure 3.1

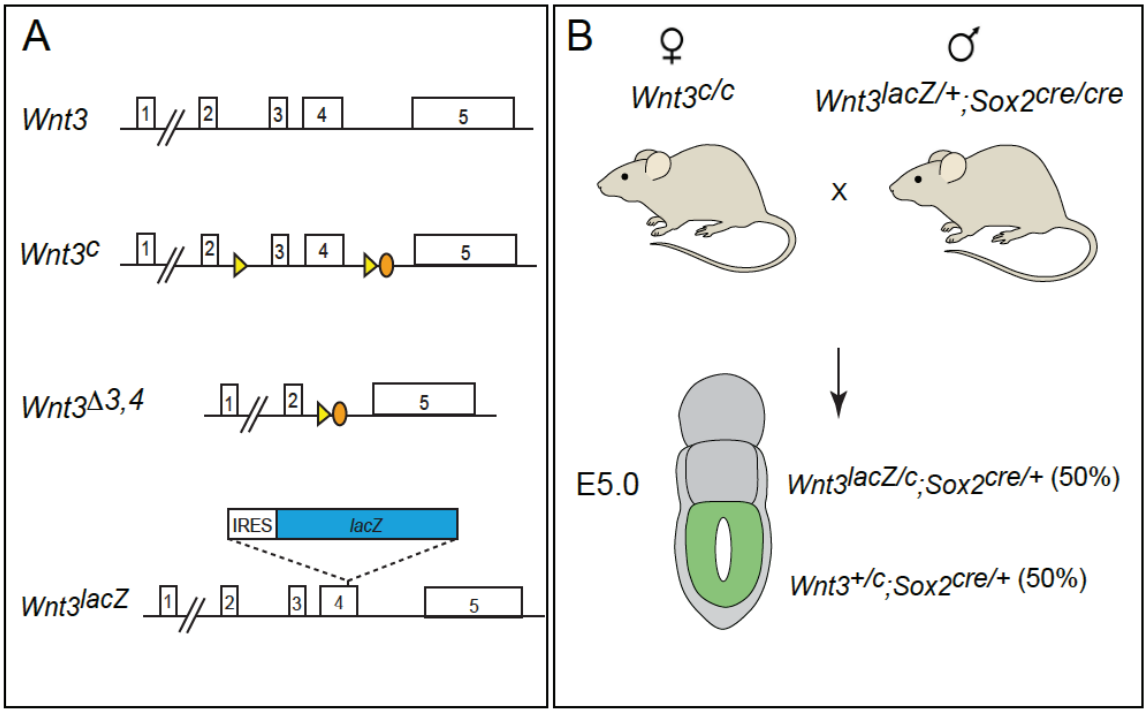
A
Wnt3
Wnt3c
Wnt3Δ3,4
IRES
lacZ
Wnt3lacZ
1
2
3
4
5
B
♀
♂
Wnt3c/c
Wnt3lacZ/+;Sox2cre/cre
x
E5.0
Wnt3lacZ/c;Sox2cre/+ (50%)
Wnt3+/c;Sox2cre/+ (50%)

Figure 3.1: Representation of *Wnt3* alleles and breeding strategy utilized to ablate *Wnt3* in the epiblast of mouse embryos. A. Schematic representation of *Wnt3* alleles. *Wnt3*, wild-type; $Wnt3^{c}$, floxed allele. The *loxP* sites are marked as yellow triangles and a residual *FRT* site is marked as an orange oval. $Wnt3^{\Delta 3,4}$, recombined null allele; $Wnt3^{lacZ}$, null allele. An *IRES-lacZ* cassette was inserted in the unique *ClaI* site present in the exon 4 of the *Wnt3* locus creating a null allele. B. Breeding strategy to generate embryos lacking *Wnt3* expression specifically in the epiblast. Using this strategy half of the embryos obtained will carry null mutations in the *Wnt3* locus solely in the epiblast and the other half will be heterozygous controls ($Wnt3^{c/+}$).

In order to do so, we performed experiments to genetically ablate the *Wnt3* locus specifically in the epiblast using *Sox2*^{cre} transgenic mice and a floxed allele of *Wnt3* (Figure 3.1A,B). Mice caring the *Sox2*^{cre} transgene show *cre* recombinase expression exclusively in the epiblast layer, when the transgene is inherited from the paternal side (Hayashi et al. 2002; Hayashi, Tenzen, and McMahon 2003). The efficiency and specificity of this line have been tested previously (Barrow et al. 2007; Hayashi et al. 2002; Kwon, Viotti, and Hadjantonakis 2008; Miura, Singh, and Mishina 2010).

In order to generate embryos lacking *Wnt3* expression in the epiblast, but retaining its expression in the visceral endoderm, we bred male mice heterozygous for a null allele of *Wnt3* and homozygous for the *Sox2*^{cre} transgene (*Wnt3*$^{lacZ/+}$;*Sox2*$^{cre/cre}$) and females homozygous for a floxed allele of *Wnt3* (*Wnt3*$^{c/c}$) (Figure 3.1B). The rationale of this breeding strategy was that embryos lacking *Wnt3* expression in the epiblast would phenocopy *Wnt3* null embryos (Liu et al. 1999) if its function in the epiblast was required for primitive streak specification.

Mouse embryos lacking *Wnt3* expression specifically in the epiblast were smaller than littermates and had no morphological signs of the presence of a primitive streak at E6.5. We used wholemount *in situ* hybridization to test whether any residual *Wnt3* expression could be detected in the epiblast of these epiblast-specific *Wnt3* null embryos. We could not observe any detectable level of *Wnt3* expression in the epiblast of epiblast-specific *Wnt3* knockout embryos

(n=11) (Figure 3.2A). This data indicated that we achieved highly efficient recombination of the *Wnt3* locus in the epiblast. Interestingly, *Wnt3* expression was also absent in the posterior visceral endoderm of epiblast-specific *Wnt3* knockout embryos at E6.5 (Figure 3.2A).

To determine whether the primitive streak had been specified we analyzed the expression of four primitive streak markers: *Brachyury (T), Axin2, Sp5,* and *Fgf8*. Expression of *T* (n=10), *Axin2* (n=6), *Sp5* (n=8), and *Fgf8* (n=7) in the epiblast of E6.5 mutant embryos was either faint or non-detected (Figure 3.2A-E).

In the majority of the mutant embryos analyzed, *T* expression was absent in the posterior epiblast (7/10). However, weak *T* expression could be detected in three mutant embryos (Figure 3.3, black arrowhead). *T* expression in the extra-embryonic ectoderm region of mutant embryos was present but weaker than that of control embryos (red arrowhead Figure 3.2B and Figure 3.3), indicating that expression of *T* in this region of the embryo is partly dependent of *Wnt3* function in the epiblast. Expression of *Axin2, Sp5,* and *Fgf8* was similarly absent or barely detectable in epiblast-specific *Wnt3* null embryos (Figure 3.3C-E).

Since markers of the primitive streak are evident in the posterior epiblast at E6.0 (Rivera-Perez and Magnuson 2005), these results suggest that embryos lacking *Wnt3* function in the epiblast are developmentally delayed by at least 12 hours.

Figure 3.2

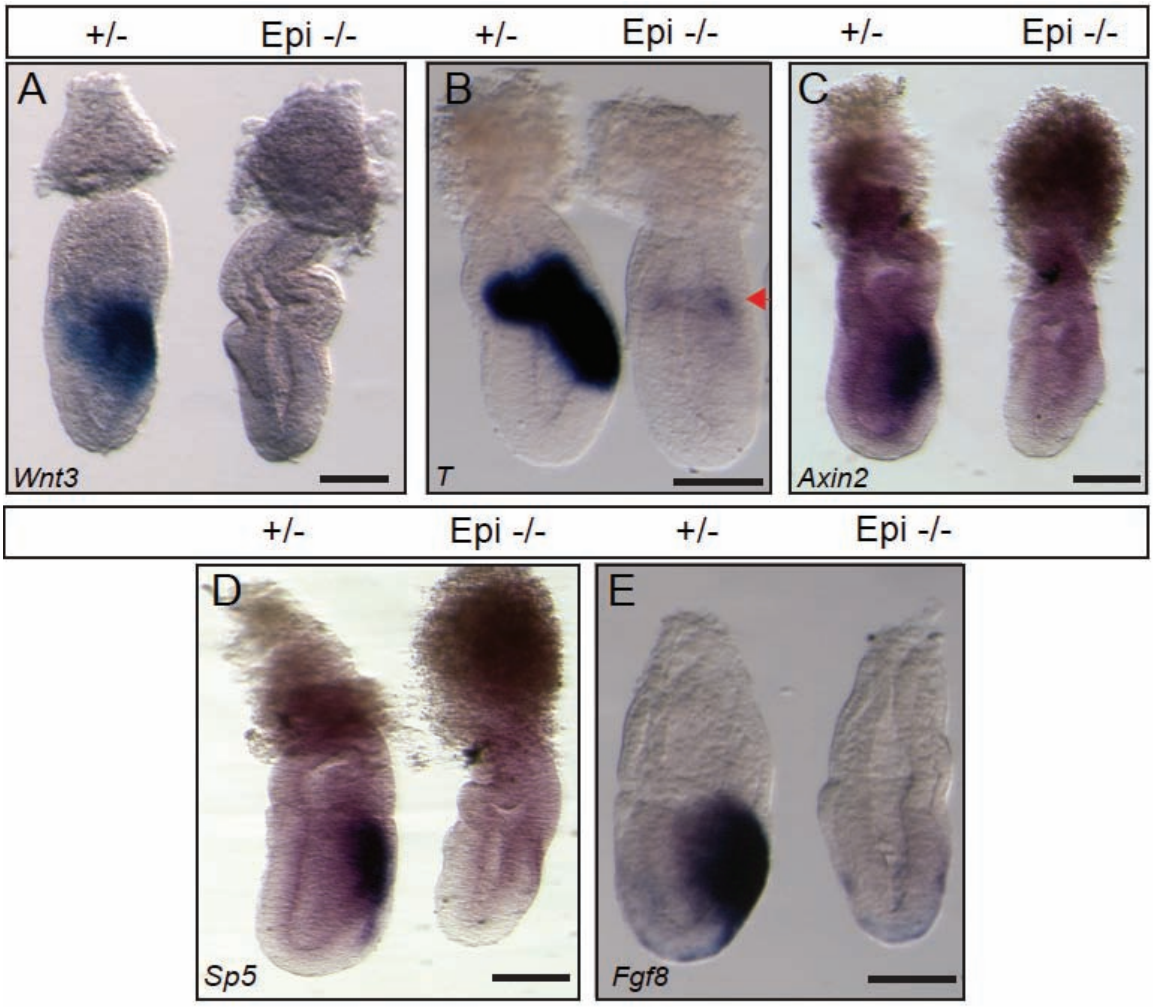

Figure 3.2: Molecular analysis of primitive streak formation in epiblast-specific *Wnt3* null embryos at E6.5. A-E. Characterization of epiblast-specific *Wnt3* knockout (Epi -/-) or heterozygous (+/-) control littermates. A. *Wnt3* RNA is not detected in the mutant embryos. B. *T* expression is nearly absent in the posterior epiblast but not in the extra-embryonic ectoderm (red arrowhead) in epiblast-specific *Wnt3* knockout embryos. C-E. Expression of *Axin2, Sp5* and *Fgf8* is absent in the posterior epiblast of mutant embryos. Scale bar 100 µm.

Figure 3.3

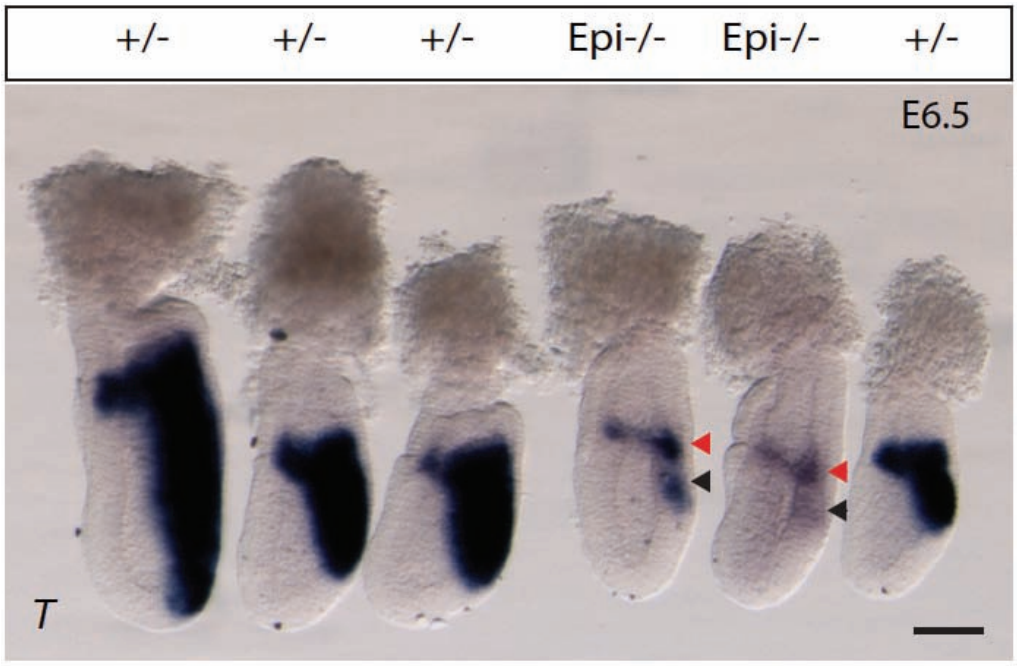

Figure 3.3: *T* expression in epiblast-specific *Wnt3* null embryos. Heterozygous (+/-) or epiblast-specific *Wnt3* knockout embryos (Epi-/-) dissected at E6.5 were hybridized with *Brachyury* probe. Epiblast-specific *Wnt3* mutants show reduced expression of *Brachyury* in the epiblast (black arrowhead) and in the extra-embryonic ectoderm (red arrowhead). Scale bar 100 µm.

The AVE is Properly Positioned in Epiblast-Specific *Wnt3* Null Embryos.

The specification of the primitive streak on the posterior side of the mouse embryo, and the positioning of the anterior visceral endoderm (AVE) on the prospective anterior side are the two major events that establish the anterior-posterior axis of the embryo (Arnold and Robertson 2009; Beddington and Robertson 1999). Thus, we inquired whether the AVE was present and properly located in epiblast-specific *Wnt3* null embryos by analyzing the expression of *Dkk1*, *Hesx1* and *Hex*, three markers of the AVE (Glinka et al. 1998; Thomas and Beddington 1996; Thomas, Brown, and Beddington 1998).

Dkk1, a Wnt antagonist, was present in all epiblast-specific *Wnt3* nulls (n=6) (red arrow Figure 3.4A). However, its domain of expression in wild-type embryos appeared wider compared to the mutant embryos (Figure 3.4A). The expression of *Hesx1* (n=5) and *Hex* (n=8) was also detected on the anterior side of all epiblast-specific *Wnt3* nulls, providing additional evidence that the formation and positioning of the AVE is not altered in these mutants (Figure 3.4B,C). *Hex* is also expressed at the anterior primitive streak in the nascent mesendodermal tissue (Thomas, Brown, and Beddington 1998). Epiblast-specific *Wnt3* null mouse embryos show faint expression of *Hex* in this region of the embryo (black arrows Figure 3.4C-D), indicating that continuous Wnt3 signaling in the epiblast is required for progression of mesendoderm formation.

Figure 3.4

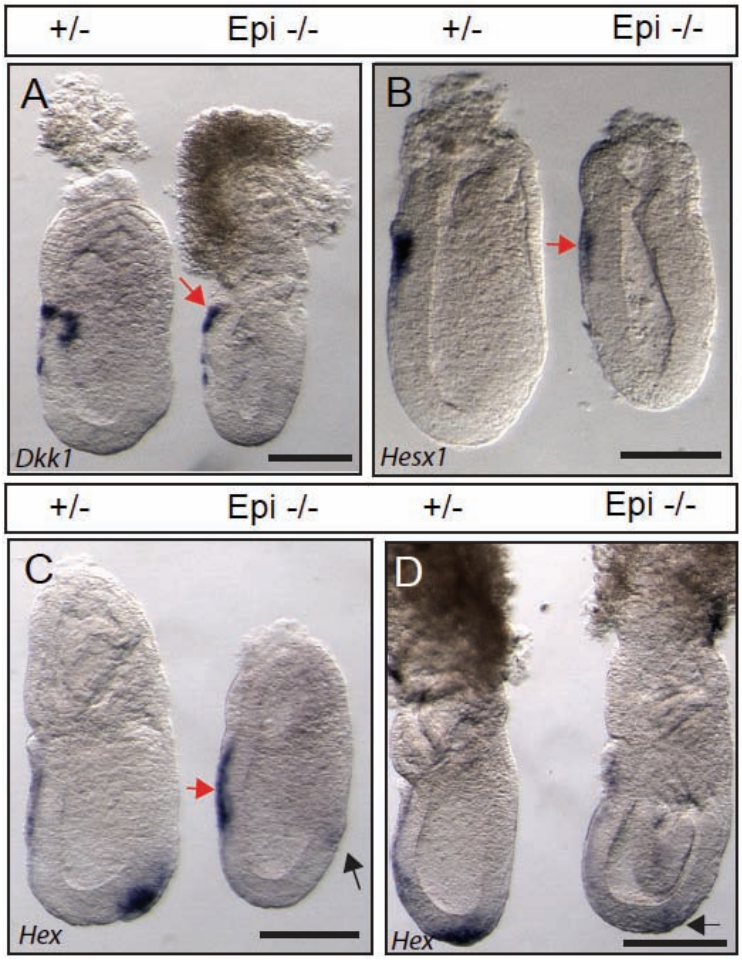

Figure 3.4: Molecular analysis of AVE formation in epiblast-specific *Wnt3* null embryos at E6.5. A-D. Characterization of epiblast-specific *Wnt3* knockout embryos using AVE markers. *Dkk1, Hesx1* and *Hex* are expressed in the AVE of mutant embryos (red arrows). C,D. *Hex* is expressed in the nascent mesendoderm of control embryos, but its expression is greatly diminished in mutant embryos (black arrows). Note the presence of a bulge protruding from the posterior epiblast into the proamniotic cavity in the mutant embryo shown in D. Scale bars 100 µm.

Wnt3 Expression in the Epiblast is Required for Proper Completion of Gastrulation but not its Initiation.

The absent or weak expression of primitive streak markers observed in epiblast-specific *Wnt3* null embryos dissected at E6.5 suggested they were delayed in development, therefore, we dissected mutant embryos at E7.5 to assess whether the molecular and morphological identity of the primitive streak was specified. We hypothesized that if the E6.5 mutant embryos were delayed in the development, then embryos dissected at E7.5 should unequivocally show hallmarks of primitive streak formation. Our results showed clear expression levels of *T* (n=13), *Axin2* (n=6), and *Sp5* (n=8) in the primitive streak of epiblast-specific *Wnt3* null embryos (Figure 3.5A-C). At this stage, however, additional defects became evident in the mutant embryos. Mutant embryos had a primitive streak that varied in length, with the more developmentally advanced embryos showing *T* expression in the axial midline region (black arrow Figure 3.5A). In these embryos, however the axial midline expression of *T* failed to extend to the same rostral levels observed in control littermates (black arrows Figure 3.5A). Mutant embryos often showed a bulging mass of cells in the primitive streak region that projected into the pro-amniotic cavity (n=12/27) (black arrowheads; Figure 3.5A,B). The node was not morphologically evident nor was the notochord as indicated by the expression pattern of *T*. In addition, the posterior amniotic fold failed to fuse with the anterior margin of the egg cylinder (red arrows Figure 3.5A-C).

Figure 3.5

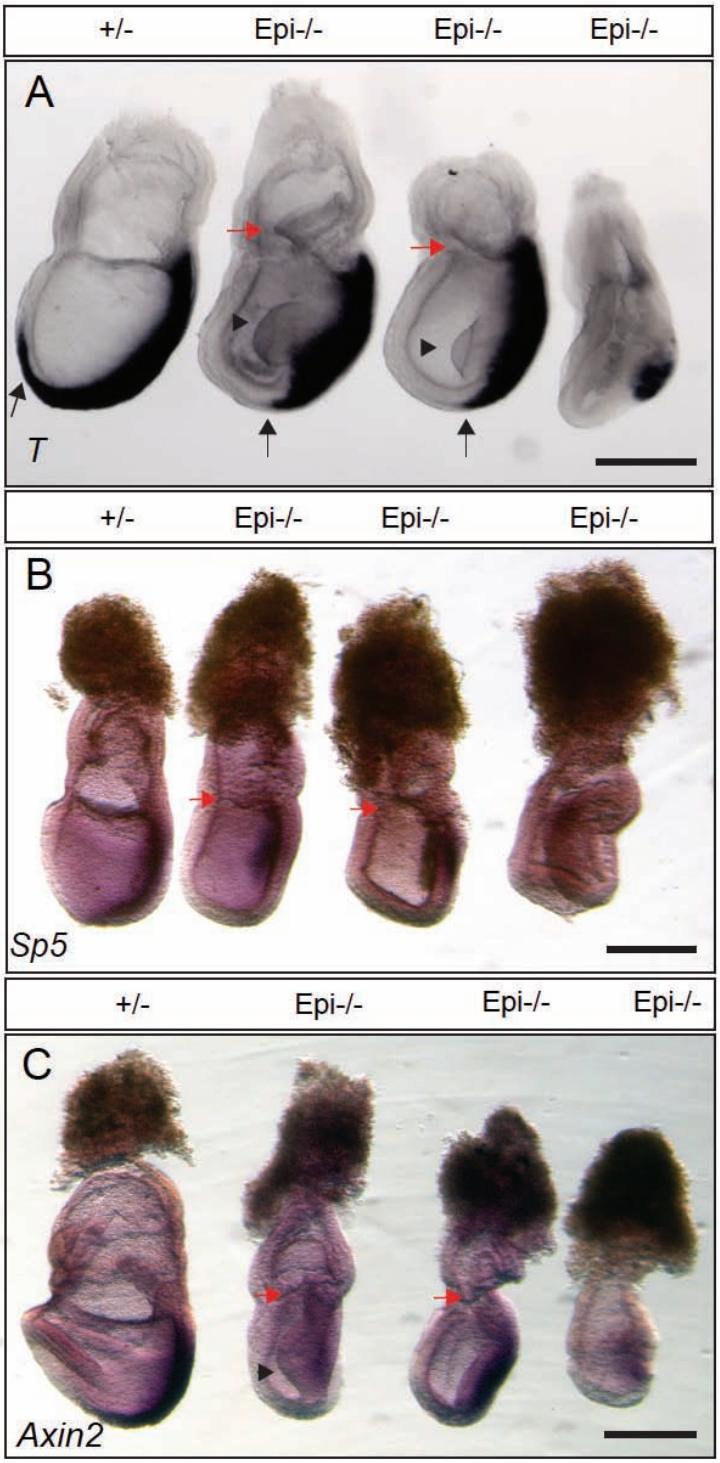

Figure 3.5: Molecular analysis of primitive streak formation in epiblast-specific *Wnt3* null embryos at E7.5. *Wnt3* heterozygous embryos (+/-) and epiblast-specific *Wnt3* knockout embryos (Epi-/-) were dissected at E7.5 and hybridized with three primitive streak markers: *T, Sp5* and *Axin2*. Epiblast-specific *Wnt3* mutant embryos initiate gastrulation as evidenced by the presence of primitive streak markers and axial mesendoderm. Axial mesoderm fails to extend anteriorly (black arrows in A). The posterior amniotic fold fails to fuse to the anterior margin and form the amnion in the mutant embryos (red arrows). A bulge protruding from the primitive streak region is visible (black arrowheads). B-C. Mutant embryos show diminished *Axin2* and *Sp5* expression. Scale bars 200 μm.

Litters dissected at E8.5 showed that abnormal embryos were in the process of being resorbed (not shown) and genotype analysis revealed that they were *Wnt3* epiblast mutants (8/8). The number of abnormal embryos also corresponded to the expected 50% Mendelian ratio (8/18). At E9.5 we recovered 11 normal-looking embryos and observed 9 resorption sites. Genotyping of the normal-looking embryos revealed that they were control heterozygous embryos. Analysis of the number of heterozygous embryos and resorption sites matched the expected Mendelian ratios suggesting that the resorption sites represented mutant embryos.

These results indicate that epiblast-specific *Wnt3* null embryos can initiate gastrulation but fail to complete it resulting in lethality and resorption by ~E9.5.

The *Wnt3* Locus in the Epiblast is Efficiently Recombined.

The expression of primitive streak markers in the epiblast of epiblast-specific mutant embryos raised two possibilities: First, that *cre*-mediated recombination in the epiblast was not carried out in all epiblast cells, resulting in a few non-mutant epiblast cells capable supplying neighboring cells with limited quantities of Wnt3. This would explain the failure to complete but not to initiate gastrulation in epiblast-specific *Wnt3* null embryos. The second possibility is that the *cre*-mediated recombination in the epiblast was 100% efficient, and that the initiation of gastrulation was due to Wnt3 molecules originating in the adjacent posterior visceral endoderm.

To test whether the *Wnt3* locus had been thoroughly recombined in the epiblast of the mutant embryos we performed tissue specific PCR genotyping of pre-streak embryos (Figure 3.6A). To conduct these experiments, the ectoplacental cone was removed and the egg cylinder was severed at the epiblast/extraembryonic ectoderm boundary. The region containing the epiblast was treated with pancreatin and trypsin to separate the epiblast from the visceral endoderm layer as described before (Nagy et al. 2003). The epiblast tissue and the extra-embryonic ectoderm piece were then processed for PCR analysis. We genotyped a total of 21 embryos, using primers that amplify the wild type ($Wnt3^{+}$), floxed ($Wnt3^{c}$) and null ($Wnt3^{lacZ}$) alleles, plus a set of primers that amplify the $Sox2^{cre}$ transgene (Figure 3.6B).

The genotyping results reflected the expected 50% Mendelian ratio. In 11 embryos, we detected the *Wnt3* floxed allele in the extra-embryonic ectoderm region of epiblast-specific *Wnt3* null embryos (Figure 3.6C) but not in the epiblast where $Sox2^{cre}$ is expressed (Figure 3.6D upper panel), indicating that efficient recombination of the floxed allele in the epiblast took place. These embryos were also positive for the $Wnt3^{lacZ}$ null allele (n=11), which allowed us to unequivocally identify the mutant embryos (Figure 3.6D lower panel). In ten embryos, the floxed allele was amplified in the extra-embryonic ectoderm but not the *lacZ* allele indicating they were heterozygous $Wnt3^{c/+}$;$Sox2^{cre/+}$ control embryos.

Figure 3.6

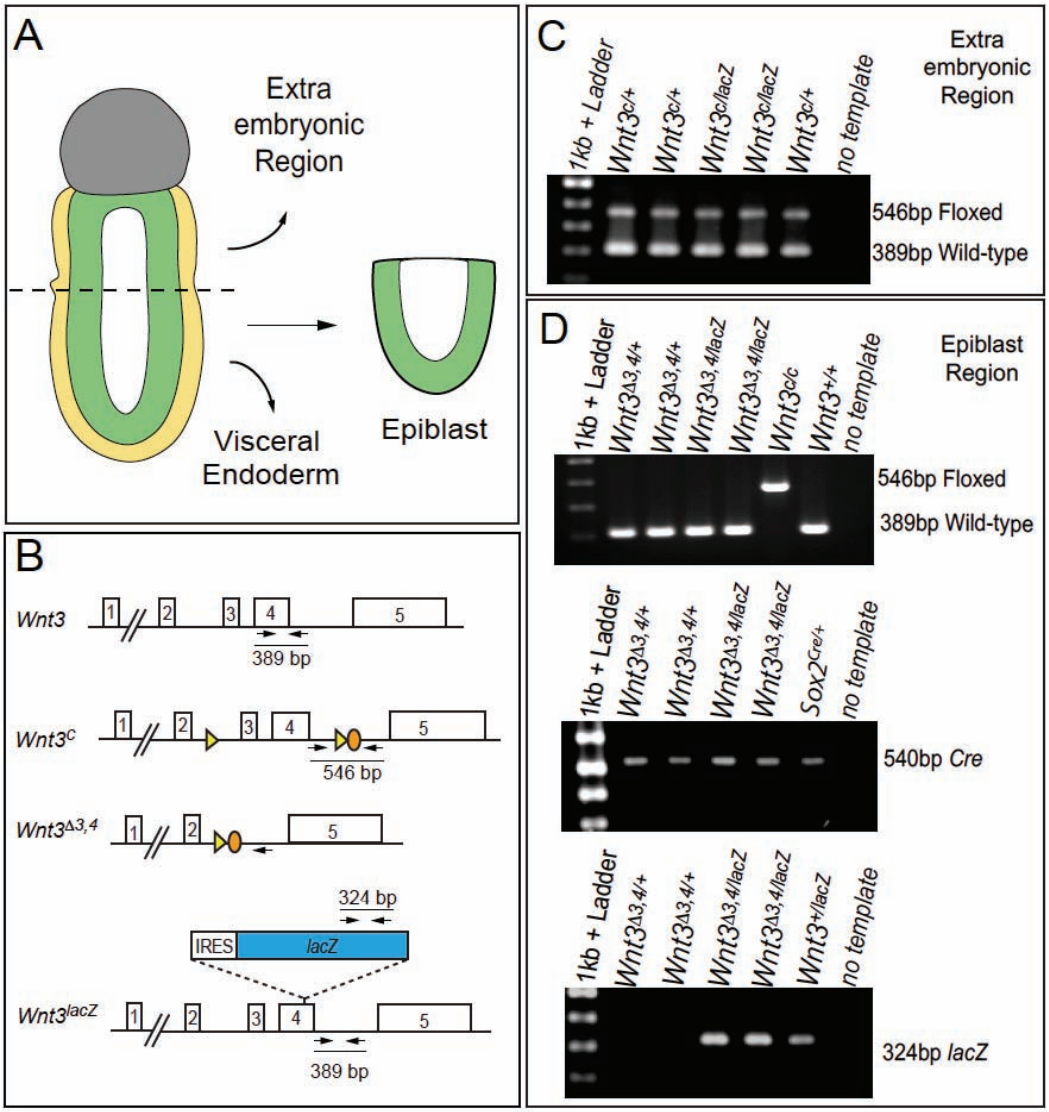
A
Extra embryonic Region
Visceral Endoderm
Epiblast
B
Wnt3
389 bp
Wnt3C
546 bp
Wnt3Δ3,4
324 bp
IRES
lacZ
Wnt3lacZ
389 bp
C
1kb + Ladder
Wnt3c/+
Wnt3c/+
Wnt3c/lacZ
Wnt3c/lacZ
Wnt3c/+
no template
Extra embryonic Region
546bp Floxed
389bp Wild-type
D
1kb + Ladder
Wnt3Δ3, 4/+
Wnt3Δ3, 4/+
Wnt3Δ3, 4/lacZ
Wnt3Δ3, 4/lacZ
Wnt3c/c
Wnt3+/+
no template
Epiblast Region
546bp Floxed
389bp Wild-type
1kb + Ladder
Wnt3Δ3, 4/+
Wnt3Δ3, 4/+
Wnt3Δ3, 4/lacZ
Wnt3Δ3, 4/lacZ
Sox2Cre/+
no template
540bp Cre
1kb + Ladder
Wnt3Δ3, 4/+
Wnt3Δ3, 4/+
Wnt3Δ3, 4/lacZ
Wnt3Δ3, 4/lacZ
Wnt3+/lacZ
no template
324bp lacZ

Figure 3.6: Germ layer genotype of epiblast-specific *Wnt3* null embryos. A. Schematic representation of the strategy utilized to isolate the epiblast layer for genotyping. B. Diagram showing the different *Wnt3* alleles and position of PCR primers (black arrows). C. PCR amplification of the floxed ($Wnt3^{c}$) and wild-type *Wnt3* alleles in the extra-embryonic ectoderm region. The oligos used to amplify the $Wnt3^{c}$ allele (546 bp) also amplify the wild-type *Wnt3* allele (389 bp). All embryos maintain a non-recombined $Wnt3^{c}$ allele in the extra-embryonic ectoderm. D. PCR amplification of the floxed and wild-type *Wnt3* alleles (upper panel) and the $Sox2^{cre}$ allele (middle panel) in the epiblast. The absence of the 546 bp fragment indicates that the floxed *Wnt3* allele is absent in the epiblast (upper panel). All embryos carry the $Sox2^{cre}$ allele. Amplification the *lacZ* allele (324 bp) indicates the presence of the null embryos (lower panel). All embryos are derived from $Wnt3^{c/c}$ females and $Wnt3^{lacZ/+;}$ $Sox2^{cre/cre}$ males crosses.

This set of experiments excluded the possibility that incomplete recombination of the *Wnt3* floxed allele in the epiblast was responsible for the expression of primitive streak markers in that tissue. Therefore, these results strongly suggest that the expression of primitive streak markers in the epiblast of epiblast-specific *Wnt3* null embryos is due to Wnt3 emanating from the visceral endoderm.

Wnt3 Utilizes the Canonical Wnt Pathway to Drive the Expression of Primitive Streak Markers.

Wnt3 has been suggested to act through the canonical Wnt pathway using morphological analysis and reporter assays *in vitro* (Bhat et al. 2010; Shimizu et al. 1997). Previously, we used the reporter BAT-Gal and a null allele of Wnt3 (*Wnt3*$^{\Delta 3,4}$) to provide genetic evidence that Wnt3 activates the canonical Wnt pathway in mouse embryos during gastrulation. To provide direct evidence that Wnt3 acting through the canonical Wnt pathway modulates the expression of primitive streak markers, and to further understand the molecular basis of Wnt3 signaling during gastrulation we assessed whether Wnt3 was capable of activating the canonical Wnt pathway by driving expression of the BAT-Gal transgene *in vitro*. To do so, we generated primary mouse embryonic fibroblasts (MEF) from E13.5 embryos heterozygous for the BAT-Gal transgene and treated them with medium containing Wnt3 (Wnt3-conditioned medium), or control medium.

We generated Wnt3-conditioned medium by overexpressing *Wnt3* in HeLa cells using a construct in which the CMV promoter drives expression of a bicistronic *Wnt3*-eGFP mRNA. As a negative control, we generated conditioned medium from HeLa cells transfected with a CMV-eGFP construct (GFP-conditioned medium). MEF cells were treated overnight with Wnt3-conditioned, GFP-conditioned or MEF medium (control medium).

Treatment with Wnt3-conditioned medium led to increased β-galactosidase expression in BAT-Gal MEFs (Figure 3.7A-B). Unexpectedly, BAT-Gal MEFs treated with control medium had less prominent, yet detectable levels of β-galactosidase activity (Figure 4A). This observation could not be attributed to the medium containing FBS, since MEF cultured in medium made with KO replacement serum showed the same phenomenon (not shown).

Although this “background” activity was considerably less than the one observed in BAT-Gal MEFs treated with Wnt3, over 3 consecutives experiments, it did not allow us to clearly verify whether Wnt3 treatment was an effective direct way to activate the canonical Wnt pathway. Therefore, we decided to measure activation of the canonical Wnt pathway in a more direct way.

β-catenin is a central player in the canonical Wnt pathway, and its nuclear accumulation is a hallmark of activation of the pathway (Archbold et al. 2012; Tolwinski and Wieschaus 2004). Thus, we decided to measure the ability of the treatment with Wnt3-conditioned to promote nuclear localization of β-catenin.

Figure 3.7

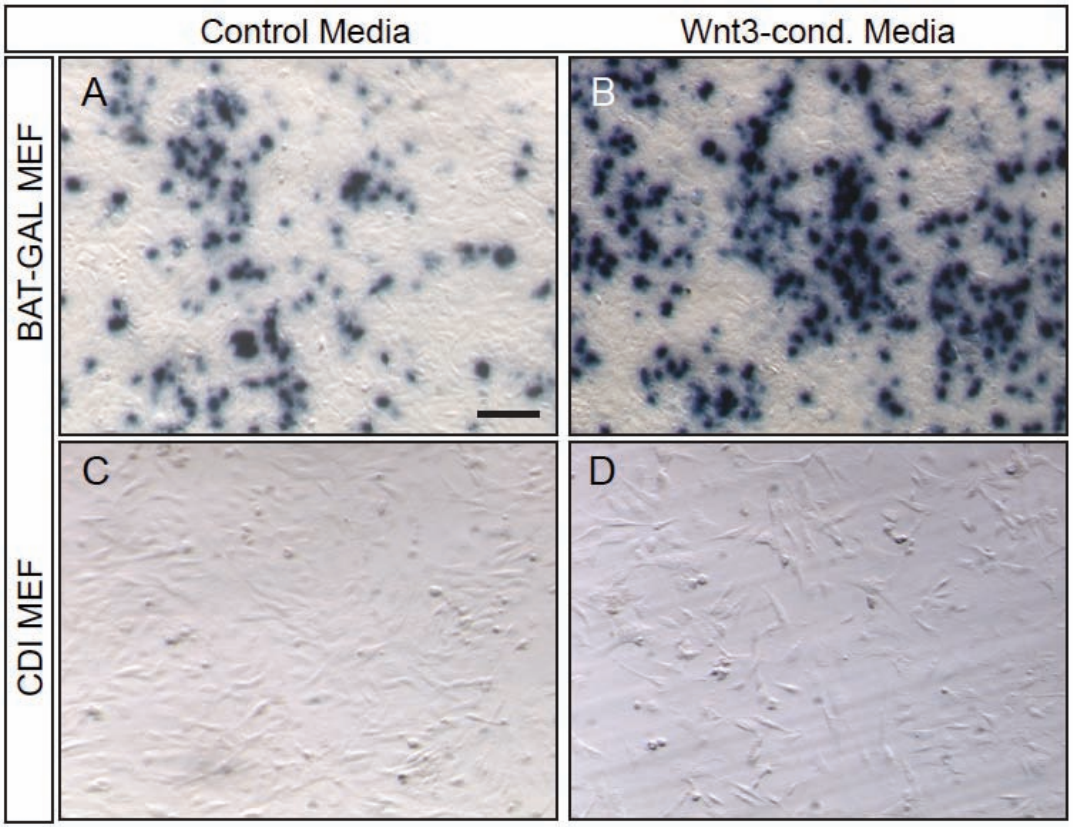

Figure 3.7: Wnt3 treatment leads to activation of BAT-Gal transgene in MEFs. A-D. Primary MEF cells obtained from either embryos hemizygous for the BAT-Gal transgene or CDI wild type embryos were incubated overnight in presence or absence of Wnt3-conditioned medium and assayed for β-galactosidase activity. A. BAT-Gal MEFs treated overnight with control medium. B. BAT-Gal MEFs treated overnight with Wnt3-conditioned medium. C. CDI MEFs treated overnight with control medium. D. CDI MEFs treated overnight with Wnt3 conditioned medium. Scale bar 100 µm.

To this end, we generated MEF cells from E13.5 CD1 embryos and treated them either with Wnt3-conditioned or control medium. The treatment of MEFs with Wnt3-conditioned medium promoted a significant redistribution of the cellular contents of β-catenin (Figure 3.8A). Using confocal microscopy we observed that a large portion of β-catenin relocated to the cell nucleus after overnight treatment with Wnt3-conditioned medium (Figure 3.8A, lower panel). The same phenomenon was not observed when either GFP-conditioned medium or control medium was used (Figure 3.8A, middle and upper panel). Treatment with Wnt3-conditioned medium increased the frequency of nuclear localization of β-catenin by about 6-fold compared to controls (Figure 3.8B). This data indicated that Wnt3 is a direct activator of the canonical Wnt pathway. A question that remained, however, was: can treatment with Wnt3-conditioned medium drive the expression of primitive streak markers?

In the nucleus β-catenin acts as a transcription co-factor altering the rate of transcription of primitive streak markers such as *T* and *Axin2* (Jho et al. 2002; Yamaguchi et al. 1999). Thus, we used qPCR to assess whether overnight treatment with Wnt3-conditioned medium would lead to changes in the transcription rates of these genes in MEFs. We also analyzed the expression of *Cyclin D1*, a gene involved in the regulation of the cell cycle that is a well-known target of the canonical Wnt pathway (Tetsu and McCormick 1999).

Figure 3.8

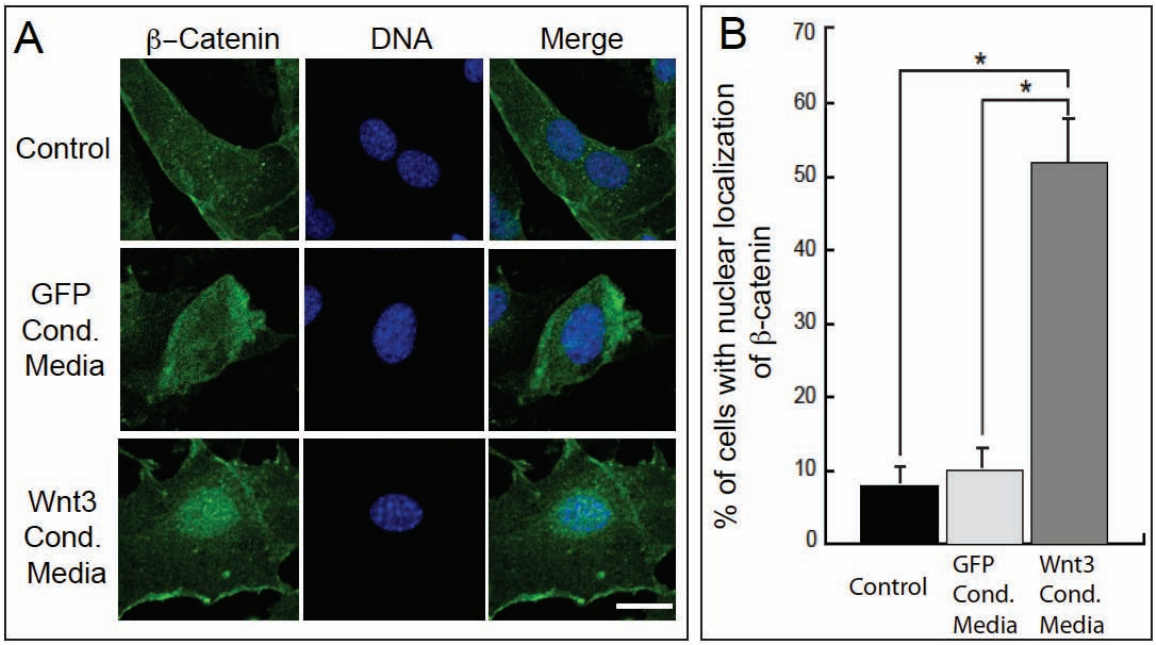
A
β–Catenin
DNA
Merge
Control
GFP
Cond.
Media
Wnt3
Cond.
Media
B
% of cells with nuclear localization
of β-catenin
70
60
50
40
30
20
10
0
*
*
Control
GFP
Cond.
Media
Wnt3
Cond.
Media

Figure 3.8: Wnt3 treatment leads to nuclear accumulation of β-Catenin in MEFs. A. MEF cells treated overnight with control medium, GFP-conditioned medium or *Wnt3*-conditioned medium were immunostained with anti-β-catenin antibody. Only MEFs treated with *Wnt3*-conditioned medium show accumulation of β-catenin (green) in their nuclei (blue) indicating activation of the canonical Wnt pathway. B. Quantification of cells with nuclear localization of β-catenin. All cells with accumulation of β-catenin (green) in their nuclei (blue) were counted and plotted as a percentage of the total number of cells counted. The graph shows the mean percentage of β-catenin positive nuclei in each group (mean ± SEM), from three independent experiments. Student *t*-test was used to compare levels of nuclear accumulation of β-catenin in control samples and *Wnt3*-treated samples. Treatment with *Wnt3*-conditioned medium significantly enhanced the level of nuclear localization of β-catenin when compared either with control medium or GFP-conditioned medium, (*$p < 0.001$). Differences between treatment with control medium and GFP-conditioned medium were not significant. All pictures are shown at the same magnification. Scale bar, 20 μm.

Our results showed an increase of *T* (10-fold), *Axin2* (12-fold) and *Cyclin D1* (35-fold) after overnight treatment with Wnt3-conditioned medium, but not when control medium was used (Figure 3.9A), suggesting that Wnt3 treatment directly modulate the transcription of these target genes.

Due to its pattern of expression and the results described before we wondered whether the treatment of Wnt3 would affect the rate of transcription of its own locus. Interestingly, the *Wnt3* locus was even more sensitive to changes in transcription rates (400 fold), than those observed for *T, Axin2* and *Cyclin D1,* in MEF cells treated Wnt3-conditioned medium (Figure 3.9A,B)

These results suggest that Wnt3 can control *Wnt3* expression and the expression of genes involved in gastrulation in the epiblast via activation of the canonical Wnt pathway.

Treatment of MEF cells with Wnt3-Conditioned Medium Promotes β-Catenin Occupancy of the *Wnt3* Promoter.

The data presented above suggest that Wnt3 utilizes the canonical Wnt pathway to drive the expression of primitive streak markers, but does not provide information about whether Wnt3 directly activates its own promoter in a β-catenin-dependent manner.

To address this possibility, we performed an *in silico* promoter analysis of the *Wnt3* locus using Genomatix software (http://www.genomatix.de). We

analyzed a 5 kb long segment of DNA in the vicinity of the transcription start site of *Wnt3* and found a total of eight TCF binding sites in this DNA segment. Five of which clustered on a DNA segment of 680 bp around the transcription site of *Wnt3* (Figure 3.10A); therefore, we concentrated our analysis in this region.

Analysis of the 5 TCF-binding sites using a nucleotide position preference graphic showed the presence the highly conserved CAAAG sequence at the core of the TCF-binding sites (Figure 3.10B), similar to what is found in other loci targeted by the activation of the canonical Wnt pathway (Jho et al. 2002; Tetsu and McCormick 1999). This observation suggested that the Wnt3 locus is a direct target of the canonical Wnt pathway.

In order to determine whether β-catenin occupies the *Wnt3* promoter after activation of the canonical Wnt pathway, we carried out chromatin immunoprecipitation experiments using antibodies against β-catenin and primers designed to detect the TCF-binding-site-rich region on MEF cells treated overnight either with Wnt3-conditioned medium or control medium. Our results showed that β-catenin occupies the proximal promoter of *Wnt3* in cells treated with Wnt3-conditioned medium, but not when control medium was used (Figure 3.10C). As a positive control, we showed that β-catenin occupies the *Brachyury* promoter. The *IgH* promoter was used as a negative control. This gene contains similar sequences to TCF-biding sites in its promoter but it is not expressed in MEFs. As expected, β-catenin was not detected at the *IgH* locus when cells were treated either with control medium or *Wnt3*-conditioned medium (Figure 3.10C).

Figure 3.9

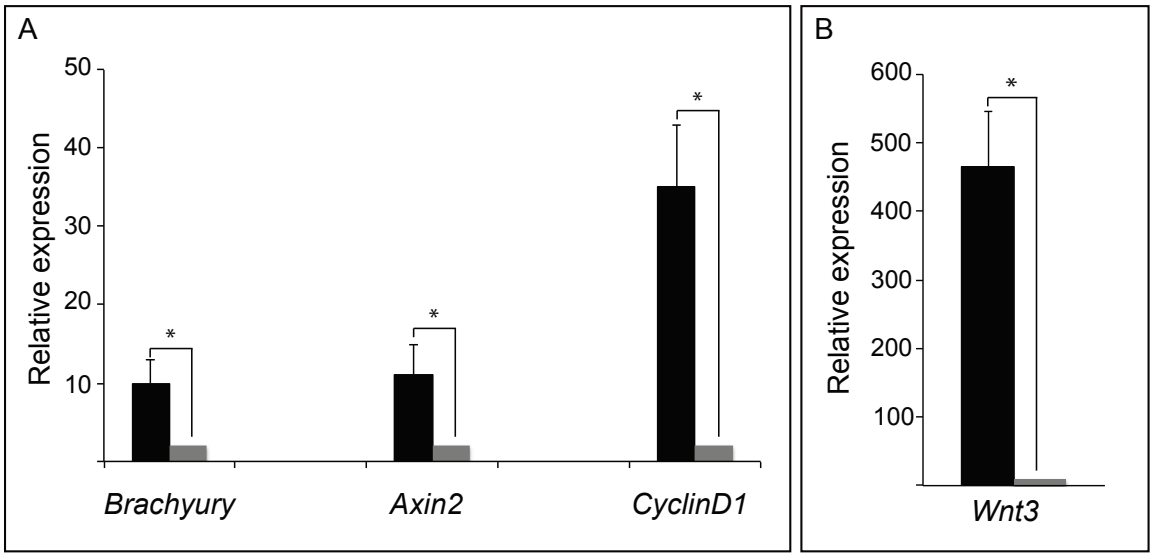
A
Relative expression
50
40
30
20
10
*
*
*
Brachyury
Axin2
CyclinD1
B
Relative expression
600
500
400
300
200
100
*
Wnt3

Figure 3.9: Wnt3 treatment leads to expression of primitive streak markers in MEFs. A. MEF cells treated overnight either with *Wnt3*-conditioned medium (black bars) or control medium (grey bars) were assayed for *Brachyury, Axin2* and *Cyclin D1* RNA expression. All three genes are targets of the canonical Wnt signaling pathway. The primitive streak markers *Brachyury* and *Axin2* show about ten-fold elevated transcription levels relative to controls. The effects on the expression levels of *Cyclin D1* are higher (35 fold). B. *Wnt3* mRNA levels are highly upregulated after *Wnt3*-mediated activation of the canonical Wnt pathway. Graph shows results (mean ± SEM) from three independent experiments. Control samples were compared with *Wnt3*-treated samples using student *t*-test (*p< 0.001).

These results indicate that occupancy of the *Wnt3* promoter is due to specific recruitment of β-catenin after *Wnt3*-mediated activation of the canonical Wnt pathway.

β-Catenin Occupies the *Wnt3* Promoter in E6.5 Embryos.

Since treatment with Wnt3-conditioned medium leads to occupancy of the *Wnt3* promoter by β-catenin in MEFs, and thus we wondered whether this was also the case in the Wnt3 promoter of gastrulating mouse embryos. To answering this question, we dissected 140 E6.5 wild-type embryos from fifteen litters and conducted chromatin immunoprecipitation using antibodies against β-catenin. We hypothesized that if *Wnt3* plays a role in the activation of its own locus during mouse gastrulation, one should expect to find β-catenin present at the *Wnt3* promoter at the onset of primitive streak formation at E6.5.

Our results showed that β-catenin is indeed present at the *Wnt3* promoter *in vivo* in embryos dissected at E6.5 (Figure 3.11A,B). In contrast, when parietal yolk sac (PYS) tissue was used there was no indication of the presence of β-catenin in the *Wnt3* promoter (Figure 3.11A,B). Once again, the *IgH* promoter was probed as a negative control. As expected, β-catenin was not found at the *IgH* locus in any tissue tested (Figure 3.11A,B). As a positive control, we quantified the occupancy of *Brachyury* and *Axin2* promoters by β-catenin (Figure 3.11A). The presence of β-catenin at the *Wnt3, Brachyury* and *Axin2* promoters is in agreement with the pattern of expression of these genes in mouse embryos

at E6.5 (Herrmann 1991; Jho et al. 2002; Rivera-Perez and Magnuson 2005). Taken together, these results provide a mechanism by which *Wnt3* regulates its own locus *in vivo* and initiates the process of gastrulation.

Discussion.

The *Wnt3* gene is known to be essential for specification of the primitive streak and gastrulation (Liu et al, 1999). However, since *Wnt3* is expressed in two different tissues of the early post implantation embryo, the epiblast and adjacent posterior visceral endoderm, it is not possible to differentiate the specific requirement of *Wnt3* in either tissue in knockout experiments. In this study, we observed that embryos lacking *Wnt3* exclusively in the epiblast are able to form a primitive streak and proceed through gastrulation, yet they are delayed in development and have morphological abnormalities that lead to lethality and resorption by E9.5. These results led us to conclude that *Wnt3* is necessary in the epiblast for the maintenance but not the initiation of gastrulation. A previous study that also used the *Sox2*^{Cre} transgene to ablate *Wnt3* in the epiblast of early post-implantation mouse embryos found that *Wnt3* epiblast knockout embryos phenocopy embryos with a full *Wnt3* knockout and concluded that *Wnt3* is essential in the epiblast for gastrulation (Barrow et al. 2007). In our experiments, we observed variation in the penetrance of the *Wnt3* Epi-KO phenotype.

Figure 3.10

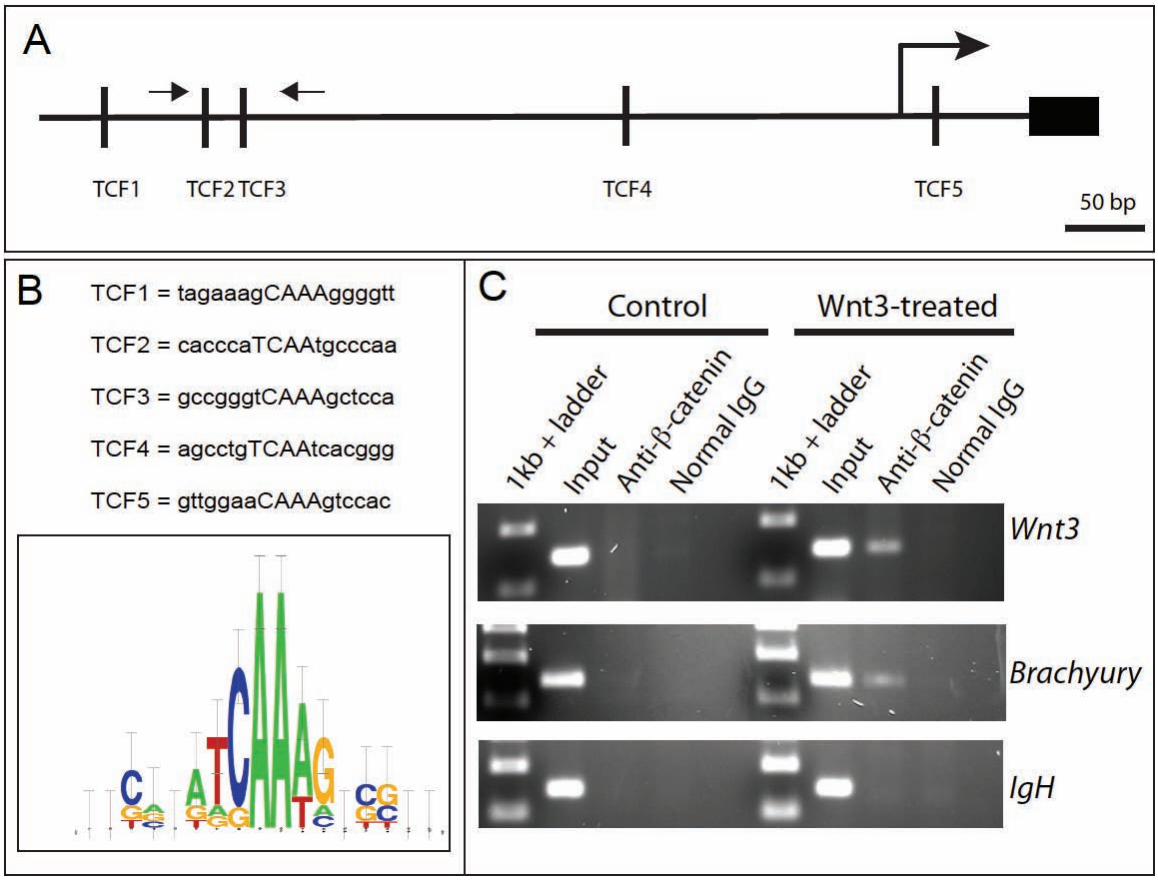
A
TCF1
TCF2 TCF3
TCF4
TCF5
50 bp
B
TCF1 = tagaaagCAAAggggtt
TCF2 = cacccaTCAAtgcccaa
TCF3 = gccgggtCAAAgctcca
TCF4 = agcctgTCAAtcacggg
TCF5 = gttggaaCAAAgtccac
C
Control
Wnt3-treated
1kb + ladder
Input
Anti-β-catenin
Normal IgG
Wnt3
Brachyury
IgH

Figure 3.10: Wnt3-conditioned medium treatment leads to β-Catenin occupancy of the *Wnt3* promoter in MEF cells. A. Schematic representation of the proximal promoter region of *Wnt3* showing five TCF/LEF binding sites within 700 bp around the transcription start site (arrow). B. Sequence of each TCF biding site and sequence logo showing conserved nucleotides at the core of the TCF-biding site. C. Overnight treatment of MEF cells with *Wnt3*-conditioned medium results in β-catenin occupancy of the *Wnt3* and *Brachyury* (*T*) promoters. *IgH* promoter (negative control) does not show occupancy by β-catenin.

Figure 3.11

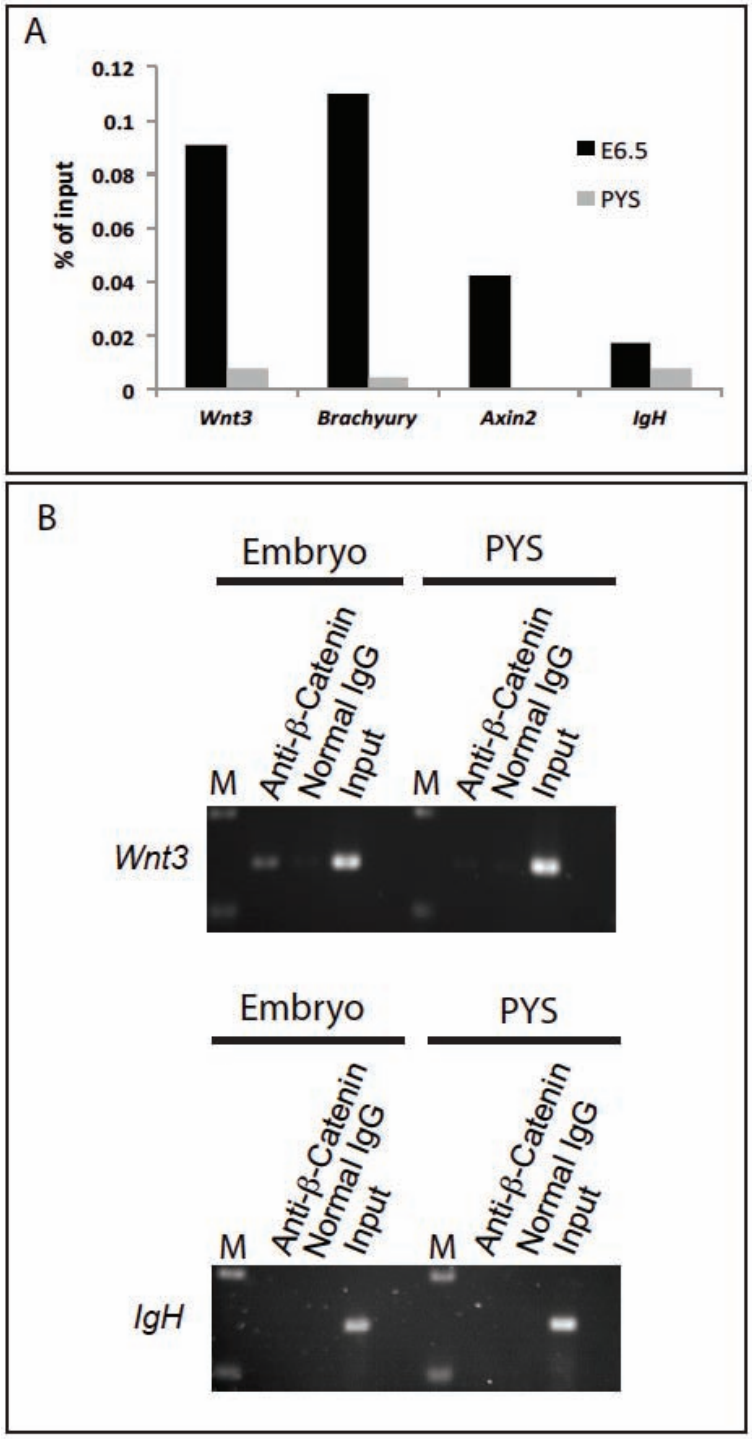
A
0.12
0.1
0.08
0.06
0.04
0.02
0
% of input
E6.5
PYS
Wnt3
Brachyury
Axin2
IgH
B
Embryo
PYS
Anti-β-Catenin
Normal IgG
Input
M
Wnt3
IgH

Figure 3.11: β-catenin occupancy of the *Wnt3, Axin2* and *Brachyury* promoters *in vivo*. Wild-type mouse embryos dissected at E6.5 subjected to chromatin immunoprecipitation using monoclonal antibodies against β-catenin. A. Graph shows quantification of β-catenin occupancy on *Wnt3, Brachyury, Axin2* and *IgH* promoters in embryos (black bars) and parietal yolk sac (PYS) (grey bars). *Axin2* and *Brachyury loci* were used as positive control. The *IgH* locus was used as a negative control. B. PCR analysis of β-catenin occupancy on *Wnt3* locus (upper panel) and *IgH* locus (lower panel). M, 1kb (+) marker.

In some E6.5 mutant embryos *T* is indeed absent in the posterior epiblast suggesting an absence of primitive streak specification, however, we found weak *T* expression in other embryos. Moreover, all E7.5 embryos analyzed show expression of *T* and gastrulation is evident, although at different levels of progression. Hence, *Wnt3* epiblast knockout embryos show delayed, but not a lack of gastrulation. We have addressed the possibility that a partial knockout of *Wnt3* in the epiblast is responsible for our results by showing that *Wnt3* expression is absent in *Wnt3* epiblast knockout embryos and by noting the absence of the *Wnt3* floxed allele in the epiblast. Therefore, the contradictory conclusions derived from our results and those of Barrow and co-workers can be attributed to delayed development of *Wnt3* epiblast knockout embryos, which incorrectly appeared to indicate absence of gastrulation.

An interesting observation is that ablation of *Wnt3* in the epiblast leads to absence of *Wnt3* expression in the posterior visceral endoderm. *Wnt3* is initially expressed in the posterior visceral endoderm at E5.5 and a few hours later in the adjacent posterior epiblast (Rivera-Perez and Magnuson, 2005). Since the only difference between our results and those of a full *Wnt3* knockout embryo is solely an absence of *Wnt3* in the epiblast, we believe that the posterior visceral endoderm in *Wnt3* epiblast knockout embryos provides the initial signal to activate its own expression in the epiblast and that *Wnt3* activity in the epiblast is required to signal back to the posterior visceral endoderm to maintain *Wnt3* expression in this tissue. In support of this hypothesis, we have shown that *Wnt3*

ablation in the posterior visceral endoderm is essential for its own expression in the epiblast and that these *Wnt3* visceral endoderm-knockout embryos fail to generate a primitive streak and gastrulate (Tortelote et al., *in preparation*).

We also noticed that the *Wnt3* locus contains five TCF/LEF-binding sites within a 700 bp DNA segment immediately upstream of the transcription start site. Using chromatin immunoprecipitation we were able to show that β-catenin occupies the *Wnt3* promoter, indicating that the *Wnt3* locus is a target of the Wnt3-mediated activation of the canonical Wnt pathway. The posterior visceral endoderm is a source of Wnt signaling (Rivera-Perez and Magnuson 2005) and also a target of the canonical Wnt signaling in the gastrulating mouse embryo as shown by the activation of the canonical Wnt signaling pathway in the posterior visceral endoderm using a GFP reporter line (Ferrer-Vaquer et al. 2010).

Mice mutant for *Gpr177* or *Porcupine*, two genes required for Wnt secretion, show gastrulation defects similar to those observed for *Wnt3* epiblast knockout (Fu et al. 2009; Nusse et al. 1991). *Gpr177* is a direct target of the canonical Wnt pathway as well. Thus, it is possible that the lack of *Wnt3* in the epiblast affects genes involved in the production, maturation and secretion of Wnts required for further progression of gastrulation. Interestingly, embryos mutant for both genes show expression of *Wnt3* in the epiblast, revealing that the initial expression of *Wnt3* is not dependent on these genes in the epiblast.

The canonical Wnt signaling pathway has been implicated in mesendoderm induction in *Xenopus laevis* and in zebrafish (Tao et al. 2005; Lu,

Thisse, and Thisse 2011). Our results in mouse embryos suggest that the Wnt signaling role in gastrulation is conserved in the majority if not all vertebrates. Moreover, a *Wnt3* autoregulatory feedback loop has been described in *Hydra* head organizer formation (Nakamura et al. 2011). Therefore, the intracellular mechanism by which *Wnt3* controls its own expression may be part of an ancient axial regulatory mechanism conserved among metazoans.

Materials and Methods

Embryo staging and mouse strains: Embryos were staged using morphological landmarks as previously described (Downs and Davies 1993; Rivera-Perez, Jones, and Tam 2010) or described as days of development. Noon of the day that a mating plug was observed was considered embryonic day 0.5 (E0.5) of development. CD-1 mice were obtained from Charles River Laboratories. *Sox2*^{cre} mice were obtained from the Jackson laboratory (Stock No. 004783). *Wnt3*^{c} mice were obtained from Dr. Jeff Barrow (Barrow et al. 2003). *Wnt3*^{lacZ} mice were provided by Dr. Richard Behringer. These mice carry an *IRES-lacZ* cassette was inserted in the unique ClaI site of exon 4 of the *Wnt3* locus creating a null allele (to be published elsewhere). All embryos analyzed were derived from mixed stock mice.

Whole-mount RNA *in situ* analysis: We performed whole-mount *in situ* hybridization as described (Rivera-Perez and Magnuson 2005). Briefly, embryos

were dissected using forceps and fixed overnight at 4^{o}C in 4% paraformaldehyde prepared in PBS. After fixation, the embryos were dehydrated in methanol series and stored in 100% methanol at -20℃. Hybridization was conducted at 70℃. The probes were: *Wnt3*, a 0.8 kb cDNA fragment containing exons 3–5 (Liu et al. 1999). *Wnt3* 3'F, a 1,050 bp fragment of exon 5 containing a piece of the 3'UTR. *Brachyury*, full-length cDNA probe of 1,308 bp (Herrmann 1991). *Fgf8,* full length cDNA, 1,100 bp. *Axin2,* 2,420 bp cDNA piece containing part of exon 2, exons 3 – 9 and a portion of exon 10. *Sp5,* full length cDNA, 1,540 bp. *Hesx1,* cDNA piece containing part of exon1, exon2 and a portion of exon 3, 394 bp (Thomas and Beddington 1996). *Hex,* 527 bp fragment from 281 – 818 bp of cDNA sequence (Thomas, Brown, and Beddington 1998) and *Dkk1,* full cDNA sequence, 1235 bp (Miura, Singh, and Mishina 2010). All riboprobes were prepared using a digoxigenin RNA labeling kit (Roche Cat. No.1175025).

Preparation of mouse embryonic fibroblasts: MEFs were prepared as previously described (Nagy et al. 2003). CD1 females were selected based upon estrus cycle and mated to CD1 or BAT-Gal male mice. The next day, the pregnancy was confirmed by appearance of vaginal plug (E0.5). The pregnant females were then sacrificed at E13.5 and the embryos dissected in dissection medium (low glucose DMEM, 10% FBS, 1x Penicillin/streptomycin). The head and internal organs of each embryo were surgically removed and the remaining part of the embryo was moved to another plate and washed twice with PBS. The

embryos were separated in groups of ten. Each group was transferred to a 50 ml conical tube with 5 ml of 0.25% Trypsin-EDTA in PBS. Next the embryos were forced through an 18-gauge needle for about 5 times. The resulting tissue suspension was incubated at 37 ˚C with 5% CO2 for 15 min, every 5 min the suspension was lightly vortexed for 5 sec. An equal volume of MEF medium (High glucose DMEM, 10% FBS, 1x Glutamax, 1x Penicillin/streptomycin) was added to each 50 ml tube. The final tissue suspension was plated on 15 cm plates (3 ml/plate) containing 27 ml of MEF medium. Two to three days later when the MEFs had reached 90% confluence they were harvested and frozen. This was passage zero (P0). All the experiments were conducted with P2-P3 MEFs. Before each experiment we thawed a new aliquot of P0 MEF and expanded it in order to have enough plates at P2 or P3. MEF cells were maintained in MEF medium at 37 ˚C with 5% CO2. All experiments were performed using semi-confluent plates (roughly 75-85%).

Preparation of *Wnt3*-conditioned medium: To obtain *Wnt3* conditioned medium, we transfected HeLa cells with pCMV-*Wnt3*-2a-eGFP. This plasmid produces a bicistronic message that contains the self-cleaving 2A peptide (Szymczak and Vignali 2005), inserted between *Wnt3* and eGFP. To generate pCMV-*Wnt3*-2a-eGFP, a 1.2 kb fragment containing the *Wnt3* coding region was PCR-amplified from a *Wnt3* cDNA plasmid obtained from Open Biosystems (Cat. No. 40039305). The amplified fragment was digested with XhoI and BspEI and

subcloned into the pCAG-SH-RG plasmid (Stewart et al. 2009). Next the *Wnt3*-2a-EGFP cassete was PCR-amplified, digested with NheI and BglII and subcloned into pCMV-GFP (Clontech, Cat. No. 6082-1) followed by digestion with NheI and BamHI. All transfections were carried out using FUGENE HD reagent (Promega Cat. No. E2311). The transfection success was evaluated by the presence of eGFP, using fluorescence microscopy. The efficiency of the transfection, using HeLa cells was 60 to 70%. Twenty-four hours after transfection, the medium was replaced by MEF medium composed of DMEM (Gibco, Cat. No. 12100-046), 10% FBS, 1x Glutamax (Invitrogen, Cat. No. 35050-061), penicillin (100 U/ml), streptomycin (100 μg/ml) (Invitrogen, Cat. No. 15140-122). Medium was collected every 24 hours for three days, stored at 4˚C and used for no longer than 4 days. Before use Wnt3-conditioned medium was cleared by centrifugation (5 min, 1000g) and filtered through a 0.45 μm filter. Control conditioned medium (GFP-conditioned medium), was generated by transfecting HeLa cells with a pCMV-eGFP construct.

***Wnt3* treatment and immunofluorescence:** MEFs cells were grown on cover slips placed in 12-well plates containing MEF medium. When the cells reached about 70% confluence the medium was replaced either with MEF medium (control), *Wnt3*-conditioned medium or GFP-conditioned medium and incubated overnight. The cells were then fixed in 4% PFA for 40 minutes and washed in TBS-1 (10 mM Tris-HCl, pH7.7, 150 mM NaCl, 3 mM KCl, 1.5 mM $MgCl_2$, 0.05%

Tween 20, 0.1% bovine serum albumin and 0.2% glycine). Cells were blocked in TBS-1 for 1h and then incubated overnight at 4^{o}C with mouse anti-β-catenin antibody diluted 1:50 (BD Transduction lab, Cat. No. 610154) in TBS-1. The next day, cells were washed three times for 15 minutes with TBS-1 and incubated for one hour with goat anti-mouse Alexa 488 secondary antibody (Molecular probes, Cat. No. A10680) diluted 1:1500 at room temperature. Cells were then washed twice for 15 minutes in TBS-1 and incubated with DAPI (Sigma Cat. No. D9564) for 30 minutes. Cells were mounted on glass slides using prolong gold mounting medium (Molecular Probes, Cat. No. P36930) and visualized using fluorescence microscopy (Leica DMI 6000B). Quantification of cells with nuclear localization of β-catenin was performed by selecting five adjacent fields per treatment (Control medium, GFP-conditioned medium, *Wnt3*-conditioned medium). The experiments were repeated three times and were scored by two investigators. One investigator scored the cells blindly. Each field averaged around 20 cells. Cells were assessed based on their nuclear staining (blue) and β-catenin staining (green). All cells with accumulation of β-catenin in their nuclei (blue) were counted and plotted as a percentage of the total number of cells. The scoring of β-catenin positive nuclei was validated by confocal microscopy (Leica SP1 laser scanning confocal microscopy). Cells that were scored as negative had fluorescence above or below the nucleus, but did not have nuclear fluorescence in an optical section through the nucleus. Cells scored as positive had a high level of nuclear fluorescence in optical sections through the nucleus.

RNA isolation and quantitative PCR: For quantitative PCR analysis, P3 MEF cells were grown in 10 cm tissue culture plates, until they reached 70% confluence. At this point, they were incubated overnight in control or *Wnt3*-conditioned medium. The next day cells were harvested and the RNA was isolated using TRIzol (Invitrogen, Cat. No.15596-026). The RNA was digested with DNAse I for 30 min at 37^{o}C to yield DNA-free RNA samples and purified using a DNA-free RNA kit (Zymo Research, Cat. No. R1013). One microgram of total RNA was utilized for reverse transcription reactions to generate cDNA using the Superscript III First Strand kit (Invitrogen, Cat. No. 18080-051). Quantitative PCR was performed with Fast SYBR Green Master Mix (Applied Biosystem, Cat. No. 4385612). The primers used for qPCR were designed with at least one of the primers spanning an intronic region in order to prevent amplification of genomic DNA contaminants. The following primers were used: *Axin2*: 5'-CTCCTTGGAGGCAAGAGC-3' and 5'-GGCCACGCAGCACCGCTG-3' (Jho et al. 2002). *Brachyury*: 5'-TACCCCAGCCCCTATGCTCA-3' and 5'-GGCACTCCGAGGCTAGACCA-3'; *Cyclin D1*: 5′-TCGTGGCCTCTAAGATGAAG-3' and 5′-TTTTGGAGAGGAAGTGTTCG-3' (Zhang et al. 2009); *Gapdh* (5′-AAGGTCATCCCAGAGCTGAA-3' and 5′-CTGCTTCACCACCTTCTTGA-3' (Zhang et al. 2009); *Wnt3*-P1 5'-CAAGCACAACAATGAAGCAGG-3' and 5'-TCGGGACTCACGGTGTTTCTC-3' (Binnerts et al. 2007); *Wnt3*-P2 (Forward 5'-GACAAAGCCACCCGTGAATC-3' and 5'- ACTTCCAGCCTTCTCCAGGT-3'. Amplification was performed using the

ABI StepOne Plus Real Time PCR System (Applied Biosystems). Relative expression of each gene was measured based upon the ΔΔCt method normalizing to *Gapdh* (Livak and Schmittgen 2001).

Chromatin immunoprecipitation: MEF cells were cultured in 10 cm plates (~3x10^6 cells/plate) for 12 hours either in MEF medium or *Wnt3*-conditioned medium. The cells were then cross-linked in 1% formaldehyde (Fisher), lysed in buffer containing 1% SDS, 10 mM EDTA, 50 mM Tris-HCl, (pH 8.1), incubated on ice, and sonicated to obtain DNA fragments of approximately 600 bp. Next, 150 µg of sonicated DNA was diluted 10-fold in immunoprecipitation buffer (0.01% SDS, 1.1% Triton-X100, 1.2 mM EDTA, 16.7 mM Tris (pH 8.1), 167 mM NaCl) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml pepstatin A). One tenth of the sample was stored at -20°C to be used as input control. The remaining sample was precleared with 30 µl of protein A beads (50% slurry) (Amersham) containing 20 µg of sonicated salmon sperm DNA and 1 mg/ml BSA in TE (10 mM Tris-HCl pH 8.1, 1 mM EDTA) at 4°C for 1 h. Cleared lysates were incubated with 3 µg of mouse anti-β-catenin antibody (BD Transduction Laboratories, Cat. No. 610154) overnight. Normal mouse IgG (Santa Cruz Biotechnology, Cat. No. sc-2015.) was used as an immunoprecipitation specificity control. To precipitate immune complexes 50 µl of a 50% slurry of protein A beads were added to the cell lysates and incubated for 2 h at 4°C, and then centrifuged at 1000 x g for 1 min. The sepharose precipitate

complex was washed 5 times using the following buffers for 5 min each at 4°C. Buffer A: 20 mM Tris-HCl (pH 8.1), 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 150 mM NaCl. Buffer B: 20 mM Tris-HCl (pH 8.1), 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 500 mM NaCl. Buffer C: 0.25 M LiCl, 1% NP-40, 1% sodium deoxycolate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.1). Next the samples were washed twice with TE (pH 8.0). Immune complexes were eluted from the beads by adding 1% SDS in 0.1M $NaHCO_3$, mixed by quickly vortexing and incubated at room temperature for 30 min with rotation. The samples were then centrifuged at 1000 g for 1 minute to pellet the agarose beads and the supernatant was carefully transferred to another tube. Protein-DNA cross-links of both sample and the inputs were reversed by adding NaCl (200 mM final concentration) and heating at 65°C overnight. Samples were then treated with 100 µg/ml proteinase K for 1 hour at 37°C and heated up to 95°C. The DNA was purified using the Qiaquick PCR purification kit (QIAGEN, Cat. No. 28704). Analysis of immunoprecipitated DNA was performed by PCR amplification or by qPCR using a Fast SYBR Green PCR kit (Applied Biosystems, Cat. No. 4385612). Amplification was performed using an ABI system (ABI StepOne Plus Real Time PCR System, Applied Biosystems). The following primers were used: *Wnt3*: 5'-GATCCCAGTCGCGCGATC-3' and 5'-GAGCCAGGTTTAGGGAGCTG-3'. *Brachyury*: 5'-CTTTGATGGAGGTGCAAACA-3' and 5'-GCGGCCATATCAGACTGG-3' and IgH 5'-GCCGATCAGAACCAGAACACC-3' and 5'-TGGTGGGGCTGGACAGAGTGTTTC-3'.

Chromatin immunoprecipitation in mouse embryos: For embryo chromatin immunoprecipitation (ChIP), 140 E6.5 embryos were dissected from fifteen litters of wild-type CD1 females. The embryos were dissected in low glucose DMEM (Gibco Cat. No. 31600-034) containing 10% fetal bovine serum and penicillin (100 U/ml), streptomycin (100 μg/ml) (Invitrogen, Cat. No.15140-122). After dissection, the embryos were cross-linked in 1% formaldehyde for 10 min. Crosslinking was stopped by adding glycine (125 mM final concentration) for 5min. The embryos were then washed twice in 1 ml ice-cold PBS containing protease inhibitors (aprotinin 1μg/ml, pepstatin 1μg/ml and 1mM PMSF) and pelleted for 30 sec using a microcentrifuge. After centrifugation, the excess supernatant was removed and the embryos were stored in liquid nitrogen. For ChIP assay, the embryos were resuspended in 350 μl of lysis buffer and processed according to the protocol above.

Genotyping: Mice were genotyped using a 2 mm piece of the tail tip. The tail tips were incubated overnight at 56˚C in 200 μl of PBND buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.3; 25 mM MgCl2; 0.1 mg/ml bovine gelatin; 0.45% IGEPAL; 0.45% Tween 20) containing 100 μg of proteinase K. The next day, proteinase K was heat inactivated at 95˚C for 5 min. One microliter of the lysate was used for PCR reactions. PCR reactions were carried out using GoTaq Flexi DNA polymerase (Promega Cat. No. M829B). Embryos were genotyped retrospectively after whole-mount *in situ* hybridization and removal of the

ectoplacental cone or after isolation of germ layers. To isolate the epiblast we severed the embryos at the extra-embryonic ectoderm/ectoplacental cone and epiblast/extra-embryonic ectoderm junctions. The epiblast was separated from the visceral endoderm layer enzymatically as previously described (Nagy et al. 2003). The following primers were used for genotyping: *Wnt3* floxed and wild type alleles: 5'-TGGCTTCAGCATCTGTTACCTTC-3' and 5'-AAGATC CCCATACTGCCATCAC-3'; *Cre*: 5'-TCCAATTTACTGACCGTACACCAA-3' and 5'-CCTGATCCTGGCAATTTCGGCTA-3'); *lacZ*: 5'-TGGCGTTACCCAACTTAATCG-3' and 5'-ATGTGAGCGAGTAACAACCCG-3'.

CHAPTER IV:

General Discussion and Considerations.

Developmental biology during the twentieth century was marked by the pioneering idea of a center that organizes the development of the embryo by controlling tissue specification and allocation of cells within the body. The experiments carried out by Hans Spemann and Hilde Mangold in 1924 demonstrating that a region of the amphibian embryo, named the dorsal lip of the blastopore, is able to direct and organize development, not only by instructing surrounding cells to differentiate into different tissues, but also by organizing their allocation and orientation regarding their final positioning in the embryos, are often regarded as one of the major landmarks in the history of developmental biology (Harland and Gerhart 1997; Gerhart 1999; De Robertis 1999). The publication of Spemann and Mangold in 1924 was followed by a number of studies that led to a better understanding of the development of the embryo, culminating with a Nobel Prize in 1935 (Harland and Gerhart 1997; Gerhart 1999).

Later, in the 1960's and 70's, Pieter Nieuwkoop, also working with amphibians embryos, showed that Spemann organizer activity could be restored by recombination of animal and vegetal hemispheres, and that neither alone could give rise to mesoderm. Perhaps more importantly he demonstrated that the formation of the Spemann organizer depended upon a small group of cells located in the dorsovegetal area of the blastula today known as the Nieuwkoop center in honor of its discoverer (Gerhart 1997, 1999). These early studies were carried out with a very specific goal: to understand the basis of differentiation and

cell fate decision required for the formation of complex organisms. Amphibians were chosen as a model of study due to developmental characteristics that allowed easy scientific manipulation such as large and numerous eggs and embryos, and development of a whole organism that occurs in a short time period of time.

The vertebrate embryo undergoes gastrulation in order to reshape its body and make mesoderm and endoderm from epiblast cells. The three germ layers observed during gastrulation are used as a source raw of material for production of every tissue in the adult body (Arnold and Robertson 2009; Harland and Gerhart 1997). Thus, it is clear that the process of gastrulation should be looked at in detail in order to understand the development of complex organisms from a single fertilized cell.

Early studies in amphibian embryos have paved the way for the generation of the three signals model that has been used to describe the organizer and mesoderm formation (De Robertis and Kuroda 2004; Vonica and Gumbiner 2007). In amphibians, the identity of this mesoderm inducer remained elusive for 81 years. It had been recently identified as a Wnt11 in *X. leavis* (Gerhart 1999; Tao et al. 2005). Similar studies have also put the Wnt signaling pathway at the core of gastrulation and organizer formation in other organism as well, such as the cnidarian *Hydra,* and the zebrafish (Lu, Thisse, and Thisse 2011; Nakamura et al. 2011).

This scenario is not different in mouse embryos in which a clear dependence on Wnt signaling for axial specification and gastrulation has been shown over the years (Liu et al. 1999; van Amerongen and Berns 2006).

But, how is the A-P axis specified in mice? To answer this question many groups have searched for the first evidence of asymmetric gene expression that could influence acquisition of a posterior versus anterior identity in mouse embryos (Arnold and Robertson 2009; Kimelman 2006; Beddington and Robertson 1999).

It has been hypothesized Bmp4, a TGF-β super family member, would be the agent that breaks the symmetry of the post-implantation embryo, starts gastrulation and establishes the anterior-posterior axis (Ben-Haim et al. 2006; Beddington and Robertson 1999). This observation was somewhat counter intuitive since some Bmp4 null embryos gastrulate and no ectopic expression experiments showed Bmp4 induction of a primitive streak (Winnier et al. 1995).

We rejected this view that states that Bmp4 molecules emanating from the extra-embryonic ectoderm dictates the time of gastrulation in the epiblast (Ben-Haim et al. 2006), and turned our attention to the *Wnt3* locus in that tissue. The reason was that the Bmp4 model often failed to fit experimental evidence, for instance the presence of gastrulation hallmarks (primitive streak, mesoderm) in *Bmp4* null embryo (Winnier et al. 1995), when the model would predict otherwise. However, the presence of morphological defects in *Bmp4* nulls embryos right around when gastrulation takes place suggests that the expression of this gene

plays an important role during this period of development but by no means demonstrates that Bmp4 is required for initiation of gastrulation.

An alternative hypothesis for how gastrulation starts in mouse embryos has emerged from two different sets of data. First, a paper was published in the late 1990's showing that posterior visceral endoderm, but not extra-embryonic ectoderm explants, were able to re-specify neuro-ectoderm to a mesodermal fate (Belaoussoff, Farrington, and Baron 1998). Second, a study showed that, in mouse embryos *Wnt3* is expressed in a sequential manner, first in the posterior visceral endoderm and later in the epiblast (Rivera-Perez and Magnuson 2005).

We hypothesized that, *Wnt3* that is first expressed in the visceral endoderm gets secreted towards the epiblast and activates its own locus and other primitive streak markers in that tissue, and in doing so, *Wnt3* starts gastrulation on the posterior side of the embryo. We tested this hypothesis by genetically inactivating the *Wnt3* locus in the visceral endoderm.

The rationale for it was that if the expression of *Wnt3* in the visceral endoderm is required for its own expression in the epiblast and gastrulation then the lack of *Wnt3* specifically in the visceral endoderm should phenocopy the Wnt3 null embryos phenotype.

We found that mouse embryos lacking *Wnt3* expression specifically in the visceral endoderm do not form a primitive streak. They also fail to show expression of several streak markers. Interestingly, the expression of *Bmp4* is maintained in visceral endoderm *Wnt3* null embryos. This fact goes against the

hypothesis that Bmp4 derived from the extra-embryonic ectoderm controls *Wnt3* expression in the epiblast, and therefore gastrulation (Ben-Haim et al. 2006). Our data provide support to the idea that the posterior visceral endoderm plays a significant role for mesoderm specification in mice as previously suggested (Belaoussoff, Farrington, and Baron 1998; Beddington and Robertson 1999).

Furthermore, we took advantage of a null allele of *Wnt3* (*Wnt3*^{lacZ} allele) that behaves as a reporter for the activation of the *Wnt3* locus, to show that although the *Wnt3* locus in the visceral endoderm remained responsive to upstream signals, it was unable to propagate the signal to the epiblast. These data indicate that the block indeed happened at the visceral endoderm level and that the phenotype was not due to non-specific effects caused by widespread *cre* activity as observed by others (Naiche and Papaioannou 2007).

In addition, if Bmp4 emanating from the extraembryonic ectoderm was indeed responsible for the activation the *Wnt3* locus in the epiblast, one should expect to see *lacZ* expression in the epiblast of the visceral endoderm-specific *Wnt3* mutant embryos, since, once again, in our breeding strategy, the mutant embryos carry a copy of the *Wnt3*^{lacZ} allele. However, we were unable to observe *lacZ* expression in the epiblast of visceral endoderm-specific *Wnt3* mutant embryos, indicating that Bmp4 was dispensable for initiation of gastrulation and that an initial Wnt signaling event in the visceral endoderm is absolutely required for expression of *Wnt3* in the epiblast.

Yet, Bmp signaling appears to be quite important for proper development of the embryos. For instance, embryos lacking *Bmp4* expression initiate gastrulation, but they all stall the development at some point at around E8.5-9.5 and die presenting a variable phenotype, with some even developing a heart (Winnier et al. 1995). The variation in the phenotype observed in the Bmp4 null embryos could perhaps be an indicative of a partial rescue by another Bmp family member expressed in the extraembryonic ectoderm or due to signaling received from the mother (Winnier et al. 1995). This scenario made difficult to assess the importance of Bmp4 for the initiation of gastrulation in mice. Therefore a different approach was taken. Bmpr1/Alk3 receptor is a type I TGF-β receptor utilized for propagate Bmp signaling inside the cells (Zhao 2003). When Bmpr1/Alk3 receptor was ablated, the embryos lacked mesoderm and any derivative (Mishina et al. 1995), and this phenotype was 100% penetrant with no variation in the expressivity, indicating that TGF-β signaling is necessary for proper embryonic development in mice. Again, because this receptor is also involved in signaling events other than Bmp signaling itself that raises the possibility that some other signaling pathway could have been affected in Bmpr1/Alk3 receptor knockout mouse.

Nodal is a TGF-β family member that also signals through Bmpr1/Alk3 receptor (Shen 2007). And some of the developmental defects in Bmpr1/Alk3 receptor knockout mouse are very symptomatic of Nodal signaling, such as: smaller epiblast size at E6.5, reduced cell proliferation in the epiblast, AVE fails

to shift position to the anterior side (Shen 2007; Whitman 2001; Conlon, Barth, and Robertson 1991). Thus, it is hard to draw any conclusion regarding Bmp signaling looking at the Bmpr1/Alk3 receptor knockout phenotype. Perhaps in the future one will generated a triple knockout mouse embryo lacking expression of *Bmp4, Bmp2* and *Bmp8a* specifically in the extraembryonic ectoderm to examine the requirement of Bmp signaling for initiation of gastrulation. One could also genetically engineer Bmpr1/Alk3 receptor that is responsive to Nodal signaling but not to Bmp signaling to address the possibility raised above.

When I was conducting the promoter analysis of the *Wnt3* locus I found that putative smad-binding sites on it. Smads are the downstream effector of Bmp/Nodal signaling. Therefore, these results brought up the possibility that Bmp signaling could to some extend regulate the activation of the *Wnt3* locus in the visceral endoderm. Once again, in that scenario, the activation of the *Wnt3* locus in the visceral endoderm would depend on Bmp signaling regardless the Bmp ligand available. What could to some extend explain the phenotypic variability observed in the *Bmp4* nulls, since other Bmp ligands are also expressed in the extra-embryonic ectoderm at the same time (Winnier et al. 1995).

For the future, one could revisit the data of the promoter analysis of the *Wnt3* locus and perhaps pinpoint pathways that could be involved on the activation of the *Wnt3* locus in the visceral endoderm. At this moment we strongly believe that if Bmp signaling is involved with initiation of gastrulation it needs to go through activation of the Wnt3 locus in the visceral endoderm first.

In other model organisms such as *X. laevis* and zebrafish, the specification of the anterior-posterior axis and gastrulation are events comprising large cytoskeleton rearrangements that asymmetrically concentrate Wnt RNAs on one side of the embryo (Tao et al. 2005; Lu, Thisse, and Thisse 2011). Perhaps, it would be possible that a molecule that regulates cytoskeleton rearrangements to be involved in local activation of the *Wnt3* locus in the visceral endoderm initiating gastrulation in mouse embryos, however this still need to be tested.

In vitro studies have suggested that Wnt3 activates the canonical Wnt pathway in order to carry out its functions inside the cells (Bhat et al. 2010; Lako et al. 2001). We tested this possibility *in vivo* by generating $Wnt3^{\Delta 3,4}$ mutant embryos caring a copy of the BAT-Gal reporter transgene (reporter for the activation of the canonical Wnt pathway). These data confirm that *Wnt3* turns on the canonical Wnt pathway in the epiblast of mouse embryos and utilizes it to set in motion the process of gastrulation.

Another interesting observation obtained from visceral endoderm-specific *Wnt3* null embryos was the lack of primordial germ cells. This phenomenon recapitulates the absence of primordial germ cells observed in *Wnt3* null embryos (Ohinata et al. 2009). Whether this is because of Wnt3 signaling is required for primordial germ cell formation or because the presence of a mesodermal niche is required to promote/nourish the presence of primordial germ cells remains an open question. Nonetheless, the data indicates an

involvement of a Wnt3-dependent inductive event for the formation of primordial germ cells.

As stated before, *Wnt3* is sequentially expressed in two tissues of the early post-implantation mouse embryo, the posterior visceral endoderm at E5.5 and 6 hours later in the epiblast (Rivera-Perez and Magnuson 2005). In the second chapter we studied the requirement of the expression of *Wnt3* in the posterior visceral endoderm. However, a few questions arose from those studies. First, what is the function of *Wnt3* expression in the epiblast? Is *Wnt3* expression in the epiblast required for gastrulation? How does Wnt3 emanating from the posterior visceral endoderm control its own expression in the epiblast?

To answer these questions we utilized a variety of genetically engineered mouse lines and several molecular approaches. Our data indicate that the expression of *Wnt3* in the epiblast is required for maintenance of gastrulation but not for its initialization. In epiblast-specific *Wnt3* null embryos dissected at E6.5, primitive streak markers were absent or weakly expressed. However, a day later, at E7.5 epiblast-specific *Wnt3* mutant embryos have clear expression of primitive streak markers. This delayed expression of primitive streak markers in epiblast-specific *Wnt3* null embryos, may have lead others to a mistaken interpretation of the importance of *Wnt3* expression in the visceral endoderm for gastrulation in mouse embryos.

A previous publication has suggested that *Wnt3* expression in the visceral endoderm is dispensable for gastrulation, and embryos lacking *Wnt3* expression

in the epiblast but retaining its expression in the visceral endoderm phenocopy *Wnt3* null embryos (Barrow et al. 2007). We cannot reconcile these two different sets of observations. We think that in the case of the epiblast-specific ablation of *Wnt3,* the different conclusions arises from differences in the time points chosen for embryo analysis. Our results and those of Barrow and co-workers agree that the expression of gastrulation markers can be absent in embryos dissected at around E6.5. However, Barrow and co-workers failed to extend their analysis of mutant embryos to later time points in which gastrulation hallmarks are clearly evident Thus, we think the difference in the dissection time or embryonic stage of the analyzed embryos played a pivotal role in the diametrically different conclusions of both studies.

Regarding the different outcomes we think that distinct experimental approaches may have led to different outcomes. In our studies, we utilized a visceral endoderm-specific *cre* driver, the *Ttr-cre* (Kwon, Viotti, and Hadjantonakis 2008), to ablate the *Wnt3* locus specifically in the visceral endoderm. Barrow and co-workers relied on the ability of ES cells to contribute to embryonic tissues only in a chimeric situation. Such concept has been questioned in the literature since ES cells can sporadically contribute for extraembryonic tissues (Eakin et al. 2005).

Additionally, the identification of mutant embryos at later stages (E9.5) carried out by Barrow and co-workers was based on the isolation of the visceral endoderm-derived or embryo-derived cell layer of the visceral yolk sac for

genotyping (Barrow et al. 2007). We think that such approach is daunting and error prone since it relies on the ability of the investigator to separate the two cell layers of the visceral yolk sac without leaving traces of ES cell-derived tissues.

Wnt3 has been classified as a canonical Wnt due its ability promote nuclear accumulation of β-catenin and activation of the canonical Wnt pathway (Bhat et al. 2010; Shimizu et al. 1997). In chapter two of this dissertation, we have shown the ability of *Wnt3* to activate the canonical Wnt pathway *in vivo*. However, it was not clear whether this activation directly result in expression of *Wnt3* itself and of primitive streak markers, or whether it results from an indirect, unknown mechanism. To investigate these possibilities we generated Wnt3-conditined medium and treated MEF cells with it. MEF cells treated with Wnt3-conditioned medium showed increasing levels of β-catenin accumulation in the nucleus, and augmented expression of primitive streak markers. Interestingly, the largest increase was detected when *Wnt3* mRNA was quantified, suggesting the *Wnt3* locus is one of the main targets of Wnt3-mediated activation of the canonical pathway.

However, there was still the possibility that the expression of primitive streak markers in MEF cells was due to something other than the presence of Wnt3 in the medium or the ability of Wnt3 to activate the canonical Wnt pathway. We addressed this possibility by showing β-catenin occupancy of the Wnt3 promoter in E6.5 embryos. We believe that this was the first time this experiment has been done. We were able to replicate the *in vitro* results *in vivo*. Some well-

known primitive streak markers are also downstream targets of the canonical Wnt pathway, such as Brachyury and Axin2. They contain in their promoter region *cis*-regulatory elements called TCF-binding sites (Jho et al. 2002; Yamaguchi et al. 1999). We were able to find β-catenin at the promoter region of these genes in both *in vitro* and *in vivo* situations. The remaining question was how can Wnt3 from the posterior visceral endoderm turn on its own locus in the epiblast? One possibility is through a direct mechanism as demonstrated for primitive streak markers such as *Gpr177, Axin2,* and *Barchyury* (Fu et al. 2009; Jho et al. 2002; Tetsu and McCormick 1999; Yamaguchi et al. 1999). Therefore, we conducted an analysis of the murine *Wnt3* promoter *in silico* to investigate whether the response to Wnt3-mediated activation of the canonical Wnt pathway was due to the presence of an autoregulatory loop. We confirmed that was the case by performing chromatin immunoprecipitation on E6.5 mouse embryos, in which Wnt3 is highly expressed and the expression of genes involved with primitive streak formation has been shown to be upregulated as well (Beddington and Robertson 1999; Jho et al. 2002; Yamaguchi et al. 1999; Rivera-Perez and Magnuson 2005).

The presence of five functional TCF-binding sites as well as the ability of β-catenin to occupy the TCF-binding site-rich region indicated the presence of an ancient autoregulatory loop that is at work during murine gastrulation. It seems that the activation of the canonical Wnt pathway via Wnt11 in amphibians (Tao et al. 2005), Wnt8a in zebrafish (Lu, Thisse, and Thisse 2011) and Wnt3 in mice

(Liu et al. 1999) is at the core of axial specification and gastrulation in vertebrates.

The Wnt signaling pathway is as conserved across metazoans as is the formation of a signaling center that coordinates axial specification and gastrulation (Holstein 2012; Lee et al. 2006; Joubin and Stern 2001). A recent publication has emphasized the importance of Wnt signaling and in particular *Wnt3* requirement for axial specification in the cnidarian fresh water polyp *Hydra* (*Hydra magnipapillata*). In this report, *Wnt3* is shown to be absolutely required for organizer formation and formation of new individuals in *Hydra.* Again the activation of the canonical Wnt pathway is pivotal in this process (Nakamura et al. 2011). This data together with the well-established role of the Wnt pathway in other organisms (Beetschen 2001; Cadigan and Nusse 1997; Chien, Conrad, and Moon 2009; De Robertis 2010; Guger and Gumbiner 1995), once again indicates that the canonical Wnt pathway is the signaling pathway of choice for axial specification in metazoans.

In mice, the organizer activity of the canonical Wnt pathway appears to be orchestrated by *Wnt3*. Other Wnt ligands are expressed around the same time as *Wnt3*, such as *Wnt5a, Wnt5b* and *Wnt2b* (Kemp et al. 2005). However, ablation of these Wnt ligands do not lead to a gastrulation phenotype (van Amerongen and Berns 2006), nor they can rescue for the lack of *Wnt3* in the *Wnt3* null embryos (Liu et al. 1999).

The Nieuwkoop center in amphibians induces the formation of mesendoderm utilizing Wnt11 and the canonical Wnt signaling pathway (Tao et al. 2005). Our data shows that the posterior visceral endoderm induces the formation of the primitive streak using *Wnt3* to activate the canonical Wnt signaling pathway in a similar fashion of that of the amphibian Nieuwkoop center. Since the primitive streak is the source of mesendoderm in mice, then the posterior visceral endoderm is a strong candidate to be Nieuwkoop center equivalent of mice.

Perhaps, the most straightforward approach to confirm this possibility would be to place explants of posterior visceral endoderm on the animal cap of amphibians embryos and to observe whether the posterior visceral endoderm explants could change the fate of the targeted cells in a similar fashion of that of the Nieuwkoop center in the transplantation experiments reported by Nieuwkoop in the 1960's (Gerhart 1997, 1999). It would be interesting to also use posterior visceral endoderm explants from *Wnt3* null embryos in order to show that in the absence of *Wnt3*, there is not formation of ectopic axis. This strategy has the advantage of using the original animal model to prove the posterior visceral endoderm is the murine equivalent of the Nieuwkoop center, however, such experiments are laborious and there is no guarantee that an interspecific recombination experiment would work.

We thought that perhaps, it would be interesting to drive expression of *Wnt3* in an ectopic site of the mouse embryo in order to see whether the

expression of *Wnt3*, *per se*, is sufficient to drive formation of an ectopic axis as shown before for other canonical Wnt, *Wnt8c* (Popperl et al. 1997). Thus, we built a DNA construct in which the *CMV* promoter is positioned immediately upstream the *Wnt3* cDNA followed by a 2A peptide-GFP cDNA. In this construct, the expression of *Wnt3* and *GFP* are under control of the *CMV* promoter. The construct was microinjected in one of the two pro-nuclei of the mouse zygote. A total of 150 embryos were transferred into 7 surrogate mothers. However, only 51 embryos were recovered at E6.5. None was a transgenic embryo, as far as one could tell by GFP fluorescence. We then decided to PCR the embryos in order to assess whether the transgene was inserted in a silent site of chromatin. A few embryos (n=7) turned out to be positive for the *CMC-Wnt3-GFP* transgene by PCR, but they did not have any green florescence indicating that the transgene was indeed silent in those embryos. Based on the data, we think that the activation of the transgene in early stages of development could be lethal to the embryos since we did not recovered the expected number of transgenic embryos at E6.5. Perhaps a better strategy engineering the transgene could be helpful towards this end.

Another possibility would be to direct microinject the cDNA construct in specific sites of the early post-implantation mouse embryo and to culture the injected embryo outside the uterus, in an incubator, for 12 hours or so, before probing the embryo for ectopic expression of primitive streak markers, an indicative that ectopic expression of *Wnt3* is sufficient to initiate gastrulation.

Such design is currently being conducted in our laboratory. At this point we do not have an answer whether this strategy will work. But, based on a similar approach conducted with *Wnt8c* (Popperl et al. 1997) we believe that soon we will be able to assess whether Wnt3 is sufficient to drive formation of an ectopic axis.

We propose a two-compartment model for initiation of gastrulation in mouse embryos (Fig4.1). In this model, the posterior visceral endoderm (yellow) and the epiblast (red) are the sites of induction and the targets of Wnt3 (Fig4.1A). *Wnt3* expression in the visceral endoderm is necessary for activation of its own locus in the epiblast and initiation of gastrulation (Fig. 4B). The conditional inactivation of *Wnt3* in the visceral endoderm leads to gastrulation failure in a similar fashion to that of *Wnt3* knockout embryos (Fig. 4.1C). However, in embryos with conditional inactivation of *Wnt3* in the epiblast, but retaining *Wnt3* expression in the posterior visceral endoderm, gastrulation is initiated but the embryos present defects associated with the progression of gastrulation (Fig.4.1D), indicating that the sequential activation of the *Wnt3* locus in the posterior visceral endoderm and the epiblast are the molecular events that prelude the morphological initiation of gastrulation at E6.5.

Although primates have been used for research, and even ES cells have been derived from primate species (Thomson and Marshall 1998), it is still not known the mechanism that coordinates axial specification and gastrulation in primates. Therefore, it would be interesting to find which Wnt molecule, if any,

controls gastrulation in primates. A recent publication showed human fetuses, homozygous for a nonsense mutation in the *WNT3* locus, were grossly malformed, with defects in almost all internal organs, and lacking limbs (Niemann et al. 2004). Although, these reported fetuses were highly affected for the mutation in the *WNT3* locus, it is clear that gastrulation has taken place, and therefore, it appears that *WNT3* is not the Wnt ligand for human gastrulation and perhaps to non-human primates. A study to find the molecular basis of gastrulation in human fetus would meet ethical barriers. But, perhaps, studies being carried out in non-human primate model systems will help to answer this question.

The final differentiation state of a cell of is highly dependent of its position along the anterior-posterior axis (Arnold and Robertson 2009). Thus, the understanding of the initial steps that lead to the differentiation of a epiblast cell into any other cell type will have a major impact on whether or not we will be able to culture and differentiate ES cells that can be used for therapies such as: organs transplant, limb regeneration or any other application in the field of regenerative medicine. More studies aiming to understand how embryos orchestrate development in a progressive and controlled manner are necessary in order to move the ES cell research from bench to bedside.

Figure 4.1

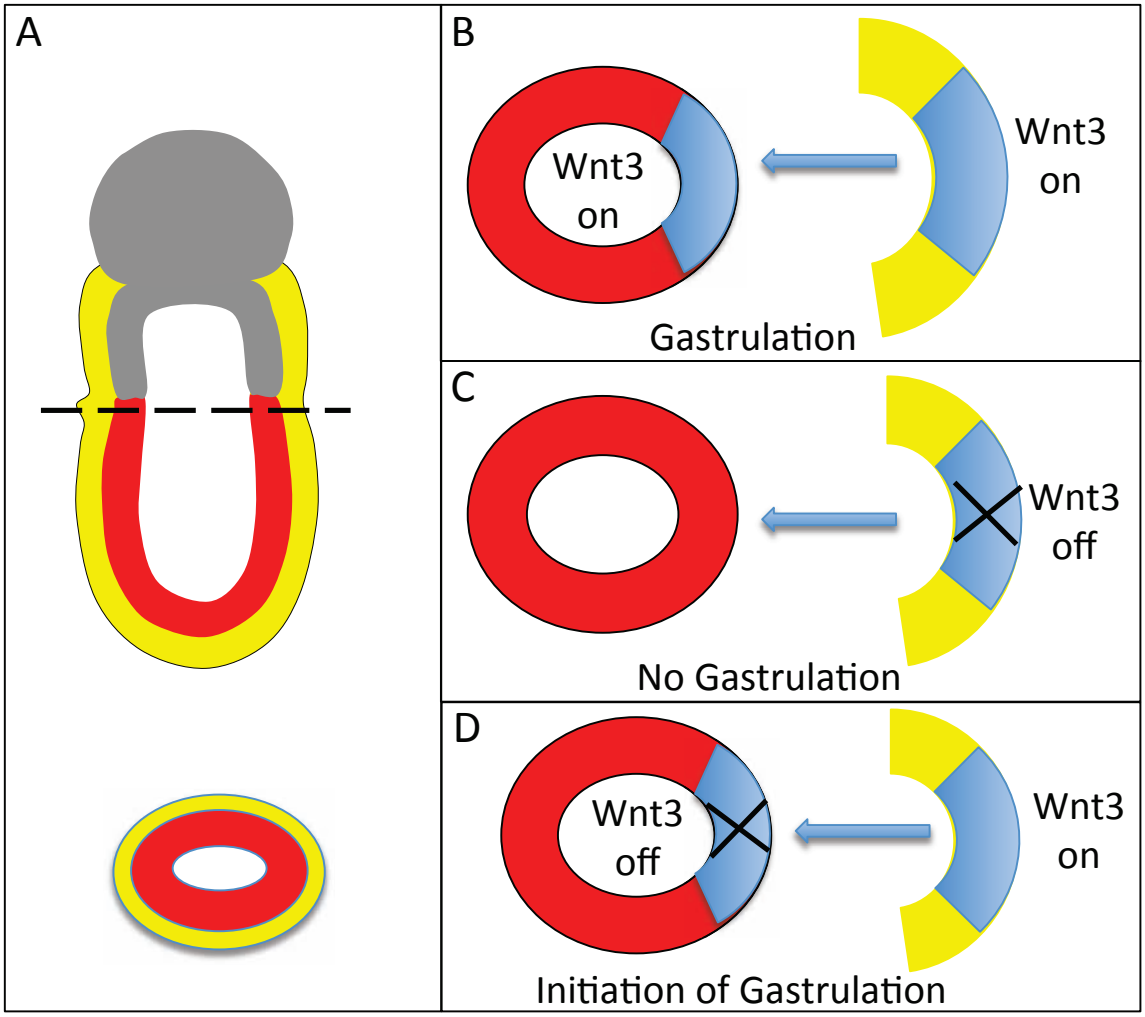
A
B
Wnt3
on
Wnt3
on
Gastrulation
C
Wnt3
off
No Gastrulation
D
Wnt3
off
Wnt3
on
Initiation of Gastrulation

Figure 4.1: PVE-Wnt3 model for initiation of gastrulation. A. E6.5 mouse embryo (upper) and transversal section obtained at the level of the dashed line shown below. The visceral endoderm (yellow) covers the extra-embryonic ectoderm (grey) and the epiblast (red). B. In wild type embryos, the *Wnt3* locus is sequentially activated in the posterior visceral endoderm and the epiblast. This sequential activation of the *Wnt3* locus initiates gastrulation in the epiblast. C. Conditional inactivation of the *Wnt3* locus in the posterior visceral endoderm leads to absence of gastrulation. D. When the *Wnt3* locus is conditionally inactivated in the epiblast gastrulation is initiated but the progression of it is impaired.

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