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GENETIC ANALYSIS OF LIGAND-RECEPTOR INTERACTIONS IN THE TGF-β SUPERFAMILY DURING EARLY EMBRYONIC DEVELOPMENT

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It is not birth, marriage, or death, but gastrulation,
which is truly the most important time in your life.

Wolpert, 1986

ABSTRACT

During early development, cells differentiate and acquire specific fates according to their position in concentration gradients of morphogens. These substances often spread from a local region, termed an organizer, and exert different effects at distinct thresholds. The transforming growth factor beta (TGF-β) superfamily comprises more than 30 factors that have previously been shown to induce specific cell-types and to act as morphogens. The work presented here identified critical roles for ligand-receptor interactions within the TGF-β superfamily during formation and functioning of organizing centers in the mouse embryo.

TGF-β ligands bind and signal through a heteromeric receptor complex consisting of type I and type II receptors. Although most of the TGF-β ligands were identified more than ten years ago, interactions with their cognate receptors remain largely unknown. The number of ligands exceeds the number of receptors, indicating the existence of redundancy among the ligands. Indeed, *in vitro* experiments performed during the course of these studies revealed that three ligands, Nodal, GDF1, and GDF3, could all signal through the type I receptors ALK4 and ALK7 in a complex with the type II receptors Acvr2 and Acvr2b. The most critical time during embryogenesis is prior to- and during gastrulation, which is a phase of development that is characterized by extensive cell migration and morphological change. Data presented here show that, in the pre-gastrulation embryo, GDF1 and GDF3 cooperate during formation of anterior visceral endoderm, a tissue that is responsible for initiation of the anterior-posterior axis and forebrain induction. At gastrulation, GDF1 was shown to synergize with Nodal during the specific allocation of mesendoderm precursors into prechordal plate and foregut endoderm, tissues that are necessary for maintenance of the anterior axis and forebrain identity. Despite the ability of these ligands to signal through both ALK4 and ALK7 *in vitro*, analysis of compound mutants indicated that ALK4, but not ALK7, was responsible for anterior axis development. Since these three ligands have overlapping expression patterns and signal through a common pathway, it was not surprising that functional redundancy occurred at multiple sites, as revealed by genetic interactions in mutant mice. Thus, Nodal, GDF1, and GDF3 form a robust signaling network of major importance during early embryonic development. Given the high degree of convergence on a limited set of receptors, this mode of action may be widespread among other members of the TGF-β superfamily.

Subsequent to gastrulation, during which the basic body plan is set up, elongation and regionalization of the embryo is coordinated by structures in the tail bud. GDF11 is a ligand that is expressed in the tail bud, and has been shown to affect these processes in a dose-dependent manner by signaling through Acvr2b, although the identity of the corresponding type I receptor was unknown. Therefore, several distinct biochemical assays were undertaken to examine ligand binding and signaling properties, and complemented with *in vivo* experiments to establish physiological relevance. Despite *in vitro* evidence showing that GDF11 can signal promiscuously, signaling specificity was revealed *in vivo* by a genetic interaction between *Alk5* and *Acvr2b*, which resulted in an enhanced homeotic transformation. Thus, functional specificity was only observed when taking a genetic approach to a mechanistic question.

Taken together, this thesis provides insight into the mechanisms by which TGF-β ligands cooperate and are functionally integrated during embryonic development.

LIST OF PUBLICATIONS

I. Reissmann E, Jornvall H, Blokzijl A, Andersson O, Chang C, Minchiotti G, Persico MG, Ibáñez CF, Brivanlou AH. (2001) The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. Genes & Dev. 1;15(15):2010-22.

II. Jornvall H, Reissmann E, Andersson O, Mehrkash M, Ibáñez CF. (2004) ALK7, a receptor for nodal, is dispensable for embryogenesis and left-right patterning in the mouse. Mol. Cell. Biol. 24(21):9383-9.

III. Andersson O, Reissmann E, Jornvall H, Ibáñez CF. (2006) Synergistic interaction between Gdf1 and Nodal during anterior axis development. Dev. Biol. 15;293(2):370-81.

IV. Andersson O, Reissmann E, Ibáñez CF. (2006) Growth differentiation factor 11 signals through the transforming growth factor-beta receptor ALK5 to regionalize the anterior-posterior axis. EMBO Rep. 7(8):831-7.

V. Andersson O, Ibáñez CF. (2006) Distinct and cooperative roles of mammalian Vg1 homologs GDF1 and GDF3 during embryonic patterning. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ActRIA	Activin receptor type IA (ALK2)
ActRIB	Activin receptor type IB (ALK4)
ActRII	Activin receptor type II (Acvr2)
ActRIIB	Activin receptor type IIB (Acvr2b)
Acvr2	Activin receptor type 2 (ActRII)
Acvr2b	Activin receptor type 2b (ActRIIB)
ALK	Activin receptor-like kinase
AMH	Anti-Müllerian hormone
AVE	Anterior visceral endoderm
BMP	Bone morphogenetic protein
BMPR1A	BMP receptor type 1A (ALK3)
BMPR1B	BMP receptor type 1B (ALK6)
BMPR2	BMP receptor type 2
BRE	BMP responsive element
Cerl	Cerberus-like
Cre	Cyclization recombinase
Dkk1	Dickkopf1
DVE	Distal visceral endoderm
EGF-CFC	Epidermal growth factor–Cripto/FRL-1/Cryptic
ES-cell	Embryonic stem cell
Fc	constant region of an immunoglobulin heavy chain
FGF	Fibroblast growth factor
FRL-1	FGF receptor ligand 1
GDF	Growth/differentiation factor
Gsc	Goosecoid
HA	Haemagglutinin
LoxP	Locus of crossover of P1 phage
Luc	Luciferase
MAD	Mothers against Decapentaplegic
PAGE	Polyacrylamide gel electrophoresis
Shh	Sonic hedgehog
Sma	Small phenotype
Smad	Sma/MAD
T	Brachyury
Tgfbr2	TGF-β receptor 2 (TGFβRII)
TGFβRI	TGF-β receptor type I (ALK5)
TGFβRII	TGF-β receptor type II (Tgfbr2)
TGF-β	Transforming growth factor β
Vg1	Vegetal-1
Xnr	*Xenopus* Nodal-related

1 INTRODUCTION

1.1 GETTING ORGANIZED – LESSONS FROM EXPERIMENTAL EMBRYOLOGY

Establishment of the body plan involves formation of the three germ layers, ectoderm, mesoderm, and endoderm, and patterning of the embryonic axes, anterior-posterior, dorsal-ventral, and left-right. In mammalian embryos, the basic body plan becomes apparent after about one third of the gestational period. Although different model systems, including invertebrates, share many features and signaling pathways, it should be noted that development of mammalian embryos spans over a much longer time frame. Therefore, patterning of the mouse, which essentially starts five days after fertilization, is less likely to be affected by maternal mRNA and protein than, for example, patterning of amphibian embryos. Nevertheless, it should be appreciated that much of the present knowledge about embryonic development of the mouse is based on experiments initially performed in amphibian or chick embryos.

During the last century, several key experiments were performed before the introduction of molecular biology and genetics. The pioneering work done by Spemann and Mangold included transplanting small pieces of tissues from one embryo to a different position in another embryo. These studies identified a region of the amphibian embryo that, after grafting, gave rise to a double axis, and was therefore referred to as an organizer (Spemann, 1924). It is of course intriguing that a small group of cells contains such a large amount of information. Spemann defined the organizer as something that "creates an organization field of a certain orientation and extent, in the indifferent material in which it is normally located or to which it is transplanted". Spemanns organizer corresponds to the node in developing mouse and chick embryos. Amphibian and chick embryos consist of robust cells that are useful for transplantation and explant experiments, contrary to the mouse, in which such experiments are more challenging. Thus, early studies were most often performed in chick or amphibian embryos whereas more recent genetic experiments most often utilized mouse, drosophila, and zebrafish model systems.

Although many developmental processes are conserved between species, the amphibian embryo differs from most other model systems in that it does not contain extra-embryonic tissues. By 1970 it was already appreciated that extra-embryonic tissues have inducing capacities during development of chick embryos (Eyal-Giladi, 1970). It was shown that the chick hypoblast could induce forebrain independently of the node. In the end of the last century these findings were also confirmed in the mouse embryo, first by transplantation and ablation studies, and later through genetic approaches (Thomas and Beddington, 1996). An extra-embryonic cell population present in the pre-gastrulation mouse embryo, called the anterior visceral endoderm (AVE), was shown to be responsible for the expression of forebrain markers in the epiblast. The mouse

node was shown to induce a second ectopic axis when grafted, although this ectopic axis lacked head structures (Beddington, 1994). Thus, the development of the mouse is in this respect more similar to chick than to amphibian development, since both the chick and the mouse require an early inducing capacity from the extra-embryonic tissues. It was then suggested that the AVE and node derivatives synergize during formation of anterior structures, in which the AVE induces forebrain fate, and the node derivatives maintain forebrain identity (Tam and Steiner, 1999). The node derivatives include mesendoderm (mesoderm and endoderm), which comprises precursors of notochord, prechordal plate, and foregut endoderm (Kinder et al., 2001). Both the prechordal plate and the foregut endoderm contribute to the patterning of the forebrain, whereas the notochord patterns the neural tube (Camus et al., 2000; Shawlot et al., 1999; Withington et al., 2001). It now becomes important to define induction and patterning as two different events, such that the AVE induces forebrain fate by directing the naïve cells into an anterior fate, and the node derivatives maintain and pattern the forebrain into different embryonic structures (Fig. 1). Thus, the murine AVE can promote forebrain specification but does not have all the features of a classical organizer since it requires the synergistic activities of the node derivatives for proper forebrain development and patterning.

As a continuation of gastrulation, cells from Spemanns organizer, as well as the node in chick and mouse, form the tail bud (Cambray and Wilson, 2002; Gont et al., 1993; Knezevic et al., 1998). The tail bud displays classical organizer activities since transplantation of tail bud tissues can induce the formation of ectopic tail-like structures (Gont et al., 1993; Knezevic et al., 1998). This signaling center also couples axis elongation with segmentation of the body plan. However, the molecular events that take place in the tail bud organizer are not nearly as well characterized as in the node.

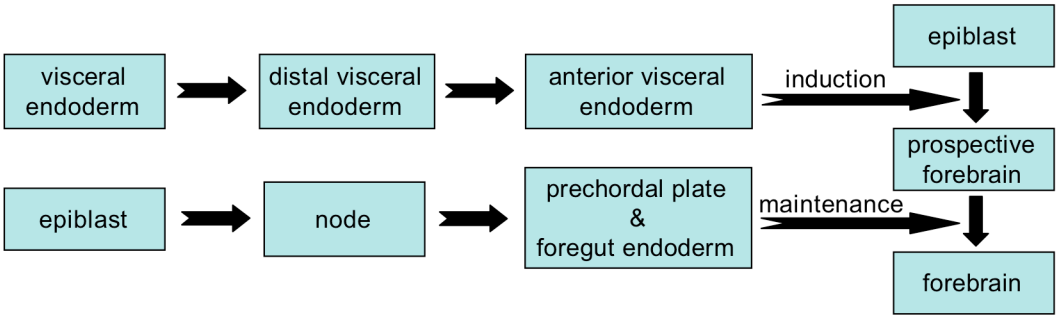

Fig. 1. Schematic illustration of the sequential formation of tissues that induce and maintain forebrain identity. The localization of each tissue and the molecular interactions that take place between tissues are depicted in Fig. 3 and Fig. 4.

All of these organizers are signaling centers where multiple pathways intersect. One signaling pathway that has been shown to affect several critical events during embryogenesis comprises the Transforming Growth Factor beta (TGF-β) superfamily of ligands and receptors. This thesis describes how TGF-β signaling affects the formation of organizing centers and the organizers' subsequent effects during induction and patterning of structures in the mouse embryo.

1.2 THE TGF-β SUPERFAMILY

1.2.1 Ligands

The TGF-β superfamily comprises a large family of evolutionarily conserved ligands with pleiotropic functions. They control cell differentiation, specification, proliferation, and apoptosis, both during embryonic development and in adult tissues. The TGF-β ligands are divided into subfamilies based on their activities and sequence identities, including TGF-βs, bone morphogenetic proteins (BMPs), Activins, Inhibins, growth/differentiation factors (GDFs), Leftys, Nodal, Müllerian inhibiting substance (MIS), and the distantly related glial cell-line derived neurotrophic factor (GDNF) subfamily. The fact that they affect an array of biological events is reflected in the way they were originally found. The first members of the family were identified through their effects on cells in culture. The founder molecule, TGF-β1, was identified after it had been shown to induce phenotypic transformation of cells in various cultures (Derynck et al., 1985). Activins and inhibins were isolated and cloned due to their effects on secretion of follicle-stimulating hormone (Ling et al., 1986; Mayo et al., 1986). Members of the BMP subfamily were originally derived from bone extracts, and identified for their ability to form cartilage and bone after subcutaneous injection (Wozney et al., 1988).

The second-generation ligands were cloned before their functions were known. A screen for maternal mRNAs that were localized in the *Xenopus* oocyte identified Vegetal-1 (Vg1), which was shown to be 10-20 times more abundant in the vegetal than in the animal pole of the unfertilized egg (Rebagliati et al., 1985). This mRNA was later identified as a TGF-β ligand, and proposed to be a mesoderm inducer (Weeks and Melton, 1987). This prompted other researchers to look for Vg1-related ligands that were expressed during mouse embryogenesis. Mouse embryonic cDNA libraries were screened by low stringency hybridization with a *Xenopus* Vg1 probe, resulting in the identification of "Vg-related" (Vgr) 1 and 2 (Jones et al., 1992; Lyons et al., 1989). Vgr-1 and Vgr-2 were concurrently cloned by others who named them BMP6 and GDF3, which are also their present names (Celeste et al., 1990; McPherron and Lee, 1993). GDF1 and GDF11 were also cloned from DNA libraries, whereas Nodal was identified in a mutant mouse line carrying a retroviral insertion (Gamer et al., 2001; Lee, 1990; Nakashima et al., 1999; Zhou et al., 1993). There are obviously many ways to isolate ligands, and these efforts have lead to the identification of more than 30 members belonging to the TGF-β superfamily.

Despite the fact that TGF-β ligands generate very diverse effects, they share an overall conserved structure. Members of TGF-β superfamily are secreted as large pro-proteins consisting of a signal peptide, a pro- and a mature-domain. A phylogenetic comparison of the amino acid sequences of the TGF-β ligands is shown in Fig. 1A in paper V. The mature-domain of TGF-β ligands is around 110 amino acids long, and has a size of approximately 15kDa. However, these ligands form dimers, resulting in an active complex of double the size. The

common structural features originate from seven conserved cysteines within the mature domain. Six of these cysteines form three intra-molecular disulfide bonds, which together shape the three dimensional structure into a characteristic cysteine-knot (McDonald and Hendrickson, 1993). The fourth cysteine forms an inter-molecular disulfide bond, which holds the dimer together. However, this inter-molecular bond does not seem to be necessary since GDF3, GDF9, BMP15, Lefty1, and Lefty2 lack a cysteine at that position.

The TGF-β pro-proteins are cleaved by seven members of the pro-protein convertase family, which are enzymes predominantly localized in the Golgi membrane (Thomas, 2002). However, soluble forms of pro-protein convertases have been detected and can mediate extracellular cleavege (Beck et al., 2002). The most well studied pro-protein convertase, Furin, cleaves the pro-protein after a consensus sequence encoding -Arg-X-Lys/Arg-Arg$^{\downarrow}$-. However, Furin can also cleave -Arg-X-X-Arg$^{\downarrow}$-, albeit with less efficiency (Molloy et al., 1992). The features of the pro-protein convertases, i.e. enzymatic properties, localization, and substrate specificity, are critical for the spatial and temporal regulation of mature ligand activation. The C-terminal mature-domain constitutes the active ligand, whereas the N-terminal part forms a pro-domain that is thought to be essential for proper folding. Moreover, following cleavage, the pro-domain can non-covalently bind to the mature-domain and thus form a latent complex that stabilizes/inhibits the mature ligand, as was first shown for TGF-β1 (Miyazono et al., 1988; Wakefield et al., 1988). Stabilization of Nodal by such a complex has been shown to affect its signaling range in zebrafish embryos (Le Good et al., 2005). This may be very important for ligands that form a concentration gradient by which they can act as morphogens. The pro-domains of GDF8 and GDF11 have also been shown to inhibit their corresponding mature-domains (Ge et al., 2005; Zimmers et al., 2002). The latent complex is degraded by another cleavage of the pro-domain, which thereby releases the active mature ligand (Wolfman et al., 2003). Targeted deletion studies in mice have shown that the pro-protein convertases Furin and Spc4 are the most important convertases during embryonic development (Beck et al., 2002; Constam and Robertson, 2000a; Constam and Robertson, 2000b; Roebroek et al., 1998). It was suggested that Furin and Spc4 normally regulate cleavage of Nodal, such that failure of left-right and anterior-posterior patterning is observed in *Furin* and *Spc4* deficient mice. Thus, the activity of TGF-β ligands is tightly regulated by secretion, processing, and release from the latent ligand complex.

The activity of TGF-β ligands is also regulated by a large number of extracellular antagonists, including Follistatin, Chordin, Noggin, Cerberus-like (Cerl), and many more (Belo et al., 1997; Esch et al., 1987; Sasai et al., 1994; Smith and Harland, 1992). These proteins are multifunctional inhibitors that antagonize several different ligands. For example, Follistatin and its related proteins inhibit the activity of Activins, GDF8, GDF11 and some BMPs. Chordin and Noggin inhibit several BMPs, and Cerl inhibits Nodal, BMPs, and members of the Wnt-family. Moreover, some TGF-β ligands do not generate any downstream effect but rather inhibit other ligands of the family: Lefty1 and

Lefty2 block Nodal and GDF3, whereas BMP3 and GDF10 seem to inhibit Activin and BMP4 (Chen and Shen, 2004; Cheng et al., 2004; Gamer et al., 2005). Therefore, at any given time there is a whole plethora of ligands, inhibitors, and enzymatic activities that modulate the outcome of TGF-β signaling.

1.2.2 Receptors and signal transduction

The TGF-β receptors are classified into type I and type II receptors according to their different properties. All TGF-β ligands bind and signal through a heteromeric receptor complex consisting of both type I and type II receptors (Wrana et al., 1992). Both the type I and the type II receptors have an N-terminal extra-cellular ligand binding domain and an intracellular serine/threonine kinase domain. There are seven type I and five type II receptors in mammals, which contain divergent ligand binding domains but conserved kinase domains. The type I receptors were originally named Activin receptor-like kinase (ALK), although some investigators named the receptors according to the first ligand that was found to interact with the identified receptor. For example, ALK5 was shown to interact with TGF-β1 and is therefore also called TGF-β receptor type I (TGFβR1). However, since several ligands have now been found to interact with the same receptor, this nomenclature is no longer clear. Therefore, for simplicity, the type I receptors are called ALK1-7 in this thesis. The five type II receptors were also named according to their first identified ligands, such that the TGF-β type II receptor was called TGFβRII, the Activin type II receptors ActRII and ActRIIB, the BMP type II receptor BMPRII, and the Anti-Müllerian hormone type II receptor AMHRII. However, the new naming of genes introduced the following abbreviations, Tgfbr2, Acvr2, Acvr2b, Bmpr2, and Amhr2, which are used in this thesis.

Ligand specificity can be achieved through different combinations of type I and type II receptors, although most often one particular combination of type I and type II receptors can be used by several different ligands. It should also be taken into account that all receptors are not likely to be expressed in the same cell, which also limiting the number of possible receptor combinations. Even if a ligand can use several combinations of type I and type II receptors, it may preferentially use one combination and be a partial agonist for the other receptor combinations. For example, Activin A has been shown to have higher affinity for Acvr2b than for Acvr2, although it can use either of these type II receptors in combination with ALK4 (Attisano et al., 1992; Carcamo et al., 1994). Moreover, Activin B can use Acvr2 or Acvr2b in combination with either ALK4 or ALK7 for downstream signaling (Tsuchida et al., 2004). The ligand-receptor interaction map within the TGF-β superfamily is not entirely clear since most studies have not tested many different combinations of type I and type II receptors, while other ligands have not been examined at all. The experimental conditions may also affect the outcome of some assays, since a ligand may need a co-receptor for efficient signaling. For example, Nodal needs an Epidermal growth factor–Cripto/FRL-1/Cryptic (EGF-CFC) co-receptor, Cripto or Cryptic, for efficient signaling through the Acvr2/Acvr2b in combination with ALK4 or ALK7

Ligand	Type I	Type II	References
TGF-β1-3	ALK1/5	Tgfbr2	(Attisano et al., 1993; Goumans et al., 2002; Wrana et al., 1992)
Activin A	ALK4	Acvr2/Acvr2b	(Attisano et al., 1993; Attisano et al., 1992; Carcamo et al., 1994; Macias-Silva et al., 1998)
Nodal GDF1 GDF3	ALK4/7	Acvr2/Acvr2b	(Andersson et al., 2006b; Chen et al., 2006; Cheng et al., 2003; Reissmann et al., 2001; Sakuma et al., 2002; Yeo and Whitman, 2001)
BMP2 BMP4	ALK3/6	Bmpr2	(Koenig et al., 1994; Nishitoh et al., 1996)
BMP7	ALK2	Acvr2b	(Greenwald et al., 2003; Macias-Silva et al., 1998)
AMH	ALK2/3	Amhr2	(Belville et al., 2005; Clarke et al., 2001; Jamin et al., 2002; Mishina et al., 1996)
GDF8 GDF11	ALK4/5	Acvr2b	(Andersson et al., 2006a; Ho et al., 2006; Rebbapragada et al., 2003)

Table 1. Combinations of type I and type II receptors used by different ligands in the TGF-β superfamily. Note that some of the interactions also need a co-receptor for signaling, such that Nodal, GDF1, and GDF3 require Cripto or Cryptic, and signaling by TGF-β1 to 3 is facilitated by endoglin and betaglycan.

(Reissmann et al., 2001; Yeo and Whitman, 2001). Other examples of accessory receptors include betaglycan and endoglin, which present TGF-β1 to 3 to Tgfbr2 in combination with ALK5 or ALK1 (Bertolino et al., 2005; Cheifetz et al., 1992; Lopez-Casillas et al., 1991; Lopez-Casillas et al., 1993). It is also important to distinguish between affinity and downstream responses, since a ligand may bind without causing any intracellular effect. Some of the most well characterized ligand-receptor combinations are listed in table 1.

Activins and TGF-βs show high affinity for their type II receptors and can only bind their type I receptors when already bound to the type II receptor. Conversely, BMPs have in general a stronger affinity for their type I receptors than for their type II receptor (Shi and Massague, 2003; Wrana et al., 1994). The ligand-receptor complex consists of a dimeric ligand, two type I receptors, and two type II receptors. The type I and type II receptors can form preassembled homodimeric complexes in a ligand independent manner, although the presence of a ligand is necessary to bring the type I and type II receptors together (Derynck and Zhang, 2003). The type II receptors are constitutively active and phosphorylate a glycine/serine-rich region, called the GS-domain, present in the type I receptors. Phosphorylation of the GS-domain in ALK5 has been shown to shift its binding preference from the inhibitory protein FKBP12, to the downstream activator Smad2 (Huse et al., 2001). FKBP12 normally bind to the unphosphorylated type I receptor to inhibit leaky basal activity, which enables a sharp increase of phosphorylated Smads upon stimulation with a ligand. The signal has by this general mechanism been transmitted from the extra-cellular compartment into the cell.

The main intra-cellular mediators of TGF-β signaling comprise members of the Smad family. There are eight Smads in mammals, which can be classified into 3 sub-groups, five receptor-regulated Smads, two inhibitory Smads, and one common-partner Smad. All Smads have two conserved domains called MH1 and MH2, which are connected by a linker region. The receptor-regulated Smads are substrates for the type I receptors, which phosphorylate serine residues located in the C-terminus of the MH2 domain (Abdollah et al., 1997; Souchelnytskyi et al., 1997). When phosphorylated, and thereby activated, the receptor-regulated Smad binds Smad4 (the only common-partner Smad) and translocates to the nucleus to modulate transcription (Fig.2 A). Recently, an alternative common-partner protein was identified, transcriptional intermediary factor γ (TIF1γ), which can enable nuclear translocation of phosphorylated Smad2/3 (He et al., 2006). However, the general importance of this separate pathway remains to be defined. The inhibitory Smads include Smad6 and 7, which can interact with the type I receptor kinase without being phosphorylated, and thereby block activation of the receptor-regulated Smads by preventing them from associating with the receptor (Kavsak et al., 2000). Smad6 can also inhibit a subset of receptor-regulated Smads by preventing their association with Smad4 (Hata et al., 1998). Thus, Smads exert both positive and negative effects to modulate the impact of a given signal.

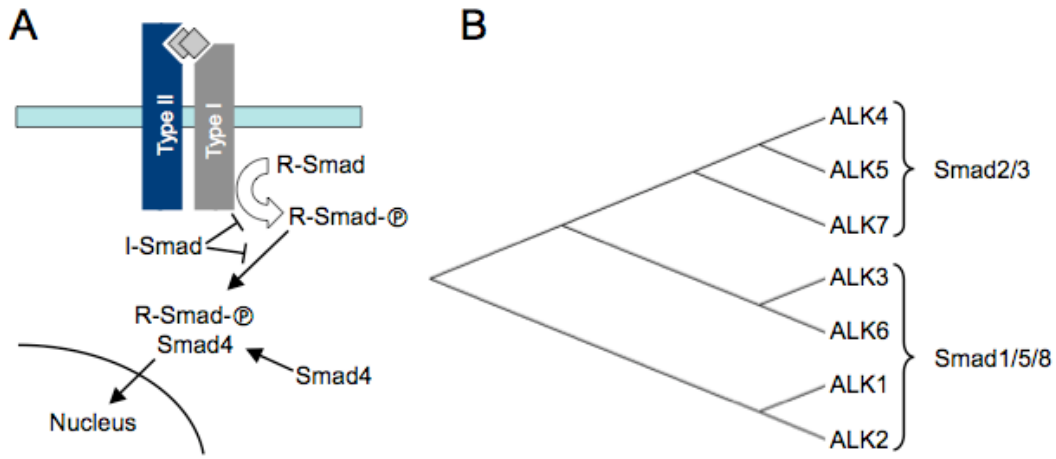

Fig. 2. (A) General signaling mechanism for ligands belonging to the TGF-β superfamily. Once the ligand has formed a complex with the type I and type II receptors, the type II receptor phosphorylates the GS-region of the type I receptor, which makes it active. Subsequently, the type I receptor phosphorylates receptor-regulated Smads (R-Smad), and thereafter the phosphorylated Smad (R-Smad-P) forms a complex with Smad4. This complex translocates to the nucleus, where it recruits transcription factors, co-activators, and co-repressors, which together affect transcription of target genes. There is also negative regulation of the pathway: inhibitory Smads (I-Smad) can either bind to the type I receptor and prevent the R-Smads from being phosphorylated, or block the association of R-Smads with Smad4. (B) Phylogenetic comparison of the type I receptors ALK1-7, using ClustalW. The analysis reveals that receptors activating a common set of R-Smads group together.

The type I receptors have distinct preferences for receptor-regulated Smads, such that ALK4/5/7 phosphorylate Smad2 and 3, and ALK1/2/3/6 phosphorylate Smad1, 5, and 8. A phylogenetic comparison of the type I receptors also groups them according to their Smad recognition preferences (Fig.2 B). Thus, Smads can be divided into "BMP-regulated Smads" and "TGF-β/Activin-regulated Smads". Ligands belonging to the GDF subfamily activate either of these two pathways, namely GDF1/3/8/9/11 activate TGF-β/Activin-regulated Smads, whereas GDF5/6/7 activate BMP-regulated Smads (Andersson et al., 2006a; Chen et al., 2006; Cheng et al., 2003; Mazerbourg et al., 2004; Mazerbourg et al., 2005; Nishitoh et al., 1996; Rebbapragada et al., 2003). Nevertheless, a given ligand is generally restricted to one of these two pathways through interactions with its receptors. However, there is one exception to the rule, since TGF-β1-3 can signal through either ALK1 or ALK5 and thereby phosphorylate both BMP- and TGF-β/Activin-regulated Smads (Goumans et al., 2002). TGF-β1-3 may thus perform a balancing act in cells that express both ALK1 and ALK5 (Goumans et al., 2003). The underlying cause of differential Smad-specificity among the type I receptors has been attributed to a region in the type I receptors, called the L45 loop, which interact with the L3 loop in the Smads (Chen et al., 1998; Chen and Massague, 1999; Persson et al., 1998). The type I receptors can be divided into three groups based on their amino acid sequence in the L45 loops, i.e. ALK1/2, ALK3/6, and ALK4/5/7, with ALK4/5/7 differing in their ability to bind Smad2 and 3. The crystal structure of ALK5 and Smad2 revealed that the Smads interact with both the GS-region and the L45 loop of the type I receptors (Wu et al., 2001). However, Smad specificity relies on the L45 loop, since the GS-region is similar in all type I receptors.

Once the phosphorylated Smads translocate into the nucleus they interact with a large number transcriptional components. The combination of binding partners depends very much on the cellular context, which may explain why TGF-β signaling can elicit such a rich variety of responses. The first transcription factor that was shown to interact with Smads was the winged-helix protein FoxH1 (previously called FAST-1), which was shown to form a complex with phosphorylated Smad2 on the Mix.2 promoter during development of *Xenopus* embryos (Chen et al., 1996). Thus, FoxH1 is dependent on ligands that phosphorylate Smad2/3 in order to exert its activities. FoxH1 does not only bind to the Mix.2 promoter but is likely to have many other target genes that together direct the formation of mesoderm and endoderm. Interestingly, FoxH1 was later shown to bind an enhancer regulating Nodal expression, which creates a positive feedback loop for this ligand (Norris and Robertson, 1999; Osada et al., 2000). Since this first example of a Smad binding partner, many other transcription factors, transcriptional co-activators/repressors, and adaptor proteins, have been shown to modulate the transcriptional program elicited by TGF-β signaling (Moustakas et al., 2001).

1.3 EMBRYONIC DEVELOPMENT

1.3.1 Pre-implantation

The entry of the sperm determines the first asymmetry of the fertilized egg and later the second polar body marks the asymmetry of the blastocyst. Cell labeling experiments imply that these early asymmetries are lost after implantation in the uterus due to cell mixing (Fujimori et al., 2003; Gardner and Cockroft, 1998). However, it is still under debate as to whether these pre-implantation asymmetries have any influence on later post-implantation development of the mouse embryo (Rossant and Tam, 2004). Nevertheless, one can conclude that pre-implantation patterning is not necessary for embryonic development since mice can be generated entirely from embryonic stem cells (Nagy et al., 1990). Whichever the case, this thesis only discuss post-implantation development of the mouse, although comparisons are made to amphibian and chick development, which do not involve implantation.

1.3.2 Post-implantation pre-gastrulation

The main patterning event that precedes gastrulation in the post-implantation embryo involves the formation of the AVE. At this stage, the epiblast consists of a uniform cup of embryonic ectodermal cells surrounded by overlying visceral endoderm. Distal visceral endoderm (DVE) is induced in the tip of the epiblast and subsequently migrates to the prospective anterior side of the epiblast to form the AVE (Fig. 3). Recent advances in molecular biology have identified an array of genes expressed in the AVE, node, and its derivatives. Some of these genes do not seem to be required for the function of these tissues but are still useful as cellspecific markers during development of certain cell-types and structures. Other genes have, by targeted deletion studies in mice, been shown to play important roles during embryogenesis. However, in most cases, a single gene is expressed in several structures and has multiple functions during development of the embryo. This may be advantageous for the embryo but makes life harder for the researcher, since it can be difficult to distinguish functions that take place in close proximity to each other. However, one can determine if a gene is necessary in either embryonic or extra-embryonic tissues by making chimeric embryos. In such experiments, ES cells are injected into blastocysts or aggregated with tetraploid cells, in which the ES will contribute to the embryo but will not colonize the extra-embryonic visceral endoderm or trophoblasts (Gardner, 1968; Tam and Rossant, 2003; Tarkowski et al., 1977). These techniques have been used to show that a number of genes, including *Alk4, Smad2, Smad4, Otx2, FoxA2 (HNF3-β), Cerl (Cerberus-like),* and *Lefty1*, are necessary in the extra-embryonic tissues during formation or function of the AVE (Dufort et al., 1998; Gu et al., 1998; Perea-Gomez et al., 2002; Rhinn et al., 1998; Sirard et al., 1998).

It has been suggested that Nodal signals from the epiblast to the extra-embryonic visceral endoderm by binding to ALK4, leading to the phosphorylation of Smad2 (Ang and Constam, 2004). The DVE is thereby induced and starts to

express Cerl, and Lefty1 (Belo et al., 1997; Oulad-Abdelghani et al., 1998), as well as other genes that are characteristic for this cell-type but are not necessary for its capability to induce the prospective forebrain, such as the transcription factor Hex, and the Wnt-antagonist Dickkopf-1 (Dkk1) (Pearce et al., 1999; Thomas et al., 1998). However, recent findings have shown that GDF1 and GDF3 contribute to induction and migration of the DVE, and thus to the formation of the AVE (Chen et al., 2006; paper V). It has been shown that the extra-embryonic ectoderm inhibits the formation of the AVE, which may explain why the DVE is induced at the tip of the epiblast (Rodriguez et al., 2005). This is also supported by the fact that the distance between the extra-embryonic ectoderm and the apex of the epiblast needs to have reached a length of 70μm to be capable of forming DVE (Mesnard et al., 2006). The AVE also expresses Wnt antagonists, such as Cerl and Dkk1, although there is no evidence for a direct role for Wnt signaling during forebrain induction (Glinka et al., 1998; Piccolo et al., 1999). Wnt signaling may instead, at a later stage, transform forebrain into more posterior identities (Nordstrom et al., 2002). The mechanism regulating migration of these cells from the distal to the anterior side of the epiblast is less clear, although it seems to involve Otx2, Wnt, and Nodal signaling (Kimura et al., 2000; Kimura-Yoshida et al., 2005; Yamamoto et al., 2001).

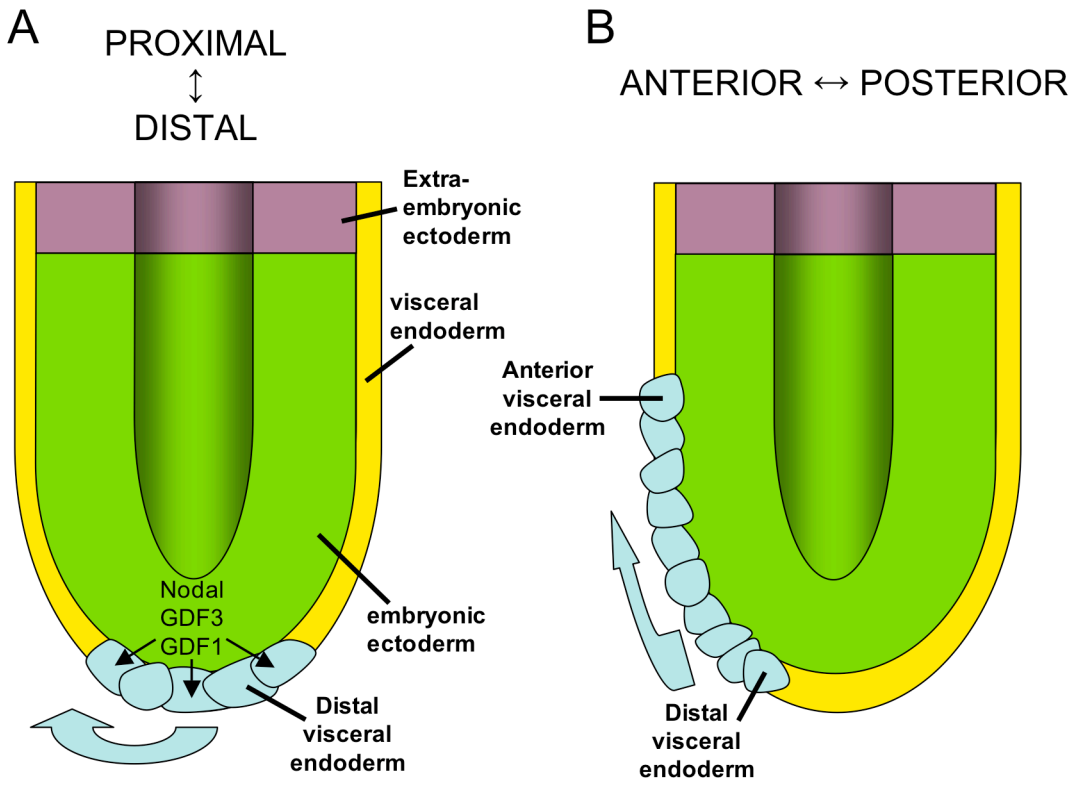

Fig. 3. (A) At E5.5, the epiblast consists of a uniform cup of embryonic ectodermal cells surrounded by overlying visceral endoderm. Nodal, GDF1, and GDF3 signal from the epiblast to the visceral endoderm, which induces the formation of distal visceral endoderm (DVE). (B) At E6, the distal visceral endoderm migrates to the anterior side of the epiblast, and is then termed anterior visceral endoderm (AVE). This rotation converts the proximal-distal axis into an anterior-posterior axis.

Once the AVE has formed it induces forebrain markers, such as Six3, Otx2, and Hesx1, in the embryonic ectoderm on the anterior side of the epiblast (Oliver et al., 1995; Simeone et al., 1992; Thomas and Beddington, 1996). Forebrain induction has been an intense area of research in attempts to explain the molecular nature of Spemanns organizer. In *Xenopus* it seems to involve Noggin and Chordin, which, by inhibiting BMP signaling, allow neuronal differentiation along the so-called "default pathway" (Vonica and Brivanlou, 2006). The theory behind this pathway favors a permissive rather than an instructive role for neuronal differentiation. However, instructive signals have been implicated in the induction of chick forebrain, including FGF in combination with Wnt- or BMP-antagonists (Albazerchi and Stern, 2006). This heated debate between chick and *Xenopus* developmental biologists can be studied in the following reviews (Bottcher and Niehrs, 2005; De Robertis, 2006; Stern, 2006). In mouse, Cerl and Lefty1 are secreted from the AVE and block BMP and Nodal signaling, which allows forebrain formation (Fig. 3A) (Chen and Shen, 2004; Cheng et al., 2003; Piccolo et al., 1999). This notion is also supported by targeted deletion of either *Alk3* or *Nodal*, which promotes premature and widespread neuronal differentiation in pre-gastrulation mouse embryos (Camus et al., 2006; Davis et al., 2004). Thus, although much remains to be resolved, forebrain induction seems to involve inhibition of BMP signaling.

1.3.3 Gastrulation

Gastrulation is a phase of development that is characterized by extensive cell migration and morphological change. This re-arrangement of the embryo results in the establishment of the three germ layers, from which the embryo is formed. Gastrulation starts with the formation of the primitive streak at the posterior end of the embryo, at about E6.5 (Fig. 4A). The primitive streak is formed in the posterior region of the proximal epiblast, but will move distally as the streak elongates. Epiblast cells undergo an epithelial-mesenchyme transition, and cell adhesion molecules are downregulated to enable motility. Cells from the epiblast detach and migrate through the primitive streak to different positions on the posterior side of the epiblast cup, which then form the mesodermal layer. The streak elongates until it has reached the apex of the epiblast, where the node will form. The cells in and around the node generate the axial mesendoderm, which then migrates anteriorly (Fig. 4B). The axial mesendoderm will later give rise to prechordal plate, notochord, and foregut endoderm.

The induction of the primitive streak is thought to depend on several signals derived from both embryonic and extra-embryonic structures. Nodal expression becomes restricted to the posterior side of the epiblast by the secretion of Cerl and Lefty1 from the AVE (Perea-Gomez et al., 2002; Waldrip et al., 1998). Wnt3 is first expressed in the posterior visceral endoderm from E5.5 and onwards, and later in the posterior side of the epiblast at E6.5 (Rivera-Perez and Magnuson, 2005). It is possible that Wnt3 expression is restricted to the posterior side in a similar manner to Nodal, i.e. by secretion of Wnt-antagonists from the AVE. The anterior primitive streak shows characteristic expression of

FoxA2 and Goosecoid (Gsc), two transcription factors that co-operate in the anterior part of the streak (Filosa et al., 1997). Embryos carrying a targeted deletion of *Foxa2* lack node and axial mesendoderm, and *Foxa2*$^{+/-}$;*Gsc*$^{-/-}$ compound mutants exhibit weaker defects (Ang and Rossant, 1994; Filosa et al., 1997; Weinstein et al., 1994). Moreover, all mesodermal cells in primitive streak and axial mesoderm express T (Brachyury), which is a transcription factor necessary for mesoderm formation, and is a widely used marker for mesoderm (Herrmann, 1991). *Nodal* mutant mice do not form a primitive streak, as shown by the absence of T and Gsc expression, which demonstrates the importance of Nodal during initiation of gastrulation (Conlon et al., 1994). *Wnt3* mutants do not form mesoderm but, contrary to *Nodal* mutants, do form AVE (Liu et al., 1999). The importance of Wnt signaling during onset of gastrulation has also been confirmed in double mutants of the co-receptors *Lrp5/6* (Kelly et al., 2004).

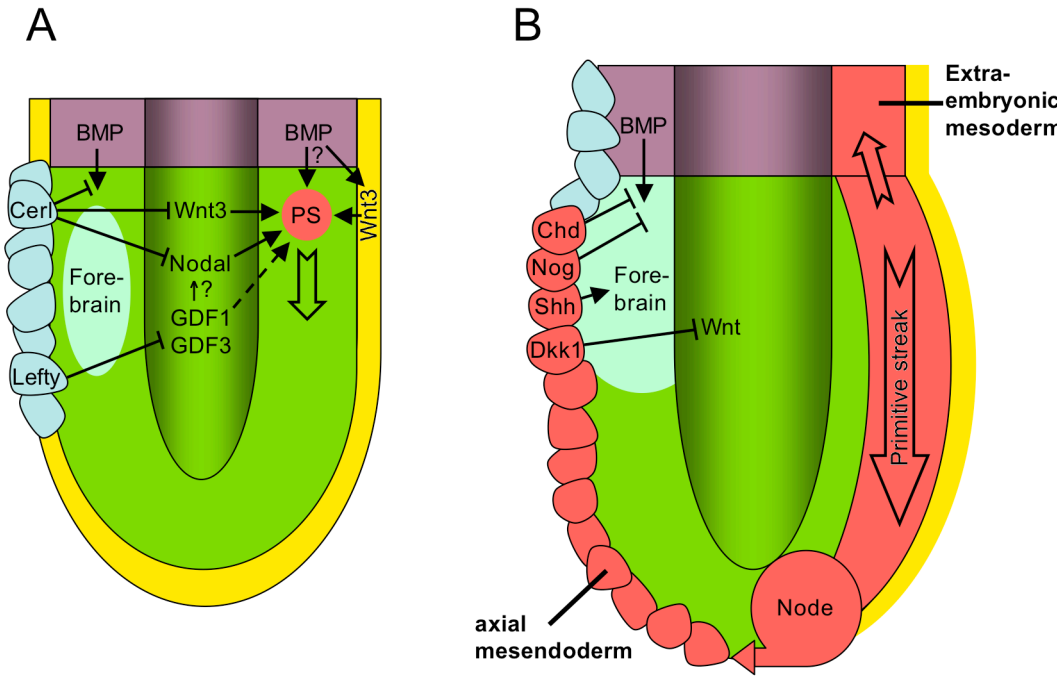

Fig. 4. (A) The prospective forebrain is induced by secretion of Lefty1 and Cerberus-like (Cerl) from the AVE on the anterior side of the epiblast. Lefty1 blocks Nodal, GDF3, and most likely also GDF1, whereas Cerl inhibits BMP, Wnt, and Nodal signaling. The induction of the primitive streak (PS) occurs at E6.5 on the posterior side of the epiblast. Nodal expression becomes restricted to the posterior side of the epiblast by the secretion of Cerl and Lefty1 from the AVE. Wnt3 is expressed in the posterior visceral endoderm and in the posterior side of the epiblast. GDF1 and GDF3 may act directly or upstream of Nodal during initiation of the streak. BMP signaling from the extra-embryonic ectoderm is also required, but the mechanism by which BMP affects mesoderm formation is unclear. The anterior primitive streak elongates in the direction of the open arrow. (B) At E7.5 of development, the elongation of the primitive streak has ended and the node is formed in the apex of the epiblast. Axial mesendoderm migrates from this location to the anterior side, and thereby displaces the visceral endoderm. Extra-embryonic mesoderm also starts to form at the posterior side. The axial mesendoderm secretes the BMP-inhibitors Chordin (Chd) and Noggin (Nog), Wnt-antagonist Dickkopf-1 (Dkk1), and Sonic hedgehog (Shh), to maintain forebrain identity.

Additional complexity is added by the Nodal-related factors GDF1 and GDF3. Approximately half of *Gdf3* mutants display absent or diminished expression of anterior primitive streak markers (Chen et al., 2006). However, the temporal expression of GDF3 is still unclear, since one study has shown that GDF3 expression ceases prior to the onset of gastrulation, and another that GDF3 is expressed in the node at E7.5 (Chen et al., 2006; Levine and Brivanlou, 2006). RNAse protection assays also failed to detect GDF3 expression at E7.5 (Jones et al., 1992). It is still possible that GDF3 protein persists at the onset of gastrulation, even in the absence of mRNA expression. In paper III of this thesis, *Gdf1* was shown to genetically interact with *Nodal* during formation of mesendoderm, but the primitive streak was consistently initiated in *Gdf1*$^{-/-}$*;Nodal*$^{+/-}$ compound mutants. However, in paper V of this thesis, *Gdf1* was shown to genetically interact with *Gdf3* during formation of mesoderm, although it remains to be determined if this effect is direct or due to a downregulation of Nodal expression. BMP4 is highly expressed in the extra-embryonic ectoderm at E6.5, and could therefore affect primitive streak initiation (Lawson et al., 1999). Indeed, mutant mice for *Bmp4* and its receptors, *Alk3* and *Bmpr2,* fail to form mesoderm (Beppu et al., 2000; Mishina et al., 1995; Winnier et al., 1995). However, aggregation of BMP4 mutant ES-cells with wild-type tetraploid cells rescues gastrulation defects, suggesting that BMP4 is necessary in the extra-embryonic region (Fujiwara et al., 2002). Perhaps BMP4 acts upstream of Wnt3 in the visceral endoderm or epiblast, where phosphorylated Smad1 has been detected (Davis et al., 2004; Gotoh et al., 2005). Taken together, initiation of the primitive streak is mainly dependent on Nodal and Wnt3 signaling, although other players may also participate.

As the primitive streak elongates, FGF signaling up-regulates Snail in epiblast cells, which then down-regulates the cell adhesion molecule E-cadherin, enabling epiblast cells to migrate through the streak and form mesoderm (Ciruna and Rossant, 2001; Deng et al., 1994; Sun et al., 1999; Yamaguchi et al., 1994). The elongation of the primitive streak has also been attributed to Nodal signaling. Targeted deletion of the transcription factor *Foxh1* has been very instrumental in the understanding of streak elongation. It was shown that Nodal can act in both FoxH1-dependent and FoxH1-independent pathways (Hoodless et al., 2001; Yamamoto et al., 2001). Mesoderm induction was *Foxh1*-independent, whereas streak elongation and formation of node and mesendoderm was *Foxh1*-dependent. Therefore, elongation of the primitive streak may depend on the ability of FoxH1 to propagate Nodal expression in the anterior portion of the streak. Forebrain markers, such as Otx2, were progressively down-regulated in *Foxh1* mutants, since mesendoderm precursors of the prechordal plate and foregut were not correctly specified. The prechordal plate has been shown to express several instructive and permissive cues, including the BMP inhibitors Chordin and Noggin, the Wnt antagonist Dkk1, and Shh. All of these proteins have, in mutant mice models, been shown to be necessary for stabilization of anterior identity (Bachiller et al., 2000; Chiang et al., 1996; Mukhopadhyay et al., 2001). Together with the formation of the AVE,

the morphological changes during gastrulation establish the anterior-posterior axis.

1.3.4 Regionalization of the anterior-posterior axis

Subsequent to gastrulation, during which the basic body plan is set up, the posterior body is formed. The elongation and segmentation of the embryo is coordinated from structures in the developing tail (Dubrulle and Pourquie, 2004a). The tail bud is formed from remnants of the regressing primitive streak and node (Tam and Trainor, 1994). It comprises a stem cell zone from which progenitors exit in a sequential manner. Cells that leave the stem cell zone early will form more anterior structures than cells that exit later during development, due to progressive axis elongation. The remains of the node form a structure, called the "chordoneural hinge", which will give rise to hindbrain, spinal cord, and notochord, whereas the regressing primitive streak forms the tail bud mesoderm, which gives rise to paraxial mesoderm (Cambray and Wilson, 2002). Paraxial mesoderm constitutes pre-somite mesoderm and somites, which visualize segmentation of the embryo. Somites give rise to the vertebral column, dermis, and skeletal muscle within the trunk. Therefore, the formation and patterning of the spinal cord and vertebral column along the anterior-posterior axis share many characteristics.

The tail bud expresses several ligands, including FGF8, Wnt3a, and GDF11, that together form a gradient of signaling from posterior to anterior (Aulehla et al., 2003; Dubrulle et al., 2001; McPherron et al., 1999; Nakashima et al., 1999). This gradient works against an opposing gradient of retinoic acid produced by somitic mesoderm (Niederreither et al., 1999). Retinoic acid synthesis is regulated by several retinal dehydrogenases, of which RalDH2 is expressed in the somitic region (Niederreither et al., 1999). Retinoic acid represses FGF8 expression in the tail bud, and, conversely, FGF8 down-regulates RalDH2 expression in the somitic region (Diez del Corral and Storey, 2004). FGF8 transcription is restricted to the stem cell zone where it maintains the progenitors through a Notch-dependent pathway (Akai et al., 2005). However, FGF8 transcription ceases as the progenitors exit the tail bud and enter newly formed tissues, which generates a gradient of mRNA from the posterior part of the embryo (Dubrulle and Pourquie, 2004b). Thus, the stability of FGF8 mRNA, and the translation of this mRNA, form a gradient of FGF8 protein. It is likely that a similar mechanism generates a gradient of GDF11 along the anterior-posterior axis since *in situ* hybridization of GDF11 mRNA seems to decay progressively from posterior to anterior (Gamer et al., 2001; Liu et al., 2001; McPherron et al., 1999; Nakashima et al., 1999; paper IV).

The anterior-posterior axis is subsequently divided into segments that acquire specific spatial fates. This segmentation process is governed by Hox genes, a structurally and functionally related class of homeodomain transcription factors (Dubrulle et al., 2001). The Hox gene family comprises 39 members, organized into four genomic clusters, that act in concert to regionalize the embryo along the anterior-posterior axis (Deschamps and van Nes, 2005; Dubrulle and

Pourquie, 2004a). The specific combination of Hox-genes is thought to generate particular fates. Abnormal expression of Hox genes can result in a shift of fate, although not always since there is substantial redundancy among Hox-gene paralogs expressed from different Hox-gene clusters. A shift of fate along the anterior-posterior axis is referred to as a homeotic transformation.

The formation of Hox-gene expression domains is determined by the opposing gradients of retinoic acid from the anterior end of the embryo, and FGF8, Wnt3a, and GDF11 from the posterior end (Deschamps and van Nes, 2005; Diez del Corral and Storey, 2004). Indeed, deletions of *Gdf11* and *Raldh2* in mice, and hypomophic mutants for *Wnt3a* and *Fgfr1* (encoding an FGF receptor), affect Hox-gene expression and display homeotic transformations (Ikeya and Takada, 2001; McPherron et al., 1999; Niederreither et al., 1999; Partanen et al., 1998). GDF11 has been shown to be necessary for normal Hoxc6, Hoxc8, Hoxc10, and Hoxc11 expression in mouse, and can ectopically induce Hoxc6, Hoxc8, Hoxc9, and Hoxc10 expression when over-expressed in chicken (Liu, 2006; McPherron et al., 1999). It has also been shown that GDF11 synergizes with FGF signaling during the induction of Hoxc10 in explants from chick spinal cord (Liu et al., 2001). Moreover, targeted deletion of *Follistatin*, which encodes an antagonist of GDF11, results in a homeotic transformation that are in the opposite direction of those seen in *Gdf11* mutant mice (Gamer et al., 2001; Matzuk et al., 1995b). Over-expression of Follistatin in chick spinal cord can also oppose the effects of GDF11, confirming the significance of GDF11 signaling during organization of the Hox-gene code (Liu, 2006). Therefore, it is possible that GDF11 fine-tunes the outcome of FGF signaling during the induction of specific Hox-genes. Thus, the coordination of regionalization and elongation of axial and paraxial structures in the developing embryo is regulated by an integration of several signaling pathways.

2 AIMS

The main objectives of this thesis were to study ligand-receptor interactions in the TGF-β superfamily and their roles during embryonic development. Specifically:

1. Identify ligands that can signal through the type I receptor ALK7, and investigate the functions of ALK7 during embryonic development.

2. Explore the functions of Nodal-related ligands during embryonic development.

3. Compare the functions of the closely related type I receptors ALK4, ALK5, and ALK7.

3 GENERAL METHODOLOGY

3.1 RESEARCH APPROACH

One of the aims of the present work was to identify specific ligand-receptor interactions in the TGF-β superfamily. Several of the main papers in this thesis were initiated in a similar way and therefore share technical characteristics. Before the onset of these thesis studies, in the first report describing the cloning and identification of rat ALK7, it was suggested that GDF1 could be a ligand to ALK7 due to their co-expression in the central nervous system (Ryden et al., 1996). There are many other leads and methods, in addition to co-expression, that can be used in the search for a specific ligand-receptor interaction. Previously, ligands have been found through activity-based assays, in which the active component was isolated during purification steps, and then identified through sequencing. One can also make use of the interaction between the ligand and the receptor and use the receptor as bait during affinity based identification. Alternatively, a list of candidate ligands may be gleaned from the literature, and then studied one by one. The present investigations made use of the latter methodology since, at the outset of these studies, there were many orphan ligands in the TGF-β superfamily. By scanning the literature and the amino acid structure of the ligands, a list of possible ligands for ALK4, ALK5, and ALK7, was compiled based on educated guesses. The initial list of candidate ligands included GDF1, GDF3, GDF10, GDF11, and, BMP3. During the course of these thesis studies GDF8, GDF15, Activin A, and Activin B were also analyzed for their receptor specificity. Of these, GDF8, GDF15, BMP3, Activin A, and Activin B were commercially available and were therefore studied by using the mature peptide ligand. The remaining ligands, namely GDF1, GDF3, GDF10, and GDF11, were not available as peptides and were therefore cloned into expression vectors for production in cell-lines. However, later during these studies GDF1, GDF3, and GDF11 became available as purified recombinant peptides, although no activity could be detected in commercial batches of GDF1 and GDF3 peptides. The methods employed differ slightly between the ligands tested as peptides versus those produced by expression vectors, although the general methodology can be outlined in five steps:

3.1.1 Acquisition of the ligand

When the ligand was not commercially available, the mature-domain was ligated into 2 different vector backbones carrying a pro-domain of either *Xenopus* Activin B or *Xenopus* BMP2, with or without fusing the N-terminal part of the mature ligand to a HA-tag (Piccolo et al., 1999; Wall et al., 2000). It should be noted that both the choice of pro-domain and the presence of a HA-tag affected the ability of the ligands to elicit activity in receptor reconstitution experiments. While the HA-tag occasionally reduced the activity of the ligand, it was useful for verification of efficient proteolytic maturation, i.e. cleavage of the pro-protein and thereby release of the mature ligand, as visualized by Western blotting.

3.1.2 Characterization of signaling specificity of a ligand

Receptor reconstitution experiments were performed in cell-lines transfected with different receptor combinations, a ligand and a Smad-dependent luciferase reporter. If the ligand was in the form of a peptide it was added to the cells one day after transfection. The choice of cell-line is critical for the outcome of these experiments, i.e. to obtain a good signal-to-noise ratio. Thus, the cell-line should have low background signaling activity due to endogenously expressed receptors and ligands, but still contain the intracellular signaling machinery to enable it to be highly responsive when transfected with the cognate receptors of the studied ligand. After testing approximately 15 cell-lines, HepG2 cells were found to be most highly responsive to ligands activating a Smad3-dependent reporter (Dennler et al., 1998). However, R4-2 was the cell-line that had the lowest background activity due to endogenously expressed components. The cell-lines responded somewhat differently to ligands activating a Smad1-dependent reporter (Hata et al., 2000), such that C2C12 and Mg87 cells were most responsive to BMP4 signaling, whereas HepG2 cells displayed good signal-to-noise ratio.

3.1.3 Assessment of ligand binding to specific receptors

When the ligands were available as peptides they could be labeled with ^{125}I and subsequently cross-linked to cells over-expressing different combinations of HA-tagged type I receptors, together with a type II receptor. Receptor complexes were immuno-precipitated from cell lysates with anti-HA antibodies, separated with SDS-PAGE, blotted onto polyvinylidinedifluoride membranes, and visualized using a phosphorimager. This methodology turned out to be very useful for studying ligand-receptor interactions in the TGF-β superfamily, since most often ligands only engaged in a complex with a cognate type I receptor after they having bound to a type II receptor (Shi and Massague, 2003). Therefore, ^{125}I-labeled ligands were cross-linked to COS cells that had been transfected with different combinations of HA-tagged ALK4, ALK5 or ALK7, together with Acvr2b receptors. While batches of GDF1 and GDF3 were inactive and did not bind to any receptor tested, GDF11 and Activin B bound directly to Acvr2b and via cooperativity to type I receptors (paper IV, and data not shown).

The remaining ligands, which were expressed in cell-lines, were not purified and could therefore not be labeled with ^{125}I. However, ligands that were fused to a HA-tag were used for studying binding to soluble receptors (Acvr2b-Fc, ALK4-Fc, ALK5-Fc and ALK7-Fc), which were added to the conditioned media, prior to pull-down with Protein-G Sepharose beads. Ligand-receptor interactions were detected by Western blotting with anti-HA antibodies. The assay analyzing interactions in solution differ from the cross-linking experiments in that only direct interactions could be determined and no binding cooperativity between the receptors could take place. Therefore, direct interactions between a ligand and a type I receptor should be determined by cross-linking experiments.

3.1.4 Establishing functional links *in vivo*

From using the biochemical methods described above it became clear that there was substantial promiscuity among both ligands and receptors within the TGF-β superfamily. In order to claim that a ligand signals through a specific receptor *in vivo*, one would like to observe similar phenotypes when deleting either the ligand or the receptor. However, targeted deletion of most genes encoding TGF-β receptors generates mouse models that are embryonic lethal. Since each receptor is likely to have several different ligands and functions, due to the described promiscuity, mutant mice may not display all phenotypes if an early phenotype results in embryonic lethality. Thus, establishing the *in vivo* functions of ligands and receptors in the TGF-β superfamily has been hampered by embryonic lethal phenotypes, redundancy and compensatory effects.

The most common way to circumvent limitations of mouse models caused by early embryonic lethality is to generate conditional mutant mice, in which a cre-enzyme enables a tissue specific deletion of a gene flanked by loxP sites. This method can be very informative when applied correctly, but is not always straightforward, and is limited by the availability of specific promoters driving the expression of the cre-enzyme. This approach can then generate an incomplete deletion, which in the worst case can lead to the masking of potential phenotypes.

Since TGF-β ligands can act as morphogens, i.e. generate differential effects along a concentration gradient, one could speculate that decreasing signaling strength, by deleting several alleles of different signaling components that act together in a pathway, should reveal additional functions for individual genes. If a receptor and a ligand genetically interact and potentiate phenotypes previously found when only one of the two genes were mutated, this ligand-receptor combination is likely to confer signaling *in vivo*. Other possible links include interactions between two ligands or two receptors, which would then suggest that the interacting genes promote a common signaling pathway. If one copy of a gene is mutated and transcription from the other copy is not sufficient to mediate normal function, a state called haploinsufficiency ensues. These types of mutations can be very informative when studying gene function, redundancy and compensatory mechanisms. This strategy has previously been exploited to reveal Nodal-dependent phenotypes in compound mutant mice carrying mutations in *Nodal, Acvr2*, *Acvr2b*, and *Smad2*, indicating that Nodal function is susceptible to gene-dosage effects (Nomura and Li, 1998; Oh and Li, 2002; Song et al., 1999). In the present thesis it has been shown that, despite the promiscuity of GDF11 signaling *in vitro,* specificity was revealed *in vivo* by a genetic interaction between *Alk5* and *Acvr2b* (paper IV). Thus, functional specificity was only observed when taking a genetic approach to a mechanistic question.

3.1.5 Localizing the origin of malformations

In order to pinpoint the origin of a specific malformation, the expression of tissue and cell-type specific markers can be examined. At early stages of development, i.e. up till embryonic day 9.5, this is most commonly done by

using whole-mount *in situ* hybridization. The choice of probes can be critical, and it is advantageous to apply commonly used probes if findings are to be correlated to previously published results. It is important to examine embryos at early stages, during the onset of the original defect, to avoid misleading secondary defects which are not directly linked to the targeted signaling pathway. It can be problematic if the studied gene is likely to have multiple roles within a tight time-frame, or in close proximity to each other. In such cases it may be even more important to time breedings, and to stage embryos according to morphological features instead of according to age (Downs and Davies, 1993). It should be noted that even wild-type embryos within one single litter differ in progress of development, often by up to half a day. The description of a morphological phenotype by characterization of tissue and cell-type specific markers is necessary for understanding the underlying defect.

4 RESULTS AND DISCUSSION

4.1 NODAL SIGNALS THROUGH ALK4 AND ALK7

At the outset of these thesis studies no ligand had been described to signal through ALK7. A collaboration with a group at Rockefeller University was initiated in order to characterize the function of ALK7 during embryogenesis (paper I). Injections of a constitutively active version of the ALK7 receptor into *Xenopus* embryos induced the expression of the mesodermal markers T, cardiac actin, collagen type II, and HoxB9, the endodermal markers GATA-4 and Xlhbox8, as well as the mesendodermal markers Xnr1 and Xnr2. Together these data suggested that ALK7 normally functions as a mesendoderm inducer, similar to what has previously been shown for ALK4 (Chang et al., 1997).

Mesendoderm induction has also been attributed to a group of ligands called *Xenopus* Nodal-related (Xnr), which indicated that ALK7 could be mediating signaling by binding to Nodal ligands (Jones et al., 1995). Indeed, a dominant-negative version of ALK7 could inhibit the effects of injection of Xnr1 and Nodal, but not *Xenopus* Activin B, Xnr2, or Xnr4. In addition, ALK7 was shown to mediate Nodal signaling when receptor complexes were reconstituted *in vitro*, and directly bind to Nodal and the co-receptor Cripto. ALK4 was also found to bind Cripto and mediate Nodal signaling, which indicated that the type I receptors ALK4 and ALK7 share a similar signaling mechanism. However, all these experiments were generated by using different ALK7 constructs made from rat cDNA. If endogenous *Xenopus* ALK7 mediates some of the functions regulated by Nodal proteins during *Xenopus* embryonic development it had to be present in a temporal and spatial expression pattern that correspond to these functions. A fragment of *Xenopus* ALK7 was cloned through degenerative PCR and used for expression studies, verifying ALK7 localization in the organizer region from which mesendoderm originates.

Xenopus Activin B could not signal through rat ALK7, despite the fact that mammalian Activin B has later been shown to elicit downstream signals via ALK7 (Tsuchida et al., 2004). Perhaps there exists substantial co-evolution between ligands and receptors, such that a mutation in the ligand may lead to a mutation in its corresponding receptor in order to retain an efficient interaction. Thus, it is possible that *Xenopus* Activin B and rat ALK7 are too divergent to produce functional ligand-receptor interactions, and downstream signaling.

The initial study of ALK7 functions during *Xenopus* development directed the subsequent work of this thesis towards embryonic development and mesendoderm formation. Also, several of the methods used in this first study, like the receptor reconstitution assays and production of ligands, were instrumental techniques re-utilized throughout the following work.

4.2 ALK7 IS DISPENSABLE FOR EMBRYOGENESIS

As a continuation of the first study, which essentially characterized roles for ALK7 during gain and loss of function in *Xenopus laevis*, the second study of this thesis addressed the consequences of *Alk7* loss of function in mice. Most other gene ablations in the TGF-β superfamily have resulted in severe developmental defects, and of those components that prior to this study had been shown to the act in concert with ALK7, i.e. Smad2, Smad3, Nodal, Cripto, and Acvr2b, only *Smad3* mutant mice survive birth (Jornvall et al., 2001; Reissmann et al., 2001; Watanabe et al., 1999). A targeted deletion in the mouse *Alk7* gene was generated and, surprisingly, the homozygote mutant mice survived until adulthood. Nevertheless, *Alk7* mutant mice were examined for morphological defects previously described in *Nodal* and *Acvr2b* mutants, although no phenotypes correlating to Nodal functions were found. This could be due to overlapping functions of the closely related ALK4 receptor, with which ALK7 shares all of its ligands (Andersson et al., 2006b; Reissmann et al., 2001; Tsuchida et al., 2004). In order to examine redundancy, *Alk7* mutants were crossed with *Alk4* and *Nodal* mutants; however, the survival rates did not differ from expected Mendelian distribution even among the compound mutant mice $Alk4^{+/-};Alk7^{-/-}$ and $Nodal^{+/-};Alk7^{-/-}$. The possibility that ALK4 function rescues *Alk7* mutant mice during development is therefore less likely, although one allele of *Alk4* may be sufficient to compensate for the absence of *Alk7*. It remains possible, however, that ALK7 mediates Nodal signals during *Xenopus* development, even though it is dispensable for mouse embryogenesis.

In an attempt to find other functions related to ALK7, its expression was examined by whole-mount *in situ* hybridization through development. Using this technique, ALK7 mRNA was only found to be expressed in the developing limb. The expression patterns in the limb partly overlap with previously described expression of GDF11 (Gamer et al., 2001; Nakashima et al., 1999). However, no phenotypes, such as webbing, limb or digit malformations, were found in *Alk7* mutants analyzed at birth. Since ALK7 expression has previously been described in perinatal and adult cerebellum, cortex, and hippocampus, possible histological abnormalities were investigated in brains of adult *Alk7* mutant mice (Lorentzon et al., 1996; Ryden et al., 1996; Tsuchida et al., 1996). However, the histological organization of those areas appeared normal in ALK7 mutant mice.

Together, these analysis suggested that ALK7 may instead function during adulthood. Given that no prominent expression of Nodal has been found during adulthood, putative ALK7 functions in the adult mouse should be mediated by other ligands. This prompted the investigation of other possible ligand-receptor interactions and thereby broaden the studies to encompass several ligands of the TGF-β superfamily.

4.3 GDF1 SYNERGIZES WITH NODAL

Since ALK7 signaling could induce mesendoderm in *Xenopus* embryos similarly to Nodal during both *Xenopus* and mouse development, it became important in our studies to understand how the precursors of the mesendoderm responded to graded Nodal signaling (Jones et al., 1995; Vincent et al., 2003). Since the preceding studies of this thesis had described possible redundant functions among type I receptors, it could be speculated that there may also exist substantial redundancy among ligands belonging to the TGF-β superfamily. Both *Gdf1* and *Nodal* had previously been shown to regulate left-right patterning of the lateral plate mesoderm, but are also co-expressed in other regions of the developing embryo, such as in the epiblast and the node (Collignon et al., 1996; Rankin et al., 2000; Wall et al., 2000; Zhang et al., 2001). Therefore, genetic interactions and haploinsufficiency experiments were performed, through the generation of compound mutant mice, to elucidate cooperative roles.

Mutant mice lacking Nodal die at gastrulation, whereas mice that are heterozygote for this mutation appear normal (Collignon et al., 1996). In contrast, *Gdf1*$^{-/-}$ single mutant embryos are viable up to at least E14.5, although they display defects in patterning of the left-right axis (Rankin et al., 2000). By crossing *Gdf1* and *Nodal* mutant mice to generate *Gdf1*$^{-/-}$;*Nodal*$^{+/-}$ double mutants, putative synergistic functions could be revealed through examining effects of gene-dosage. A decline in the observed frequency of *Gdf1*$^{-/-}$;*Nodal*$^{+/-}$ compound mutants with respect to the expected Mendelian ratio was already detected at E13.5, indicating partial embryonic lethality during the second week of gestation. The embryonic lethality of *Gdf1*$^{-/-}$;*Nodal*$^{+/-}$ embryos correlated with abnormalities that were not present in either *Gdf1*$^{-/-}$ or *Nodal*$^{+/-}$ single mutant littermates. Approximately 2/3 of *Gdf1*$^{-/-}$;*Nodal*$^{+/-}$ embryos displayed anterior neural defects and closely resembled previously described mice with reduced Nodal signaling.

Malformations of mesendoderm and its derivatives, including notochord prechordal plate, and foregut endoderm, were confirmed using markers such as FoxA2, goosecoid, T, and Shh. The prechordal plate and the foregut endoderm are organizing centers implicated in the development of the anterior head and branchial arches (Camus et al., 2000; Kirby et al., 2003). Thus, consistent with these deficits, *Gdf1*$^{-/-}$;*Nodal*$^{+/-}$ mutant embryos displayed a number of axial midline abnormalities, including holoprosencephaly, anterior head truncation, cleft lip, fused nasal cavity, and lack of jaws and tongue. The absence of these defects in single mutants indicated a synergistic interaction between *Nodal* and *Gdf1* in the node, from which mesendoderm originates, and where the two factors are normally co-expressed. This indicated that GDF1 synergizes with Nodal either by acting upstream of Nodal or by independently activating similar intracellular pathways.

GDF1 has previously been shown to act directly upstream of Nodal during left-right patterning of the mouse embryo by controlling Nodal expression in the lateral plate mesoderm (Rankin et al., 2000). It is likely that GDF1 controls Nodal expression in the lateral plate mesoderm by affecting a positive feedback

loop, regulated by a complex consisting of phosphorylated Smads 2 or 3 and the transcription factor Foxh1, that bind an intronic enhancer in the *Nodal* gene (Norris et al., 2002). Whether this mechanism affected Nodal expression at other sites was unclear. By monitoring the activity of the *lacZ* reporter gene inserted into the *Nodal* locus in *Nodal*$^{+/-}$ heterozygous and *Gdf1*$^{-/-}$*;Nodal*$^{+/-}$ double mutants, GDF1 was shown not to significantly affect Nodal expression in areas that could have an influence on the development of malformed tissues.

Receptor reconstitution experiments, and previously published findings, indicated that GDF1 could signal through the same type I receptors as Nodal (Cheng et al., 2003). However, since ALK4 mutant mice display defects already prior to mesendoderm formation (Gu et al., 1998), and ALK7 is dispensable for Nodal signaling during embryogenesis in mice (Jörnvall et al., 2004), the precise functions of these two receptors remained to be defined. In order to assess the individual contribution of these type I receptors during anterior axis development, compound mutant mice between *Alk4, Alk7, Nodal,* and *Gdf1* were generated. In triple mutants *Alk4*$^{+/-}$*;Gdf1*$^{+/-}$*;Nodal*$^{+/-}$ and *Alk7*$^{+/-}$*;Gdf1*$^{+/-}$*;Nodal*$^{+/-}$*,* only 3 out of 19 *Alk4*$^{+/-}$*;Gdf1*$^{+/-}$*;Nodal*$^{+/-}$ triple mutants displayed anterior malformations; the low incidence could possibly be due to compensations by the remaining alleles. Among *Alk4*$^{+/-}$*;Gdf1*$^{-/-}$ and *Alk7*$^{+/-}$*;Gdf1*$^{-/-}$ double mutants, in which GDF1 were absent, 33% of embryos carrying a mutant allele of *Alk4* showed an anterior truncation, but no contribution was found for ALK7. Despite the partial penetrance of defects, the analysis of compound mutants indicated that ALK4, but not ALK7, was responsible for the effects of GDF1 and Nodal during formation of mesendoderm. Thus, GDF1 was found to activate similar receptors as Nodal, but did not affect Nodal expression, indicating that GDF1 and Nodal act independently. Together, these results suggested that GDF1 and Nodal converge on ALK4 in the anterior primitive streak to control the formation of organizing centers that are necessary for normal forebrain and branchial arch development.

4.4 GDF1 COOPERATES WITH GDF3

The distinct phenotypes among mice carrying targeted deletions for *Nodal, Gdf1, Smad2,* and *Cripto* suggested that there should be additional players in this signaling network (Conlon et al., 1994; Ding et al., 1998; Nomura and Li, 1998; Rankin et al., 2000; Waldrip et al., 1998; Weinstein et al., 1998). Also, Cripto has been shown to be expressed in several adult mouse tissues that do not express Nodal or GDF1 (Dono et al., 1993; Johnson et al., 1994). Therefore, a search for ligands that signal through ALK4 and ALK7 in a Cripto-dependent fashion was initiated. This search identified GDF3 based on its activities in reporter assays.

GDF3 shares a high amino acid identity with *Xenopus* Vg1, a ligand that indeed also has been shown to be dependent on Cripto to elicit downstream signaling (Cheng et al., 2003; McPherron and Lee, 1993). However, synteny analyses suggest that GDF1 is the true ortholog of Vg1 based on its position in the genome, despite the fact that GDF3 has a higher sequence identity to Vg1 than does GDF1 (paper V). Vg1 has been shown to function as a mesoderm inducer during early *Xenopus* embryogenesis, and to later regulate development of the left-right axis (Hyatt and Yost, 1998; Joseph and Melton, 1998; Kessler and Melton, 1995; Thomsen and Melton, 1993). Depletion of Vg1 mRNA using antisense oligonucleotides in frog embryos has also shown that Vg1 is required for Smad2 phosphorylation and mesoderm formation, such that antisense-treated embryos lacked notochord, had fused somites along the midline, and reduced and abnormal neural structures (Birsoy et al., 2006). Note that these defects are similar to the malformations found in $Gdf1^{-/-};Nodal^{+/-}$ mutants, described in paper III of this thesis, suggesting a common function of these ligands during mesoderm formation.

The close sequence identity between Vg1, GDF1, and GDF3 suggests that they all may signal in a similar fashion. Previous studies of these ligands have been hampered by poor processing of the pro-protein. However, by co-expressing GDF1 or GDF3 with the pro-protein convertase Furin, these ligands were shown to share a similar signaling mechanism to Vg1, which requires Cripto. Some of these assays displayed background activity, which was most likely due to low endogenous expression of unknown signaling components in the cell-line used. In order to circumvent this problem, chimeric ALK4 and ALK7 receptors in which the L45-loop was replaced with the corresponding sequence from the BMP receptor ALK3 were generated. The L45 loop of the type I receptor interacts with Smad proteins and determines the Smad specificity of the receptor (Chen et al., 1998; Persson et al., 1998). Using this assay, GDF3 could significantly activate the reporter only in the presence of either ALK4 or ALK7 chimeric receptors together with Cripto. Additional characterization of GDF3 signaling revealed its similarities to GDF1 signaling in terms of its ability to utilize the type I receptors ALK4 and ALK7, and the type II receptors Acvr2 and Acvr2b, to activate Smad-dependent reporter genes.

To assess the function of GDF3, mutant mice were generated from ES-cells carrying a gene trap in the single intron of *Gdf3*. Approximately half of the mice homozygous for the gene trap insertion survived until adulthood and were fertile. During the course of this study, another group published the independent generation of *Gdf3* mutant mice, in which they also found a partially penetrant defect during early embryonic development (Chen et al., 2006). The partial penetrance of malformations found in *Gdf3* mutants could be due to redundant functions of GDF1, since both ligands have been shown to be expressed in the embryonic ectoderm (Chen et al., 2006; Rankin et al., 2000; Wall et al., 2000).

GDF3 was previously found to regulate both the formation and the movement of the AVE from the distal tip to the prospective anterior side of the epiblast (Chen et al., 2006). In addition, GDF3 was shown to affect the formation of the primitive streak and mesoderm. To examine whether deletion of *Gdf1* can augment the phenotypes found in $Gdf3^{-/-}$ mutants, $Gdf1^{-/-};Gdf3^{-/-}$ double null mutants were generated. Whole-mount *in situ* hybridization with markers specific to the AVE (Lefty1), primitive streak and axial mesoderm (T), and anterior primitive streak and axial mesoderm (FoxA2) were performed. Lefty1 staining revealed two distinct phenotypes among the mutants when analyzed at E6.5, one in which AVE was formed but did not migrate to the anterior side (referred to as a type I), and another in which the AVE was totally absent (type II). All affected embryos carried a homozygous mutation in *Gdf3* and the incidence of defects increased with a homozygous mutation of *Gdf1,* although, *Gdf1* deletion by itself did not seem to generate any defects. Approximately 50% of the embryos carrying $Gdf1^{+/+};Gdf3^{-/-}$ or $Gdf1^{+/-};Gdf3^{-/-}$ mutations displayed either type I or type II defects, while 83% of $Gdf1^{-/-};Gdf3^{-/-}$ mutants were affected in this way. The severity of the defects was also augmented in the $Gdf1^{-/-};Gdf3^{-/-}$ mutant embryos, such that 58% displayed type II defects, compared to $Gdf1^{+/+};Gdf3^{-/-}$ or $Gdf1^{+/-};Gdf3^{-/-}$ mutants in which approximately 20% presented with type II defects. Thus, *Gdf1* and *Gdf3* have partially redundant functions during formation of the anterior visceral endoderm.

Two distinct phenotypes were also found at E7.5, one in which the embryos displayed a constriction at the extra-embryonic/embryonic junction (referred to as a type I), and another with normal extra-embryonic tissues but no or very small and disorganized embryo proper (type II). Using FoxA2 and T as markers for mesoderm, it was found that type I embryos were able to form mesoderm, but showed impaired elongation of the primitive streak and abnormal axial mesoderm. Type II embryos did not show any staining for FoxA2 or T, indicating a lack of mesoderm. Among $Gdf3^{-/-}$ single mutants at E7.5, 27% were shown to be type I embryos, but no type II embryo was found. In contrast, type II embryos were found in 13% of $Gdf1^{+/-};Gdf3^{-/-}$ compound mutants and in 47% of $Gdf1^{-/-};Gdf3^{-/-}$ mutants. Thus, mutations in *Gdf1* can potentiate the defects in mesoderm formation found in *Gdf3* mutant mice.

One possibility is that GDF1 and GDF3 act upstream of Nodal during these processes, since Nodal mutant mice have been shown to display similar phenotypes (Brennan et al., 2001). Alternatively, these effects could be due to

altered BMP signaling since Nodal and GDF3 have been shown to inhibit BMP7 and BMP4, respectively, through the formation of heterodimeric ligand complexes (Levine and Brivanlou, 2006; Yeo and Whitman, 2001). Loss of this inhibition could lead to excess BMP signaling in *Gdf3* and *Nodal* mutant mice, which may account for some of the effects on AVE formation observed in these mutants. This notion is supported by a conditional inactivation of the BMP type I receptor, *Alk3,* in the epiblast, obtained by crossing mice carrying *Mox2-cre* with a mouse line of *Alk3* flanked with loxP sites, which generates embryos with an expanded AVE (Davis et al., 2004). Thus, BMP signaling through ALK3 in the epiblast can negatively regulate generation of the AVE. However, there is no direct evidence for increased phosphorylation of Smad1/5/8 in *Nodal* or *Gdf3* mutant mice, or for the existence of endogenous heterodimeric ligand complexes during embryogenesis.

Pro-protein convertases have been shown to be critical for the formation of AVE and mesendoderm in *Furin;Spc4* double mutant mice, which was attributed to insufficient cleavage/activation of Nodal (Beck et al., 2002). The secretion of soluble Furin and Spc4 from the extra-embryonic ectoderm was proposed to affect cleavage of Nodal in the epiblast. Since our data shows that Furin can also process GDF1 and GDF3, it is possible that insufficient cleavage of these ligands may contribute to the observed defects found in *Furin;Spc4* compound mutants. Moreover, the fact that around 20% of $Gdf1^{-/-};Gdf3^{-/-}$ compound mutants develop normally suggests that there must be another ligand –most likely Nodal- that can occasionally compensate for the absence of GDF1 and GDF3. Together, this indicates that GDF1, GDF3, and Nodal form a signaling network with partially redundant functions during early embryogenesis.

4.5 GDF11 REGIONALIZES THE ANTERIOR-POSTERIOR AXIS

The orphan ligand GDF11 was investigated for its receptor specificity and functions as a follow-up from paper II, due to its possible signaling through ALK7. However, it soon became clear that GDF11 predominantly uses ALK4 and ALK5 to mediate downstream effects, at least when assessed *in vitro*. GDF11 had been shown to regionalize the anterior-posterior axis by its expression in the tail bud, although it was not known through which receptors this was mediated (McPherron et al., 1999).

Receptor binding experiments showed that GDF11 can interact with ALK4, ALK5 and ALK7 in a Acvr2b-dependent manner, i.e. GDF11 could only engage in a complex with type I receptors after it had already bound to Acvr2b. Furthermore, receptor reconstitution experiments revealed that GDF11 predominantly utilizes ALK4 and ALK5 to activate a downstream reporter gene.

To identify the type I receptor that mediates GDF11 signaling *in vivo*, the incidence of *Gdf11* null phenotypes in mice carrying inactivating mutations in the *Alk4*, *Alk5*, or *Alk7* genes was investigated. Unfortunately, the early embryonic lethality of $Alk4^{-/-}$ and $Alk5^{-/-}$ homozygote mutants prevented a direct assessment of phenotypes in these mice (Gu et al., 1998; Larsson et al., 2001; Seki et al., 2006). However, we speculated that heterozygocity of either *Alk4* or *Alk5*, which *per se* gives no visible defects, could possibly potentiate the phenotypes observed in $Acvr2b^{-/-}$ mice, which display malformations that are similar to —but less severe than— those seen in $Gdf11^{-/-}$ mutants.

Wild type mice normally have 26 presacral vertebrae in a characteristic pattern that comprises 7 cervical (C), 13 thoracic (T), and 6 lumbar (L), referred to as C7/T13/L6. In contrast, and due to defects in anterior-posterior axis formation, most $Gdf11^{-/-}$ mutants have 33 presacral vertebrae in a C7/T18/L8 pattern (McPherron et al., 1999). On the other hand, $Acvr2b^{-/-}$ mice most often displayed 29 presacral vertebrae in a C7/T16/L6 pattern, consistent with what has previously been described (Oh and Li, 1997). Interestingly, the severity of this phenotype was potentiated in $Alk5^{+/-};Acvr2b^{-/-}$ mutants, in which the number of presacral vertebrae was increased to 30. Potentiation of this homeotic transformation contributed to an increase in either thoracic or lumbar segments, which manifested as either C7/T16/L7 or C7/T17/L6 vertebral patterns in the compound mutants. In contrast, neither $Alk4^{+/-};Acvr2b^{-/-}$ or $Alk7^{-/-};Acvr2b^{-/-}$ compound mutants showed any enhanced homeotic transformation compared to littermates $Acvr2b^{-/-}$ single mutants. In order to delineate the mechanism by which *Alk5* affects anterior-posterior patterning, the induction of specific Hox genes was examined. *Alk5* homozygous mutant embryos displayed no expression of the posterior determinant Hoxc10 at E9, which resembles the defects found in *Gdf11* null mutants.

Recent findings have shown that phosphorylation of Smad2 is only decreased by 50% in *Gdf11* mutant tail buds, raising the possibility that other TGF-β ligands that phosphorylate Smad2 may cooperate with GDF11 in this structure (Liu, 2006). Although both GDF1 and Nodal have been shown to be expressed in the

tail bud at E9.5 (Varlet et al., 1997; Wall et al., 2000), *Gdf1*$^{-/-}$;*Nodal*$^{+/-}$ compound mutants did not display defects in vertebral patterning (data not shown). Nevertheless, the importance of TGF-β signaling during regionalization may be under-estimated since overlapping functions of ligands could possibly mask the full effect of TGF-β signaling.

The role of TGF-β signaling during gastrulation and regionalization of the anterior-posterior axis has also been studied in *Xenopus* and Zebrafish. One study made use of the inhibitor SB-431542, which inhibits ALK4/5/7 by targeting the ATP-binding pocket of the type I receptors (Ho et al., 2006; Inman et al., 2002). By maintaining embryos in culture media and adding the inhibitor before or after gastrulation, the phenotypic consequences of TGF-β inhibition can be assessed. To rescue these phenotypes, the investigators over-expressed type I receptors carrying point mutations that make them insensitive to the inhibitor. They found that an inhibitor-resistant ALK4 receptor can rescue gastrulation and left-right patterning defects, while either inhibitor-resistant ALK4 or ALK5, but not ALK7, receptors could rescue signaling in the tail bud. These findings correlate well with our findings in mutant mice, i.e. ALK4 mutant mice display gastrulation defects (paper III) and ALK5 mutant mice display defects arising from the tail bud (paper IV).

Additional defects observed during the course of this project involved kidney organogenesis and palate closure. GDF11 has been shown to direct the initial outgrowth of the ureteric bud during E11, and mice lacking *Gdf11* consequently display kidney agenesis at birth (Esquela and Lee, 2003). The incidences of kidney agenesis and cleft palate were also increased in *Alk5*$^{+/-}$;*Acvr2b*$^{-/-}$ mutants compared to *Acvr2b*$^{-/-}$ single mutants. Whereas the kidney agenesis is likely to be due to reduced GDF11 signaling, it is less clear whether the defect in closure of the palate depends solely on GDF11 signaling since several other ligands belonging to the TGF-β superfamily, such as TGF-β1, TGF-β3, and Activin A, have also been implicated in this process (Kaartinen et al., 1995; Matzuk et al., 1995a; Proetzel et al., 1995; Sanford et al., 1997). Indeed, over-expression of ALK5 in palate epithelium from *Tgf-β3* mutant mice can rescue palate fusion (Dudas et al., 2004). The importance of ALK5 during craniofacial development has been confirmed *in vivo* using conditional *Alk5/Wnt1*-cre mice (Dudas et al., 2006). Interestingly, it was shown that *Alk5/Wnt1*-cre display a stronger phenotype than *Tgfbr2/Wnt1*-cre, suggesting that ALK5 also acts in conjunction with type II receptors other than the typical binding partner, TGFβR2 (Ito et al., 2003). This study also described GDF11 expression in the craniofacial primordia, which suggests that GDF11 plays a role during closure of the palate, possibly via the receptor combination ALK5/Acvr2b.

In addition to the phenotypes described above, targeted deletion of *Gdf11* has been associated with abnormal development of the spleen, as well as defective regulation of progenitor cells in retina, nasal epithelium, and pancreas (Dichmann et al., 2006; Harmon et al., 2004; Kim et al., 2005; Wu et al., 2003). However, the formation of the spleen was not examined since Nodal and GDF1 signaling through Acvr2b also affects spleen development, making these effects

difficult to separate from each other (Oh and Li, 2002; Rankin et al., 2000). Alterations in the development of progenitor cells first occur at E14 in *Gdf11* mutants, which, unfortunately, is after *Alk5* mutant embryos arrest development, and were therefore not analyzed.

In conclusion, ALK5 plays an important role in anterior-posterior regionalization, kidney, and palate development, and our findings also support a role for this type I receptor in mediating GDF11 activities.

5 CONCLUDING REMARKS

This thesis describes mechanisms by which a group of morphogens affects the development of the mouse embryo. The use of biochemical methods enabled the characterization of the pathways by which Nodal, GDF1, GDF3, and GDF11 transduce their signals. By examining phenotypes susceptible to gene-dosage effects, the signaling mechanisms and specific functions of these ligands were analyzed *in vivo* through genetic interactions between ligands and receptors. The main findings were that:

1. GDF1 and Nodal cooperate to establish a graded continuum of signaling strength for the specific allocation of mesendoderm precursors.

2. GDF1 and GDF3 act together during the formation of anterior visceral endoderm and the formation of mesoderm.

3. GDF1, GDF3, and Nodal can all signal via ALK4 and ALK7, although their effects during embryogenesis rely on ALK4.

4. In contrast, GDF11 signals through ALK5 to regionalize the body-plan along the anterior-posterior axis.

Different scenarios can be envisioned to explain the observed cooperativity among ligands. One reason for overlapping functions may be that superfluous signaling is desirable in order to ensure that a critical event does not fail to take place. This could be the case for Nodal, GDF1, and GDF3 signaling during formation of anterior visceral endoderm. If these three ligands act in parallel, a mutation in one allele of any one of these ligands is less likely to alter the outcome of their common function. This type of redundancy may be particularly important in inter-cellular signaling that is affected by intrinsic variations in tissue morphology, as a certain signaling strength may be critical for a given signal to reach its final destination.

Localized expression of several ligands with different properties may affect the shape of a merged gradient. For example, it is possible that Nodal signals over a longer range than does GDF1, although they are both expressed in the node. In such a case, GDF1 may affect the node derivatives to a greater extent than other distantly localized mesoderm precursors. Perhaps there are significant differences in the stability of Nodal and GDF1, since the stability of mature Nodal has been shown to be increased by formation of a latent complex with its pro-domain (Le Good et al., 2005). The range of Nodal signaling may also be influenced by its positive feedback loop, which enables Nodal to spread via a relay mechanism from cell to cell. These mechanisms are of course hard to examine in mutant mice, but would be feasible to examine in other model systems, such as in zebrafish or *Xenopus*. Nevertheless, it is likely that Nodal and GDF1 form a merged gradient that promotes the generation of distinct cell types in a spatial order. However, this mode of action does not necessarily need

to be restricted to a single signaling pathway. It is possible that GDF11 and FGF8 signaling cooperate during regionalization of the anterior-posterior axis by forming overlapping gradients from the posterior end of the embryo. More complex genetic interactions could potentially be uncovered by combining targeted deletions of different components in the GDF11 and the FGF8 signaling pathways.

Cells within different compartments, such as the visceral endoderm and the epiblast, do not inter-mingle but still affect each other in an ordered fashion. As mentioned before, Nodal, GDF1, and GDF3 signal from the epiblast to the visceral endoderm, resulting in the induction of distal visceral endoderm, which in turn migrates to the anterior side of the epiblast from where it signals back to the epiblast to induce the prospective forebrain. It is important to note that this reciprocal interaction between the epiblast and the visceral endoderm occurs in a hierarchical fashion, in which the visceral endoderm needs to change cell fate in order to return the signal. Moreover, the prospective forebrain subsequently needs to be stabilized by receiving maintenance signals from the prechordal plate. This mode of sequential induction/maintenance of specific cell fates may be common during coordination of embryonic development.

The data presented in this thesis significantly contribute to the understanding of how different TGF-β ligands are functionally integrated during embryonic development. The studied ligands share common downstream signaling components, which allows for functional redundancy. Given the high degree of convergence in this pathway, this type of signaling may be widely applicable to other members of the TGF-β superfamily.

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7 REFERENCES

Abdollah, S., Macias-Silva, M., Tsukazaki, T., Hayashi, H., Attisano, L. and Wrana, J. L. (1997). TbetaRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2-Smad4 complex formation and signaling. *J Biol Chem* **272**, 27678-85.

Akai, J., Halley, P. A. and Storey, K. G. (2005). FGF-dependent Notch signaling maintains the spinal cord stem zone. *Genes Dev* **19**, 2877-87.

Albazerchi, A. and Stern, C. D. (2006). A role for the hypoblast (AVE) in the initiation of neural induction, independent of its ability to position the primitive streak. *Dev Biol*.

Andersson, O., Reissmann, E. and Ibanez, C. F. (2006a). Growth differentiation factor 11 signals through the transforming growth factor-beta receptor ALK5 to regionalize the anterior-posterior axis. *EMBO Rep* **7**, 831-7.

Andersson, O., Reissmann, E., Jornvall, H. and Ibanez, C. F. (2006b). Synergistic interaction between Gdf1 and Nodal during anterior axis development. *Dev Biol* **293**, 370-81.

Ang, S. L. and Constam, D. B. (2004). A gene network establishing polarity in the early mouse embryo. *Semin Cell Dev Biol* **15**, 555-61.

Ang, S. L. and Rossant, J. (1994). HNF-3 beta is essential for node and notochord formation in mouse development. *Cell* **78**, 561-74.

Attisano, L., Carcamo, J., Ventura, F., Weis, F. M., Massague, J. and Wrana, J. L. (1993). Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* **75**, 671-80.

Attisano, L., Wrana, J. L., Cheifetz, S. and Massague, J. (1992). Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. *Cell* **68**, 97-108.

Aulehla, A., Wehrle, C., Brand-Saberi, B., Kemler, R., Gossler, A., Kanzler, B. and Herrmann, B. G. (2003). Wnt3a plays a major role in the segmentation clock controlling somitogenesis. *Dev Cell* **4**, 395-406.

Bachiller, D., Klingensmith, J., Kemp, C., Belo, J. A., Anderson, R. M., May, S. R., McMahon, J. A., McMahon, A. P., Harland, R. M., Rossant, J. et al. (2000). The organizer factors Chordin and Noggin are required for mouse forebrain development. *Nature* **403**, 658-61.

Beck, S., Le Good, J. A., Guzman, M., Ben Haim, N., Roy, K., Beermann, F. and Constam, D. B. (2002). Extraembryonic proteases regulate Nodal signalling during gastrulation. *Nat Cell Biol* **4**, 981-5.

Beddington, R. S. (1994). Induction of a second neural axis by the mouse node. *Development* **120**, 613-20.

Belo, J. A., Bouwmeester, T., Leyns, L., Kertesz, N., Gallo, M., Follettie, M. and De Robertis, E. M. (1997). Cerberus-like is a secreted factor with neutralizing activity expressed in the anterior primitive endoderm of the mouse gastrula. *Mech Dev* **68**, 45-57.

Belville, C., Jamin, S. P., Picard, J. Y., Josso, N. and di Clemente, N. (2005). Role of type I receptors for anti-Mullerian hormone in the SMAT-1 Sertoli cell line. *Oncogene* **24**, 4984-92.

Beppu, H., Kawabata, M., Hamamoto, T., Chytil, A., Minowa, O., Noda, T. and Miyazono, K. (2000). BMP type II receptor is required for gastrulation and early development of mouse embryos. *Dev Biol* **221**, 249-58.

Bertolino, P., Deckers, M., Lebrin, F. and ten Dijke, P. (2005). Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders. *Chest* **128**, 585S-590S.

Birsoy, B., Kofron, M., Schaible, K., Wylie, C. and Heasman, J. (2006). Vg 1 is an essential signaling molecule in Xenopus development. *Development* **133**, 15-20.

Bottcher, R. T. and Niehrs, C. (2005). Fibroblast growth factor signaling during early vertebrate development. *Endocr Rev* **26**, 63-77.

Brennan, J., Lu, C. C., Norris, D. P., Rodriguez, T. A., Beddington, R. S. and Robertson, E. J. (2001). Nodal signalling in the epiblast patterns the early mouse embryo. *Nature* **411**, 965-9.
Cambray, N. and Wilson, V. (2002). Axial progenitors with extensive potency are localised to the mouse chordoneural hinge. *Development* **129**, 4855-66.
Camus, A., Davidson, B. P., Billiards, S., Khoo, P., Rivera-Perez, J. A., Wakamiya, M., Behringer, R. R. and Tam, P. P. (2000). The morphogenetic role of midline mesendoderm and ectoderm in the development of the forebrain and the midbrain of the mouse embryo. *Development* **127**, 1799-813.
Camus, A., Perea-Gomez, A., Moreau, A. and Collignon, J. (2006). Absence of Nodal signaling promotes precocious neural differentiation in the mouse embryo. *Dev Biol* **295**, 743-55.
Carcamo, J., Weis, F. M., Ventura, F., Wieser, R., Wrana, J. L., Attisano, L. and Massague, J. (1994). Type I receptors specify growth-inhibitory and transcriptional responses to transforming growth factor beta and activin. *Mol Cell Biol* **14**, 3810-21.
Celeste, A. J., Iannazzi, J. A., Taylor, R. C., Hewick, R. M., Rosen, V., Wang, E. A. and Wozney, J. M. (1990). Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone. *Proc Natl Acad Sci U S A* **87**, 9843-7.
Chang, C., Wilson, P. A., Mathews, L. S. and Hemmati-Brivanlou, A. (1997). A Xenopus type I activin receptor mediates mesodermal but not neural specification during embryogenesis. *Development* **124**, 827-37.
Cheifetz, S., Bellon, T., Cales, C., Vera, S., Bernabeu, C., Massague, J. and Letarte, M. (1992). Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. *J Biol Chem* **267**, 19027-30.
Chen, C. and Shen, M. M. (2004). Two modes by which Lefty proteins inhibit nodal signaling. *Curr Biol* **14**, 618-24.
Chen, C., Ware, S. M., Sato, A., Houston-Hawkins, D. E., Habas, R., Matzuk, M. M., Shen, M. M. and Brown, C. W. (2006). The Vg1-related protein Gdf3 acts in a Nodal signaling pathway in the pre-gastrulation mouse embryo. *Development* **133**, 319-29.
Chen, X., Rubock, M. J. and Whitman, M. (1996). A transcriptional partner for MAD proteins in TGF-beta signalling. *Nature* **383**, 691-6.
Chen, Y. G., Hata, A., Lo, R. S., Wotton, D., Shi, Y., Pavletich, N. and Massague, J. (1998). Determinants of specificity in TGF-beta signal transduction. *Genes Dev* **12**, 2144-52.
Chen, Y. G. and Massague, J. (1999). Smad1 recognition and activation by the ALK1 group of transforming growth factor-beta family receptors. *J Biol Chem* **274**, 3672-7.
Cheng, S. K., Olale, F., Bennett, J. T., Brivanlou, A. H. and Schier, A. F. (2003). EGF-CFC proteins are essential coreceptors for the TGF-beta signals Vg1 and GDF1. *Genes Dev* **17**, 31-6.
Cheng, S. K., Olale, F., Brivanlou, A. H. and Schier, A. F. (2004). Lefty blocks a subset of TGFbeta signals by antagonizing EGF-CFC coreceptors. *PLoS Biol* **2**, E30.
Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383**, 407-13.
Ciruna, B. and Rossant, J. (2001). FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. *Dev Cell* **1**, 37-49.
Clarke, T. R., Hoshiya, Y., Yi, S. E., Liu, X., Lyons, K. M. and Donahoe, P. K. (2001). Mullerian inhibiting substance signaling uses a bone morphogenetic protein (BMP)-like pathway mediated by ALK2 and induces SMAD6 expression. *Mol Endocrinol* **15**, 946-59.
Collignon, J., Varlet, I. and Robertson, E. J. (1996). Relationship between asymmetric nodal expression and the direction of embryonic turning. *Nature* **381**, 155-8.

Conlon, F. L., Lyons, K. M., Takaesu, N., Barth, K. S., Kispert, A., Herrmann, B. and Robertson, E. J. (1994). A primary requirement for nodal in the formation and maintenance of the primitive streak in the mouse. *Development* **120**, 1919-28.
Constam, D. B. and Robertson, E. J. (2000a). SPC4/PACE4 regulates a TGFbeta signaling network during axis formation. *Genes Dev* **14**, 1146-55.
Constam, D. B. and Robertson, E. J. (2000b). Tissue-specific requirements for the proprotein convertase furin/SPC1 during embryonic turning and heart looping. *Development* **127**, 245-54.
Davis, S., Miura, S., Hill, C., Mishina, Y. and Klingensmith, J. (2004). BMP receptor IA is required in the mammalian embryo for endodermal morphogenesis and ectodermal patterning. *Dev Biol* **270**, 47-63.
De Robertis, E. M. (2006). Spemann's organizer and self-regulation in amphibian embryos. *Nat Rev Mol Cell Biol* **7**, 296-302.
Deng, C. X., Wynshaw-Boris, A., Shen, M. M., Daugherty, C., Ornitz, D. M. and Leder, P. (1994). Murine FGFR-1 is required for early postimplantation growth and axial organization. *Genes Dev* **8**, 3045-57.
Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huet, S. and Gauthier, J. M. (1998). Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *Embo J* **17**, 3091-100.
Derynck, R., Jarrett, J. A., Chen, E. Y., Eaton, D. H., Bell, J. R., Assoian, R. K., Roberts, A. B., Sporn, M. B. and Goeddel, D. V. (1985). Human transforming growth factor-beta complementary DNA sequence and expression in normal and transformed cells. *Nature* **316**, 701-5.
Derynck, R. and Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**, 577-84.
Deschamps, J. and van Nes, J. (2005). Developmental regulation of the Hox genes during axial morphogenesis in the mouse. *Development* **132**, 2931-42.
Dichmann, D. S., Yassin, H. and Serup, P. (2006). Analysis of pancreatic endocrine development in GDF11-deficient mice. *Dev Dyn* **235**, 3016-3025.
Diez del Corral, R. and Storey, K. G. (2004). Opposing FGF and retinoid pathways: a signalling switch that controls differentiation and patterning onset in the extending vertebrate body axis. *Bioessays* **26**, 857-69.
Ding, J., Yang, L., Yan, Y. T., Chen, A., Desai, N., Wynshaw-Boris, A. and Shen, M. M. (1998). Cripto is required for correct orientation of the anterior-posterior axis in the mouse embryo. *Nature* **395**, 702-7.
Dono, R., Scalera, L., Pacifico, F., Acampora, D., Persico, M. G. and Simeone, A. (1993). The murine cripto gene: expression during mesoderm induction and early heart morphogenesis. *Development* **118**, 1157-68.
Downs, K. M. and Davies, T. (1993). Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. *Development* **118**, 1255-66.
Dubrulle, J., McGrew, M. J. and Pourquie, O. (2001). FGF signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* **106**, 219-32.
Dubrulle, J. and Pourquie, O. (2004a). Coupling segmentation to axis formation. *Development* **131**, 5783-93.
Dubrulle, J. and Pourquie, O. (2004b). fgf8 mRNA decay establishes a gradient that couples axial elongation to patterning in the vertebrate embryo. *Nature* **427**, 419-22.
Dudas, M., Kim, J., Li, W. Y., Nagy, A., Larsson, J., Karlsson, S., Chai, Y. and Kaartinen, V. (2006). Epithelial and ectomesenchymal role of the type I TGF-beta receptor ALK5 during facial morphogenesis and palatal fusion. *Dev Biol* **296**, 298-314.
Dudas, M., Nagy, A., Laping, N. J., Moustakas, A. and Kaartinen, V. (2004). Tgf-beta3-induced palatal fusion is mediated by Alk-5/Smad pathway. *Dev Biol* **266**, 96-108.

Dufort, D., Schwartz, L., Harpal, K. and Rossant, J. (1998). The transcription factor HNF3beta is required in visceral endoderm for normal primitive streak morphogenesis. *Development* **125**, 3015-25.
Esch, F. S., Shimasaki, S., Mercado, M., Cooksey, K., Ling, N., Ying, S., Ueno, N. and Guillemin, R. (1987). Structural characterization of follistatin: a novel follicle-stimulating hormone release-inhibiting polypeptide from the gonad. *Mol Endocrinol* **1**, 849-55.
Esquela, A. F. and Lee, S. J. (2003). Regulation of metanephric kidney development by growth/differentiation factor 11. *Dev Biol* **257**, 356-70.
Eyal-Giladi, H. M., W. (1970). The inducing capacities of the primary hypoblast as revealed by transfilter induction studies. *Wilhelm Roux Archiv* **165**, 226-241.
Filosa, S., Rivera-Perez, J. A., Gomez, A. P., Gansmuller, A., Sasaki, H., Behringer, R. R. and Ang, S. L. (1997). Goosecoid and HNF-3beta genetically interact to regulate neural tube patterning during mouse embryogenesis. *Development* **124**, 2843-54.
Fujimori, T., Kurotaki, Y., Miyazaki, J. and Nabeshima, Y. (2003). Analysis of cell lineage in two- and four-cell mouse embryos. *Development* **130**, 5113-22.
Fujiwara, T., Dehart, D. B., Sulik, K. K. and Hogan, B. L. (2002). Distinct requirements for extra-embryonic and embryonic bone morphogenetic protein 4 in the formation of the node and primitive streak and coordination of left-right asymmetry in the mouse. *Development* **129**, 4685-96.
Gamer, L. W., Cox, K. A., Small, C. and Rosen, V. (2001). Gdf11 is a negative regulator of chondrogenesis and myogenesis in the developing chick limb. *Dev Biol* **229**, 407-20.
Gamer, L. W., Nove, J., Levin, M. and Rosen, V. (2005). BMP-3 is a novel inhibitor of both activin and BMP-4 signaling in Xenopus embryos. *Dev Biol* **285**, 156-68.
Gardner, R. L. (1968). Mouse chimeras obtained by the injection of cells into the blastocyst. *Nature* **220**, 596-7.
Gardner, R. L. and Cockroft, D. L. (1998). Complete dissipation of coherent clonal growth occurs before gastrulation in mouse epiblast. *Development* **125**, 2397-402.
Ge, G., Hopkins, D. R., Ho, W. B. and Greenspan, D. S. (2005). GDF11 forms a bone morphogenetic protein 1-activated latent complex that can modulate nerve growth factor-induced differentiation of PC12 cells. *Mol Cell Biol* **25**, 5846-58.
Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C. and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357-62.
Gont, L. K., Steinbeisser, H., Blumberg, B. and de Robertis, E. M. (1993). Tail formation as a continuation of gastrulation: the multiple cell populations of the Xenopus tailbud derive from the late blastopore lip. *Development* **119**, 991-1004.
Gotoh, N., Manova, K., Tanaka, S., Murohashi, M., Hadari, Y., Lee, A., Hamada, Y., Hiroe, T., Ito, M., Kurihara, T. et al. (2005). The docking protein FRS2alpha is an essential component of multiple fibroblast growth factor responses during early mouse development. *Mol Cell Biol* **25**, 4105-16.
Goumans, M. J., Lebrin, F. and Valdimarsdottir, G. (2003). Controlling the angiogenic switch: a balance between two distinct TGF-b receptor signaling pathways. *Trends Cardiovasc Med* **13**, 301-7.
Goumans, M. J., Valdimarsdottir, G., Itoh, S., Rosendahl, A., Sideras, P. and ten Dijke, P. (2002). Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *Embo J* **21**, 1743-53.
Greenwald, J., Groppe, J., Gray, P., Wiater, E., Kwiatkowski, W., Vale, W. and Choe, S. (2003). The BMP7/ActRII extracellular domain complex provides new insights into the cooperative nature of receptor assembly. *Mol Cell* **11**, 605-17.
Gu, Z., Nomura, M., Simpson, B. B., Lei, H., Feijen, A., van den Eijnden-van Raaij, J., Donahoe, P. K. and Li, E. (1998). The type I activin receptor

ActRIB is required for egg cylinder organization and gastrulation in the mouse. *Genes Dev* **12**, 844-57.
Harmon, E. B., Apelqvist, A. A., Smart, N. G., Gu, X., Osborne, D. H. and Kim, S. K. (2004). GDF11 modulates NGN3+ islet progenitor cell number and promotes beta-cell differentiation in pancreas development. *Development* **131**, 6163-74.
Hata, A., Lagna, G., Massague, J. and Hemmati-Brivanlou, A. (1998). Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad4 tumor suppressor. *Genes Dev* **12**, 186-97.
Hata, A., Seoane, J., Lagna, G., Montalvo, E., Hemmati-Brivanlou, A. and Massague, J. (2000). OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. *Cell* **100**, 229-40.
He, W., Dorn, D. C., Erdjument-Bromage, H., Tempst, P., Moore, M. A. and Massague, J. (2006). Hematopoiesis controlled by distinct TIF1gamma and Smad4 branches of the TGFbeta pathway. *Cell* **125**, 929-41.
Herrmann, B. G. (1991). Expression pattern of the Brachyury gene in whole-mount TWis/TWis mutant embryos. *Development* **113**, 913-7.
Ho, D. M., Chan, J., Bayliss, P. and Whitman, M. (2006). Inhibitor-resistant type I receptors reveal specific requirements for TGF-beta signaling in vivo. *Dev Biol* **295**, 730-42.
Hoodless, P. A., Pye, M., Chazaud, C., Labbe, E., Attisano, L., Rossant, J. and Wrana, J. L. (2001). FoxH1 (Fast) functions to specify the anterior primitive streak in the mouse. *Genes Dev* **15**, 1257-71.
Huse, M., Muir, T. W., Xu, L., Chen, Y. G., Kuriyan, J. and Massague, J. (2001). The TGF beta receptor activation process: an inhibitor- to substrate-binding switch. *Mol Cell* **8**, 671-82.
Hyatt, B. A. and Yost, H. J. (1998). The left-right coordinator: the role of Vg1 in organizing left-right axis formation. *Cell* **93**, 37-46.
Ikeya, M. and Takada, S. (2001). Wnt-3a is required for somite specification along the anteroposterior axis of the mouse embryo and for regulation of cdx-1 expression. *Mech Dev* **103**, 27-33.
Inman, G. J., Nicolas, F. J., Callahan, J. F., Harling, J. D., Gaster, L. M., Reith, A. D., Laping, N. J. and Hill, C. S. (2002). SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. *Mol Pharmacol* **62**, 65-74.
Ito, Y., Yeo, J. Y., Chytil, A., Han, J., Bringas, P., Jr., Nakajima, A., Shuler, C. F., Moses, H. L. and Chai, Y. (2003). Conditional inactivation of Tgfbr2 in cranial neural crest causes cleft palate and calvaria defects. *Development* **130**, 5269-80.
Jamin, S. P., Arango, N. A., Mishina, Y., Hanks, M. C. and Behringer, R. R. (2002). Requirement of Bmpr1a for Mullerian duct regression during male sexual development. *Nat Genet* **32**, 408-10.
Johnson, S. E., Rothstein, J. L. and Knowles, B. B. (1994). Expression of epidermal growth factor family gene members in early mouse development. *Dev Dyn* **201**, 216-26.
Jones, C. M., Kuehn, M. R., Hogan, B. L., Smith, J. C. and Wright, C. V. (1995). Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* **121**, 3651-62.
Jones, C. M., Simon-Chazottes, D., Guenet, J. L. and Hogan, B. L. (1992). Isolation of Vgr-2, a novel member of the transforming growth factor-beta-related gene family. *Mol Endocrinol* **6**, 1961-8.
Jornvall, H., Blokzijl, A., ten Dijke, P. and Ibanez, C. F. (2001). The orphan receptor serine/threonine kinase ALK7 signals arrest of proliferation and morphological differentiation in a neuronal cell line. *J Biol Chem* **276**, 5140-6.
Joseph, E. M. and Melton, D. A. (1998). Mutant Vg1 ligands disrupt endoderm and mesoderm formation in Xenopus embryos. *Development* **125**, 2677-85.
Jörnvall, H., Reissmann, E., Andersson, O., Mehrkash, M. and Ibáñez, C. F. (2004). ALK7, a receptor for nodal, is dispensable for embryogenesis and left-right patterning in the mouse. *Mol Cell Biol* **24**, 9383-9389.

Kaartinen, V., Voncken, J. W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N. and Groffen, J. (1995). Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet* **11**, 415-21.
Kavsak, P., Rasmussen, R. K., Causing, C. G., Bonni, S., Zhu, H., Thomsen, G. H. and Wrana, J. L. (2000). Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* **6**, 1365-75.
Kelly, O. G., Pinson, K. I. and Skarnes, W. C. (2004). The Wnt co-receptors Lrp5 and Lrp6 are essential for gastrulation in mice. *Development* **131**, 2803-15.
Kessler, D. S. and Melton, D. A. (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. *Development* **121**, 2155-64.
Kim, J., Wu, H. H., Lander, A. D., Lyons, K. M., Matzuk, M. M. and Calof, A. L. (2005). GDF11 controls the timing of progenitor cell competence in developing retina. *Science* **308**, 1927-30.
Kimura, C., Yoshinaga, K., Tian, E., Suzuki, M., Aizawa, S. and Matsuo, I. (2000). Visceral endoderm mediates forebrain development by suppressing posteriorizing signals. *Dev Biol* **225**, 304-21.
Kimura-Yoshida, C., Nakano, H., Okamura, D., Nakao, K., Yonemura, S., Belo, J. A., Aizawa, S., Matsui, Y. and Matsuo, I. (2005). Canonical Wnt signaling and its antagonist regulate anterior-posterior axis polarization by guiding cell migration in mouse visceral endoderm. *Dev Cell* **9**, 639-50.
Kinder, S. J., Tsang, T. E., Wakamiya, M., Sasaki, H., Behringer, R. R., Nagy, A. and Tam, P. P. (2001). The organizer of the mouse gastrula is composed of a dynamic population of progenitor cells for the axial mesoderm. *Development* **128**, 3623-34.
Kirby, M. L., Lawson, A., Stadt, H. A., Kumiski, D. H., Wallis, K. T., McCraney, E., Waldo, K. L., Li, Y. X. and Schoenwolf, G. C. (2003). Hensen's node gives rise to the ventral midline of the foregut: implications for organizing head and heart development. *Dev Biol* **253**, 175-88.
Knezevic, V., De Santo, R. and Mackem, S. (1998). Continuing organizer function during chick tail development. *Development* **125**, 1791-801.
Koenig, B. B., Cook, J. S., Wolsing, D. H., Ting, J., Tiesman, J. P., Correa, P. E., Olson, C. A., Pecquet, A. L., Ventura, F., Grant, R. A. et al. (1994). Characterization and cloning of a receptor for BMP-2 and BMP-4 from NIH 3T3 cells. *Mol Cell Biol* **14**, 5961-74.
Larsson, J., Goumans, M. J., Sjostrand, L. J., van Rooijen, M. A., Ward, D., Leveen, P., Xu, X., ten Dijke, P., Mummery, C. L. and Karlsson, S. (2001). Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. *Embo J* **20**, 1663-73.
Lawson, K. A., Dunn, N. R., Roelen, B. A., Zeinstra, L. M., Davis, A. M., Wright, C. V., Korving, J. P. and Hogan, B. L. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev* **13**, 424-36.
Le Good, J. A., Joubin, K., Giraldez, A. J., Ben-Haim, N., Beck, S., Chen, Y., Schier, A. F. and Constam, D. B. (2005). Nodal stability determines signaling range. *Curr Biol* **15**, 31-6.
Lee, S. J. (1990). Identification of a novel member (GDF-1) of the transforming growth factor-beta superfamily. *Mol Endocrinol* **4**, 1034-40.
Levine, A. J. and Brivanlou, A. H. (2006). GDF3, a BMP inhibitor, regulates cell fate in stem cells and early embryos. *Development* **133**, 209-16.
Ling, N., Ying, S. Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M. and Guillemin, R. (1986). Pituitary FSH is released by a heterodimer of the beta-subunits from the two forms of inhibin. *Nature* **321**, 779-82.
Liu, J. P. (2006). The function of growth/differentiation factor 11 (Gdf11) in rostrocaudal patterning of the developing spinal cord. *Development* **133**, 2865-74.
Liu, J. P., Laufer, E. and Jessell, T. M. (2001). Assigning the positional identity of spinal motor neurons: rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. *Neuron* **32**, 997-1012.

Liu, P., Wakamiya, M., Shea, M. J., Albrecht, U., Behringer, R. R. and Bradley, A. (1999). Requirement for Wnt3 in vertebrate axis formation. *Nat Genet* **22**, 361-5.
Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J. L., Lane, W. S. and Massague, J. (1991). Structure and expression of the membrane proteoglycan betaglycan, a component of the TGF-beta receptor system. *Cell* **67**, 785-95.
Lopez-Casillas, F., Wrana, J. L. and Massague, J. (1993). Betaglycan presents ligand to the TGF beta signaling receptor. *Cell* **73**, 1435-44.
Lorentzon, M., Hoffer, B., Ebendal, T., Olson, L. and Tomac, A. (1996). Habrec1, a novel serine/threonine kinase TGF-beta type I-like receptor, has a specific cellular expression suggesting function in the developing organism and adult brain. *Exp Neurol* **142**, 351-60.
Lyons, K., Graycar, J. L., Lee, A., Hashmi, S., Lindquist, P. B., Chen, E. Y., Hogan, B. L. and Derynck, R. (1989). Vgr-1, a mammalian gene related to Xenopus Vg-1, is a member of the transforming growth factor beta gene superfamily. *Proc Natl Acad Sci U S A* **86**, 4554-8.
Macias-Silva, M., Hoodless, P. A., Tang, S. J., Buchwald, M. and Wrana, J. L. (1998). Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. *J Biol Chem* **273**, 25628-36.
Matzuk, M. M., Kumar, T. R., Vassalli, A., Bickenbach, J. R., Roop, D. R., Jaenisch, R. and Bradley, A. (1995a). Functional analysis of activins during mammalian development. *Nature* **374**, 354-6.
Matzuk, M. M., Lu, N., Vogel, H., Sellheyer, K., Roop, D. R. and Bradley, A. (1995b). Multiple defects and perinatal death in mice deficient in follistatin. *Nature* **374**, 360-3.
Mayo, K. E., Cerelli, G. M., Spiess, J., Rivier, J., Rosenfeld, M. G., Evans, R. M. and Vale, W. (1986). Inhibin A-subunit cDNAs from porcine ovary and human placenta. *Proc Natl Acad Sci U S A* **83**, 5849-53.
Mazerbourg, S., Klein, C., Roh, J., Kaivo-Oja, N., Mottershead, D. G., Korchynskyi, O., Ritvos, O. and Hsueh, A. J. (2004). Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. *Mol Endocrinol* **18**, 653-65.
Mazerbourg, S., Sangkuhl, K., Luo, C. W., Sudo, S., Klein, C. and Hsueh, A. J. (2005). Identification of receptors and signaling pathways for orphan bone morphogenetic protein/growth differentiation factor ligands based on genomic analyses. *J Biol Chem* **280**, 32122-32.
McDonald, N. Q. and Hendrickson, W. A. (1993). A structural superfamily of growth factors containing a cystine knot motif. *Cell* **73**, 421-4.
McPherron, A. C., Lawler, A. M. and Lee, S. J. (1999). Regulation of anterior/posterior patterning of the axial skeleton by growth/differentiation factor 11. *Nat Genet* **22**, 260-4.
McPherron, A. C. and Lee, S. J. (1993). GDF-3 and GDF-9: two new members of the transforming growth factor-beta superfamily containing a novel pattern of cysteines. *J Biol Chem* **268**, 3444-9.
Mesnard, D., Guzman-Ayala, M. and Constam, D. B. (2006). Nodal specifies embryonic visceral endoderm and sustains pluripotent cells in the epiblast before overt axial patterning. *Development* **133**, 2497-505.
Mishina, Y., Rey, R., Finegold, M. J., Matzuk, M. M., Josso, N., Cate, R. L. and Behringer, R. R. (1996). Genetic analysis of the Mullerian-inhibiting substance signal transduction pathway in mammalian sexual differentiation. *Genes Dev* **10**, 2577-87.
Mishina, Y., Suzuki, A., Ueno, N. and Behringer, R. R. (1995). Bmpr encodes a type I bone morphogenetic protein receptor that is essential for gastrulation during mouse embryogenesis. *Genes Dev* **9**, 3027-37.
Miyazono, K., Hellman, U., Wernstedt, C. and Heldin, C. H. (1988). Latent high molecular weight complex of transforming growth factor beta 1. Purification from human platelets and structural characterization. *J Biol Chem* **263**, 6407-15.
Molloy, S. S., Bresnahan, P. A., Leppla, S. H., Klimpel, K. R. and Thomas, G. (1992). Human furin is a calcium-dependent serine endoprotease that

recognizes the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. *J Biol Chem* **267**, 16396-402.
Moustakas, A., Souchelnytskyi, S. and Heldin, C. H. (2001). Smad regulation in TGF-beta signal transduction. *J Cell Sci* **114**, 4359-69.
Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsukui, T., Gomer, L., Dorward, D. W., Glinka, A., Grinberg, A., Huang, S. P. et al. (2001). Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. *Dev Cell* **1**, 423-34.
Nagy, A., Gocza, E., Diaz, E. M., Prideaux, V. R., Ivanyi, E., Markkula, M. and Rossant, J. (1990). Embryonic stem cells alone are able to support fetal development in the mouse. *Development* **110**, 815-21.
Nakashima, M., Toyono, T., Akamine, A. and Joyner, A. (1999). Expression of growth/differentiation factor 11, a new member of the BMP/TGFbeta superfamily during mouse embryogenesis. *Mech Dev* **80**, 185-9.
Niederreither, K., Subbarayan, V., Dolle, P. and Chambon, P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat Genet* **21**, 444-8.
Nishitoh, H., Ichijo, H., Kimura, M., Matsumoto, T., Makishima, F., Yamaguchi, A., Yamashita, H., Enomoto, S. and Miyazono, K. (1996). Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5. *J Biol Chem* **271**, 21345-52.
Nomura, M. and Li, E. (1998). Smad2 role in mesoderm formation, left-right patterning and craniofacial development. *Nature* **393**, 786-90.
Nordstrom, U., Jessell, T. M. and Edlund, T. (2002). Progressive induction of caudal neural character by graded Wnt signaling. *Nat Neurosci* **5**, 525-32.
Norris, D. P., Brennan, J., Bikoff, E. K. and Robertson, E. J. (2002). The Foxh1-dependent autoregulatory enhancer controls the level of Nodal signals in the mouse embryo. *Development* **129**, 3455-68.
Norris, D. P. and Robertson, E. J. (1999). Asymmetric and node-specific nodal expression patterns are controlled by two distinct cis-acting regulatory elements. *Genes Dev* **13**, 1575-88.
Oh, S. P. and Li, E. (1997). The signaling pathway mediated by the type IIB activin receptor controls axial patterning and lateral asymmetry in the mouse. *Genes Dev* **11**, 1812-26.
Oh, S. P. and Li, E. (2002). Gene-dosage-sensitive genetic interactions between inversus viscerum (iv), nodal, and activin type IIB receptor (ActRIIB) genes in asymmetrical patterning of the visceral organs along the left-right axis. *Dev Dyn* **224**, 279-90.
Oliver, G., Mailhos, A., Wehr, R., Copeland, N. G., Jenkins, N. A. and Gruss, P. (1995). Six3, a murine homologue of the sine oculis gene, demarcates the most anterior border of the developing neural plate and is expressed during eye development. *Development* **121**, 4045-55.
Osada, S. I., Saijoh, Y., Frisch, A., Yeo, C. Y., Adachi, H., Watanabe, M., Whitman, M., Hamada, H. and Wright, C. V. (2000). Activin/nodal responsiveness and asymmetric expression of a Xenopus nodal-related gene converge on a FAST-regulated module in intron 1. *Development* **127**, 2503-14.
Oulad-Abdelghani, M., Chazaud, C., Bouillet, P., Mattei, M. G., Dolle, P. and Chambon, P. (1998). Stra3/lefty, a retinoic acid-inducible novel member of the transforming growth factor-beta superfamily. *Int J Dev Biol* **42**, 23-32.
Partanen, J., Schwartz, L. and Rossant, J. (1998). Opposite phenotypes of hypomorphic and Y766 phosphorylation site mutations reveal a function for Fgfr1 in anteroposterior patterning of mouse embryos. *Genes Dev* **12**, 2332-44.
Pearce, J. J., Penny, G. and Rossant, J. (1999). A mouse cerberus/Dan-related gene family. *Dev Biol* **209**, 98-110.
Perea-Gomez, A., Vella, F. D., Shawlot, W., Oulad-Abdelghani, M., Chazaud, C., Meno, C., Pfister, V., Chen, L., Robertson, E., Hamada, H. et al. (2002). Nodal antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks. *Dev Cell* **3**, 745-56.
Persson, U., Izumi, H., Souchelnytskyi, S., Itoh, S., Grimsby, S., Engstrom, U., Heldin, C. H., Funa, K. and ten Dijke, P. (1998). The L45 loop in type I

receptors for TGF-beta family members is a critical determinant in specifying Smad isoform activation. *FEBS Lett* **434**, 83-7.
Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T. and De Robertis, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397**, 707-10.
Proetzel, G., Pawlowski, S. A., Wiles, M. V., Yin, M., Boivin, G. P., Howles, P. N., Ding, J., Ferguson, M. W. and Doetschman, T. (1995). Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet* **11**, 409-14.
Rankin, C. T., Bunton, T., Lawler, A. M. and Lee, S. J. (2000). Regulation of left-right patterning in mice by growth/differentiation factor-1. *Nat Genet* **24**, 262-5.
Rebagliati, M. R., Weeks, D. L., Harvey, R. P. and Melton, D. A. (1985). Identification and cloning of localized maternal RNAs from Xenopus eggs. *Cell* **42**, 769-77.
Rebbapragada, A., Benchabane, H., Wrana, J. L., Celeste, A. J. and Attisano, L. (2003). Myostatin signals through a transforming growth factor beta-like signaling pathway to block adipogenesis. *Mol Cell Biol* **23**, 7230-42.
Reissmann, E., Jornvall, H., Blokzijl, A., Andersson, O., Chang, C., Minchiotti, G., Persico, M. G., Ibanez, C. F. and Brivanlou, A. H. (2001). The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. *Genes Dev* **15**, 2010-22.
Rhinn, M., Dierich, A., Shawlot, W., Behringer, R. R., Le Meur, M. and Ang, S. L. (1998). Sequential roles for Otx2 in visceral endoderm and neuroectoderm for forebrain and midbrain induction and specification. *Development* **125**, 845-56.
Rivera-Perez, J. A. and Magnuson, T. (2005). Primitive streak formation in mice is preceded by localized activation of Brachyury and Wnt3. *Dev Biol* **288**, 363-71.
Rodriguez, T. A., Srinivas, S., Clements, M. P., Smith, J. C. and Beddington, R. S. (2005). Induction and migration of the anterior visceral endoderm is regulated by the extra-embryonic ectoderm. *Development* **132**, 2513-20.
Roebroek, A. J., Umans, L., Pauli, I. G., Robertson, E. J., van Leuven, F., Van de Ven, W. J. and Constam, D. B. (1998). Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase Furin. *Development* **125**, 4863-76.
Rossant, J. and Tam, P. P. (2004). Emerging asymmetry and embryonic patterning in early mouse development. *Dev Cell* **7**, 155-64.
Ryden, M., Imamura, T., Jornvall, H., Belluardo, N., Neveu, I., Trupp, M., Okadome, T., ten Dijke, P. and Ibanez, C. F. (1996). A novel type I receptor serine-threonine kinase predominantly expressed in the adult central nervous system. *J Biol Chem* **271**, 30603-9.
Sakuma, R., Ohnishi Yi, Y., Meno, C., Fujii, H., Juan, H., Takeuchi, J., Ogura, T., Li, E., Miyazono, K. and Hamada, H. (2002). Inhibition of Nodal signalling by Lefty mediated through interaction with common receptors and efficient diffusion. *Genes Cells* **7**, 401-12.
Sanford, L. P., Ormsby, I., Gittenberger-de Groot, A. C., Sariola, H., Friedman, R., Boivin, G. P., Cardell, E. L. and Doetschman, T. (1997). TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* **124**, 2659-70.
Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. M. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-90.
Seki, T., Hong, K. H. and Oh, S. P. (2006). Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development. *Lab Invest* **86**, 116-29.

Shawlot, W., Wakamiya, M., Kwan, K. M., Kania, A., Jessell, T. M. and Behringer, R. R. (1999). Lim1 is required in both primitive streak-derived tissues and visceral endoderm for head formation in the mouse. *Development* **126**, 4925-32.
Shi, Y. and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.
Simeone, A., Acampora, D., Gulisano, M., Stornaiuolo, A. and Boncinelli, E. (1992). Nested expression domains of four homeobox genes in developing rostral brain. *Nature* **358**, 687-90.
Sirard, C., de la Pompa, J. L., Elia, A., Itie, A., Mirtsos, C., Cheung, A., Hahn, S., Wakeham, A., Schwartz, L., Kern, S. E. et al. (1998). The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev* **12**, 107-19.
Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* **70**, 829-40.
Song, J., Oh, S. P., Schrewe, H., Nomura, M., Lei, H., Okano, M., Gridley, T. and Li, E. (1999). The type II activin receptors are essential for egg cylinder growth, gastrulation, and rostral head development in mice. *Dev Biol* **213**, 157-69.
Souchelnytskyi, S., Tamaki, K., Engstrom, U., Wernstedt, C., ten Dijke, P. and Heldin, C. H. (1997). Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling. *J Biol Chem* **272**, 28107-15.
Spemann, H. M., H. (1924). Uber induktion von embryonalanlangen durch implantation artfremder organisatoren. *Roux Arch. EntwMech. Org.* **100**, 599-638.
Stern, C. D. (2006). Neural induction: 10 years on since the 'default model'. *Curr Opin Cell Biol*.
Sun, X., Meyers, E. N., Lewandoski, M. and Martin, G. R. (1999). Targeted disruption of Fgf8 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev* **13**, 1834-46.
Tam, P. P. and Rossant, J. (2003). Mouse embryonic chimeras: tools for studying mammalian development. *Development* **130**, 6155-63.
Tam, P. P. and Steiner, K. A. (1999). Anterior patterning by synergistic activity of the early gastrula organizer and the anterior germ layer tissues of the mouse embryo. *Development* **126**, 5171-9.
Tam, P. P. and Trainor, P. A. (1994). Specification and segmentation of the paraxial mesoderm. *Anat Embryol (Berl)* **189**, 275-305.
Tarkowski, A. K., Witkowska, A. and Opas, J. (1977). Development of cytochalasin in B-induced tetraploid and diploid/tetraploid mosaic mouse embryos. *J Embryol Exp Morphol* **41**, 47-64.
Thomas, G. (2002). Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat Rev Mol Cell Biol* **3**, 753-66.
Thomas, P. and Beddington, R. (1996). Anterior primitive endoderm may be responsible for patterning the anterior neural plate in the mouse embryo. *Curr Biol* **6**, 1487-96.
Thomas, P. Q., Brown, A. and Beddington, R. S. (1998). Hex: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. *Development* **125**, 85-94.
Thomsen, G. H. and Melton, D. A. (1993). Processed Vg1 protein is an axial mesoderm inducer in Xenopus. *Cell* **74**, 433-41.
Tsuchida, K., Nakatani, M., Yamakawa, N., Hashimoto, O., Hasegawa, Y. and Sugino, H. (2004). Activin isoforms signal through type I receptor serine/threonine kinase ALK7. *Mol Cell Endocrinol* **220**, 59-65.
Tsuchida, K., Sawchenko, P. E., Nishikawa, S. and Vale, W. W. (1996). Molecular cloning of a novel type I receptor serine/threonine kinase for the TGF beta superfamily from rat brain. *Mol Cell Neurosci* **7**, 467-78.

Varlet, I., Collignon, J., Norris, D. P. and Robertson, E. J. (1997). Nodal signaling and axis formation in the mouse. *Cold Spring Harb Symp Quant Biol* **62**, 105-13.
Vincent, S. D., Dunn, N. R., Hayashi, S., Norris, D. P. and Robertson, E. J. (2003). Cell fate decisions within the mouse organizer are governed by graded Nodal signals. *Genes Dev* **17**, 1646-62.
Vonica, A. and Brivanlou, A. H. (2006). An obligatory caravanserai stop on the silk road to neural induction: inhibition of BMP/GDF signaling. *Semin Cell Dev Biol* **17**, 117-32.
Wakefield, L. M., Smith, D. M., Flanders, K. C. and Sporn, M. B. (1988). Latent transforming growth factor-beta from human platelets. A high molecular weight complex containing precursor sequences. *J Biol Chem* **263**, 7646-54.
Waldrip, W. R., Bikoff, E. K., Hoodless, P. A., Wrana, J. L. and Robertson, E. J. (1998). Smad2 signaling in extraembryonic tissues determines anterior-posterior polarity of the early mouse embryo. *Cell* **92**, 797-808.
Wall, N. A., Craig, E. J., Labosky, P. A. and Kessler, D. S. (2000). Mesendoderm induction and reversal of left-right pattern by mouse Gdf1, a Vg1-related gene. *Dev Biol* **227**, 495-509.
Watanabe, R., Yamada, Y., Ihara, Y., Someya, Y., Kubota, A., Kagimoto, S., Kuroe, A., Iwakura, T., Shen, Z. P., Inada, A. et al. (1999). The MH1 domains of smad2 and smad3 are involved in the regulation of the ALK7 signals. *Biochem Biophys Res Commun* **254**, 707-12.
Weeks, D. L. and Melton, D. A. (1987). A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for a growth factor related to TGF-beta. *Cell* **51**, 861-7.
Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M. and Darnell, J. E., Jr. (1994). The winged-helix transcription factor HNF-3 beta is required for notochord development in the mouse embryo. *Cell* **78**, 575-88.
Weinstein, M., Yang, X., Li, C., Xu, X., Gotay, J. and Deng, C. X. (1998). Failure of egg cylinder elongation and mesoderm induction in mouse embryos lacking the tumor suppressor smad2. *Proc Natl Acad Sci U S A* **95**, 9378-83.
Winnier, G., Blessing, M., Labosky, P. A. and Hogan, B. L. (1995). Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev* **9**, 2105-16.
Withington, S., Beddington, R. and Cooke, J. (2001). Foregut endoderm is required at head process stages for anteriormost neural patterning in chick. *Development* **128**, 309-20.
Wolfman, N. M., McPherron, A. C., Pappano, W. N., Davies, M. V., Song, K., Tomkinson, K. N., Wright, J. F., Zhao, L., Sebald, S. M., Greenspan, D. S. et al. (2003). Activation of latent myostatin by the BMP-1/tolloid family of metalloproteinases. *Proc Natl Acad Sci U S A* **100**, 15842-6.
Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M. and Wang, E. A. (1988). Novel regulators of bone formation: molecular clones and activities. *Science* **242**, 1528-34.
Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X. F. and Massague, J. (1992). TGF beta signals through a heteromeric protein kinase receptor complex. *Cell* **71**, 1003-14.
Wrana, J. L., Attisano, L., Wieser, R., Ventura, F. and Massague, J. (1994). Mechanism of activation of the TGF-beta receptor. *Nature* **370**, 341-7.
Wu, H. H., Ivkovic, S., Murray, R. C., Jaramillo, S., Lyons, K. M., Johnson, J. E. and Calof, A. L. (2003). Autoregulation of neurogenesis by GDF11. *Neuron* **37**, 197-207.
Wu, J. W., Hu, M., Chai, J., Seoane, J., Huse, M., Li, C., Rigotti, D. J., Kyin, S., Muir, T. W., Fairman, R. et al. (2001). Crystal structure of a phosphorylated Smad2. Recognition of phosphoserine by the MH2 domain and insights on Smad function in TGF-beta signaling. *Mol Cell* **8**, 1277-89.
Yamaguchi, T. P., Harpal, K., Henkemeyer, M. and Rossant, J. (1994). fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev* **8**, 3032-44.

Yamamoto, M., Meno, C., Sakai, Y., Shiratori, H., Mochida, K., Ikawa, Y., Saijoh, Y. and Hamada, H. (2001). The transcription factor FoxH1 (FAST) mediates Nodal signaling during anterior-posterior patterning and node formation in the mouse. *Genes Dev* **15**, 1242-56.
Yeo, C. and Whitman, M. (2001). Nodal signals to Smads through Cripto-dependent and Cripto-independent mechanisms. *Mol Cell* **7**, 949-57.
Zhang, X. M., Ramalho-Santos, M. and McMahon, A. P. (2001). Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R symmetry by the mouse node. *Cell* **106**, 781-92.
Zhou, X., Sasaki, H., Lowe, L., Hogan, B. L. and Kuehn, M. R. (1993). Nodal is a novel TGF-beta-like gene expressed in the mouse node during gastrulation. *Nature* **361**, 543-7.
Zimmers, T. A., Davies, M. V., Koniaris, L. G., Haynes, P., Esquela, A. F., Tomkinson, K. N., McPherron, A. C., Wolfman, N. M. and Lee, S. J. (2002). Induction of cachexia in mice by systemically administered myostatin. *Science* **296**, 1486-8.